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GP award

Lignins and Lignification: New Developments and Emerging Concepts

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MAIN CONCLUSION

Lignin biosynthesis is highly malleable, allowing a variety of phenolic monomers to be incorporated into the polymer. Certain phytochemicals may be produced on massive scale (for incorporation into lignins). Strategic tailoring of lignin's structure, reactivity, and value, and actual 'design' of the polymer can be contemplated, permitting an ideal polymer for lignin valorization to be envisioned.

INTRODUCTION

Evidence continues to mount regarding lignins' inherent structural malleability from studies investigating lignin pathway mutants and transgenics, as well as on various 'natural' plants discovered to possess unusual lignins.[1,2] Most of the monomers previously considered were derived from the monolignol biosynthetic pathway itself. More recently, phenolics from beyond the monolignol pathway have been shown to be authentic monomers in some plants, including the flavone tricin in all grasses (and beyond), various hydroxystilbenes in some palm endocarp tissues, and other components. As we noted some time back, but not in print until 2008: "any phenolic transported to the lignifying zone of the cell wall can, subject to simple chemical compatibility, be incorporated into the polymer."[3] That does not automatically mean that the resulting polymer will be well tolerated by the plant, but the paradigm opens up many opportunities allowing researchers to contemplate some degree of actual design of lignins for improved utilization and value, and muse over what might constitute a lignin that is ideally suited for conversion to phenolic monomers.

MATERIALS & METHODS

This paper presents an update on some aspects of lignin and lignification – A materials and methods section therefore makes little sense.

Structures, newly elucidated by modern but standard means, reveal fresh details about lignification *in planta*. Newly identified appendages on the polymer that can be readily clipped off are realizing their value.

RESULTS & DISCUSSION

Just how expansive the definition of lignin may need to be is being illuminated as remarkable lignins are discovered and characterized. Researchers now muse over how lignins may be designed to deconstruct more readily, examples of 'ideal lignins' are revealed, and it is opportune to contemplate the ramifications of lignification using non-canonical monomers.

Weak bonds (esters) have been engineered into the lignin backbone,[4] facilitating lignin depolymerization during pretreatment or processing. Nature is already naturally making such 'sip-lignins' at low levels in a variety of plants.

For the depolymerization of the polymer to monomers, one 'ideal lignin' ideotype has three characteristics. First, it should be stable under acidic conditions to prevent condensation and the generation of undesired additional C–C bonds, during pretreatment. Second, it should contain only ether (C–O) inter-unit linkages in its backbone so that it can be fully depolymerized. Finally, it should be generated *in planta* from a single phenylpropanoid monomer to allow the production of the simplest array of compounds. **C**-lignin, such as that found in vanilla seed coats,[5] is one such example, as it is essentially a homopolymer synthesized almost purely by β–O–4-coupling of caffeyl

alcohol with the growing polymer chain, producing benzodioxanes as the dominant unit in the polymer, and has been successfully converted to monomers in near-quantitative yields.

CONCLUSIONS

Because of the revelations into how pathways interact to supply monomers for lignification, the most staid of classical phytochemistry researchers are beginning to find some intrigue in this traditionally troublesome polymer. Even at just a few percent of the polymer, some established phytochemicals are being realized to be produced on a previously unimaginable scale. Lignin may never enchant all plant researchers, but lignin research has certainly become increasingly fascinating of late as it crosses into new territories.

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O1.1

Nupharanin, a novel dehydroellagitannin from *Nuphar japonicum*

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MAIN CONCLUSION

Nupharanin is the first dehydroellagitannin identified with an α- instead of the usual β-glucopyranose core. This study demonstrated how conversion to semisynthetic derivatives has been useful in complete structure elucidation, thus giving us a broader, yet in-depth understanding on the effects of substituents in the conformation of the glucopyranose core. This study encourages further investigation on the biosynthesis of ellagitannins with an unconventional sugar conformation.

INTRODUCTION

Dehydroellagitannins are a subgroup of hydrolyzable tannins biogenetically derived from ellagitannins where the HHDP moiety is oxidized to DHHDP. These are widely distributed in the plant kingdom and some displayed an array of biological activities. The DHHDP moiety contains a hemiketal group formed between the cyclohexenetrione and pyrogallol groups. This makes the moiety reactive, as previously demonstrated with experimental conversion of geraniin to chebulagic acid (1), elaeocarpusin (2), and acetone derivatives.(3) A common structural feature among reported dehydroellagitannins is the β-D-glucopyranose core adopting a $^1C_4$ conformation as a consequence of HHDP or DHHDP substitution at 1,6-, 3,6-, or 2,4-positions. Isoterchebin is an exception to this since it adopts $^4C_1$ conformation instead, as a consequence of bridging of DHHDP group at 4,6 positions.

Nuphar japonicum DC (Nymphaceae) is the source of the crude drug Nupharis Rhizoma—a component of the Kampo medicine *jidabokuippo*, indicated for contusion-induced swelling and pain. Freeze-dried rhizomes of this aquatic plant have been previously demonstrated to contain hydrolyzable tannins bearing the unusual α-D-glucopyranose core. Moreover, both (*S*)- and (*R*)-HHDP were found to coexist, in the forms of nupharins A, B, and C. As a consequence, both $^1C_4$ and a skewed boat conformation of α-D-glucopyranose also exist. Preliminary HPLC analysis of ethanolic extract of the fresh rhizome revealed a broad peak (Figure 1a), which disappeared and replaced with sharp peak at 375 nm (Figure1b). This signified the formation of formation of phenazine derivative of the DHHDP moiety (Scheme 1). In this study, we intended to isolated and elucidate the structure of the dehydroellagitannin.

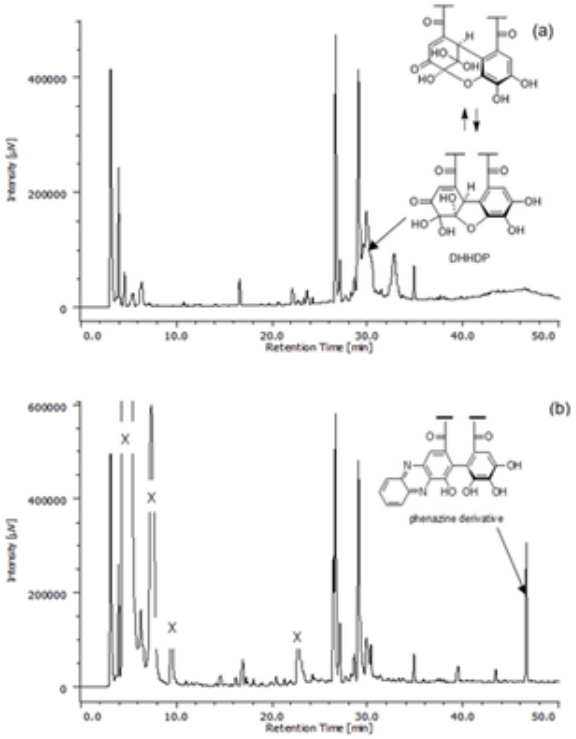
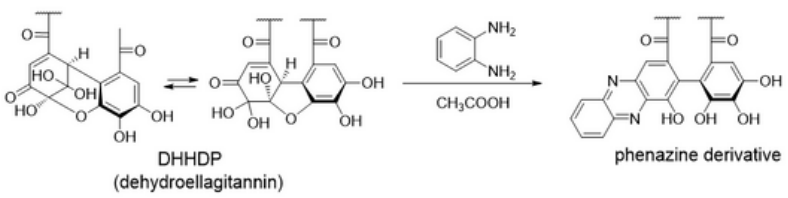


Figure 1. HPLC profiles of 60% EtOH extracts of fresh *N. japonicum* rhizome untreated (a) and treated with *o*-phenylenediamine (b).

Scheme 1. Reaction of DHHDP of a dehydroellagitannin towards formation of phenazine derivative.



MATERIALS & METHODS

Fresh rhizomes of N. japonicum were extracted with acetone, concentrated *in vacuo*, subjected to a series of chromatographic separation on Diaion HP20SS, Sephadex LH-20, then Chromatorex ODS on gradient elution with methanol. The acetonyl derivative prepared and isolated in the same manner as nupharanin, except, ammonium formate was the additional extracting solvent.

The acetonyl derivative was then reacted with TFA, cysteine in pyridine at 60°C for 1h, then tolylisothiocyanate at 60°C for 1h. the resulting thiazolidine derivative was analyzed by HPLC-PDA and the retention time was compared to those of L- and D-glucose derivatives.

Nupharanin was heated in pH 6 McIlvaine buffer at 50°C for 4.5 h, cooled, acidified with TFA, fractionated on Sephadex LH-20, then Chromatorex ODS on gradient elution with methanol, giving the pure oxidation product.

HRFABMS, ^{1}H-,^{13}C-, 2D NMR, and CD spectroscopy were employed for structure elucidation. DFT calculations, and DP4+ analyses were also done.

RESULTS & DISCUSSION

Nupharanin, the first ellagitannin with dehydrohexahydroxydiphenoyl (DHHDP) ester at the 1,4-positions of α-D-glucose, was isolated from the fresh rhizome of *Nuphar japonicum*. The structure was unambiguously determined to be 1,4-(*S*)-dehydrohexahydroxydiphenoyl-2-galloyl-3,6-(*R*)-hexahydroxydiphenoyl-α-D-glucopyranose based on HR FABMS, ^{1}H-, ^{13}C- and 2D NMR data (Figure 2). The coexistence of the two tautomers (**1a** & **1b**) in solution complicated the NMR data of nupharanin and this prompted the conversion to the more stable acetonyl derivative (**2**), a characteristic reaction product of DHHDP moiety with acetone in the presence of ammonium formate. This led to establishment of linkage of the DHHDP group at 1,4 position of the glucose core. Meanwhile, the sugar moiety was established as D-glucopyranose through comparison of HPLC chromatogram of the thiocarbamoyl-thiazolidine derivative of the acid hydrolysate of **2** to those of L- and D-glucose. The stability of the DHHDP moiety was investigated by subjecting nupharanin to 50˚C at pH 6 buffer and yielded an unusual oxidation product (**3**). The stereochemistry of this product was assigned using scaled, unscaled and summative DP4+ probabilities from differences of GIAO ^{13}C-NMR/^{1}H-NMR values and experimental data. Establishment of the absolute configuration as (*R*)-HHDP and (*S*)-DHHDP were made possible through comparison of the actual CD spectroscopic data to those previously reported in literature and to data generated for another dehydroellagitannin, acetylfurosin. Lastly, the glucose moiety was identified to have adopted a skewed boat ($^{3}S_{1}$) conformation by comparison of the experimental coupling values (*J*) of nupharanin and acetonyl derivatives to those previously reported and values generated through DFT calculation.

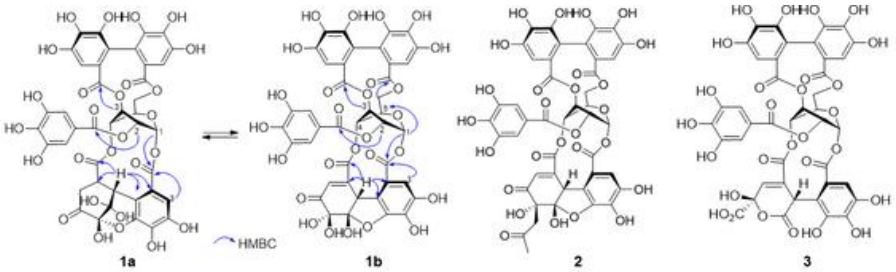


Figure 2. Tautomers of nupharanin (**1a** & **1b**), acetonyl derivative (**2**), and oxidation product (**3**).

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O1.2

Identification and quantification of molecular ellagitannins in Cognac eaux-de-vie by mass spectrometry method: evolution over time towards new compounds

Mathilde Gadrat[1,2,3], Yoan Capello[4], Joël Lavergne[5], Catherine Emo[3], Stéphane Quideau[6], Michael Jourdes[1,2], Pierre-Louis Teissedre[1,2], Kléopatra Chira[1,2]

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MAIN CONCLUSION

The identification and quantification of individual ellagitannins in cognac eaux-de-vie, as well as their evolution kinetics during ageing, led to the discovery of new evolution ellagitannin in the eaux-de-vie. These were identified and quantified for the first time.

INTRODUCTION

Ellagitannins are the main extractible phenolic compounds in oak wood with the monomers vescalagin and castalagin being the predominant ones. Derivatives of these monomers are dimers (roburin A, roburin D) as well as xylose/lyxose derivatives (grandinin, roburin B, roburin C, roburin E). These compounds are responsible for high wood durability and they also contribute to wine organoleptic quality (color, astringency, bitterness). Given their important properties as well as lack of knowledge regarding their composition and evolution in spirits, the aims of our study were firstly to determine and quantify the ellagitannins content and composition in Cognac eaux-de-vie and secondly to estimate their evolution during spirits aging.

MATERIALS & METHODS

For achieving these objectives, an ellagitannins extraction protocol was established from Cognac samples prior to the analysis on a Triple Quadrupole mass spectrometer (HPLC-QQQ). Once the method was validated according to the OIV regulations [1], three set of sample Cognac eaux-de-vie from different terroirs and multiple vintages were analyzed. New evolution ellagitannins compounds were identified by UPLC-Q-ToF. In order to purify these compounds, a model solution of eau-de-vie doped with ellagitannins was made and then freeze-dried. The dry extract was fractionated on a TSK 50F gel column in order to simplify the matrix. The fractions containing the compounds of interest were then injected into semi-preparative HPLC in order to purify the ellagitannins evolution compounds and to elucidate their structure by 1D/2D-NMR.

RESULTS & DISCUSSION

The 8 native ellagitannins of oak wood evolve rapidly in Cognac eaux-de-vie. Indeed, the vertical analysis of eaux-de-vie from 1 to 40 years old has shown that these molecules degrade over time and undergo transformations like oxidation, reduction or hydrolysis reactions that give rise to many unknown evolution compounds. Castalagin is the most stable ellagitannin over time, as it is still present after 40 years, whereas the other native ellagitannins have been transformed since the first years of ageing. A selection of several ellagitanins-derived compounds containing characteristic MS/MS ellagitannin fragments (i.e., ellagic acid/vescalin) were detected A model solution of eau-de-vie enriched with oak ellagitannins to produce these compounds in high concentration and to simplify the matrix compare to Cognac in order to purify them. Among them, the whiskey tannins B and

A [2] ([M-H]$^-$ ion peak at *m/z* 977.0896 and [M-H]$^-$ ion peak at *m/z* 675.0833 respectively) were found for the first time in Cognac (Figure 1.). The whiskey tannin B results from the ethanol/castalagin reaction and the whiskey tannin A result from the ellagic acid hydrolysis of the whiskey tannin B. These compounds could also be quantified in eaux-de-vie for the first time and their concentration decreased during aging.

Other derivative which showed [M-H]$^-$ ion peak at *m/z* 1005,1212 was produced in 25 years old Cognac and it structure has been elucidated by NMR analysis. This work brings new insights on the ellagitannins composition associated to spirits ageing and quality. Further sensory analysis will be performed in order to understand the organoleptic impact of these new compounds.

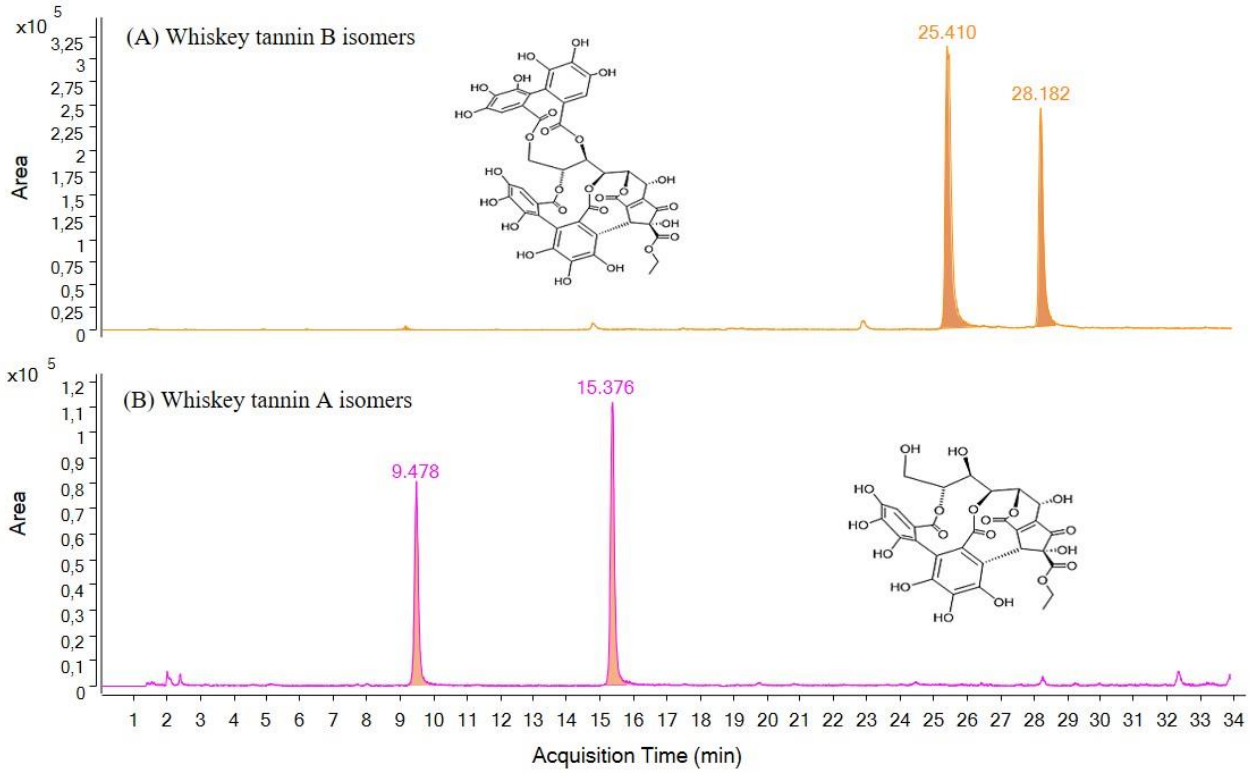

Figure 1: Negative HPLC-QQQ corresponding to [M-H]$^-$ ions of the two isomers of Whiskey Tannin B (A) and the two isomers of Whiskey Tannin A (B) respectively.

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O1.3

New Insights of Pectin-Procyanidin Interactions: Structure/Function Relationships

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MAIN CONCLUSION

All pectins preferentially interact with procyanidins with higher degree of polymerization (DPn). Pectins with high linearity and homogalacturonan (HG) content, had higher affinity and aggregation capacities with procyanidins. On the contrary, high rhamnogalacturonan (RG) I side-chains and ferulic acid content limited pectins affinity for procyanidins. The DPn of procyanidins appears to affect the interactions more than the structure of pectins.

INTRODUCTION

Procyanidins, condensed tannins, are polymers and oligomers of flavan-3-ol units, which presence in fruit and vegetables is related to protective health effects. The chemical structure of procyanidins and their interactions with the food matrix can modulate their fermentescibility by colon microbiota and bioavailability of their metabolites, but this is further influenced by food processing. Pectins are the polysaccharide in plant cell walls which have the highest affinity for procyanidins; they are also susceptible to enzymatic and non-enzymatic reactions during plant-based production processing, modifying their structure and, hence, their physicochemical properties. While impact of procyanidin structure on the affinity for pectins is relatively clear[1], it is not the case for impact of the structural determinants in pectins. Pectins are complex branched heteropolysaccharides, which can notably be differentiated in terms of ratio of homogalacturonans to neutral side-chains, substitution and size. However, the intrinsic relationship between the pectins structure and their interaction with procyanidins is still unclear, although it is known to be related to their conformation and composition.

A deep understanding of the molecular mechanisms that drive the interaction between pectins and procyanidins can enable us to better bridge the gap between food processing and the bioavailability of commensal microbiota fermentation products of pectin and procyanidins. Further, this promotes the design of more rational processing conditions for healthier and more nutritious foods.

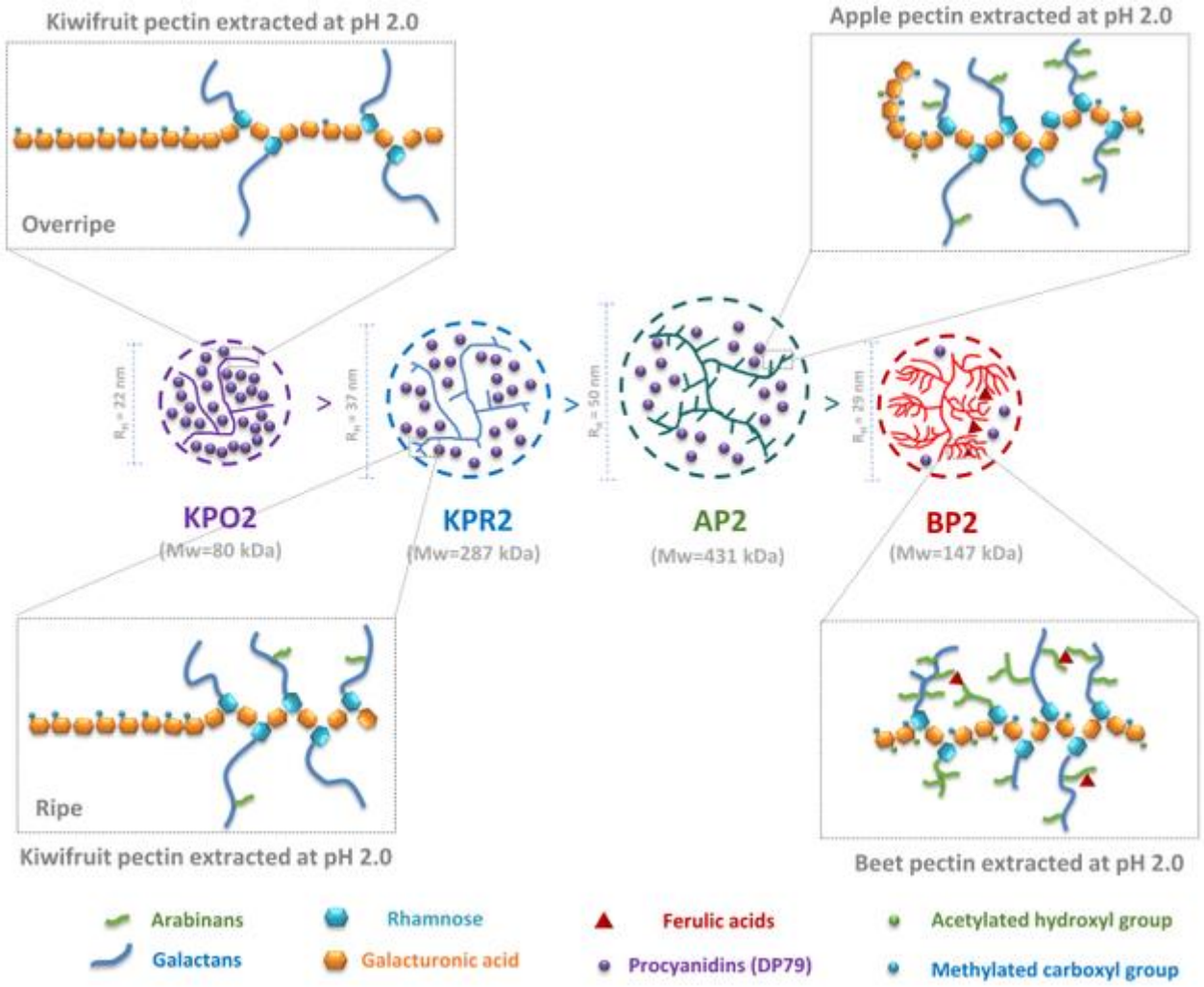
MATERIALS & METHODS

Procyanidins from two apples ('Marie Menard' and 'Avrolles' cultivars) were extracted and purified. Two procyanidin fractions were obtained: DP9 and DP79. Pectin fractions were extracted from apple, beet and kiwifruits cell walls of flesh tissue after heating at 100 °C during 20 min at pH 2.0, 3.5 and 6.0[2]. Good diversities and variabilities were obtained in this sample set for pectin linearity, length of side chains, arabinans / galactans ratio, degree of acetylation, molar mass and conformation. No pectins with a low degree of methylation (DM < 30) were present. Aggregate's formation and thermodynamic nature of their interactions were analyzed by UV−visible spectroscopy and isothermal titration calorimetry (ITC), respectively. The conformational properties before and after pectin-procyanidin interactions were determined by HPSEC-MALLS.

RESULTS & DISCUSSION

Using both isothermal titration calorimetry and turbidity, pectins with different linearity (or side-chain abundance) and macromolecular characteristics were shown to interact with procyanidins (DP9 and DP79). These two methods are complementary, allowing higher sensitivity for detection of the interactions (haze formation) on the one hand and access to stoichiometric ratio and binding enthalpy

(ITC) on the other hand. KPO2 is a linear polymer chain and less branched polymer structures with KPR2 less long-chain branches, AP2 moderate RG content with long/short-chain mixture branches, and BP2 both much RG region with short-chain and long-chain branches, and some covalently bound ferulic acid (Fig.1).



The highest affinities for procyanidins DP9/79 were obtained for kiwifruit pectins KPO2 and KPR2, with both the highest K_a and the most marked aggregate formation, and the lowest affinities were observed for beet pectins. Higher linearity and higher HG ratio of pectins strengthen their binding to procyanidins. The low affinity performance of beet pectins may be not only due to their complex arabinan side chain structures, but also to the presence of ferulic acid covalently linked to arabinans, and to their acetylation. Ferulic acid cross-linking, by further rigidifying the arabinan side-chains, might lower interactions with procyanidins due to steric hindrance. Moreover, the interactions were higher with the procyanidins of the highest DPn. With procyanidins DP79, marked aggregation appeared for all pectin fractions at the maximum concentration of both, while with procyanidins DP9 formation of cloud or aggregate were relatively lower. However, BP2 had a lower affinity with DP79 than DP9. Higher number average DP of procyanidins might hinder the adsorption of pectins with high side chain branching.

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O1.4

Dendrimers as color-stabilizers of anthocyanin-type dyes: how the structure and concentration of the dye modulates the interaction mechanisms

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MAIN CONCLUSION

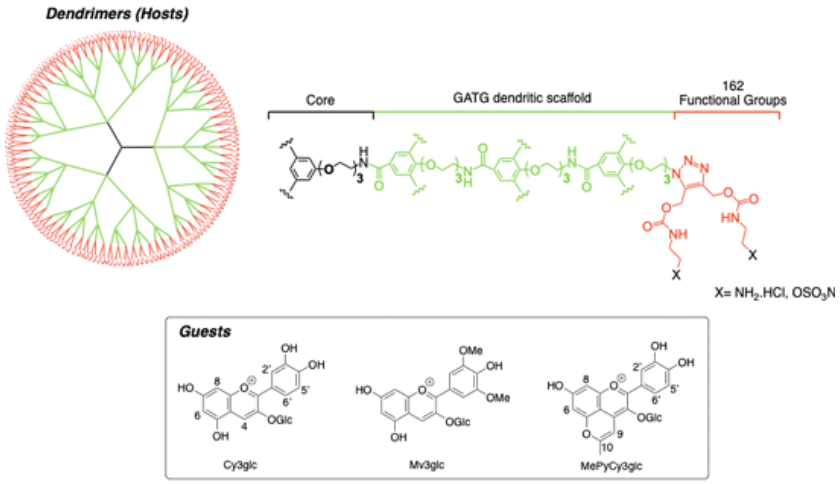
Our results provide insights about the ability of a polyanionic GATG-based dendrimer to host structurally diverse flavylium-based dyes and how the structure and the concentration of the dye of the modulates the type of interactions involved.

The tuning, color stabilization of anthocyanins and encapsulation of pyranoanthocyanin-type dyes using this biocompatible dendrimer envisage novel host-guest sensing applications such as pH-responsive systems used for example in food smart packaging.

INTRODUCTION

Flavylium-based dyes represent a huge family of natural and synthetic pigments, which includes anthocyanins, 3-deoxyanthocyanins and anthocyanin-derived pigments (e.g., pyranoanthocyanins) with interesting applications as colorants for textiles, foods, cosmetics, as well as for smart and functional materials because of their pH- and light-responsiveness. While for anthocyanins a great color fading occurs at moderated acidic to neutral pH essentially due to the hydration reaction of the flavylium cation to form an uncolored hemiketal species, pyranoanthocyanins are much more stable because the presence of additional pyranic ring impedes the production of hemiketal colourless species. This leads to a more evident colour changes than anthocyanins in response to pH variations, which constitutes an advantage for sensing applications. Furthermore, the color tuning and the physico-chemical properties improvement of these guests can be achieve through the interaction with host structure receptors (e.g., crown ethers, porphyrins, cyclodextrins, cucurbiturils, nanoparticles, nanotubes, liposomes, dendrimers). Because of their globular architecture at the nanoscale, high functional surface and inherent multivalency, dendrimers are considered archetypal macromolecular hosts.[1]

In this work, the molecular interactions between natural anthocyanin derivatives and a four generation of a gallic acid triethylene glycol(GATG)-based dendrimer decorated with positive, anionic and neutral functional groups (NH_2.HCl, OSO_3Na and OH, respectively) (Scheme 1) were studied by means of UV-Vis, stopped-flow and NMR spectroscopy techniques (^1H, T_2, DOSY, NOESY).[2]



Scheme 1: Chemical structures of hosts and guests used.

MATERIALS & METHODS

For the NMR titration, a 261 µM solution of MePyCy3glc (or 124 µM solution of Cy3glc) was prepared in D_2O and the pH was adjusted to 1 (pD 1.4) and transferred into 5 mm NMR tubes. TSP (5 µL, 2 mg/mL in D_2O) was used as an internal standard for chemical shift measurements. Successive volumes of dendrimers stock solutions in D_2O at pD 1.4 were added to the NMR tube to obtain different guest: dendrimer molar ratios (G/H) during the titration. All ^{1}H NMR spectra were recorded under the same experimental conditions of previous work.[5a]

The chemical shift variations ($\Delta\delta_{obs}$) of guest protons as a function of G/H ratio can be expressed through equation 1:[3]

$$\Delta\delta_{obs} = \frac{\Delta\delta_{max}}{2}\left\{\left(1+\frac{1}{K[Guest]}+\frac{n[Dend]}{[Guest]}\right)-\left[\left(1+\frac{1}{K[Guest]}+\frac{n[Dend]}{[Guest]}\right)^2-\frac{4n[Dend]}{[Guest]}\right]^{1/2}\right\} \quad (1)$$

$\Delta\delta_{max}$ is the maximum chemical shift variation of the guest molecule in NMR titration experiment, *K* is the binding affinity or association constant The number of binding sites (*n*) was obtained by fitting the titration data with equation 1 using a nonlinear least-squares method within the software program Microsoft Excel.

RESULTS & DISCUSSION

The preliminary results obtained by UV-Vis spectroscopy showed only a significant and interesting interaction of Cy3glc and MePyCy3glc in the presence of the anionic dendrimer rather than with the cationic and neutral dendrimers. Bearing this, a deeper interaction mechanism study was performed for Cy3glc and MePyCy3glc with the polyanionic dendrimer by NMR using T_2, DOSY, NOESY and ^{1}H titration (Figure 1).

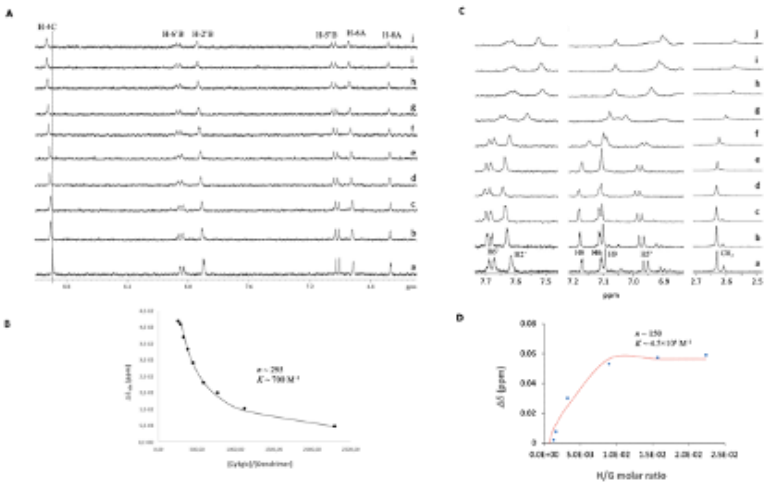


Figure 1. (**A**) ^{1}H NMR titration of Cy3glc (124 µM) with increasing concentrations of host dendrimer: a) no dendrimer; b) 0.054 µM; c) 0.11 µM; d) 0.16 µM; e) 0.21 µM; f) 0.27 µM; g) 0.32 µM; h) 0.37 µM; i) 0.42 µM; j) 0.47 µM. (**B**) Chemical shift variation of H-4C from Cy3glc as a function of G/H molar ratio. (**C**) the same for MePyCy3glc (261 µM): a) no dendrimer; b) 0.12 µM; c) 0.16 µM; d) 0.29 µM; e) 0.33 µM; f) 0.41 µM; g) 0.80 µM; h) 2.2 µM; i) 3.6 µM; j) 5.0 µM. (**D**) Chemical shift variation of CH_3 from MePyCy3glc as a function of H/G molar ratio.

When cy3glc was titrated with the dendrimer, significant downfield shifts of all aromatic protons of cy3glc were observed. The interaction parameters (K~700 M^{-1}, n~295) indicate the binding of *ca.* two anthocyanin molecules by each sulfate group at the dendrimer periphery, forming reversibly contact ion pairs with the sulfate group. On the other hand, for MePyCy3glc two distinct interaction modes were observed: at very low concentrations of dendrimer all protons of the guest were shifted downfield suggesting the formation of reversible contact ion-pairs; at higher dendrimer concentrations a larger upfield shift of the guest protons was observed accompanied with an intensity decrease of the signals which is compatible with an encapsulation near the anionic surface facilitated by the hydrophobicity of the dye.[3] The interaction parameters of the encapsulation process (K~4.5×10^4 M^{-1}, n~150) indicate the binding of *ca.* one pyranoanthocyanin molecule by each sulfate terminal group.

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O1.5

Color expression and stability of cis and trans p-coumaric acylated cyanidin-derivatives and their UV-induced isomerization

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MAIN CONCLUSION

Cyanidin-glycosides with *cis*-configured *p*-coumaric acid exhibited sharper peaks, greater $\lambda_{vis\text{-}max}$, larger absorbance; and expressed darker and more saturated color than their *trans* counterparts with broader color expression. The *cis* isomers also retained more color at pH 4-6 and had longer half-lives under most pH. UV irradiation induced the *trans-to-cis* isomerization in both aqueous and ethanolic solutions, and could help expand the use of the *cis* isomers in solutions.

INTRODUCTION

Anthocyanins are water-soluble flavonoids that have been widely used as synthetic dye alternatives due to their favorable view by consumers and potential health benefits. However, their applications may be restricted by difficulties with color matching and quick degradation during processing and storage [1]. The aglycone, glycosylation and acylation all determine anthocyanins' coloring properties and stability, impacting their application in food system [1]. Acylation of anthocyanins has been commonly recognized to increase their stability and impact color properties [2]. Due to the presence of double bonds on their side chains, anthocyanins acylated with *p*-coumaric acid can be either *cis* or *trans*-configured [3]. A study [4] of malvidin and delphinidin derivatives indicated that the *cis* isomers exhibited larger molar absorptivity than the *trans*, and it experienced less hydration at mildly acidic pH. Nevertheless, the *trans* configured anthocyanins are abundant in nature while the *cis* configured isomers are very rare. Although a *trans-to-cis* isomerization is rarely seen *in vivo*, it has been reported to happen *in vitro* under UV irradiation, with a much higher conversion rate obtained in methanol than in water [5]. So far, the impact of *p*-coumaric acid configuration on anthocyanin coloration and stability were mostly evaluated at acidic pH. Most relevant studies have been conducted on delphinidin, malvidin, or petunidin derivatives, despite cyanidin being the most abundant aglycone in nature. Our knowledge on the anthocyanin photoisomerization is still limited. Therefore, the objective of this work was to investigate the impact of *p*-coumaric acid conformation on the color expression and stability of cyanidin-derivatives and their photoisomerization in aqueous and ethanolic solvents.

MATERIALS & METHODS

Anthocyanins were extracted from American elderberry using acetone and partitioned with chloroform. ***Color and stability***: *Cis* and *trans* cyanidin-3-*p*-coumaroyl-sambubioside-5-glucoside were isolated on a semi-prep HPLC. Each isolate was diluted to 100M using pH 1–9 buffers, loaded on a microplate and stored at 4°C in dark up to 28 days. The visible spectrum data (380–700 nm) was monitored over time using a microplate reader. The colorimetric data (CIE L^*, a^*, b^*, c^*_{ab}, h^*_{ab}) were obtained from the spectral data using ColoBySpectra software. Half-lives were calculated by the attenuation of absorbance at $_{vis\text{-}max}$. ***Solvent effect:*** *Cis* and *trans* isolates were diluted to 100M with ethanol or DI water, placed in quartz cuvettes and irradiated 20min in a UV Crosslinker (254 nm). The *cis*/*trans* ratio after irradiation was monitored by uHPLC-PDA. All treatments were triplicated. Statistical comparison and half-lives were computed using RStudio. Color swatches were generated by Adobe Photoshop.

RESULTS & DISCUSSION

Similar $\lambda_{vis\text{-}max}$ (522–526 nm) were displayed by the two isolates at pH 1–4. At pH 7–9, the *cis* isomers displayed a $\lambda_{vis\text{-}max}$ (577–603 nm) 5–13 nm larger than the *trans* isomers. Higher absorbance was consistently displayed by the *cis* isomers at all pH, with the largest difference (>2.5 times higher than the *trans*) obtained at pH 4–6. Spectral differences of *cis* and *trans* cyanidin-derivatives, previously reported [4] mostly for acidic pH, were extended in this study to a broader range. At pH ≥3, the *cis* isomers expressed darker colors (L^* 5.1–24 units smaller than the *trans*) (Figure). The higher absorption and darker color of the *cis* isomers may ascribe to closer proximity between acid and aglycone, offering better protection against hydration reaction. C^*_{ab} of the *cis* was 1.6–32.6 units larger than the *trans*, indicating more saturated color expression. The h^*_{ab} of the *cis* isomers was ~10° larger than the *trans* at pH 1-3 (more orange-red tones) and ~10° smaller at pH 8–9 (more cyan-blue hue), covering a wider span of color hues. The *trans* isomer had very low color expression at pH 3–4. At pH 7–9, half-lives of the *trans* isomers were <2 days, and up to 7 days for the *cis* isomers. Both *cis* and *trans* cyanidin-derivatives displayed blue hues at pH 8, a color that remains challenging to reproduce in foods, and the half-life of the *cis* was nearly 4 times longer than the *trans*. The extended half-lives, along with its darker, more saturated color expression make the *cis* isomers a good natural blue color candidate. Irradiation of *cis* and *trans* isomers in ethanol reached *cis*/*trans* ratios ~0.63–0.75, significantly higher than the ratio obtained in water (~0.3). The *trans-to-cis* isomerization was favored in ethanol, and photoirradiation could be used to modulate their ratios and the extract's color expression.

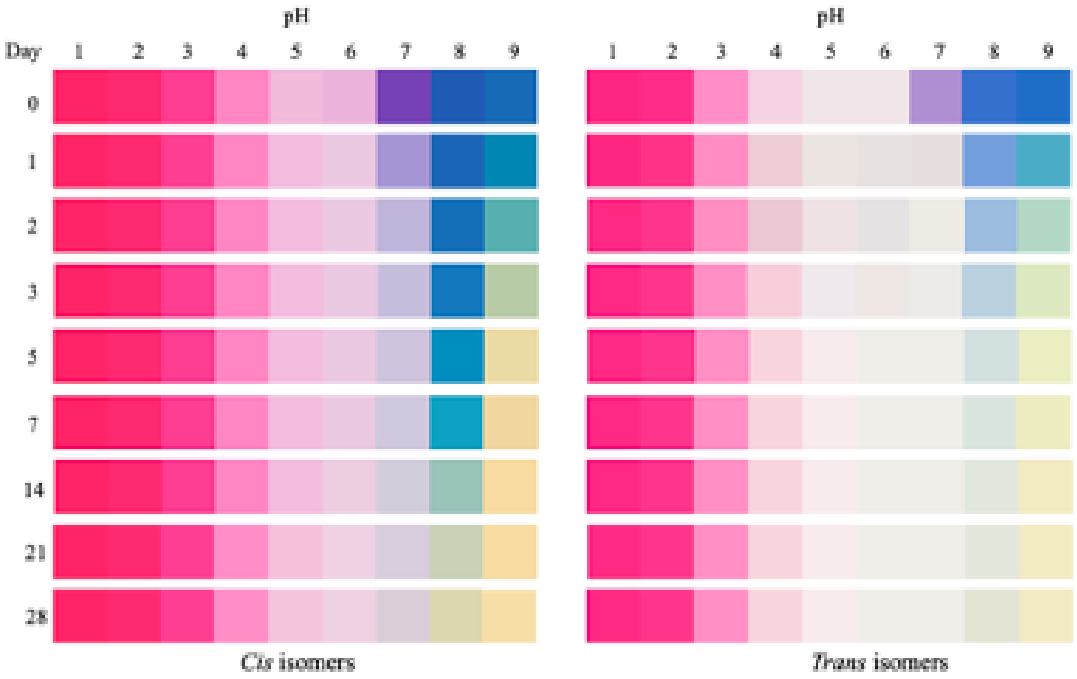

Figure. Color swatches of *cis* and *trans* cyanidin-3-*p*-coumaroyl-sambubioside-5-glucoside isolates in pH 1-9 buffers during storage.

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O1.6

Photochemical cyclization of stilbenes isolated from Norway spruce root bark

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MAIN CONCLUSION

Natural stilbenes were extracted from the bark of Norway spruce roots. Photochemical stabilities of the extrated stilbenes were studied. Stilbenes were shown to be unstable under fluorescent and UV light. Trans-stilbenes rapidly isomerized to cis-stilbenes upon UV irradiation. Under extended exposure cis-stilbenes cyclized to phenanthrene structures which were elucidated by MS and NMR spectrometry. This is the first time that phenanthrene derivatives are found from stilbenes of Norway spruce.

INTRODUCTION

Polyphenolic stilbene compounds exist in the bark of Norway spruce, and provide protection against ultraviolet(UV)light and fungal pathogens or other microorganisms. Stilbenes have potential antioxidative properties against pathogens. Thus stilbenes as secondary metabolites are important natural compounds in the tree defense mechanism [1].

Stilbenes may exist as the trans and cis stereoisomeric forms, but due to the higher thermodynamical stability of the trans isomers, they exist naturally as trans isomers. Välimaa [2] recently reported that stilbenes from spruce bark showed increased radical scavenging activity after UVA-modification. This indicates that the stilbenes have potential biological properties. However there is still some lack of information on the structure of certain stilbene compounds which are formed after photoisomerization. Resveratrol is one of the derivatives that has been studied. The trans-resveratrol is fully photoisomerized to cis-resveratrol at UV light 366nm. This result was also obtained by Montsko et al [3] and Francioso et al [4] when analyzing the structures in the photoisomerization. Both groups suggested possible cyclisation reaction based on the UV and MS spectra data results. However the characterization of ultimate compounds after UV irradiation still need to be done by using some other analytical methods like NMR. This could be more meaningful in future studies because stilbenes showed potential biological properties against fungal pathogens or microorganisms under UV light conditions. In this study, we mainly focused on other stilbenes derivatives namely astringin and isorhapontin and their aglucones piceatannol and isorhapontigenin. We isolated stilbenes and did stability studies. We characterized the new compounds by spectroscopic techniques (UV, MS, NMR). We report in detail about the photoisomerization of astringin, isorhapontin, piceatannol and isorhapontigenen which are extracted from the bark Norway spruce roots

MATERIALS & METHODS

1. Bark: Norway spruce root samples from Finnish forest. Stilbenes were sequentially extracted of the powdered bark. Hydrophobic constituents with hexane and hydrophilic constituents then using ASE 350 in 2 steps by hexane and then C_2H_5OH: H_2O (95:5 V/V). The crude extracts: purified by an XAD-7HP column. Finally the stilbene glucosides: extracted by C_2H_5OH-H_2O.
2. HPLC-DAD for separation of extracted stilbenes: A Model 1100 series LC H-P system. Agilent 1100 series diode array detector coupled to H-P Chem Station. Xbridge-C18 column with H_2O and MeOH mobile phase.
3. Isolation of stilbenes by preparative HPLC Waters 600 HPLC instrument on a Waters Xbridge-C18: preparative isolation.

4. HPLC-DAD/ESI-MS and HPLC-DAD/ESI-MS/MS analyses Negative ion HPLC-DAD/ESI-MS method for the identification of stilbenes.
5. The UV stability studies: UV lamp/366 nm
6. HRMS ESI-TOF-MS in the negative ion mode: stilbene glucosides and HRMS EI-MS stilbene aglucones. 500MHz and 300MHz NMR: new cmpds.

RESULTS & DISCUSSION

1. Stability assessments were conducted under different conditions.
1.1 Stability of stilbenes in solution (dissolved in MeOH).
The solutions were monitored for 2 weeks under light protected and light unprotected conditions. Trans-piceid, trans-resveratrol and trans astringin were unstable under light unprotected conditions, whereas trans isorhapontigenin and trans-isorhapontin were stable. This might be due to the MeO substituent in the isorhapontin structure and its aglucone being sterically hindered from isomerization.
1.2 Stability of stilbenes in solid crude extract: they are stable under light unprotected conditions.
Our results are in agreement with the other studies.
1.3 UV stability of stilbenes: stilbenes solutions were highly sensitive to UV irradiation.
Trans-stilbenes were isomerized to cis-stilbenes after 10-30 min of UV exposure. With extended exposure time, cis-stilbenes started to form other new compounds: loss of 2 Daltons (GC-MS).
2. Isolation and identification of the new compounds formed during UV irradiation: The new compounds were fractionated by preparative HPLC and then identified by NMR and MS. The NMR & MS data confirmed our hypothesis that stilbenes lost two hydrogens under a longer time of irradiation.
3. Structural elucidation of astringin and isorhapontin: 1H NMR and 2D NMR spectra clearly showed the absence of alkene H and ^{13}C signals found in the trans- and cis-stilbenes. Instead the new signals appeared in the aromatic region and showed couplings characteristics of aromatic protons. According to our further analysis, we confirmed that trans-astringin and trans-isorhapontin firstly undergo photoisomerization to cis-astringin and cis-isorhapontin and are then cyclized to phenanthrene molecules by the loss of two hydrogens. Our study confirmed that the new compounds that had lost two Dalton mass units are phenanthrene molecules. We are the first to report of phenanthrenes formed from Norway spruce stilbenes under light irradiation.

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O1.7

A reaction mechanism of photo-oxidation process of catechin in relation to its bactericidal activity

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MAIN CONCLUSION

Photoirradiation to (+)-catechin induces oxidation of (+)-catechin and reduction of dissolved oxygen resulting in generation of hydrogen peroxide (H_2O_2). The H_2O_2 is then homolytically cleaved to hydroxyl radical (·OH) which shows bactericidal activity. (+)-Catechin is finally converted into two intramolecular cyclization products that are different from the quinone structure of the B-ring which is reported previously as a product of autoxidation and enzymatic oxidation of catechin.

INTRODUCTION

It is well known that polyphenolic compounds have the antioxidative activity. In addition, polyphenolic compounds also have the prooxidative activity, which is the potential to promote oxidation. The prooxidative action of polyphenols is mediated by the reactive oxygen species (ROS) which are generated by the autoxidation of phenolic hydroxyl groups. A typical example of their prooxidative activity is the antibacterial activity of catechins, which is mediated by H_2O_2 generated from the autoxidation. It has been proposed that autoxidation and enzymatic oxidation of catechins lead to the quinone structure formation of the B-ring with generation of H_2O_2 [1].

Novel disinfection techniques using artificially generated ·OHs have been developed in our laboratory. In this method, ·OH is generated by photolysis of H_2O_2, and it kills the pathogenic bacteria by causing the oxidative damage to microbial cellular components such as cell membrane, nucleic acids, and subcellular organelle. Because of these backgrounds, we combined the prooxidative activity of polyphenols and photolysis of H_2O_2, and reported as a novel antimicrobial technique utilizing ·OH generated by photo-oxidation of polyphenols, such as flavan-3-ols [2]. That is, exposing an aqueous solution of polyphenols to blue light leads to the generation of H_2O_2, which is in turn homolytically cleaved to ·OH. The resultant ·OH causes oxidative damage leading to bacterial death. However, final products of photo-irradiated flavan-3-ols are unclarified. In the present study, we used (+)-catechin as an authentic flavan-3-ol, and aimed to analyze the photo-oxidation process along with ROS generation and resultant antimicrobial activity.

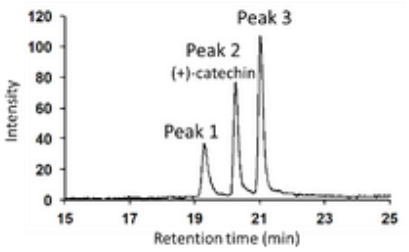
MATERIALS & METHODS

(+)-Catechin aqueous solution at a concentration of 1 mg/mL was irradiated with LED at a wavelength of 400 nm. Generation of H_2O_2 and ·OH was analyzed using a colorimetric method and electron spin resonance analysis, respectively. The substances derived from photo-oxidation of (+)-catechin were mass-analyzed using liquid chromatography-high-resolution-electrospray ionization-mass spectroscopy (LC-HRESI-MS). Furthermore, nuclear magnetic resonance (NMR) analysis was performed to determine the molecular structures of the final products. Bactericidal activity of photo-irradiated (+)-catechin was evaluated using *Staphylococcus aureus*. The bacterial suspension containing (+)-catechin was photo-irradiated, and viable bacterial counts were enumerated by a

colony-counting method. To determine whether the bactericidal effect of photo-irradiated (+)-catechin is caused by ·OH or not, thiourea was added to the reaction system as a ·OH scavenger.

RESULTS & DISCUSSION

Yield of H_2O_2 and ·OH increased in an irradiation-time dependent manner. These results suggest that photo-irradiated (+)-catechin reduces dissolved oxygen to H_2O_2, then the H_2O_2 is homolytically cleaved to ·OH. LC-HRESI-MS showed that photo-irradiation resulted in appearance of two peaks of a substance with molecular formula $C_{15}H_{12}O_6$ dependently on irradiation time (Fig. 1). NMR indicated that the final two products were generated by intramolecular cyclization and they were possibly tautomers of each other. To reveal their tautomerization, an additional analysis was performed. The peak 1 and 3 were isolated and stored 4℃ for 7 days followed by high performance liquid chromatography (HPLC) analysis. Since both samples showed the peak 1 and 3, it is suggested that they are keto-enol tautomers. In conclusion, unlike the autoxidation and enzymatic oxidation of (+)-catechin in which the putative final product has the quinone structure of the B-ring, photo-oxidation induces different structures as shown in Fig. 2.



peak	retention time (min)	m/z	molecular formula	ppm error
1	19.26	289.0718	$C_{15}H_{13}O_6$	3.9271
2	20.23	291.0876	$C_{15}H_{15}O_6$	4.4149
3	21.01	289.0727	$C_{15}H_{13}O_6$	4.6189

Figure 1

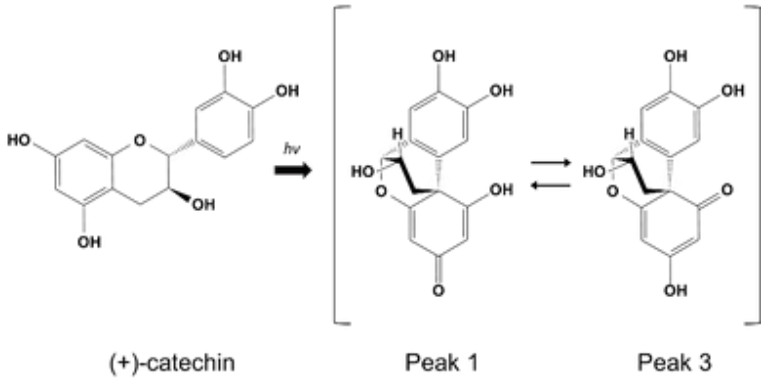


Figure 2

Figure 1. Representative LC chromatograms of (+)-catechin solution, which was photo-irradiated for 4 hours, with information of HRMS analysis. LC and HRMS stand for liquid chromatography and high-resolution mass spectrometry, respectively.

Figure 2. Chemical structures of the final products generated by photoirradiation of (+)-catechin.

Viable bacterial counts also decreased in an irradiation-time dependent manner, and over 5-log reduction was observed within 20 min. When thiourea was added to the reaction system, the antimicrobial activity was attenuated, indicating that the bactericidal effect would be attributable to ·OH rather than the final products of photo-oxidized (+)-catechin.

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O1.8

NMR structural determination of (+)-catechin-laccase reaction dimeric products: potential oxidation markers in grapes and wines

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MAIN CONCLUSION

- Eight different dimeric markers resulting from the reaction between (+)-catechin and laccase from Trametes vesicolor were identified by UPLC-MS²
- Different enzymes have been investigated: Polyphenoloxidase extracted from grapes, laccase from *Trametes vesicolor* and laccase from *Brotytis cinerea*. They all lead to the same products based on UV-MS chromatograms.
- Five of the eight dimeric markers were unambiguously characterized by 1D and 2D NMR spectroscopy.

INTRODUCTION

In Enology, polyphenols are primary targets to oxidation which their impact organoleptic properties. Wine oxygen uptakes will influence their aging and remain a long-standing issue for winemakers.

Two types of polyphenol oxidation can occur in wine: enzymatic and chemical oxidation. Enzymatic oxidation mainly occurs in grape must and is usually the one responsible for browning. This enzymatic activity is catalyzed by oxidoreductases: polyphenol oxidase/PPO (naturally present in grapes) and laccase (produced by molds).

In wine, benzoquinones produced by oxidation (PPO or laccase) can easily undergo further reactions depending on their redox properties and electronic affinities. They can act either as electrophiles and react with amino derivatives, or as oxidants and react with other polyphenolic substrates. Depending on their chemical conformation (quinone or semi-quinone), benzoquinones can lead to different oxidation reaction products. At neutral pH, (+)-catechin will be oxidized in quinone and lead to the formation of six possible isomers between B-ring (position C2'; C5' or C6') of the upper catechin unit and A-ring of lower unit (position C6 or C8). Dehydrodicatechins are well-known products of this coupling [1]. Under acidic conditions, catechin B-ring can also have a semi-quinone conformation (position OH3' or OH4') and can lead to the formation of four possible isomers [2] by connecting to the A-ring of a lower catechin unit (position C6 or C8).

The aim of the present research was first to compare (+)-catechin oxidation products catalyzed by three oxydoreductases: PPO extracted from grapes, laccase from the fungus *Botrytis cinerea* present in Botrityzed sweet wines and laccase from *Trametes versicolor*. Second, laccase from *Trametes versicolor* was used as a biological reagent to hemisynthesize and to obtain (+)-catechin dimeric oxidation products. The products were characterized by both UPLC-MS and NMR spectroscopy.

MATERIALS & METHODS

A hemi-synthesis procedure was designed using laccase from *Trametes versicolor* and (+)-catechin in wine model solution pH 3.6. Reaction was then stopped and purified in two steps: a flash liquid chromatography (Puriflash diol 50 µm f0025 column) followed by a semi-preparative chromatography system (C18 Microsorb 3 µm 21.2 × 250 mm). Eight distinct fractions were collected, corresponding to pure UPLC signals at 280 nm.

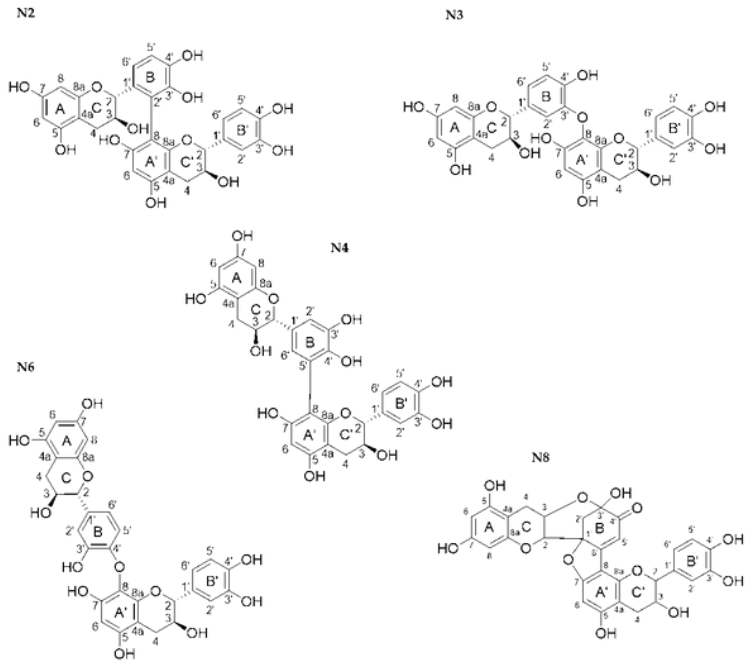
For NMR analysis, the lyophilized samples were dissolved in acetone-d_6 containing a trace of cadmium nitrate ($Cd(NO_3)_2$). All NMR experiments were conducted on an Agilent DD2 500 MHz

spectrometer (Agilent Technologies, Santa Clara, CA, U.S.A.). 1D and 2D NMR experiments were obtained using classical pulse sequences and spectra analyzed using Mnova software. ^{1}H DOSY measurements were acquiered using a DgcsteSL pulse sequence and 2D DOSY spectra were constructed from the peak height measurement using VNMRJ4.2 software process as previously described [3].

RESULTS & DISCUSSION

Oxidation of (+)-catechin in presence of Laccase from *Trametes vesicolor* revealed the presence of 8 dimeric compounds. The electrospray mass spectra in positive mode gave ion peaks $[M+H]^+$ at m/z 579 for N1 to N6, hypothetically corresponding to a single bound between two catechin units, and $[M+H]^+$ at m/z 577 for N7 and N8, hypothetically corresponding to the formation of a supplementary bound. Three different enzymes (grape PPO, B.cinerea and T. versicolor laccases) were used at pH 3.6 with catechin in model wine solution. For each of the eight compounds, the retention times were almost identical with the different enzymes, and identical m/z were determined with MS analysis. These results support the hypothesis that same compounds were obtained for each experiment, with possible products formed similar to those found and partially characterized by Guyot et al. [2]

Structural characterization of such dimers *(fig 1)* can be obtained by NMR spectroscopy. If the linkage is of ether type, the attribution of the hydroxyl signal protons is mandatory to determine the exact linkage position. This may also be true in the case of C-C bonds. However, even in aprotic solvent, hydroxyl protons of polyphenols often appear as broad signals from which no structural information can be obtained. This issue was addressed by the addition of traces of $Cd(NO_3)_2$ in the sample solutions giving rise to sharp and resolved phenol signals in the low-field region of the spectrum. Furthermore, a decrease of the acquisition temperature from 25 to 15°C lead to a downfield shift of exchangeable protons. This effect allowed to resolve some overlapped phenol and aliphatic OH signals making their identification more obvious. Thanks to this methodology, the linkage positions between units of six of these dimers were formally determined using both 1D and 2D NMR experiments. The assessment of the hydroxylation patterns in both A, B and C rings was performed and all ^{1}H and ^{13}C chemical shifts were assigned.



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O1.9

On laccase-catalyzed polymerization of alkaline lignin fractions in aqueous alkaline solution

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MAIN CONCLUSION

The correlation between alkaline lignin (AL) structural characteristics and performance of alkaliphilic-laccase-catalyzed lignin polymerization was established based on the molar-mass-dependent differences of the initial lignin fractions. The laccase-treated lignin exhibited higher glass transition temperature (Tg) values (all around 160 °C) than the initial counterparts, which endows the advantages of thermal stability and chemical tolerance to the laccase-polymerized lignins.

INTRODUCTION

Oxidative enzymes, i.e., laccase and peroxidase, have long been proposed as a promising biological green tool for lignin substrate oxidation, depolymerization, and polymerization.[1] One major obstacle for valorizing lignin with aid of enzymatic catalysis is the limited efficient pH range of most of the industrially available laccases (mainly fungal-derived), usually retaining their activity under acidic reaction conditions, where lignin is hard to dissolve. In comparison to widely studied fungal laccases, the bacterial-derived laccase is known to have an extended pH working range and higher thermostability and robustness, which makes it attract significant research attention. Recently, a new industrial genetically evolved laccase of bacterial origin with a trademark of MetZyme® has been reported to efficiently oxidize the hardwood mid acidolysis lignin under highly alkaline conditions (pH 10.5).[2] The effectiveness of the MetZyme® treatment on lignin oxidation and depolymerization, more specifically oxidative demethylation has been confirmed, which highlights this bacterial-derived laccase with great potential in upgrading lignin by increasing the number of functional hydroxyls. Noteworthy, in the valorization of technical lignins, while substantial research is on the decomposition of lignin to aromatic monomers and oligomers, the main industrial interests and developments are in the direct use and application of polymeric lignin. Considering the unique advantages of MetZyme® alkaline laccase and aiming at investigating the correlations between lignin structural characteristics and laccase-assisted lignin oxidation/polymerization performance. Hardwood and softwood AL were both subjected to a sequential solvent fractionation, in order to derive lignin fractions with well-defined characteristics in terms of the molar mass and lignin macromolecular structure, for use in the enzymatic oxidation and polymerization experiment under lignin soluble state (pH 10).

MATERIALS & METHODS

Birch and spruce alkaline lignin (AL) were supplied by CH-Bioforce Oy (Espoo, Finland). They were produced through a pressurized hot water extraction followed by an alkaline extraction process under oxygen-starved conditions. Birch or spruce AL was subdivided into three alcohol-soluble lignin fractions by sequential dissolution in isopropyl alcohol (*i*-PrOH), ethanol (EtOH), and methanol (MeOH).[3] The industrial bacterial-derived alkaliphilic laccase (MetZyme®) was supplied by MetGen Oy (Kaarina, Finland). The protein concentration was determined as 9.7 mg mL^{-1}. The enzyme activity was determined as 357 U mL^{-1}. The oxidation experiment of lignin (10 mg mL^{-1}) by laccase (1 U mg^{-1} lignin) was performed in sodium hydroxide solution (NaOH, pH 10) at 39 °C with ambient air (O_2) circulation under gentle stirring (400 rpm). The laccase-treated lignin was isolated from the reaction medium by acid precipitation using hydrochloric acid (HCl, 2M) to pH 2.5 and was centrifuged (8000 rpm, 10 min).

RESULTS & DISCUSSION

Solvent fractionation strategy provides two series of lignin fractions with narrow molar mass dispersity ($Đ_M$ 1.2–1.4) and gradually increasing molar mass as well as lignin condensation degree from birch and spruce AL. In addition, the lowest molar mass lignin fraction (*i*-PrOH-soluble fractions) is characterized by a high amount of phenolic-OH. It has to be noted that the aryl-vinyl (stilbene and aryl enol ether) moieties were detected in the HSQC NMR spectra of spruce AL and its fractions in appreciable amounts, whereas these structures were not detectable in birch AL. The alkaliphilic-laccase-assisted medium significantly catalyzed the oxidation and polymerization of birch AL in mild conditions (**Figure 1a**). Compared with birch AL fractions, spruce AL fractions exhibited rather prominent oxidation of aryl-vinyl moieties even without the laccase mediation, leading to less pronounced molecular polymerization (**Figure 1b**). Coupling and resonance of phenoxy radicals, cleavage of α-aryl ether interunit linkages, and electron-donating effect of methoxyl (-OCH_3) groups substantially induced the lignin-lignin condensation/polymerization at aromatic rings. In addition, lignin demethylation and benzylic oxidation also occurred during the course of laccase treatment, highlighting the complexity of chemoenzymatic interactions. The correlation between alkaline lignin (AL) structural characteristics and performance of alkaliphilic-laccase-catalyzed lignin polymerization was established based on molar mass, amount of phenolic-OH, and lignin condensation degree. For instance, the polymerization degree of lignin fractions increased with decreasing molar mass and condensation degree as well as with increasing amount of phenolic-OH groups in the initial AL fractions.

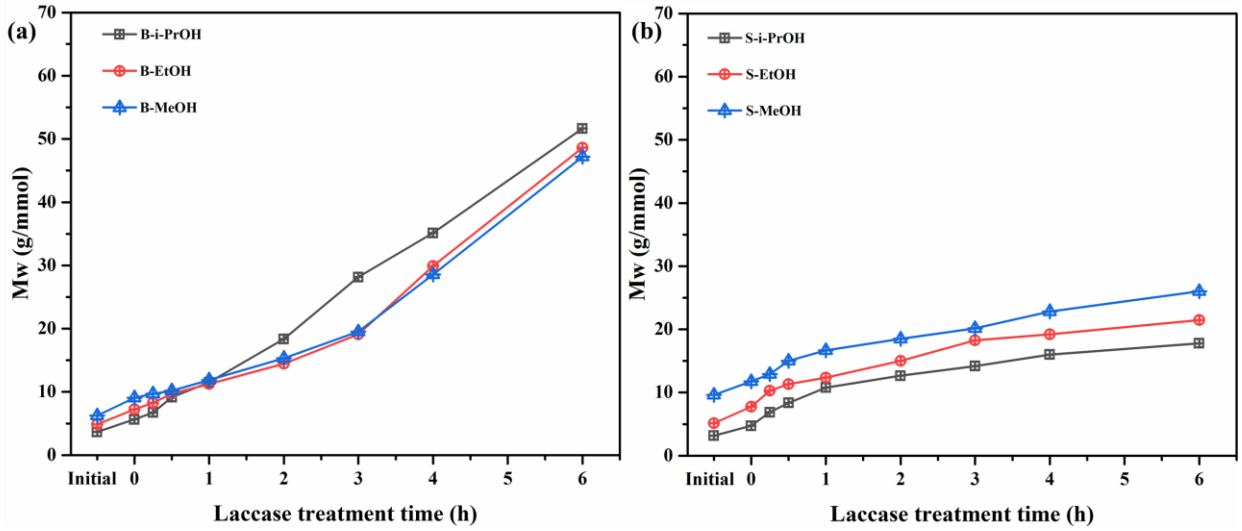

Figure 1. Weight-average molar mass (M_w) of laccase-treated AL (a) and spruce AL (b) fractions, measured by size exclusion chromatography.

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O1.10

Orthogonal annulation strategy, enabling an efficient assembly of doubly-linked oligoflavans

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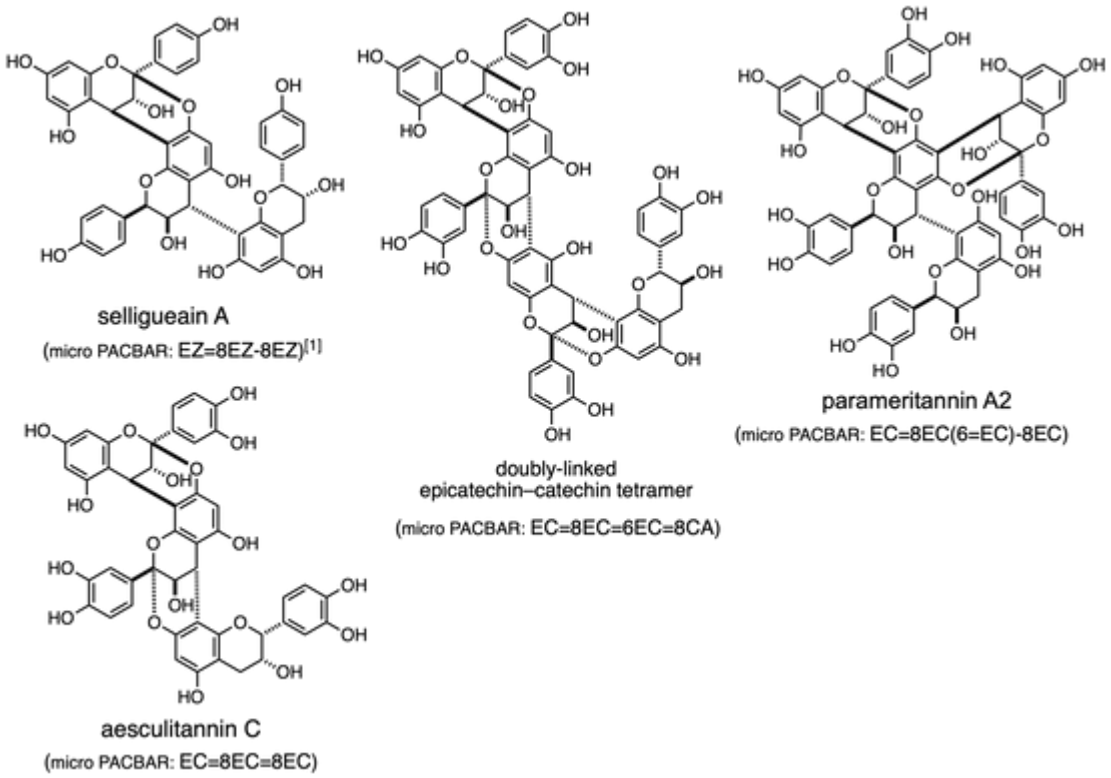
MAIN CONCLUSION

This paper describes our efforts to synthesize doubly-linked oligomeric proanthocyanidins, e.g. selligueain A, cinnamtanint B1, aesculitannin C, parameritannin A2, and doubly-linked epicatechin–catechin tetramer.

INTRODUCTION

The oligomeric proanthocyanidins (OPAs), condensed tannins, constitute a huge natural product library composed of diverse polyphenols. Although potential bioactivities are expected, a majority of compounds are left unexplored, as the natural plant extracts are often intractable mixture of closely-related compounds, rendering isolation difficult even with modern separation techniques. As with the well-known flavan oligomers formed by a linear array of singly-linked (B-type) interflavan connectivity, an increasing number of oligomeric PAs, having doubly-linked (A-type) interflavan connectivity, has been identified. Although these compounds are attractive in terms of potential activities associated with the unique molecular scaffolds, detailed studies have been limited by the difficulty in obtaining pure samples, calling for the availability by organic synthesis.



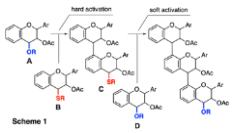
MATERIALS & METHODS

In planning the synthesis of complex oligomeric proanthocyanidins, we investigated two general synthetic methods.

RESULTS & DISCUSSION

#1 Orthogonal activation strategy

For the iterative formation of an interflavan linkage, we examined the orthogonal activation method (Scheme 1). Two distinct monomer units, *i.e.* C(4)-oxy and C(4)-thio flavan units, **A** and **B**, are selectively activated to execute reiterative union of flavan units. We found that an alkoxy- and an arylthio-substituted substrates, **A** and **B**, as a set of substrates amenable to selective activation under mutually orthogonal conditions. The oxy group in **A** is smoothly activated under hard acidic conditions, while the thio group in **B** is unaffected, and vice versa, soft Lewis-acidic conditions activates the thio group on **C**, the oxy group in **D** remains intact.

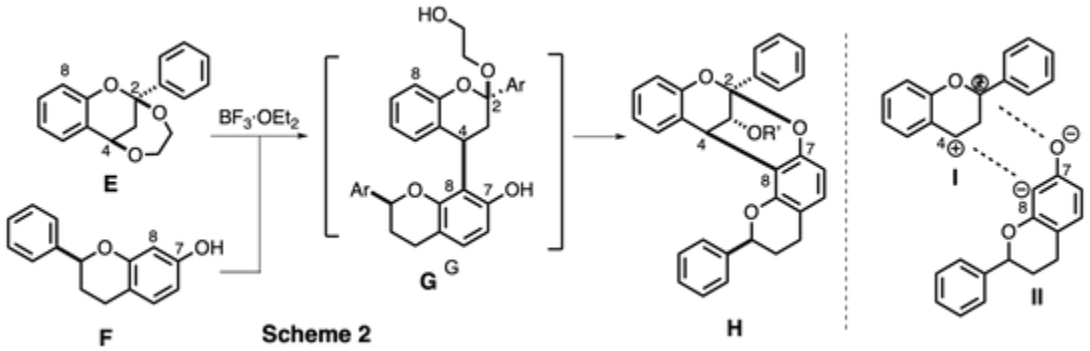


Scheme 1

#2 Flavan annulation

We also launched a study to develop effective means of constructing the A-type double linkages (Scheme 2).[2] The key is to use the 2,4-ethylenedioxy flavan **E**, which served as an equivalent of dication synthon **I** and phenol **F** as that of a dianion synthon **II**.

Among two oxy-leaving groups in **E**, the one at C4 is initially activated by Lewis acid, generating the C4-cation, that is captured by the C8 nucleophilic center in **F** to form the intermediate **G** with a rigorous regioselectivity. Subsequent activation at C2 allows the second C2–O-C7 linkage, giving the annulation product **H**.



Scheme 2

In this paper, we will discuss applicability of these methods for accessing complex oligomeric proanthocyanidins.

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O1.11

Enzymatic synthesis, structures, interactions with saliva proteins and quantification in juices of a series of dehydrodicaffeoylquinic acids, one of the main classes of oxidation products in apple-based beverages

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MAIN CONCLUSION

Dehydrodicaffeoylquinic acids (DDCQAs) are among the main phenolic products resulting from enzymatic oxidation occurring during apple processing. Accounting for dozen milligrams per liters in some apple juices, they present original chemical structures including benzodioxane, dihydronaphtalene, dihydrobenzofuran or dicatechol patterns. Interestingly, those oxidation products exhibited unusual properties regarding their ability to aggregate salivary proteins.

INTRODUCTION

Mainly located in the flesh and in the skin, apples polyphenols show a great diversity of molecules belonging to the classes of hydroxycinnamic acids, catechins, procyanidins oligomers and polymers, dihydrochalcones, flavonols and anthocyanins. When apple are processed into juices and ciders, a great diversity of newly formed polyphenolic molecules is generated by enzymatic oxidation. Some of those oxidation products contribute to the colour of the juice. However, a great majority are colourless and their detailed structures, their real concentrations in the juices or their contribution to sensory, antioxidant and other nutritional properties in the final products are still scarcely known. Previously, we showed that newly-formed products resulting from enzymatic oxidation of 5′-O-caffeoylquinic acid (CQA), also commonly known as chlorogenic acid, still presented high antioxidant activity *in vitro* [1]. However, we have no information regarding their possible involvement in the bitter taste and the sensation of astringency that may be related to the presence of polyphenols in fruit-derived beverages. We remember that, in this particular case, astringency is a consequence of the precipitation of salivary proteins due to the tanning properties of polyphenols. We present here our main recent results related to the oxidation products of CQA focusing on their UV, MS and NMR structural characterisation, their LC-MS quantification in a panel of commercial juices and their capacity to aggregate salivary proteins.

MATERIALS & METHODS

Synthesis, purification and structural analysis: DDCQAs were synthesized from CQA in model solution using a crude apple polyphenoloxidase (PPO) extract. Ten DDCQAs were purified by centrifugal partition chromatography and RP18 HPLC at the semi-preparative scale [2]. Their complete structural elucidation was achieved by MS and 1D and 2D NMR ^{1}H and ^{13}C.

Quantification in apple juices: a new LC-UV-MS method was developped using the purified standards to quantify native polyphenols, the series of DDCQAs and a series of CQA-epicatechin dimers in 45 commercial and experimental apple juices.

Aggregation to salivary proteins (SP): a mixture of soluble DDCQAs was incubated with acidic saliva. After centrifugation, the supernatant was analyse by RP18 HPLC-UV and compared to the initial mixture in order to quantify the remaining SP and DDCQAs [3].

RESULTS & DISCUSSION

A series of ten dehydrodicaffeoylquinic acids (DDCQAs) were synthesized by enzymatic oxidation of a model solution of 5′-O-Caffeoylquinic acid (CQA), using a crude apple polyphenoloxidase extract. Then, they were purified by centrifugal partition chromatography and RP-18 HPLC at the semi-preparative scale [2]. Their complete structural elucidation was achieved by 1D and 2D NMR ^{1}H and ^{13}C revealing original dihydrobenzofuran, benzodioxan and dihydronaphtalen polyphenolic skeletons. In addition, for the first time a new symmetric structure exhibiting two free catechol groups was identified.

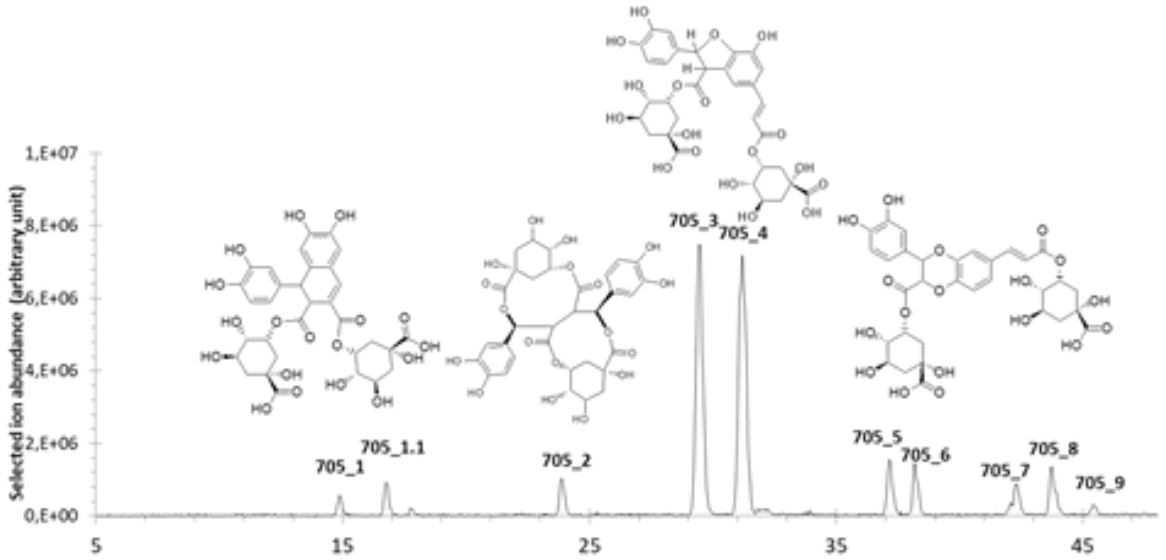

Figure - Extracted ion (m/z) LC-MS chromatogram revealing the series of DDCQAs oxidation products

The capacity of DDCQAs to aggregate salivary proteins (SP) was studied using a precipitation method associated to HPLC analysis of the supernatant [3]. Experiments were conducted in model solutions in order to vary at the same time the concentrations and the ratio of both polyphenols and saliva proteins in the medium. Results showed that interactions between salivary proteins (SP) and DDCQAs are highly impacted by the ratio SP/DDCQAs, and in the tested conditions, we hypothesize that a low number of DDCQAs is necessary to significantly precipitate SP. Moreover, a specific interaction of DDCQAs with cystatins and statherin/PB was observed during these experiments. Some of these observations were confirmed by fluorescence quenching analysis. Surprinsingly, DDCQAs exhibited weak interactions with PRPs (Proline Rich Proteins) but interactions were much more significant with statherins/P-B peptide and cystatins.

Finally, polyphenol oxidation products, including the DDCQAs series completed by some epicatechin-caffeoylquinic acid dimers, were quantified for the first time in real juices by developing a new LC-MS method. Its application to 32 commercial, 13 experimental and 9 craft apple juices revealed that these families of compounds can reach several dozen mg/L apple juice contributing up to 14 % of total polyphenols.

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O1.12

Revisiting the oxidative coupling of catechol-type flavan-3- ols: dimeric and trimeric products of (−)-epicatechin with polyphenol oxidase

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MAIN CONCLUSION

Enzymatic oxidation products of (−)-epicatechin (**1**) were investigated, and six dimers (**2**–**7**) and two trimers (**8**, **9**), including two new compounds (**5**, **7**), were afforded. Structures of new compounds were elucidated on the basis of various spectroscopic data and computational calculations of ECD and NMR data. Because dehydrodi(epi)catechins were found in several fruits and plant-derived foods, **7**–**9** are also expected to be contained in such food products.

INTRODUCTION

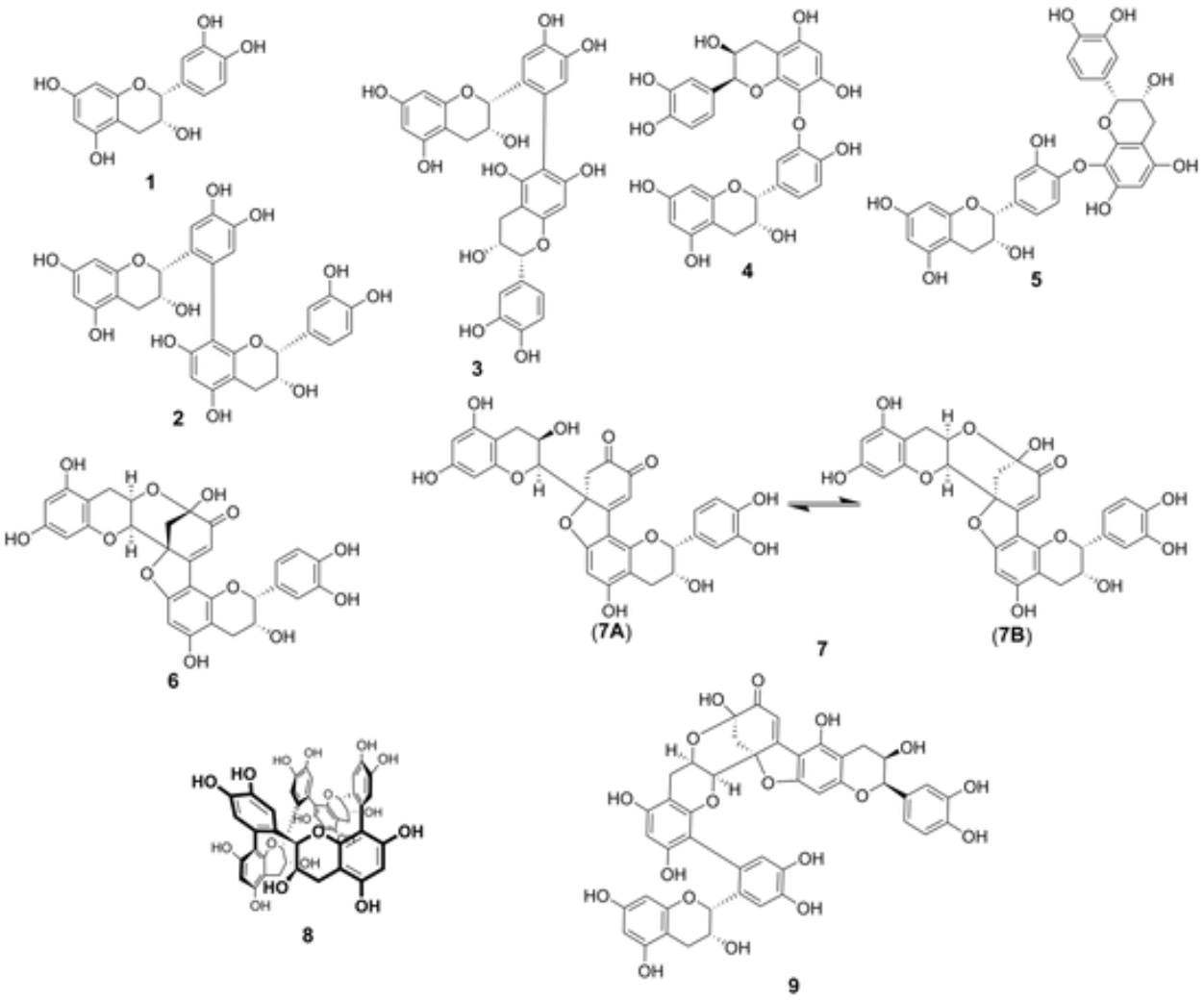
(−)-Epicatechin (**1**), one of the major tea flavan-3-ols with a catechol ring, is enzymatically oxidized during black tea production to afford dehydrodiepicatechins with a C–C bond between A- and B-rings. Dehydrodi(epi)catechins and their related oligomers are also contained in grape pomace, cocoa, and beer. Most oxidation products of flavan-3-ols chemically assigned were dimers and trimers, and the precise structures of oligomers are still unknown. Therefore, it becomes necessary to reveal the details of the oxidative coupling reactions of flavan-3-ols to clarify the structures of oligomeric products. In this study, the oxidation products of **1** with polyphenol oxidase were investigated in detail.[1,2]

MATERIALS & METHODS

Japanese pear fruits (*Pyrus pyrifolia*) were homogenized with H_2O and filtered. The filtrate was mixed with an aqueous solution of (−)-epicatechin (**1**) and vigorously stirred for 1 h at room temperature. The reaction mixture was filtered and separated using Diaion HP20SS, Sephadex LH-20, Chromatorex ODS, and Cosmosil 40C_{18}-PREP to afford **2**–**9**. Their structures were assigned on the basis of various spectroscopic data including 1D, 2D NMR, and computational calculations of ECD and NMR data.

RESULTS & DISCUSSION

An aqueous solution of (−)-epicatechin (**1**) was stirred vigorously with a Japanese pear fruit homogenate, which exhibited a strong polyphenol oxidase activity, to afford six dimers (**2**–**7**) and two trimers (**8**, **9**), two of which (**5**, **7**) were new compounds. Compound **7** was found to be a stereoisomer of dehydrodiepicatechin A (**6**) from NMR and MS analysis; however, **7** was present as an equilibrium mixture of α-dicarbonyl form (**7A**) and hemiacetal form (**7B**). Compound **8** was an C_3 symmetric trimer of **1** reported as an oxidation product with $CuCl_2$; however, only the 2D structure was proposed.[3] Its stereostructure was elucidated as (a*R*,a*R*,a*R*) using computational calculations of ECD spectra and NMR chemical shifts. Compound **9** was previously isolated as an enzymatic oxidation product of **1** by our group; however, its substituent positions and stereostructure had not been assigned.[4] In addition, its NMR spectroscopic data matched with those of dehydrotricatechin A reported as a $K_3[Fe(CN)_6]$-mediated oxidation product of **1**.[3] Therefore, the structure of **9** was reassigned in a similar manner. Plausible dimerization and trimerization pathways of **1** were discussed.



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O1.13

A new biosynthetic intermediate of cyanidin 3-*O*-glucoside in black soybean seed coat

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MAIN CONCLUSION

A new biosynthetic pathway of cyanidin 3-*O*-glucoside (Cy3G) in the seed coat of black soybean, *Glycine max*is reported. In both usual maturation in pod and quick color change upon removal of the shell, the same chemical changes as a decrease in 5,7,3′,4′-tetrahydroxyflav-2-en-3-ol 3-*O*-glucoside (2F3G) along with a simultaneous increase in Cy3G were observed. This result strongly suggests a 3-*O*-glucosylation reaction occurs prior to oxidation to the corresponding anthocyanidin.

INTRODUCTION

Anthocyanins are plant pigments responsible for the colors of flowers, fruits, vegetables, and roots. Among them Cy3G is one of the simplest and most widely distributed pigments in nature; seed coat color of the black soybean, *G. max*,is also due to Cy3G [1].

The biosynthetic pathway of Cy3G has been well studied, and almost all genes and enzymes involved have been identified. After the reaction of Phenylalanine ammonia-lyase (PAL), several redox enzymes are then involved in its conversion and the first colored compound, cyanidin, was produced by anthocyanidin synthase (ANS), for which*cis*-leucocyanidin (2*R*, 3*S*, 4*S*-leucocyanidin) is presumed to be the substrate, then, 3-*O*-glucosyltransferase (3GT) worked to give Cy3G. However, catechin was reported to form cyanidin upon treatment with ANS. Therefore, a re-examination of the biosynthetic pathway of Cy3G and the role of ANS should be done.

We are interested in the organic synthesis of Cy3G, also, and found that the oxidation of a *cis*-leucocyanidin derivative did not give Cy3G, but 3-flavenol derivative gave Cy3G by an air oxidation in a good yield [2]. Furthermore, we showed that the Clemmensen-type reduction of flavonol 3-*O*-glycoside gave a mixture of 2- and 3-flavenol compounds; these compounds are then oxidized by air to yield Cy3G [3]. At the same period, Fukami et al. reported the presence of 2F3Gin the immature green-colored seed coat of black soybean [4].

The green-colored seed matures to a black color over an approximately two-month, however, an accelerated change in the color of the beans from green to black was found only within 24 h in normal pods upon removal of the shell and exposed to air and light. This phenomenon also indicates that a precursor to Cy3G already stored at green colored seed coat and 2F3G should be a candidate. Then, we studied the levels of Cy3G and 2F3Gin the immature seed coats and monitored their contents during the rapid color change [5].

MATERIALS & METHODS

Black soybeans (*G. max*) cv. Iwaikuro were cultivated in Nagoya University. Rapid color change treatment was carried out by using an immature beans of green colored pod. The beans were removed from the pod and separated into four stages according to the seed coat color. Each bean was placed in a glass bottle and incubated at 25 °C under light conditions (20,000 lx) in a plant incubator. For quantitative analysis of Cy3G and 2F3G three immature beans were weighed and frozen using liq. N_2, and extracted with an acidic solvent (3% TFA-50% CH_3CN aq, 3.0 mL/g FW) for Cy3G and a neutral solvent (50% aq. CH_3CN) for 2F3G were used, respectively. Each extract was analyzed by HPLC and the obtained peak areas were used for construction of the calibration curve. During an

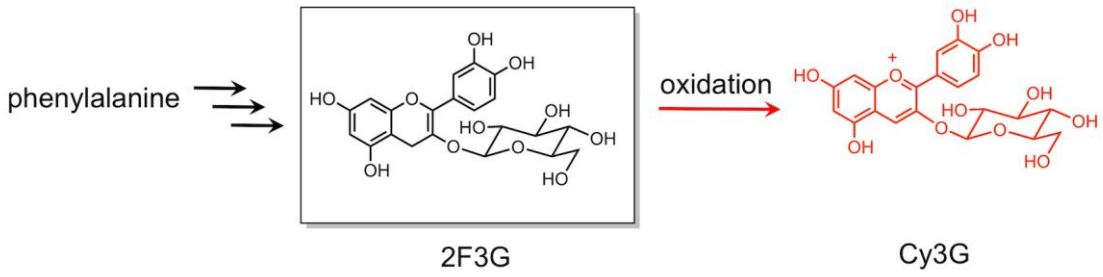
acidic extraction 2F3G converted to Cy3G, therefore, the true content of Cy3G was calculated by subtraction of the content of 2F3G from that of total Cy3G.

RESULTS & DISCUSSION

At first the contents of Cy3G and 2F3G in the beans at usual maturation stages were analyzed. In stage 1, the contents of Cy3G and 2F3G wer low, but upon maturation of the beans, Cy3G increased from 58 nmol/gFW (stage 2) to 166 nmol/gFW (stage 3) and 1200 nmol/gFW (stage 4), while the 2F3G contents were approximately 320 nmol/gFW in stage 2, 1130 nmol/gFW in stage 3, and 0 nmol/gFW in stage 4. These results suggested that the biosynthesis of 2F3G may begin around stage 2, while that of Cy3G follows.

Next, we analyzed the contents of Cy3G and 2F3G during the rapid color change outside the pod. We removed the shells of the immature beans at stages 1–3, and exposed them to air under light conditions for 12 h. The beans at stage 1 did not show any remarkable color change, but the beans at stages 2 and 3 turned black following incubation. At stage 2, the content of 2F3G changed form 305 (0 h) to 2 nmol/gFW (12 h). In contrast, the content of Cy3G increased from 65 nmol/gFW (0 h) to 415 nmol/gFW (12 h). The total amount of Cy3G and 2F3G at each incubation time were 370 and 417 nmol/gFW, respectively, indicating that the total amount was constant. These results strongly indicate that the conversion of 2F3G to Cy3G occurs in the seed coat. At stage 3, a similar phenomenon was observed. These results strongly support that conversion from 2F3G to Cy3G during the color change.

We detected the presence of 2F3G in the immature seed coat, and during the color change, a decrease in 2F3G with a simultaneous increase in Cy3G was observed. It was also found that *in vitro*2F3G was oxidized to Cy3G in the presence of air and Fe^{2+}at pH 5.0. These results indicate that 2F3G may exist in vacuoles, and that it is oxidized non-enzymatically by the ferrous ion. Based on the results, a new biosynthetic pathway to Cy3G was proposed via a 3-*O*-glucosylation reaction prior to oxidation to the corresponding anthocyanidin.



A proposed new biosynthetic pathway

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O1.14

Hydrolyzable tannins inhibit pore-forming toxin pneumolysin

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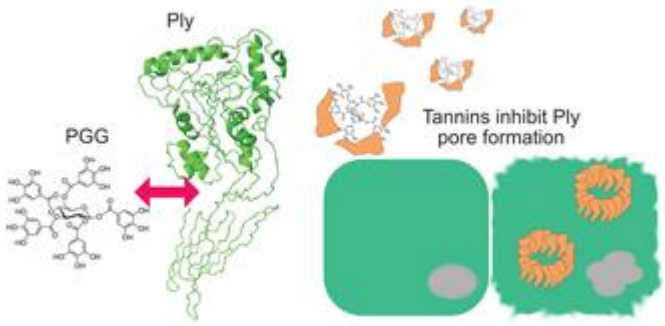
MAIN CONCLUSION

Hydrolyzable tannins pentagalloyl glucose (PGG) and Gemin A, were the strongest inhibitors of Ply cytolytic activity. The structure-activity analysis suggests that the compounds with flexibility of the galloyl groups and the formation of flexible oligomers are the most powerful Ply inhibitors. PGG was shown *in vitro* to inhibit Ply oligomerization and cytotoxicity to A549 cells. Therefore tannins are potential therapeutic compounds against *S. pneumoniae* infections (Fig 1.).



INTRODUCTION

Streptococcus pneumoniae is a major human pathogen causing pneumonia, otitis media and meningitis. Due to the emergence of *S. pneumoniae* resistance to antibiotics and rapid increase of non-vaccine serotypes, it remains a serious global health problem [1]. Therefore development of alternative non-bacteriosidic compounds is important. Compounds targeting essential virulence factors, such as pneumolysin (Ply), are promising candidates that could be used in conjunction with other antibiotics.

Pneumolysin is a 53 kDa protein of *S. pneumoniae*, belonging to the family of cholesterol-dependent cytolysins [2]. Pneumolysin binds to membrane cholesterol, is oligomerized, creates a pore onto the membrane and kills the target cell. It is composed of domains 1, 2, and 3 required for oligomerization and domain 4 responsible for host cell recognition. Ply is a highly multifunctional toxin, which in addition to its hemolytic and cytolytic activity induces a number of signaling effects and also play a role in *S. pneumoniae* transmission from host to host. Therefore it is an important target for the prevention of pneumococcal infections. Recent study performed with hydrolyzable tannins has revealed that structural features such as number of galloyl groups, flexibility, degree of oxidative coupling between the galloyls, positional isomerism, and cyclic vs acyclic glucose core were the major structural features effecting protein precipitation capacity [3].

Here, we purified 27 hydrolysable tannins and studied how they inhibit Ply cytotoxicity. The results show that the hemolysis, Ply oligomerization and cytotoxicity of Ply is inhibited by hydrolysable tannins.

MATERIALS & METHODS

The polyphenols were purified and characterized as described in Engström et al. [3]. The methods used in this study have been described in Maatsola et al. [4]. In summary, pneumolysin gene was cloned into a vector pET46EkLIC for expression and purification of 6xHis-tagged Ply. Ply hemolysis inhibition assays with tannins were performed with human erythrocytes. The released hemoglobin from erythrocytes was measured at 570 nm. The Ply precipitation assay with tannins were done in microtiter plates and the precipitation was measured at 414 nm. The hemolysis inhibition IC_{50} values and minimum concentration precipitating Ply were calculated with Origin. Modelling of Ply-PGG interaction was performed with GLIDE and NAMD2.13 programs.

Ply oligomerization assay was done by incubating erythrocytes with Ply and the oligomerization was visualized by Western blotting using anti-His antibody. Inhibition of Ply cytotoxicity to A549 cells by PGG was analysed with Cytotoxicity Detection assay (Roche).

RESULTS & DISCUSSION

Inhibition of hemolysis by hydrolyzable tannins

27 hydrolyzable tannins were tested for their inhibitory activity against pneumolysin hemolytic activity. Pentagalloylglucose (PGG), and gemin A as nanomolar inhibitors were chosen for more extensive characterization of the inhibitory properties of these compounds. Weaker inhibitors Oenothein B and Vescalagin were chosen as control compounds.

Inhibitory power of PGG and Gemin A

PGG was the strongest inhibitor of hemolysis with an IC_{50} of 18 nM. The most potent compound causing precipitation was Gemin A with the minimum concentration causing precipitation of 6 mM. The docking and energy minimization results show that PGG interacts with Glu42, Ser256, Asp257, Glu277 and Arg359 of Ply. These results suggest that the inhibitory mechanism of tannins could be based on the targeting of the domains that are required for the oligomerization.

PGG abolishes oligomerization of pneumolysin

Ply oligomerization assay was developed using erythrocytes as target cells. Ply recognizes cholesterol on the erythrocyte cell surface resulting in the membrane pore formation and cell lysis. The monomeric Ply (55 kDa) and the oligomerized Ply (2500 kDa) were detected with Western blotting. PGG was tested for its ability to inhibit Ply oligomerization by testing dilutions of PGG with Ply and the oligomerization was detected by Western blotting. The concentration that still inhibited Ply oligomerization was 30 µM.

Inhibition of Ply cytotoxicity to A549 cells by PGG

The Ply cytotoxicity to lung pneumocyte A549 cells was analyzed based on the release of cytoplasmic lactate dehydrogenase. Lower concentrations of PGG (≤ 250 nM) caused a moderate inhibition whereas 500, 1000 and 2000 nM of PGG inhibited LDH release by 29, 76 and 89 % compared to the cells incubated with pneumolysin alone.

In summary the results show that hydrolyzable tannins inhibit the cytotoxicity of Ply and could be used in the prevention of *S. pneumoniae* infections.

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O1.15

Synthesis and evaluation of lipophilic alkyl-polyphenols as new therapeutics toward retinal degeneration

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MAIN CONCLUSION

Rational design allowed the development of three synthetic pathways leading to alkyl-lipophenol derivatives. Among them, the most promising derivative is the quercetin lipophenol which shown best anti-carbonyl and antioxidant properties, and was the most efficient to protect retina cells against light induced toxicity of A2E. The *in vivo* evaluation of quercetin lipophenol in a mouse model of Stargardt disease showed its ability to conserve photoreceptors and their functionality.

INTRODUCTION

Carbonyl and oxidative stresses (COS) have been highlighted as toxic mechanisms occurring in many neurodegenerative diseases. Among those pathologies, dry age-related macular degeneration (AMD) and Stargardt disease are going through this toxic pathway and have currently no treatment. COS mechanisms are responsible for the accumulation of a toxic *bis*-retinoid conjugate called A2E in retinal pigment epithelium (RPE). Pathologic A2E biosynthesis occurs when a reactive carbonyl species (RCS), the all-*trans*-retinal (a*t*RAL), rather than undergoing reduction to retinol in the RPE, accumulates abnormally in photoreceptors. One of the mechanism involved in A2E cytotoxicity is related to an increase of oxidative stress, by the generation of singlet oxygen during exposition to blue light, and the production of A2E-oxidized metabolites. Limitation of A2E biosynthesis (by clearance of a*t*RAL) and reduction of the oxidation are therefore attractive targets to slow the progression of macular degeneration. Natural polyphenols, specialized metabolites of plants, have been reported to protect cells from oxidative stress through their capacity to scavenge reactive oxygen species (ROS), or to stimulate enzymatic antioxidant defenses. Furthermore, their potential to trap toxic RCS (such as a*t*RAL) has been validated recently. Unfortunately, their weak bioavailability limit their therapeutic potential and therefore, we aimed to increase the lipophilicity of natural polyphenols in order to ameliorate their absorption and their protective effects, using coupling with docosahexaenoic acid, which can bring protection to retinal tissue. Preliminary *in vitro* studies have identified a leading phloroglucinol-isopropyl-DHA conjugate. This lipophenol was able to reduce carbonyl stress in retina cells. [1] However, as its antioxidant properties were still weak, we focused on designing different families of lipophenol derivatives to conserve anti-carbonyl activity and improve antioxidant properties.

MATERIALS & METHODS

We developed synthetic pathways to access various isopropyl-polyphenol derivatives (phloroglucinol, resveratrol and quercetin) linked to the DHA moiety. The strategies elaborated go through orthogonal protection/deprotection steps of phenolic positions allowing the introduction of isopropyl and DHA moieties in specific positions for each polyphenol backbone. For all lipophenol derivatives, their corresponding polyphenol bearing only isopropyl function or only lipid moiety was produced, to evaluate the impact of those substituents on *in vitro* properties. Synthetic compounds were evaluated *in vitro* on ARPE-19 cell lines for their cytotoxicity, their anti-carbonyl stress activity

and their antioxidant potential. [2,3] Finally, the most promising lipophenol was evaluated *in vivo* by intravenous administration in a murine model of Stargardt disease.

RESULTS & DISCUSSION

Synthesis of three families of lipophenols were developed acceding to a library of isopropyl-polyphenols and lipophenols (bearing an isopropyl moiety or a free phenol) from phloroglucinol, resveratrol and quercetin (figure 1).

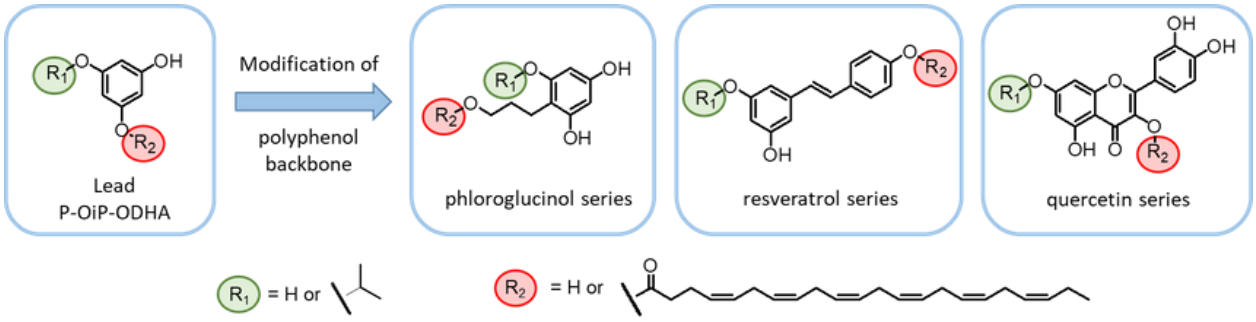

Figure 1. Chemical modifications performed on phloroglucinol, resveratrol and quercetin polyphenol backbones.

For all lipophenols bearing an isopropyl and a DHA moieties, no cytotoxicity was observed up to 160 µM. On the contrary, resveratrol and quercetin bearing only an isopropyl moiety were very toxic with no more viability at 80 µM only. Furthermore, our results confirmed the necessity of the presence of isopropyl group as well as the lipid part in the lipophenol structure to observe efficient anti-carbonyl stress activity. Antioxidant potential of lipophenols was evaluated through two experiments targeting oxidative stress in AMD. First, the potential of lipophenols to limit hydrogen peroxide induced ROS production in cells was evaluated. New lipophenols from phloroglucinol, resveratrol and quercetin showed increased ROS scavenging properties than the leading P-OiP-ODHA. Secondly, the ability of lipophenols to protect cells against A2E light induced toxicity was evaluated and, again, new lipophenols showed better protecting effect than the P-OiP-ODHA. The most promising candidate, a quercetin lipophenol, was evaluated in a murine model of acute light induced degeneration (Abca4KO) and was able to protect integrity and functionality of the photoreceptors of mice treated intravenously at 30 mg/kg prior light exposure (figure 2).

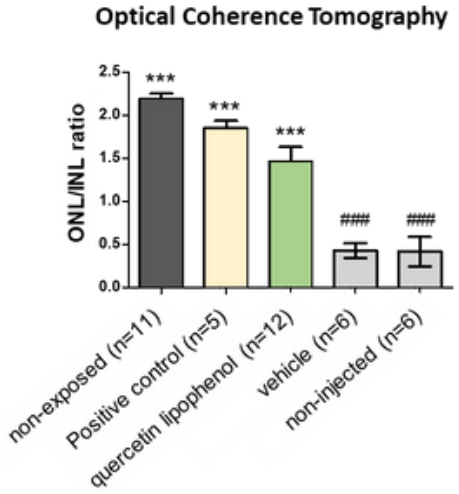

Figure 2. Measure of photoreceptor nuclear layer thickness. Comparison of mice non-exposed to intensive light, mice exposed and treated with positive control, quercetin lipophenol, and vehicle only and mice non-injected. Oneway ANOVA, ***p < 0.001 versus non-injected mice; ###p < 0.001 versus non-exposed mice.

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O1.16

The Evolution of the Color systems in Plants. A physical chemical approach

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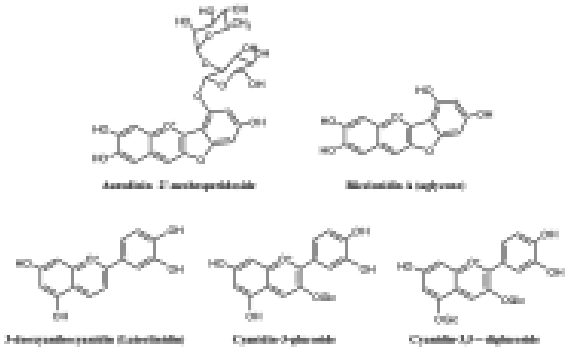
MAIN CONCLUSION

Anthocyanins are on the basis of the angiosperms colour, 3-deoxyanthocyanidins play the same role in mosses and ferns and auronidins are responsible for the colour in liverworts. The separation of the ancestors of each of these lineages occurred in different periods of land plant evolution, therefore, our thesis is that chemical evolution of the color systems accompanied plant evolution.

INTRODUCTION

The colour system of cyanidin-3-*O*-glucoside (kuromanin) as a representative compound of simpler anthocyanins was fully characterized by stopped flow. This type of anthocyanins cannot confer significant colour to plants without intra or intermolecular interactions, complexation with metals or supramolecular structures as in *Commelina communis*. The anthocyanin's colour system was compared to the one of 3-deoxyanthocyanidins and riccionidin A, the aglycone of auronidins. The three systems follow the same sequence of chemical reactions, but the respective thermodynamic and kinetics is dramatically different.



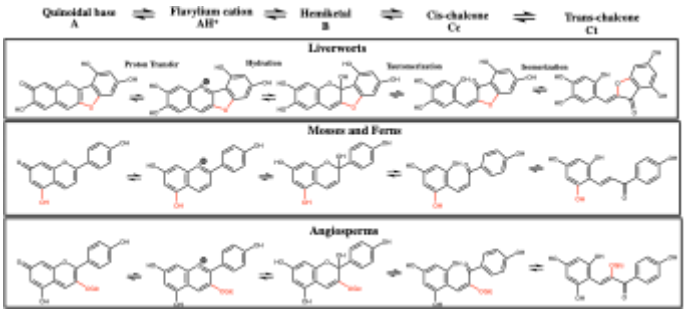
In this communication we compare the three systems and explain why anthocyanins were selected by angiosperms, the plants on the top of the evolution.

MATERIALS & METHODS

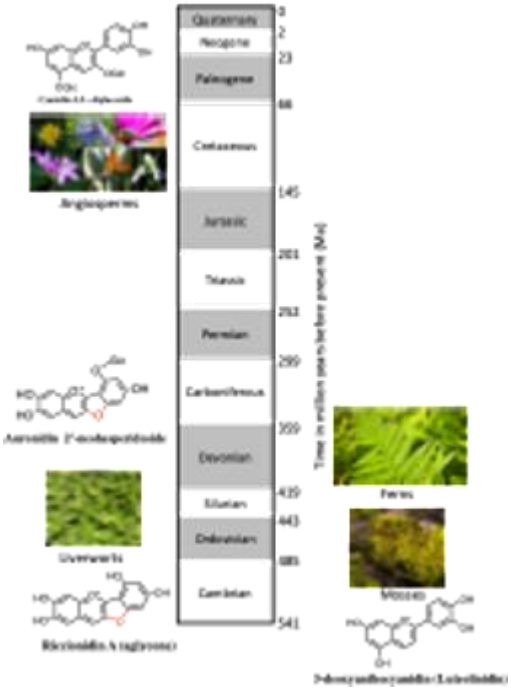
The direct pH jumps were carried out by mixing a stock solution of kuromanin in HCl 0.1 M (3×10^{-5} M) with a solution containing NaOH 0.1 M and Theorell and Stenhagen universal buffer at the desired final pH using the stopped flow (SX20, Applied Photophysics; Surrey, UK) spectrometer equipped with a PDA.1/UV photodiode array detector. Spectroscopic measurements were performed using Milli-Q water at a constant temperature of 20 ± 1 °C using Varian-Cary 100 Bio or Varian-Cary 5000 spectrophotometers. Reverse pH jumps were carried out by stopped flow (pseudo-equilibrated solutions) and common spectrophotometer (equilibrated solutions), adding enough HCl to reach pH = 1 in equilibrated solution of the anthocyanidin/anthocyanin at different pH values. The final pH of the solutions was measured in a Radiometer Copenhagen PHM240 pH/ion meter (Brønshøj, Denmark).

RESULTS & DISCUSSION

It was proved that riccionidin A, luteolinidin and anthocyanins follow the same multistage of chemical species, exhibiting the same type of molecules reversibly interconnected by the same type of chemical reactions, Scheme 1.



Scheme 1. Riccionidin A, 3-deoxyanthocyanidins and anthocyanins: the same multistate of chemical species.



Scheme 2. Estimated origin of liverworts, mosses, ferns, and angiosperms. Jason Hollinger, Charles Peterson, Peter and Joyce Grace, Sarah Vanderweele, Camelia TWU, and Rafael Medina are credited for the photographs presented in Scheme 1, depicting several plant species.

The physical chemistry of riccionidin A and 3-deoxyanthocyanins unequivocally shows that the former was significantly less versatile than the latter. Although anthocyanins are limited a priori to express color by themselves, they have a complete color pallet available and can give expression to these colors by means of intra- and intermolecular copigmentation, coordination with metals, and the combination of these effects, as in the case of metalloanthocyanins. It is clear that the color systems have evolved from auronidins to 3-deoxyanthocyanins to anthocyanins, by widening the color range they can cover. We hope the results reported in this work can contribute to the current discussion regarding the phylogenetic hypotheses about the divergence between mosses and liverworts, and between this group and vascular plants

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O2.1

Improvement of bone health condition by oral administration of proanthocyanidin-rich grape seed extract in ovariectomized animals

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MAIN CONCLUSION

Daily oral administration of proanthocyanidin-rich grape seed extract (GSE) prevented bone loss of the femur and the lumber spine in ovariectomized (OVX) mice used as a model of osteoporosis caused by estrogen deficiency. In addition, GSE facilitated bone healing and implant osseointegration in OVX rats. Thus, oral administration of GSE may be applicable as a safe complementary alternative not only to osteoporosis itself but to dental and orthopedic implant therapy in osteoporosis patients.

INTRODUCTION

GSE abundantly contains proanthocyanidin, a type of bioactive polyphenol, composed of oligomers of flavan-3-ols with a range of polymerization between 2 and 17. Epidemiological studies indicated that intake of bioactive polyphenols ameliorated bone health, especially in postmenopausal women with the cessation in ovarian hormone production, such as estrogen [1]. Because estrogen suppresses bone resorption via regulation of osteoclasts, estrogen deficiency results in excessive bone resorption and thus osteoporosis, that is characterized by low bone mineral density and increased risk of bone fracture. Although the mechanism of action has not been fully understood, intake or oral administration of bioactive polyphenols seems to alleviate the osteoporosis. Animal studies using OVX rats, a model of osteoporosis in postmenopausal women, and rats fed with a low-calcium diet, a model of osteopenia, showed beneficial effects of bioactive polyphenols on bone health [2], supporting the human epidemiological evidence. If proanthocyanidin-rich GSE improves bone health in postmenopausal women, it may be useful not only for prevention of bone fracture but also for improvement of osseointegration implant therapy. Osseointegration is defined as the direct connection between bone tissue and an implant. The success of dental and orthopedic implant therapy depends on the establishment and maintenance of adequate osseointegration. Thus, we hypothesized that GSE could improve bone health and bone healing including implant osseointegration in postmenopausal women. Therefore, the aim of present study was to confirm if proanthocyanidin-rich GSE had an ability to prevent bone loss in OVX mice and rats, and to evaluate the potential effects of GSE on the healing of bone defects as well as implant osseointegration in OVX rats.

MATERIALS & METHODS

Female C57BL/6J mice and Wistar rats were used. The mouse experiment was performed to analyze bone volume, and osteoclastogenic and osteogenic parameters, while the rat experiment was carried out to evaluate bone healing and implant osseointegration. The animals underwent sham or OVX operation. GSE suspended in water was daily administered by oral gavage to the animals at a dose of 100 mg/kg. The mice were sacrificed 13 weeks after OVX surgery, and femurs and lumbar vertebrae were collected for micro-CT analyses and histological evaluation. In the rat experiment, one week after OVX surgery, the second surgery was performed to create calvarial bone defects. In addition, one week after the second surgery, implant surgery was performed to install titanium mini-screws in tibiae. The rats were sacrificed 35 days after OVX surgery, and calvaria and tibia were subjected to micro-CT analysis, implant removal torque test, and histological evaluation of bone-to-implant contact (BIC).

RESULTS & DISCUSSION

In the mouse experiment, the micro-CT analysis of femur and the histological analyses of lumber spine revealed that OVX decreased trabecular bone volume. Daily oral administration of GSE attenuated the trabecular bone loss caused by OVX. Histomorphometric analysis indicated osteoclastogenesis in lumbar spine bone increased in OVX group, and GSE prevented this dynamization. In contrast, osteoblastic parameters did not show any significant differences between the groups. This indicated that neither OVX nor OVX + GSE affected osteoblastic activity and the bone loss observed in OVX mice was likely due to accelerated osteoclastogenic activity induced by estrogen deficiency. As such, GSE would counteract the accelerated osteoclastogenic activity, resulting in the prevention of this imbalanced bone turnover.

In the rat experiment, micro-CT analysis confirmed that OVX decreased trabecular bone volume in femur, and oral administration of GSE attenuated the bone loss. As for bone healing, OVX tended to impair healing of the defects created on the calvaria, and GSE counteracted the OVX-induced impairment in bone healing. Similarly, GSE increased the implant removal torque and BIC in OVX rats (Figure 1). Osseointegration was negatively affected by OVX, resulting in a lower implant removal torque and lower BIC as previously reported [3]. Regarding the mechanism by which GSE attenuated the OVX-induced impaired bone healing and implant osseointegration, GSE may not mimic estrogen effects because GSE did not attenuate OVX-induced uterine atrophy. Although it was reported that polyphenols such as resveratrol, kaempferol, anthocyanins, and quercetin would act as phytoestrogens [4], proanthocyanidin-rich GSE may not mimic the effects of estrogen. Though the underlying mechanism is still unclear, GSE may partly exert its therapeutic effects via modification of the intestinal microflora as recently suggested [5].

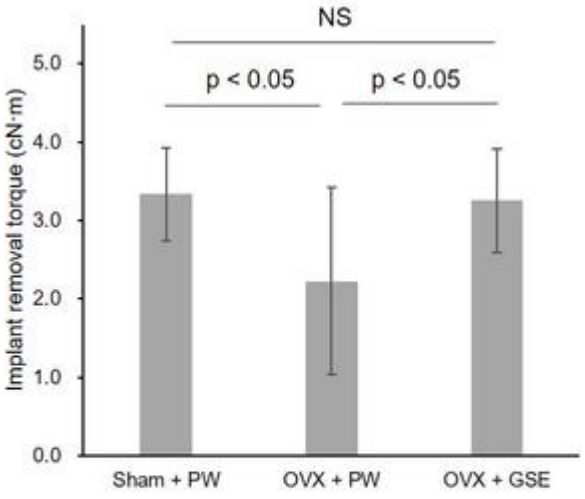

Figure 1. Implant removal torque of screw-shaped implant installed in the rat tibia. OVX: ovariectomy, PW: pure water, GSE: grape seed extract suspended in pure water, NS: no significant difference.

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O2.2

Metabotypes of flavan-3-ol colonic metabolites after cranberry intake

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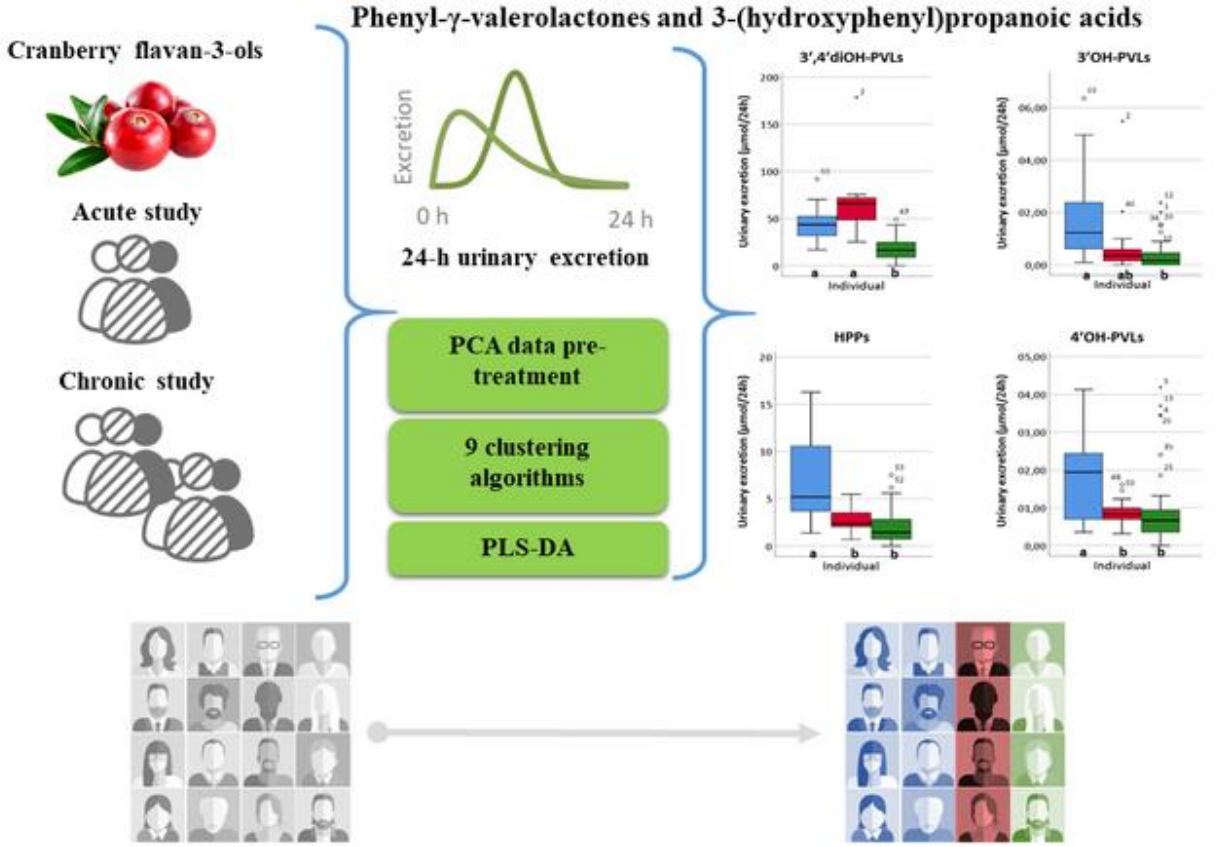
MAIN CONCLUSION

This work shed light on the existence of metabotypes in the urinary excretion of flavan-3-ol metabolites, which are not characterized by the dichotomic production/non-production of specific metabolites, but by different quali-quantitative metabolic profiles. A series of univariate and multivariate tools, all broadly accessible to the research community, highlighted the importance of data pre-treatment and clustering methods on the final outcomes for a given dataset.



INTRODUCTION

An extensive inter-individual variability is reported in the production of flavan-3-ol colonic metabolites, possibly affecting, at individual level, the health benefits associated with this class of compounds [1].

This variability might be due to personal differences in gut microbiota composition, resulting in different metabolic phenotypes (metabotypes). In a preliminary study, three putative metabotypes after green tea flavan-3-ol consumption were defined on the basis of a different production of phenyl-γ-valerolactones and 3-(hydroxyphenyl)propanoic acids, through explorative partial least squares-discriminant analysis (PLS-DA) models [2]. However, there is a lack of information on how to handle the inter-individual variability in the production of phenolic metabolites to define metabotypes in those cases where all the subjects produce all the phenolic metabolites of a catabolic pathway, but in different proportions, as it happens for flavan-3-ols and for the main dietary classes of (poly)phenols.

The primary aim of the present study was to evaluate the existence of metabotypes, based on the urinary excretion of flavan-3-ol metabolites after consumption of flavan-3-ols from cranberry products. Secondly, this work aimed at investigating the impact of the statistical techniques used for the definition of phenolic metabotypes, defining an approach to specifically seek for metabotypes when they are not characterized by the dichotomic production/non-production of specific phenolic metabolites.

MATERIALS & METHODS

Data on urinary concentrations of several gut microbiota-derived metabolites of flavan-3-ols, namely monohydroxyphenyl-γ-valerolactones (isomers 3′ and 4′), dihydroxyphenyl-γ-valerolactones (3′,4′), and 3-(hydroxyphenyl)propanoic acids from two human interventions with cranberry products was used. Different multivariate statistics were considered to assess the impact of the statistical technique used for metabotyping on the evidence observed: principal component analysis (PCA) focusing on the effects of data pre-treatment methods, cluster analysis testing several clustering algorithms and a principal component score-based method, and partial least square-discriminant analysis (PLS-DA). Lastly, univariate statistics served to confirm the results from PLS-DA models.

RESULTS & DISCUSSION

Different profiles in the urinary excretion of phenyl-γ-valerolactones and 3-(hydroxyphenyl)propanoic acids were observed upon cranberry consumption in two diverse experimental settings. These metabolic profiles were related either to specific pathways of phase II metabolism or the type of metabolites produced at colonic level. Data pre-treatment played a major role on resulting PCA models: mean centering and mean centering + Pareto scaling highlighted different patterns of phase II metabolism (sulfation vs. glucuronidation), while centering + unit variance scaling showed different patterns of colonic metabolism. These facts emphasised the importance of data pre-treatment when analysing datasets of flavan-3-ol metabolites, as it is well acknowledged that pre-treatment procedures in metabolomics studies may greatly influence the biological relevance of the results [3]. Regarding clustering, k-means and a final consensus algorithm highlighted differences in the overall production of phenyl-γ-valerolactones and 3-(hydroxyphenyl)propanoic acids, leading to quantitative-based models whereby the distribution of the clusters was due to the amount of metabolites excreted (high vs. low). Differently, the expectation-maximization algorithm and clustering according to principal component scores yielded well-defined metabotypes characterized by quali-quantitative differences in the excretion of the colonic metabolites. PLS-DA, together with univariate analyses, served to validate the urinary metabotypes in the production of flavan-3-ol metabolites and to confirm the robustness of the methodological approach. This metabotyping strategy may be key to manage the inter-individual variability reported in the colonic metabolism of flavan-3-ols [1] and to further investigate its consequences in the impact on the observed health effects attributed to this class of compounds.

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O2.3

Anthocyanin-mediated cardioprotection: an insight into molecular mechanisms

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MAIN CONCLUSION

A C3G-rich extract (RED) promotes HL-1 cardiomyocytes survival upon Doxo treatment. RED prevents Doxo-induced cardiotoxicity through the FGF21-AMPK-SIRT1-p53 pathway. RED averts the Doxo-induced AMPK activation, avoiding the increase of the NAD/NADH ratio, leading to SIRT1 inactivation. SIRT1 is activated upon Doxo treatment, deacetylates p53 at Lys379, which induces cell death, increasing apoptosis markers. Instead, the increase of Ac-p53 upon RED treatment leads to cell survival.

INTRODUCTION

Epidemiological studies indicate that consumption of polyphenol-rich foods reduces the risk of cardiovascular disease, cancer and neurodegenerative diseases[1]. Among polyphenols, in addition to having a strong in vitro antioxidant capacity, anthocyanins (ACNs) may regulate cellular signalling pathways involved in antioxidant and anti-inflammatory response. Doxo is one of the most effective chemotherapeutic drugs; however, it can lead to cardiomyopathy and heart failure even 10-15 years after the end of the treatment[2]. Our previous studies showed that an ACN-rich diet from purple corn, which mainly contains cyanidin 3-glucoside (C3G) and its acetylated derivatives, proved to be effective in reducing the Doxo-induced cardiotoxicity in mice compared to an ACN-free control diet from yellow corn[3]. Therefore, ACNs could be a beneficial supplement to guarantee a better life style to cancer patients. Aiming at unveiling the molecular mechanisms involved in ACN protection, we decided to consider the FGF21-AMPK-SIRT1-p53 pathway that recently gained interest for its cardioprotective role. Fibroblast growth factor 21 (FGF21), which is mainly secreted by liver and adipose tissue, is also produced and secreted by cardiomyocytes[4]. AMPK, a conserved energy sensor involved in stress response and cellular homeostasis, is activated through phosphorylation of Thr172 by LKB1, a downstream target of FGF21[5]. Activated AMPK can phosphorylate both SIRT1 and its inhibitor DBC1, causing SIRT1 detachment and activation. It can also lead to SIRT1 activation modulating NAD/NADH ratio[6]. SIRT1 is a NAD-dependent deacetylase expressed in the heart, with important roles in longevity, cellular senescence, cell differentiation and cell survival[7]. One of the most interesting targets of SIRT1 is P53. The deacetylation of P53 at Lys382 promotes cell survival, reducing the expression of genes involved in apoptosis and cell cycle arrest (like *Puma* and *p21*) mediated by P53 itself[8].

MATERIALS & METHODS

HL-1 murine cardiomyocytes were treated with two different concentrations of Doxo in presence or absence of a purple corn extract (RED) obtained from cobs, which mainly contains cyanidin-3-glucoside and its acetylated derivatives [3]. Total protein levels and post-translational modifications were analysed through western blot. Expression of different target genes was analysed using qPCR. Silencing of *Fgf21* and *Sirt1* was performed using lipofectamine-mediated siRNA transfection. NAD/NADH ratio was measured using a colorimetric assay.

RESULTS & DISCUSSION

Our results showed that in HL-1 cardiomyocytes RED treatment reduced Doxo-induced cytotoxicity lowering cleaved caspase 3 and preventing apoptosis. More specifically, AMPK activation through phosphorylation of Thr172 was increased by Doxo alone and restored to the control level by RED. In addition, Fgf*21* silencing caused a significant reduction of active AMPK and led to significant reduction of cleaved caspase 3 protein level, together with reduced expression of genes involved in apoptosis (*p53* and *Puma*) and cell cycle arrest (*p21*), mimicking the effect of RED extract. Once activated, AMPK in turn activates SIRT1 through phosphorylation. Despite SIRT1 total protein level is not altered, an indirect indication of SIRT1 activity was obtained measuring the NAD/NADH ratio, which is increased by Doxo and maintained at control level by the RED cotreatment, suggesting a SIRT1 activation upon Doxo treatment. Moreover, the deacetylation of p53 at Lys382 (corresponding to Lys379 in mouse), which is SIRT1-dependent, was maintained at the control level by Doxo and increased by the RED treatment. *Sirt1* silencing led to a slight increase in p53 acetylation in all conditions, validating their connection. In addition, Ac-p53(Lys379) was predominantly localized in the cytoplasm and the levels of cleaved caspase 3 were clearly reduced by RED treatment, suggesting a reduction in apoptosis. As reported in literature, acetylated Lys379 in P53 increases the transactivation of genes involved in apoptosis and cell cycle arrest[9], but it may have opposite roles of Ac-p53(Lys379) in different cell types[10]. Lys382 acetylation, following DNA damages, seems to promote cell survival of neurons and apoptosis in cancer cells, where genes, such as *Puma*, are induced. Similarly, in cardiomyocytes, Ac-p53(Lys379) could promote cell survival, as supported by the qPCR results on *Puma* and *P21*, whose transcript levels were significantly lower upon Doxo and RED treatment with respect to Doxo alone.

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O2.4

A Polyphenol From *Corema Album* L. Reduces Alpha-Synuclein Aggregation And Toxicity In Cellular And Animal Models Of Parkinson's Disease

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MAIN CONCLUSION

A bio-guided fractionation was used to identify CAL_X as a potent bioactive small molecule present in *Corema Album* L. leaves. CAL_X interferes with α-synuclein aggregation and acts on cell metabolism to decrease the deleterious effects of α-synuclein. These findings open new avenues for the exploitation of CAL_X as a lead molecule for PD cases associated with α-synuclein accumulation.

INTRODUCTION

Parkinson's Disease (PD) is the most common neurodegenerative movement disorder affecting millions of people worldwide. In the brain, PD is characterized by the loss of dopaminergic neurons at the *substantia nigra pars compacta*. Molecularly, it is characterized by the deposition of proteinaceous inclusions called Lewy Bodies (LB) and Lewy neurites in the surviving neurons that are mainly formed by misfolded and aggregated α-synuclein (αSyn). The aggregation of αSyn is one of the several molecular hallmarks, alongside with increased oxidative stress and vesicular trafficking impairments. Huge efforts have been made both to understand the molecular basis of PD and to try to tackle disease progression. Thus far, there is no cure for PD, and the lack of disease-modifying strategies gives PD a public health priority status. Phenolic compounds have emerged as potent molecules targeting key pathological processes underlying neurodegeneration. We have previously shown that a (poly)phenol-enriched fraction (PEF) of *Corema album* L. leaves modulates central events in PD pathogenesis, namely αSyn aggregation and clearance [1]. To identify the main bioactive compound against PD present in PEF, we performed a bio-guided fractionation using a well-established yeast model of PD and characterized the mechanism by which it exerts its biological activity.

MATERIALS & METHODS

PEF fractionation and identification of components was performed by Liquid Chromatography (LC) as described [2]. Yeast PD model, based on the overexpression of human αSyn fused with GFP (αSyn-GFP) under the control of the inducible promoter GAL1, was used [3]. Yeast growth was monitored for 24 h, as described [3]. All the following experiments were conducted after 6h of expression in the presence or not of CAL_X. Toxicity, inclusions counting and Size Exclusion Chromatography (SEC) was performed as previously described [4]. For RNAseq analysis, 20 million reads per sample was recorded and S288C strain was used as the reference genome. *In vitro* αSyn aggregation was monitored by Dynamic Light Scattering and by ThT fluorescence. CAL_X interaction with αSyn

monomer was assessed by NMR. Drosophila model of PD is based on the expression of human αSyn-GFP as previously described [5]. Lifespan and a decline of motor capabilities in flies fed or not with CAL_X were assessed over time.

RESULTS & DISCUSSION

The original PEF extract was fractionated into 16 different fractions and their activity in the yeast PD model was assessed. Only two fractions were able to improve cell growth and the compounds present in the fractions were identified. We identified CAL_X, a (poly)phenol whose activity was further investigated. Importantly, CAL_X was confirmed to decrease αSyn-GFP toxicity and to reduce the percentage of cells with αSyn-GFP inclusions. Moreover, we assessed the size of the inclusions by SEC and found that CAL_X decreased the size of the inclusions. We then assessed the capacity of CAL_X to directly interact with αSyn *in vitro*. We allowed pure human αSyn to aggregate and incubated it or not with CAL_X. The results show that CAL_X interacts with αSyn and prevents the formation of bigger aggregates and fibrils *in vitro*.

Gene expression was assessed by RNA-seq in the yeast model of αSyn aggregation, and the targets of CAL_X were analyzed. Transcriptomics revealed that the effect of CAL_X is dependent on the presence of αSyn since no differences in gene expression were observed in control cells incubated with CAL_X. Gene Ontology data suggests that carbon metabolism, lipid homeostasis and vesicular trafficking are central processes involved in the mechanism of CAL_X protection. Given the importance of lipid homeostasis and vesicular trafficking in PD pathology we went to investigate CAL_X impact on these processes.

Importantly, we proved that CAL_X impacts on lipid storage and vesicular trafficking in order to decrease the deleterious effects of αSyn-GFP. Attempting to prove that CAL_X can exert beneficial effects in motor dysfunction promoted by human αSyn expression we used a Drosophila model of PD. Importantly, after the expression of human αSyn-GFP in the fly nervous system a decrease in lifespan and a decline of motor capabilities were observed. Furthermore, CAL_X was able to prevent the motor defects caused by expression of αSyn-GFP and to extend life span.

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O2.5

Gnetol and oxyresveratrol glucuronide metabolites: Chemical production, structural identification, metabolism by human and rat liver fractions and *in vitro* anti-inflammatory properties

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MAIN CONCLUSION

This work shows for the first time the unambiguous structural characterization of ORV and GN glucuronide metabolites. Additionally, the *in vitro* metabolisation of ORV and GN by human and rat S9 fractions have shown that human enzymes glucuronidation was preferably performed at 2' position whereas rat enzymes do it at 3 position. ORV, GN and mainly the metabolites glucuronidated at 3 position have proven to be effective to decrease (> 50%) NO and ROS production in LPS-stimulated macrophages.

INTRODUCTION

Oxyresveratrol (ORV) and gnetol (GN) are stilbenes monomers which differ from resveratrol in the number and position of -OH groups. GN has been extensively isolated in the genus *Gnetum* whereas ORV has been identified in mulberry fruits and in wine. Both compounds are gaining importance in human health, as was confirmed by some *in vitro* and *in vivo* studies (1-2). However, most stilbenes are poorly absorbed at intestinal level, highly metabolized and consequently practically absent in plasma and tissues (3). The major metabolites found in biological fluids are the phase II metabolites, which, are formed by the parent compound conjugates to glucuronide, sulphate and methyl groups. Thus, the study of the biological properties of the metabolized forms represents a more realistic situation.

For all explained above, the objectives of this study are: i) the chemical hemi-synthesis of ORV and GN glucuronides ii) their structural elucidation by NMR spectroscopy iii) the *in vitro* study of ORV and GN metabolism by human and rat liver S9 fractions and, iv) the study of the potential anti-inflammatory properties of ORV, GN and their glucuronide metabolites in LPS-stimulated macrophages.

MATERIALS & METHODS

The production of ORV and GN glucuronide metabolites was performed by chemical *O* glucuronidation using acetobromo-α-D-glucuronic acid methyl ester in alkaline conditions. After their identification by UPLC-Q-TOF, metabolites were purified by preparative-HPLC and finally the structural characterization was carried out by NMR spectrometry. In order to study the *in vitro* metabolisation of ORV and GN and the interspecies differences, S9 fractions of rat and humans were incubated during 15 minutes at 37°C with both compounds at different concentrations (0 to 3000 µM) in presence of UDPGA and other cofactors. Afterwards, samples were analysed by UPLC-MS and

kinetic parameters were calculated (V_{max}, K_m, K_i). Finally, the cytotoxicity and the anti-inflammatory properties of ORV and GN and their metabolites were evaluated. For this, MTT assay, nitric oxide (NO) production (Griess reaction) and the intracellular ROS production ($DCFH_2$-DA probe) were performed in LPS-stimulated RAW264.7 macrophages.

RESULTS & DISCUSSION

Three glucuronide metabolites for ORV were formed by hemi-synthesis: *t*-ORV-4'G, *t*-ORV-3G and *t*-ORV-2'G. Moreover, 2 glucuronide metabolites were obtained for GN: *t*-GN-2'G and *t*-GN-3G. All compounds were purified by preparative HPLC-DAD and structurally elucidated by 1D and 2D NMR for the first time.

After the *in vitro* metabolisation by human and rat S9 fractions of ORV, two of the three metabolites obtained by hemi-synthesis were identified: *t*-ORV-3G and *t*-ORV-2'G. In addition, we have confirmed the presence of 2 *cis*- metabolites: *Cis*-Oxyresveratrol-3-*O*-glucuronide (*c*-ORV-3G) and *cis*-Oxyresveratrol-*O*-2'glucuronide (*c*-ORV-2'G) which derive from their corresponding *trans*-forms. Related to the *in vitro* metabolisation of GN, two main metabolites were formed: *t*-GN-2'G and *t*-GN-3G. These results also shown that the way of metabolize ORV and GN by human and rat enzymes are different. In fact, human enzymes glucuronidation was preferably at 2' position whereas rat enzymes do it at 3 position. The formation of metabolites for both stilbenes present a substrate inhibition profile. Based on the calculated kinetics parameters (K_m,V_{max},V_{max}/K_m) it has to be noted that rat enzymes exhibit a higher metabolic capacity that human enzymes.

Additionally, ORV, GN and their metabolites shown anti-inflammatory activity. Indeed, both parents compounds and the metabolites glucuronidated at 3 position were effective to decrease (>50%) NO and ROS intracellular production in macrophages. In general, fourfold concentration of the glucuronide compound is necessary to achieve the anti-inflammatory effect of his parent compound.

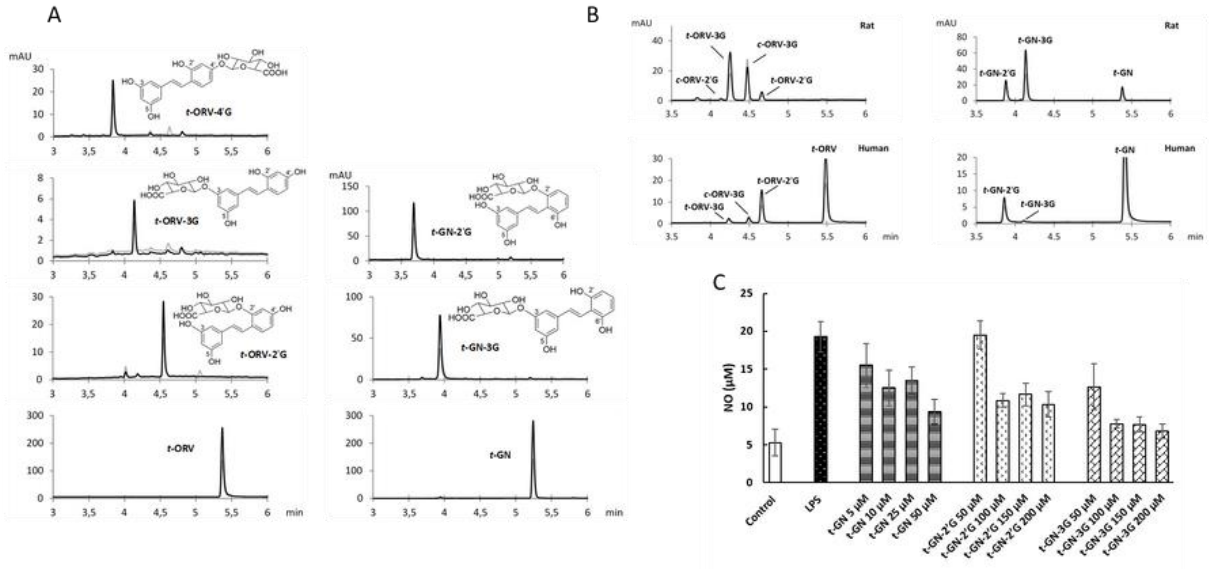

Figure 1. (A) Structure and LC-DAD chromatograms of ORV and GN glucuronide metabolites obtained by hemi-synthesis. (**B)** LC-DAD chromatograms of ORV and its metabolites and GN and its metabolites, obtained by metabolisation (50 µM). (**C**) NO (µM) production in RAW264.7 cells. Cells were treated for 24 h by LPS (0.1 µg/mL) or LPS with *t*-GN, *t*-GN-2'G and *t*-GN-3G (5-200 µM).

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O2.6

Urolithin B inhibits IAPP aggregation: a potential strategy for Diabetes therapeutics

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MAIN CONCLUSION

Urolithins are interesting (poly)phenol metabolites with powerful protective actions against pathological pathways associated with Diabetes, in particular, Islet Amyloid PolyPeptide (IAPP) aggregation. This was proven in *in silico*, in cell-free systems, in yeast models expressing human IAPP and in pancreatic beta-cells. Future studies should be done to support the exploitation of Urolithins as a tool in Diabetes management.

INTRODUCTION

Diabetes is an epidemic with frightening numbers worldwide. Despite the improvements in disease control, new strategies are still urgent. An overseen aspect in Diabetes therapeutics is IAPP aggregation and amyloid formation, a histopathological hallmark of the disease. IAPP is co-expressed and co-processed with insulin in pancreatic beta-cells from where they are released upon glucose stimulation. Its physiological role includes slowing gastric emptying and satiety, aid in glucose homeostasis. For unknown reasons, IAPP starts to oligomerize forming increasingly complex structures, ultimately depositing as amyloid fibrils. The intermediate, soluble, oligomeric forms were proven to affect virtually all cellular processes, causing cytotoxic effects that indisputably contribute for beta-cell failure. In that view, IAPP aggregation and deposition represents key features of Diabetes onset and progression, which remains largely unexplored from the therapeutic point of view.

Dietary (poly)phenols (PP), and their metabolites, have been proven effective towards a myriad of chronic pathologies, including Diabetes. Besides their protective effects towards the main features of Diabetes – insulin deficiency and high blood glucose – PP have also been associated with the inhibition of IAPP aggregation. In that view, PP represent a pool of compounds with great potential for the management of Diabetes. As PP are extensively metabolized throughout the gastrointestinal tract, attention on the physiological relevance of these compounds should be focused on the metabolites, since they are the ones circulating and reaching target tissues.

MATERIALS & METHODS

A library of PP metabolites, predicted to be found circulating in humans after PP consumption, was tested in docking studies with IAPP (NMR structure 2L86 from Protein Data Base) using Auto Dock Vina. Using Vina Score as a measure of affinity towards IAPP, the best hits were assayed in cell-free systems. Synthetic IAPP was aggregated *in vitro*, either alone or in the presence of selected metabolites. The kinetics of fibril formation was followed by Thioflavin-T (ThT) assays and Transmission Electronic Microscopy (TEM). Metabolites-mediated protection was investigated in *S. cerevisiae* models expressing immature forms of human IAPP fused to GFP, which recapitulate IAPP cytotoxicity and intracellular aggregation, using cellular and molecular biology approaches. The protective activity of the metabolites was validated in INS-1 832/13 cells challenged with toxic IAPP aggregates by means of viability, Glucose Stimulated Insulin Secretion assays, and transcriptomic analysis.

RESULTS & DISCUSSION

Urolithins (Uro), particularly UroA and UroB, were the compounds with the better scores for IAPP in the *in silico* analysis. Epigallocatechin gallate (EGCG) was used as a positive control as it has been reported to hamper IAPP aggregation. Both UroA and UroB showed similar scores to EGCG.

The *in silico* data were validated in cell free assays, by following the kinetics of aggregation of synthetic IAPP in the presence of either of the metabolites. The results indicated that both UroA and UroB interfere with IAPP aggregation. Although UroA caused a rapid increase in ThT fluorescence signals up to 6 h, these signals were kept constant until the end of the experiment. On the other hand, UroB delayed the appearance of IAPP beta-sheet structures interacting with ThT for 24 h, but the fluorescence signals were similar to the control condition at 36 h of incubation. TEM images showed that UroA and UroB differentially alter the morphology of fibril structures formed.

The metabolites were then tested in yeast models of IAPP aggregation. Viability assays show that only UroB could prevent IAPP toxicity (20.8 vs 7.6 %) at 50 µM. This seems to be associated, at least partially, with a decrease in the size of IAPP aggregates and the alteration of their solubility. Mechanistic studies reveal that UroB increased protein clearance via proteosome and autophagy pathways, and genetic analysis further indicate that central players in these pathways are essential for UroB-mediated protection. Furthermore, the improvement of cell antioxidant responses is also related to UroB protective action.

RNAseq analysis show that challenging pancreatic beta-cells with IAPP aggregates caused damage to several cell systems, namely mitochondrial respiration, autophagy, and synthesis of glycerolipids. UroB protected against IAPP-induced toxicity and the damage caused to glucose stimulated insulin secretion, possibly through the modulation of calcium channels, as revealed by the transcriptomic analysis.

REFERENCES

UroB interacts with IAPP, interfering with the aggregation process, and protects eukaryotic cells from IAPP proteotoxicity by mechanisms as proteostasis maintenance, antioxidant response and calcium signaling pathways, which is essential for the insulin secretion function of pancreatic beta-cells.

O2.7

Unravelling the insoluble hydrolysable tannin-protein complexes

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MAIN CONCLUSION

The results indicated clear relationships between structural features of hydrolysable tannins (HTs) and the ability to form insoluble complexes with the model protein bovine serum albumin (BSA). In addition, both the complex compositions and the stabilities of the formed complexes depended on the tannin structure. Altogether, our results highlighted the importance of tannin structure to understand the role of tannins in various applications.

INTRODUCTION

Tannin-protein interactions are generally recognised as a source of the beneficial effects of tannins on ruminant nutrition. However, the mechanism by which tannins reduce ruminal protein digestion and increase protein flow to the small intestine is not fully understood. Accordingly, although tannin affinities towards proteins are well studied, not much is known of the stabilities of the tannin-protein complexes in conditions relevant for protein binding.

We studied the ability of 32 purified and characterised HTs to form insoluble complexes with model protein BSA. These tannins were first tested for their protein precipitation capacities (PPC), then the complex compositions were investigated and finally, we studied the stabilities of the formed tannin-protein complexes at various pHs.

MATERIALS & METHODS

The plant material collection, extraction, and fractionation was performed as described in Engström et al. 2019 [1]. Shortly, various plant materials were collected from Southwestern Finland, macerated and extracted with aqueous acetone. Altogether, 32 HTs representing eleven biosynthetic branches of the HT pathway was isolated and purified using Sephadex LH-20 fractionation in a Büchner funnel, Sephadex LH-20 column chromatography, and preparative and semipreparative liquid chromatography. The protein precipitation capacities of the HTs with BSA were measured in various concentrations with a turbidimetry-based 96-well plate reader method [1] and complex compositions were assessed by UHPLC-DAD. The stabilities of the formed tannin-protein complexes at various pHs were measured by adjusting the pH of the reaction solution were initial complex formation occurred and measuring the remaining complex by turbidimetry and complex composition by UPLC-DAD.

RESULTS & DISCUSSION

We studied the ability of 32 purified and characterized HTs to form insoluble complexes with model protein BSA, the complex compositions and the stabilities of the formed complexes at various pHs. The results showed clear relationship between the HT structure and their PPC. In addition to molecular weight, for monomeric HTs, the main structural features affecting the PPC were number of galloyl groups, degree of oxidative coupling between the galloyls, positional isomerism and cyclic vs acyclic glucose core. As expected, oligomeric HTs were superior to monomeric HTs in their PPC. In addition, their PPC depended less on the functional groups, but mostly on their size and overall flexibility. Based on the results, we constructed an equation predicting the PPC of the studied HTs with high accuracy.

By UHPLC-DAD, a more detailed understanding of the PPC of the studied HTs was accomplished. In general, the results by UHPLC-DAD supported well the turbidimetric

measurements but some interesting differences occurred in compound-to-compound comparisons. Regarding the pH-stabilities of the formed complexes, these too depended on the exact HT structure. Again, in such degree that a mathematical model accurately estimating the complex stabilities based on nine most important structural features of the HTs could be generated.

Together with our previous structure-activity studies, this fundamental knowledge could be used to estimate e.g. the bioactivities of different plant species based on their HT composition and content.

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O2.8

Modification of proanthocyanidins and their analysis tools for the screening of potential anthelmintic drugs of natural origin

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MAIN CONCLUSION

To produce modified proanthocyanidin (PA) structures for anthelmintic activity tests under alkaline oxidative transformation, we found that typically the least active procyanidins (PC) reaction route was concluded to intramolecular conversion from B-type to A-type ether linkage. However, the more active types of PAs (high prodelphinidin levels) reaction route were both intra- and intermolecular. Moreover, modification of PAs can increase their *in vitro* anthelmintic activity against *Ascaris suum*.

INTRODUCTION

Our research focused on one of the most common classes of bioactive plant secondary metabolites, proanthocyanidins (PAs). PAs play an important role in the leather tanning, wine, food and feed, cosmetic, and pharmaceutical industries, as well as some other industrial applications such as mineral flotation and oil drilling. Natural PAs from various leftover sources, on the other hand, were still unexploited, and more research is needed to spark public interest in using these globally available resources. In addition to the possibility of using these resources to extract tannins for the aforementioned industrial purposes, these residues could be transformed into even more appealing high-value products with some additional processing steps.

The main objective was to carefully investigate a wide range of PA-containing plant samples with various PA fingerprints to identify their differences in reactivity and modification processes under alkaline conditions. Besides, we wanted to see if plant extracts containing modified PAs had better anthelmintic activities than the original extracts that had not been modified in alkaline conditions. Thus, we studied the susceptibility of PAs from various plant sources (>300 plant samples) to oxidation under alkaline conditions by ultra-high performance liquid chromatography coupled with tandem and high-resolution mass spectrometry.

As a result, we want to know (1) how and why plant proanthocyanidins (PAs) are modified under alkaline conditions, (2) how to characterize PA oxidation products using high-resolution mass spectrometry, and (3) how oxidized and non-oxidized PA-rich plant extracts perform *in vitro* anthelmintic bioactivity against *Ascaris suum* nematodes. In the big picture, we want to learn more about the relationships between structure and activity in PAs so that we can create new types of active PAs from inactive ones.

MATERIALS & METHODS

In total, 300 plant samples were collected from the Botanical Garden of the University of Turku, Finland. In addition, to increase the heterogeneity from the PA point of view, some additional samples were collected from Barro Colorado Island, Panama, and 7 commercial tannin preparations were investigated. From the preliminary screening, 102 plant samples found to contained procyanidin and prodelphinidin rich oligomers and polymers. The tannins in these plant samples were extracted and oxidized, as shown by Imran et al. [1]. The PA oligomer and polymer fingerprints in oxidized and non-oxidized pieces were analyzed by the PA group-specific Engström method [2] and by a UPLC-Orbitrap high-resolution mass spectrometer in order to better characterize the individual modified tannin structures in more details [3]. Finally, we studied the *in vitro* anthelmintic effects of oxidized and non-oxidized extracts against *Ascaris suum* by a highly reproducible L3 stage larval migration inhibition assay.

RESULTS & DISCUSSION

We categorized all the studied samples into four different groups based on their UHPLC-UV area at 280 nm: The category (A) non-modified PAs without a loss of PA concentration; (B) non-modified PAs with a loss of PA concentration; (C) modified PAs without a loss of PA concentration; and (D) modified PAs with a loss of PA concentration, see for example Figure 2 in Imran et al. [1]. This study found two main reaction route for PAs in alkaline conditions: In most of the PC-rich samples, the reported PA hump retention time was constant, but, in most of the samples, the drop in the UV peak area was moderate, but the drop in the MS/MS detection varied a lot. Conversely, most of the PD-rich samples and the ones containing galloylated PAs demonstrated a distinct reaction route by UHPLC-DAD. Also, in these samples, the quantitative MS/MS levels decreased, while the UV peak area varied a lot. It suggests that category A and B samples may have involved intramolecular events, while in the C and D samples, both intra- and intermolecular events were occurring [1].

When observed by ultrahigh-resolution UHPLC-MS/MS, we found that B-type PCs in various plant extracts were relatively stable, with no or modest changes due to alkaline oxidation. The intramolecular reactions of PCs creating A-type ether linkages were found in several samples. A-type PCs were likewise relatively stable with no or little modifications, though the creation of extra ether linkage was observed in some plants. Plant extracts with PD units in PAs were more reactive (either pure PDs or PC/PD mixes). Even though a different type and/or delayed PA hump was still found by UV at 280 nm after oxidation [3]

Moreover, analysis of direct anthelmintic effects against *Ascaris suum* nematodes showed that the oxidized sample typically having higher inhibition values (Fig. 1). This suggests that the oxidation of some PAs increases their anthelmintic activity.

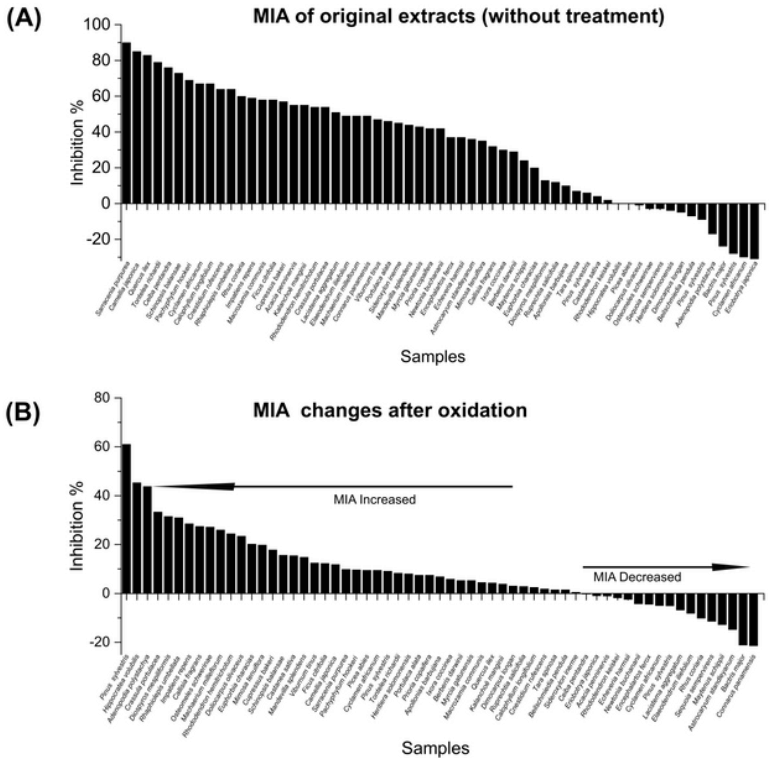

Figure 1. Migration inhibition activity of studied plant species.

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O2.9

Modulation of inflammatory responses in RAW 264.7 macrophages by purified condensed tannins and possible implication in a parasitized mouse-model

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MAIN CONCLUSION

Condensed tannins (CT) were purified by novel chemical techniques[1] and were screened for their bioactivity. We found that the mean degree of polymerization (mDP) and procyanidin/prodelphinidin (PC/PD) ratio may play an important role in the observed anti-inflammatory bioactivity in LPS-activated macrophages. This was also confirmed in mice dosed with CT. We next investigated the anti-parasitic effect of CT samples in *Heligmosomoides polygyrus* infected mice but observed no effect on worm burdens.

INTRODUCTION

Parasites are common pathogens found in humans causing substantial morbidity and mortality each year. Notably, soil-transmitted nematodes, more commonly known as roundworms, cause important co-morbidities such as malnutrition and reduced growth. Nematodes are also responsible for disease and great economic losses in livestock worldwide. Due to the increasing prevalence of drug resistance in predominantly livestock nematode infections, new and alternative intervention strategies are of great relevance. Thus, phytochemicals have been assessed for their potential as anti-parasitic with promising results *in vitro*. Notably, CT have been shown to significantly reduce migratory and feeding abilities in larvae, and CT had direct anti-parasitic effects on the pig nematode *Ascaris suum*[2].Furthermore, studies conducted in goats and sheep have also shown convincing results of CT supplementation in the reduction of worm burdens[3]. However, only limited research has assessed the importance of the chemical characteristics of purified CTs in their potential anti-parasitic properties. Here, we thus purified and characterized CT by novel chemical techniques to evaluate bioactivity of these compounds *in vitro* and *in vivo*.

MATERIALS & METHODS

Purification methods involved sephadex separation and semi-preparative liquid chromatography[1]. We characterized each samples by UPLC-MS/MS to assess mDP and PC/PD ratios (%). Samples derived from alpine currant (*Ribes alpinum*), grape pomace (*vitis vinifera*) and cocoa were obtained and initially screened for their bioactivity in LPS-activated macrophages. Their capacity to reduce secretion of the pro-inflammatory interleukin (IL)-6 was measured by ELISA. Transcriptional profiling was conducted by the analysis of extracted RNA from stimulated cells.

Animal studies were conducted with C57BL/6 mice, which were dosed on alternate days with 200 mg/kg BW PC-rich CT diluted in 200 µl or water alone for 10 days (n = 5 mice per treatment groups). Another mouse study was conducted with mice dosed with either 200 mg/kg BW PC-rich CT (medium mDP) or water, on alternate days for 4-weeks and infected with *H. polygyrus*. Necropsy was performed 2 week post infection (n = 5 mice per treatment groups).

RESULTS & DISCUSSION

The relative mean degree of polymerization (mDP) of the purified samples varied between 1.7 and 32.3. Samples derived from cocoa had the lowest mDPs and the CT were mostly PC-rich. On the

other hand, higher mDPs were observed in CT samples derived from both alpine currant and grape, which were generally rich in PDs and PCs, respectively. Interestingly, samples derived from grape pomace and alpine currant showed higher activity than samples derived from cocoa in reducing the secretion of the pro-inflammatory cytokine IL-6 in LPS-activated macrophages. Transcriptomic data from LPS-activated macrophages demonstrated that cells stimulated with PC-rich CT with a medium mDP caused substantial transcriptional changes, including downregulation of inflammatory genes and pathways. Conversely, gene expression in macrophages stimulated with samples with lower or higher mDP did not markedly differ from control cells stimulated with LPS only.

Consistent with our *in vitro* results, transcriptomic data from murine ileum tissues demonstrated a pronounced effect of medium mDP PC-rich CT while this was not observed in mice dosed with low mDP PC-rich CT. The genes that were significantly regulated were involved in diverse biological pathways including lipid metabolism and inflammatory responses. These findings suggest that mDP plays a key role in the bioactivity of PC-rich CT in mice. Finally, we assessed the anthelminthic effect of PC-rich CT in mice infected with *H. polygyrus.* However, no impact on worm burdens was observed in mice infected with *H. polygyrus* and dosed with PC-rich CT. These findings suggest a strong potential of bioactive diets in reducing inflammatory responses. Although previous studies have indicated anti-parasitic properties of CT *in vitro,* these effects were not confirmed in parasitized mice. Further research should focus on the cellular mechanisms of how CT modulate inflammation and metabolism in different models of infection and immunity.

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O2.10

Beer effects on biochemical outcomes and gut microbiota: Alcoholic vs non-alcoholic

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MAIN CONCLUSION

Our data obtained from a 4-week randomized, double-blinded, three arm parallel-group trial show that both alcoholic and non-alcoholic beer (0.0%) increase gut microbiota diversity, suggesting an effect of beer polyphenols. Beer and non-alcoholic beer did not change weight, BMI and cardiometabolic markers. Given the absence of ethanol, non-alcoholic beer (0.0%) may be safe to be consumed as part of a balanced diet, as a source of polyphenols, B-complex vitamins, minerals and prebiotics.

INTRODUCTION

Beer, a fermented extract of malted barley grains, is the most widely consumed alcoholic beverage in the world. The consumption of low to moderate doses of beer is protective against cardiovascular risk, as shown by epidemiologic studies [1].

The ethanol content of a beer varies from 3.5 to 10% w/v [1]. Numerous mechanisms have been proposed to mediate the protective effect of alcohol (ethanol) in cardiovascular disease [2]. Nevertheless, the protective effects of alcohol consumption for ischaemic heart disease and diabetes are offset by monotonic associations with cancer, which reinforces the importance of studying non-alcoholic beer effect.

Beer is the main (and probably the only) source of hop polyphenols in the human diet. Hop confers beer aroma and bitterness and contain interesting amounts of prenylflavonoids, namely xanthohumol [3]. Several preclinical studies suggest that xanthohumol lowers the risk of development and the progression of oxidative stress-related diseases, such as chronic diseases, including obesity and diabetes [4]. During the brewing process, xanthohumol undergoes a ring-closing reaction, being converted (isomerized) into isoxanthohumol which also has biological activity [3].

Limited data are available concerning the human bioavailability of beer polyphenols, but similarly to other phenolic compounds, they might reach the gut and modulate bacterial growth. The Flemish Gut Flora Project has shown that beer consumption is a key influence on the overall microbiota composition. Therefore, given the importance of the gut microbiota in the pathophysiology of obesity, cardiovascular disease and diabetes, gut microbiota modulation might constitute another mechanism mediating the effects of beer on health [5].

The aim of this pilot study was to evaluate the effect of beer with alcohol (5.2%) and without alcohol (0.5 and 0.0%) on metabolic markers and gut microbiota composition, in healthy men.

MATERIALS & METHODS

Healthy volunteers were recruited and signed their written informed consent (Approved by the Ethics Committee of NOVA Medical School and clinicaltrials.gov NCT03513432).

A 4-week randomized, double-blinded, three arm parallel-group pilot trial aimed to study the effect of different beers on biochemical outcomes and gut microbiota. Participants were assigned into one of the intervention groups (ratio 1:1:1), that daily consumed 330 ml beer with 0.0% alcohol (Group A, n=9), 330 ml beer with 5.2% alcohol (Group B, n=10) or 330 ml beer with 0.5% alcohol (Group C, n=7) during 4 weeks. At the beginning and end of the intervention, blood and faecal samples were collected, body composition was evaluated and adhesion to Mediterranean diet and ethanol consumption were evaluated.

Biochemical parameters were determined using standard protocols in an official clinical laboratory. Microbiota was characterized using 16S rRNA gene sequence. Statistical analysis was performed using SPSSV.23 software.

RESULTS & DISCUSSION

Study participants were healthy men, mean age of 34 years (range: 19–58 years). Baseline characteristics of study participants in each group did not significantly differ at baseline (P>0.05).

Drinking alcoholic beer (5.2% alcohol) or non-alcoholic beer (0.0% or 0.5% alcohol) daily for 4 weeks did not change body weight, body fat mass neither changed biochemical parameters. As a source of phenolic compounds, B-complex vitamins and minerals, beer, especially non-alcoholic beer, may be included as part of a balanced diet. Hepatic transaminases were not changed but alkaline phosphatase (ALP), a marker of hepatic, kidney or bone injury was decreased after 4 weeks of daily beer intake, independently of beer ethanol content (74.6±15.1 to 71.4±13.1, P=0.028 and from 84.5±22.7 to 78.6±17.4, P=0.015 and from 92.4±25.0 to 83.9±24.8, P=0.028). “Lager” type beer has showed a strong inhibitory effect on ALP activity in vascular smooth muscle cells. Also, in volunteers drinking alcoholic beer, serum potassium levels were increased whereas sodium levels were decreased. The effect on potassium was significantly different from that caused by non-alcoholic beers. Nevertheless, the levels were still within the reference values for adults.

Alcoholic beer and non-alcoholic beer 0.0% increased the bacterial diversity revealed by Shannon diversity index, from 2.7±0.3 to 2.9±0.3, P=0.037 and from 2.8±0.2 to 3.0±0.2, P=0.021, respectively. Beer includes a range of polyphenols such as flavonoids and phenolic acids and is the richest dietary source of isoxanthohumol which may modulate bacterial growth. *Prevotella*, *Bacteroides* and *Faecalibacterium* were the main features associated with the principal components that explain the variation of the gut microbiota.

Alcohol consumption has been shown to decrease bacterial diversity [6]. Nevertheless, since the consumption of alcoholic beer increased bacterial diversity, beer polyphenols seem to surpass the effect of alcohol on this outcome.

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O2.11

Interactions between trans-resveratrol and CpLIP2 lipase/acyltransferase: evidenced by fluorescence and in silico

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MAIN CONCLUSION

We have examined the *trans*-resveratrol/lipase interaction by analyses of fluorescence spectra, molecular docking and quantum-chemical calculations. Spontaneous interactions are confirmed by a strong redshift with a major contribution of tryptophan residues and with binding energy of -24 kJ/mol. *trans*-resveratrol competitively inhibited CpLIP2 activity. Molecular docking and quantum-chemical calculations were consistent with a strong binding of *trans*-resveratrol to the CpLIP2 catalytic site.

INTRODUCTION

Trans-resveratrol, a bioactive polyphenol of the stilbene class is found in many fruits, has been studied extensively over decades. A lipolytic activity has been shown to be a factor involved in the virulence and the pathogenicity of some fungi and bacteria [1]. Two putative lipase genes have been identified in *C. parapsilosis* CBS 604 [2], but only CpLIP2, codes for an active protein, with lipase/acyltransferase activity [3]. Secreted lipase activity has been demonstrated to be involved in the mechanism of infection by some *C. parapsilosis* and *C. albicans* strains [3]. Here, we have explored the *trans*-resveratrol/lipase interaction in aqueous medium by analyses of emission fluorescence spectra. Molecular docking and quantum-chemical calculations at the DFT level were performed to support the analysis of the interaction and inhibition. Understanding the interaction between *trans*-resveratrol and CpLIP2 could support to develop the screening of efficient stilbenes for target proteins.

MATERIALS & METHODS

Stock solutions of CpLIP2 (2 µM) and *trans*-resveratrol (0 to 45 µM) in buffer at pH 6.5 were incubated for 1 h. Emission fluorescence spectra were scanned from 285 nm to 500 nm with an excitation wavelength of 280 nm. Synchronous spectra were measured by keeping the deviation between excitation and emission monochromators ($\Delta\lambda = \lambda_{emission} - \lambda_{excitation}$) at 15 nm and 60 nm. *trans*-resveratrol was dissolved in absolute ethanol, and then mixed to buffer to obtain 1.6% (v/v) ethanol in the final mixture. Each microtube contained 2 - 10 µM 4-methylumbelliferyl acetate (4-MuAc), 0.05 µM CpLIP2 and *trans*-resveratrol used at different concentrations of 0, 1.67, 6.67, 13.34 (µM). The fluorescence emission spectra were recorded at 440 nm (λexcitation = 310 nm) at 90 s intervals. Quantum chemical calculations of the interaction were based on the density functional theory (DFT). Molecular docking was performed with Autodock Vina v1.1.2 using the UCSF Chimera v1.13.1 [4] interface.

RESULTS & DISCUSSION

The interaction of CpLIP2 and *trans*-resveratrol were characterized by the fluorescence emission intensity in the presence of *trans*-resveratrol from 0 to 45 µM. The fluorescence quenching rate is proportional to the *trans*-resveratrol concentrations. The binding process of *trans*-resveratrol to CpLIP2 involves both the dynamic and the static quenching. The fluorescence quenching of

tryptophan was higher than that of tyrosine by about one order of magnitude indicated that interactions seem to result in strong conformation changes of 7 tryptophan residues. The distance between CpLIP2 and *trans*-resveratrol by the Förster theory was calculated to be 29Å to 32Å.

The thermodynamic process through the fluorescence quenching and the effect of temperature on the binding constant from 0°C to 35°C were investigated. D*H, DS, and* DG were calculated to be -8.5 kJ/mol and 54.19 J/mol and from -23.3 kJ/mol to -25.2 kJ/mol, respectively.

We have studied its effect on the kinetics of hydrolysis of substrate, 4-methylumbelliferone acetate into the product, 4-methylumbelliferone, at pH 7.0 and 30 °C. The calculated results are compatible with a competitive inhibition model, with a constant value of V_{max} around 0.50 mM/s and a slight variation of K_M with *trans*-resveratrol concentration.

A molecular docking study was performed using the CpLIP2 structure model equilibrated in a water box at 30 °C. *trans*-resveratrol was successfully docked in the active site of CpLIP2. Fig. 1A showed *trans*-resveratrol in the catalytic site of the lipase/acyltransferase. The calculated binding energy was -102 kcal/mol.

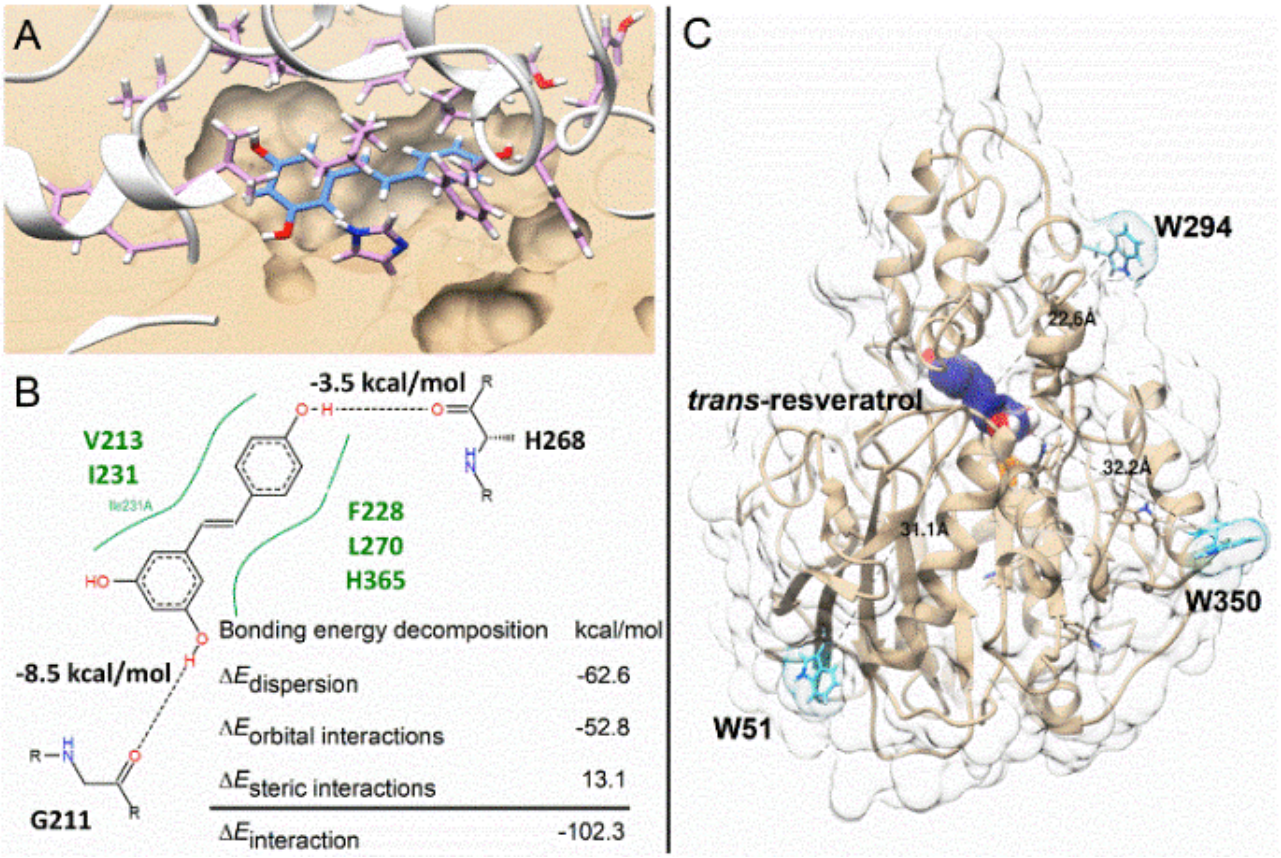

Figure 1. A. trans-resveratrol, in blue, bound to CpLIP2. B. Scheme of the main interactions of trans-resveratrol with CpLIP2 active site residues, energy decomposition was calculated. C. Distance between trans-resveratrol docked in the catalytic pocket of CpLIP2 and the three tryptophan residues exposed to solvent.

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O2.12

Human metabolism of flavan-3-ols: highlights from the EU-JPI project "FOODPHYT- Food phytochemicals matter for cardiometabolic health"

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MAIN CONCLUSION

Flavan-3-ols, arguably the main class of flavonoids in the human diet, are associated with the production of a huge variety of metabolites due to the extensive metabolism to which they are subjected upon consumption. These findings will represent an important achievement in the study of the bioavailability of these key plant bioactives. This work will also help to better understand the bioactivity of flavan-3-ols, as it is strongly correlated to the capacity to produce bioavailable metabolites.

INTRODUCTION

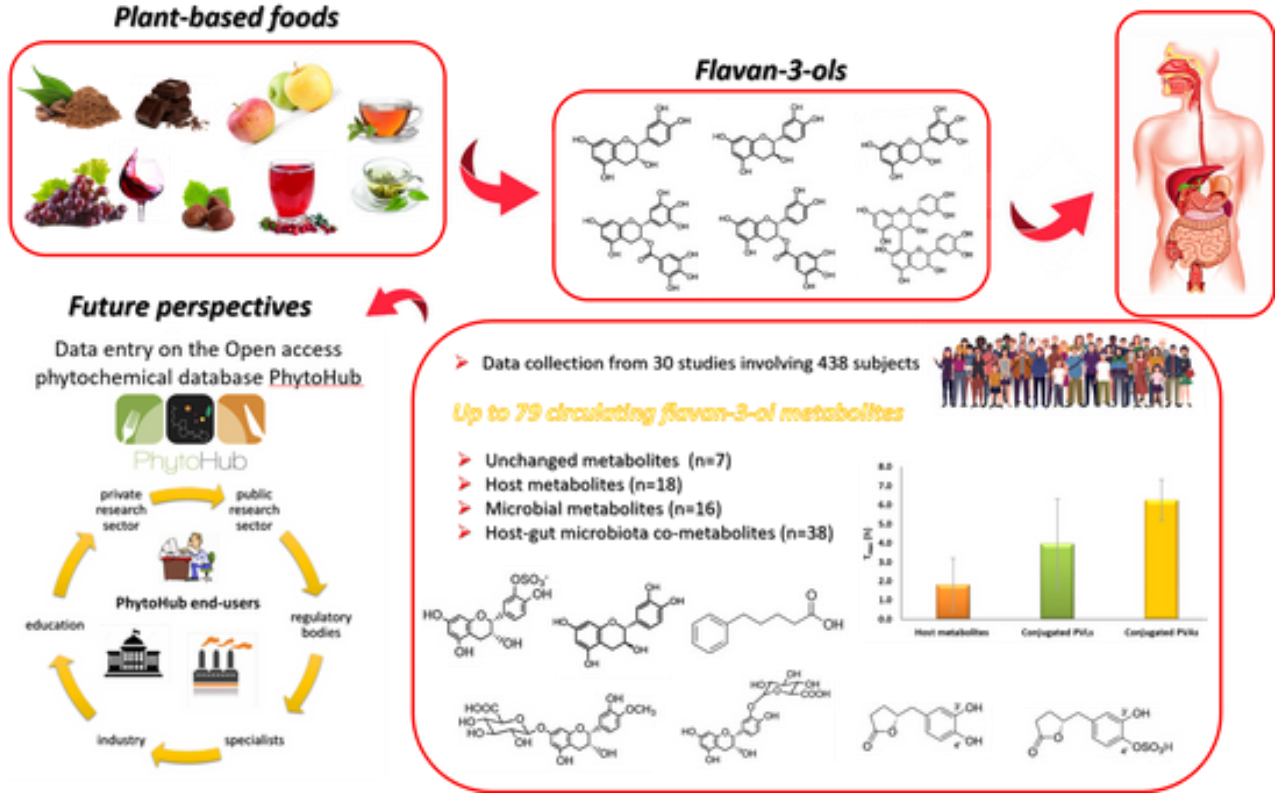
Dietary guidelines worldwide strongly recommend the adherence to plant-based dietary patterns, which provide various beneficial compounds i.e. vitamins, minerals, dietary fiber and hundreds of phytochemicals (PCs). (Poly)phenols are the most widely distributed PCs in the human diet, and among them, flavan-3-ols represent a major class of flavonoids. Flavan-3-ol intake has been associated with positive effects on cardiometabolic outcomes, mainly due to the putative bioactivity of their circulating metabolites [1]. According to their chemical structure, flavan-3-ols may range from simple monomers to oligomeric and polymeric proanthocyanidins (PACs), also known as condensed tannins. Fruits, pulses, nuts, cereals, cocoa and derived-products, spices and some beverages (i.e., green tea, wine) are among the main contributors to the dietary intake of flavan-3-ols in the human diet [2]. After their ingestion, simple flavan-3-ols are rapidly metabolised in the upper gastro-intestinal (GI) tract, yielding to the production of phase-II conjugates [1]. The unabsorbed part of simple and polymerized flavan-3-ols reach undigested the colon, where they undergo an extensive catabolism by gut microbiota, resulting into peculiar 5C-ring fission metabolites (5C-RFMs), namely phenyl-γ-valerolactones (PVLs) and phenylvaleric acids (PVAs) [1]. The present work aimed to collect the available knowledge on absorption, metabolism, distribution and excretion (ADME) of flavan-3-ols through an extensive literature search focused on human studies. This work is inserted in an EU-JPI project named "FOODPHYT- Food phytochemicals matter for cardiometabolic health", which aims at consolidating the available knowledge on metabolism, cardiometabolic health effects and mechanisms of action of dietary PCs and plant-based foods in an open access database named PhytoHub (http://phytohub.eu/).

MATERIALS & METHODS

Literature searches were performed in PubMed, Scopus, and Web of Science. Human studies were properly selected for data collection considering specific inclusion criteria (i.e., healthy subjects, no enzymatic hydrolysis of biospecimens, etc.). Data regarding the I) taxonomy and metabolic origin of flavan-3-ol metabolites, II) biospecimens where metabolites were detected (plasma, serum, or urine), III) sources of ingested flavan-3-ols (food, extract, or pure compound), IV) amount and dose type (single or multiple) of ingested compound(s), V) study characteristics (population and duration), VI) pharmacokinetic parameters (maximum plasma concentration (C_{max}), time to reach Cmax (T_{max}), Area Under the Curve (AUC), and half-life of elimination ($t_{1/2}$)) and urinary excretion (recovery at 24h, excreted amount and time interval) were collected from studies that met inclusion criteria. The existence of potential inter-individual variability in ADME of flavan-3-ols was also investigated.

RESULTS & DISCUSSION

Data on flavan-3-ol metabolites were collected from 30 studies involving a total of 438 subjects. A high heterogeneity in terms of experimental design and consumption manner of flavan-3-ols was found among studies. Grape derivatives (i.e. red wine and red grape pomace), cocoa and dark chocolate, tea (green and black), fruit derivatives (i.e. cranberry, apple, hazelnut skin) and pure extracts were the main dietary vehicles of the ingested flavan-3-ols. Up to 79 plasma and urinary circulating forms of flavan-3-ol metabolites were found. Based on their metabolic origin and molecular weight, metabolites were classified into: unchanged (n=7), host (i.e., phase II metabolites, n=18), microbial (PVLs (n=3), PVAs (n=1) and phenolic acids (PAs, n=12)), and host-gut microbiota co-metabolites (phase-II conjugates of PVLs (n=14), PVAs (n=10), and PAs (n=14)). C_{max} of host metabolites ranged from 4.6 to 3,400 nmol/L (for (+)-catechin-glucuronide and (−)-epicatechin-sulfate, respectively) and varied accordingly to the ingested doses of flavan-3-ols. Similarly, C_{max} of conjugated PVLs ranged from 0.08 to 1,171 nmol/L (for 5-(5′-hydroxyphenyl)-γ-valerolactone-3′-sulfate and 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-glucuronide, respectively), while the C_{max} of PVAs varied from 11.0 to 56.0 nmol/L (for 5-phenylvaleric acid-sulfate-glucuronide and 5-(4′-hydroxyphenyl)-γ-hydroxyvaleric acid-3′-sulfate, respectively). According to the time of appearance of the different metabolites, it should be assumed that they were metabolised in distinct sites along the human GI tract. Indeed, host metabolites reached their C_{max} at about 1.8 h (T_{max}), while conjugated PVLs and PVAs displayed a T_{max} between 4.0 and 6.3 h. A notable variability in qualitative and quantitative profiles of produced metabolites was reported following flavan-3-ol intake. As it is well known, inter-individual differences in phase II enzymes and gut microbiota profile lead to a variation in phenolic metabolism.



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O2.13

Identification of plant dihydrophenanthrenes as direct activators of AMP-activated protein kinase through the allosteric drug and metabolite binding site

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MAIN CONCLUSION

AMP-activated protein kinase (AMPK) is an evolutionary conserved energy sensor that functions to maintain energy homeostasis through coordinating metabolic pathways. We report here that Lusianthridin and Lusianthrin, members of the dihydrophenanthrene and phenanthrene chemical series, can now be added to the short list of natural compounds, including salicylic acid, that directly activate AMPK through the allosteric drug and metabolite (ADaM)-binding pocket.

INTRODUCTION

A plethora of natural plant compounds and their derivatives, including resveratrol, quercetin, berberine, metformin and canagliflozin, have been shown to activate the energy-sensing enzyme, AMP-activated protein kinase (AMPK), raising the possibility that some of their beneficial effects may be mediated by activation of AMPK (Grahame Hardie, 2016; Steinberg and Carling, 2019). AMPK is an enzyme that plays a major role in maintaining cellular homeostasis, and is a potential therapeutic target for treating metabolic diseases, including type 2 diabetes, fatty liver disease and obesity.

However, due to the fact that metformin, canagliflozin and natural plant compounds indirectly activate AMPK, as a consequence of their ability to inhibit mitochondrial ATP production, altering the AMP/ADP:ATP ratio and thus activate AMPK through nucleotide binding to the γ subunit (Hawley *et al.*, 2016; Hawley *et al.*, 2010). As a consequence, it is likely that these compounds have a combination of AMPK-dependent and -independent effects (Steinberg and Carling, 2019). Therefore, the potential role of AMPK in mediating the health-benefits of natural compounds and anti-diabetic drugs has stimulated interest in this enzyme as a therapeutic target for treating various metabolic disorders. The natural plant compound, salicylic acid, was the first natural compound shown to directly activate AMPK through the novel allosteric drug and metabolite (ADaM) site on AMPK. This discovery raises the possibility that other natural plant compounds activate AMPK through the ADaM-binding site. This site is the target of pharmaceutical companies to specifically activate AMPK for treating metabolic diseases and natural compounds (or their derivatives) that share a common mechanism could also be used as therapeutic agents targeting AMPK. Therefore, we conducted a high-throughput screen to identify potential direct natural AMPK activators.

MATERIALS & METHODS

We performed a high throughput screen with a natural pure compound and extract library to identify direct activators of human AMPK (α2β1γ1). We used a range of cellular expression models and mouse primary hepatocytes to elucidate their cellular effects and mechanism of action. There are two key regulatory sites in AMPK that direct activators have been shown to bind to, i) the CBS domains in the γ subunit and ii) the ADaM site located at the interface between the α and β subunits. but its effects were abolished in various AMPK complexes including, i) a β1 S108A mutant, ii) a mutant with a deletion of the CBM of the β1 subunit and iii) β2-containing complexes.

RESULTS & DISCUSSION

We identified a natural plant dihydrophenathrene, Lusianthridin, which allosterically activates and protects AMPKα2β1γ1 from dephosphorylation by binding to the ADaM site and not through the nucleotide binding sites in the γ subunit. Similar to other ADaM-site activators, including A-769662 and 991, Lusianthridin showed preferential activation of AMPK β1-containing complexes in intact cells as well as deficient in its ability to activate an AMPK β1 S108A mutant stably expressed in β1β2 double knockout cells. Lusianthridin dose-dependently increased phosphorylation of acetyl-CoA carboxylase (ACC) in mouse primary hepatocytes and this led to a corresponding decrease in de novo lipogenesis. The ability of Lusianthridin to inhibit lipogenesis was impaired in mouse primary hepatocytes taken from S108A knockin mice and mice with a mutation at the AMPK phosphorylation site of ACC1/2. Finally, we show that activation of AMPK by natural compounds extends to a number of analogues of Lusianthridin as well as the related chemical series, phenanthrenes. The emergence of natural plant compounds that regulate AMPK through the ADaM-site, including the previously reported salicylic acid, raises the distinct possibility that other natural compounds share a common mechanism of regulation. Furthermore, this study provides growing evidence that there may be an, as of yet unidentified, endogenous mammalian metabolite that regulates AMPK through the ADaM site.

O2.14

Ellagitannin-lipid interactions by HR-MAS-NMR spectroscopy

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MAIN CONCLUSION

We studied ET-lipid interactions by HR-MAS-NMR spectroscopy using 12 ETs and pentagalloylglucose as model tannins and determined their interactions with a lipid extract of *Escherichia coli*. HR-MAS-NMR proved to be very suitable for studying these interactions as it tolerates semisolid emulsions. The structure of ET affected significantly its ability to penetrate the lipid bilayer. All ETs that showed interactions with lipids by NMR inhibited the growth of *E. coli* in antimicrobial tests.

INTRODUCTION

Dietary tannins can affect animal nutrition and health in many ways, for example, by improving the uptake of amino acids from feed proteins, by increasing anthelmintic effects against parasitic intestinal nematodes or by lowering gaseous ammonia and methane emissions [1, and references therein]. Tannins also have antimicrobial effects against several pathogens and they have been studied as an alternative for synthetic anthelmintics. In living plants, tannins and macromolecules are usually compartmentally separated but during the plant harvesting, processing and eating, tannins are released from the vacuoles and they will have the opportunity to contact and interact with different macromolecules. Tannins bind proteins and macromolecules and one explanation for some of their observed bioactivities could be their interactions with lipids and membranes, which is so far unknown. Tannin-lipid interactions may play an important role in understanding the capability and the mechanisms with which tannins inhibit, for instance, the growth of different bacteria and their possibilities as antimicrobial agents in general [2, and references therein].

High-resolution magic angle spinning (HR-MAS) NMR spectroscopy has opened new possibilities to study lipids, lipid membranes, and their potential interactions with other compounds [2]. A notable benefit of the HR-MAS probe is that it tolerates the kind of semisolid emulsions that ETs and lipids form in a solution, while still enabling ordinary liquid-state NMR experiments. We studied the interactions of 12 ETs and pentagalloylglucose with a lipid extract of *Escherichia coli* by HR-MAS-NMR. ETs were selected from different branches of the ET biosynthetic pathway and based on their hydrophobicity [3]. The main aims were to study whether there are ET-lipid interactions and if they can be studied by HR-MAS-NMR. In addition, we tested the growth inhibition effect of individual ETs and of pentagalloylglucose on *E. coli*.

MATERIALS & METHODS

The isolation, purification and characterization of selected ETs and pentagalloylglucose followed our previously reported methods including extraction, Sephadex LH-20 funnel and column chromatography, preparative and semipreparative HPLC, UPLC-DAD-ESI-MS/MS and NMR [1-3]. For HR-MAS measurements, the lipid extract/tannin mixture was dissolved in 100 µL of D_2O and subsequently handled via a freeze–thaw method in order for the lipids to form a bilayer (a hazy emulsion). The emulsion was transferred into an HR-MAS insert for HR-MAS-NMR analysis, see details in [2]. We utilized the nuclear Overhauser effect to measure the cross relaxation rates between ET and lipid protons. In addition, the shifting of lipid signals in ^{1}H NMR spectra of ET–lipid mixture due to ring current effect was also observed. The antimicrobial testing of ETs and pentagalloylglucose was performed on Luria-Bertani plates using *E. coli* (APEC46) according to [1].

RESULTS & DISCUSSION

The use of HR-MS-NMR allowed us to study and determine the possible interactions between ETs and lipids [3]. HR-MAS NMR proved to be very suitable for the study of the tannin–lipid mixtures as it tolerates the sort of semisolid emulsions formed in an aqueous measurement environment. The results showed that some ETs were more inclined to penetrate into the lipid bilayer than other ETs. The structure of ET seemed to significantly affect its ability to penetrate the lipid bilayer [3]. All ETs that had interactions with lipids had glucopyranose cores. The highest level of lipid interaction was detected for tellimagrandin I, casuarictin, tellimagrandin II, pentagalloylglucose, sanguiin H-6, and lambertianin C (Fig. 1).

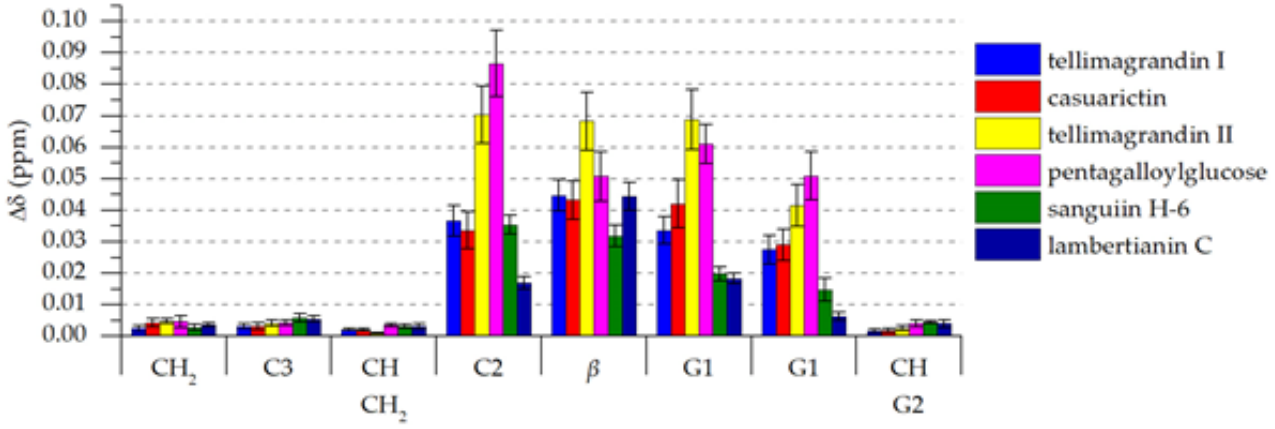

Figure 1. ^{1}H NMR chemical shift deltas (Δδ, ppm) of *E. coli* lipid extracts in the presence of ellagitannins and pentagalloylglucose. The lipid proton assignations refer to Fig. 5 in [2]. Values are presented as Δδ (average values and standard error, n=4).

In addition to the central polyol, the most important structural feature affecting the interaction seemed to be the structural flexibility of the ET. Even dimeric and trimeric ETs could penetrate to the lipid bilayers if their structures were flexible with free galloyl and hexahydroxydiphenoyl groups. The results were well in line with the studied hydrophobicities of these ETs meaning that more hydrophobic ones interacted more with lipids [3].

All ETs that showed interactions with lipids by NMR were also able to inhibit the growth of *E. coli* in antimicrobial tests [1]. There seemed to be a correlation between the molecular size of ETs and the intensity of their antimicrobial activity against *E. coli*. Oligomeric ETs with molecular weight > 1700 Da inhibited over 90% of the *E. coli* growth [1]. In addition, the inhibitory effects of the ET against *E. coli* seemed to be enhanced by additional free galloyl groups.

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O2.15

In vitro bioaccessibility and protective activity of an anthocyanin-rich extract from bilberry and blackcurrant against TNF-α-induced inflammation in intestinal epithelial cells

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MAIN CONCLUSION

This study demonstrates that anthocyanins (ACNs) exert in vitro intestinal protective effects against proinflammatory stimuli-induced cell damage, also at concentrations physiologically achievable following dietary supplementation, as demonstrated by the bioaccessibility findings. These data confirm that ACNs could provide a wide contribution to intestinal health when introduced through the diet or as a food supplement, and thus could represent a possible approach for the prevention of IBDs.

INTRODUCTION

Epidemiological studies suggest that the consumption of food rich in anthocyanins (ACNs) is associated with a reduced risk of different diseases, and support their beneficial effects in various chronic inflammatory diseases, such as inflammatory bowel diseases (IBDs). Indeed, different studies have demonstrated their antioxidant activity, mostly through the scavenging of free radicals, and antiinflammatory properties, thanks to the inhibition of NF-κB pathway or through activation of the Nrf2-mediated adaptive response (Speciale et al., 2011). In vivo studies have reported that a diet rich in polyphenols is associated with a lower risk of IBD (Tian et al., 2017). In line with this, other studies demonstrated that the ACNs can positively modulate tight junctions, and thus reduce intestinal permeability.

The aim of this work was to evaluate the in vitro beneficial effects of ACNs in counteracting the alterations caused by TNF-α in an in vitro model of acute intestinal inflammation to confirm their role in the prevention of gut pathological conditions associated with inflammation and oxidative stress. Nevertheless, the bioavailability of these substances is reported to be very low, mainly due to poor stability during gastrointestinal digestion. It has been in fact reported that the bioavailability of ACNs is very poor, ranging from 0.5-1% of the ingested part (McGhie and Walton, 2007).

Therefore, with the aim to have a more realistic knowledge about the potential protective effect of ACNs, an in vitro simulated gastrointestinal digestion of a purified and standardized bilberry and blackcurrant extract rich in ACNs was performed. We further studied the in situ effect of the intestinal phase of the simulated digestion on the in vitro model of intestinal inflammation above described. In particular, we assessed NF-κB proinflammatory pathway and the activation of the antioxidant and detoxifying response modulated by Nrf2 pathway.

MATERIALS & METHODS

The blackcurrant and bilberry extract (BBE) used herein is an ACNs-enriched dietary supplement consisting mainly of 17 purified ACNs. In vitro gastrointestinal digestion experiments were carried out with the method described by Minekus et al. (2014). Total anthocyanins content was evaluated by the pH differential method. ACNs profiles were determined by HPLC-DAD. The antioxidant activity was determined by the FRAP assay.

The anti-inflammatory activity of the intestinal digested BBE was evaluated in an in vitro model of acute intestinal inflammation using Caco-2 cells. Since we aimed to study the effects of physiological intestinal concentrations, Caco-2 were pre-treated for 24 h with low concentrations of the BBE intestinal phase (0.18-1.5 µg/mL expressed as C3G), and then exposed to TNF-α for 6 h. Inflammatory pathway and adaptive cellular response were studied in Caco-2 cells evaluating NF-κB and Nrf2 pathways.

RESULTS & DISCUSSION

With the aim to evaluate ACNs stability after gastrointestinal digestion, a static in vitro digestion of a purified and standardized BBE was performed. After each step we analyzed the total amount of ACNs by the differential pH method and a Recovery Index (RI) of digesta vs sham digested sample was evaluated. The bioaccessibility results, obtained through HPLC-UV/Vis analysis, confirmed the high instability of anthocyanins in the mild alkaline environment of the small intestine, reporting a 13% of recovery index. This high loss in anthocyanins corresponded to the decrease in the reducing power observed by the FRAP assay. The Pearson's correlation coefficient showed a strong correlation between ACNs content and FRAP for the undigested extract, as well as for the extracts undergone oral and gastric digestion. However, it evidenced a weak correlation between FRAP assay and total anthocyanins content of the GI digested, suggesting that newly formed metabolites could probably contribute to the antioxidant power.

Moreover, although the high loss of anthocyanins, the digested product maintained part of its bioactivity in the in vitro Caco-2 model. BBE-IP pretreatment dose-dependently prevented TNF-α-induced nuclear translocation of p65 and significantly reduced the level of IL-6 and OL-8 gene expression, and the highest concentration tested was able to restore it to levels similar to those found in control cells.

Moreover, treatment with BBE-IP was able to increase Nrf2 translocation in TNF-α-exposed Caco-2 cells. In addition, BBE-IP alone was able to induce Nrf2 pathway. Cells pretreatment with BBE-IP was also able to induce NQO-1 gene expression, and this effect was still evident even in cells exposed to TNF-α. Taken together, these results support the hypothesis that Nrf2 signaling activation is involved in BBE-IP protective effect on epithelial inflammation induced by TNF-α.

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O2.16

Silymarin flavonolignans: news about their bioactivity, bioavailability and safety

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MAIN CONCLUSION

Although silymarin is used for centuries and studied for decades, new bioactivities are still being discovered for its components. Moreover, individual optically pure silymarin (2,3-dehydro)flavonolignans displayed distinct biological activities and metabolic profiles in an array of models. Therefore, in further studies, it is imperative to work with (optically) pure flavonolignan components or (in vivo) with well-defined silymarin preparations.

INTRODUCTION

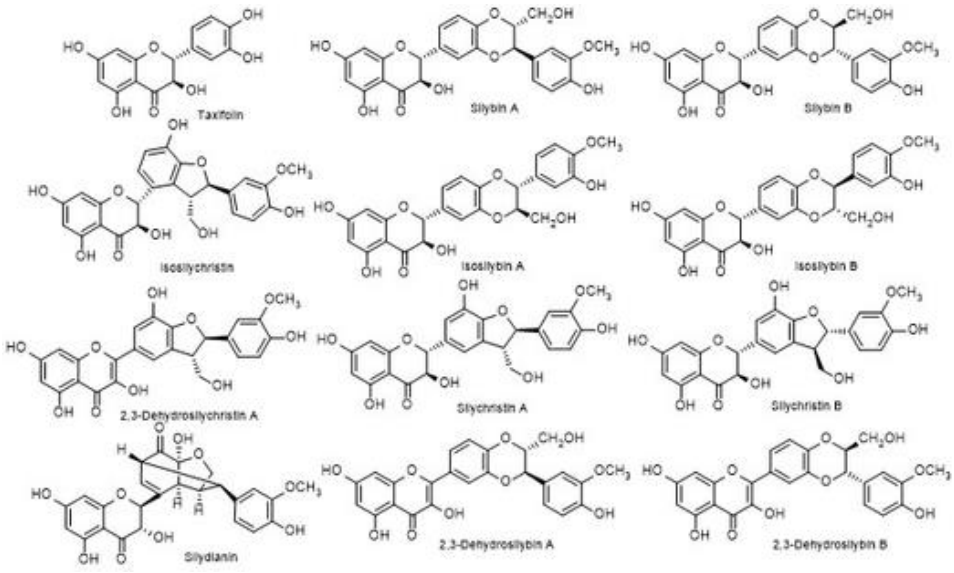
Silymarin, an extract from the fruits of the milk thistle (*Silybum marianum* (L.) Gaertn, Asteraceae) displays various biological activities such as hepatoprotective, neuroprotective, cardioprotective, antioxidant, anti-inflammatory, immunomodulatory, estrogenic, antidiabetic, antiviral, antiparasitic, anticancer, and multidrug resistance modulatory.

Silybum marianum (L.) Gaertn

Depending on the plant cultivar and extraction method used, silymarin consists of a mixture of structurally closely related flavonolignans (flavonoids with fused lignan part) and 2,3-flavonolignans together with their biogenetic precursor taxifolin (flavanonol). The main silymarin constituents are silybin (silibinin) A, silybin B, isosilybin A, isosilybin B, silychristin A, silydianin, taxifolin together with minor flavonolignans isosilychristin, silychristin B, 2,3-dehydrosilybin A, 2,3-dehydrosilybin B, 2,3-dehydrosilychristin A and approximately 30% of undefined polymeric polyphenolic fraction[1]. The composition of silymarin is highly variable, depending on the source of plant material (chemoraces), agrotechnical and climatic conditions, technology, extraction procedure, and many other factors. Bioactivities of silymarin are attributed mostly to silybin (and mostly the mixture of its diastereomers A and B) as it is the most abundant and the most easily obtainable silymarin component. However, other flavonolignans and 2,3-dehydroflavonolignans can be specifically responsible for some activities. Recently, a new preparatory separation method using Sephadex LH-20 was developed, which provides multigram amounts of pure silychristin A, silydianin, and a fraction containing silybin and isosilybin. Moreover, the methods for the preparation of 2,3-dehydroflavonolignans were optimized. The present contribution aims to compare the biological activities of pure silymarin components in various models.

MATERIALS & METHODS

Silybin A, silybin B, silychristin A, and silydianin were isolated from silymarin (Liaoning Senrong Pharmaceutical, China). 2,3-Dehydrosilybin, 2,3-dehydrosilychristin and 2,3-dehydrosilydianin were prepared from respective flavonolignans by optimized oxidative methods in the presence of organic bases. Sulfated flavonolignans and 2,3-dehydroflavonolignans were prepared using arylsulfotransferase from *Desulfitobacterium hafniense* heterologously expressed in *E. coli* and using *p*-nitrophenyl sulfate as sulfate donor. Their 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and *N*,*N*-dimethyl-*p*-phenylenediamine (DMPD)



Structures of silymarin components.

radical scavenging; ferric (FRAP) and Folin–Ciocalteu reagent (FCR) reducing; anti-lipoperoxidant; metal chelating, cytotoxic, cytoprotective, anticancer, anti-inflammatory, pro-longevity, and multidrug resistance modulating activities, but also metabolism and bioavailability of silymarin components were measured and compared.

RESULTS & DISCUSSION

All the compounds were successfully prepared in multimiligram up to gram amounts, enabling a detailed study of their properties. 2,3-Dehydroderivatives and their sulfated metabolites were more efficient antioxidants, anti-lipoperoxidant, and cytoprotective agents than major silymarin flavonolignans[2]. 2,3-Dehydrosilybin and 2,3-dehydrosilychristin chelated iron and copper ions and intensified hydroxyl radical production *via* Fenton reaction, but they both were strongly protective against the copper-triggered lysis of red blood cells. 2,3-Dehydroisosilybin inhibited *Leishmania* promastigotes and 2,3-dehydrosilydianin activated nuclear factor erythroid 2–related factor 2 (Nrf2) and upregulated NAD(P)H quinone dehydrogenase 1 (NQO1). Silychristin, 2,3-dehydrosilychristin, silybins, and 2,3-dehydrosilybins A and B all inhibited P-glycoprotein (P-gp) and sensitized doxorubicin-resistant ovarian carcinoma cells, but the mechanism of P-gp inhibition differed[3,4]. Various flavonolignans and their sulfates had vasodilatory properties, silychristin-A-19-*O*-sulfate was the most potent[5]. 2,3-Dehydrosilybin modulated inflammation in dermal fibroblasts, exhibited UVA protection, displayed anti-collagenase, and anti-elastase activity. It also displayed lifespan-extension in *Caenorhabditis elegans*, inhibited basal cell carcinoma allograft growth, and affected bilirubin concentrations in mice. Also, bioavailability and metabolism of individual flavonolignans differ; silybin diastereomers A and B have different metabolic profiles in rat plasma. Membrane permeability (mimicking gastrointestinal absorption) and dermal delivery of silybin, 2,3-dehydrosilybin, and isosilybin were higher than that of taxifolin, silychristin, and silydianin. Biotransformation of individual silymarin components by human liver microsomes and fecal microbiota was compound- and subject-dependent[6].

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O2.17

Polyphenol-bearing probes for unveiling polyphenol-proteins interactions: Synthesis and applications

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MAIN CONCLUSION

We developed an experimental protocol relying on the use of polyphenol-bearing probes to identify proteins targeted by the ellagitannin vescalagin in bone cells, more specifically in osteoblasts and osteoclasts, through proteomic and bioinformatic analyses. This methodology is suitable for the utilisation of other polyphenol-bearing probes and lysates from other cell types towards a better understanding of the interactions between polyphenols and proteins.

INTRODUCTION

Polyphenols are known to be beneficial to human health. Their properties are not only due to their activity as antioxidants, but also to their abilities to bind to proteins.[1] However, the nature of the proteins involved and the mechanisms by which these molecular interactions translate into positive biological responses remain obscure. Numerous studies have shown the implication of different polyphenols (*e.g.*, resveratrol or epigallocatechin gallate (EGCG)) as inhibitors or activators of enzymes and/or antagonists or agonists of cell receptors. However, the use of polyphenols as therapeutics is still decried by the scientific community, notably because of their lack of selectivity. Nonetheless, the multi-target nature of polyphenols make them interesting candidates to consider in the search for compounds capable of achieving specific biological effects through interactions with different target proteins. We developed a proteomic method based on the use of polyphenolic probes to explore the interaction of polyphenolic compounds, here the ellagitannin vescalagin, with proteins extracted from cells, here osteoclastic and osteoblastic bone cells.

MATERIALS & METHODS

A biotinylated vescalagin-bearing probe was synthetised in 6 steps from purified vescalagin and commercially available compounds in a 18 % overall yield. This polyphenolic probe was incubated with lysates from bone cells. An oxidation of the pyrogallolic units of the vescalagin-derived moiety is performed using sodium periodate to create covalent bonds with proteins.[2] The biotinylated polyphenol-protein complexes are isolated from the reaction mixtures using streptavidin magnetic beads, further processed and analysed using electrophoresis (*e.g.*, SDS-PAGE gels and western blots), and shotgun proteomics. The resulting data sets were finally submitted to bioinformatic analyses to afford a list of statistically significantly captured proteins.

RESULTS & DISCUSSION

The high number of proteins obtained after LC-MS/MS analysis were submitted to different statistical filters to highlight a shorter list of proteins. Theses filters consider two different controls used during the protein capture and pull-down process, which were the absence of probe and the absence of oxidant in order to disregard proteins non-specifically captured by the polyphenol. Proteins that were

considered as significant targets for the polyphenol vescalagin were bioinformatically examined through different approaches, and notably through biological network analysis using data from GWAS (*i.e.*, genome-wide association study), in the aim of revealing their potential correlation with the development of diseases, here osteoporosis. Through this methodology, we highlighted a pool of proteins which are thus, in one hand, specifically interacting with the polyphenol vescalagin, and on the other hand, potentially involved in the development of the bone disease osteoporosis.

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O2.18

Boosting the bioaccessibility of dietary polyphenols by delivery as colloidal aggregate protein-polyphenol particles

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MAIN CONCLUSION

When flavonoid-rich fruit extracts are complexed with plant proteins to create stable, colloidal protein-polyphenol particles (powders), the bound (microencapsulated) flavonoids are effectively protected through gastrointestinal digestion *in vitro*, reaching the lower GIT intact. As a result, the bioaccessibility of bioactive phenolic metabolites is significantly enhanced. Digestibility of the protein component of colloidal particles may be decelerated *or* hastened, depending on protein source.

INTRODUCTION

It was an enigma to reconcile the diverse health benefits of dietary flavonoids with their (apparently) poor bioavailability, until the fairly recent revelation that most (>90%) are not actually absorbed in the upper GIT, but instead are biotransformed by commensal intestinal microbiota into smaller molecular weight phenolic and aromatic catabolites [1]. While some deglycosylation and chemical degradation occurs in the small intestine, it is the colonic catabolites that subsequently enter circulation in sufficiently high concentrations to exert health-relevant bioactivities.

Flavonoids that ultimately reach the colon have a dual impact on human health. First, as prebiotics, they have a dramatic influence on the composition and the functionality of the gut microbiome. Ingestion of prebiotic flavonoids favorably alters gut microbial community structure, supporting beneficial commensal bacteria and reducing levels of opportunistic species [2]. Prebiotic flavonoids and their metabolites influence inflammation in the gut, improving the epithelial barrier's integrity and activating tight junctions. Interestingly, the flavonoids do not even need to be absorbed to exert these benefits; as xenobiotic compounds they induce cellular stress and an overcompensation reaction to maintain homeostasis, producing a hormetic response that improves cell and barrier function.

Second, the biotransformation of dietary flavonoids by the intestinal microbiota drives the ultimate delivery of active metabolites into circulation where they elicit health-protective mechanisms [3]. The bioavailability of these flavonoids is largely dependent on their catabolism by the gut microbiome and subsequent secondary xenobiotic biotransformation in the liver before entering circulation in the form of phenolic and aromatic metabolites.

This work examines bioaccessibility & digestibility of both the flavonoid and the protein components in stably bound protein-polyphenol colloidal aggregate particles.

MATERIALS & METHODS

Plant materials: cranberry (Ocean Spray), wild blueberry (Wyman's), muscadine grape (Muscadine Products) pomaces, pea protein isolates (Axiom & Roquette), rice protein concentrate (Nutra Food).

Protein-polyphenol aggregation: 10% (w/v) protein mixtures complexed with pomace extracts, wet solid pelleted material retrieved. Total phenolics (TP) determined spectrophotometrically, anthocyanins (ANC) by HPLC-DAD and proanthocyanidins (PAC) by DMAC method. Aggregate particles evaluated in terms of radical scavenging activity and anti-inflammatory bioactivity.

In vitro gastrointestinal digestion: a static method performed with simulated salivary fluid, gastric fluid, and intestinal fluid, in sequence, and recovery index for TP, ANC and PAC determined. For

protein digestibility, aliquots were removed hourly for amine quantification, increase in free amino groups in the supernatant due to digestion was measured, and SDS-PAGE determined solubility of protein-polyphenol particles.

RESULTS & DISCUSSION

Abundant residual flavonoids trapped in fruit pomaces were efficiently recovered and repurposed as food ingredients by complexing them with plant protein isolates. In an in vitro gastrointestinal model, the bioaccessibility of three berry pomace extracts (blueberry, cranberry and muscadine grape), measured as percentage of input, were 25%–35% for total phenolics, 14%–45% for proanthocyanidins, and 6%–20% for anthocyanins. After complexation with rice and pea protein isolates, the bioaccessibility increased to 47%–75% for total phenolics (about 3-fold higher for blueberry, 1.5-fold higher for cranberry, and 2-fold higher for muscadine grape), 56%–71% for proanthocyanidins (about 2-fold for blueberry, 1.5-fold for cranberry, and 2-fold for muscadine), and 28%–52% for anthocyanins (about 8-fold for blueberry, 1.4-fold for cranberry, and 3-fold for muscadine). Pomace-derived flavonoids were protected in the colloidal aggregates during simulated digestion, affording them a higher level of bioaccessibility (and bioactivity) compared to flavonoids without a protein carrier.

The effect of protein-polyphenol particle formation on protein digestibility was dependent on the protein source – soy protein digestibility was slightly enhanced (faster digestion rate) in bound particles, as was peanut protein digestibility, while complexation with polyphenols slowed the digestion rates for pea proteins (both pepsin and pancreatin simulated digestions), especially when higher concentrations of polyphenols were stably bound [5]. Since the health benefits of ingested flavonoids greatly depend on their stability and structural integrity during transit through the gastrointestinal tract (GIT), successful release in the lower GIT and the subsequent absorption of microbial metabolites into circulation, delivery of these compounds in the form of protein-polyphenol colloidal ingredients can potentially heighten bioactive metabolite bioaccessibility.

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O3.1

Comparison of different extraction techniques to determine the phenolic compound concentration in olive mill waste water

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MAIN CONCLUSION

Among the literature, the most favoured method of determining phenolic content of OMWW is through the use of ethyl acetate extraction with hexane defatting. Our study targeted 25 phenolic compounds and their isomers and it was found that with freeze drying and resuspension of the solid in methanol while shaking or ultrasonification, or acidification with ultrasonification, much higher concentrations of phenols could be detected.

INTRODUCTION

Bio-based phenolic compounds are the subject of increasing scientific interest because of their possible beneficial effects on human health from a renewable source. One such source of bio-based phenolics is found in the olive oil production industry. During three-phase olive oil production olive oil, olive pomace, and olive mill wastewater (OMWW) are generated. Olive oil is the principal fat source of the traditional Mediterranean diet and due to its high content of polyphenols, has been associated with numerous beneficial-human health properties [1]. However, only 2% of the total phenolic content of the milled olive fruit goes to the oil phase, while most resides in the liquid OMWW (≈53%) and solid pomace (≈45%) [2]. In this way, OMWW can act as a cheap source of valuable phenolic compounds which have been investigated by several research groups using different techniques to recover and utilize them as source in natural food additives, pharmaceuticals, and cosmetics [3].

Prior to phenolic compound recovery, the quantity and identification of phenols present in OMWW must be determined. Various procedures to determine the phenol content in OMWW have been studied, but most rely on maximizing the recovery of one compound, hydroxytyrosol, and thus the complexity of the biophenols may be underrepresented. The analysis of phenols in OMWW generally follows similar methods as for phenols from other sources. However, the fact that OMWW is the result of a process that breaks cell walls and exposes the matrix to enzymes, oxygen, and mild heat (during malaxation of the paste) means that the phenol profile undergoes significant changes prior to sampling. This creates a challenge in stabilising the profile at the point of sampling to minimize further changes prior to analysis. The objective of this study was to assess various OMWW extraction methods and their effect on the identification of phenolic compounds.

MATERIALS & METHODS

OMWW samples were collected from the Oljarna Krožera Franka Marzi olive oil mill (Srgaši, Slovenia) from a three-phase decanter centrifuge. Immediately after sampling, OMWW samples were stored in a freezer (-18 °C) prior to analysis.

Samples were treated via different methods to extract the phenolic compounds. These were characterized by high-performance liquid chromatography (HPLC) coupled to electrospray ionisation and quadrupole time-of-flight mass spectrometer (ESI-QTOF-MS). HPLC equipment incorporated a Poroshell 120 column (EC-C18; 2.7 µm; 3.0 × 150 mm). An elution gradient of 100% water/ formic acid (99.05: 0.5, v/v) (A) towards 100% acetonitrile/ methanol (50: 50, v/v) is used over a period of 20 minutes (flow rate: 0.5 mL/min; injection volume: 1 uL). Extracts were screened for phenolic

compounds previously reported for Olea europaea L. Their presence was confirmed based on accurate mass and fragmentation profiles with literature data and analytical grade standards.

RESULTS & DISCUSSION

Phenols were extracted via different methods:

- Freeze drying of OMWW. Adding dry matter in methanol while shaking. Filter.
- Freeze drying of OMWW. Adding dry matter in methanol:water (1:1) while shaking. Filter.
- Freeze drying of OMWW. Adding dry matter in methanol. Ultrasonification. Filter
- Ethyl acetate liquid-liquid extraction of OMWW
- Filter OMWW. Get filtrate. Dissolve residue in methanol + filter.

Twenty-five phenolic compounds and their isomers were determined. It was found that the ethyl acetate extraction method, which is the most frequent method used in literature to measure phenolic compounds in OMWW detected lower amounts of phenols than other methods, even the filtration method performed better. Acidifying the OMWW didn't lead to a better performance. The highest phenol concentrations were obtained with freeze drying of OMWW and resuspension of the dry matter in methanol via shaking or ultrasonification. Freeze drying of OMWW and resuspension of the dry matter in methanol:water delivered concentrations for certain phenols similar to the filtration technique, for other compounds similar to the freeze drying with methanol resuspension technique.

Also the influence of acidification and ultrasonification was examined. Ethyl acetate liquid-liquid extraction of acidified OMWW didn't result in higher phenol detection than of normal OMWW. Also acidification of OMWW and filtration didn't increase the phenol yield. However, acidification and 5 min of ultrasonification of OMWW gave two to ten times higher phenol concentrations, depending on the phenolic compound.

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O3.2

Hyperglycemia alters the polyphenol metabolome in lipoproteins: putative implications from lipoprotein's lipid environment

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MAIN CONCLUSION

Analysis of lipoprotein populations in diabetic patients and age-matched controls show that hyperglycemia induces significant changes to the cargo polyphenol metabolites in circulation, which is more prominent in triglyceride-rich samples (VLDL) (>50%) but not in cholesterol-rich samples (LDL, and HDL). Our findings suggest that changes to lipid composition could play a role in the adsorption of polyphenol to lipoprotein particles.

INTRODUCTION

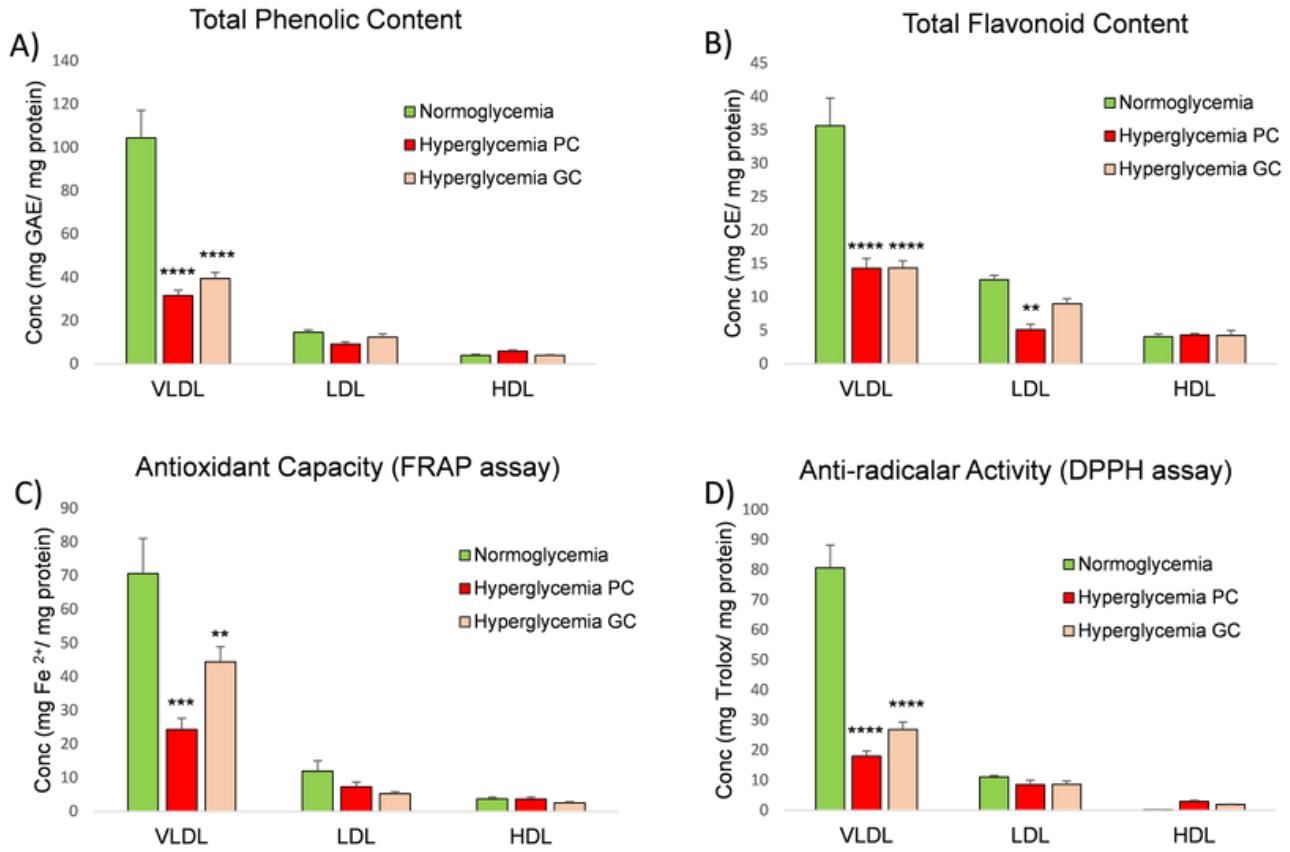
The 20th century brought massive changes to people's eating habits. Processed and ready-to-eat foods became routinely consumed resulting in a sharp rise of sugar intake in people's daily diets. According to the World Health Organization (WHO) over 422 million people have diabetes [1] but even more alarming is the forecasted number of undiagnosed people and the increasing incidence of diabetes among children and young adults [2]. The WHO recommends the inclusion of (poly)phenol-rich fruits, vegetables and nuts typical of Mediterranean- and Nordic-type diets to prevent and manage diabetes and to ease the burden of health-associated costs. While intervention studies have shown that (poly)phenol-rich diets improve fasting glucose levels and other blood parameters in healthy individuals helping to reduce the risk of diabetes complications, very little is known about the polyphenol metabolome in disease and particularly how (poly)phenol metabolites are transported in circulation, as well as the changes to the panel and cargo of circulating (poly)phenol metabolites occurring with disease such as diabetes.

MATERIALS & METHODS

Pooled plasma samples collected from diabetic patients (n=15) before (Poor Glycemic Control, HbA1c > 8.5%) and after drug and diet hypoglycemic treatment (Good Glycemic Control, HbA1c < 7%), and age-matched controls (n=15) were used in this study. Plasma lipoproteins were isolated by salt-gradient ultracentrifugation [3] and purity of lipoprotein populations was confirmed by SDS-PAGE. Lipoprotein lipid and protein content were conducted as described earlier [4]. (Poly)phenol metabolites were extracted from lipoproteins by solid-phase extraction (SPE). Extracts were characterized by UV/Vis methods (total phenolics and flavonoid content); antioxidant assay (FRAP) and anti-radicalar assay (DPPH)). Circulating (poly)phenol metabolites were quantified by targeted MS-approach (SIM) coupled to reverse-phase chromatography (UPLC-MS, LTQ Orbitrap XL) in negative ion mode. Quantification of OxPC was achieved by targeted MRM MS-based approach in positive ion mode, as described earlier [4].

RESULTS & DISCUSSION

It is widely known that ingested (poly)phenols and transported in circulation are responsible for health-promoting effects. In a tentative approach to understand how (poly)phenols are transported in circulation, analysis of extracts obtained from lipoprotein populations (HDL, LDL and VLDL) and lipoprotein-depleted fraction (LPDF) revealed that while (poly)phenol metabolites are transported by both low- (albumins) and high-molecular weight plasma proteins (lipoproteins) data obtained after



normalization of absolute values to the protein content reveals that high-molecular weight proteins (ApoB-100) appear to be the main carriers of (poly)phenol metabolites in normo- and hyperglycemia (data not shown). Spectrophotometric characterization of lipoprotein extracts (total phenolics and flavonoid content, antioxidant (FRAP) and anti-radicalar assay (DPPH)) revealed that (poly)phenols appear to be mainly distributed in VLDL (Figure 1A). Data also shows that hyperglycemia induces a marked decrease of phenolics content in VLDL (>70%) that may be due to changes in flavonoid content (Figure 1B). Interestingly, the content of phenolics and flavonoids is slightly improved upon diet and drug treatment (patients in hyperglycemia good control). The decrease in phenolics and flavonoid content in VLDL with hyperglycemia is accompanied by a decrease in antioxidant capacity (65%) (Figure 1C) as well as anti-radicalar activity (Figure 1D). Targeted quantification of (poly)phenol metabolites in VLDL and LDL populations by UPLC-(SIM)-MS approach confirms the spectrophotometric data showing that (poly)phenol metabolites are heterogeneously distributed among lipoproteins in normoglycemic and hyperglycemic conditions (data not shown). Considering the data obtained and the changes observed to lipid composition (data not shown) we suggest that the lipid environments may impact the adsorption and transport of ingested (poly)phenols in circulation.

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O3.3

Decrypting bacterial polyphenol metabolism in an anoxic wetland soil

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MAIN CONCLUSION

Challenging the idea that polyphenols are not bioavailable under anoxia, we showed metabolite evidence that CT was depolymerized, resulting in (epi)catechin monomer accumulation, followed by the generation of small phenolic degradation products. We identified the enzymes that likely catalyzed these transformations and the microbial genomes that encoded them in our metaproteome. In summary, we provided chemical and enzymatic evidence that some soil microbiota can degrade polyphenols under anoxia.

INTRODUCTION

Condensed tannins (CT) have been implicated as important "latches" on soil carbon stores under fluctuating redox conditions. Specifically, the enzyme latch paradigm posits that polyphenols in peat soils resist microbial degradation under anoxic conditions and accumulate, diminishing microbial activity and consequently biogeochemical processes [1]. This model assumes that polyphenols are microbially unavailable under anoxia, a supposition that has not been thoroughly investigated in any soil type. Here, we used anoxic soil reactors amended with and without a chemically defined CT to test this hypothesis, employing metabolomics and genome-resolved metaproteomics to interrogate soil microbial polyphenol metabolism.

MATERIALS & METHODS

We selected plant-covered, mineral soils from a microbially well-studied temperate, freshwater wetland as a model soil for studying microbial polyphenol degradation. Using these soils as the inoculum, we amended anoxic laboratory microcosms with and without a model polyphenol, Sorghum-purified condensed tannin. Our experimental design included two control treatments: (i) an unamended soil control to discern polyphenol-stimulated responses from native, background soil microbial activity, and (ii) an autoclaved soil control to differentiate microbially-mediated CT degradation from abiotic CT degradation resulting from reactions with the soil matrix. From the triplicate, anoxic soil reactors, 16S rRNA genes, metabolites, and genome-resolved metaproteomes were sampled on days 1, 3, 7, 10, 14, and 20. For more detailed information, see [2].

RESULTS & DISCUSSION

This study provided evidence that the anoxic soil microbiome is capable of polyphenol metabolism that includes depolymerization of a condensed tannin polymer and subsequent monomer degradation. We offered a new multi-omics enabled view of the soil microbiome's response to a high molecular weight polyphenol under anoxia. Together our data support a model in which polyphenols in soils are not as microbially inert as previously claimed [1]. Importantly, our findings provide a new scaffolding that others can leverage. We expanded the definition of soil polyphenol degrading enzymes, and we highlighted canonically aerobic enzymes (i.e., peroxidase) that may play unrecognized roles in anoxic transformations of polymeric carbon. While our study demonstrated that under anoxic conditions the soil microbiome in a freshwater wetland can degrade polyphenols, translating this finding to relevant

field ecosystems like peatlands requires: quantifying the kinetics and environmental constraints of these transformations on the overall carbon budget, expanding research to other relevant polyphenol substrates, and investigating the effects of abiotic and biotic polyphenol transformations associated with diverse soil types. Our findings pave a way for these research avenues, providing metabolite and enzyme framework for mining these processes from complex systems. Collectively, our results highlight the promise of modern soil microbiome technologies for uncovering the ecological and biochemical mechanisms underlying long-held soil biogeochemical paradigms.

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O3.4

Spatiotemporal Modulation of Flavonoid Metabolism in Vaccinium berries

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MAIN CONCLUSION

In bilberry, accumulation of anthocyanins and flavonols proceeds from the exocarp to the mesocarp during ripening and general mechanisms of biosynthesis are synchronised between skin and flesh. In contrast, flavonoid biosynthesis is tissue-specific in blueberries and declines in fruit flesh during ripening. While anthocyanin biosynthesis is dependent on co-expression of multiple structural genes, three enzymes (PAL, CHS, ANS) are postulated to act as bottlenecks for anthocyanin production.

INTRODUCTION

Cultivated blueberries and wild bilberries (*Vaccinium* spp.) are distinguished by their purple-blue colour, which is derived from a characteristic composition of flavonoid-derived anthocyanin pigments. Health benefits have been assigned to these attractive anthocyanins and structurally related polyphenolic compounds, contributing to the 'superfood' status of these berries [1]. Different stages of *Vaccinium* fruit development are commonly based on berry expansion and colour change, and it is well established that anthocyanins accumulate in parallel with the expression of structural genes of the flavonoid pathway [2, 3]. In bilberry, anthocyanins are present in both, skin and flesh while blueberry flesh is usually devoid of these pigments. The genetic mechanisms that regulate flesh colour in *Vaccinium* flesh are largely unknown and we do not know whether anthocyanin biosynthesis in blueberry flesh is limited primarily at final steps within the pathway or results from reduced flavonoid biosynthesis overall. The biosynthesis of flavonoids is regulated at the transcriptional level and distinct control over the expression of biosynthetic genes is regulated by an 'MYB-bHLH-WD40' transcription factor complex [4]. Since our current knowledge is generally based on homogenised whole fruit studies, little is known, about the spatiotemporal biosynthesis of flavonoids across tissue types and distribution patterns within fruit flesh. Understanding how phytochemical accumulation is spatially orchestrated in these tissues over development can provide insights on fundamental physiological responses during ripening. Here, we integrate accumulation patterns of flavonoid-derived metabolites with transcript abundances of their respective structural genes to compare tissue-specific anthocyanin biosynthesis in blueberry and bilberry. We also show, for the first time, the locations of three major classes of flavonoids in bilberry fruit using FT-ICR (Fourier Transform Ion Cyclotron Resonance)-MSI.

MATERIALS & METHODS

An interdisciplinary approach was employed by linking targeted analysis of metabolites and gene transcripts. The commercial blueberry cultivars 'Nui' (*V. corymbosum*) and 'Velluto Blue' (*V. virgatum*) and a North-Norwegian bilberry (*V. myrtillus*) accession were sampled at five developmental stages. For the quantitative analysis of flavonoids in *Vaccinium* tissues, fruit skin was separated from berry flesh while frozen. UHPLC-ESI-QTOF-HRMS was used for analysis of anthocyanins and polyphenols following solvent extraction (ethanol: water: formic acid; 80:20:1). In parallel, RNA was isolated from these tissues and sequenced using the Illumina NovaSeq 6000 platform for gene expression studies. Candidate genes were identified by combination of co-

correlation analyses with metabolites and differential gene expression analysis. In bilberry fruit, FT-ICR-MSI was used to determine the spatial distribution of flavonoids in three distinct ripening stages using both positive and negative ion modes.

RESULTS & DISCUSSION

We found that concentrations of flavonoids were generally comparable between bilberry and commercial blueberries, and higher in skin compared with flesh. In all species, anthocyanins and trihydroxylated flavonols increased rapidly from the time of berry colour change whilst procyanidins were highest in unripe fruit and concentrations declined during ripening. The compound composition, however, was distinct between species. For example, malvidin-derived anthocyanins were prevalent in blueberries but cyanidin-and delphinidin-3-*O*-glycosides in bilberry. The glycosidation pattern was also distinct and dependent on the respective aglycone in bilberry fruit, whilst anthocyanidins were predominantly linked with glucose in *V. corymbosum* and with galactose in *V. virgatum*. In bilberry, anthocyanins and flavonols were present in skin and flesh, and their accumulation trends were synchronised between tissues despite fruit skin accumulating 12-fold higher concentrations of both flavonoids. MSI revealed a polarised distribution for these compounds which altered over development.

The flesh of ripe blueberries was not only devoid of anthocyanins but also of flavonoids in general. A common set of structural genes was identified to drive anthocyanin production across species, reflecting increased transcript abundance in bilberry compared with blueberry flesh. In mature blueberries, gene expression was not detected for enzymes of the phenylpropanoid pathway (PAL, C4'H) and significantly reduced at entry points to the flavonoid pathway (CHS, F3H) as well as anthocyanidin biosynthesis (ANS). Thus creating potential bottlenecks for flavonoid biosynthesis in general. Co-expression of the structural genes with a set of transcriptional regulators, including the activators MYBA, MYBPA1 and bHLH2 together with the repressor MYBC2, was characteristic for pigmentation in blueberry skin, indicating an interdependent role of these transcription factors in regulating anthocyanin production [5].

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O3.5

Two-dimensional chromatographic fingerprints of oligomeric proanthocyanidin–malvidin glycoside adducts provide new insight into the complex world of red wine chemistry

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MAIN CONCLUSION

Group-specific mass spectrometric methodology provided a new way to approach the analytics of oligomeric adducts in red wines. We could, for instance, verify many of the hypotheses made about the evolution of the oligomeric pigments that were previously made by monitoring individual small oligomers. As a second example, the qualitative information provided by the chromatographic fingerprints made it possible to draw conclusion about compositional evolution, which has not been achieved previously

INTRODUCTION

The various sub-groups of adducts consisting of proanthocyanidins (PAs) and anthocyanins in wines are often referred to as polymeric pigments. As the names suggests, these pigments are generally thought to exist in wines as mixtures of oligomers and polymers, but their analytics often focuses only on the most abundant individual dimers and trimers. In other words, the nature of the polymeric pigments is already acknowledged in the nomenclature – they are oligomeric and polymeric pigments – but the conventional utilization of modern analytical equipment in targeted analysis does not give a comprehensive qualitative or quantitative picture of the polymeric pigments in red wines. A change in analytical approach is evidently needed to improve our knowledge in this challenging field of red wine chemistry.

We achieved this by developing group-specific tandem mass spectrometric method which relies on detecting marker ions of the targeted compound groups, i.e., fragment ions that are specific to different polyphenolic compound groups [1]. This approach provides both quantitative and qualitative information of the pigment composition in the form of two-dimensional chromatographic fingerprints, of which the qualitative information provides a completely new insight to the pigment composition in wines. The method can separately detect three different sub-groups of oligomeric adducts consisting of PAs and malvidin glycosides (Mv) and in total 18 different monomeric pigment groups. We have now analyzed a wine set of 317 commercial red wines with the developed methods to answer basic questions about the properties of the oligomeric pigments in wines, such as how their composition differs in wines, how do they evolve as wines age, which factors contribute to their formation during wine making and how they affect sensorial properties of wines [2, 3]. Some selected results are reported here, with the emphasis being on the oligomeric adducts.

MATERIALS & METHODS

317 commercial red wines were analyzed with the previously developed UPLC-MS/MS method [1]. All wines were filtered through a 0.2 µm PTFE filter prior to the analyzes but no other pre-treatments were done. The detected compound groups were semi-quantified against a dilution series, that were prepared from a single reference wine and the concentrations were reported as percentages compared to the reference. This approach was solely adopted to account for the non-linear responses of some of the compound groups. The perceived tannicity of 201 red wines was sensorially evaluated either as “tannic” or “medium tannic” to study the link between the composition of the PAs and PA–Mv adducts and the perceived tannicity. The sensorial evaluations were made by Alko Inc, a Finnish

national alcoholic beverages retailing company. The color intensities of the red wines were measured using a well plate reader and the color intensity was determined as sum of absorbances at 415 nm, 520 nm and 620 nm.

RESULTS & DISCUSSION

Qualitatively, many common wine types, such as Cabernet Sauvignon, Merlot and Shiraz wines were remarkably similar to one another. Only clear exception was the Pinot Noir wines. For instance, the fingerprints of the acetaldehyde-mediated PA–methylmethine–Mv^+ adducts showed that the Pinot Noir wines had a clear main compound in this compound group whereas the fingerprints of these pigments were dominated by a hump-like shape in the other wine types [3]. There were, however, quantitative differences between the wine types with similar qualitative compositions. As an example, the concentration of the directly linked PA–Mv^+ type adducts were approximately 60% higher in Shiraz and Cabernet Sauvignon wines than in Merlot wines, even though the chromatographic fingerprints were similar.

The evolution of the pigment groups was studied next [3]. We found that the PA–Mv^+ adducts were more stable than the PA–methylmethine–Mv^+ adducts, whose concentration decreased approximately by 75% from the 1-year-old wines to 5-8 years old wines. Compositionally all groups of oligomeric adducts showed similar trends as the average size of the adducts seemed to increase, albeit with different pace.

Finally, the oligomeric PA–Mv^+ and PA–methylmethine–Mv^+ pigments were shown to be major contributors to color intensity [2]. Interestingly, not only their concentration had a positive impact on the color intensity but so did the sizes of the pigments. The sensorial data suggested that the oligomeric pigments also contributed to the mouthfeel of the wines, along with the PAs. However, the metrics related to the composition of the PAs (degree of polymerization and sub-unit composition) and oligomeric PA–Mv adducts (size distribution of the adducts) were more substantial variables in separating the tannicity categories than the concentrations (manuscript in preparation).

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O3.6

Polyphenol targeted metabolomics to predict rosé wine color

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MAIN CONCLUSION

The prediction models established for light and dark rosé wines indicated that they have different pigment compositions. Indeed, polyphenols responsible for light rosé wines redness are native anthocyanins and their hydroxycinnamic acid derivatives, phenylpyranoanthocyanins. In contrast, dark rosé wine a* is explained by anthocyanins, pyranoanthocyanins, carboxypyranoanthocyanins and flavanol derivatives. Yellow pigments involved in the b* parameter require further investigation.

INTRODUCTION

Color is the key element in the marketing of rosé wines. It is due to the presence of red anthocyanin pigments extracted from the grape skin during the controlled maceration and a range of derived pigments formed from them during winemaking [1]. These reactions involve other polyphenols such as flavan-3-ols oligomers and hydroxycinnamic acids, whose concentration depend both on the grape variety and the extraction conditions as they are localized in different grape compartments. Reactions of flavan-3-ols with anthocyanins yields colored tannin – anthocyanin (T-A) adducts and colorless anthocyanin-tannin (A-T). Anthocyanin oligomers (A-A) have also been detected in wine. Indirect condensations can also occur to form methylmethine bridged adducts (tannin-methylmethine-anthocyanin) in presence of acetaldehyde. In parallel, anthocyanins can react with hydroxycinnamic acids to form phenylpyranoanthocyanins pigments. In addition, anthocyanin reactions with various yeast metabolites such as acetaldehyde and pyruvic acid lead to the formation of pyranoanthocyanins (i.e. pyranoanthocyanins and carboxypyranoanthocyanins, respectively). Native anthocyanins, conventionally represented under their red form (flavylium cation), are mainly found under colorless forms (hydrated forms, bisulfite adducts) in wines, especially at higher pH values. In contrast, some derived pigments (e.g. pyranoanthocyanins) are resistant to hydration and sulfite bleaching reactions and remain colored in wine. However, the drivers of rosé wine color, including in particular the pigment composition and the balance between the different anthocyanin pigment forms, are not fully established. This research aims at understanding links between color and phenolic (especially pigment) composition by analyzing 268 wines from the “Rosé du monde” collection of Centre du Rosé.

MATERIALS & METHODS

268 commercial wines from several regions of 21 different countries were collected by the Centre du Rosé. Classical oenological data (pH, sulfite concentration and alcohol content) has been recorded. Color and the different pigment forms were characterized by multiple spectrophotometer analysis using the CIELab system in addition to absorbance data [2]. In parallel, the concentration of 125 polyphenolic compounds including 85 anthocyanins and derived pigments was assessed by UPLC—QqQ-ESI-MS in the MRM mode [3]. The content of oligomeric flavan-3-ols was determined by UPLC—QqQ-ESI-MS in the MRM mode after phloroglucinolysis [4].

Principal Component Analysis (PCA), ANalysis of VAriance (ANOVA) using Student-Newman-Keuls (SNK) test and correlation matrix were performed using R Studio software. Prediction models

using CovSel variable selection method and Analysis of partial least squares regression (PLSR) were carried using Scilab with Free Access Chemometric Toolbox.

RESULTS & DISCUSSION

PCA performed on the whole data set revealed anticorrelation between L* and a* parameters from the CIELab system and correlation of rosé wines redness (a*) with the concentration of flavan-3-ols and anthocyanins related to the extent of extraction. Redness (a*) could be predicted from flavan-3-ol and anthocyanin content, modulated by oenological variables (pH, sulfites).

Examination of the correlation matrix performed on the polyphenol composition revealed similar behavior of molecules sharing the same polyphenolic pattern which were therefore grouped to compare three classes of wines defined by their Color Intensity (CI): light (IC<0.5), intermediate (0.5<IC<1) and dark (IC>0.5).

ANOVA showed differences in the proportion of the various groups of pyranoanthocyanins. Phenyl-pyranoanthocyanins were enhanced in light rosé wines, whereas pyranoanthocyanins and carboxy-pyranoanthocyanins were more abundant in the darker class.

Two distinct models based on the polyphenolic composition and oenological data were developed to predict redness (a*) for the light and dark rosé wines. These models shared a large contribution of native anthocyanins, pH, and sulfite levels. However, the light rosé wines model showed a strong contribution of phenylpyranoanthocyanins whereas dark rosé wines redness was linked to higher contents of carboxypyranoanthocyanins, pyranoanthocyanins and flavanol units released by phloroglucinolysis.

The yellow component (b*) is linked to flavan-3-ol content but poorly predicted, suggesting the benefit of untargeted analyses to investigate additional variables involved in browning.

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O3.7

Identification of prenyl number, configuration, and position in (iso)flavonoids in complex plant extracts by IT-MS and HR-MS

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MAIN CONCLUSION

We developed a decision guideline that characterizes prenylated (iso)flavonoids with ESI-IT-MS^n. We elucidated fragmentation pathways and spectral features of various subclasses of prenylated (iso)flavonoids, as well as fragmentation pathways and corresponding neutral losses. HR-MS confirmed molecular formulas of fragments and led to new insights. Our guideline aids identification of (1) an (iso)flavonoid backbone; prenyl (2) number; (3) configuration; and (4) position in complex plant extracts.

INTRODUCTION

Prenylated (iso)flavonoids are secondary metabolites found in *i.e.* the *Fabaceae* plant family. Prenylation alters bioactivity of (iso)flavonoids, generally enhancing *e.g.* antibacterial activity. Prenyl groups can have various configurations, with 3,3-dimethylallyl (3,3-DMA) and 2,2-dimethylpyran (2,2-DMP) the most common (**Fig. 1A**). So far, >1000 prenylated (iso)flavonoids have been identified from plants, and a PubChem search for 3,3-DMA and 2,2-DMP prenylated (iso)flavonoids revealed ~2000 and ~700 compounds, respectively (**Fig. 1B**). Thus, prenylated (iso)flavonoids are important secondary metabolites, and tools that assist their annotation in complex plant extracts could speed up studies in the areas of plant metabolomics. In this respect, liquid chromatography (LC) with ion trap (IT) mass spectrometry (MS) is a cost-effective general-purpose tool that is widely used in secondary metabolite identification. Fragmentation of (prenylated) (iso)flavonoids is characterized by *retro*-Diels Alder (RDA) fragmentation in which the C-ring of the (iso)flavonoid is cleaved, resulting in specific A- and B-ring fragments that provide information on the number and type of substituents (**Fig. 1A**). Fragments $^{i,j}A^+$–prenyl or $^{i,j}B^+$–prenyl moiety are indicative for A- or B-ring prenylation, respectively[1]. Additionally, prenyl configuration (3,3-DMA and 2,2-DMP) can be determined based on the ions corresponding to neutral losses (NLs) of 42 ($[M+H-C_3H_6]^+$) and 56 u ($[M+H-C_4H_8]^+$); 56:42>1 indicates 3,3-DMA and 56:42<1 2,2-DMP prenylation.[2]

Besides 3,3-DMA and 2,2-DMP prenylations, more prenyl configurations are possible, however, little is known whether NLs 42 and 56 u can be used to annotate other configurations. Furthermore,

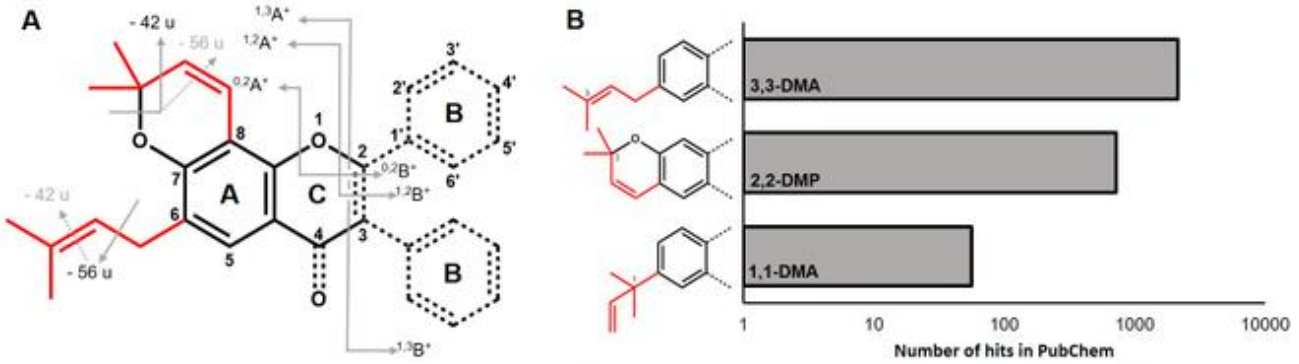

Figure 1. (A) (Iso)flavonoid backbone with 3,3-DMA and 2,2-DMP prenylation. Notations $^{i,j}A^+$ and $^{i,j}B^+$ are shown, where superscripts i and j represent the bonds that are cleaved. Common cleavages with 3,3-DMA and 2,2-DMP prenylation are shown, where solid and dashed arrows indicate major and minor cleavage sites, respectively. (B) Main configurations of prenyl groups on (iso)flavonoids, as retrieved from PubChem. Prenyl groups are indicated in red.

rapid identification of single or double prenylated compounds and prenyl position in different backbones is essential for characterizing complex plant extracts. Therefore, we aimed to develop a guideline that identifies prenyl number, configuration, and position.

MATERIALS & METHODS

Standards. A collection of 23 different prenylated (iso)flavonoid standard compounds belonging to 7 subclasses and having different prenyl configurations (*i.e.* 3,3-DMA, 2,2-DMP, 1,1-dimethylallyl (1,1-DMA)) were used to build the analytical guideline.

Methods. Standards were separated on a Thermo Vanquish UHPLC system (Thermo Scientific, San Jose, CA, USA) with an Acquity UPLC BEH C18 (150 mm x 2.1 mm, i.d. 1.7 µm) with a VanGuard (5 mm x 2.1 mm, 1.7 µm) guard column of the same material (Waters, Milford, USA). Mass spectrometric data were acquired on a LTQ Velos Pro linear ion trap MS (Thermo Scientific) and accurate mass data was acquired on a Thermo Q Exactive Focus hybrid quadrupole-Orbitrap Fourier transform MS. Data were processed using Xcalibur 4.1 (Thermo Scientific).

RESULTS & DISCUSSION

Prenyl number. Prenyl number was identified based on the *m/z* of the parent ion; a *m/z* of at least 305 indicated single prenylation and a *m/z* >373 possibly double prenylated compounds (based on the smallest natural prenylated flavonoid with a molecular weight of 304 g mol^{-1}).

Prenyl configuration. The ratio of NLs 56 and 42 u (56:42) in IT-MS^n identified prenyl configuration correctly for all 3,3-DMA and 2,2-DMP prenylated standards; a ratio >1 indicated 3,3-DMA and <1 indicated 2,2-DMP prenylation. The less common 1,1-DMA prenylation in licochalcone A (**Fig. 2A**) was not correctly identified based on this ratio; NL of 42 u was not associated with the prenyl but with a ketene moiety, as confirmed with HR-MS^2. IT-MS^2 and HR-MS^2 showed fragment $[M+H-prenyl]^+$, indicating cleavage of the whole 1,1-DMA. Presence of ions $[M+H-prenyl]^+$ and *m/z* 69 were crucial for correct identification of 1,1-DMA. Furan prenylated compounds (*e.g.* glyceofuran, **Fig. 2B**) were also analyzed and the ratio 56:42 falsely identified chain configuration; NLs were not associated with the prenyl, but with loss of ketene (42 u) and double carbonyl (56 u) moieties. For correct identification of these less common configurations, using HR-MS is essential.

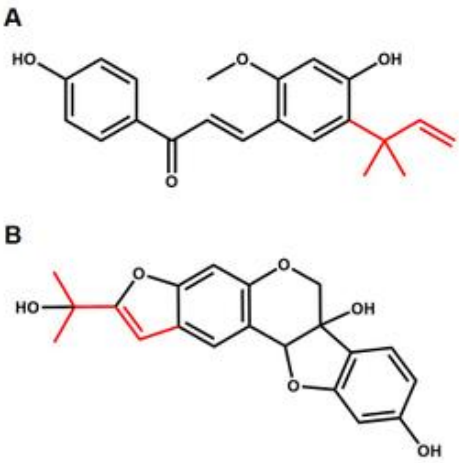

Figure 2. Molecular structures of (A) licochalcone A and (B) glyceofuran. Prenyl groups are indicated in red.

Prenyl position. A- or B-ring prenylation was identified by RDA-fragments $^{i,j}A^+$−prenyl and $^{i,j}B^+$−prenyl, respectively. Also position of the prenyl on the A-ring was determined; a new rule was established based on the ratio of the parent ion $[M+H]^+$ and fragment $[M+H-C_4H_8]^+$ in IT-MS^2. For molecules prenylated at position *C8*, the ratio favored the parent ion; for molecules prenylated on position *C6*, the ratio favored towards the fragment.

Decision guideline. With all obtained spectral data, a widely applicable decision guideline was developed that identifies (1) presence of an (iso)flavonoid, (2) prenyl number (0, 1, 2), (3) prenyl configuration (3,3-DMA, 2,2-DMP, 1,1-DMA), and (4) prenyl position (A- or B-ring, *C6* or *C8*) in complex plant extracts.

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O3.8

An efficient strategy to boost stilbene production in Vitis vinifera cv. Gamay Red cell suspension

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MAIN CONCLUSION

Co-transformation of the grape cell culture with *AroG** and *STS* resulted in increased levels of resveratrol and viniferin. External feeding with Phe further increased stilbene levels, reaching 26-fold increase in resveratrol and 620-fold increase in viniferin. Further increase stilbenes was achieved by Phe feeding and elicitation with sucrose, UV-C or MeJA, reaching up to 3.2 mg/g DW resveratrol and 13.43 mg/g DW viniferin, which is the highest accumulation reported in cell cultures.

INTRODUCTION

Stilbenes, a group of phenylpropanoids derived from *trans*-resveratrol, are effective phytoalexins, and have pharmacological and health-promoting bioactivities. Resveratrol, the most studied stilbene, has anticancer, antioxidant, anti-inflammatory, and neuroprotective characteristics. ε-Viniferin, a dehydrodimer of resveratrol, is more stable, with comparable, and in some cases stronger activities. While both stilbenes accumulate to relatively low levels in plants, there is an increasing demand for them in the pharmacological industry. Their production in plant cell suspensions offers optimized protocols of growth with rapid yield and relatively uniform quality.

Metabolic engineering is a valuable approach to enhance the productivity of stilbenes. Overexpression of the feedback-insensitive bacterial form of DAHPS, *AroG** in several plants including the *Vitis vinifera* cv. Gamay Red cell suspension, resulted in an increased production of Phe, the precursor of stilbenes [1]. Additionally, transformation of cell cultures, including *V. vinifera*, *V. amurensis*, and *Silybum marianum* with *STS*, resulted in resveratrol accumulation [2].

Here, we present the metabolomic effect of combining overexpression of *AroG** with external Phe feeding for increasing Phe levels, with diversion of Phe metabolism towards stilbene production by overexpressing *STS*. This three-step strategy resulted in a dramatic increase in stilbenes and in particular ε-viniferin in comparison to non-Phe-fed lines transformed with vector control. For further increase in stilbenes, the transformed lines, treated simultaneously with Phe and elicitors, including UV-C light, sucrose or MeJA, all known to induce stilbene production. Both UV-C and MeJA treatments caused induction of genes along the phenylpropanoid and stilbene biosynthetic pathways, including *STS* and *MYB14*. All three elicitation treatments resulted in a synergistic increase in stilbene levels, with no significant effect on flavonoids.

MATERIALS & METHODS

Plant material and cell growth

V. vinifera cv. Gamay Red cell suspensions were established from young grape berries as described previously [3]. Cell culture growth, RNA isolation and cloning of STS cDNA, Binary Vector Construction and Transformation in Agrobacterium, Generation of Stable Transgenic Gamay Red Cell Lines, Immunoblot Analysis, Metabolite Extraction and Profiling by LC−MS, Phe Feeding, Real Time PCR Analysis and Statistical analysis according to [4].

Elicitation treatments

Feeding and elicitation experiments were conducted on 1 week-old transgenic cells. A total of 5 g of cells were subculture, Phe (5mM), sucrose (5%), MeJA (100 µM), Phe + sucrose, Phe + MeJA were treated 4

h after subculturing. To avoid the cell death, UV-C irradiation was conducted 30 min on day 7 when the cell growth reached the stationary phase. Samples were collected on day 9 for metabolites analysis.

RESULTS & DISCUSSION

Metabolic engineering is a valuable approach to enhance the productivity of stilbenes. Stilbenes are derived from Phe, whose increased cellular availability was shown to affect several of the metabolic pathways associated with it. Here we produced 13 stable transgenic lines of *V. vinifera* cv. Gamay Red, overexpressing both *AroG** and *STS* genes. Three of the 48 *STS* genes in grape, were chosen for this study, including *VvSTS5*, *VvSTS10*, and *VvSTS28*. They were selected as the functional representative from each of the major clades of grape *STS* family and cloned.

All transgenic lines had increased levels of Phe and stilbenes, and in particular viniferin (74-fold higher viniferin reaching 0.74 mg/g DW), with no significant effect on flavonoids [4]. External Phe feeding of the transgenic *AroG** + *STS* lines caused a synergistic effect on resveratrol and viniferin accumulation, achieving a 26-fold (1.33 mg/g DW) increase in resveratrol and a 620-fold increase (6.2 mg/g DW) in viniferin, which to date is the highest viniferin accumulation reported in plant cultures. This strategy of combining higher Phe availability and *STS* expression generates grape cell cultures as potential factories for sustainable production of stilbenes with a minor effect on the levels of flavonoids (**Figure 1**).

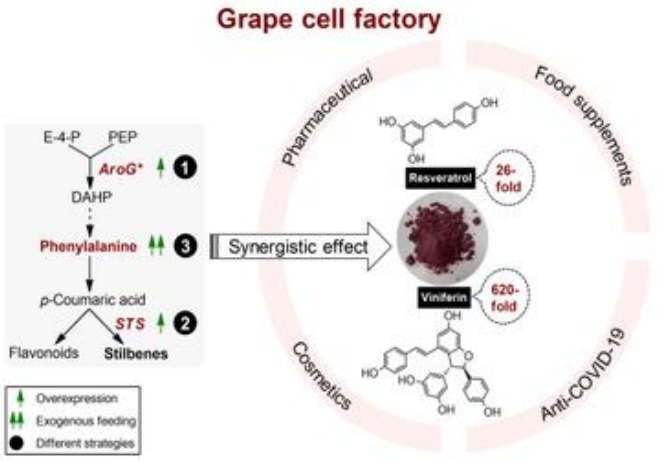

Figure 1. Schematic representation of the unique combination of strategies resulting in a "grape cell factory" for the production of stilbenes and in particular resveratrol and viniferin.

Further increase in stilbenes was achieved by simultaneously treating the *AroG** + *STS* lines with Phe and elicitors (**Table 1**). All treatments resulted in a synergistic increase of viniferin. Moreover, the combined treatments of Phe + UV-C resulted in the highest increase in resveratrol (64-fold, 3.23 mg/g DW), ε-viniferin (1343-fold, 13.43 mg/g DW) and total stilbenes (38-fold, 17.4 mg/g DW) in comparison to controls (non-treated lines transformed with an empty vector) (**Table 1**). These combined treatments of Phe feeding and elicitation may be implemented in large-scale production of viniferin.

Table 1. Content of stilbenes (mg/g Dry Weight) in the control (empty vector) and *AroG + *STS28* transgenic cell line after elicitation treatments.** Values are presented as mean ± SE (n = 4). Numbers in bold font indicate a significant increase of the level of metabolite in corresponding treatments as compared to non-treated control line ($P < 0.05$, Student's t test). The number in yellow shade indicate significant synergistic up-regulation the level of metabolite in combination treatments (Phe + elicitors) as compared to corresponding treatment alone ($P < 0.05$, Tukey HSD test).

	Treatments	*cis*-piceid	*t*-piceid	resveratrol	ε-viniferin	Total stilbenes
Control line	Control	0.20±0.01	0.20±0.02	0.05±0.01	0.01±0.002	0.46±0.03
	Phe	0.22±0.02	0.23±0.03	**0.10±0.02**	**0.15±0.02**	**0.70±0.07**
	Sucrose	0.22±0.01	0.23±0.02	**0.24±0.03**	**0.19±0.03**	**0.88±0.05**
	Phe + Sucrose	0.22±0.02	0.26±0.03	**0.09±0.01**	**0.39±0.01**	**0.97±0.05**
	UV	0.23±0.03	0.23±0.03	**0.14±0.03**	**0.03±0.01**	0.63±0.09
	Phe + UV	0.23±0.03	0.26±0.03	**0.31±0.04**	**0.23±0.03**	**1.03±0.13**
***AroG** + STS* line**	Control	**0.27±0.01**	**0.37±0.01**	**0.23±0.03**	**0.21±0.04**	**1.08±0.07**
	Phe	0.24±0.02	**0.45±0.02**	**0.94±0.1**	**3.44±0.33**	**5.07±0.44**
	Sucrose	**0.29±0.01**	**0.4±0.01**	**0.43±0.01**	**1.23±0.17**	**2.35±0.15**
	Phe + Sucrose	0.24±0.01	**0.42±0.01**	**0.44±0.02**	**6.76±0.27**	**7.86±0.25**
	UV	**0.31±0.02**	**0.45±0.02**	**1.29±0.14**	**2.8±0.84**	**4.84±0.93**
	Phe + UV	0.25±0.01	**0.48±0.02**	**3.23±0.30**	**13.43±1.75**	**17.39±2.01**
	MeJA	**0.39±0.04**	**0.78±0.08**	**0.58±0.05**	**2.05±0.38**	**3.80±0.38**
	Phe + MeJA	**0.34±0.01**	**0.58±0.02**	**1.36±0.24**	**9.08±1.49**	**11.35±1.72**

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O3.9

Metabolomics investigation of Antioxidant Proprieties, Polyphenolic profile and, Anthocyanin content in Commercial, Ancient and Red-fleshed apple varieties

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MAIN CONCLUSION

By balancing the intake of different apples cultivars, several classes of polyphenols beneficial are provided to human diet. The maximum efficiency in predicting the antioxidant properties using the polyphenolic profile is achieved in old cultivars, where the variability in the concentration of phytochemicals is less marked, contrary to what is observed in the commercial and red-fleshed apples. The aim is to contribute a new understanding for selecting promising fruits with health features.

INTRODUCTION

The World Health Organization encourages a 400 g per day of fruits and vegetables due to the presence of healthy compounds such as polyphenols. Apples are 12.5% of all consumed fruits in the world [1]. The antioxidants have a potential role to prevent the formation of reactive oxygen species (ROS), which are implicated in the onset of chronic disease and inflammation. In apples, the concentration of the antioxidants compounds is differently distributed in the fruit pulp and in the fruit peel. Agronomic, environmental, and genetic factors influenced the levels of phenolic compounds in both sections. The environmental and the agronomic conditions in the present investigation are kept as uniform as possible [1]. To date there are a few numbers of studies where apple varieties, grown in the same site, are compared. In this study, twenty-two apple varieties are fingerprinted and validated, and they are categorized in three groups: old, commercial, and red-fleshed [2]. The old varieties originated between 1900 and 1950, however, they have lost the acceptability from the farmers and consumers due to the deficits in production, storability and/or shelf life. The commercial group gathers the most consumed apple fruits, and they are currently available on the world apple market. The red-fleshed apples are characterized by a pigmented pericarp, and they are considered as promising apple cultivars for their nutritional properties. Since, the breeding process had a important effect on the content of certain phytochemicals. The red-fleshed apple cultivars were not taken into consideration in these extensive surveys. Indeed, it would be considerable to promote new apple cultivars with a reliable ratio between polyphenols and anthocyanins in pulp and peel compartment. In this study a metabolomic investigation is carried out on the characterization of twenty-two apple cultivars to profile the antioxidant properties and the polyphenol composition of the pulp and peel of apples.

MATERIALS & METHODS

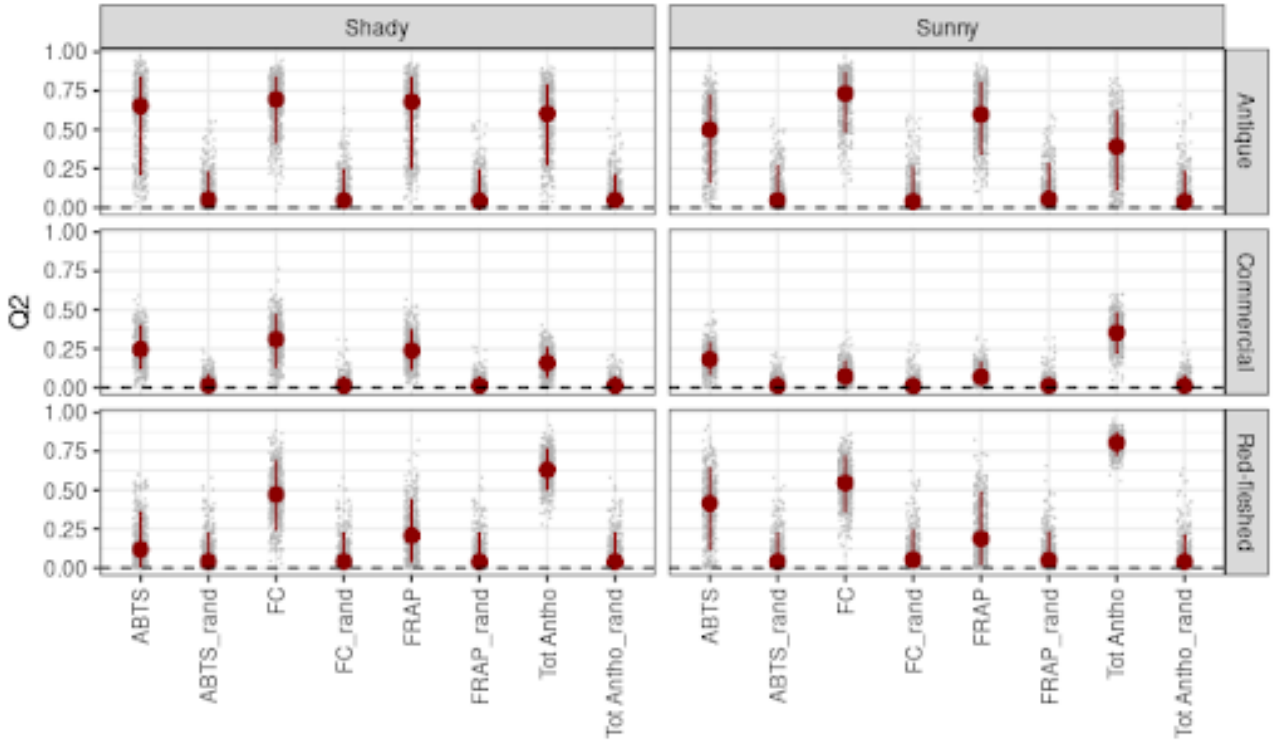
Twenty-two cultivars (10 biological replicates for each) were harvest in 2015 [4] Antioxidant activity, total polyphenolic content, total anthocyanin content assays were evaluated on the sunny and the shady sides of apple skin by FRAP, ABTS, Folin-Ciocalteu, and pH differential methods. Single polyphenols in pulp were quantified by UHPLC–MS/MS. Principal component analysis evaluated the separation between the three apple groups (old, commercial, and red-fleshed). The variability in concentration of flavan-3-ols and anthocyanins amongst groups is investigated. The partial least squares method predicted the antioxidant activity in the apple peel using the polyphenolic profile in apple pulp. The procedure of generating data, the splitting in train and the test and fitting models were

repeated 500 times. Q2 values of each model were compared to the same data in random order. The overlapping of the confidence intervals defined the reliability of the prediction power.

RESULTS & DISCUSSION

The PC1 and PC2 of the PCA (principal component analysis) applied on the whole dataset accounts for 49% of the total variance and the plot shows a good separation between the three groups. The PCA shows that the old varieties contain the highest amount of flavan-3-ols and antioxidant metabolites than the other samples [5]. The red-fleshed cultivars have a higher amount of anthocyanins and quercetin glycosides. In contrast, Y103 and Y102, two red-fleshed cultivars, are separated from the other red-fleshed varieties. The concentration of flavan-3-ols is less variable in old apple varieties than the other groups, where a marked variability in the concentration of flavan-3-ols is observed [5]. Y102 show the highest concentration of these metabolites and it could be linked to the red fruit-flesh phenotypes. The highest concentration of anthocyanins is found in the red-fleshed group, expect for Y102. The occurrence and the distribution of flavan-3-ols and anthocyanins in the apple pulps is attributed to the competitive pathway between these two polyphenolic classes. The presence of metabolites in the apple pulps seems to be more homogenous in old apple varieties [5].

The shady side of apples showed a lower antioxidant, total polyphenolic, and total anthocyanin content than the sunny side due to an up-regulation enzymatic activity in the sun-exposed side. A notably higher content of anthocyanins in the peel than in the pulp is found. Again, red-fleshed and old varieties are able to develop biochemical characteristics despite the exposure to sunlight. The best predictive power for almost all assays considered is achieved in the old group as show in the picture, which highlights a higher effectiveness in predicting the antioxidant properties of the peel using the polyphenolic profile [5]. In contrast, the evidence that there is a more diverse metabolism within the commercial and red-fleshed groups is confirmed by the large variability in the prediction ability [5].



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O3.10

Phenolic compounds in agricultural residues from olive, tomato and citrus industries

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MAIN CONCLUSION

In order to support the development of innovative extraction methodology, phenolic compounds were determined in different agricultural residues by HPLC-DAD-ESI-QTOF. In this study many different phenolic compounds were determined confirming that valuable phenolic compounds were not degraded and were still present in food processing by-product streams. This study also showed the importance of the determination of each phenolic compounds and not just the total amount.

INTRODUCTION

The conversion of raw fruits and vegetables into processed food creates side streams and by-products that go underutilized and that can place a burden on the environment. However, these by-products can still be valuable as they are rich in bioactive compounds. Among the biologically active compounds present in agricultural production residues are phenolic compounds. These compounds are secondary metabolites and are present in plants to act as a defense mechanism. Within humans, they have been shown to have anti-inflammatory, antimicrobial and antioxidant effects and can have a protective role against various chronic degenerative and cardiovascular diseases and cancer [1]. The by-products generated during production of plant food are primarily the seeds and peels that often contain the highest amount of phenolic compounds among all the parts of the plant [2].

In an effort to utilize agricultural production by-products and generate value from them, the Pro-Enrich project was developed. This project is funded from the Bio-Based Industries Joint Undertaking under the EU's Horizon 2020 research and innovation programme and was designed to develop valuable sources of protein and bioactive ingredients (e.g., phenolic compounds) to support new bio-based value chains within the EU. Bioactive compounds were targeted in olive, tomato, and citrus feedstock and extracted at laboratory and demonstration scales. The aim of this study was to support the development of extraction methodologies by identifying phenolic compounds present in olive pomace, olive mill waste water, orange peels and tomato agricultural by-products.

MATERIALS & METHODS

The extraction of phenolic compounds was performed according to Obied *et al.* [3] and Barros *et al.*, [4] with minor modifications.

Phenolic compounds were characterized using a high-pressure liquid chromatography system interfaced with a qTOF mass spectrometer (HPLC-ESI-QTOF/MS). The system was equipped with a Poroshell 120 column (EC-C18; 2.7 µm; 3.0 × 150 mm). The elution gradient of water/formic acid (99.05: 0.5, v/v) (A) and acetonitrile/ methanol (50: 50, v/v) (B), starting at 3.0% B and increased to 100.0% B in 15 min and held for 5 min. The separation was performed at a flow rate 0.5 mL/min, using a 1 µL injection volume. The separation compounds were first monitored using a diode array detector with a wavelength of 280 nm. This was followed by MS scans performed in the range of m/z 40–1000. The Automated MS/MS data-dependent acquisition was done for ions detected in the full scan above 2000 counts using fixed collision energies of 10, 20 and 40 eV.

RESULTS & DISCUSSION

Forty-five different phenolic compounds were identified in olive pomace and waste mill water samples. The prevalent group of phenolic compounds found were complex secoiridoids that comprised round 50–70% (564–2953 mg/kg d.w.) of the total phenolic content. The fact that a low amount of simple phenolic compounds was found (45–154 mg/kg d.w.) might be the consequence of relatively low phenolic breakdown as a consequence of post-harvest treatments and storage of the samples. Many different phenolic compounds were also found in tomato pomace samples with seeds including simple phenolic acid derivatives, hydroxycinnamoylquinic acid derivatives, flavone derivates, flavonone derivates, flavonol derivatives and dihydrochalcone derivatives. Furthermore, in the protein extract from tomatoes, most of these compounds were still present. Thirty phenolic compounds were found in orange peel samples with the heights peak identified as hesperidin. Overall, this results showed the importance of identification of each phenolic compound rather than the total phenolic compounds and they confirmed that various valuable phenolic compounds are still present in different agricultural residues from olive, tomato and citrus.

High variation of phenolic compounds were observed that could be due to different genetic and environmental factors. In the case of phenolic compounds in pomace the determined levels of total phenolic compounds amount from 800 mg/kg d.w. to 4000 mg/kg d.w. The highest content of phenolic compounds was found in the pomace from local variety “Istrska Belica”. No significant difference in yearly distribution was found, although the phenolic compounds content was higher in pomace obtained from a two phase decanter system compared to a three phase decanter system. Additional studies must be carried on to further determine the main factors that affect the variation of phenolic compounds in specific samples from certain areas.

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O3.11

Identification of AMPK activators analogues in plant extracts

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MAIN CONCLUSION

Plant secondary metabolites are almost unlimited sources of bioactive compounds with potential health benefit applications. Such a wide structural and physiochemical diversity is of great interest for food and pharmaceutical industries, but their ease of sourcing and cost are important parameters to consider. Investigating and developing natural extracts containing these bioactives might be a convenient and cheap way to source or explore chemical analogues. To address the chemical complexity of plant extracts, modern chromatography hyphenated to high resolution mass spectrometry (HRMS) appears to be a valuable strategy. The use of cutting-edge technology including ion mobility MS and data independent acquisition (DIA) mode improves the quality of the acquired information. However, such approach also increases the amount and the complexity of data and requires powerful bioinformatic tools for data processing. In this sense, molecular networking representation allows the reorganization of tandem mass spectrometry (MS/MS) data according to their spectral similarities. Compounds belonging to the same chemical family tend to cluster together facilitating their chromatographic localization, while their identification is ensured by direct comparison with in-house spectral databases.

The present study focuses on the identification of lusianthridin analogues, direct AMP-activated protein kinase (AMPK) activators previously identified by screening of the internal library of natural compounds. Dihydrophenanthrene derivatives were chemically synthesized and used to build an in-house HRMS-IMS-MSMS library. The database was used for the annotation of molecular networking representation of plant extracts. Then, the deep investigation of the cluster related to dihydrophenanthrenes allowed the identification of several analogues of the originally discovered bioactive. Moreover, this approach led to the discovery of new direct AMPK activators.

In summary this approach allowed implementing a rapid method for the discovery of direct AMPK activators in natural extracts. These structures further completed our set of dihydrophenanthrene analogues and allowed more comprehensive structure-activity relationship (SAR) investigations.

O3.12

Flax tissue cultures and elicitation as a strategy for bioactive compounds production

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MAIN CONCLUSION

Three tissue cultures, calli (Cc), adventitious roots (ARc) and hairy-roots (HRc), were developed from *L. austriacum*. ARs and HRs were the best producer of justicidin B and elicitation with Me-JA and COR induced its synthesis more than three times. Microscopic analyses highlighted, for the first time, that justicidin B was mainly located in the cytoplasm. Collectively the results obtained will enrich the knowledge for future scale-up process.

INTRODUCTION

Lignans are the main secondary metabolites synthetized by Linum species as plant defense compounds but they are also valuable for human health [1]. In particular, arylnaphthalene lignans such as justicidin B could be considered potential compounds for novel therapeutics [2]. The production of these molecules is often low in plant, less than 1%. However, plant cell cultures provide an excellent system for secondary metabolites production. The major advantage of this method is the continuous, reliable source of bioactive molecules and the possibility of using elicitors to improve their synthesis. Jasmonate derivatives, such as Methyl jasmonate (Me-JA) play many important roles in plant defence response by acting as signal molecules [3] and they could be effective elicitors when added exogenously in the culture medium. Coronatine (COR), produced by pathovars of plant bacteria Pseudomonas syringae, is a molecular mimic of the isoleucine-combined form of jasmonic acid, a plant growth regulator and an elicitor that induces plant secondary metabolites. In this context we developed three in vitro tissue cultures from L. austriacum seedling: calli (Cc), adventitious roots (ARc) and hairy roots (HRc) with the aim to select the most promising tissue along with the most promising elicitor treatment between Me-JA and COR in term of justicidin B production. The total phenol contents and the antioxidant activity of cell and root extracts were evaluated, and justicidin B was identified by NMR spectroscopy and quantified by HPLC analysis. Furthermore, we investigated, for the first time, the intracellular localization of justicidin B in ARc of L. austriacum through microscopic analysis.

MATERIALS & METHODS

L. austriacum seeds were obtained from USDA (U.S. Department of Agriculture).

Calli, adventitious and hairy roots were obtained as described in Mascheretti et al. 2021 [4]. Suspension cultures of Cc, ARc and HRc were elicited with 100 µM Me-JA and 10 µM COR. Lignans extraction was performed from powdered lyophilized tissues and extracted with 80% (v/v) methanol. The methanolic extracts were dried under nitrogen and then dissolved in chloroform for TLC and HPLC chromatography, in $CDCl_3$ for ^{1}H-NMR analyses.

The amount of total soluble phenolics was evaluated by Folin-Ciocalteu assay using gallic acid (GA) as standard. The DPPH radicals scavenging assay was determined following the method

described by Cheng et al. 2006. Fluorescence and Confocal Microscopic Analysis of Justicidin B was performed after fixing ARc in 4% (w/v) paraformaldehyde in phosphate buffer 0.1 M pH 7.4. Autofluorescence of samples was obtained by exciting at 366 nm and the emission filter was set at 430–490 nm.

RESULTS & DISCUSSION

Three different *in vitro* cultured tissues from *L. austriacum* were developed: Cc, ARc, HRc. The elicitor treatments increased the total phenol content in each tissue culture. HRc showed the highest phenols' content, whereas Cc and ARc exhibited a similar phenolics level. However, similarly to what described for several plant tissue cultures, in our experiments the growth of both cell and root biomass significantly decreased (21%) after the elicitation [4]. The antioxidant capacity of the extracts from the three tissues correlated to their phenolic content. In particular, HRc showed the highest antioxidant power among the examined tissues, and COR-treated HRc were the most effective [4].

As far as justicidin B identification and quantification, this molecule was detected in each tissue culture, but ARc and HRc produced four times more justicidin B (3.94 and 4.89 mg/g DW, respectively) than conventional calli. ARc achieved the maximum content of justicidin B when elicited with COR (15.74 mg/g DW) whereas the HRc reached the highest content when elicited with MeJA (14.71 mg/g DW). The elicitors induced the synthesis of other two compounds: isojusticidine B and a not yet identified one. IsojusticidinB was produced mainly by the COR-treated HRc.

ARc of L. austriacum examined by conventional fluorescence microscopy with UV light showed a bright blue autofluorescence. The fluorescence was visible only with the excitation filter of 330–385 nm but not with higher wavelengths, confirming that it was due to justidicin B deposition. Confocal laser scanning microscope revealed that the spots were in turn formed by much smaller ones mainly located on the periphery of the cortical parenchymatic cells of the root, thus likely in the cytoplasm, but not on the wall or in the vacuole, suggesting that justidicin B is not secreted. The results obtained together with those regarding the cellular localization of justicidin B will enrich the knowledge for future scale-up process.

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O4.1

Multi-method approach for extensive characterization of gallnut tannin extracts

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MAIN CONCLUSION

Characterisation of three commercial gallnut tannin extracts using a combination of approaches (NMR, HPLC-DAD-MS/MS and GPC) has shown large composition differences, concerning gallic acid content, distribution of X-galloylglucose molecular weight, and substitution patterns of the glucose and galloyl units, which may impact their properties. This highlights the need for detailed characterization of tannin extracts in the frame of quality control and selection for specific applications.

INTRODUCTION

Tannin extracts from various botanical sources are used for different applications and in particular as enological tannins, added during winemaking to help wine clarification and stabilisation against protein haze, discoloration and oxidation. However, their effects on color and mouthfeel are controversial due to a lack of knowledge and large variability of their composition.

Enological tannins include gallnut extracts from *Quercus infectoria* (Turkish or Alep gall) or *Rhus semialata* (Chinese gall) which are rich in gallotannins consisting in complex mixtures of polygalloyl esters of glucose. Gallotannins from Chinese galls are composed of a 1,2,3,4,6-pentagalloylglucose core, to which additional galloyl groups are linked by depsidic linkages, forming up to a nonagalloylglucose or eventually larger structures. Those from Turkish galls were described as mixtures of galloylglucoses based on both 1,2,3,6-tetragalloylglucose and 1,2,3,4,6-pentagalloylglucose cores. Analysis using high performance liquid chromatography coupled to UV-visible spectrophotometry and mass spectrometry (HPLC-DAD-MS), gel permation chromatography (GPC) or nuclear magnetic resonance (NMR) has provided evidence of large diversity of structures and molecular weights in a given extract. In addition, different gallnut extracts differ in their tannin composition and thus potentially in their properties. For instance, protein precipitation capacity was found to vary with the composition of the gallotannin mixture and in particular to increase with the degree of galloylation.

The present study describes the implementation of a multi-method approach, involving UPLC−DAD−MS/MS, one dimension (1D) and two-dimension (2D) NRM, and GPC and its application for detailed characterisation of three commercial gallnut extracts (TAN A, TAN B1, TAN B2) [1].

MATERIALS & METHODS

The compounds present in the crude extracts and in the extracts after acidic methanolysis (120°C, 260 min) were analysed by UPLC−DAD−MS/MS, on a C18 column. The detection was with both the DAD from 250 to 600 nm and MS equipped with an electrospray-ionization source and an ion trap analyzer, operated in the negative ion mode. The quantification was based on peak areas at 280 nm, using gallic acid and methyl gallate as calibration standards.

Structural and quantitative information was obtained by NMR conducted on an Agilent DD2 500 MHz spectrometer, using 1D (^{1}H and ^{31}P) and 2D (^{31}P TOCSY, ^{1}H/^{13}C HSQC and HMBC)

experiments. For ^{31}P experiments, phosphitylation of OH groups was carried out as described earlier [2].

Size distribution was obtained by 2D ^{1}H DOSY NMR spectroscopy and by GPC using detection at 280 nm, and gallic acid, β-glucogallin, and 1,2,3,4,6-pentagalloylglucose as size distribution standards [3].

RESULTS & DISCUSSION

Differences in the structure and molecular weight distributions of gallotannins were demonstrated between the three extracts.

HPLC-DAD-MS/MS analysis detected mono-, di-, tri- and tetra-gallic acids in all extracts, along with X-galloylglucose (XG-Glc) polymers containing up to 12, 18, and 17 galloyl units respectively, in TANA, B1, and B2. It also distinguished galloylglucose isomers based on tetra-O-galloylglucose in TANA and on penta-O-galloylglucose in TANB1 and B2. This was confirmed by ^{31}P NMR analysis after phosphitylation of the hydroxyl groups, the presence of a phosphitylated aliphatic OH indicating that glucose was not fully substituted by gallic acids in TAN A. After identification of the correlation between phosphitylated OH based upon 2D TOCSY analysis, ^{31}P NMR distinguished free gallic acid, terminal gallic acids and gallic acids involved in depside bonds which enabled calculation of the average number of galloyl units per glucose residue (8.5, 12.2, and 12.4, in TANA, TANB1, and TANB2, respectively). Polymers detected by SEC were larger in TANB1 and B2 than in TAN A, congruent with the NMR and MS data.

Finally, the structures appeared more complex than expected. NMR provided evidence of para-substituted gallic acid units in addition to the well-established meta-depside structures. Detection of methyl galloylglucose derivatives by LC-MS after methanolysis indicated that some of the glucose units were linked to gallic acid through ether rather than ester bonds. These structures were more abundant in TANB than in TANA and in TANB2 than in TANB1, which also contained significantly larger amounts of gallic, digallic, and trigallic acids.

Our results demonstrate the relevance of the multi-method approach for detailed characterization of complex tannin mixtures and show variability in the composition of the three commercial gallnut extracts studied, likely impacting their properties.

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O4.2

Oral cell-line based model to understand phenolic compounds astringency perception: insights from single compounds to real food matrix

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MAIN CONCLUSION

Interaction of different families of polyphenols within oral cavity is driven by different oral constituents

INTRODUCTION

Astringency is described as a tactile and diffuse sensation of puckering, tightening and dryness in the mouth, which has been widely explained by the interaction and/or precipitation of salivary proteins (SP) [1] by food tannins. Among the major SP, acidic proline-rich proteins (aPRPs), basic PRPs, P-B peptide and statherin have been reported as the most reactive toward tannins. In spite of the undoubtedly importance of SP interaction and/or precipitation induced by tannins in the astringency perception, some other (polyphenol) compounds able to elicit astringency fail to precipitate SP. Currently, astringency is recognized as a trigeminal sensation although the molecular pathway responsible for its onset has not been yet fully established. Moreover, it is unknown if the many different astringency mouthfeel sub-qualities such as velvet, puckering, harsh, among others, are perceived by different mechanisms.

In food, astringent compounds are by excellence the polyphenols, in particular tannins. In addition to tannins, numerous studies confirmed that astringency could also be elicited by flavonols. Since SP precipitation is not able to explain the astringency of all polyphenols, the interaction with other oral constituents can contribute to astringency as well. Also, different mechanisms of astringency perception and the involvement of different oral constituents could explain the different astringent mouthfeel subqualities. Different oral models have been developed in our work [4, 5], comprising different oral epithelia (buccal mucosa (TR146) and tongue (HSC-3)) and other main oral constituents (human saliva and mucosal pellicle). These models, have been used to study the interaction with polyphenol extracts as well as with food matrices, such as coffee. The studied extracts were rich in different families of polyphenols, namely flavanols (a green tea extract), anthocyanins (a red wine extract), gallotannins (a tannic acid extract) and flavonols (an onion extract).

MATERIALS & METHODS

Saliva from seventeen healthy volunteers was collected and treated as reported. A flavanols extract was obtained from green tea leaves of Camellia sinensis from Portuguese origin as described previously [3]. An anthocyanins extract was obtained from concentrated red wine as described previously [3]. Flavonols extract was prepared from yellow onions (*Allium cepa L.*) as described in [4]. All extracts were analyzed and characterized by LC-DAD/ESI-MS analysis.

Two human epithelial-like cell lines derived from tongue (HSC-3) and buccal mucosa (TR146) were used. The oral models were established following a similar approach to a previous study [2]. 15 µL of three stock concentrations of each extract were deposited in the respective wells, comprising six experimental conditions, with and without each cell line, covering all the control conditions and

all the oral constituents: +Extract, +Extract+cell, +Extract+saliva, +Extract+saliva+cell, +Extract+mucin+cell, and +Extract+mucin+saliva+cell.

RESULTS & DISCUSSION

The obtained results suggest that the engagement of different oral constituents into the interactions could be related to the perception of different astringency sub-qualities.

From our results, it can be conjectured that the flavanols present in the food matrix seem to interact more than anthocyanins. This work showed that when all the oral constituents occur together, the oral interactions of flavanols seems to be driven by salivary proteins, leading to their significant depletion. On the other hand, oral cells were shown for the first time to be the oral constituent with the major interaction toward anthocyanins. This work also gathered first-time evidences that the studied flavanols and anthocyanins seem to bind in the same way to two oral cell lines.

Regarding the gallotannins and flavonols studied, they seem to bind in a different way to the several oral models used here. The interactions of high molecular weight and galloylation degree of galloylglucoses seem to be also driven by salivary proteins, mainly statherin, P-B and aPRPs. The two oral cell lines (HSC-3 and TR146) seem to bind these compounds in a different way but these differences seem to be totally reverted by the presence of salivary proteins. On the other hand, for gallic acid, it seems to occur a synergy between all the oral constituents studied. Moreover, gallic acid was found to be the only compound within gallotannins mixture to have a higher interaction toward human buccal mucosa cell line. This result could be interesting in the light of one possible mechanism for astringency perception, the penetration of some small phenolics on the surface of the oral epithelia. On the other hand, a totally different behavior was observed for the flavanols studied here. It was found that the interaction of quercetin and its derivatives with the oral constituents seems driven by oral cells, in particular by tongue cell line, while the contribution of salivary proteins seems neglectable.

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O4.3

Separation of Pyranoanthocyanins from Precursor Anthocyanins Using Cation-Exchange Chromatography

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MAIN CONCLUSION

Cation exchange chromatography (CXC) in combination with controlled anion formation produced highly purified (≥95%) pyranoanthocyanin (PACN) extracts facilitating the study of their coloring characteristics and bioactivity and opening the door to similar technologies to be adapted for large-scale industrial applications. This technology allowed for the separation of PACNs from precursor anthocyanins (ACNs) in mixtures containing both, increasing the PACN content after treatment.

INTRODUCTION

PACN are ACN-derived pigments with vivid colors, better stability, and enhanced resistance to degradation, making them ideal alternatives to artificial colorants. PACN are scarce in nature being reported in few sources such as red onions and strawberries [1]. This scarcity hinders their use as viable pigments by the industry. However, studies have shown that 10-catechyl-PACN can be formed efficiently through the reaction between select ACNs and caffeic acid, yet conversion over a short time is not complete [2]. Extended incubation times can result in a slight increase in PACN yields, however, ACN and PACN degradation, ACN polymerization, and undesirable changes also may occur depending on the incubation conditions. After formation, remnant caffeic acid can be removed using solid-phase extraction (SPE) with C_{18} resin, but further separation of PACN from their ACN precursors is mostly conducted using semi-preparative HPLC or countercurrent chromatography. These are efficient processes for analytical purposes but tedious and expensive for large-scale preparation. Studies on the equilibria of PACNs in aqueous solutions showed that these pigments have higher p*K*a values than their ACN counterparts [3,4]. At low pH, ACN and PACN are expected to be in their protonated form. As pH increases, deprotonation and subsequent anionization of ACN are expected to occur at a lower pH than with PACN (Fig 1). It was hypothesized that this p*K*a differential could be used for the separation of 10-catechyl-PACN from their ACN precursors. Therefore, the objective of this study was to develop a method to separate PACN from their ACN precursor based on their p*K*a differential using controlled anion formation and cation-exchange chromatography.

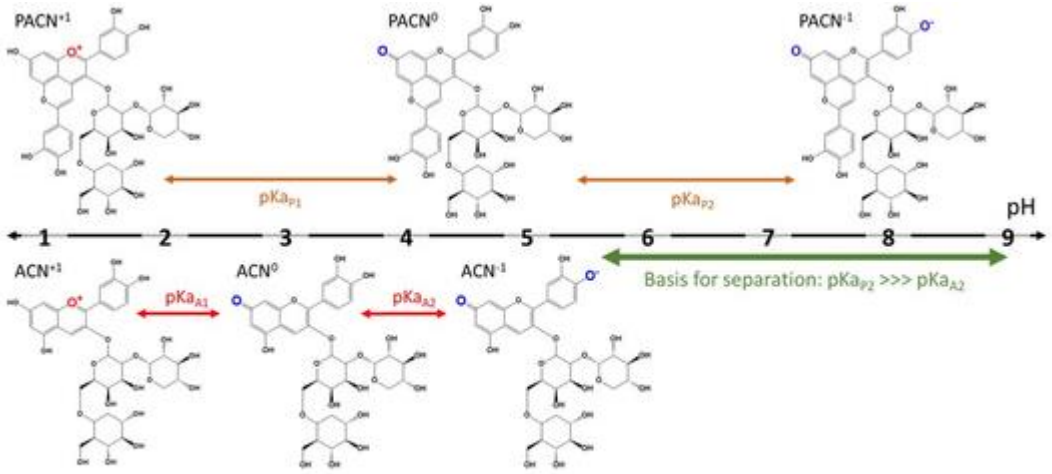

Figure 1. Proposed basis for pyranoanthocyanin (PACN) separation from precursor anthocyanins (ACN) using cation exchange chromatography.

MATERIALS & METHODS

An ACN and PACN mixture from saponified black carrot was used for separations and as control. Briefly, saponified ACN extracts were incubated for 3 days at 45°C with caffeic acid using an ACN:cofactor molar ratio of 1:30 at pH 3.1. Experimental solutions were then semi-purified using SPE with C_{18} cartridges, and pigment proportions and identities were determined using UHPLC-PDA-ESI-MS/MS. CXC experiments were performed using Oasis® mixed mode-cation exchange cartridges (30 mg, 3 cc; Waters Corp., Milford, MA, USA). Samples (1mL) containing ACN and PACN were loaded onto pre-activated cartridges and then washed with three volumes of an aqueous buffer (either citric acid-Na_2HPO_4 at pH 5, 6, or 7; or Na_2HPO_4-NaH_2PO_4 for pH 8 or 9) to elute ACN. After washing, the remaining pigments were eluted using 1% NH_4OH in methanol. Eluents were collected at each step into vials containing HCl to reacidify the medium, filtered, and analyzed using HPLC-PDA. All experiments were conducted in triplicate.

RESULTS & DISCUSSION

The control mixture of PACN and ACN obtained after incubation was composed of ~25% ACN (cyanidin-3-xylosyl-galactoside, C3XG, and cyanidin-3-xylosyl-glucosyl-galactoside, C3XGG) and ~75% of their 10-catechyl-PACN derivatives (PC3XG and PC3XGG, respectively). After CXC purification the PACN proportion significantly increased regardless of the pH of the buffer used in the washing step (Fig 2A), with this being the highest in purified fractions washed with buffer at pH 8 or pH9. Pigment content analyses in purified fractions (Fig 2B) showed that washing with buffers at pH 5–8 resulted in a significantly higher PACN content. Additionally, washing with buffers at pH 7–9 resulted in a significantly lower ACN content.

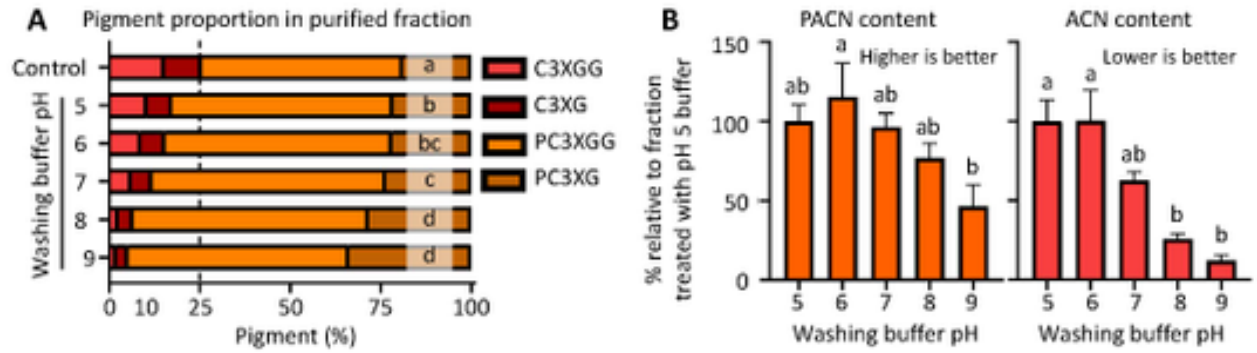

Figure 2. Pigment proportion in the purified fraction (A), different letters indicate significant differences in pyranoanthocyanin (PACN) proportion. PACN and anthocyanin (ACN) content in purified fractions (B). Content relative to the purified extract after washing with pH 5 buffer. Different letters indicate significant differences in pigment content.

Analyses of washing buffers showed that the ACN proportion in them was higher than in the control (Fig 3A). These were significantly higher in buffers at pH 5–7, consistent with previous research showing that the p*K*a of ACN is ~5.5 [4]. Analyses of pigment content in washing buffers (Fig 3B) showed that PACN loss during the washing step was significantly lower when washing with buffers at pH 5–7. Moreover, ACN removal was significantly higher when washing with buffers at pH 9, followed by buffers at pH 7 and 8.

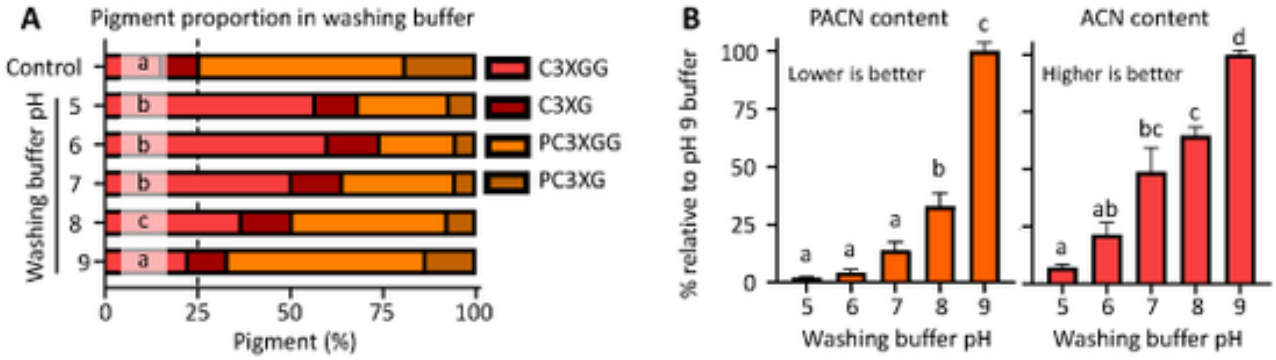

Figure 3. Pigment proportion in the recovered washing buffers (A), different letters indicate significant differences in anthocyanin (ACN) proportion between treatments. Pyranoanthocyanin (PACN) and ACN content in purified washing buffers (B). Content relative to recovered pH 9 buffer. Different letters indicate significant differences in pigment content between treatments.

Overall, washing with pH 7 buffer resulted in an increased PACN proportion in the purified extract, yielding the highest content of PACNs. Additionally, washing with this buffer resulted in low PACN loss during the washing step while removing the second highest amount of ACNs from the column. The use of buffers at pH 8 or 9 resulted in significant PACN loss during the washing step, demonstrating that buffers at pH ≥ 8 eluted both ACN and PACN. This can be explained by the p*K*a of 10-catechyl-PACN with similar structures to the ones used in this study that has been reported to be between 7.8 and 8.4 [3], although exact values for the pigments used in this study have not yet been reported.

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O4.4

Development of a cell-based quartenary system to unveil the effect of polysaccharides on oral astringency

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MAIN CONCLUSION

The present work showed evidence that polysaccharides can interact with several oral constituents and reduce salivary protein-tannin interactions in a quaternary system.

INTRODUCTION

Consumer's demand for healthy foods has increased the ingestion of plant-based foodstuffs rich in polyphenols which are highly recognized as bioactive compounds. However, these products have been also correlated with unpleasant taste properties, such as astringency and bitterness, when perceived at high intensities. Among polyphenols, tannins are usually associated with flavor and particularly with astringency [1,2].

Astringency is defined as a very complex phenomenon resulting of a complex group of tactile sensations, including dryness, puckering, and tightening felt in the oral cavity. Several mechanisms have been discussed to explain its onset. Over the years, the most accepted mechanism refers to the interaction and precipitation of salivary proteins, mainly proline-rich proteins, by food polyphenols [1,2]. However, other mechanisms (e.g., activation of mechanoreceptors and/or the polyphenols interaction with oral cells) can be at the origin of astringency perception [3].

Modulating these untasty properties could be important to promote the intake of healthy foodstuffs while keeping consumer's approval. Food industry has some strategies to balance astringency and bitterness, usually leading to a decrease of nutritional properties and the removal of potential health benefits, rising concerns about food allergies, safety and security. Polysaccharides have been an emerging natural and sustainable option to be used on taste properties modulation. In fact, polysaccharides can influence salivary protein-tannin interactions and they could be used to modulate astringency and bitterness [4].

The aim of this work was to study the effect of polysaccharides on salivary protein-tannin interactions present in a quaternary system composed by several oral constituents, namely oral cells (HSC-3 cell line), saliva and oral mucosa pellicle (saliva, HSC-3 cell line and mucin).

MATERIALS & METHODS

The experimental approach described herein intends to simulate the process of food ingestion in which polysaccharides and tannins interact with the constituents of the oral cavity (oral cells, saliva and mucosal pellicle) after interacting both in food matrices.

This work was divided into different parts: a) isolation and characterization of pectic polysaccharides fractions from grape skins obtained by sequential extraction with several solvents; b) isolation of human saliva; c) interaction of polysaccharides with two classes of hydrolysable tannins (punicalagin and tannic acid); d) evaluation of the effect of the polysaccharides on salivary protein-tannin interactions using a recent developed oral cell-based model composed by saliva, HSC-3 cell line and mucosal pellicle.

RESULTS & DISCUSSION

Considering the interaction with polyphenols, polysaccharides have a great effect on reducing precipitation of punicalagin and the most galloylated structures of tannic acid. This is particularly evident for the highest concentrations (2.0 and 3.0 $g.L^{-1}$) of some polysaccharides such as CSP and Pect (Figure 1). On the other hand, polysaccharides can also reduce precipitation of some salivary protein families such as statherin/P-B peptide and acidic proline-rich proteins. The observed polysaccharide´s effect can be explained by the structural features of the compounds involved: polysaccharides, tannins and salivary proteins.

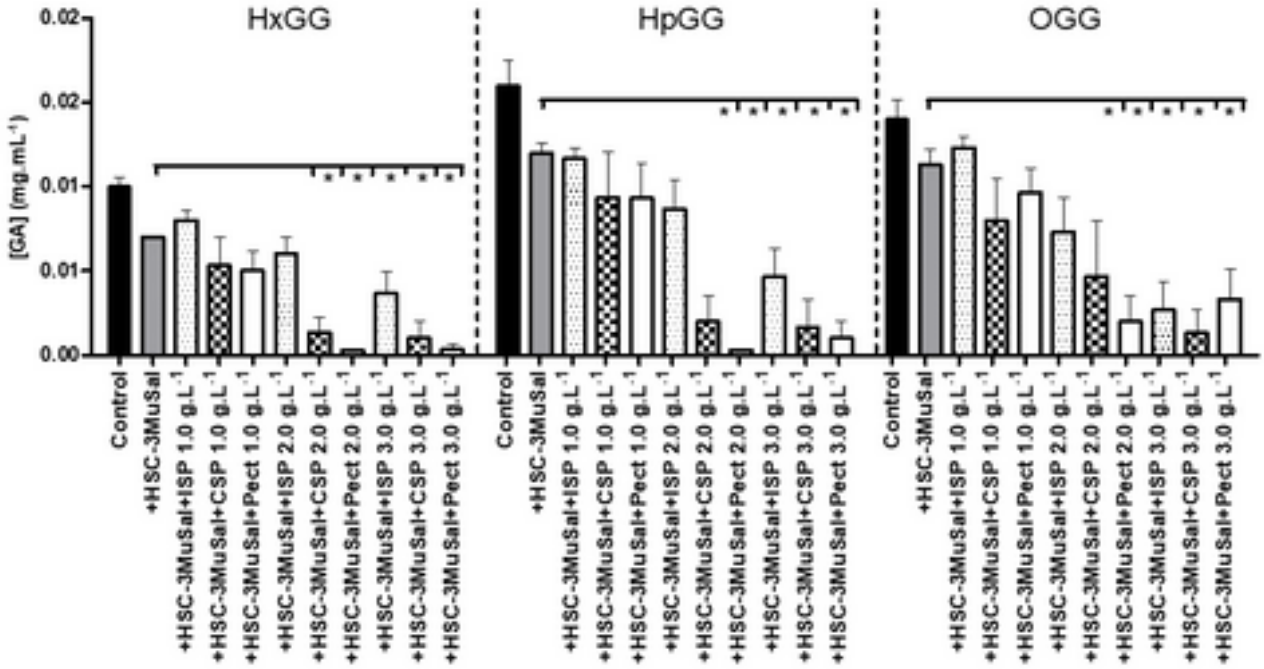

Figure 1. Concentration of the most galloylated compounds from tannic acid that were retained in the HSC-3 oral model in the absence and presence of several pectic polysaccharides (ISP – imidazole soluble pectins; CSP - carbonate soluble pectins; Pect – commercial pectins). Control, concentration in the tannic acid extract; HSC-3MuSal, concentration in the absence of polysaccharides. Compounds in tannic acid extract: HxGG, hexagalloylglucoses; HpGG, heptagalloyglucoses; OGG, octagalloylglucoses. The compounds are expressed in equivalents of gallic acid ($mg.mL^{-1}$). The data are presented as mean and SEM values for at least three independent experiments; values statistically different are indicated (*$p<0.05$).

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O5.1

Auronidins are a novel group of cell-wall bound red flavonoid pigments that contribute to liverwort abiotic stress tolerance

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MAIN CONCLUSION

Liverworts produce cell-wall-bound flavonoid pigments called auronidins when exposed to environmental challenges. Our previous studies suggest auronidins assist liverworts in tolerating abiotic stresses such as high light and nutrient stress. Using the model species *Marchantia polymorpha*, we are now examining how auronidins are associated with the cell wall and how they alter cell wall properties for stress tolerance functions.

INTRODUCTION

The flavonoid biosynthetic pathway may have evolved during colonization of land by plants for providing protection against abiotic stresses. In angiosperms, flavonoids such flavonols, flavones, and the anthocyanin pigments protect plants from adverse environmental conditions, whereas lignin, produced by another branch of the phenylpropanoid pathway, provides structural support. The bryophytes, comprising three of the major land plant lineages, liverworts, mosses and hornworts, are non-vascular plants thought to lack both anthocyanins and lignin. However, liverworts, as exemplified by the model species *Marchantia polymorpha* (Marchantia), is able to produce alternative red flavonoid pigments called auronidins [1]. Auronidins are produced from a distinct biosynthetic branch from anthocyanins and are cell-wall-bound [1]. Here we are investigating whether or not auronidins provide not only beneficial elements of anthocyanins (screening of excess light) but also structural elements of lignin, including the formation of a phenolic polymer integrated into the cell wall.

Bryophytes are thought to have retained characteristics that were present in the early land plants that have been lost during angiosperm evolution. It is thus possible that auronidins, and not anthocyanins, were the red pigments present in the ancestral land plant. Alternatively, auronidins may have evolved specifically in liverworts as part of adaptations that enable them to colonise extreme environments.

MpMYB14 regulates auronidin production in Marchantia in response to abiotic stresses such as excess white light and nutrient deprivation [2]. By using 35S:MYB14 transgenic lines (OE, over producing auronidins) and the *myb14* knockout mutant (lacking auronidins) we can examine the binding of auronidins to the cell walls and how this alters cell wall properties.

MATERIALS & METHODS

Marchantia polymorpha L. accessions Sey-1 (male) and Aud-2 (female) were grown as previously described [2]. Primary cell walls (PCW) were prepared from 10 g of frozen Marchantia tissues as described [3]. Flavonoid extraction and UHPLC analysis was performed as described previously [2].

RESULTS & DISCUSSION

The primary cell walls (PCW) were prepared from 35S:MYB14 (OE), *myb14* mutant and wild type (WT) plants. The PCW of OE plants were highly pigmented, suggesting that auronidins are tightly bound to cell wall components as they remain after the extraction procedures involved in PCW

preparation (Figure 1A). PCW of *myb14* were colourless, indicating the colour is from MYB14 activated auronidin production. Pigment in the PCW was extracted in methanol and analysed on UHPLC, confirming the pigment was the auronidin riccionidinA (Figure 1B and C), as previously reported [2]. Next, we sequentially separated the PCW polysaccharides by using solvents such as CDTA, Na_2CO_3 and KOH. CDTA and Na_2CO_3 remove pectin polymers, whereas the alkali solution releases hemicelluloses. We found that although some of the PCW pigment was released with hemicelluloses, the majority of it was attached to the insoluble residue pallet (IR) obtained after sequential extractions (Figure 1D). In vascular plants, cell wall material left insoluble after the sequential extraction of polysaccharides is generally cellulose and lignin with small amounts of pectin and hemicelluloses. As the walls from the OE are still coloured at this stage, we hypothesise that auronidins in Marchantia are associated with cellulose in a similar manner to lignin in vascular plants, perhaps by covalent interactions or entrapment as a pigment polymer within cell wall polysaccharide polymers. Further experiments are in progress to test this hypothesis.

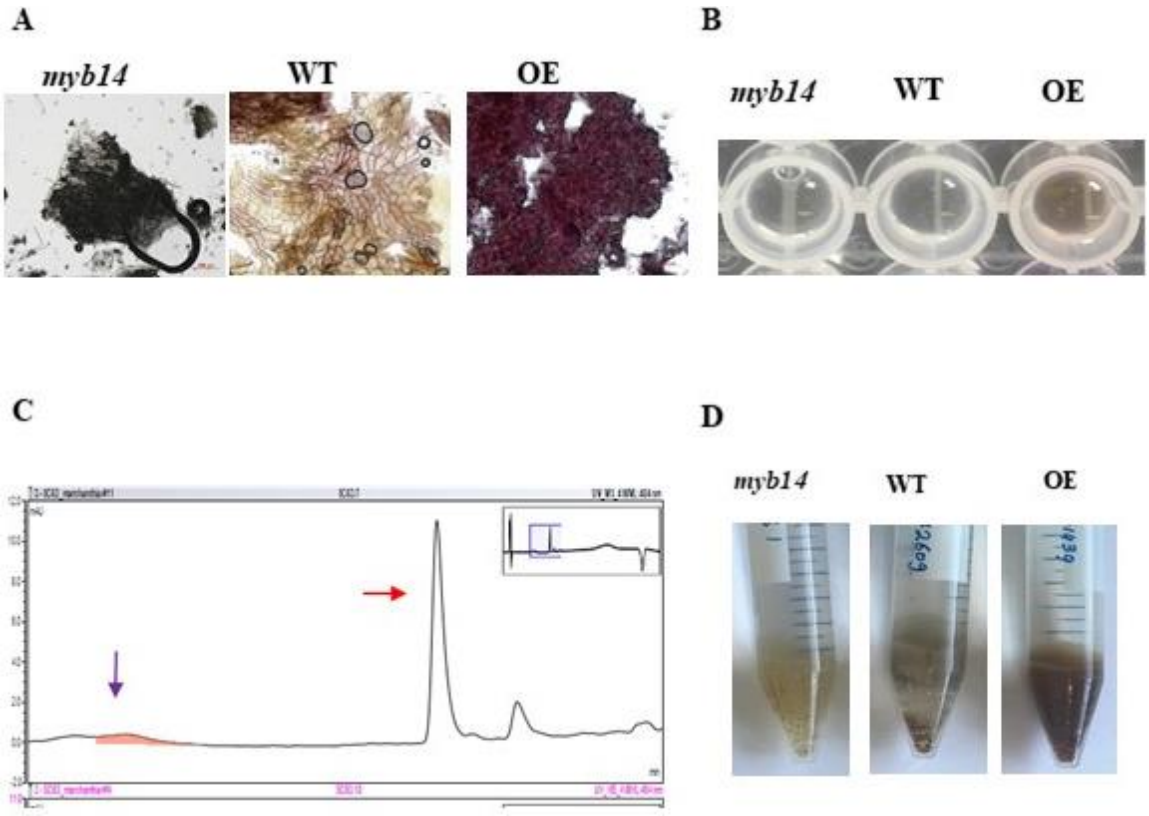

Figure 1. Analysis of red- and non-pigmented cell walls. A. Primary cell walls (PCW) prepared from 6-weeks old myb14, wild-type (WT) and 35S-MYB14 (OE) plants. B. Extracts of OE, WT and myb14 cell walls in methanol. C. UHPLC profiles (484 nm) showing Auronidin 4-neohesperidoside (purple arrows) and the riccionidin A aglycone (red arrows) as described previously 2.D. Insoluble residue of myb14, WT and OE plants after sequential extractions of cell wall polymers.

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O5.2

Overexpression of dahlia chalcone reductase candidate gene in tobacco

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MAIN CONCLUSION

In order to confirm function of dahlia chalcone reductase candidate gene *c25599*, overexpression lines were produced in *Nicotiana tabacum* and transient overexpression analysis was performed in *N. benthamiana*. Single overexpression of *c25599* did not induce isoliquiritigenin accumulation, whereas transient overexpression of the *c25599* with *Am4'CGT* and *CaMYBA* in *N. benthamiana* leaves successfully induced isoliquiritigenin accumulation, indicating *c25599* functions as a chalcone reductase.

INTRODUCTION

Butein (2′,3,4,4′-tetrahydroxychalcone) is a bright yellow flavonoid pigment, which is detected in limited species including dahlia (*Dahlia variabilis*), *Cosmos sulphureus* and *Coreopsis grandiflora* Butein is presumed to be synthesized from malonyl CoA and 4-coumaroyl CoA, which are converted to 2′,4,4′-trihydroxychalcone (isoliquiritigenin) by chalcone synthase (CHS) and chalcone reductase (CHR), and then hydroxylated by chalcone 3-hydroxylase (CH3H). Previous studies suggested CHR is the determinant factor for butein biosynthesis in dahlia. In soybean (*Glycine max*), *GmCHRs*, which belong to aldo-keto reductase 4A sub-family, regulate isoliquiritigenin biosynthesis in isoflavone biosynthesis pathway. However, in dahlia, no orthologous gene of aldo-keto reductase 4A sub-family was found, suggesting a gene encoding CHR in dahlia is completely different from *GmCHR*. By comparative RNA-seq analysis using a red-white bicolor cultivar ‘Shukuhai’ and its lateral mutant purple-white bicolor cultivar ‘Rinka’, we isolated *c25599* as a candidate CHR gene [1]. Expression level of *c25599* was corelated to butein content in dahlia ray florets, indicating *c25599* have functions as CHR. In this study, to confirm the function of *c25599*, we produced stable overexpression plants in *N. tabacum* and performed transient overexpression analysis by agroinfiltration in *N. benthamiana* leaves.

MATERIALS & METHODS

c25599-1, *c25599-2*, *DvCHS2*, *DvCH3H*, *GmCHR5*, *GmCHR6*, *Am4'CGT* or *CaMYBA* cDNA was cloned to a pGWB2 binary vector. *c25599-1*, *c25599-2*, and *DvCH3H* were cloned from ray florets of dahlia ‘Shukuhai’, *DvCHS2* was cloned from ray florets of dahlia ‘Yuino’, *GmCHR5* and *GmCHR6* were cloned from *G. max* and *Am4'CGT* was cloned from *Antirrhinum majus*. *CaMYBA* was cloned from *Capsicum annuum* ‘Peruvian Purple’ [2]. Transgenic tobacco (*N. tabacum*) plants were obtained by standard *Agrobacterium* leaf disc transformation. T_0 transgenic plants harboring one copy of the transgene were selected by genomic PCR, qPCR or DNA gel blot analysis. T_0 or T_1 plants were crossed to obtain multiple transgene overexpression lines. Transient overexpression in *N. benthamiana* plants were performed according to [3]. *Agrobacterium* harboring transgene were infiltrated solely or co-infiltrated to 3–4 weeks seedling leaves, and leaves were collected for subsequent pigment extraction and RT-PCR at 5–7 days after infiltration.

RESULTS & DISCUSSION

At first, we produced stable *c25599* overexpression plants, but we could not detect isoliquiritigenin in leaves or flowers. However, isoliquiritigenin was neither detected from plants with overexpression of known soybean CHRs (*GmCHR5* or *GmCHR6*), suggesting single overexpression of *CHR* is not sufficient for isoliquiritigenin accumulation in tobacco. We also produced co-overexpression plants of *c25599* with *DvCHS2* or *DvCH3H*, but isoliquiritigenin was not detected. Generally, flavonoids are accumulated as a glycoside in plants, and dahlia accumulates isoliquiritigenin and butein as a 4′-glucoside. Glucosyltransferase for isoliquiritigenin or butein in dahlia has not been identified yet, but snapdragon chalcone 4′-glucosyltransferase (*Am4′CGT*) was predicted to glycosylate the same position. We performed transient co-overexpression of *c25599* and *Am4′CGT* by agroinfiltration in *N. benthamiana* leaves. Again, we could not detect isoliquiritigenin, but we neither detect any other flavonoids, thus we inferred flavonoid biosynthesis activity is not sufficient in *N. benthamiana* leaves. To intensify flavonoid biosynthesis in leaves, we co-overexpressed *c25599* and *Am4′CGT* with *CaMYBA*, a MYB transcription factor for anthocyanin biosynthesis in pepper. Eventually, isoliquiritigenin and anthocyanins were detected in the infiltrated leaves, suggesting that *c25599* has function as a chalcone reductase, and that *CaMYBA* promoted expression of genes which are important for isoliquiritigenin accumulation in *N. benthamiana*.

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O5.3

Comparing the effect of targeting a specific-phloretin glycosyltransferase in apple by RNA silencing and CRISPR/Cas9 genome editing

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MAIN CONCLUSION

In this work we studied the effects of targeting the gene *UGT88F1* encoding a key glycosyltransferase involved in the synthesis of phloridzin in apple (*Malus* x *domestica*), by RNA silencing and CRISPR/Cas9 genome editing. Both approaches resulted in a decrease of phloridzin, although noticeable differences in growth phenotype were found. Phytohormone and molecular analyses further extended the identification of common and distinctive patterns following different strategies.

INTRODUCTION

Dihydrochalcones (DHCs) constitute a class of metabolites derived from the phenylpropanoid pathway, which have been shown to exert beneficial properties for both human health and plants. In contrast to the more common polyphenols, DHCs have a limited natural distribution in plants. Among DHCs, phloridzin is the most abundant polyphenolic compound in apple *(Malus* x *domestica*), accounting for up to 10% of leaf dry weight (Gosch *et al.*, 2010). DHC synthesis diverges as a side branch of the phenylpropanoid pathway starting from *p*-coumaroyl-CoA, to produce dihydro-*p*-coumaroyl-CoA as the result of the postulated action of a double-bond reductase (DBR). Chalcone synthases (CHS) catalyse the condensation of one molecule of dihydro-*p*-coumaroyl-CoA with three molecules of malonyl-CoA to produce phloretin (Gosch *et al.*, 2010). Finally, a 2'-*O*-UDP-glycosyltransferase (UGT88F1) transfers a glucose moiety to the aglycone phloretin, resulting in the synthesis of phloridzin.

The elucidation of genes involved in phloridzin biosynthesis has provided the basis to perform functional analyses that may contribute to a better understanding of its role *in planta*. Dare *et al.* (2013) reported that down-regulation of CHS by *RNAi*-mediated gene silencing led to stunted plants with reduced internode length and smaller leaves. A similar effect was found in *UGT88F1* knockdown apple lines, indicating that *UGT88F1* RNA silencing also perturbs plant development (Dare *et al.*, 2017). More recently, another *RNAi*-based study to silence *UGT88F1* showed that knockdown lines had increased salicylic acid and reactive oxygen species (Zhou *et al.*, 2019). In the current study we aimed to evaluate the roles of phloridzin in apple by targeting *UGT88F1* through RNA silencing and CRISPR/Cas9-mediated genome editing.

MATERIALS & METHODS

UGT88F1 was cloned into pK2WG7 vector to produce a silencing vector pK2WG7:UGT88F1-AS. A specific gRNA for the coding sequence (KX639791) was designed and cloned into the vector C504p9ioR-Uq10CasWToi. Both constructs were transformed into 'Golden Delicious' leaves. LC-MS analysis of dihydrochalcones was performed as previously described. Leaves of transgenic lines were subjected to hormone profiling. RNA from young leaves was extracted using Spectrum™ Plant Total RNA kit (Sigma-Aldrich). cDNA was synthesised using SuperScript™ III Reverse

Transcriptase (Invitrogen) and used to conduct gene expression analysis by qRT-PCR in representative lines of each approach.

RESULTS & DISCUSSION

Five transgenic apple lines were obtained following transformation with pK2WG7:*UGT88F1*-AS, showing a reduced expression level. In addition, seven lines transformed with the construct for CRISPR/Cas9 genome editing were regenerated, and three lines were successfully edited. Lines of both strategies grew normally during *in vitro* regeneration. When grown in glasshouse conditions, however, knockdown lines exhibited a characteristic dwarf phenotype, which has been reported previously (Dare *et al.*, 2017; Zhou *et al.*, 2019). Interestingly, this phenotype was not observed in genome edited lines. Lines of both approaches showed a significant decrease in phloridzin. The precursor phloretin showed an increase in knockdown lines, whilst edited lines exhibited a significant reduction.

To better understand the difference in growth phenotype, phytohormone and molecular changes were assessed in transgenic lines. Jasmonic acid (JA) and JA derivatives were significantly increased in knockdown lines, whereas edited lines showed similar levels to controls. In addition, a significant increase in salicylic acid (SA) was found only in knockdown lines. Indole-3-acetic acid (IAA) and abscisic acid (ABA) showed contrasting patterns among lines. Moreover, RT-qPCR expression analyses showed a differential regulation of transcripts involved in the metabolism of IAA, SA and JA. In addition, genes involved in flavonoid and phenylpropanoid pathways were transcriptionally modulated in response to *UGT88F1* down-regulation. Altogether, these results suggest that phloridzin regulates a range of integrated processes, including stress-related hormones and secondary metabolism, ultimately impacting plant development. Silencing *UGT88F1* by conventional silencing and CRISPR/Cas9 genome editingalso provides insights into the common and distinctive features underlying specific effects of phloridzin on apple development.

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O5.4

Identification of arbutin synthases in Rosaceae

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MAIN CONCLUSION

The last step in arbutin biosynthesis, the glucosylation of *p*-hydroquinone, is active in apple leaves indicating the lack of intermediate steps from phenylpropanoid pathway towards this precursor.

INTRODUCTION

The Rosaceae family includes many economically important fruit crops, among which the subfamily Pyreae comprises a number of genera and species known as ‘‘pome fruit’’. Pome fruits are valuable crops for human nutrition, vitamin supply and health. The most significant health benefits of these fruits are attributed to the (poly)phenolic compounds which are represented by phenolic acids, flavonoids (flavonols, flavan-3-ols, anthocyanins) and tannins (proanthocyanidins), but also *p*-hydroquinone and dihydrochalcone derivatives. Generally, these metabolites are considered as protective micronutrients and quality parameter for edible fruit. The genera-specific distribution of high concentrations of phloridzin (*Malus*) and arbutin (*p*-hydroquinone *ß*-D-glucoside) (*Pyrus*) is well established, while dihydrochalcones in general are only a minor compounds in other Rosaceae fruits (e.g., berries). However, an apple-pear hybrid accumulates both compounds including the respective aglycons (phloretin and *p*-hydroquinone) in leaves [1]. These facts provide an excellent opportunity to study the arbutin biosynthesis, which is not understood completely so far. The only characterized step is the glycosylation of *p*-hydroquinone to arbutin (Fig. 1)

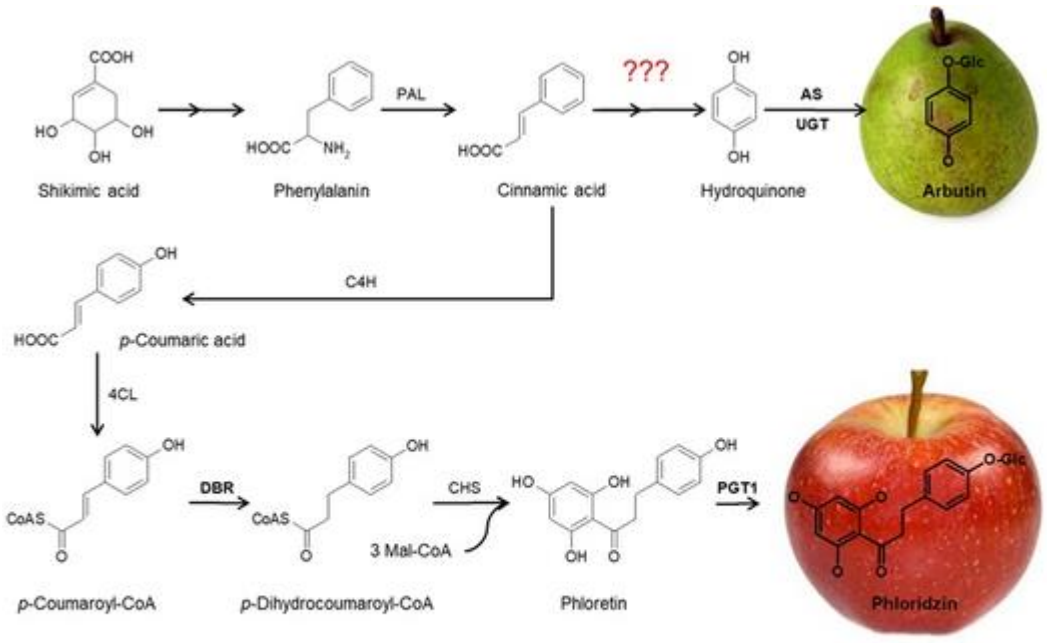

Fig. 1. Pathway scheme to genera specific metabolites in apple and pear

MATERIALS & METHODS

M. domestica “Golden Delicious”, “Kalko”, *P. communis* “Conference”, “Williams” and the apple-pear hybrid “Zwintschzers Hybrid” were grown in the experimental field at Edmund Mach Foundation. Plant material were collected, directly shock frozen and stored at -80°C. Candidate genes were amplified with gene specific primer derived from genome screening from cDNA and further cloned into bacterial expression vector. Recombinant protein was obtained after induction with IPTG and used in *in vitro* enzyme assays. Supplementation of leaves and *in vitro* plants were performed according to [2] and by including the precursor into the media. All analytics were done using targeted LS-MS/MS method optimised for “Pome fruit” phenolics according [3].

RESULTS & DISCUSSION

Homologues genes of the glycosyltransferase encoding arbutin synthase described from Indian snakeroot (*Rauvolfia serpentine*, Apocynaceae) where identified in available Rosaceae genomes. The candidate genes from apple and pear where cloned, heterologous expressed in *E coli* and the resulting recombinant proteins were analyzed in *in vitro* enzyme assay. Together with supplementation experiments of apple leaves and *in vitro* plantlets these results show that apple is in principle be able to produce arbutin but due to missing *p*-hydroquinone synthesis it lacks this compound. Furthermore, this indicates that one or more steps from phenylpropanoid precursor, e.g., cinnamic acid, towards *p*-hydroquinone are not expressed or missing in the genome of apple (Fig. 1). Using RNA seq data generated from apple, pear and apple-pear hybrid leaves are now the basis for mining for differentially expressed candidate genes to elucidate this branch for the first time in pear.

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O5.5

Flavonoid-tannin pathway and growth of silver birch

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MAIN CONCLUSION

Decreased growth of genetically modified silver birches shows that an undisturbed channeling of foliar phenylpropanoid metabolites from flavonoids to condensed tannins is required for normal growth. Our results could be explained by essential ecological functions of foliar condensed tannins, or by over-accumulating flavonoids interacting with primary pathways. This may have implications for the mechanisms of metabolic cross-talk between primary and secondary pathways in plants faced with stress.

INTRODUCTION

Plant secondary (or specialized) metabolites can be defined as taxonomically restricted phytochemicals with specialized ecological functions, while primary metabolites are shared by all plants and required for normal development and growth. The traditional division of plant metabolism into primary and secondary components was challenged with the discovery that certain flavonoid aglycones can limit polar auxin transport [1], required for the normal development of several plant tissues. Although the mechanistic understanding of flavonoid-auxin interactions is still hampered by the unresolved questions on glycosylation and compartmentalization of flavonoids [2], interactions of these environmentally responsive compounds with plant hormones could explain the stress-induced morphogenic responses of plants to, for example, ultraviolet B (UVB) radiation.

Genetic modification of biosynthetic pathways enables studying how plants respond to endogenously altered accumulation of phytochemicals. For example, RNA interference (RNAi) restricting the expression of genes that code biosynthetic enzymes in the late flavonoid-tannin pathway may reduce the accumulation of condensed tannins (also known as proanthocyanidins) [3], which are the final products of the pathway. At the same time, this modification increases the accumulation of flavonol glycosides, the alternative end products, or various intermediary flavonoids, as was shown in silver birch [3] – a boreal deciduous tree that naturally accumulates both flavonoids (especially flavonol glycosides and *trans*-flavan-3-ols) and condensed tannins (CTs).

To study how altered flavonoid-tannin balance or condensed tannin structure affects the growth of genetically modified *B. pendula*, we expand the results from an earlier experiment where RNAi was used to limit the levels of dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS) or anthocyanidin reductase (ANR) in early-flowering silver birch [3].

MATERIALS & METHODS

After growing 5 plants from 11 RNAi-modified lines and an unmodified control line of *B. pendula* for 14 weeks in a greenhouse (first without, then with ambient UVB for 51 days) as described in [3], we measured total biomasses and characterized phenolics from both leaves and stems. Low-molecular weight phenolics were identified with UHPLC-Q-TOF/MS and quantified with HPLC-UV-DAD, while acid butanol assay was used for CTs. Material from a subset of lines was thiolysed and analyzed with HPLC to define the average CT structure (see [3] for details).

Pearson correlation coefficients were calculated between means of variables measured from each line (n=13 for biomass; n=5–8 for tannin structure). Foliar flavonoid-tannin ratio was defined as the ratio of pre-DFR flavonoids to CTs and their monomers. The relationship between aboveground biomass and foliar flavonoid-tannin ratio measured from 51 individual plants was explored with linear mixed models, with random intercepts for each line.

RESULTS & DISCUSSION

Blocking the flavonoid-tannin pathway from DFR was earlier shown to severely reduce CT production, while pre-DFR flavonoids strongly accumulate [3]. Restricting the *cis*-flavan-3-ol-producing pathway (ANS or ANR) only moderately affects flavonoids and CTs, but decreases the size of CT polymers and increases their *trans*-% [3].

Strongly negative correlations between biomass and pre-DFR flavonoids in leaves ($r = –0.92$) and stems ($r = –0.72$) would support the hypothesis that overaccumulation of flavonoids inhibited growth in DFRi and ANRi plants. Oxidative inactivation or reduced transport of auxins [2] due to over-accumulating flavonoids could have constrained normal organ development in strongly growth-inhibited DFRi plants.

Biomass correlated strongly with CTs or their precursors in leaves ($r = 0.94$) but not in stems ($r = 0.31$), suggesting that unimpaired production of foliar CTs may be essential for normal growth of young *B. pendula*. Corresponding relationship between soluble CTs and leaf growth has been found in *B. pubescens* ssp. *czerepanovii* [4]. Since overaccumulation of CTs seems to increase resistance to oxidative conditions in transgenic *Populus* [5], DFR-restricted *B. pendula* plants might have suffered from oxidative stress, as supported by their chlorophyll fluorescence characteristics [3].

Only weak correlations were found between mDP and trans-% of leaf and stem CTs with biomasses of respective organs ($|r| < 0.45$).

When modelling the square root of aboveground biomass based on foliar flavonoid-tannin ratio, a negative relationship was found (estimate ± SE: –0.46 ± 0.21, df=48, $p=0.029$). Our results show that a metabolic shift from CTs and their precursors into pre-DFR flavonoids can have severe consequences for growth in plants. Corresponding metabolic cross-talk potentially occurring in unmodified plants could contribute to the mechanisms by which environmental conditions induce morphogenic changes in deciduous trees.

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O5.6

Creating CRISPR knockouts for two MYBs that regulate Proanthocyanidins biosynthesis in poplar

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MAIN CONCLUSION

This study used CRISPR/Cas9 technique to knock out two MYB genes in order to generate mutants for testing the roles and tissue specific interactions of MYB115 and MYB134 in PA biosynthesis in hybrid poplar. The editing efficiency of CRISPR/Cas9 system on several gRNAs for these two MYBs was compared. We found that gRNA2 triggered mutations more frequently than gRNA1 in both genes, and that mutation types of these two genes were also distinct. The poplar genotype transformed was also important.

INTRODUCTION

Tree defense is highly dependant on secondary metabolites, which are small organic plant constituents, often with bioactive properties. Many secondary metabolic pathways have been studied and their regulation is an active research area. Proanthocyanidins (PAs), also known as condensed tannins (CTs), are one of the most common secondary metabolites, and widely distributed in trees and woody plants, for example, poplar (*Populus spp*). *Populus* species contain abundant and diverse phenolic compounds, including phenolic glycosides, flavonoids and PAs, making this genus an excellent system for studies of the functional and ecological properties of phenolic secondary metabolites. The biological functions of PAs in leaves and shoots have been extensively investigated, suggesting anti-pathogen or anti-herbivore effects, as well as anti-oxidant functions. PAs are often abundant in roots of woody plants, including poplar. However, little research attention has been given to the potential physiological and ecological roles of these compounds in roots. Potential functions of root PAs could include defence against soil pest and pathogen, or chelation of ions and nutrients.

In previous work, we characterized two MYB transcription factors, MYB134 and MYB115, that specifically regulate PA biosynthesis in poplar leaves [1]. High-PA lines with overexpressed MYB134 or MYB115 and MYB134-RNAi knockout lines have been engineered in our lab. Studies of these transgenic plants revealed the importance of these two MYBs in regulating PA synthesis in leaves, but their roles and interactions in roots are not known. In order to test the regulation roles and tissue specific priority of these two MYBs in vivo, CRISPR knockouts for each MYB were generated. This study aims to test the function of both MYBs in the regulation of PAs in poplar roots, and to determine if they interact or work cooperatively to control PA biosynthesis in roots as they do in leaves.

MATERIALS & METHODS

Both MYB genes from wildtype poplars were first cloned and sequenced in order to identify both alleles in poplar hybrid clones. Potential gRNAs targets for each gene were selected to be free of single nucleotide polymorphisms (SNPs) from AspenDB. CRISPR constructs were prepared as described previously [2]. Two gRNAs in different exons were inserted into one construct in order to create large deletions in coding sequence. MYB knockouts were generated in both hairy roots and whole plants. Positive hairy root mutants were identified using a GFP marker, while independent whole plant lines were screened by antibiotic resistance. To characterize mutations, genomic DNA of each transgenic line was extracted and both MYB genes were amplified and sequenced. Resulting sequences were aligned with wild-type sequences and analyzed using the TIDE web tool

(https://tide.nki.nl/) to determine mutation types. PCR product was cloned into T-vector and sequenced again to confirm mutations in both alleles.

RESULTS & DISCUSSION

CRISPR/Cas9 technology has recently been adapted to poplar and has been applied in both poplar hairy roots and whole plants generation. Hairy roots transformation is a faster and more efficient strategy for obtaining transgenic material compared to whole plant transformation, and an approach for testing gRNA specificity and efficiency in advance. Because poplar roots accumulate large amounts of PAs, the hairy root expression system is ideal for testing the roles of MYB115 and MYB134 in PA biosynthesis.

The rates of successful target gene edits in hairy roots or whole plants was calculated on the basis of target gene sequencing results. Altogether, 87.5–92.3% of all GFP-positive hairy roots were edited. However, only 30.8–37.5% of all transgenic events in hairy roots led to biallelic mutations. Another 30% of mutant lines were monoallelic, while some were chimeric cultures with three mutated alleles or with two mutated alleles and one WT allele simultaneous in the same tissue. For whole plant mutants, the editing ratio of MYB134 and MYB 115 were 66.7% and 100% respectively. The MYB134 CRISPR construct generated more large deletions than MYB115. Short indel mutations were the most prevalent mutations identified in MYB115 transgenics. For both MYBs, we identified more mutations and indels in gRNA2 compared to gRNA1, suggesting that gRNA2 possessed higher editing efficiency than gRNA1.

As expected from the CRISPR/Cas9 system, we found not only short deletions and insertions, but also large deletions between two gRNAs, although this typically occurred only in one of the alleles. Most deletions were monoallelic or biallelic with only one large deleted allele, and no homozygous large deletions were found. Based on the genotyping results, the editing efficiency of CRISPR/Cas9 system appeared gene- and gRNA-dependent. Mutants generated on hairy roots seemed to have more mutation types than whole plants, indicating that the Cas9 capacity may change in different tissues.

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O5.7

Interaction between root tannins and soil fungi stabilizes carbon in the soil

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MAIN CONCLUSION

The understanding of mechanisms underlying carbon (C) stabilization is urgently needed due to the threat of climate change. Here I unravel a mechanism behind C stabilization via the interaction of root-derived tannins with fungal necromass. This interaction between plant (tannins) and fungal (chitin) compounds sheds light on the new paradigm of microbial control over soil organic matter stabilization.

INTRODUCTION

Plants produce tannins in high abundance and of large chemical diversity [1]. The role of tannins in the ecosystem spans interaction with herbivores, microorganisms, and control of C and nitrogen (N) cycling [1]. Tannins act on soil processes via two main routes: -direct toxicity to microbes, - formation of chemically resistant complexes with peptides/proteins, including enzymes [1]. Tannin effects relate mainly to their ability to bind proteins forming stable complexes, a property of tannins used for millennia to manufacture leather. Discovery of interaction between chitin and tannins similar to these of tannin-protein provides a new twist to the understanding of C stabilization [2]. Fungal cell wall chitin is a polymer of amino sugar *N*-acetyl-D-glucosamine, and similarly to proteins, it has amide groups adjacent to the carbonyl group, which reinforces the hydrogen bonding with tannins producing stable complexes [2]. As the newest studies point to roots and fungal residues (=fungal necromass) as main sources of stable soil organic matter [3] interaction between compounds originating from roots and fungi could unravel high soil organic matter accumulation in some ecosystems. This mechanism of C stabilization could be of crucial importance especially in tannin-rich plant communities and ecosystems with abundant ectomycorrhizal symbiont biomass, like boreal forests.

MATERIALS & METHODS

To study tannin-fungal residue interaction a set of experiments was conducted. These include laboratory and field experiments. A laboratory experiment with pine seedlings aimed to study the effect of tannin-fungal necromass interaction on fungal residue decomposition [2]. A field experiment with fungal necromass placed in mesh bags aimed to understand the effect of root tannins on fungal necromass decomposition and to prove that tannin-fungal necromass interaction takes place also under natural conditions [2]. Following field experiment with soil organic layer placed in mesh bags aimed to explain if roots may increase soil organic matter build-up via tannin-fungal necromass interaction [4].

RESULTS & DISCUSSION

A new theory of soil organic matter stabilization based on the ability of tannins to form complexes with fungal proteins and chitin has been confirmed in a set of experiments. Laboratory experiment proved that interaction between tannins and fungal necromass slows down its decomposition [2]. Field experiments proved that tannins interact with fungal necromass also under natural conditions and that such interaction promotes build-up of soil organic matter [4]. Taken together, these experiments provide mechanistic explanation for carbon stabilization derived from roots and fungal

necromass. Moreover, this route of C stabilization explains the prevalence of peptide-like and amino sugar compounds in stable soil organic matter [3].

Future studies should estimate stability of tannin-fungal necromass interactions and their commonness across different ecosystems. Formation of complexes between root-derived tannins and fungal residues can be a crucial C stabilization mechanism in ecosystems with tannin-rich plants and abundant mycorrhization. However, it is also possible that in other ecosystems, like tropical forests with high amounts of tannins [1], the same mechanism can occur. This route of soil organic matter stabilization should be tested for interactions with other soil organic matter stabilization mechanisms, i.e. stabilization of organic matter on soil minerals. For example, it is possible that tannin-fungal necromass complexes interplay with minerals, increasing their stability. Further studies should include differences in tannin chemistry and also fungal necromass chemistry. Moreover, climatic conditions, soil physicochemistry and the ability of microbial communities to decompose tannin-fungal necromass complexes should be taken into account.

All in all, the interaction between tannins and fungal necromass leading to stabilization of microbial-derived C could be important, though hitherto overlooked, mechanism of soil organic matter build-up.

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O5.8

Breeding for novel flower colour in poinsettia (Euphorbia pulcherrima) via Genome editing and classical transgenic approaches

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MAIN CONCLUSION

Genome edited poinsettia plants show loss-of-function mutations in the *F3'H* gene introduced by CRISPR/Cas9. This is sufficient to change the bract colour from red to reddish orange. This is the first report of successful use of CRISPR/Cas9 for genome editing in poinsettia.

INTRODUCTION

Poinsettia (*Euphorbia pulcherrima*) belongs to the economically most important potted ornamentals, specially associated with the Christmas time. Typically, poinsettia are characterised by intense red bract colour, which is based on the presence of cyanidin based anthocyanins carrying two hydroxyl groups in the B-ring. There is, however, a growing demand for cultivars with novel bract colours. Rare orangered poinsettias accumulate pelargonidins (one hydroxyl group in the B-ring) instead of cyanidins. Recently, we have shown that the rare orange-red bract colouration of poinsettia (*Euphorbia pulcherrima*) is associated with a prevalence of pelargonidin-type anthocyanins and a somewhat decreased anthocyanin concentration in general. We also reported that accumulation of pelargonidin-based pigments in poinsettia is caused by different mechanisms, of which one is a strong reduction of flavonoid 3'-hydroxylase (F3′H) activity in the bracts [1].

F3′H (EC 1.14.13.21) is a membrane bound enzyme associated with the cytosolic site of the endoplasmic reticulum [2] and is responsible for the introduction of a second hydroxyl group in the B-ring thereby leading to the formation of cyanidin type anthocyanins.

To obtain poinsettias, which accumulate prevalently pelargonidins, we decided to silence *flavonoid 3'-hydroxylase* (*F3'H*), which introduces a second hydroxyl group in the B-ring during anthocyanin biosynthesis. We attempted to knockout the poinsettia *F3'H* gene by applying the CRISPR/Cas9 method to obtain poinsettias prevalently accumulating pelargonidin type anthocyanins in their bracts. To our knowledge, this is the first report of a genome-edited poinsettia.

MATERIALS & METHODS

Poinsettia *Euphorbia pulcherrima* cultivar 'Christmas Eve' (Klemm + Sohn GmbH & Co. KG, Germany) was used for transformation. Plants were grown in the greenhouse under long day conditions (16 h day/8 h night). For *Agrobacterium*-mediated transformation internode stem explants were used as recently described [3]. For transformation, the binary vector pDe-Sa_Cas9 [4], carrying a kanamycin resistance gene (*nptII*) to facilitate selection was used (Fig. 2). A sgRNA sequence was designed based on the sequences available in the Gene Bank (KY273440.1). Details of sgRNA design and cloning, transformation and regeneration, screening and analysis of regenerated plants were provided recently [3]. Bract colouration was induced by cultivation for 8 weeks at short day conditions (11h day, 13h night). Poinsettia bract pigment analysis was performed by HPLC as previously described [5].

RESULTS & DISCUSSION

In the regeneration process, three independent transgenic lines were obtained, in which the presence of *nptII*, *cas9* and gRNA was confirmed at gDNA and RNA level. Positive lines named B2, B158

and B284 were transferred to the greenhouse for further cultivation. After tinduction of bract colour development, plants propagated from line 2 revealed brighter red bract colour (B2.1), or a chimeric phenotype with bright-red areas on the bracts (B2.2). To analyse the *F3'H* sequence of transgenic plants, next generation sequencing (NGS) was performed. 24 % of the reads for line B2 reddish orange (B2.1) and 19 % of line B2 chimera (B2.2) was modified. For lines B158 and B284, almost no modifications were found. The most prevalent modification for line B2 was an insertion of a thymine (T) located three nucleotides before the PAM sequence. Nevertheless, in all transgenic lines, the most prevalent sequence was that of the WT, which suggests that the genome editing approach was not efficient enough. HPLC analysis confirmed that the reddish orange line contained mostly pelargonidins, whereas in the chimeric plant the pelargonidin:cyanidin ratio was approximately 1:1. Accordingly, the expression level of *F3'H* was lower than in the wild type. In the other two lines, anthocyanin composition and *F3'H* expression was similar to the wild type and no colour differences visible.

The CRISPR/Cas9 system is without doubt an interesting alternative for targeted mutagenesis in poinsettia. It should, however, be taken into account that poinsettia transformation and regeneration is a challenging process and protocol optimization for each variety seems to be essential. Poinsettias are propagated vegetatively, and selected varieties are frequently marketed as a series with a range of colours. This study puts forward CRISPR/Cas9 as an alternative to the process of irradiated mutation for breeding of colour variation, which is currently the standard practice in commercial poinsettia breeding.

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O5.9

Seed-coat protective neolignans are produced by the dirigent protein AtDP1 and the laccase AtLAC5 in Arabidopsis

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MAIN CONCLUSION

Neolignans are plant-derived compounds that have several health benefits, however, the biosynthetic genes remain unknown. We determined the precise structures of neolignans in Arabidopsis seeds. Additionally we identified genes for a dirigent protein and a laccase that play essential roles for neolignan biosynthesis and revealed the neolignan biosynthetic pathway via sinapoylcholine/feruloylcholine. Seed coat permeability test suggests a protective role of neolignans in Arabidopsis seeds.

INTRODUCTION

Lignans and neolignans are a class of dimeric phenylpropanoid metabolites [$(C_6C_3)_2$] that are generally synthesized from coniferyl alcohol in the cinnamate/monolignol pathway by oxidation to generate the corresponding radicals with subsequent stereoselective dimerization aided by dirigent proteins (DIRs). The biosynthetic pathways of lignans and neolignans diverge at the step for oxidative coupling of monolignols by DIRs. The dimers with a C8-C8′ linkage are lignans and those with all other types of linkages are called neolignans. Compared to lignans, the enzymes, genes and pathways for neolignan biosynthesis remain largely unknown.

Based on the gene-metabolite correlation analyses, we found an orphan *DIR*, *AtDP1* that showed no significant correlation with putative lignans/neolignans and other *DIR*s [1]. The mutant deficient in *AtDP1* lacked a putative neolignan compound but the structure remained to be verified.

MATERIALS & METHODS

The details for plant materials, neolignan analysis using LC–ESI–Q-Tof–MS, chemical synthesis of authentic standards for neolignans, confocal laser scanning microscopy, light microscopy, and electron microscopy, seed coat permeability test, proanthocyanidin analysis, and lignin analysis were described in [2].

RESULTS & DISCUSSION

We determined structure of a putative neolignan compound missing in the *atdp1* mutant as a sinapoylcholine (SC)-conjugated neolignan, *erythro*-3-{4-[2-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-1-hydroxymethylethoxy]-3,5-dimethoxyphenyl}acryloylcholine using a chemically synthesized standard. A complementation test of the *atdp1* mutant showed that the dirigent protein AtDP1 plays an essential role for the 8-*O*-4′ coupling in neolignan biosynthesis. Phylogenetic analyses showed that AtDP1 belongs to the DIR-a subfamily comprised of DIRs for lignan biosynthesis. *AtDP1* transcripts are specifically accumulated in outer integument 1 cells in developing seeds. As an oxidase for neolignan biosynthesis, we focused on *AtLAC5*, a laccase gene coexpressed with *AtDP1*. In *lac5* mutants, the abundance of feruloylcholine (FC)-conjugated neolignans decreased to a level comparable to those in the *atdp1* mutant. These results strongly suggest that AtLAC5 are also involved in neolignan biosynthesis. In addition, SC/FC-conjugated neolignans were missing in

the seeds of mutants defective in SCT/SCPL19, an enzyme that synthesizes SC, suggesting that the neolignans in Arabidopsis seeds are synthesized via SC/FC.

A penetration assay using tetrazolium salt showed that seed coat permeability increased in the *atdp1* mutant that is deficient in neolignans but has comparable accumulation levels of proanthocyanidins and lignins as the wild type. This result suggests a protective role of neolignans in Arabidopsis seeds.

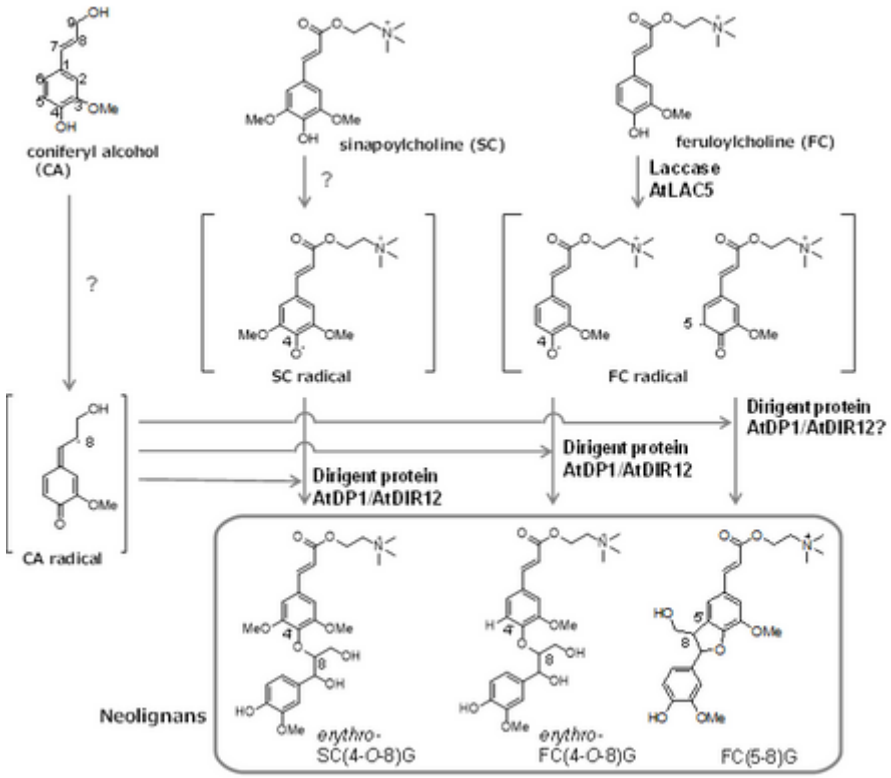

Figure 1. The proposed biosynthetic pathways of neolignans in Arabidopsis. DIR, dirigent protein; laccase, LAC; G, guaiacyl moiety; SC, sinapoylcholine; FC, feruloylcholine; CA, coniferyl alcohol.

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O5.10

Towards understanding the role and regulation of condensed tannin during ectomycorrhizal symbiosis development in *Populus* roots

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MAIN CONCLUSION

Condensed tannin (CT) levels decrease in *Populus* root epidermis cells during symbiosis formation with the ectomycorrhizal fungus *Laccaria bicolor*. Transgenic increase of CT levels hampers ectomycorrhiza formation, suggesting that CT-decrease is a prerequisite for root colonization.

INTRODUCTION

Most boreal and temperate forest trees live in symbiosis with ectomycorrhizal soil fungi via their root system. Ectomycorrhiza formation involves the formation of a fungal mantle around roots, followed by the formation of a labyrinthine network of hyphae in between root epidermis and cortex cells, the so-called Hartig Net [1]. This process requires cell wall loosening and deconstruction of the middle lamella between adjacent root cells, a process thought to involve dampening of plant defense responses as well as pectin remodelling [2]. Pectin is a polysaccharide highly present in the middle lamella where it has an essential role in cell-to-cell adhesion. Condensed tannins (CTs) are secondary plant metabolites that function as defence compounds during pathogen attacks and herbivory [3], and can interact with pectin [4]. Their presence in roots and their regulation upon ECM formation, as well as their regulatory function for pectin remodelling during ECM formation is poorly understood, however. We are studying the regulation and potential role of CTs in the symbiotic interaction of *Populus* roots with the fungus *Laccaria bicolor* using wildtype and transgenic *Populus* lines [5] with altered CT levels and profiles. Combining quantification, localization, gene expression and ECM formation studies, we investigate CT dynamics, biosynthesis and role during ECM formation.

MATERIALS & METHODS

Biological material: *Populus tremula x tremuloides* (wild type and MYB115 overexpressors which overaccumulate CTs) [5] and *Laccaria bicolor* were grown *in vitro.* ECM were generated using a plate-based culture system described in [1].

Staining and microscopy Root were stained with p-dimethylaminocinnamaldehyde (DMACA) according to [6], followed by rehydration, agarose embedding and vibratome sectioning (70µm), counterstained with Wheat-Germ-Agglutinin, WGA, for fungal chitin, mounted in 50% glycerol and observed using a confocal microscope (Zeiss LM780, excitation 633nm, emission 650–690 nm for DMACA and 488nm and 500-590nm for WGA). Micrographs were analyzed using ZEN black software (ZEISS).

CT quantification Condensed tannin levels were quantified using the acid-butanol assay [7].

RESULTS & DISCUSSION

Using DMACA staining in combination with fluorescence imaging (Chowdhury et al. in preparation), we localized and quantified the abundance of CTs in the epidermal layer of *Populus* lateral root cross sections, colonized or not by *L. bicolor*. We observed a decrease of wall-bound CTs at 14 d of colonization, corresponding to the appearance of mature ECM (Fig 1a). These results were confirmed using purification and spectroscopic quantification of CTs from roots and ECM over a time course.

This showed that CT decrease occurs from early stages during ECM formation (Fig 1b). We hypothesized that CT reduction was a requirement for ECM formation and expected that ECM formation would be hampered in transgenic *Populus* trees with constitutively elevated CT production. Using transgenic PtMYB115 overexpressors which were previously shown to have increased CT levels [5], after contact with *L. bicolor* we noted a significant decrease in ECM root tips by 10% as compared to wt plants (Fig 1c). This suggests that the decrease of CT levels observed during ECM formation is a prerequisite for ECM formation in *Populus*. Plausible mechanisms behind this could be (i) release of pectin-CT complexes that allow pectin remodelling, middle lamella deconstruction and Hartig Net formation and (ii) a reduction of potential anti-fungal activities of CTs.

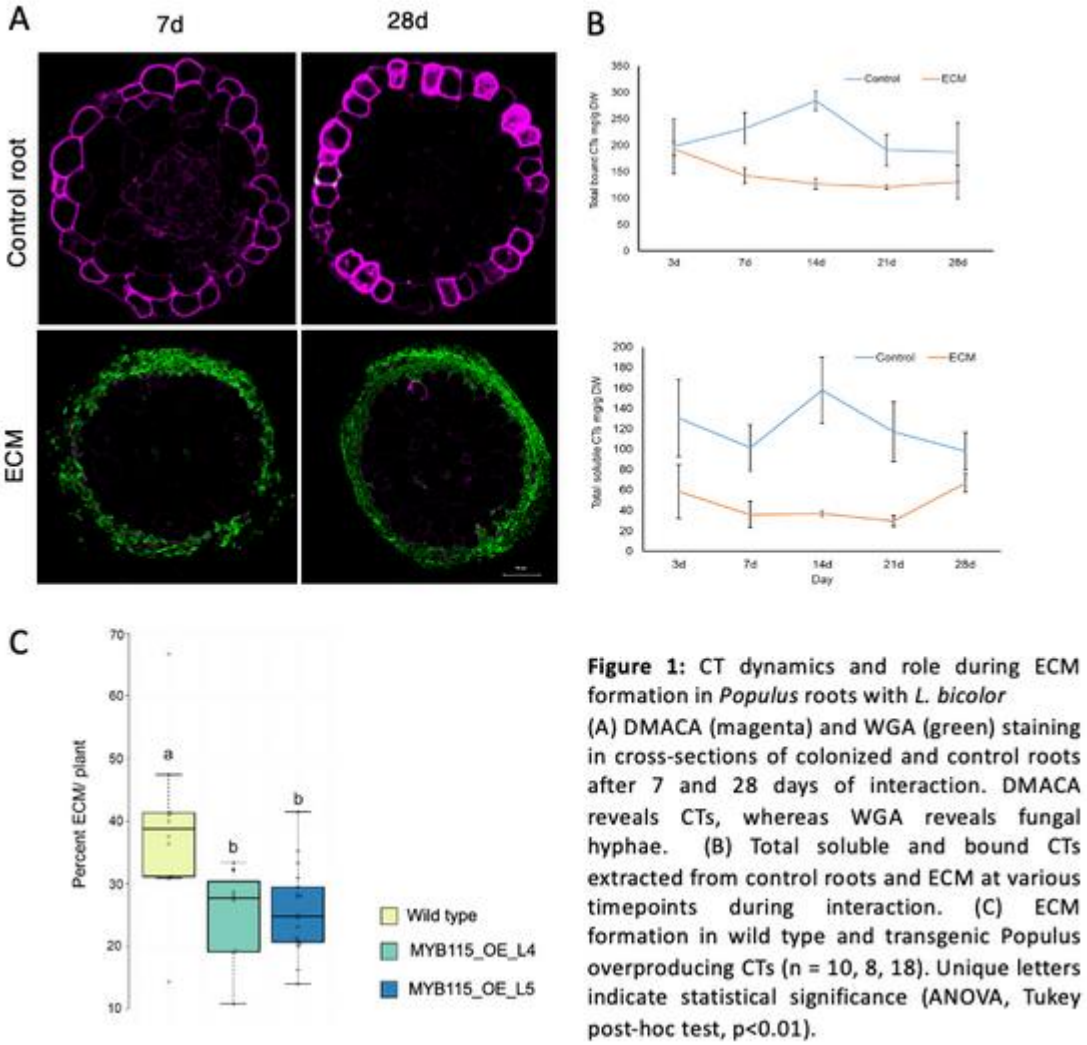

Figure 1: CT dynamics and role during ECM formation in *Populus* roots with *L. bicolor*
(A) DMACA (magenta) and WGA (green) staining in cross-sections of colonized and control roots after 7 and 28 days of interaction. DMACA reveals CTs, whereas WGA reveals fungal hyphae. (B) Total soluble and bound CTs extracted from control roots and ECM at various timepoints during interaction. (C) ECM formation in wild type and transgenic Populus overproducing CTs (n = 10, 8, 18). Unique letters indicate statistical significance (ANOVA, Tukey post-hoc test, $p<0.01$).

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O5.11

Dehydroquinate dehydratase/shikimate dehydrogenases from *Eucalyptus camaldulensis* involved in shikimate pathway, quinate metabolism, and gallate formation

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MAIN CONCLUSION

Functional analysis of four dehydroquinate dehydratase/shikimate dehydrogenase enzymes (DQD/SDH) from the aluminum-resistant tree *Eucalyptus camaldulensis* revealed distinct functions. EcDQD/SDH1 is the major shikimate pathway enzyme, EcDQD/SDH2 and 3 can be involved in both gallate biosynthesis and shikimate pathway, and EcDQD/SDH4a is a NAD-dependent quinate dehydrogenase (QDHa).

INTRODUCTION

Plant dehydroquinate dehydratase/shikimate dehydrogenases (DQD/SDHs) were first described as bifunctional shikimate pathway enzymes catalyzing the dehydration of 3-dehydroquinate (3-DHQ) to 3-dehydroshikimate (3-DHS; DQD activity) and the reversible reduction of 3-DHS to shikimate (SDH activity). Recent analyses revealed that many seed plants contain multiple DQD/SDH enzymes, some of which displayed quinate dehydrogenase (QDH) or gallate formation activities, which may link the shikimate pathway to quinate metabolism and the synthesis of hydrolyzable tannins (HTs) [1]. In *Eucalyptus camaldulensis*, HT formation has attracted considerable interest because HTs contribute to the exceptional aluminum tolerance of this tree species [2]. We used *in vitro* enzyme assays to characterize the distinct DQD, SDH, QDH and gallate formation activities of four DQD/SDH candidates from *E. camaldulensis*. Model structures were generated to interpret catalytic activities at the molecular level. Enzyme activities and gene expression support distinct roles of the investigated enzymes *in planta*.

MATERIALS & METHODS

EcDQD/SDH enzymes were produced in *E. coli* as N-terminal GST-tagged proteins, affinity-purified and subjected to thrombin cleavage for tag removal. Enzymatic activities were assessed at 30°C and optimal pH in the presence of saturating substrate-cofactor concentrations. Kinetic constants were calculated from reaction velocities with at least eight substrate concentrations by nonlinear fitting to the Michaelis-Menten equation. SDH and QDH activities were measured by monitoring of NAD(P)H; DQD, QDH and gallate formation activities by HPLC-quantification of reaction products. EcDQD/SDH model structures were generated by homology modeling with crystal structures of AtDQD/SDH as template. *E. camaldulensis* was cultured hydroponically, and Al stress was applied by 1.5 mM $AlCl_3$ for 5 days. Gene expression was assessed by qRT-PCR normalized against *EcActin* expression. Metabolite concentrations in *E. camaldulensis* plant organs were analyzed by GC/MS of methanolic extracts.

RESULTS & DISCUSSION

Enzyme assays and kinetic constants revealed that EcDQD/SDH1 is a “classical” shikimate dehydrogenase catalyzing the third and fourth steps of the shikimate pathway. EcDQD/SDH2 and 3 displayed gallate formation, DQD and SDH activities. They are candidates for providing the essential

gallate required for HT biosynthesis in *E. camaldulensis*. This is further supported by coexpression with UGT84A25/26 involved in HT biosynthesis. Structure modeling suggests that the gallate formation activity of EcDQD/SDH2 and 3 is correlated with specific amino acid substitutions in the substrate-binding pocket which support a changed orientation of the substrate 3-DHS toward the active center. EcDQD/SDH4a is a NAD-dependent quinate dehydrogenase (EcQDHa). This enzyme has lost DQD activity by amino acid substitutions affecting substrate binding and catalysis. QDH activity of this enzyme is correlated with specific amino acid substitutions in the SDH domain. Under aluminum stress conditions the gene expression of EcDQD/SDH2 and 3 in roots of *E. camaldulensis* was not induced, and the concentration of substrates and products of EcDQD/SDH enzymes were not affected. This indicates that HT biosynthesis provides a rather steady protection against increased aluminum concentrations in soil.

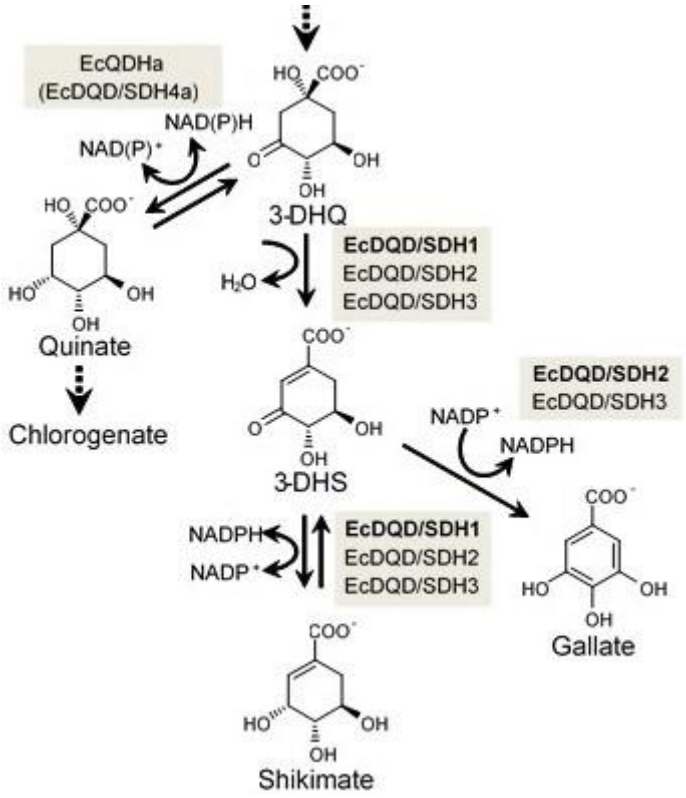

Figure 1. The proposed role of EcDQD/SDH enzymes in shikimate pathway, quinate metabolism and gallate biosynthesis

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O5.12

Aluminum detoxification abilities of hydrolyzable tannins identified in *Eucalyptus camaldulensis*

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MAIN CONCLUSION

In addition to oenothein B, we identified five hydrolyzable tannins in the aluminum-resistant tree *Eucalyptus camaldulensis* (Figure 1). These six hydrolyzable tannins showed aluminum detoxification abilities, which showed a tendency to be greater in tannins with higher molecular weights. These results suggest that hydrolyzable tannins besides oenothein B contribute to aluminum detoxification in *E. camaldulensis*.

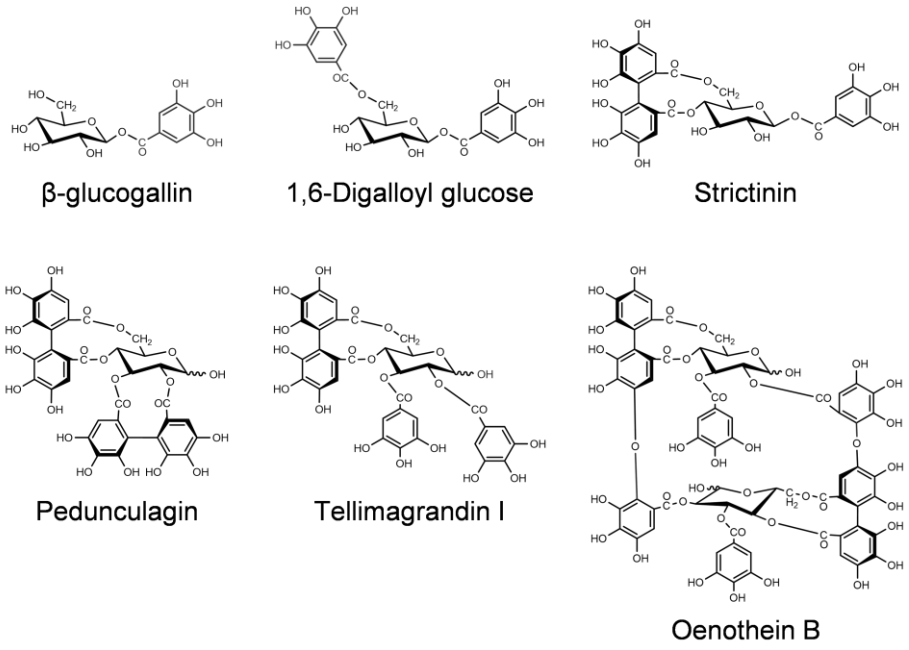

Figure 1. Hydrolyzable tannins identified in *Eucalyptus camaldulensis*.

INTRODUCTION

Aluminum toxicity is a major factor limiting plant productivity in acidic soils, which comprise approximately 30% of the global ice-free land area. Aluminum inhibits plant root elongation, leading to decreased water and nutrient uptake and subsequent growth restriction of the entire plant. *Eucalyptus camaldulensis* is a Myrtaceae tree species that is highly resistant to aluminum toxicity. Previously, we identified oenothein B in *E. camaldulensis* roots; this hydrolyzable tannin contributes to aluminum detoxification [1]. The present study aimed to identify additional hydrolyzable tannins in *E. camaldulensis* and assess their abilities to detoxify aluminum.

MATERIALS & METHODS

Roots and leaves were sampled from an *E. camaldulensis* clone propagated by cutting and cultured hydroponically. Aqueous acetone homogenate of roots and leaves was successively extracted with diethyl ether, ethyl acetate, and *n*-butanol. Hydrolyzable tannins in the *n*-butanol extract were then isolated by column chromatographies and identified based on NMR and MS analyses. Root extracts

were also analyzed by GC–MS. To evaluate the aluminum detoxification abilities of the fractionated extracts and hydrolyzable tannins, we performed bioassays in which the aluminum-sensitive plant *Arabidopsis thaliana* was grown in culture medium containing aluminum and fractionated extracts or hydrolyzable tannins [1]. The concentration of soluble aluminum in the culture medium used in the bioassays was determined by ICP–MS.

RESULTS & DISCUSSION

The results of the bioassays showed that *n*-butanol extract effectively detoxified aluminum. Five hydrolyzable tannins were identified from the *n*-butanol extract, namely, 1,6-digalloyl glucose, strictinin, tellimagrandin I, pedunculagin, and oenothein B (Figure 1). Gallic acid and β-glucogallin, which are the precursors of hydrolyzable tannins, were also identified in the *E. camaldulensis* root extract. The aluminum detoxification abilities of 13 hydrolyzable tannins and precursors, including the compounds identified in *E. camaldulensis*, were analyzed. We found that all compounds showed aluminum detoxification abilities, which showed a tendency to be greater in compounds with higher molecular weights. The soluble aluminum concentration in the culture medium decreased when a high-molecular-weight compound was added, indicating that these compounds can insolubilize aluminum. Taken together, these findings suggest that hydrolyzable tannins in addition to oenothein B contribute to aluminum detoxification in *E. camaldulensis*.

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This work was supported by KAKENHI from the Japan Society for the Promotion of Science (JP18H02246).

O6.1

Polyphenols from pecan nut shell as multifunctional compounds for active packaging, food colorant stabilization and synthesis of silver nanoparticles

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MAIN CONCLUSION

The results of these studies open new perspectives for the exploitation of pecan nut shell as a low cost source of multifunctional polyphenols, endowed with efficient antioxidant and reducing properties for food industry applications or for the preparation of photocatalytic and antimicrobial devices.

INTRODUCTION

The search for natural and sustainably produced antioxidant additives for use in health, food, or cosmetic applications has received considerable attention in recent years, prompted also by the increasing need for green approaches to innovative functional materials. In this context, agri-food byproducts represent an easily accessible source of antioxidant phenolic compounds, which have found increasing applications in materials science. Remarkable examples include incorporation into polymers for both stabilization and functionalization purposes for application in active food packaging, which is an important sector of the food industry aimed at avoiding or delaying food deterioration. Phenolic compounds from agri-food byproducts are also being employed as reducing agents for the preparation of silver nanoparticles (AgNP) under environmentally friendly conditions, for applications in catalysis, photonics, electronics as well as in medicine, as bactericidal, antifungal and antitumor agents. Among agri-food byproducts, pecan nut shell (PNS), which is produced in high amounts (ca. 420,000 tons per year) from pecan nut (*Carya illinoinensis* (Wagenh.) K. Koch) processing, occupies a prominent role, since it contains significant amounts of antioxidant and antimicrobial phenolic compounds. In this context, we report herein new data concerning the possible use of PNS-derived phenolic compounds in the food industry and in the development of AgNP-based photocatalytic and antibacterial devices.

MATERIALS & METHODS

PNS hydroalcoholic extract (PNSE) was prepared and characterized as reported [1]. PNSE-functionalized polylactic acid (PLA) and whey protein (WP) films were prepared by extrusion and/or solvent casting, and characterized by scanning electron microscopy (SEM), attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy, and tensile tests. Gas permeability properties were also measured. AgNP were prepared following a wet chemical and a mechanochemical approach, and characterized by SEM, transmission electron microscopy (TEM), dynamic light scattering (DLS) and X-ray diffraction (XRD). Antioxidant properties were evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing/antioxidant power (FRAP) assays. Cell viability and antimicrobial properties were determined by validated assays. Enzymatic browning inhibition and anthocyanin stabilization properties were evaluated as reported [1].

RESULTS & DISCUSSION

PNSE was found to contain condensed, prodelphinidin-type tannins as the main phenolic components, as demonstrated by chemical degradation and electron paramagnetic resonance (EPR) experiments. It showed remarkable antioxidant properties in the DPPH assay (EC_{50}= 0.004 mg/mL) and exhibited a quite low IC_{50} value (0.055 mg/mL) against mushroom tyrosinase. In addition, PNSE was found to be effective as an anthocyanin stabilizer at concentrations as low as 0.05 mg/mL. In order to explore the possibility of using PNSE as a functional additive for active packaging, PLA films containing PNSE were prepared by solvent-casting. These films showed remarkable antioxidant properties (DDPH reduction >60% with a 3% w/w loading, at a dose of 1 mg/mL in the DPPH solution) and were able to delay the onset of browning of apple smoothies (ca. 30% inhibition with a 10% w/w loading). WP-based edible films functionalized with PNSE were also prepared and characterized. PNSE was able to improve film mechanical and barrier properties, likely as a result of tannins-WP interactions, and also to impart antioxidant properties to the film, as particularly evident in the FRAP assay. WP/PNSE films also inhibited the growth of the foodborne bacteria *E. faecalis* and *S. enterica subsp. enterica ser. Typhimurium*.

Finally, a low-cost, scalable and straightforward mechanochemical protocol for the production of AgNP based on the use of PNS as reducing agent was developed. Under the optimized conditions, a complete reduction of silver ions was achieved, leading to a material containing ca. 30% w/w Ag(0). Model photocatalytic experiments indicated a good efficiency of AgNP/PNS in the degradation of methylene blue, with a good recycling stability. AgNP/PNS exhibited also antimicrobial properties against *E.coli* and *S. mutans*.

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O6.2

Polyphenols as additives for eco-friendly and bio-inspired adhesives from soy proteins

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MAIN CONCLUSION

It was shown that addition of phenols to thermally denaturated SPI improve its adhesive properties. All the resulting materials showed better water resistance and glued wooden supports higher shear strength with respect to denatured SPI. The involvement of the NH_2 and SH group of SPI in the reaction with polyphenols was demonstrated by conventional chemical assays. A polyphenol containing agarose/SPI gel were prepared to assess the applications of these materials in wound healing treatment.

INTRODUCTION

In the last decades, the chemical industry has devoted growing interest to the manufacturing of improved adhesive products. In fact, adhesives are widely used in almost all products of everyday life, such as in consumer goods, in the paper and packaging industries, in the construction sector and in transport. Synthetic adhesives are widely used and find applications in different fields. Largely used are the resins based on urea or formaldehyde that thanks to the high reactivity and cost efficiency are preferred by the wood panels industry. Nevertheless, their main disadvantage is the low tolerance to humidity and the carcinogenic formaldehyde emission, also occurring in the finished goods. In order to have less toxic materials, possibly from renewable sources and with a low environmental impact, the preparation of adhesives by use of natural substances has been actively investigated.

Nature is obviously a source of great inspiration, there are in fact many efficient natural adhesion systems, which allow permanent or temporary, reversible, or irreversible adhesion and in different environmental conditions. Examples include the locomotor organs in various animal species, eg. insects, spiders and geckos or the extraordinary wet adhesion properties of mussel byssus proteins. In this latter case the presence of catechol system gives rise to a cross-linked network. Moreover, soy proteins isolates (SPI) are of particular interest since they are low cost, easy to handle and above all, they are one of the main industrial waste in soybean processing. However, soy proteins have relatively low strength and water resistance. Therefore, research efforts have been recently directed to improve their adhesives properties. On these bases, the aim of the present work is to improve the adhesive properties of soy proteins with natural phenols additives pursuing a green approach.

MATERIALS & METHODS

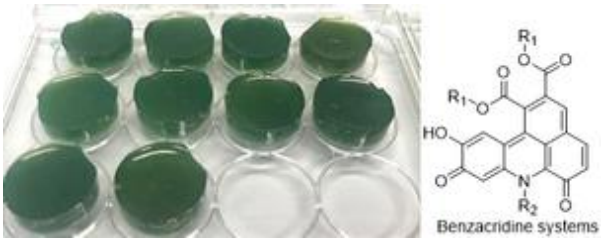
1 g of SPI was dissolved in 10 ml of distilled water in a glass flask and taken at 85°C for 1h under stirring. Then the resulting solution was brought to pH 9, CGA (28mM) was added and stirred for 2h at 50°C. The resulting solution was centrifuged, and the precipitate was placed between two rough surfaces of soft fir wood, pressing them with a clamp for 24 hours. The same procedure was followed for each phenol such as gallic acid, caffeic acid and its methyl ester (CAME). The obtained samples were immersed in a beaker containing distilled water.

The agarose/SPI gel was prepared heating 50 mg of agarose in 5 mL water solution at 70°C and adding 1 mL of the previously thermally denaturated SPI (100 mg/ml). After cooling in a petri dish, the resulting gel was immersed in a 10 mM water solution of each phenol at pH 9 for 24 h.

RESULTS & DISCUSSION

In a first series of experiments, it was shown that SPI thermal denaturation is critical to get adhesive capacity as evaluated by a simple model in which the adhesive is spread on wood specimens that are then pressed together and immersed in water. The effect of polyphenols such as caffeic acid and its methyl ester, gallic acid and chlorogenic acid (CGA) as additives was evaluated. SPI was dissolved in water (at 10% w/w) and taken at 85°C for 1 h, then the phenol component at 28 mM was added and the final solution brought to 50°C and to pH 9, in order to favour oxidation of the catechol to the quinone capable of interacting with the residues of the protein. The water resistance of wood supports glued with this material proved higher compared to that obtained using SPI alone. Determination of amount of free SH and NH_2 groups on the final material as lyophilized powder by Ellman's reagent and *o*-phthalaldehyde (OPA) reagent assay indicated the substantial involvement of these groups in the formation of cross-linking between polyphenols and SPI. In addition, shear strengths of wood specimens were determined by an Instron testing machine, showing in all cases values higher than control samples of SPI with Urea (2.07±0.70) and in particular the SPI with caffeic acid showed the highest value equivalent to 3.21±0.19.

To assess the potential of these materials for biomedical applications in wound treatment, agarose/SPI gel was prepared at 70°C starting from 5 mL of agarose water solution and adding thermally denaturated SPI at 1:2 w/w ratio. Then the obtained solution was cooled in a petri dish and immersed in 10 mM water solution at pH 9 of the appropriate polyphenol. The resulting gel obtained from CGA with agarose/SPI showed a green color, due to the formation of benzacridine systems (figure), indicating the reaction of CGA with lysine residues of proteins present inside the preformed gel. The cytocompatibility of these gels is currently under assessment.[1]



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O6.3

Eco-friendly recovery of antioxidant phenolic compounds from chestnut wood fiber by optimized deep eutectic solvents (DES) extraction

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MAIN CONCLUSION

In conclusion, we have developed a straightforward, smart, and green protocol based on a two-step DES-based treatment of CWF for the selective recovery of a EA-rich and a lignin-rich material. The disclosed approach looks amenable in view of a full exploitation and valorization not only of CWF but also of other agri-food by-products containing both low- and high-molecular weight phenolic compounds for different applications.

INTRODUCTION

Agri-food industry is responsible for the generation of high volumes of organic by-products, which represent a largely available and low-cost source of value-added compounds [1]. In this context, a prominent role is occupied by phenolic compounds and their polymers, above all tannins and lignin, endowed with potent antioxidant properties which have prompted their use as functional additives in a variety of sectors. Phenol-rich agri-food by-products include exhausted woods, that is the residual biomasses from wood tannin industrial extraction. In this context, chestnut wood fiber (CWF), produced from steamed exhausted chestnut wood, has been recently described as a material endowed with good antioxidant properties and able to adsorb pollutants such as toxic gases, organic dyes and heavy metals [2]. The main phenolic components of CWF are residual, non-extracted ellagitannins, lignin, and low molecular weight phenolic compounds, such as ellagic acid (EA), whose anticancer, anti-inflammatory and antimicrobial properties are well known. However, the possibility to practically exploit CWF as a source of high-value, antioxidant phenolic compounds has still remained unexplored.

Solid-liquid extraction is the most commonly used method to extract phenolic compounds from bioorganic matrices. This methodology typically involves the use of organic solvents that show many intrinsic drawbacks. Therefore, the development of cost-effective and environmentally friendly extraction, involving deep eutectic solvents (DESs), is the focus of increasing interest [1]. DESs are composed of Lewis and Brønsted acids and bases to form a new solvent with a lower melting point than those of the individual components, and show many advantages, including negligible vapor pressure, non-flammability, low toxicity, and biocompatibility.

In this scenario, the present study was carried out to establish DES-based extraction protocols for CWF phenolic compounds.

MATERIALS & METHODS

DES screening

CWF (66.7 g/Kg of solvent) was kept under stirring in different DESs containing 30% w/w of water, at 40°C, for 60 min. For optimization, treatment of CWF with 2:1 mol/mol ChCl/tartaric acid (ChCl:TA2) was repeated by varying solid-to-liquid ratio, time, temperature or amount of water.

Harsh treatment

CWF (100 g/Kg of solvent) was added to 1:2 mol/mol ChCl/lactic acid (ChCl:LA2) or ChCl:TA2 (both containing 20% w/w water) and taken under stirring at 120 °C for 8 h. After cooling, lignin was precipitated by addition of 0. 1 M HCl.

Sequential two-step treatment

CWF (100 g/Kg of solvent) was added to ChCl:TA2 containing 20% w/w water and taken under stirring at 50°C for 90 min (mild conditions). The EA-enriched sample was precipitated by addition of a 1% KCl solution. The residual CWF was then added to ChCl:LA2 containing 20% w/w water and kept under stirring at 120°C for 8 h (harsh conditions). The lignin-enriched sample was precipitated by addition of 1% KCl solution.

RESULTS & DISCUSSION

Forty-seven choline chloride- and sugar-based DESs were prepared and comparatively evaluated for extraction of antioxidant compounds from CWF. Further to an initial screening based on total phenolic content (TPC) and antioxidant properties of the extracts, ChCl:TA2 was selected as the most promising DES. Then, the effects of different parameters were evaluated in order to optimize the extraction conditions. Under the optimal settings (20% w/w water, 100 g/kg solid/solvent ratio, 50 °C, 90 min) ChCl:TA2 provided extracts with antioxidant properties up to 6-fold higher than those obtained with conventional solvents. HPLC and UV-Vis analysis indicated an EA extraction yield of ca. 3% w/w, which was comparable to that obtained using DMSO.

In a second series of experiments, a harsher protocol was developed for extracting and purifying lignin from CWF, based on treatment with ChCl:TA2 or ChCl:LA2 for 8 h at 120 °C and subsequent precipitation with 0.1 M HCl. Under these conditions a brown solid was recovered in 10% w/w yield, exhibiting stronger antioxidant properties and higher TPC than the starting CWF. UV-Vis, HPLC, FT-IR, NMR and EPR analysis confirmed the presence of lignin in the sample, which however was accompanied by >15% w/w EA.

Then, the possibility to apply a sequential two-step DES-based treatment to selectively obtain an EA- and a lignin-enriched sample was explored. Firstly, a mild treatment of CWF with ChCl:TA2 was performed using the optimized “mild” protocol initially developed. The addition of 1% KCl aqueous solution led to the precipitation of a light brown solid containing >30% w/w EA. Subsequently, the residual, undissolved CWF was treated with ChCl:LA2 under the harsh conditions previously reported (120°C, 8 h) to give, further to addition of 1% KCl, a brown solid composed mainly of lignin, as determined by spectroscopic and chemical degradation analysis.

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O6.4

Host-guest chemistry: γ-cyclodextrin interaction with pyranoanthocyanins.

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MAIN CONCLUSION

The interaction of 4-(dimethylamino)-cinnamyl-10-vinylene-pyranomalvidin-3-*O*-glucoside with γ-cyclodextrin in aqueous solution showed that the neutral and anionic quinoidal bases of the pigment were the equilibrium forms that mainly interact with the cyclodextrin.

INTRODUCTION

Anthocyanins are natural pigments that contribute to the red, purple and blue colours found in plants, especially flowers, fruits, and tubers [1]. These features associated with the many beneficial health effects are the main reasons why anthocyanins can be of great interest for different industries. However, the application of this pigments is still a challenge since in aqueous solutions anthocyanins are very instable and their colour is depending of the pH of the medium. On the other hand, pyranoanthocyanins present a fourth ring that is responsible for the higher chromatic stability of these anthocyanin-derived compounds and can display different colours according to the substitution pattern of the fourth ring. These pigments represent a promising alternative concerning their application in different matrices, although some of these pyranoanthocyanins are known for their low solubility in aqueous solutions [2]. Cyclodextrins (CDs) are oligosaccharides widely applied in different industrial matrices and the formation of molecular inclusion complexes between these molecules and anthocyanins are known to affect important properties of the pigments, namely water solubility and equilibrium and rate constants [3]. Therefore, the formation of inclusion complexes between CDs and pyranoanthocyanins could be a key way of improving its solubility and stability and simplifying its application in food and cosmetic products. In this context, the aim of this work was to study the influence of molecular inclusion complexes of different pyranoanthocyanins with CDs on their acid-base equilibrium and solubility.

MATERIALS & METHODS

4-(dimethylamino)-cinnamyl-10-vinylene-pyranomalvidin-3-*O*-glucoside was synthesized from the reaction of the carboxypyranomalvidin-3-*O*-glucoside with 4-(Dimethylamino)-cinnamic acid (1:10 molar ratio) in an aqueous solution of 20% (v/v) ethanol at pH 1.5.

A stock solution of the pigment (1.2×10^{-4} M) was prepared in ethanol. Then, to a 1 cm quartz cuvette were added: 1 mL of 0.1 M NaOH, 0.9 mL of 0.11 M HCl, 1 mL of Theorell and Stenhagen universal buffer solution at different pH values ranging from 2 to 9 and 0.1 mL of the pigment stock solution (final concentration of 1.2×10^{-5} M). UV-Visible spectra were recorded instantly after the addition of the pigments in a Thermo Scientific Evolution Array UV-Visible spectrophotometer at 25ºC. Solutions at different pH values were left in the dark for one day and then the UV-Visible spectra recorded. Similar experiments were performed in the presence of 7.5×10^{-3} M of γ-cyclodextrins.

RESULTS & DISCUSSION

4-(dimethylamino)-cinnamyl-10-vinylene-pyranomalvidin-3-*O*-glucoside (1.2×10^{-5} M) was titrated in aqueous solution at different pH values (-0.7 – 10) in the presence of γ-cyclodextrin (7.5×10^{-3} M).

We observed by UV-Visible spectroscopy that, at equilibrium, the most affected pK_a values were pK_{a2} and pK_{a3}. pK_{a2} and pK_{a3} decreased from 5.4 and 8.3, to 4.0 and 7.7, respectively, in the presence of cyclodextrin (Figure 1A and B). This indicates that that the neutral and anionic species of the pigment are the ones that mainly interact with γ-cyclodextrin. It was also observed the increase of the solubility of the pigment (Figure 2).

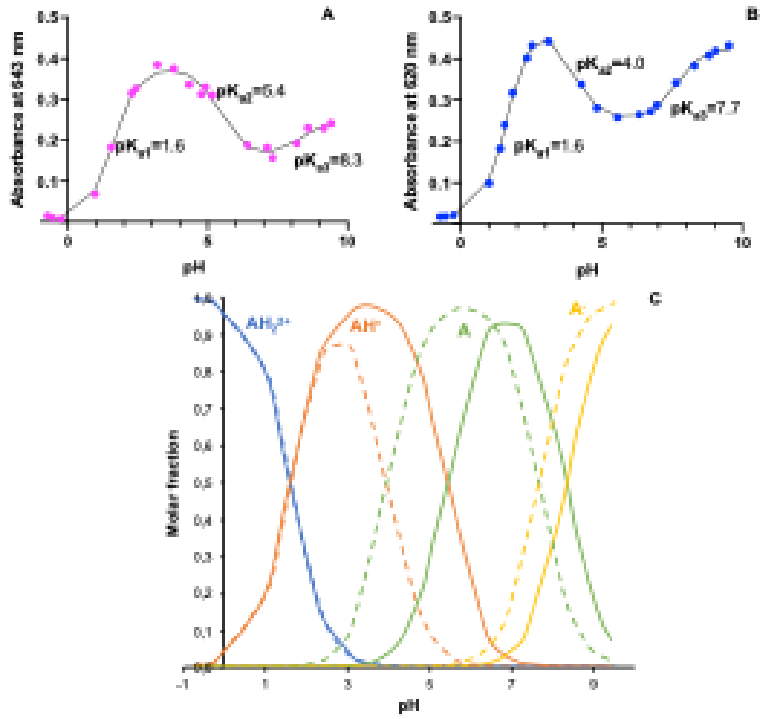

Figure 1. A) Representation of the absorbance at 643 nm (pink) and respective fitting (black curve) of 4-(dimethylamino)-cinnamyl-10-vinylene-pyranomalvidin-3-*O*-glucoside ($1.2x10^{-5}$ M) at different pH values (-0.7 – 10); **B)** in the presence of γ-cyclodextrin (7.5 × 10−3 M) (blue) and respective fitting (black curve); **C)** Molar fraction distribution of the pigment at different pH values in the absence (full lines) and presence of γ-cyclodextrin (dotted lines).

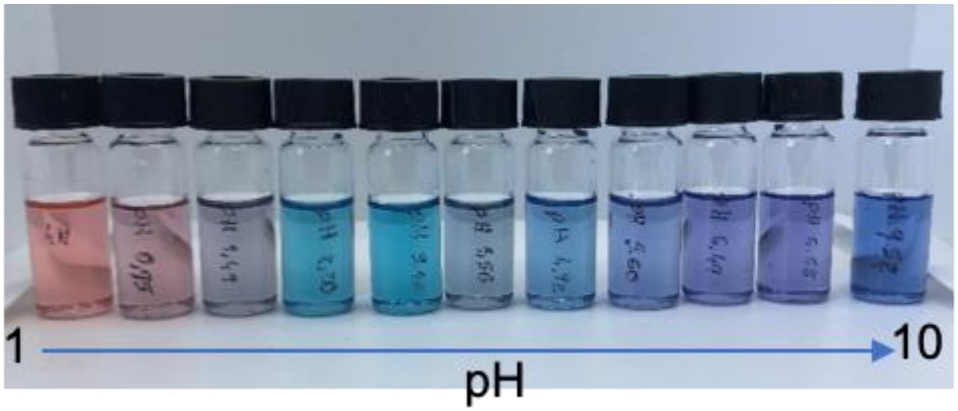

Figure *2*. Colours displayed by the pyranoanthocyanin-*γ*-cyclodextrin system at different pH values.

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O6.5

Impact of processing technology and storage on proanthocyanidins and sensory properties of blackcurrant juices

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MAIN CONCLUSION

Due to the significant changes in proanthocyanidin composition, it is important store black currant juices in refrigerated conditions rather in room temperature.

INTRODUCTION

Commercial berry juices rich in anthocyanins are typically stored in room temperature for long time. Monomeric anthocyanins are well known to be unstable and their contents decrease during storage. The decrease has a significant impact on the perceived color intensity of the juice [1]. However, the fates of other phenolic compounds, such as proanthocyanidins or phenolic acids, have not been equally well reported. Contents of various hydroxycinnamic acid derivatives decrease during storage resulting in increased contents [1].

Proanthocyanidins in food and beverages are typically associated with astringent mouthfeel and bitter taste. In our earlier study, these compounds correlated positively with mouth-drying astringent sensation also in black currant juices prepared with enzymatic assistance [2]. However, there are only few studies focusing on the potential changes in sensory quality, especially in bitterness or astringency, during storage and the roles of juice proanthocyanidin composition in the sensory changes.

MATERIALS & METHODS

In this study, three blackcurrant (cultivar ‘Mortti’) juice types (processed with a commercial pectinolytic enzyme, without the enzyme, with the enzyme from berry skins) were stored for 12 months in +4 °C or room temperature (in dark or exposed to light) after pasteurization [1].

The proanthocyanidin compositions of stored juice samples were investigated using a UPLC-MS/MS method [3] previously applied to similar juices without storage [2]. A part of stored juices (no enzymatic assistance; +4 °C or room temperature samples) was included in sensory evaluations using generic descriptive analysis focusing on the bitterness and astringency [1]. A panel (n=11) was trained to evaluate taste and astringency attributes from black currant juices with aid of reference samples. Principal component analysis (PCA) models were used to investigate correlations within datasets.

RESULTS & DISCUSSION

In general, the three juice types differed significantly, as the juice prepared from berry skins contained the most procyanidins (PC) and prodelphinidins (PD) and had the highest mean degree of polymerization (mDP), whereas the juice without enzymatic assistance had the highest PC to PD ratio. Enzymatic treatment in juice processing significantly increases astringency due to the increase in phenolic compound contents by releasing the compounds from the berry skins and breaking the berry pectins potentially masking the astringency [2]. Thus, the juice prepared from the berry skins in this study is potentially more astringent than the enzyme aided juice.

Storage of twelve months resulted in significant decreases in total PC and PD contents, as well as, in most of the dimers and trimers. However, the storage in refrigerator (+4 °C) had significantly

less impact on the proanthocyanidins in comparison to room temperature, which is in accordance with the significant changes in other phenolic compound classes at higher temperature [1]. PC to PD ratio was highest in juices stored at RT for 12 months in all juice types.

An increase in bitterness was observed in the juices without enzymes and stored room temperature, which correlated negatively with most of the proanthocyanidin variables. At the same time, PC to PD ratio correlated positively with bitterness. Moreover, the mDP correlated closely with mouth-drying astringency in the samples stored at +4 °C. The mDP variable correlated with mouth-drying astringency also in our earlier study [2].

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O6.6

Investigation of protein-polyphenol conjugates in almond blanch water in food production

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MAIN CONCLUSION

Blanch water from almond processing is rich in polyphenols. Its visible cloudiness hints at the interaction of polyphenols with proteins. Polyphenol extracts yielded after an adsorption step at XAD resin may be complex mixtures of polyphenols and protein-polyphenol complexes. The protein-polyphenol conjugates were detected via SDS-PAGE coupled to NBT staining.

INTRODUCTION

Almonds are processed into various products in food industry. The kernels contain plenty of nutrients such as proteins (18%), lipids (54%) and carbohydrates (16%). Polyphenols, mainly procyanidins, which can have positive effects on health, are components (up to 30%) of the almonds skin. The first step in industrial almond processing is often the blanching of almonds in hot water to peel off the skin. The resulting blanch water waste fractions are rich in water-soluble polyphenols, but contain also proteins, lipids and other components.

Cost-efficient and selective recovery steps of such polyphenols are needed to valorize the waste water of almond processing. Our concept for recovery of polyphenols from blanch water [1] is realized by an enrichment step, here an adsorption step on e.g. XAD resins. Subsequent elution with organic solvents yields the so called polyphenol extracts with high antioxidative activity. Regarding the optimization of the adsorption step, it is important to investigate the formation of protein-polyphenol conjugates in the blanch water for the first time. The visible cloudiness of the blanch water similar to the turbidity known from beverages such as beer hints at the interaction between proteins and polyphenols [2]. Protein-polyphenol complexes are already described in food technology and may cause a reduced shelf life and an impaired appearance. Investigations of the formation of such complexes in the blanch water of almonds will help to estimate the availability of the target polyphenols in the course of the downstream processing, especially in the adsorption step.

Key questions consider the formation of protein-polyphenol conjugates in blanch water samples as well as the appearance of such conjugates in the yielded polyphenol extracts.

MATERIALS & METHODS

The samples from blanch water and polyphenols extracts [1] were preconcentrated, if suited and subjected to SDS PAGE using Mini-Protean TGX 4–20% gels (BioRad) and Laemmli running buffer (Tris, glycine, SDS) using a BioRad Mini Protean Tetra Cell system. For each sample two SDS PAGE were achieved. The first gel gel was stained with Coomassie Brilliant Blue R250 Staining Solution (BioRad). The bands on the second gel were visualized by activation with UV light. The gel was blotted on to a nitrocellulose membrane using Trans-Blot SD semi dry cell (BioRad) Blotting system. Staining was achieved with with 0.6 mg/mL nitro blue tetrazolium (NBT) in 1M glycine potassium salt, pH 10 for 45 min. The membrane was washed with water.

RESULTS & DISCUSSION

The literature describes a SDS-PAGE indicated MW distribution of almond kernel proteins within the range of 21–245 kDa [3]. Gel electrophoresis of almond blanch water with subsequent Coomassie

staining results in bands according to MW > 250 kDa, 250 kDa, 37–50 Da and 20–25 kDa. The suggested assignments for corresponding proteins are due to the literature. The storage protein amandin is one of the major proteins in the almond kernel [4].

Since amandin contains two polypeptide chains with MW of 42–46 kDa and 20–22 kDa, the bands corresponding to 37–50 Da and 20–25 kDa are preliminary assigned to amandin.

Furthermore, high molecular weight compounds of 250 kDa and> 250 kDa could be assigned as protein-polyphenol complexes. Albumins and globulins (240 kDa) are the only high MW proteins described so far from almonds [3]. After separation by SDS-PAGE with a stain-free system followed by electroblotting and subsequent staining of the nitrocellulose membrane with NBT, some of the occurring aggregates could be identified as conjugated protein-polyphenol complexes due to the color for protein-bound quinones.

Comparing the SDS page of blanch water samples and of their corresponding polyphenol extracts shows that not only the polyphenols, but also protein-polyphenol complexes found in the blanch water are also adsorbed during the production of the phenol extracts. The phenol extracts are complex mixtures of oligomeric and polymeric procyanidins also containing protein-polyphenol complexes.

However, for optimization of the adsorption step, the Influence of protein-polyphenol conjugates and of free proteins on the performance of adsorption step will be investigated.

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O6.7

Oxidative coupling of chlorogenic acid with tryptophan: toward a natural product-based food dye

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MAIN CONCLUSION

A facile and expedient procedure was developed for preparation of a red pigment by oxidative coupling of chlorogenic acid (CGA) with tryptophan (TRP) featuring a cyanine-type chromophore integrated in a benzochromeno[2,3-*b*]indole scaffold. The pigment exhibited excellent stability at different pH (1–12) and temperatures (≤ 90°C) and proved not toxic on hepatic and colonic cell lines. The sodium salt imparted a natural red color to both lipophilic and hydrophilic food matrices at 0.01%.

INTRODUCTION

The search for food dyes based on natural compounds with high stability profile and low toxicity is nowadays very active. Interaction of proteins with polyphenols in natural matrices leading to pigmented species has been described since a long time and has now been considered a valuable approach to get dyes. A remarkable example is the greening process of defatted sunflower proteins, ascribed to the reaction of the amino acid residues with oxidized chlorogenic acid (CGA). Exploitation of this pigment-forming reaction for the preparation of food-grade green coloring agents is becoming increasingly attractive. We recently reported an expedient procedure for the preparation of green benzacridine pigments by reaction of CGA with lysine, glycine or protein-rich food matrices (e.g. egg albumen).[1] Notably, a systematic investigation of the reactivity of CGA with proteinogenic amino acids reported in a previous study revealed the peculiar course of the reaction with tryptophan (TRP), leading to the development of an intense red color. This reaction appeared highly attractive for the preparation of a red food dye.

Synthetic red dyes e.g. allura red AC (E129), amaranth (E123) and carmoisine (E122) are commonly used for food coloring because of high stability to light, oxygen and pH, low microbiological contamination, and relatively low production costs. Yet, their toxicity has directed research toward the exploitation of pigments from natural sources.

Presently the most widespread red food colorants are anthocyanins (E163) and beetroot red (E162). However, anthocyanins are unstable at pH>3, and sensitive to light, temperature, and food matrix composition while betanin and related betalains benefit from a higher pH and thermal stability compared to anthocyanins, but are difficult to isolate.

We report herein the preparation and structural characterization of a red pigment from oxidative coupling of CGA and TRP, and the assessment of its potential as food colorant.[2]

MATERIALS & METHODS

CGA (0.65 g) was reacted with TRP (5 mol. eqs) in water with pH adjusted to 9 over the first 30 min. After 64 h under stirring, the reaction mixture was acidified to pH 1 and the red precipitate collected by centrifugation and freeze-drying was purified on Sephadex G-10 (eluant: water). The combined fractions (abs 540 nm) were acidified to pH 1 and the precipitate collected as above, to give ca. 0.24 g (37% w/w yield with respect to CGA) of a red powder. Fractions with a 1:1 abs at 280 and 550 nm obtained by further semipreparative HPLC fractionation (0.1% formic acid with 20% to 80% methanol gradient) were collected and acidified. The pigment that separated was characterized by

NMR and MS analysis. For assessment of thermal stability, solutions of the pigment (acetate buffer, pH 3.6 or phosphate buffer, pH 7.0) were taken at 90 °C and analyzed by UV-vis spectroscopy every 1h.

RESULTS & DISCUSSION

LC-MS analysis of the CGA-TRP pigment purified by gel filtration (abs max 540 nm) indicated the presence of a main group of products (R_T 8 to 12 min), all with the same molecular ion peak at *m/z* 840 suggesting different isomers generated by acyl migration on the quinic acid moiety as reported.[1] Further purification by semipreparative HPLC gave fractions with abs 540/280 at a 1:1 ratio, eluting as a single peak at 11.8 min and showing a molecular ion peak at *m/z* 476 $[M+Na]^+$ and *m/z* 452 $[M-H]^-$. It was concluded that CGA-TRP pigment comprised a major constituent with *m/z* 453 (CGA-TRP/A) and a minor one responsible for the cluster of peaks with *m/z* 840 (CGA-TRP/B) which was preferentially revealed by LCMS. This latter however could not be obtained in sufficient amounts for NMR analysis. Complete 1D and 2D NMR analysis of CGA-TRP/A allowed definition of the structure as a benzochromeno[2,3-*b*]indole comprising a cyanine-type chromophore (**Figure**) A unifying mechanistic route was proposed for both components supported by experiments with model precursors.

The pH and thermal stability of the CGA-TRP pigment was probed in comparison with red wine anthocyanins and commercial betanin. Differently from anthocyanins, a minimal pH-dependence of the chromophore was observed with a 578 to 546 nm shift moving from pH 1–4 to pH 5–12. The thermal stability was probed at pH 3.6 or 7.0. While anthocyanins underwent a ca. 30% abatement after 1 h, CGA-TRP pigment was stable over at least 3 h, while betanin chromophore completely decayed in 1 h. At pH 7.0 CGA-TRP pigment was stable whereas red wine anthocyanins and betanin rapidly degraded.

Food dyeing with the sodium salt of CGA-TRP pigment led satisfactory results with all the food matrices examined using amounts as low as 0.01 %w/w (**Figure**). No effects were observed on cell viability and proliferation of human hepatic cells (HepG2) and colonic cells (Caco-2 and CCD-18Co) with the pigment at 0–200 µg/mL concentrations over 24 h.

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O6.8

Chemical / colour stability and rheological properties of cyanidin-3-glucoside in deep eutectic solvents as a gateway to design task-specific bioactive compounds§

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MAIN CONCLUSION

Chemical and colour stability of anthocyanin in DES containing glycerol in their composition were improved by deceleration of their thermal decomposition and maintain colour stability when compared with the cy3glc-DES urea systems

These results underline the potential application of green solvents on the anthocyanin stability for improvement of chemical and colour protection opening new windows for designing bioactive compounds to be potentially applied in different health and food areas.

INTRODUCTION

The production of food colourants derived from renewable sources is a current topic within the food industry. This interest emerged due to the continuous use of synthetic food colourants, which can be harmful to human health as well as the environment. This situation has opened a big door of opportunity to develop eco-friendly food colourants from natural materials based on polyphenols.

Anthocyanin (water-soluble class of polyphenolic pigments present in the plant kingdom) belongs to the group of natural dyes used in various industries such as food or drink colourant. They are the glycosylated form of anthocyanidins and yield the red, blue and purple colour of plants[1]. The consumption of anthocyanins (ANC) has been positively associated with health beneficial effects, including reducing diabetes, obesity, etc. Structurally, ANC shows a very interesting pH-dependence feature (red flavylium cationic form (AH+) at low pH and blue quinoidal bases (A) at basic pH) that makes them excellent pH-sensors for applications in food quality, which could contribute to the quality of food products.

Regarding anthocyanin stability, the literature relates studies with different sources in different solutions (most prepared in weak acid or neutral aqueous media). In this sense, there is a great need to introduce non-aqueous solvents such as the Deep Eutectic Solvent (DES), not only because of the environmental benefits (replacement of water/ organic solvent) but also to a better understanding of the stability (chemical, thermal and colour) of anthocyanin.

The present work attempts the study the chemical/colour stability and rheological properties of cyanidin-3-glucoside(c3yglc) dissolved in non-aqueous liquid ionic systems. Solutions were prepared by dissolving the polyphenol in ChCl-based deep eutectic solvents. They were characterized concerning viscosity, refractive index, calorimetric and spectroscopic (UV-visible and Fourier Transform Infrared (FTIR)) properties.

MATERIALS & METHODS

Materials

Cyanidin-3-glucoside (cy3glc) was obtained by extraction from blackberries (Rubus f ruticosus L.).

Choline chloride (ChCl),; glycerol (Gly); ethylene glycol (EG), urea all from were used as received.

Sample Preparation. The binary (BDES) and ternary (TDES) used in this study was prepared according to the procedure described on the literature [6]

Methods

1) Fourier Transform Infrared Spectroscopy (FTIR2) UV−visible spectrophotometer for optical Properties, Color Evaluation and Determination of Anthocyanin Concentration.

3) Refractive Index

4) Rheological Measurements

5)Thermal Analysis (TG and DSC)

Table 1. Deep eutectic components used for the present study.

Samples	Designation
ChCl/EG	DES_1
ChCl/Urea	DES_2
ChCl/Gly	DES_3
ChCl/Urea/Gly	$TDES_4$
ChCl/Urea/EG	$TDES_5$
ChCl/Gly/EG	$TDES_6$

RESULTS & DISCUSSION

FTIR Spectra

The chemical structural information on binary and ternary DES (BDES and TDES) with or without cy3glc were analyzed by FTIR spectra.

The spectrum of lyophilized anthocyanin powder showed characteristic absorption which is attributed to its functional groups.

The formation of each BDES and TDES, which is a combination of two or three components, was interpreted based on the changes observed in their FTIR spectra relative to the FTIR spectra of their individual components in three main sections: the hydroxyl stretching, carbonyl stretching, and ether stretching regions.

After the addition of 0.1%-cy3glc, the FTIR results showed that the signal positions and the intensity of absorption bands observed for hydroxyl, carbonyl, and ether stretching regions were not apparently changed in all deep eutectic solvents used

UV−Visible Spectroscopy (Color and Thermal Evaluation)

The use of different DESs for solubilization of cy3glc (0.1% w/w) produced solutions with different colours. On the basis of the visual observation (and confirmed by the EasyRGB program, reddish liquids were obtained when cy3glc was solubilized in BDES1, BDES3, and TDES6 (ChCl/glycolic HBDs), while the colour of cy3glc dissolved in DES containing urea changed to brownish (BDES2, TDES5) or dark-blue (TDES4).

The thermal stability of the studied solutions differed according to the solvent conditions (Figure 1)

Thermal Characterization

The thermal stability of studied DES presented an intermediate behavior between BDES1 and BDES3, and Tonset increased in the following order: BDES1(125 °C) < TDES6(142 °C) < TDES5 (164 °C) < TDES4(190 °C) < BDES2 (198 °C) < BDES3 (217 °C).

The Tonset of cy3glc-DESs systems follows the order cy3glc-BDES1 (125 °C) < cy3glc-TDES5(140 °C) <cy3glc-TDES6 (142 °C) < cy3glc-BDES2 (196 °C) < cy3glc-TDES4 (202 °C) < BDES3 (209 °C).

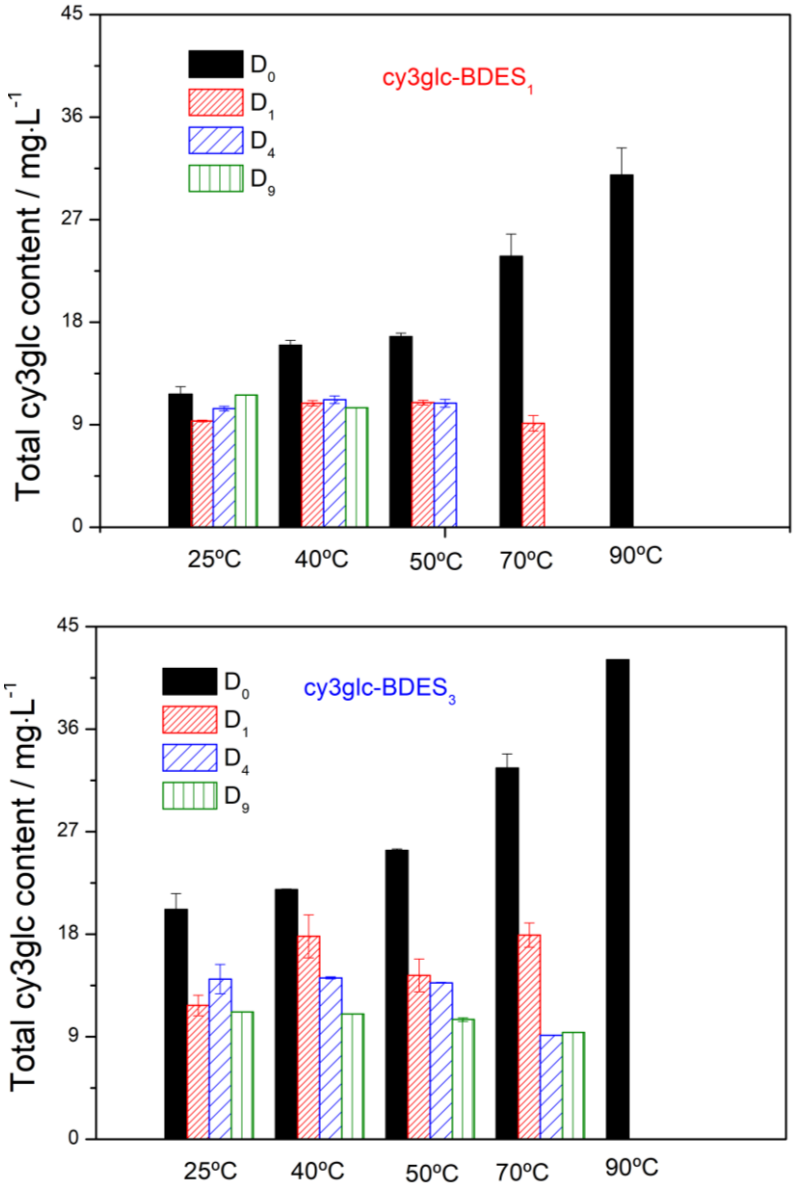

Figure 1. Effect of temperature on the stability of cy3glc anthocyanins in glycolic DES systems during the storage period.

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O6.9

Potential of industrial sweet orange waste to act as an anti-cariogenic agent

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MAIN CONCLUSION

This study has established the anti-cariogenic potential of industrial sweet orange waste. This finding will not only help in developing a novel organic antimicrobial agent but also help in the valorisation of the waste generated in juice producing industries.

INTRODUCTION

Dental caries is a disease caused by dysbiosis in the oral microflora as pathogenic microorganisms outcompete the other resident, non-pathogenic bacteria. One of the etiologies for shifting healthy plaque to a caries-causing state is a high sugar-rich diet [1]. Dietary sugars are metabolized to lactic acid by the plaque bacteria leading to low pH in the plaque. This environment provides optimal conditions for the growth of acidophilic and acidogenic microorganisms such as *Streptococcus mutans* and *Lactobacillus casei*, resulting in the formation of a caries causing biofilm.

Chlorhexidine (CHX) is still considered to be the gold standard for treating dental caries. However, it has many side-effects including staining on the teeth and tongue, soreness and dryness in the mouth, taste alteration, and mucosal desquamation. There is also a growing consumer demand for natural therapeutics. These factors have led to researchers seeking alternatives to CHX. Plant polyphenols could be such an alternative.

Citrus peel waste is a rich source of polyphenols. Among all the citrus fruits, sweet oranges (*Citrus sinensis*) are the most widely cultivated. Oranges are mainly processed for juice production resulting in the generation of huge amounts of solid waste (mainly peels) that makes up to 50% of the original processed whole fruit mass [2]. Although this waste has a potentially useful polyphenol-rich profile, it is widely used as animal feeds and also goes to landfills [2]. Further, there are constraints for citrus-waste management due to both environmental and economic factors.

This study aimed to explore the anti-cariogenicity of polyphenols from industrial sweet orange waste and for this purpose *S. mutans* and *L. casei* were used as the model cariogenic pathogens.

MATERIALS & METHODS

Industrial sweet oranges waste extracts (ISOWE) were obtained by microwave digestion system (CEM Mars 6) at 70 and 90°C for 5(A), 10(B), and 15(C) minutes. Flavonoid compounds were identified using LC-MS and quantified by HPLC. Based on preliminary findings on the MIC of the ISOWE against the two model organisms, the anticariogenicity of ISOWE was explored by developing a 7-day dual-species cariogenic-biofilm. Subsequently, the cariogenic model was treated twice a day for 4 days with different concentrations of CHX (0.1 and 0.2%), ISOWE, and combinations of both for 1 minute each time. The efficacy was monitored through viable bacterial counts (CFU/mL), and confocal laser scanning microscopy.

RESULTS & DISCUSSION

The identified flavonoids in the ISOWE included flavanones (hesperidin and narirutin), flavonols (quercetin), and flavones (sinensetin, nobiletin, and tangeretin). Among the identified flavonoids flavones were found to be the most effective antimicrobial compounds. For the two extraction temperatures, the lowest MIC of the ISOWE against *S. mutans* were ‘70_B’ (13±2 mg/ mL), and

90_A' (11±1.73 mg/ mL). A similar trend was found against *L. casei* but at a higher dose compared to *S. mutans. Based on MIC data analysis,* '70_B' (40, 80, 120 mg/ mL*) was further processed for the anticariogenic assay.* In the case of anticariogenicity of '70_B', 120 mg/mL was more effective compared to the other two concentrations, although, not as effective as CHX alone. The number of viable bacteria (65–83 x 10^2) was markedly reduced after treating the dual biofilm model with the combination of CHX (1%) and '70_B' (120 mg/mL).

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O6.10

Anthocyanin-Polysaccharide Complexes: from nature to innovative food solutions

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MAIN CONCLUSION

Interaction between anthocyanins and pectic polysaccharides resulted on anthocyanins red colour improvement and thermostabilization. Pectic polysaccharides bearing homogalacturonan domains and low amount of neutral side-chains interacted effectively with anthocyanins, mainly by electrostatic interactions and hydrophobic effect. These results, open the opportunity for the use of sustainable sources and tailor-made pectic polysaccharides for innovative functionalities of anthocyanins.

INTRODUCTION

Currently, consumers request safer, natural and healthier foodstuffs, which has stimulated the development of food products with plant-derived ingredients. Anthocyanins, one of the most important group of water-soluble pigments, are responsible for the red, purple and blue colours of flowers, fruits and vegetables. Due to their attractive colours, but also due to their broad spectrum of safety and benefitial health effects, there has been a growing interest from the industry in these pigments, particularly as food colourants. However, anthocyanins stability is affected by several factors such as pH, temperature, chemical structure, pigment concentration, light, oxygen, enzymes, the presence of other phenolic compounds, proteins and metal ions [1]. Thus, retaining a strong and stable colour of anthocyanin-derived food products can be a huge challenge during processing and storage. In the past years, the formation of pectic polysaccharide-anthocyanin complexes through the establishment of weak non-covalent interactions, like electrostatic interactions, hydrophobic effect and H-bonding has been shown [2]. Despite the potential impact of anthocyanin-pectic polysaccharide binding for anthocyanin stability and concomitant food functionality, little is known regarding anthocyanin thermostability and red colour improvement due to pectic polysaccharides interaction and about the structural domains of polysaccharides that support these bindings and potential stabilization. Additionally, the recovery of pectic polysaccharides from new natural sources and their incorporation into food products, could present an innovative and sustainable way to valorize food industry wastes. In this study, pectic polysaccharides rich fractions from *Vitis Vinifera* L. white grape skins were evaluated, aiming to assess their potential thermostabilization effect and impact on malvidin-3-*O*-ß-D-glucoside red colour, in model solution at slightly acidic pH.

MATERIALS & METHODS

Plant material

Malvidin-3-*O*-ß-D-glucoside (Mv3Glc) was extracted from a young red wine (*Vitis vinifera* L. c. v. Touriga Nacional), pre-concentrated in a nanofiltration apparatus. Cell-wall polysaccharides were recovered from *Vitis vinifera* L. white grape skins, adapting the methodology described elsewhere [3].

Isothermal Titration Calorimetry

The binding affinity and the thermodynamic parameters associated with Mv3Glc-pectic polysaccharide interactions were measured on a VP-ITC Microcalorimeter at 25ºC with an Origin 7.0 VPViewer in Milli-Q ultrapure water at pH 3.5 [3].

Spectral profile of anthocyanins

The thermodynamic and kinetic constants of Mv3Glc in the absence and presence of pectic polysaccharide fraction were determined by UV-Vis using the pH jump methodology [4].

Anthocyanins thermostability

Thermostability of mv3Glc and mv3Glc-pectic polysaccharides solutions were determined in aqueous solution (pH 3.4) according to the methodology described in the literature [5].

RESULTS & DISCUSSION

Pectic polysaccharides where recovered from the alcohol insoluble residue of white grape skins, followed by the selective extraction with chetating agents (oxalate/imidazole) and diluted alkaline solutions (carbonate, 4ºC and RT). This allowed to obtain polysaccharides with different structural features. While extraction with imidazole resulted on the recovery of polysaccharides richer in neutral side-chains sugars, extraction with diluted alkaline solutions (4ºC) allowed the recovery of homogalacturonan domains, rich in galacturonic acid residues. The binding of pectic polysaccharide extracts to Mv3Glc was fully characterized by isothermal titration calorimetry (ITC). Mv3Glc titration at pH 3.5, by the diluted alkaline fraction (4º C) gave rise to approximately 3-fold larger exothermic peaks when compared to the diluted alkaline fraction recovered at RT and to the chelating fraction, in the order diluted alkaline-4ºC (91173 M^{-1}) >diluted alkaline-RT (1627 M^{-1}) > chelating agent (1180 M^{-1}). From the calorimetric data, it could be observed a small negative enthalpy contribution, related to the establishment of hydrogen bonds or electrostatic interaction. Furthermore, a favourable entropy contribution could also be observed, suggesting that Mv3Glc-pectic polysaccharides interaction was mostly entropically driven, with the prevalence of hydrophobic effect. The global apparent equilibrium constant, K'_a and hydration constant, K_h were obtained recording the absorption spectra of equilibrated solutions at different pH values. A slightly higher pK'_a value (0.4 pK'_a units) was obtained in the presence of diluted alkaline extractable polysaccharides (4ºC). Also, when complexed with pectic polysaccharides, Mv3Glc showed a moderate reduction (≈3 times) of the K_h value from 6.00×10^{-3} to 2.20×10^{-3} M^{-1}, possibly indicating that the flavylium cation was more stabilized than the hemiketal form (**Fig.1**).

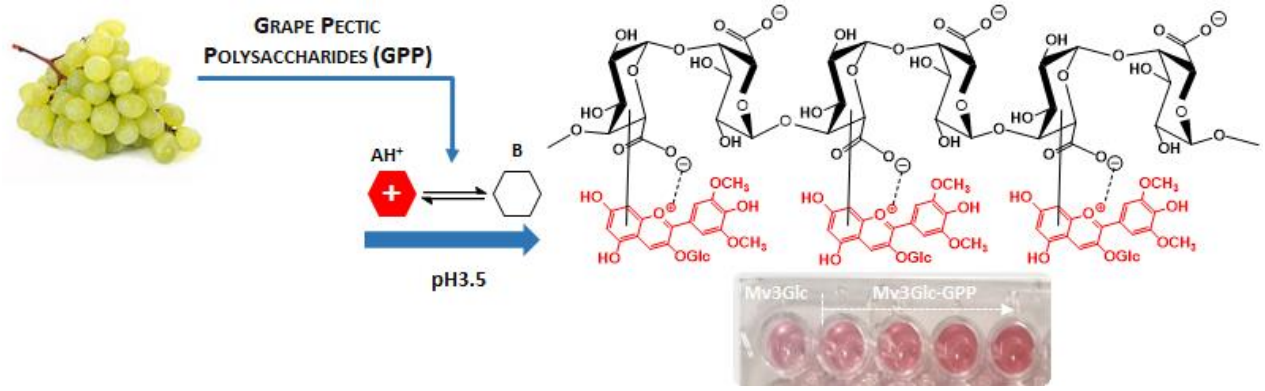

Figure1. Anthocyanins-grape pectic polysaccharides interaction.

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P1.1

Preparative isolation of apple Flavan-3-ols by pH-zone-refining centrifugal partition chromatography combined with reversed-phase liquid chromatography

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MAIN CONCLUSION

A new procedure combining centrifugal partition chromatography (CPC) and preparative reversed-phase chromatography (Prep-RP-HPLC) allowed recovering a highly purified flavan-3-ol fraction from a crude cider apple polyphenol extract. Our methodological development demonstrated the relevance of using CPC combined with a pH-displacement mode to efficiently discard hydroxycinnamic acid esters on a preparative scale. This step was followed by Prep-RP-HPLC to remove dihydrochalcones and flavonols.

INTRODUCTION

Cider apples are naturally rich in phenolic compounds that are divided in several classes including flavan-3-ols, hydroxycinnamic acid esters (HCA), flavonols (FO), and dihydrochalcones (DHC). Among them, flavanols (i.e. catechin monomers and procyanidin oligomers) are of particular interest exhibiting numerous properties in food matrix such as tanning properties, organoleptic (bitterness and astringency), nutritional effects, and biological activity (antimicrobial, antioxidant, etc.). In general, their fractionation, mainly based on liquid chromatography on solid phases, is tedious, time-consuming, and solvent-wasting, with the risk of losing a part of the tannins through irreversible adsorption. Moreover, reversed-phase HPLC with a classical gradient using a water/organic solvent mixture elutes apple procyanidins and catechin monomers throughout the chromatogram without any clear separation from HCA, FO, or DHC phenolic classes. In this study, a gentle strategy was developed to recover a highly purified flavan-3-ol fraction. Firstly, pH-zone-refining centrifugal partition chromatography (pH-ZRCPC) was optimized to discard HCA using their ionisation property. Finally, preparative reversed-phase liquid chromatography (Prep-RPLC) was used to remove the others unwanted compounds (DHC and FO).

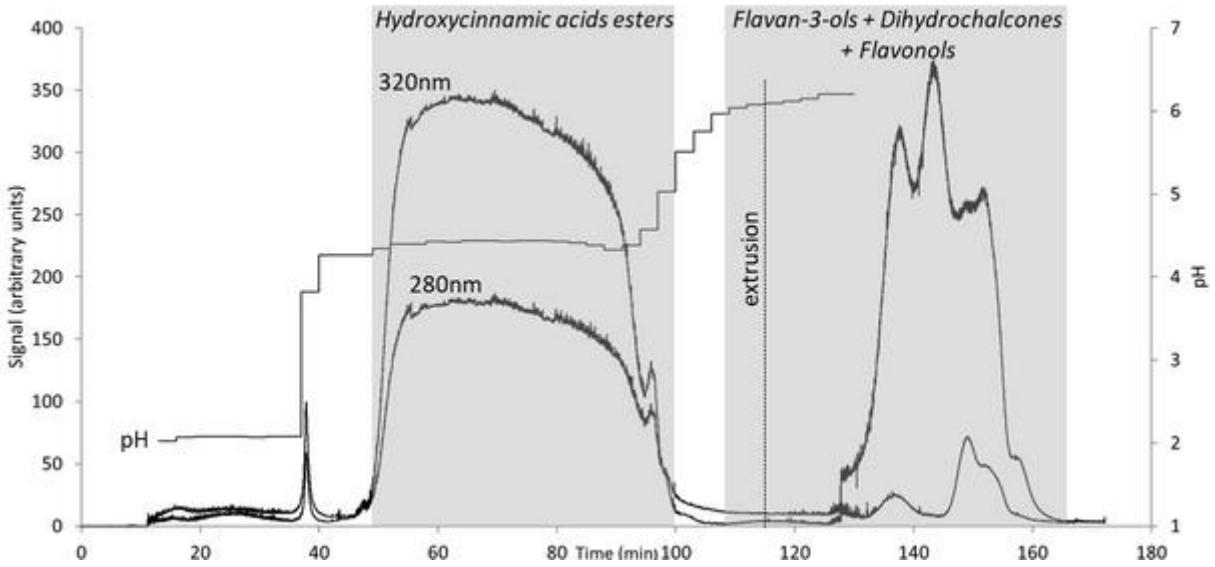
MATERIALS & METHODS

A crude extract from a polyphenol-rich French cider apple cultivar was obtained from a juice according to the method described by Millet et al. [1]. Biphasic solvent systems adapted from OKA [2] were tested and composed of ethyl acetate:n-butanol:water with 5:0:5, 3:2:5, 2:3:5 and 1:4:5 (v:v). The partition coefficients were determined using HPLC-UV-MS quantification of the target compounds in the upper and lower phases, for both conventional CPC and pH-ZRCPC by addition of a base (eluter) or an acid (retainer). Then, the suitable solvant system was selected to remove HCA using pH-ZRCPC, starting from 1 g of crude extract, in descending mode at a flow rate of 5 mL/min and a constant rotation speed of 1200 rpm. Several eluter and retainer concentrations were tested. CPC fractions were pooled according to analyses results. An ultimate fractionation on the intermediate extract was realized by Prep-RP-HPLC, using an isocratic mode (20% Acetonitrile), to recover the flavan-3-ol fraction.

RESULTS & DISCUSSION

Centrifugal partition chromatography combined with a pH shift technique during elution (pH-ZRCPC) was used to fractionate apple polyphenols and the fractionation was refined by reversed-

phase preparative HPLC. A crude polyphenol extract prepared from a polyphenol-rich cider apple juice was used as starting material. First, several biphasic solvent systems, also including variations in TFA (retainer) and NaOH (eluter) concentrations, were tested on an analytical scale for the liquid/liquid fractionation of this extract, to determine the partition coefficients (K) of the phenolic compounds. K values measured using conventional CPC showed that none of the solvent systems were appropriate to remove HCA. However, for pH-ZRCPC, K values of HCA were sufficiently different at acid and alkaline pHs to discard them, and recover the others phenolic compounds. The suitable solvent system for pH-ZRCPC was composed of ethyl acetate:n-butanol:water (3:2:5, v :v). The used retainer (TFA) concentration was 10 mM and high enough to ensure the protonation of all ionizable phenolic compounds (including HCA) and therefore to promote their trapping in the organic stationary phase. In addition, the eluter (NaOH) in the aqueous mobile phase was adjusted to 10 mM also, to enable the progressive neutralization of the retainer (H+) and the displacement of HCA only from the stationary phase to the aqueous mobile phase [3]. Finally, the elution-extrusion mode, which consists in replacing the aqueous mobile phase with the organic phase, was applied to allow the recovery of more hydrophobic compounds, including the procyanidins and catechin monomers. Their degradation was limited because the pH was not too alkaline, and the CPC fractions were acidified to pH 3 just after their collection. Finally, one single pH-ZRCPC run performed with one gram of crude extract followed by Preparative HPLC, allowed to obtained 409 mg flavan-3-ols fraction with a purity of 83% and a recovery of 73%.



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P1.2

Inter- and Intraspecies variability of polyphenols in temperate forage species

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MAIN CONCLUSION

The study showed that the selected species varied widely in terms of polyphenol composition and PA structural characteristics. However, variation within the species was found to be limited. Additionally, the variation of PA structural characteristics within the species was comparatively less than the variation of PA concentration. The relationship between PA structural characteristics and PA bioactivity needs to be investigated to determine the extent of their impact.

INTRODUCTION

Inclusion of polyphenol rich forages in grassland systems possess a strong potential in promoting animal health and productivity [1]. Particularly proanthocyanidins (PA, syn. condensed tannins) and hydrolysable tannins (HTs) have long surpassed their reputation of being antinutritional compounds. PA-rich forages, such as sainfoin (*Onobrychis viciifolia*) and birdsfoot trefoil (*Lotus corniculatus*), have been found to be benefit animal health by acting anthelmintic, preventing bloating incidences, as well as potentially improving nutrient utilisation, growth rate and milk yields [2]. Additionally, these forages can reduce environmental impact of ruminant based livestock production systems by reducing methane from enteric fermentation and nitrous oxide emissions from urine. However, the bioactivity of tannins depends on their structural characteristics. For example, PAs are the polymers of procyanidin (PC) and prodelphinidin (PD) subunits. The variation of structural in PA such as polymer length and subunit composition can influence its functional attributes [3]. In addition to PA concentration, PA structural characteristics have been found to vary with the species as well as their cultivars. However, variation in structural characteristics of tannins are rarely considered in the studies examining their impact on biological systems, which leads to contrasting results from different studies. Ultimately, this prevents the incorporation of tannin rich forages in livestock systems [2]. The current study aims to (i) investigate the variation in composition of polyphenols and tannins across the selected species and their cultivars, and (ii) characterize PA and their structural characteristics in leaves of PA containing forages.

MATERIALS & METHODS

A greenhouse experiment was established with 8 forage species and their cultivars: sainfoin (cv. Visnovsky, CPI 63750 and Esky), birdsfoot trefoil (cv. Bull, Lotar and Rocco), big trefoil (*Lotus pedunculatus*, cv. Lot 29 and Wild type), salad burnet (*Sanguisorba minor*, cv. Sang 10 and PI 308861), chichory (*Cichorium intybus* cv. Plumato and Spadona), plantain (*Plantago lanceolata* cv. PLA60 and Svatojansky), and sulla (*Hedysarum coronarium*, cv. Sudda and Grimaldi), and Lucerne (*Medicago sativa*, cv. Galaxy). Each pot (25 × 25 × 40 cm^3) was planted with five seeds of each cultivar. 68 experimental pots were arranged in a completely randomized design. Plants were harvested at the flowering stage and were divided into leaves, stems and flowers. The leaves were immediately freeze dried and ball milled into fine powder and were stored at -80℃ for further analysis. The leaves were analysed with UPLC-MS/MS to determine the polyphenol composition, and the PA structural characteristics [4].

RESULTS & DISCUSSION

The species varied significantly in terms of their polyphenol (P <.0001), tannin (P <.0001) and flavonoid (P <.0001) concentration. While salad burnet comprised mainly of HTs (39-48 mg g^{-1}DM), sainfoin, birdsfoot trefoil, big trefoil and sulla contained predominantly PA in the range of 1.5 to 29 mg g^{-1} DM. The lowest polyphenol concentration was found in plantain (0.2-0.6 mg g^{-1} DM) and chicory (1-2.5 mg g^{-1} DM) cultivars. However, while differences within the polyphenolic composition existed across species, within species the differences were much smaller (Figure 1). Still, for sainfoin, variation among cultivars was observed in the flavonols, as Visnovsky had higher amounts of kaempferol derivatives compared to CPI 63750 and Esky. This might be important, as the co-presence of flavonols and PA can exert matrix effects that enhance or reduce bioactivity [1]. Thus, analysing the complete polyphenolic profile of the forages can help provide valuable information which could help in understanding their functional attributes.

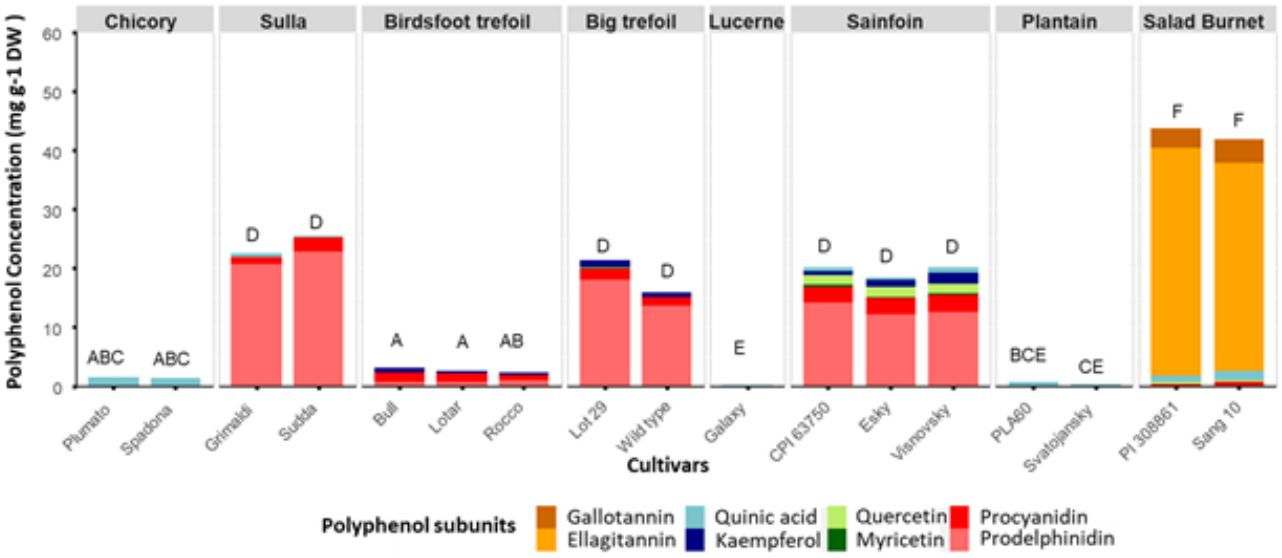

Figure 1 Polyphenolic profile of leaves in different species and their cultivars.
Means followed by different uppercase letters represent significant differences (P < 0.05) between the plants

Similarly, the differences in PA composition will be relevant, as species differed (P<0.0001) in their share of prodelphinidins (PD) and their mean degree of polymerization (mDP). Thus, both the cultivars of sulla had the highest mDP(16-19) and PD % (91-93%), while birdsfoot trefoil had the lowest mDP (7-14) and PD % values (28-52%). Variation across species again was larger than within species. Both mDP and PD% have been found to have a strong influence on structure-activity relationship in PA-protein interactions and can impact the nutritive value of the feed [1,3]. Consistent PA structural features of the forages can improve the predictability of PA traits and can accelerate their incorporation on field scale applications. This provides an opportunity for breeders to develop novel cultivars with efficacious PA traits [1].

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P1.3

Functionalization of carboxylated lignin nanoparticles with amino-flavylium derivative using EDC/NHS coupling agents

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MAIN CONCLUSION

A new bond between -COOH groups of carboxylated lignin and -NH2 groups of the amino-flavylium derivative was suggested by the presence of characteristic bands of the amide bond at 1638 cm^{-1} (amide I), 1550 cm^{-1} (amide II) and 1244 cm^{-1} (amide III) in FTIR spectrum. In addition, ^{31}P NMR analysis showed a decrease in the amount of -COOH present in carboxylated lignin after conjugation reaction, which also indicates a possible success of the amidation reaction using EDC/NHS coupling chemistry.

INTRODUCTION

Lignin is a natural phenolic polymer, one of the main components of the lignocellulosic biomass. Lignin is present in the cell walls of plants and can be obtained as a byproduct of the paper and lignocellulosic industries. It is a complex and irregular aromatic macromolecule comprised by three basic phenylpropanoid monomers: *p*-coumaroyl, coniferyl and sinapyl alcohols, whose proportion is dependent on the plant source and species (hardwood, softwood or grass), and isolation methods. The presence of different functional groups and the aromatic structure allow a wide industrial application, namely in adhesives, dispersants, surfactants, foams and resins. Nevertheless, apart from the high abundance and relatively low cost, lignin has different beneficial properties such as antioxidant and antimicrobial activities, UV-radiation absorbance, biocompatibility, biodegradability and chemical and biological degradation resistance. The abundance of the chemical sites in lignin structure offers different possibilities for new reactions and chemical modifications suggesting that lignin could play a central role as a new chemical feedstock[1,2]. The main objective of this work was a cross-coupling approach to amide bond formation from carboxylate lignin nanoparticles (CLNPs) and amino-flavylium derivative in order to study the modifications of the physical-chemical properties of both compounds. The reaction was performed using EDC/NHS coupling chemistry. The carbodiimide-mediated amidation includes the formation of a urea derivative, O-acylisourea, between the carbodiimide and carboxylic group of lignin, which a primary amine in flavylium derivative can displace, resulting in an amide bond (figure 1a). Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) spectroscopy, Fourier Transform Infrared spectroscopy (FTIR) using horizontal attenuated total reflectance (ATR) and dynamic light scattering (DLS) were used to characterized nanoparticles and reactions.

MATERIALS & METHODS

Firstly, kraft lignin was carboxylated by the reaction with succinic anhydride and 4-dimethylaminopyridine in tetrahydrofuran (THF) at room temperature for 48 h. Then, the reaction mixture was dialyzed against MilliQ-water using a dialysis bag for 24h, and then, it was freeze-dried. CLNPs was prepared dissolving carboxylated lignin in THF and introduced into a dialysis membrane. Nanoparticles were formed during the dialysis process (24h) and then, it was freeze-dried and characterized for their particle size, polydispersity index (PDI) and ζ-potential.

The conjugation reaction between the –COOH groups of carboxylated lignin and –NH2 groups of amino-flavylium pigment was performed in HEPES buffer solution (pH 7.4), using EDC/NHS coupling chemistry. The mass ratios of polymer/pigment for the conjugation reactions were chosen after the optimization of the process. Carboxylated lignin and CLNPs-amino flavylium derivative were characterized by FTIR and ^{31}P NMR.

RESULTS & DISCUSSION

FTIR spectra of carboxylated lignin (CL) and native lignin showed that both displayed the typical bands corresponding to the presence of different functional groups. However, the CL showed a stronger absorption band at 1720 cm^{-1} due to the stretching vibrations of C=O of the unconjugated –COOH groups. CLNPs-pigment showed a characteristic band absorbing near 1638 cm^{-1}, corresponding to the C=O stretching vibrations of the amide bond I (O=C–NH), a band at 1550 cm^{-1} corresponding to amide bond II (combination of the N–H in plane bending and of the C–N stretching) and a band in 1244 cm^{-1} corresponding to the amide III, which consists of more complex vibrational modes[3] (figure 1b). In the physical mixing, the absorption bands were slightly different from those observed for the CLNPs functionalized with amino-flavylium derivative.

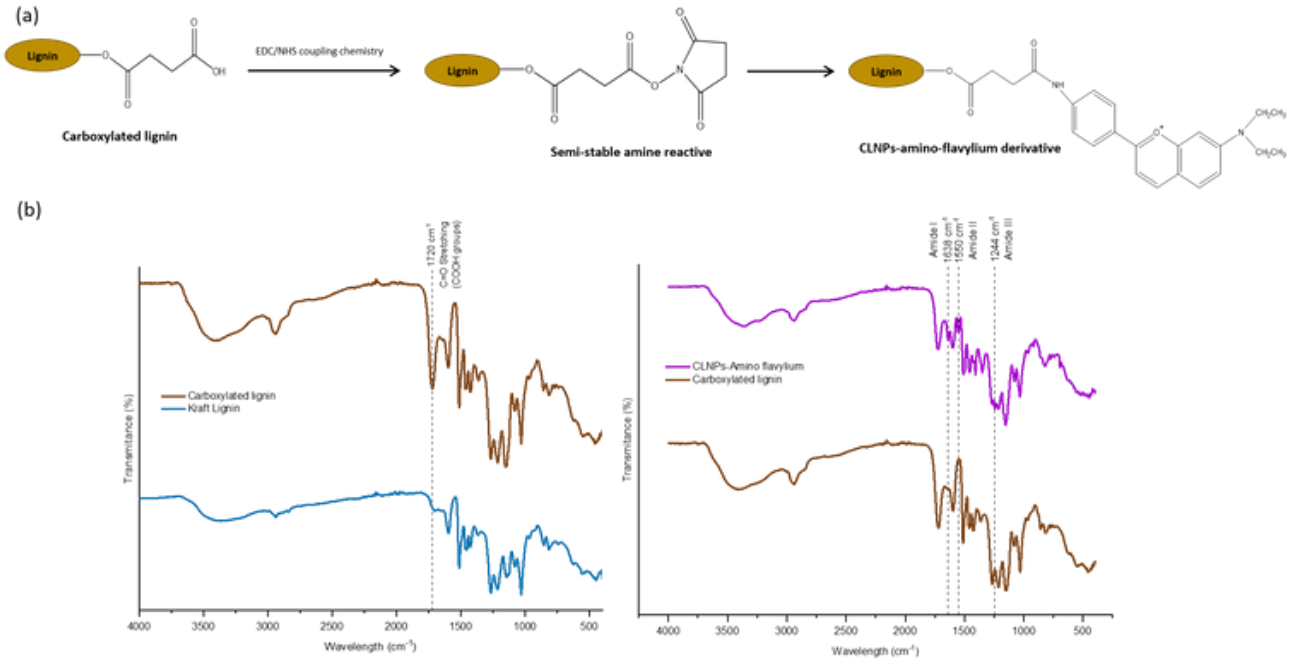

Figure1. (a) Schematic representation of the conjugation reaction (b) ATR-FTIR spectra of native lignin (blue), carboxylated lignin (brown) and functionalized lignin (purple)

The results of ^{31}P NMR showed an increase in -COOH groups amount in the carboxylated lignin from 1.250 mmol/g for the ratio 1:1. After conjugation reaction, the amount of -COOH groups decrease to 0.175 mmol/g which indicates the presence of a possible amide bond between CLNPs and amino-flavylium pigment.

CLNPs and CLNPs-amino flavylium presented an average size of 246± 3nm and 398±25nm, respectively. A slight increase in the size was observed for the functionalized CLNPs as a result of the conjugation reactions. The PDI values below 0.30 indicated a moderate dispersity of the resulting CLNPs. Regarding the surface charge, the ζ-potential value of CLPNs obtained was -40±2mV due to the presence of -COOH groups present on the surface of CLPNs. The ζ-potential value of CLNPs-pigment remained negative after functionalization.

The next step it will be analyze this sample by X-ray Photoelectron Spectroscopy in order to prove effectively the occurrence of amine bond.

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P1.4

Supramolecular study of interactions between malvidin-3-O-glucoside and wine phenolic compounds. Effect on color

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MAIN CONCLUSION

Color changes in Mv solutions at pH 1.1, due to interactions with different phenolic compounds strongly depends on the phenolic structure, showing that QG is the only one that visually affects the color of the flavylium cation solutions (to bluer and darker solutions). Moreover, ITC studies show that Mv-QG interactions are the ones in which the lowest ΔG were obtained, thus indicating that these interactions are the strongest ones. Similar results were obtained in MD simulation experiments.

INTRODUCTION

Anthocyanins are responsible for the red colour in many fruits, vegetables, juice, jams and red wines. These compounds, in mildly acidic aqueous solutions such as wine, are involved in a series of pH-dependent chemical reactions leading to both colored and colorless species. The red color is due to the existence of the flavylium cation form, which is predominant at acidic pH (pH≤2). At higher pH values, the flavylium cation can be deprotonated, leading to the blue purple quinoidal base, or involved in a hydration reaction to give the colorless hemiketal. However, the interactions between the pigments and other wine phenolic compounds may lead to the protection against those possible reactions, thus stabilizing the flavylium cation at higher pH values than expected [1]. The effectiveness of these interactions depends on different factors, such as the concentrations of anthocyanin and other phenolic compounds or their chemical structures. Although these interactions, known as copigmentation interactions, involving different phenolic compounds have been widely studied to evaluate their role in wine color [2,3] there are not information from a supramolecular point of view, about the importance of the structure of the phenolic compound on the interaction and on the color of flavylium cation solutions.

Thus, the aim of this work was to assess, by means of Isothermal Titration Calorimetry (ITC) and molecular dynamics (MD) simulations, the molecular interactions between malvidin 3-*O*-glucoside (Mv), one of the main anthocyanins in red wines, and other wine phenolic compounds (quercetin-3-*O*-glucoside (QG), (-)-epicatechin (E), (+)-catechin (C), caffeic acid (CA) and gallic acid (GA)). Furthermore, to study the potential impact on color, the effect of these interactions on the color of flavylium cation solutions have been studied.

MATERIALS & METHODS

Eleven different model aqueous solutions (pH 1.1) of Mv were prepared to evaluate the effect on the color of flavylium solutions of 5 different phenolic compounds: QG, E, C, CA and GA. Two molar ratios pigment:phenolic compound were assayed (1:1 and 1:2). Color analysis was performed using CIELAB parameters (L^*, a^*, b^*, C^*_{ab}, and h_{ab}), which were calculated using the software Cromalab™.

ITC experiments were carried out using a MicroCal PEAQ-ITC to measure the heat involved in each interaction at 298K. The software AFFINImeter was used to obtain the thermodynamic parameters of interactions (binding apparent constant, K, and changes in enthalpy, ΔH, entropy, $-T\Delta S$, and Gibbs free energy, ΔG).

MD simulations were performed using a ratio 5:11 Mv:phenolic compound to assess these interactions. From the results, just those representative geometries with a frequency> 5% were considered to account for the number and type of complexes.

RESULTS & DISCUSSION

Color parameters obtained from the Mv solutions were different depending on the phenolic compounds assayed. CA and C does not significantly modify the color of Mv solution, whereas Mv-E and Mv-GA solutions showed significant lower L* values when compared to Mv solution. On the other hand, Mv-QG solution showed significant differences for all the color parameters with regards Mv solution. These solutions showed lower L* and C_{ab}* values and an important decrease in h_{ab} values compared to Mv solutions, which pointed out to a bluish effect and darker solutions due to the interaction with QG. Color differences (ΔE^*_{ab}) showed that the color of the solutions containing phenolics cannot be discriminated by the eye from the color of Mv solution ($\Delta E^*_{ab}<3$), except for the case of Mv-QG. These results pointed out that, among the phenolic compounds assayed, only the presence of QG may modify the color of flavylium cation solutions.

ITC results showed that, although all interactions are spontaneous ($\Delta G<0$), the Gibbs free energy released differed depending on the phenolic compound structure. In all cases, the formation of 1:2 adducts released the highest amount of energy. Also, among the different phenolic compounds, Mv-QC interactions are the ones in which the lowest ΔG were obtained, which indicates that these interactions are the strongest ones. All interaction involved both hydrophobic interactions and hydrogen bonds. However, it seems that the contribution of the hydrophobic component for Mv-QG interactions is higher than for the other, because $-T\Delta S$ value is much more negative than $-\Delta H$ value (*ca.* 200 times).

MD simulations confirmed that Mv-QG interaction is the most energetically favored and that π-π stacking (i.e. hydrophobic interactions) are the main forces involved whereas, in the case of the other phenolic compound, different forces besides π-π stacking are observed, such as H-bonds in the case of Mv-GA and Mv-C and CH-methyl interactions for Mv-E and Mv-CA adducts.

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P1.5

Biomimetic intramolecular oxidative coupling between galloyl groups of pentagalloylglucose

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MAIN CONCLUSION

$CuCl_2$-mediated oxidation of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**1**) afforded trapain (isoterchebin) (**5**) with a 4,6-(*S*)-dehydrohexahydroxydiphenoyl (DHHDP) ester. Reduction of the reaction mixture of **1** afforded eugeniin (**7**) with a 4,6-(*S*)-hexahydroxydiphenoyl (HHDP) ester. These results strongly indicated that **7** with a HHDP ester were oxidatively biosynthesized from **1** via a DHHDP ester.

INTRODUCTION

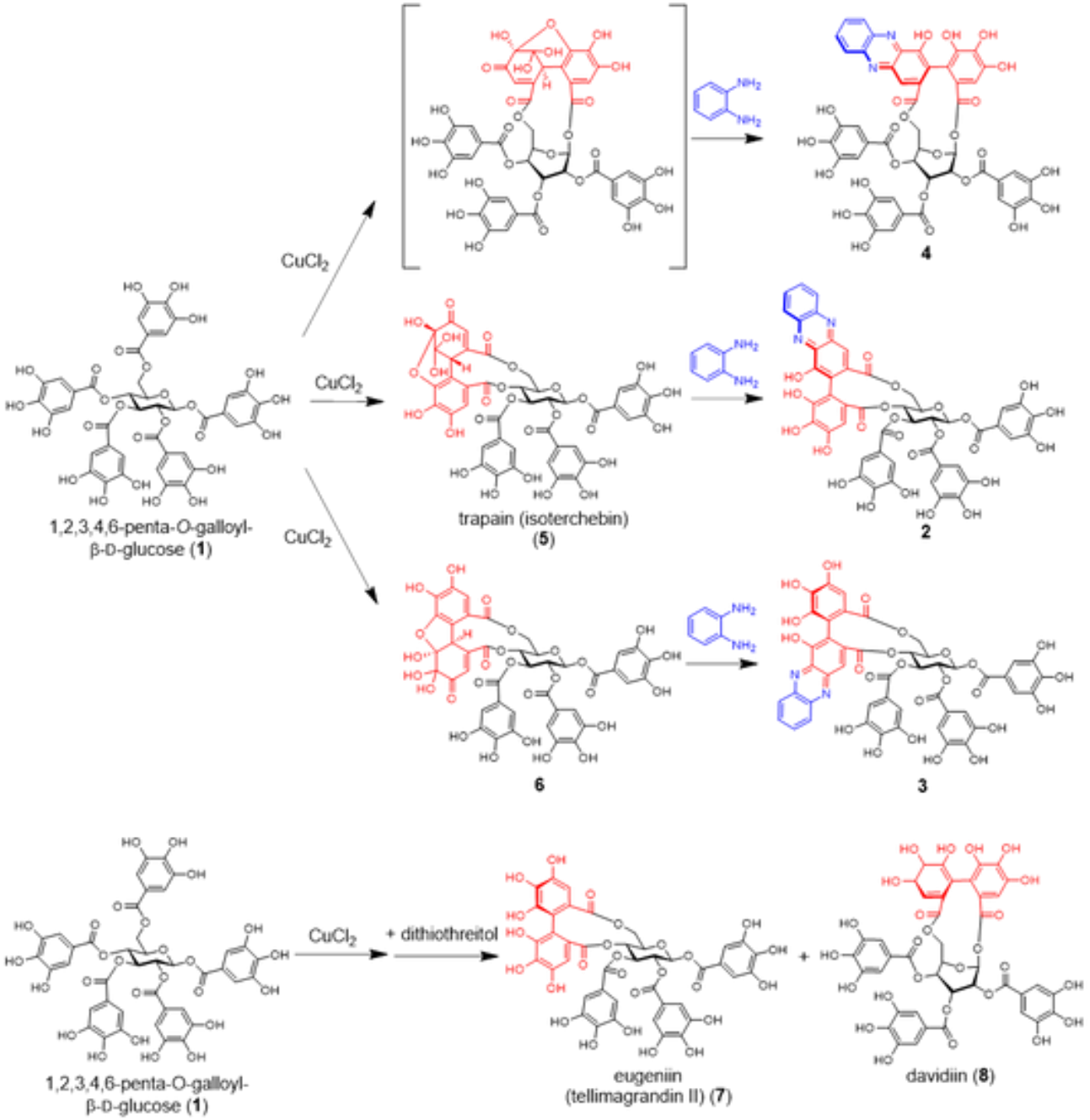
Ellagitannins are composed of polyalcohol core and acyl groups such as hexahydroxydiphenoyl (HHDP) and dehydrohexahydroxydiphenoyl (DHHDP) groups. 1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose (**1**) is a key precursor in ellagitannin biosynthesis. Gross *et al*. reported that the treatment of **1** with phenol oxidase derived from *Tellima grandiflora* yielded eugeniin (tellimagrandin II) with a 4,6-(*S*)-HHDP ester.[1] Other ellagitannins, such as pedunculagin, geraniin, vescalagin, and chebulagic acid, would also be biosynthesized from **1**. However, their biosynthetic mechanism had not been revealed yet. Recently, we found that the $CuCl_2$-mediated oxidation of galloyl ester derivatives in an aqueous medium afforded DHHDP esters, and the subsequent reduction of the products afforded HHDP esters.[2,3] In this study, the oxidation products of **1** with $CuCl_2$ in an aqueous medium were investigated to reveal its oxidative metabolism.

MATERIALS & METHODS

Compound **1** was oxidized with $CuCl_2$ in 30% aq. CH_3CN, then the reaction mixture was treated with *o*-phenylenediamine to afford **2**–**4**. $CuCl_2$-mediated oxidation products were separated with column chromatography to afford **5** and **6**. Reduction of the oxidation products with dithiothreitol afforded **7** and **8**. The structures of these products were assigned on the basis of various spectroscopic data.

RESULTS & DISCUSSION

Compound **1** was oxidized with $CuCl_2$ in 30% aq. CH_3CN, then the reaction mixture was treated with *o*-phenylenediamine to afford **2**–**4** corresponded to the phenazine derivatives of 4,6-(*S*)- and 1,6-(*S*)-DHHDP esters. The $CuCl_2$-mediated oxidation products of **1** were separated with column chromatography to afford trapain (isoterchebin) (**5**), a natural ellagitannin with a 4,6-(*S*)-DHHDP ester, along with its isomer **6**. Products **5** and **6** were produced by intramolecular oxidative coupling of galloyl groups at C-4 and C-6 of **1**. In addition, reduction of the reaction mixture of **1** afforded eugeniin (**7**) and davidiin (**8**) with 4,6-(*S*)- and 1,6-(*S*)-HHDP ester, respectively. These results strongly indicated that **7** and **8** with a HHDP ester were oxidatively biosynthesized from **1** via DHHDP esters.



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P1.6

Role of ellagitannins in the synthesis of vitisin A and in the degradation of malvidin 3-*O*-glucoside. An approach in wine-like model systems

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MAIN CONCLUSION

Ellagitannins (ET) played an important role in vitisin A synthesis and in malvidin 3-*O*-glucoside (Mv3g) degradation. Whereas the effect of ET on vitisin A synthesis has been shown to be different for castalagin and for vescalagin, the effect on Mv3g degradation did not depend on the type of ellagitannin. Castalagin increased the levels of vitisin A, vescalagin first increased and then decreased them, and both types of ellagitannins were able to modify the Mv3g degradation products profile.

INTRODUCTION

The levels of grape native anthocyanins decrease during wine ageing partly due to their transformation into anthocyanin-derived pigments and partly to their degradation. In previous studies [1,2], ellagitannins have been postulated to play a relevant role in the oxidation step [3] occurring during the synthesis of 10-carboxypyranoanthocyanins (A-type vitisins) in red wines. In addition, ellagitannins might be favouring the oxidation of ethanol to acetaldehyde, which, in turn, might be related to a greater synthesis of B-type vitisin. However, in what concerns degradation reactions, little is known about the role of these compounds in the formation of the degradation products. The complexity of wine composition makes difficult the evaluation of their factual influence in these processes. Thus, an experiment was conducted in simpler model systems to study the role of ellagitannins in the formation of these anthocyanin-derived pigments and in the degradation of grape native anthocyanins.

MATERIALS & METHODS

Eleven model systems were prepared in triplicated with Mv3g and/or pyruvic acid (PA) and/or ET (1:48:0.1) in wine-like solution (12% ethanol; 0.5% tartaric acid; pH 3.2) and kept for 122 days (23ºC, darkness, presence of air). Model systems A, B, C and D were prepared with Mv3g and PA in the absence (A) or presence of ET (B: castalagin, C: vescalagin and D: equimolar mixture of both) to study the influence of ET in the synthesis of vitisin A. Model systems E, F and G were prepared without Mv3g and only with PA and ET (E: castalagin, F: vescalagin and G: equimolar mixture) to serve as a control for the ET evolutions in B, C and D. Model systems H, I, J and K, containing Mv3g in the absence (H) or presence of ET (I: castalagin, J: vescalagin and K: equimolar mixture) were built to study the degradation of Mv3g and the formation of Mv3g-derived pigments in the absence of PA. The levels of all these compounds were monitored by HPLC-DAD-MS^n [1,4].

RESULTS & DISCUSSION

Mv3g disappeared much faster in the presence of PA than in its absence (Table 1): whereas at day 122 *circa* 16% of the initial content remained in solution in model systems A, B, C and D, more than 50% still remained in H, I, J and K. Although this disappearance of Mv3g could be due either to transformation or to degradation reactions in both types of model systems, the results of the present study showed that in the presence of PA, the synthesis of vitisin A was the main cause of this reduction. In contrast, degradation reactions were the main reactions in the model systems built without PA, in which the formation of anthocyanin-derived pigments was quite restricted. The

presence of ET in the model systems affected both vitisin A synthesis and Mv3g degradation reactions (Table 1). Respecting vitisin A, the greatest levels were obtained in the presence of castalagin, confirming its participation in the late oxidative step of the synthesis. The results of the present study confirmed again the greater reactivity of vescalagin, which showed an important decrease of its levels from the middle part of the experiment to the end. As a result, the levels of vitisin A were also reduced in vescalagin-containing model systems. In contrast, in the case of the degradation products, the levels were more affected by the presence or absence of the ET than by the type of ET. In general, in the absence of PA and in the presence of ET (I, J and K), the total content of degradation products as well as the content and proportions of the main degradation products of Mv3g, syringic acid and 2,4,6-trihydroxybenzaldehyde, were reduced. In contrast, other minor compounds related to oxidative reactions were increased. Consequently, the profile of degradation products was changed when ET were added. The co-existence of more than one type of ET in solution also caused changes in the levels of individual ET, which could affect, in turn, vitisin A synthesis or Mv3g degradation reactions.

Table 1. Percentage of Mv3g in relation to the initial content, content of Vitisin A (mg/L) and areas at 280 nm of the main degradation products of Mv3g in different model systems at different sampling points. Different letters in the same row inside the same square indicate significant differences ($p<0.05$).

	Mv3glc (%)								Vitisin A (mg/L)			
	with pyruvic acid				*without pyruvic acid*							
Days	**A**	**B**	**C**	**D**	**H**	**I**	**J**	**K**	**A**	**B**	**C**	**D**
1	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a	100.00a	0.00a	0.00a	0.00a	0.00a
5	87.56a	90.38b	90.71b	87.93a	98.55a	97.94a	99.04a	99.23a	1.03a	1.11b	1.02a	1.02a
12	69.09a	72.87b	73.92b	69.72a	96.95a	95.83a	96.43a	96.54a	1.79a	1.92b	1.82a	1.81a
21	54.79a	55.69b	57.60c	54.71a	92.26a	92.54a	91.21a	92.51a	2.50a	2.66a	2.58a	2.57a
35	42.18a	44.74b	45.46b	42.73a	89.31a	86.64a	86.22a	87.15a	3.20a	3.58b	3.29ab	3.22a
46	34.95a	36.88b	38.22b	34.62a	80.51a	81.44a	82.37a	80.81a	3.53a	3.91b	3.58a	3.34a
77	25.03a	26.54bc	27.63c	25.90ab	76.00b	69.19a	67.47a	69.44a	4.61a	5.27b	4.36a	4.22a
97	19.92a	21.94ab	23.28b	21.89ab	71.27b	64.02a	62.30a	62.95a	4.93a	5.99b	4.72a	4.47a
122	15.31a	15.68a	17.30b	16.83b	61.01c	53.33b	50.70a	51.78ab	5.70b	6.54c	4.85a	4.68a

	Degradation products (area)											
	Syringic acid				*2,4,6-Trihydroxybenzaldehyde*				*Total*			
Days	**H**	**I**	**J**	**K**	**H**	**I**	**J**	**K**	**H**	**I**	**J**	**K**
1	253.6c	248.1b	240.5a	254.4c	305.2d	263.0b	251.1a	274.4c	1157.0a	1182.2a	1136.3a	1107.7a
5	331.0a	310.6a	318.2a	323.8a	323.0b	286.9a	283.3a	294.9ab	1301.6a	1393.1b	1314.8a	1395.6b
12	418.9b	391.9ab	382.8a	400.5ab	360.6b	317.8a	290.6a	321.5a	1449.2a	1479.5a	1393.5a	1488.8a
21	510.9a	478.5a	478.3a	496.9a	376.4b	340.6ab	314.4a	354.9ab	1674.7a	1687.8a	1619.6a	1704.7a
35	624.5a	572.1a	594.6a	605.5a	430.7b	358.6a	347.6a	374.7a	1929.8a	1900.6a	1891.0a	1941.9a
46	690.9a	584.9a	685.1a	687.7a	415.7b	386.8a	374.7a	383.1a	1930.7a	1979.3a	2071.6a	2038.5a
77	1028.2b	923.2a	907.7a	905.8a	562.4b	460.7a	457.8a	446.3a	2656.0a	2478.5a	2492.3a	2410.7a
97	1248.1b	1085.3a	1085.2a	1068.1a	568.9b	484.4a	475.2a	484.9a	3116.2b	2858.0a	2804.4a	2770.3a
122	1541.6b	1253.3a	1247.8a	1237.1a	640.1c	548.1b	502.8a	523.3a	3617.8c	3219.2b	3139.2ab	3060.0a

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P1.7

Investigation of pH dependance of UV-Vis spectra of gallic and ellagic acids using combined experimental and theoretical approaches

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MAIN CONCLUSION

Experimental and calculated UV-Vis spectra of gallic and ellagic acid at low pH values are in excellent agreement, while the agreement at medium and high pH values is weaker but still good. Additional non-reversible broad bands were observed at higher pH values. Based on the comparison of experimental and calculated spectra, it was possible to select the species that are predominantly present in the solution at corresponding pH values.

INTRODUCTION

Tannins form one of the main classes of natural polyphenols. Ellagic (EA) and gallic (GA) acid represent the main hydrolysis products of hydrolysable tannins. Tannins are known to possess several health promoting properties. They have been shown to exhibit antimicrobial, antioxidant, and anticarcinogenic activities [1]. Various properties of polyphenolic compounds arise from the structural features of these compounds. Simulations of UV-Vis spectra have become an increasingly important and often crucial tool for the interpretation of experimental results, as they can provide a more understandable explanation of the electronic transitions. Moreover, they can also explain how these electronic transitions change with the pH. One can find a few experimental studies dealing with the pH influence on the spectra of polyphenols [2,3], but all of them were measured in buffer solutions. Buffers have the disadvantage of absorbing light at low wavelengths (at about 200 nm and even up to 260 nm). In contrast to buffers, aqueous solutions enable the observation of absorption bands also at low wavelengths. On the other hand, theoretical studies on this topic are still lacking. The aim of this study was to explain how the structural properties of EA and GA affect their spectra. Combined experimental and computational methods were employed to provide a better understanding of the pH dependence of the UV-Vis absorption spectra of the two aforementioned polyphenolic compounds. In addition, by comparing calculated and experimental spectra, the ionization species most likely to be present in the solution were determined.

MATERIALS & METHODS

Experimental methods: GA was dissolved in water and EA in methanol. Both were diluted with water to give the final concentrations of 2.0×10^{-5} M and 1.0×10^{-5} M, respectively. UV-Vis spectra were measured at different pH values using a Carry 50 UV-Vis spectrophotometer. The pH was adjusted with NaOH or HCl. All measurements were performed in the spectral range of 800-200 nm at a temperature of 25 °C.

Computational methods: Depending on the pH and pKa, EA and GA can exist in different ionic forms. For this reason, the structures of all possible ionic species of EA and GA were initially optimised using the B3LYP/6-311++G(d,p) level of theory in conjunction with the PCM solvation model. The time-dependent density functional theory (TDDFT) approach was further applied to simulate the UV-Vis spectra of all optimised ionic structures.

RESULTS & DISCUSSION

The experimental spectra were in an excellent agreement with the available literature data. The only differences observed were a slight shift in peak position at low wavelengths and an additional peak at 500-350 nm formed at higher pH values. To compare the experimental and calculated spectra, the superposition of the calculated absorption bands of the ionic species present at a certain pH was performed. Figure 1 shows the comparison of the calculated absorption bands and the experimental spectra of GA and EA at representative pH values. At the low pH, excellent accord was obtained between the peak position and intensity in the experimental and calculated spectra for both GA and EA. At pH 2.0 for GA and 1.8 for EA, the absorption bands of the non-ionised species G0 (see Fig. 1 for explanation) and E0, respectively, are in perfect agreement with the experimental spectra. At medium and high pH, the correlation between the spectra is weaker but still good. In case of GA at pH 6.0, the predominant species is G1. The only difference is that the calculated absorption band at »250 nm is blue shifted compared to the same peak in the experimental spectra. Where there were two or more possible ionization structures, the more stable species with absorption bands that best explained the experimental spectra was selected. In the case of GA at pH 9.0, the absorption bands of the more stable species G2 reproduced the experimental spectrum better than those of the G3 species. In the case of EA at pH 5.7, the predominant species is the more stable E2, as species E1 has an absorption band at higher wavelengths than observed in the experimental spectrum. At higher pH values (above 11) additional non-reversible broad bands at wavelengths of 500–350 nm formed, indicating that the oxidation process occurred in the case of GA and lactone ring opening in the case of EA.

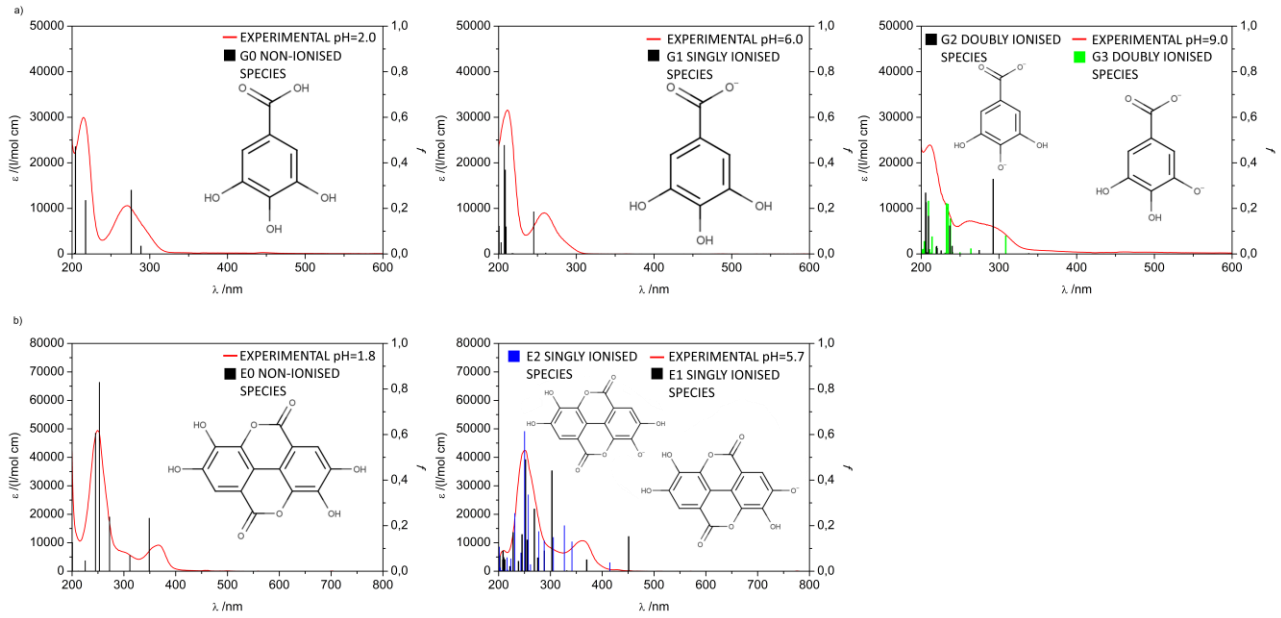

Figure 1. Comparison of experimental and calculated spectra of gallic a) and ellagic acid b) at different pH values.

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P1.8

Interactions of Fe(II) ion with gallic acid and vescalagin

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MAIN CONCLUSION

Formation of coordination compounds of iron ions with gallic acid and vescalagin was observed in the range of pH values between 3.5 and 5.5. It was seen, that the amount of coordination compounds formed is decreasing with the decreasing pH. Moreover, the stoichiometry of obtained coordination compounds of gallic acid and iron was determined to be 1:1, while each vescalagin molecule binds between 2—3 iron ions. Results were also correlated with gallic acid and vescalagin antibacterial activity.

INTRODUCTION

Tannins are plant secondary metabolites, found in several plants which they protect from oxidative and chemical stress, as well as against herbivores. They are used in leather tanning while the ability of gallic acid to form coloured coordination compounds with Fe(III) ions was early on exploited to produce ink. Because tannins possess antibacterial properties they are increasingly used as feed supplements to control the bacterial population. It is assumed, that they affect bacteria via several mechanisms, one of them being depletion of essential metal ions.[1] Next to that, interactions of tannins with iron ions are also important for preventing Fenoton-like reactions in cells and thus diminishing the oxidative damage resulting from products of Fenton-like reactions.[2]

Job diagram is an experimental technique which enables the determination of coordination compounds stoichiometry. Typical procedure is, that one mixes metal ions and ligands in various ratios, while the sum of metal and ligand concentrations are kept constant throughout the experiment. The metal/ligand ratio, at which Job plot reaches maximum, corresponds to the stoichiometry of the investigated compound. The fitting of model function enables extraction of additional information, such as extinction coefficient and formation constant.[3]

Although understanding of interactions of tannins with iron ions is crucial for understanding their biological activity, these interactions are still relatively poorly explored. The majority of studies available is performed on gallic acid, due to its easy availability. To the best of our knowledge, the studies in which equilibrium constant would be determined for interaction of pure tannins with iron ions are still lacking. The goal of this work was to, at least partly, fill this gap.

MATERIALS & METHODS

The detailed procedure of vescalagin isolation is described in our previous study [1]. Starting material was aqueous chestnut wood extract, which was initially purified on Sephadex LH-20 columns. Fractions, rich in vescalagin were further purified using high performance preparative and semipreparative liquid chromatography on C18 stationary phase. Final product purity determination and structure confirmation was performed using LC-MS quadrupole orbitrap mass spectrometer.

Coordination compound formation was observed by measuring absorbance at wavelength of 560 nm, in the cuvette with optical path of 1 cm using Carry 50 UV/Vis spectrophotometer equiped with thermostated cell holder. The pH of solutions was kept constant during the experiments with 50 mM acetate buffer, while the sum of concentrations of $FeSO_4$ and gallic acid or vescalagin was kept at 2×10^{-4} M in the case of vescalagin and 5×10^{-4} M in case of the gallic acid for all ratios between $FeSO_4$ and gallic acid or vescalagin.

RESULTS & DISCUSSION

In Fig. 1 the Job plot for the reaction of $FeSO_4$ with gallic acid or vescalagin at pH = 5,5 is presented together with corresponding model functions. Maximum values of absorbance can be observed at Fe/gallic acid ratio 1/1. Stoichiometry of Fe/vescalagin coordination compound is slightly trickier. Although highest absorbance was observed at Fe/vescalagin ratio 2/1, cross-section of extrapolation of the first 5 and last 10 points of Job diagram is located at the value of 0.25, what indicates 3/1 ratio. So, it can be concluded, that while each molecule of gallic acid binds 1 Fe ion, each molecule of vescalagin binds 2 or possibly even 3 Fe ions, which is not surprising, considering the large number of possible binding sites on vescalagin. The plots are qualitatively the same for lower pH values, but absorbances are diminishing.

Based on the ratio, determined from job plot, the model function assuming 1:1 binding ratio was fitted to the experimental data to obtain values of absorption coefficient and formation constants of coordination compounds. The agreement between experimental and model values is very good. The same procedure was performed for Fe/vescalagin ratios 2/1 and 3/1, where better agreement was observed for 2/1 Fe/vescalagin ratio (shown in Fig. 1).

Finally, the equilibrium constants of 9.6×10^{4} and 6.4×10^{8} were obtained for the formation of Fe coordination compounds with gallic acid and vescalagin, respectively. These values mean, that for the initial concentration of Fe ions 2.0×10^{-4} M at pH = 5.5 and equimolar ratio between Fe ions and ligands 79% and 95% of Fe ions are bound in the case of gallic acid and vescalagin, respectively. This can partly explain high MIC values of gallic acid compared to MIC values of vescalagin.[1] However, one should also keep in mind, that the molar mass of vescalagin is significantly higher than the molar mass of gallic acid.

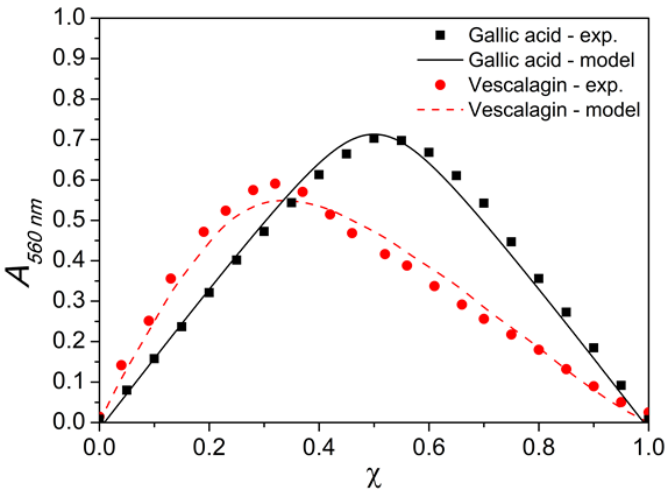

Figure 1. Job plots for the reaction of gallic acid and vescalagin with iron ions at pH = 5.5.

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P1.9

Flavan-3-ols isolated from the bark of *Bassia longifolia*

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MAIN CONCLUSION

From a methanolic extract obtained from the bark of *Bassia longifolia*, the flavan-3-ols catechin, epicatechin, procyanidin B2 and epiafzelechin-(4β→8)-epicatechin were isolated. A characterization of the proanthocyanidin pattern is performed for the first time.

Their structures were elucidated by ^{1}H- and ^{13}C-NMR including 2D-experiments. LC-MS data indicate more dimeric and rare trimeric flavan-3-ols with (epi-)afzelechin units which could be characteristic for *Bassia longifolia*.

INTRODUCTION

In Nepal, phytomedicine has a long history with approximately 1600 -1900 different species of plants used in traditional medicine [1]. One of them is *Bassia longifolia* Koenig (=Madhuca longifolia Koenig, Sapotaceae), an evergreen tree which grows about 20 m in height and is distributed throughout Nepal, India and Sri Lanka. Flowers and bark are used for both, food and medicine [2]. The bark can be applied externally as paste in the treatment of cuts and wounds [3]. To understand its therapeutic effect, it is important to know the plants chemical compounds. Until then, chemical-analytical and spectroscopic investigations are only available for the seeds [4] but data regarding the flowers and bark are not sufficiently available.

Therefore, a characterization of the proanthocyanidin pattern is performed for the first time of which four substances are shown exemplarily.

MATERIALS & METHODS

The samples were collected in the Kailali district in western Nepal. The bark was cut and dried. A voucher specimen (BL-16-DPP) was deposited at the Research Centre for Applied Science and Technology (RECAST), Tribhuvan University, Kathmandu, Nepal. Dried and powdered bark (1072.5 g) was subsequently percolated with petroleum ether, ethyl acetate and methanol. 40.0 g of the MeOH extract were fractionated by column chromatography using Sephadex®-LH20 (EtOH 70%, aceton 70%) and flash chromatography with MCI-Gel® CHP20P (MeOH 20%-->MeOH 100%). Final isolation was performed by preparative HPLC (RP-18; H_2O/MeCN).

For structure elucidation, ^{1}H-, ^{13}C- and 2D-NMR spectra were recorded on an Avance III HD 400 (Bruker) in MeOH-d_4 at 233 K. Structures were confirmed by ESI-HRMS on a Q-TOF 6540 UHD (Agilent) in positive mode. Polarimetry was performed on an UniPol L1000 (Schmidt + Haensch) and CD-spectra on a J-715 (JASCO).

RESULTS & DISCUSSION

The four substances (-)-epicatechin (**1**), (+)-catechin (**2**), procyanidin B2 (**3**) and epiafzelechin-(4β→8)epicatechin (**4**) have been isolated so far (**Fig. 1**). NMR measurement was performed at 233 K for substances 3 and 4 to avoid rotameric signals.

Structure elucidation will be shown exemplarily for compound 4. The epiafzelechin unit was identified by a d with a small coupling constant of $J_{H\text{-}2/H\text{-}3}$ = 1.8 Hz at 3.78 ppm for H-3 and a s at 5.12 ppm for H-2, which is characteristic for *cis*-configurated flavan-3-ols. The monohydroxylated B-ring was established by a d at 7.17 ppm and another d at 6.70 ppm. Both integrated for two protons and therefore indicated an AA´BB´-spin system in the B-ring.

The epicatechin unit was identified by a specific br s at 4.23 ppm for H-3 and a s at 4.95 ppm, which is also characteristic for a cis-configurated flavan-3-ol (**Fig. 2**).

The upper unit could be identified as epiafzelechin and lower unit as epicatechin by 2D-NMR experiments.

By application of the hypothesis of Barrett et al., the positive cotton-effects between 220 nm and 240 nm in the CD-spectrum indicate β-configuration (4*R*) between C-4 of the upper and C-8 of the lower unit [5].

The absolute configuration for substances **1** and **2** was determined by polarimetry.

LC-MS data indicate the presence of more dimeric and rare trimeric flavan-3-ols with (epi-)afzelechin units that could be characteristic for *Bassia longifolia*. Their identification will be object in future studies. Pharmacological investigations showed antimicrobial activity of the methanolic extract against gram-positive *Staphylococcus aureus* strains, both methicillin sensitive *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA) as well as anti-biofilm activity on these bacterial strains (see poster "*Bassia longifolia* bark extract exhibits antimicrobial activity"). It has to be further investigated how these specific proanthocyanidines contribute to the antibacterial effect of *Bassia longifolia*.

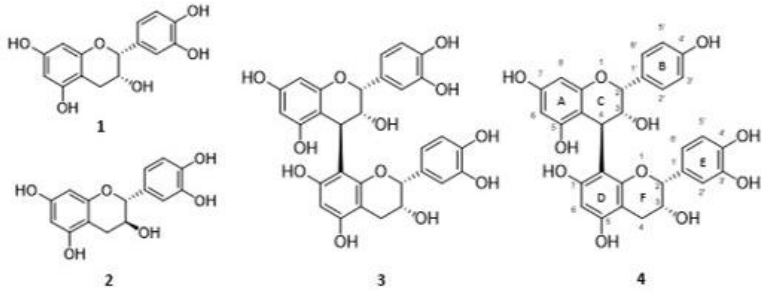

Fig. 1: Isolated flavan-3-ols from the bark of *Bassia longifolia*.

	upper unit		lower unit	
Pos.	$\delta_{C,\ major}$	$\delta_{H,\ major}$ (J in Hz)	$\delta_{C,\ major}$	$\delta_{H,\ major}$ (J in Hz)
2	77.0, CH	5.12, s	79.4, CH	4,95, s
3	73.6, CH	3.78, d (1.8)	67.1, CH	4.23, br s
4	36.7, CH	4.62, s	30.0, CH_2	2.79, d (16.4) 2.93, dd (16.4, 4.2)
4a	102.0, C		99.6, C	
5	157.8, C		157.7, C	
6	95.6, CH	5.92, d (2.3)	96.8, CH	5.87, s
7	157.8, C		157.9, C	
8	95.7, CH	5.95, d (2.3)	107.2, C	
8a	154.5, C		156.6, C	
1'	131.8, C		132.1, C	
2'	129.8, CH	7.17, d (8.7)	114.8, CH	7.09, d (1.8)
3'	115.5, CH	6.70, d (8.7)	145.9, C	
4'	156.7, C		145.5, C	
5'	115.5, CH	6.70, d (8.7)	115.7, CH	6.71, d (8.3)
6'	129.8, CH	7.17, d (8.7)	118.7, CH	6.85, dd (8.3, 1.8)

Fig. 2: ^{1}H- and ^{13}C-NMR data of **4**. 400.13 MHz (^{1}H) and 100.63 MHz (^{13}C) at 233 K in MeOH-d_4.

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P1.11

Polyphenolic composition of cold-hardy grapes and wines

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MAIN CONCLUSION

In cold-hardy red wines, monomeric phenolic compounds were extracted from grapes but the content of condensed tannins was very low compared to *Vitis vinifera* wines. Even though ultrasound-assisted extraction technique was applied on cold-hardy grape 'Marquette' cultivar, the extraction of tannins was not improved, suggesting that the chemical composition of phenolic compounds and cell wall material structure are factors retaining tannins in grapes.

INTRODUCTION

In the US Midwest region, cold-hardy interspecific hybrid grapes have been developed to be more resistant to the harsh cold winter, late spring frosts and hot and humid summer. For the wine industry, one of the top priorities is to produce high quality wines. In contrast with *Vitis vinifera* red wines, cold-hardy red wines tend to be high in acidity, low in condensed tannins and rich in anthocyanins mono- and di-glucosides. The main anthocyanins identified in 'Frontenac' and 'Marquette' grapes were delphinidin-3,5-di-O-glucoside and malvidin-3,5-di-O-glucoside, respectively [1]. In addition, cold-hardy wines contain less condensed tannins than *Vitis vinifera* wines [2]. Condensed tannins are oligomers and polymers of flavan-3-ols able to interact through non-covalent interactions with cell wall polysaccharides and proteins. It has been suggested that condensed tannins are retained in grape cell walls due to the high content of proteins in those grapes [2]. As a result of this chemical composition, cold-hardy red wines tend to be low in astringency, more prone to oxidation and with poor color stability [1,3]. Little is known about the composition of anthocyanins and tannins, and their extraction from cold-hardy grapes during the process of wine making. In this study, the phenolic compounds content and structure from grapes to wine of cold-hardy 'Marquette', 'Frontenac', 'Petite Pearl' cultivars were compared to 'Cabernet sauvignon' and 'Pinot noir' cultivars.

MATERIALS & METHODS

'Marquette', 'Frontenac' and 'Petite Pearl' grapes were provided at harvest maturity by three vineyards from Iowa. Commercial red wines from 'Marquette', 'Frontenac', 'Petite Pearl', 'Cabernet sauvignon' and 'Pinot noir' cultivars were provided and purchased in local Iowa wineries.

Total iron-reactive phenolic compounds content was evaluated in grapes and wines. The total tannin content was evaluated after methylcellulose precipitation and by UV-Visible spectrophotometry. To identify the evolution of phenolic compounds during the winemaking process of 'Marquette', the monomeric phenolic compounds were evaluated by HPLC-DAD/FLD. To identify the extraction of tannin and the color stability in 'Marquette' grapes, an ultrasound technique was applied for 10 min on crushed berries in a model wine solution (13% ethanol, 5g/L tartaric acid and a pH of 3.5). The tannin content, anthocyanins content and structure was evaluated with the same analytical method as described above.

RESULTS & DISCUSSION

Total iron-reactive phenolic compounds and tannin contents were higher in 'Petite Pearl' grapes than in 'Frontenac' and 'Marquette' grapes. In red wines, the total phenolic compounds content was the highest in 'Cabernet sauvignon' and the tannin content was higher in *Vitis vinifera* wines than 'Petite Pearl', 'Frontenac' and 'Marquette' wines as previously observed [2]. The cultivar 'Marquette' has been used in this study as a model of cold-hardy cultivar. During the 'Marquette' winemaking process

from crushing to 6 months after aging, flavan-3-ols and flavonols content increased, the anthocyanins content decreased after bottling and the tannin content remained low. In order to improve the extraction of tannins from 'Marquette' grapes, an ultrasound technique was applied after crushing. The total iron-reactive phenolic compounds content increased after the ultrasound but the tannin content remained unchanged. The main anthocyanins released in grape juice before and after ultrasounds were the malvidin-3,5-di-O-glucoside followed by the malvidin-3-O-glucoside. The total anthocyanin content increased by 3.5 fold by application of the ultrasound, which improved the color intensity of the juice. Anthocyanins are easily extracted under an aqueous solution as observed during the winemaking process and when applying the ultrasound on crushed berries. However, this technique, which breaks skin cell walls, did not improve the extraction of tannins from Marquette grapes. Tannins are able to bind strongly with cell wall polysaccharides and proteins, which might be the phenomenon occurring between tannins released from the vacuole with cell wall material disrupted by the ultrasound.

Further work will be focusing on the application of winemaking techniques including the ultrasound on other varieties and under various conditions to determine the chemistry and reactivity of tannins to cell wall material in cold-hardy grapes.

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P1.12

Kinetic and Thermodynamic characterization of 5-Hydroxy-4'-Dimethylaminoflavylium in the presence of SDS micelles

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MAIN CONCLUSION

In the present work, it is possible observed a metastable equilibrium between the flavylium cation and the quinoidal base with pK_a = 7.6. In neutral to moderately basic medium, only at 60 °C and after 5 days a new distribution involving the flavylium cation and *trans*-chalcones is achieved. The equilibrium is apparently attained between flavylium cation, neutral and anionic *trans*-chalcones respectively for p$K_{AH+/Ct}$ = 4.1 and p$K_{Ct/Ct-}$ = 10.7.

INTRODUCTION

Based on the study of many anthocyanins and related compounds it is possible to predict for any flavylium structure the chemical species that will be generate over a pH range. [1] This system is conveniently studied by addition of base to equilibrated solutions of the flavylium cation (direct pH jumps) [2] or by addition of acid to equilibrated solutions at higher pH values, in the present work the anionic *trans*-chalcone (reverse pH jumps). Upon a direct pH jump at sufficiently high pH values, the quinoidal base (**A**) is formed in sub-microseconds and the evolution of the system towards the eqilibrium takes place from the hydration of the flavylium cation ($\mathbf{AH^+}$) to give hemiketal (**B**) and not from the quinoidal base, which only in basic medium is attacked by OH^-. The hemiketal (**B**) is generally in fast equilibrium (seconds to sub-seconds) with *cis*-chalcone (**Cc**) through a ring opening/closure process (tautomerization) and *trans*-chalcone (**Ct**) is formed by an isomerization process from *cis*-chalcone.

The thermodynamic and kinetics of flavylium based systems are dramatically dependent on the nature and position of the substituents of the 2-phenylbenzopyrylium core. In the case of the newly compound 5-hydroxy-4'-methylamino, it has one hydroxyl group in carbon 5, allowing the formation of a quinoidal base exhibiting a slight red shifted of its maximum absorption in relation to flavylium cation.

The 5-hydroxy-4'-methylamino synthesized in this work, aggregates and precipitates in water, preventing the calculation of the respective kinetic and thermodynamic constants. One of the ways to overcome these drawbacks can be carrying out the study in the presence of sodium dodecyl sulphate (SDS) micelles (0.1M).

MATERIALS & METHODS

A stock solution (8.0×10^{-4} M) of 5OH4′NMe2 in ethanol acidulated with 0.1 M HCl; and a SDS 0.35 M stock solution in Millipore water were prepared. HCl, NaOH or Universal buffer were used to adjust the pH of all the solutions prepared.

A set of 5OH4′NMe2 solutions in the presence of 0.1 M SDS were prepared in individual 10 mm pathlength plastic cuvettes for different pH values along the pH scale. The pigment was added at the end, and after mixing; the direct pH jumps kinetics were followed recording the spectral variations in a Varian-Cary 100 Bio and/or 5000 spectrophotometers (Palo Alto, CA, USA). This experiment was performed at room temperature and 60 °C, controlled by a multicell block peltier carrier. The pH of

the solutions was measured after their preparation in a Radiometer Copenhagen PHM240 pH/77 ion meter.

The determination of the pK_a was performed from representation of the absorption taken immediately after a direct pH jump versus pH.

RESULTS & DISCUSSION

In spite of the existence of a significant aggregation of this compound in pure water, even at low concentrations, this phenomenon was not observed in the presence of SDS micelles.

The absorption spectra of 5OH4′NMe2 (2.56×10^{-5} M) in the presence of SDS micelles (0.1 M) taken immediately after direct pH jumps are shown in Figure 1.

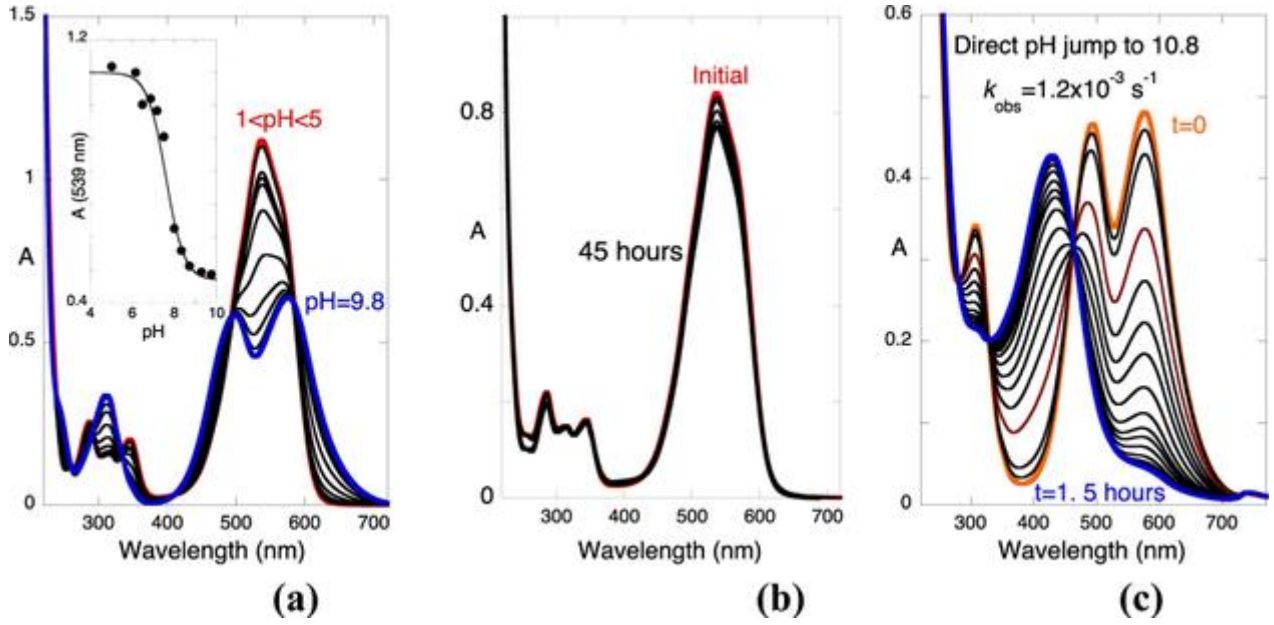

Figure 1. (a) Spectral variations of 5OH4´NMe2 (0.0000256M) in the presence of SDS micelles (0.1M) taken immediately after a direct pH jump, at room temperature. (b) Spectral variations at 60ºC after 45h. (c) Spectral variations upon a direct pH jump to pH= 10.8 at 60ºC after 90 min.

At room temperature, there is an equilibrium between AH^+ and A with pKa = 7.6 (Figure 1a) and these two species give rise to a metastable state. For very basic solutions it is necessary to heat the system at 60 °C in order to observe the kinetic processes towards the equilibrium, Figure 2b and 2c. Only at very basic pH values, for example pH = 13, the anionic form of the *trans*-chalcone is observed in a few minutes at room temperature, similarly to the behaviour of other flavylium systems bearing amine substituents.

More information regarding the meta-stability of the flavylium cation-quinoidal based system was achieved by carrying out a series of direct pH jumps at 60ºC. The absorbance of the flavylium cation after 5 days at 60ºC shows an apparent pK_a = 7.5. The quinoidal base completely disappears and at higher pH values the absorption spectra identified as *trans*-chalcones are dominant. The anionic *trans*-chalcone specie was prepared at very basic pHs and from this species a series of reverse pH jumps were carried out. The first protonation constant was $pK_{Ct+/Ct} = 2.6 \pm 0.1$ and the second equilibrium was $pK_{Ct/Ct-} = 10.7 \pm 0.1$.

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P1.13

Investigating ultraviolet-visible energies that initiate the mechanism of cis-trans photoisomerization of acylated delphinidins and its impact on color performance

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MAIN CONCLUSION

Photochromism of Dp-3-rut-5-glu (Dp) acylated with *trans*- (*t*) or *cis*-*p*-coumaroyl (*c*) occurred under UV and visible light, but at different rates, efficiency, and equilibrium. Greatest amount of $c \rightarrow t$ isomerization occurred under UV at 365nm, while $t \rightarrow c$ was greatest under visible light (F2). Both starting material and radiant energies affected the chemical equilibrium. *C*- and *t*-Dp expressed different color intensity and stability in alkaline pH. *C*-Dp was greener and more stable in pH 8-10 than *t*-Dp.

INTRODUCTION

Acylated anthocyanins (ACN) have been reported to possess higher color stability in comparison to molecules without acylation (Giusti and Wrolstad 2003). Acylated ACN extracted from fruits or vegetables can be mono-, di-, or poly-acylated with aromatic or aliphatic acids. In plants, most acylating groups exist in the *t*-configuration and may impart greater stability to the ACN by intramolecular copigmentation. Upon irradiation, acylated ACN can undergo a structural transformation to form a *c*-isomer by overcoming the isomerization barrier (Gago et al. 2015). The applied radiant energy affects this barrier, causing the molecule to adopt its excited state, and producing a molecule with different chemical characteristics. While both aromatic and aliphatic acyl-groups can undergo this transformation, most photoisomerization studies have been for aromatic acids.

Photoisomerization of hydroxycinnamic acylated anthocyanins has been shown to produce colorants of distinct hue under a broad pH range, with potential for enhanced color stability and strength (Sigurdson et al. 2018). Earlier work by Yoshida et al. (1990) showed that acylated ACN isomerization was achieved using a high-pressure mercury arc lamp peaked at 366 nm and sunlight. Albeit, the bulk of the study was on the effect of irradiating solutions and their pH on the final equilibrium of isomerization. Although studies have shown the relationship between irradiation solvent and its relative changes in isomerization, seldom have discussed the role of radiant energy on the photo-induced isomerization and its resulting chromism. This study explored the relationship between radiant energies in the UV-visible range and their respective irradiation times necessary to trigger isomerization. Specifically, four, accessible radiant energies in tandem with varying starting material (isolates or extract) were investigated, whilst using a solvent (MeOH) that has been reported to yield the greatest photoconversion rate.

MATERIALS & METHODS

Anthocyanins were extracted from frozen East Asian eggplant skins using acidified acetone, followed by partition with chloroform. The extract was loaded onto a C18 cartridge, washed with acidified H_2O, EtOAc, and ACN were recovered with acidified MeOH. The concentration was measured and adjusted to produce a 100 μM semi-crude extract (SCE) using pH differential method, using a SpectraMax microplate reader.

Pure *c*- and *t*-Dp were obtained by Semi-Preparative HPLC with a PFP column, from SCE that had been subjected to UV light. SCE, *c*-, and *t*-Dp isolates were placed in sealed quartz cuvettes and

irradiated with one of the following: D65, 3hrs; F2, 20hrs; 365nm or 254nm, 15min. Pigment isomerization was monitored on a HPLC-PDA with a C18 column. Color strength and stability of *c*- and *t*-Dp were compared using a SpectraMax microplate reader and Color By Spectra software for CIELAB colorimetric analysis. Data was collected in triplicates and analyzed by two-way ANOVA with SPSS Statistics.

RESULTS & DISCUSSION

All of the light treatments induced reversible photoisomerization between *t*- and *c*-Dp, but had varying equilibrium points and extent of isomerization. Under UV light, higher equilibrium of *t* : *c* was observed, regardless of the of starting material. Although acylated delphinidins favored existence as its *t*-isomer under UV light, specific wavelengths within the UV range varied in both *c* → *t* and *t* → *c* isomerization. Irradiation at 254 nm produced more *t* → *c* photoconversion than 365 nm did, but both energies produced substantially less isomerized *c*-Dp than isomerized *t*-Dp. Under visible light, *c*-Dp did not isomerize as efficiently nor abundantly as UV light did. Both D65 and F2 produced similar amount of isomerization of semi-crude extract and *c*-Dp. However, F2 yielded 15% more isomerization than D65 did. In summary, photoisomerization was efficiently induced in the order of: 365 nm > 254 nm > D65 ≈ F2, whereas photoisomerization was induced in the order of: F2 > D65 ≈ 254 nm > 365 nm.

Color expression of the two geometric isomers differed according to the pH. In pH 8-10, *c*-Dp showed greater color intensity (CIE c*) than the *t*-isomer; whereas in pH 7, *t*- showed greater color intensity than its *c*-counterpart. In alkaline pH 8–10, *t*- had blue-cyan hue, while *c*- displayed blue-green hue (Fig 1). Comparison of the two isolates' CIE L* a* b* colorimetric values resulted in ΔE of 10.7, 14.2, 20.9, and 22.6 for pH 7, 8, 9, and 10, respectively. After 24 hs, *c*-Dp retained its color better than *t*-Dp in pH 1 and 2.

When *c*-Dp and *t*-Dp were subjected to irradiation at 254 nm for 15 min, the resulting CIE L* a* b* values were similar in both acidic and neutral pH. However, in pH 8-10, CIE L* a* b* values of irradiated *c*- *and t*-Dp resulted in ΔE of 10, but was difficult to detect with the naked eye, perhaps due to its low saturation.

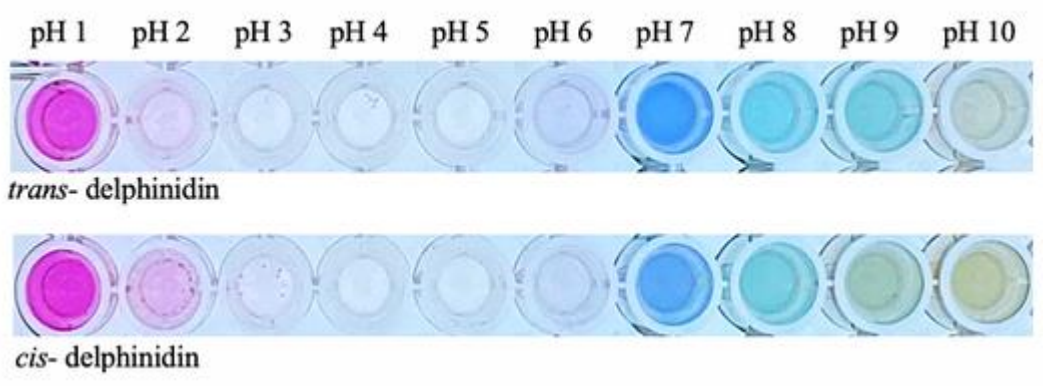

Figure 1. Comparison of Dp-3-*cis*-*p*-cou-rut-5-glu (*c*-Dp) and Dp-3-*trans*-*p*-cou-rut-5-glu *(t*-Dp*)* color expression in pH 1–10.

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P1.14

High-performance countercurrent chromatography fractionation of polymethoxy flavones by off-line electrospray mass spectrometry injection profiling of Citrus sinensis

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MAIN CONCLUSION

Polymethoxyflavones (PMFs) from the flavedo of *Citrus sinensis* were fractionated by high-performance countercurrent chromatography (HPCCC) and were monitored by *off-line* ESI-MS/MS. Co-elution effects and recovery of target compounds were visualized. The HPCCC elution of PMFs was not solely based on degree of methoxylation, as also isobars were fractionated by large retention volume differences. Mayor PMFs were purified by preparative C_{18}-HPLC and confirmed by 1D/2D-NMR spectroscopy.

INTRODUCTION

Citrus spp. belong to the family Rutaceae [1]. Worldwide the production of *Citrus sinensis* (orange fruits) is steadily increasing with approx. 78 million metric tons in 2019, including 24 mill. tons of residual non-edible peel material [2]. The valorization of side-streams such as orange peels is of mayor interest as rich source for bioactives and technologically valuable compounds: flavonoids, pectins, carotenoids, mono-terpenoic essential oils (e.g. limonene), and triterpenoids (limonoids). Principal secondary metabolites in *Citrus* spp. are flavone-/ flavanone-glycosides, and PMFs. PMFs are located in almost all tissues such as seeds and leaves and predominantly in the flavedo. In addition to *O*-glycosidic flavonoids, also *C*-linked structures are found with additional phenolic acid substitutions such as hydroxyl-cinnamic acids e.g. in orange peels. PMFs provide diverse biological activity profiles: antioxidant, anti-carcinogenic, anti-inflammatory, anti-obesity, anti-diabetes, neuro-protection [1].

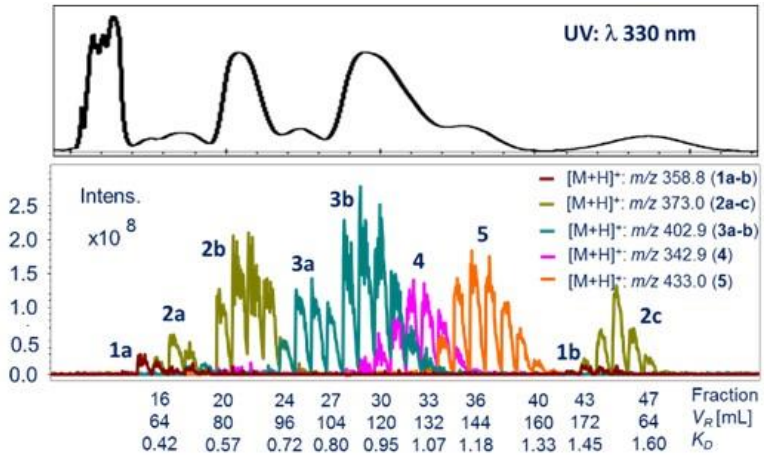
For the preparative isolation of PMFs the hyphenation of HPCCC fractionation with *off-line* ESI-MS/MS profiling was used for gentle and effective fractionation and specific detection.

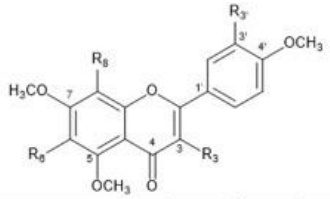
MATERIALS & METHODS

10.5 g of dried flavedo was extracted using ultrasonic extraction. Optimal conditions using ‘central composite design’ were found to be 60 °C, sample concentration of 10 mg/mL, methanol content of 66%; extraction time of 6.18 min, and amplitude of 48%. The sample was centrifuged (4500 rpm, 10 min) and the extraction was repeated two times. After liquid-liquid partitioning of the evaporated supernatant by methanol-water and ethyl acetate, the enriched PMF partition (ethyl acetate, 445 mg) was fractionated by HPCCC (Spectrum, 125 mL, Dynamic Extractions). The separation mode (*head-to-tail*) was operated at 1600 rpm, mobile phase flow 4.0 mL/min, and UV-detection (λ 330 nm). The biphasic solvent system was composed of *n*-hexane/ ethyl acetate/ methanol/ water (*HemWat*: 4/6/5/5, *v/v/v/v*) and adjusted to adequate *partition ratio values* K_D of *Citrus* PMFs. During the elution-mode, 65 fractions were submitted to ESI-MS/MS *off-line* injection profiling [3] using selected single ion-traces.

RESULTS & DISCUSSION

The semi-preparative scale HPCCC experiment using a *HemWat* System enabled the fractionation of similar PMFs (cf. Fig.). The *off-line* hyphenation by ESI-MS injections of recovered fractions labelled the PMFs by $[M+H]^+$ ion signals. The minor concentrated PMF (*m/z* 359, **1a**) eluted at low *retention volume* (V_R) or *partition ratio value* (K_D 0.41) potentially related to a demethoxy-sinensetin or -tangeretin and was separated from the very minor concentrated isobar (**1b**, K_D: 1.45–1.50). The ion trace *m/z* 373 visualized the separation of three PMF isobars (**2a-c**) whereby similar ESI-MS/MS data solely revealed the step-wise loss of methyl-groups, and PMFs were assigned to *iso*-sinensetin (**2a,** tentative), sinensetin (**2b**), and tangeretin (**2c**). The ion trace *m/z* 403 displayed the HPCCC-fractionation of a lower concentrated isomer (**3a**, K_D 0.72-0.80) from nobiletin (**3b**, K_D 0.80-1.07). However, the less methoxylated 5,7,3',4'-tetramethoxyflavone (**4**) (*m/z* 343) and potentially more polar PMF eluted at higher K_D than nobiletin (cf. Fig.). The $[M+H]^+$-signal *m/z* 433 detected 3,5,6,7,8,3',4'-heptamethoxyflavone (**5**). The hyphenation experiment revealed that *reversed-phase* (*head-to-tail*) mode HPCCC was not strictly separating the PMFs by their lipophilic character as seen by the elution of the PMFs at *m/z* 433 (**5**, K_D 1.07–1.33) and *m/z* 373 (**2c**, K_D 1.45–1.60). **5** formally counted the highest amount of lipophilicity increasing methoxy-groups. Specific methoxy-substitution pattern in PMF isobars seem to provide unexpected separation characteristics for HPCCC, as seen for the isobaric pair **1a**/**1b**. The hyphenation of preparative HPCCC to ESI-MS detection compared to previous HSCCC studies on *Citrus* PMFs [4] enabled an immediate acquisition of structural data of separated compounds, and unintentional mixing of already separated compounds was omitted. Final clean-up and confirmation of mayor PMFs (e.g. 16 mg of sinensetin and 20 mg of nobiletin) was done by preparative C_{18}-LC and 1D/2D-NMR spectroscopy [5].





Compounds	[M+H]+ : m/z	R3	R6	R8	R3'
Demethoxy-sinensetin (1a)	359	H	OH	H	OCH_3
Iso-sinensetin (2a)	373	2 x OCH_3			
Sinensetin (2b)	373	H	OCH_3	H	OCH_3
Unknown Nobelitin isobar (3a)	403	3 x OCH_3			
Nobiletin (3b)	403	H	OCH_3	OCH_3	OCH_3
5,7,3',4'-Tetra-methoxy-flavanone (4)	343	H	H	OCH_3	H
3,5,6,7,8,3',4'-Hepta-methoxyflavone (5)	433	OCH_3	OCH_3	OCH_3	OCH_3
Demethoxy-tangeretin (1b)	359	H	OH	OCH_3	H
Tangeretin (2c)	373	H	OCH_3	OCH_3	H

Figure: HPCCC-chromatogram (λ 330 nm) of PMFs in *Citrus sinensis* flavedo extract (445 mg) using the biphasic solvent system *HemWat* (4:6:5:5, *v/v/v/v*) and *off-line* ESI-MS/MS injection profiling (positive mode). Scan range *m/z* 100-2000 of the mass-spectrometer (HCT Ultra ETD II, Bruker Daltonics) used eight precursor ions for automatic MS/MS.

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P1.15

Identification of oxidation markers of the reaction of grape tannins with volatile thiols commonly found in wine

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MAIN CONCLUSION

Products of the reaction of tannins with thiols (4MSP, 3SH, FFT and EtSH) were detected and identified for the first time. Knowledge of the retention time and the fragmentation spectrum of these oxidation markers allows to progress the characterization of tannins in the oenological environment. A better follow-up of the changes of thiols in wines in terms of their reactivity with the tannins open new perspectives in the understanding of the role of tannins in the aromatic bouquet of wines.

INTRODUCTION

Thanks to their reactivity, the great diversity of condensed tannins already present in the grape berry continues to increase from the beginning of winemaking and throughout the ageing of the wine. Tannins contribute greatly to the organoleptic properties of red wines, such as astringency and colour, and also provide protection through their antioxidant properties. Currently, the reactivity of flavanols has been studied mainly from flavanol monomers, either between flavanols or between flavanols and other biomolecules such as aromas [1], but few products resulting from reactions with tannins have been identified so far. To date, our studies have focused on reactivity of condensed tannins and on the products of the reaction of tannins only. Oxidation markers from reactions between tannins have been detected and identified by UPLC-MS after chemical depolymerization [2]. These first studies need to be extended to other families of wine biomolecules in order to understand the organoleptic impact of the generated products. In this present work, the reactivity between tannins and volatile thiols commonly found in wine was studied. Thiol compounds have mainly been studied in white wines. However, those compounds have also been detected in red wines at concentrations above their perception thresholds. The reactions of tannins with these compounds could partly explain why these compounds are detected mainly in white wines and at lower concentrations in red wines (especially in young red wines). In order to gain a better understanding of the reactions of tannins with these aroma compounds, the reaction products obtained from model solutions containing catechin (C), epicatechin (EC), procyanidin B2, C1, and seed tannins have been characterized in this work. This study may further help with understanding the thiol potential of grapes and estimation of the loss of aroma due to reactions with wine components.

MATERIALS & METHODS

The samples were analyzed by UPLC-MS^2 (Waters Acquity UPLC system coupled to a Bruker AmaZon X MS). The purification process first involved flash chromatography (Interchim puriFlash430 system equipped with RP C18 column) followed by NGC BioRad semi-preparative chromatography RP Varian Dynamax C18 column). The synthesis of standards was carried out enzymatically. Reaction of tannins with thiols: 1 mL of tannins in a 12% ethanol aqueous solution (9 mM catechin unit) was added to 1 mL of a Fe(II) solution (50 mM; 5.55 eq/catechin unit). 1 mL of H_2O_2 solution (300 mM; 33.3 eq/catechin unit) followed by addition of the aroma (10 eq/catechin unit). The reaction medium was stirred at room temperature for 1 h, and the solvent was then removed using a rotary evaporator. Tannin powder was dissolved in 100 µL methanol and added to 100 µL of thioglycolysis solution in a vial, incubated for 6 min at 90°C then cooled in ice-cold water and injected into the UPLC/MS.

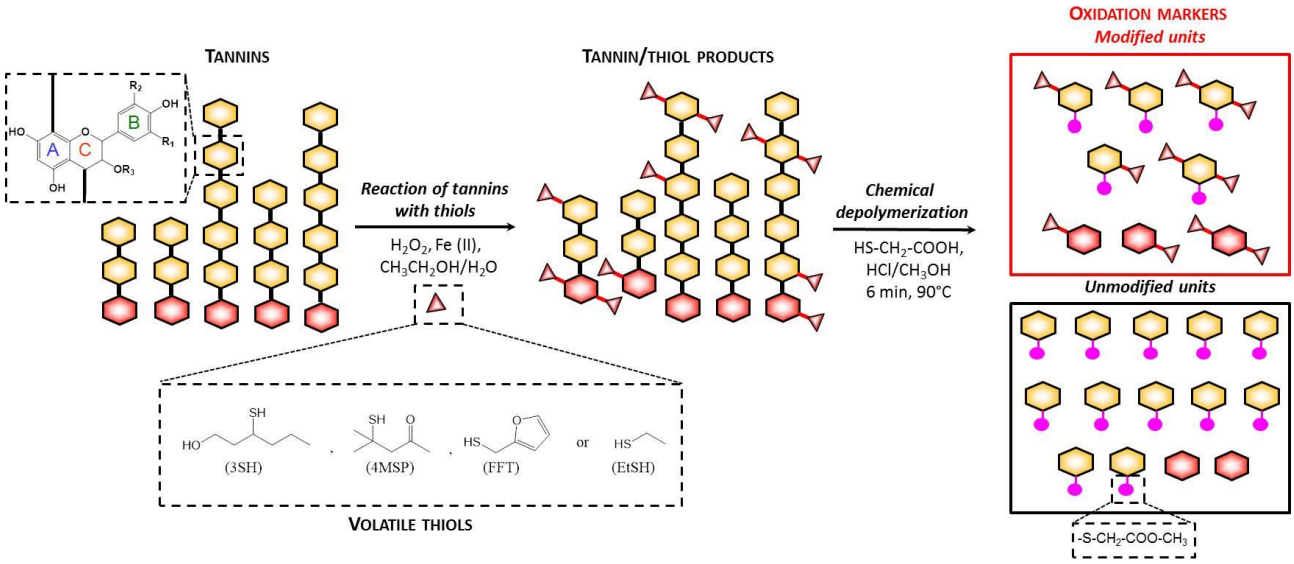
RESULTS & DISCUSSION

To specifically identify, after depolymerization, the chemical modifications induced by the reactions of the oxidized tannins and the aroma compounds within the tannin chains, monomer standards were synthesis. After UHPLC-MS^2 analysis, the retention times and the MS^2 fragmentation spectra that constitute a specific fingerprint of the products obtained with the aromas studied (3SH, FFT, EtSH, or 4MSP) and C, EC, or EC-Nu.

The reactivity of the extension and the terminal units with the aroma compounds were then studied. The reactions were carried out on the dimer B2 and the trimer C1. Regarding the proportions of modified extension terminal units, two trends were observed: 3SH, FFT, and EtSH reacted preferentially with terminal units, while 4MSP reacted mainly with extension units. The presence of an additional unit in C1 appears to favor its reactions with EtSH, whereas in the case of 4MSP, this reaction is very unfavorable. This difference in reactivity of 4MSP can be partly explained by a difference in the nucleophilicity of this compound, which is a tertiary thiol, unlike the others, which are primary (FFT and EtSH) and secondary thiols (3SH).

Finally, to detect the previously identified tannin/aroma adducts in an oenological tannin fraction, the study was performed on grape seed tannins. In addition to their greater structural diversity (the size and the composition of the tannin chains), grape seed tannins were chosen because they contain a higher proportion of ECG than that found in the skins. Thus, this study also revealed new markers of the reaction of ECG with aroma compounds. After chemical depolymerization of the modified tannins, most of the oxidation markers were detected and identified (Scheme 1)[3].

The fragment ions observed on the MS^2 spectrum obtained for each oxidation marker constitute specific fingerprints of the extension or terminal units modified in the tannins and will make it possible to identify these modifications in complex samples.



Scheme 1 : Oxidation markers of the reaction of tannins with thiols identified by UHPLC-MS^2 analysis after chemical depolymerization.

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P1.16

Influence of red wine polysaccharides profile on the flavanol composition and precipitation

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MAIN CONCLUSION

Results provide an interesting insight on the relationship between polysaccharide and flavanol composition in wines, pointing out a negative relationship between the contents of mannoproteins, dextrin or xylan, and the content of flavanols. Also, those families are being related to an important interaction with flavanoles leading to its precipitation, whereas other polysaccharides, such as arabinogalactan and rhamnogalacturonans do not seem to precipitate flavanols after their interaction.

INTRODUCTION

The macromolecular fraction of wines is constituted mainly phenolic compounds and polysaccharides; thus, both play a significant role in the colour and taste of wines. Among the phenolic compounds, flavanols have a major influence on the organoleptic properties of red wine, such as astringency. The concentration of both type of compounds depends on many parameters, such as grape culture conditions, degree of maturity, fermentation yeast, wine-making techniques and the treatments leading to increase solubilization of the macromolecular components of the grape berry cell walls.

The polysaccharides fraction of wines includes polysaccharides rich in arabinose and galactose (PRAGS) such as type II arabinogalactan (AGII) and arabinans, type I and type II rhamnogalacturonans (RGs), and homogalacturonans, which come from grape berries, and glucans, mannans and mannoproteins (MPs), which are released by yeast during fermentation [1]. On the other hand, tannins are polymeric flavan-3-ols, a wide family of polyphenolic compounds present in red wines. They are composed by the monomer units catechin, epicatechin, gallocatechin and epigallocatechin.

It has been proposed that the interactions between tannins and polysaccharides can modulate the perception of tannin astringency since the polysaccharides inhibit the precipitation of the protein-tannin complexes. [2]. Thus, the aim of this study was to characterize the polysaccharide and flavanol composition of Cabernet Sauvignon and Tempranillo red wines to determine if there is some relationship between their content in wines. Also, to obtain further insights about that possible relationship, the interactions between polysaccharides and flavanols in model solutions have been studied.

MATERIALS & METHODS

The polysaccharides from Cabernet Sauvignon (CS) and Tempranillo (T) wines corresponding to 2017 and 2018 vintages were isolated by precipitation by the addition of ethanol, following the method described by Apolinar-Valiente et al. [3]. The characterization of polysaccharide fraction was performed by NMR and HPLC-SEC. The composition of flavanols in wines was determined by HPLC-DAD-MS, according to García-Estevez et al. [4].

Interaction assays were performed between the polysaccharides extracted from CS and T wines and the flavanols extracted from grape seeds of Cabernet Sauvignon, in a ratio 1:10

(flavanol/polysaccharide). After interaction, the samples were centrifuged and the flavanol composition of the supernatants was determined. All analyses were performed in triplicate.

RESULTS & DISCUSSION

NMR study showed that all wines analysed have similar qualitative polysaccharide patterns, containing AGIIs, RGs, mannans, MPs, xylan and dextrins, being AGIIs and MPs the most important families. However, significant differences were found between the quantitate importance of each family. Thus, T wine showed a significant higher percentage of AGIIs than CS wines, which pointed out the great influence of grape variety for polysaccharide composition of wines. With regards to polysaccharide molecular weights, the statistical analysis allowed to found relationship between the concentration of the larger polysaccharides (208–295 kDa) and RGs, between the medium size polysaccharides (60–97 kDa) and AGIIs family and between the smallest polysaccharides (11–13 kDa and 6.3–7 kDA) with dextrins and xylan families.

Flavanol composition of wines also showed differences related to grape variety. T wine showed a much lower content of monomers and also lower flavanol total content than CS wine, but a higher percentage of prodelphinidins.

Principal component analysis (PCA) performed by using flavanol and polysaccharide composition of wines showed that flavanol composition and some polysaccharide families are correlated. Thus, flavanol composition is negatively correlated to mannan, mannoprotein, dextrin and xylan, which may suggest that the higher content of these polysaccharides might lead to lower contents of flavanols in wines.

Again, PCA revealed a relationship between flavanol precipitation and the content of mannan, mannoprotein and dextrin in the polysaccharide extract, which may support the hypothesis that these polysaccharides are prone to cause the precipitation of flavanols and, therefore, the reduction of their contents in wine. On the contrary, AGII and RGs families showed an important opposition to flavanol precipitation, suggesting that these type of polysaccharides do not increase flavanol precipitation or, even, could prevent it.

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P1.17

Thermal Degradation of 10-catechyl Pyranoanthocyanins Derived from Pelargonidin-, Cyanidin-, and Malvidin-3-glucosides

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MAIN CONCLUSION

The 10-catechyl pyranoanthocyanins (PACNs) displayed excellent heat stability, with 2.6 – 4.6 times greater absorbance and pigment content retention than anthocyanins (ACNs) after 4.5 hours of 90°C heating. The same B-ring break-down compounds were formed in both heated ACN and PACN samples suggesting similar degradation mechanisms. Also, a new colored compound formed (λmax = 478nm, m/z = 315) during heating of all PACNs which likely contributed to their color stability.

INTRODUCTION

The chemical structure of ACNs plays a significant role on their functional properties including color expression, stability, solubility, and bioavailability. For example, substitutions on the B-ring, defining the aglycone, impact antioxidant activity, pH stability, and the color produced with additional substitutions typically providing a bathochromic shift to the lambda max. Also, the number and orientation of glycosyl groups impacts stability with monoglucosides exhibiting limited stability despite being naturally abundant. To improve ACN stability, a possibility is to combine them with a cofactor, such as pyruvic or caffeic acid, to form a PACN [1]. These pigments contain an additional pyran ring between the carbon 4 and 5-OH group of ACNs and have greater color resistance to bleaching and pH adjustments [1]. PACNs are found in aged wine but can also be formed efficiently under accelerated conditions [2]. Adjusting the ACN chemical structure alters PACN formation efficiency, with malvidin glycosides producing greater yields than cyanidin, delphinidin, and petunidin glycosides [2].

The stability and formed degradation compounds of pigments when exposed to heat are important to consider before use. However, ACNs degrade into colorless phenolic compounds with heat, limiting their applications [3]. PACNs have been shown to have improved thermal stability compared to ACNs [1], however less is known on their degradation mechanism and how the chemical structure impacts their stability. With the importance of B-ring substitutions on ACN functional properties and PACN formation efficiency, we hypothesized that the aglycone structure would impact thermal stability of both ACNs and PACNs and that the degradation products formed would be different between pigment classes. Our objective was to evaluate the thermal stability and formed degradation compounds of three anthocyanins (pelargonidin, cyanidin, and malvidin-3-glucosides (glu)) and their respective 10-catechyl PACNs.

MATERIALS & METHODS

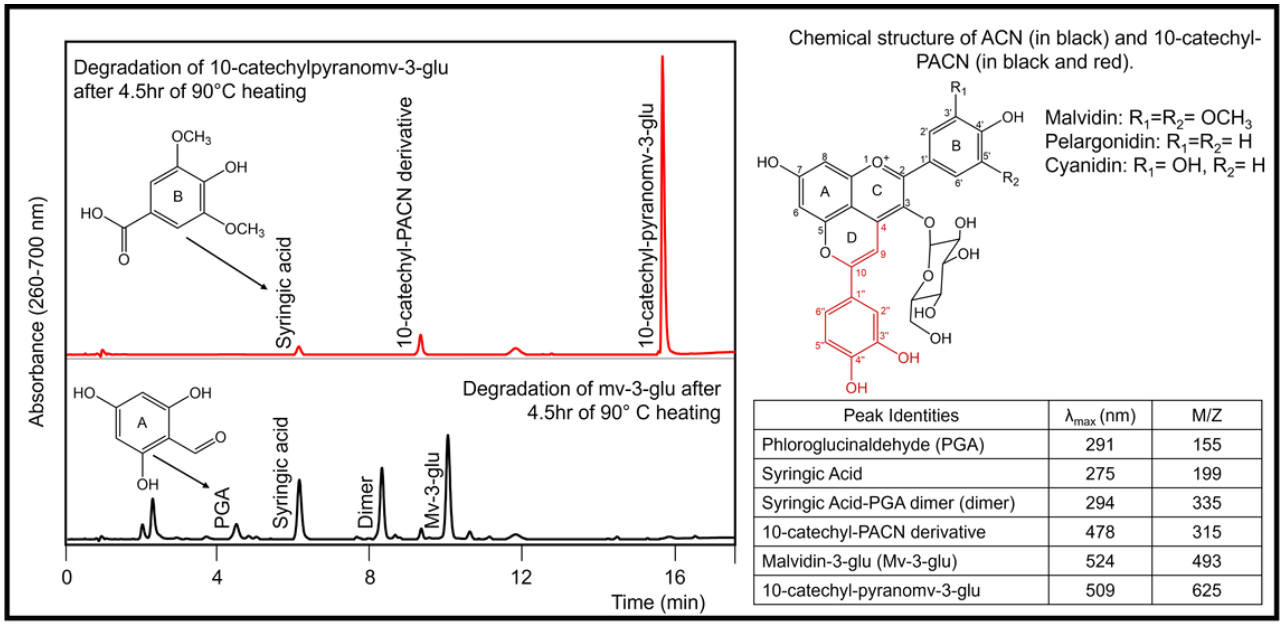
Extracts containing pelargonidin-3-glu (from strawberries), malvidin-3-glu (from *Berberis boliviana*) and cyanidin-3-glu (from *Sambucus nigra*) were each incubated with caffeic acid at 45°C in the dark to form 10-catechyl PACNs [2]. Each pigment was isolated to high purity (>90% based on HPLC area) using semi-preparatory HPLC, concentrated by solid phase extraction, and dispersed in pH 3.0 HCl water. The 6 pigment solutions were pretreated with ultrasound (4 min), standardized to equivalent pH and absorbance of 1.00 at their lambda max, aliquoted to individual tubes for each time point, and heated up to 4.5 hours in a 90°C water bath. Throughout, aliquots were removed from heat and absorbance and total pigment content measured with a spectrophotometer. Pigment degradation and product formation were monitored through uHPLC-PDA-ESI-MS/MS. A 1-way ANOVA was

used for statistic comparisons between pigments ($p<0.05$ significance level), and ACN degradation was modeled by first order kinetics.

RESULTS & DISCUSSION

ACN-3-glucosides degraded quickly, with λ_{max} absorbance (ranging from 500 – 520 nm) and pigment content decreasing after 4.5 hours by 65 – 74% and 73 – 79%, respectively, following first-order kinetics ($R^2>0.99$). In contrast, 10-catechyl PACNs were more stable, with less than a 10% reduction in λ_{max} absorbance (ranging from 495 – 505 nm) and total pigment content after 4.5 hours. Noticeable color changes ($\Delta E >5$) were observed after just 0.5 hours for all three ACNs while the threshold was not reached for any PACNs. The B-ring substitutions impacted thermal stability of ACNs with cyanidin-3-glu being the most stable, followed by pelargonidin-3-glu and malvidin-3-glu ($p<0.05$). For PACNs, 10-catechyl-pyranocyanidin-3-glu trended as the most stable, but the differences were non-significant, likely due to minimal change with heating.

ACN degradation compounds (Figure 1) included phloroglucinaldehyde, a B-ring derivative (4-hydroxybenzoic acid from pelargonidin, protocatechuic acid from cyanidin, syringic acid from malvidin), and a proposed dimer between phloroglucinaldehyde and the B-ring product as previously identified [3]. Formation of these compounds supports the proposed pathway of ACN degradation involving hydration of the flavylium cation and chalcone breakdown [3]. Small amounts of the B-ring derivatives were identified in the corresponding heated PACN samples in addition to a colored degradation compound denoted as 10-catechyl-PACN derivative (λmax = 478nm, m/z = 315). The consistent identification of the B-ring containing compound across pigments suggests a similar degradation mechanism, and therefore, the 10-catechyl-PACN derivative is hypothesized to contain part of the A and catechol rings. PACNs improved thermal stability may be due to the previously reported increased charge delocalization, disfavoring carbon 2 hydration [4], as well as the formation of the newly identified colored degradation compound.



Peak Identities	λ_{max} (nm)	M/Z
Phloroglucinaldehyde (PGA)	291	155
Syringic Acid	275	199
Syringic Acid-PGA dimer (dimer)	294	335
10-catechyl-PACN derivative	478	315
Malvidin-3-glu (Mv-3-glu)	524	493
10-catechyl-pyranomv-3-glu	509	625

Figure 1: uHPLC-PDA chromatograms for 10-catechylpyranomalvidin-3-glu (red) and malvidin-3-glu (black) after 4.5 hours of 90°C heating. Peak characteristics were obtained from uHPLC-PDA-ESI-MS/MS analysis. PACN: Pyranoanthocyanin; ACN: Anthocyanin

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P1.18

Synthesis of 6-methylflavanone and its biotransformation in cultures of entomopathogenic filamentous fungi

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MAIN CONCLUSION

The bioavailability of the flavonoids may be improved by glycosylation and methylation. The combined chemical and biotechnological methods can be used to obtain such compounds. In our studies, we synthesized and afterward biotransformed 6-methylfavanone in cultures of two entomopathogenic filamentous fungi strains *Isaria fumosorosea* KCH J2 and *Beauveria bassiana* KCH J1.5. We received glycosylated, methylated, and hydroxylated flavanones that have not been previously described in the literature.

INTRODUCTION

Flavonoid compounds are commonly known for their numerous biological activities such as antimicrobial, antioxidant, anti-inflammatory, anticancer and neuroprotective, however their pharmacological application is limited by their poor bioavailability. In nature flavonoids usually occur as products of glycosylation, methylation, and polymerization. The most common are glucosides. Sugar moiety (or moieties) attached to a flavonoid aglycone modulates its physicochemical and biological properties, primarily by improving its aqueous solubility and facilitating its absorption [1]. Likewise, methylation of a flavonoid aglycone significantly increases its metabolic stability and enhances membrane transport, resulting in easier absorption and greater oral bioavailability [2].

Extraction of natural glycosylated and methylated flavonoids or their chemical synthesis is usually inefficient. However, combined chemical and biotechnological methods can be used to obtain well-known and novel flavonoid compounds with a methyl and glycosyl moiety attached [3]. Enzymatic glycosylation of synthesized flavonoid aglycones with methyl moiety in cultures of selected microorganisms offers an environmentally friendly, one-pot, and one-step process [4].

The entomopathogenic filamentous fungi are known for their ability to perform glycosylation of flavonoids. *B. bassiana* affects a broad range of arthropods and is able to conduct reactions such as hydroxylation, glycosylation and reduction [4]. Likewise, *I. fumosorosea* has an extensive enzymatic system and was used as biocatalyst for glycosylation in our previous studies concerning biotransformation of 6-methylflavone [3].

MATERIALS & METHODS

The substrate for biotransformation, 6-methylflavanone, was obtained by cyclization of 2'-hydroxy-5'-methylchalcone, derived from the Claisen-Schmidt condensation between 2'-hydroxy-5'-methylacetophenone and benzaldehyde (both purchased from Sigma-Aldrich). Biotransformations were performed in semi-preparative scale, in 2 L flasks with 0.5 L of the liquid Sabouraud medium and *I. fumosorosea* KCH J2 and *B. bassiana* KCH J1.5 as biocatalysts. Both strains belong to the Department of Chemistry of Wrocław University of Environmental and Life Sciences Collection of microorganisms. After 3 days of microorganisms' cultivation, we added 50 mg of 6-methylflavanone to each flask. After 10 days, we extracted products from biotransformation mixtures and separated them with the use of preparative thin layer chromatography (TLC) with a mixture of chloroform and methanol (9:1 *v/v*) as eluent. Their structures were established based on spectroscopic methods (^{1}H NMR, ^{13}C NMR, COSY, HSQC, and HMBC) [4].

RESULTS & DISCUSSION

In the presented study, we synthesized 6-methylflavanone and afterwards efficiently biotransformed by two entomopathogenic filamentous fungi strains *I. fumosorosea* KCH J2 and *B. bassiana* KCH J1.5.

Biotransformation in cultures of *B. bassiana* KCH J1.5 resulted in the formation of four products: 4'-hydroxy-6-methylflavanone 3'-*O*-*β*-D-(4''-*O*-methyl)-glucopyranoside; 4'-hydroxyflavanone 6-methylene-*O*-*β*-D-(4''-*O*-methyl)-glucopyranoside; 6-hydroxymethylflavanone 3'-*O*-*β*-D-(4''-*O*-methyl)-glucopyranoside, 4'-hydroxy-6-hydroxymethylflavanone 3'-*O*-*β*-D-(4''-*O*-methyl)-glucopyranoside [4]. We repeated this biotransformation on an enlarged scale, with 100 mg of substrate, and isolated two additional products 6-methylflavanone 4'-*O*-*β*-D-(4''-*O*-methyl)-glucopyranoside and 3',4'-dihydroxy-6-hydroxymethylflavanone, along with four compounds mentioned above. Summarizing, the strain *B. bassiana* KCH J1.5 was able to hydroxylate methyl moiety at C-6 of 6-methylflavanone and to attach the glycosyl moiety at this site. Its enzymatic system also catalyzed the hydroxylation of the flavanone skeleton at C-3' and C-4', and the attachment of 4''-*O*-methyl-glycosyl moiety at these sites.

In case of biotransformation performed in cultures of *I. fumosorosea* KCH J2 we isolated two products: 6-methylflavanone 4'-*O*-*β*-D-(4''-*O*-methyl)-glucopyranoside (formed also by *B. bassiana* KCH J1.5) and 2-phenyl-6-methylchromane 4-*O*-*β*-D-(4''-*O*-methyl)-glucopyranoside. We assume that the attachment of the glycosyl moiety at C-4 was preceded by the reduction of the carbonyl group. This strain, in contrast to *B. bassiana* KCH J1.5, was not able to hydroxylate the methyl moiety [4].

All listed biotransformation products have not been described in the literature up till now. These compounds may be used in further studies of their biological activity and bioavailability and investigating their structure-bioactivity relationships.

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P1.19

Unravelling discolouration caused by iron-flavonoid interactions: complexation, oxidation, and network formation

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MAIN CONCLUSION

The current findings provide new insights in the interaction of iron with structurally different flavonoids and the contribution of complexation, oxidation, and network formation to discolouration. We demonstrate that flavonoid structure, in particular the presence of the *ortho*-diphenol group and 2–3 double bond, strongly affected the iron-flavonoid interactions that contribute to the hue and intensity of discolouration.

INTRODUCTION

Iron deficiency is a global health problem, affecting one quarter of the world's population [1]. Iron fortification of food is an effective solution to control iron malnutrition [2]. However, upon fortification of food with iron, its colour and iron bioavailability can be compromised by the reactivity of the iron ion with dietary flavonoids [3]. Hydroxyl and carbonyl groups on the flavan backbone can coordinate iron ions to form stable complexes [4]. Coordinate bond formation between Fe^{3+} and flavonoids can be followed up by a redox reaction, via electron transfer (ET) from the flavonoid to iron [5]. To date, there is a lack of integrated studies investigating the contribution of complexation, and oxidation to discolouration, and the effect of flavonoids' structural features thereon.

MATERIALS & METHODS

In this study, we monitored iron-mediated complexation, oxidation, and subsequent discolouration of ten structurally different flavonoids in aqueous environment (pH 6.5, 40°C) over time using spectrophotometric, mass spectrometric techniques. Additionally, formation of iron-flavonoid networks was investigated by microscopic, and diffraction techniques.

RESULTS & DISCUSSION

All ten investigated flavonoids demonstrated discolouration in presence of iron. Moreover, our results indicated that the electronic transitions responsible for discolouration were strongly affected by the structural features of the flavonoid, most notably by the available iron binding sites. Iron complexation to the 3–4 or 4–5 site resulted in a bathochromic shift of the spectral bands related to $\pi \rightarrow \pi^*$ transitions, whereas binding to the 3′–4′ site resulted in appearance of a LMCT band by $\pi \rightarrow d_\pi$ transitions. In the samples that showed the strongest discolouration, both of these transitions were observed in the corresponding UV-Vis spectra. Subsequent iron-mediated oxidative reactions are enhanced by presence of (i) additional hydroxyl groups on the B-ring, (ii) the 2–3 double bond, and (iii) the 3-hydroxy group. Formation of flavonoid oxidation products was monitored by RP-UHPLC-PDA-MS^n. If the 3-hydroxy group was present in conjugation with the 2–3 double bond (flavonols), degradation was most prominent. Presence of the aforementioned functional groups enables formation of the highly reactive quinone methides. In absence of the 3-hydroxy group only *ortho*-quinones can be formed, these are relatively more stable in aqueous solution, resulting in slower oxidative degradation compared to the quinone methides [6]. Oxidative coupling was more prominent in the flavonoids lacking the 2–3 double bond. Overall, iron-mediated oxidation reactions of flavonoids led to degradation or oxidative coupling of flavonoids, that resulted in a decrease or increase in visible light absorbance, respectively. Additionally, for flavonoids possessing multiple

iron-binding sites, we used XRD, SEM, and TEM to confirm the formation of dark-coloured iron-flavonoid networks that are poorly soluble and amorphous in nature.

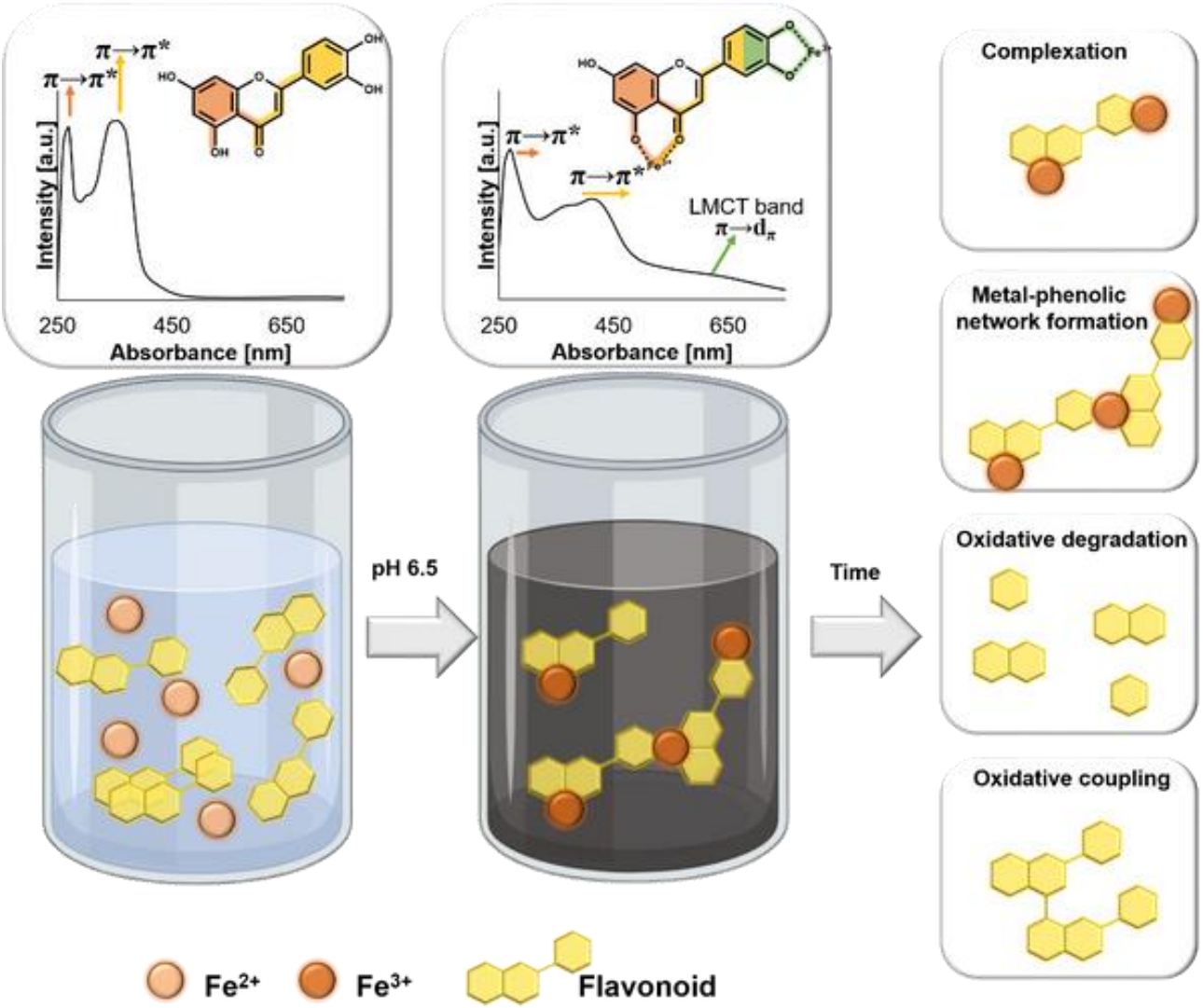

Figure 1. Schematic illustration of the initial discolouration upon iron-flavonoid complexation and the different type of reaction products that can be formed by over time.

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P1.20

Distribution of lignans and lignan mono/di glucosides in freeze-fixed stem of *Ginkgo biloba* L. by cryo-TOF-SIMS/SEM

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MAIN CONCLUSION

The distribution of lignan and lignan mono/di glucosides in freeze-fixed stem of ginkgo was investigated using the cryo-TOF-SIMS/SEM and HPLC analysis. (−)-olivil 4',4''-di-O-β-D-glucopyranoside (**4**) was the most abundant lignan glucosides in ginkgo, which was accumulated in phloem region, pith, and ray parenchyma cells in mature xylem region. These results suggest its possible synthesis sites, transport pathways, and storage sites.

INTRODUCTION

Lignans are poly-phenolic compounds structurally described by 8-8′coupling of two phenylpropanoid units. They have their roles in fight against phytopathogenic organisms, and in the defense of plants against insects [1]. They can be found either in phenolic free form or glucoside form glycosidically linked to a wide variety of different carbohydrates. To discuss biosynthesis and regulation mechanisms of lignans in plants, distributions of lignan and lignan glucosides in quick-frozen *Ginkgo biloba* L. (ginkgo) were visualized by cryo time-of-flight secondary ion mass spectrometry and scanning electron microscopy (cryo-TOF-SIMS/SEM). This is a powerful tool to reveal the distribution of chemicals in frozen-hydrated samples like higher plants [2].

In this study, 7S,8R,8'R-(+)-lariciresinol-4,4'-bis-*O*-*β*-D-glucopyranoside (**2**), *secoisolariciresinol diglucoside(**3**)*, (−)-olivil 4',4''-di-*O*-*β*-D-glucopyranoside (olivil DG, **4**) and (−)-olivil 4''-*O*-glucopyranoside (olivil MG, **4a**) were isolated from ginkgo bark. Lariciresinol (**2b**), *secoisolariciresinol (**3b**) and* (−)-olivil (**4b**) *were obtained* by *β*-glucosidase hydrolysis of the ginkgo bark aqueous extracts. Pinoresinol (**1**) was also detected in this study as confirmed in comparison with purchased one. The quantification of lignan mono/di glucosides were conducted by HPLC measurements, and the ion count distribution of cryo-TOF-SIMS was confirmed with the HPLC result. Further, the possible role of the most abundant lignan diglucoside olivil DG(**4**) in ginkgo is discussed.

MATERIALS & METHODS

The stem bark was obtained from a 30-years-old trunk of ginkgo. The sample disk for cryo-TOF-SIMS/SEM and HPLC experiments was obtained from a 3-year-old flesh shoot of ginkgo and cut into small blocks. After cutting to form a clean and flat surface, the block was transferred to the cryo-TOF-SIMS (TRIFT III spectrometer, ULVAC-PHI). The lignification stage of the cells was confirmed by microscopic observations using UV and polarized right.

Column chromatography was performed on a silica gel and Sephadex LH-20 (Pharmacia) to separate target lignans and lignan glucosides. The amount and rough distribution of lignans and lignan glucosides were confirmed by HPLC using serial tangential sections of the freeze-fixed ginkgo stem.

Water-soluble lignan glucosides and water-insoluble lignans were prepared by KClaq. Then the prepared samples were measured by cryo-TOF-SIMS to obtain their standard spectra in a frozen-hydrated state co-existing with potassium.

RESULTS & DISCUSSION

In this study, the same HPLC conditions were used to simultaneously detect lignan mono/di glucosides and free lignans. The content of lignan mono/di glucosides was obviously higher than free lignans. most of lignan mono/di glucosides distributed in phloem by HPLC. Olivil DG (**4**) was the most abundant lignan glucosides in ginkgo, and significantly higher than the sum of other lignan glucosides.

Cryo-TOF-SIMS images were obtained for the transverse surface of freeze-fixed stem of ginkgo (**Fig. 1**). The block surface contains outer bark, phloem, cambial zone, xylem, and pith regions. Potassium (**Fig. 1b**) was detected mainly in phloem, cambial zone, and pith regions. Coniferin (**Fig. 1c**) as a lignin precursor was mainly detected in the differentiating xylem region next to the cambial zone. Olivil DG (**4**) was detected as an adduct ion $[M+K]^+$ of *m/z* 739 (**Fig. 1e**) and is accumulated in phloem region, pith, and ray parenchyma cells in mature xylem region. On the other hand, the secondary ion of *m/z* 577 (**Fig. 1d**) can be derived from two targets, as a fragment ion of olivil DG $[M\text{-glu}+K]^+$ and an adduct ion of olivil MG $[M+K]^+$. Their distributions were not the same especially in the phloem and the cambial zone. Therefore, it is suggested that olivil DG (**4**) and olivil MG (**4a**) distributed differently in ginkgo stem. It should be mentioned here that TOF-SIMS can visualize only the actual storage of the target.

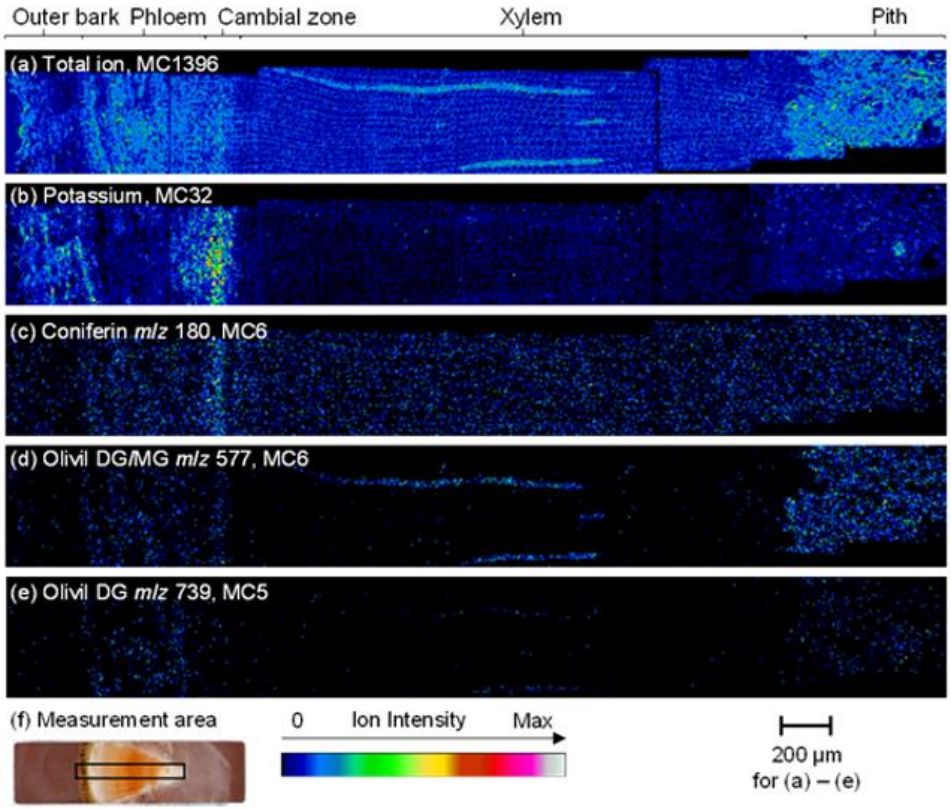

Figure 1. Transverse surface images of freeze-fixed ginkgo stem. Cryo-TOF-SIMS positive ion images of (**a**) total ion, (**b**) K^+ at *m/z* 38.96 , (**c**) coniferin at *m/z* 180, (**d**) Olivil MG $[M+K]^+$ and Olivil DG $[M\text{-glu }+K]^+$:*m/z* 577, and (**e**) Olivil DG $[M+K]^+$:*m/z* 739. (**f**) Optical microscopic image of transverse surface of freeze-fixed ginkgo stem on a cryo-TOF-SIMS sample holder showing the measurement area (ca. 3.5 × 0.4 mm). MC, Maximum ion counts per pixel.

From the view point of the chemical structure, olivil (**4b**) can be transformed to other lignan structures such as ginkool and cycloolivil detected in ginkgo. According to the above results, it's assumed that olivil DG (**4**) might be an important intermediate and storage form in the lignan biosynthesis pathway in ginkgo. Further study is needed to discuss more in detail about the specific roles of each lignan species providing a dynamic response to stem infection, mechanical damage, and other actions in tree physiological systems via multiple derivatization steps from olivil DG (**4**).

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P1.21

Interaction between salivary proteins and cork phenolic compounds able to migrate to wine model solutions

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MAIN CONCLUSION

The extracted compounds within cork fractions (M2 and M3) into wine may contribute to astringency perception.

INTRODUCTION

Cork stoppers are very important since a number of previous sensory studies [1-2] found that consumers link high quality wines to wines bottled with these stoppers instead of alternative stoppers [3]. Although the main function of a closure is to ensure a good seal and to avoid leakage, cork is a semi-permeable material that allows oxygen permeability in small amounts, which is important for a gradual and proper wine aging [1]. On the other hand, cork is not an inert material, allowing the migration of some compounds to the alcoholic matrices, such as phenolic acids, aldehydes, tannins and pectic polysaccharides [4-5]. Among the phenolic compounds present in cork, the ones that usually to extract to alcoholic beverages are phenolic acids (mainly gallic and protocatechuic acids), aldehydes (mainly vanillin and protocatechuic aldehyde), condensed tannins and ellagitannins such as vescalagin and mongolícain [4-5]. Tannins are well-known to interact with salivary protein (SP) leading to the formation of (in)soluble aggregates which are supposed to be at the origin of the astringency sensation. Astringency is an oral sensation involving dryness, tightening, and shrinking of the oral cavity. Among the different families of SP, proline-rich proteins (PRPs) are the ones mainly related to astringency perception [6-7].

Among the different families of SP, the different classes of PRPs have been widely reported to interact with different phenolic compounds and have been related to astringency perception in both *in vitro* and *in vivo* studies. This work aims to study the interaction of human salivary proteins with cork phenolic compounds able to extract into wine model solutions. This will provide more insights about the putative contribution of the migration of these phenolic compounds on taste properties of wine.

MATERIALS & METHODS

Three different phenolic fractions (M1, M2, M3) were obtained from cork after extraction with a wine model solution (12% EtOH; 5.0 g/L Tartaric Acid; pH 3.2) followed by a fractionation using low-pressure chromatography (column: Toyopearl®, 250 × 25 mm i.d.; eluent: methanol, 0.8 mL/min). Each of the fractions were characterised by HPLC-DAD/ESI-MS. The average molecular weight of each fraction was estimated based on the relative abundance of each identified compound. This allowed also to estimate the mean molarity of each fraction.

Human saliva was collected from several healthy non-smoker volunteers (males and females) and kept on ice for further processing according to described in the literature [8].

The interaction between three cork fractions (M1, M2 and M3) and human saliva was studied for four different concentrations of each fraction, 0.25, 0.5, 1.0 and 1.5 mg. mL^{-1}.

RESULTS & DISCUSSION

This study aimed to determine the interaction of three different fractions of phenolic compounds obtained from cork stoppers (M1, M2 and M3) with human salivary proteins. The experimental approach used allows to understand if these compounds are able to complex and precipitate SP, a mechanism that is described to contribute to the perception of astringency in wine. To attain this, the changes in the chromatographic profiles of the SP families and phenolic compounds were studied upon their interaction and after removal of the insoluble precipitates formed by centrifugation.

Fractions M1 and M2 are representative fractions of phenolic compounds that migrate in greater amounts to wine model solutions and include compounds such as gallic acid, protocatechuic acid and protocatechuic aldehyde. Fraction M3 is representative of ellagitannins (vescalagin and castalagin) present in cork that have already been identified in wines and reported to contribute to wine astringency.

Overall, the results of this study suggest that cork fraction M3 is the most reactive toward SP comparing to the other fractions, followed by fraction M2 and by fraction M1. Within M3, castalagin was the compound that most interacted with SP, mainly aPRPs and P-B peptide. Within fraction M2, 4-dehydrocastalagin was the most precipitated compound by all families of SP. In fraction M1, caffeic and sinapic acids were the compounds with the highest binding to SP, mainly cystatins. Within the referred fractions, castalagin, 4-dehydrocastalagin, caffeic and ellagic acids were almost always precipitated above their reported astringency thresholds, so they may contribute to astringency perception. In addition, there seems to be a matrix effect (due to the presence of other compounds) that could affect the binding of a specific compound. Here, this was observed for castalagin that was bound in higher amounts to SP in presence of other phenolic compounds.

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P1.23

Formation of dehydrohexahydroxydiphenoyl esters by oxidative coupling of galloyl esters involved in ellagitannin biosynthesis

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MAIN CONCLUSION

The hydrated form of the dehydrohexahydroxydiphenoyl (DHHDP) group was firstly synthesized by oxidative coupling of galloyl groups, and the subsequent reduction afforded hexahydroxydiphenoyl (HHDP) group. The results strongly suggested that the DHHDP group is the initial product of the oxidative coupling of two galloyl groups, and subsequent reductive metabolism affords HHDP esters in ellagitannin biosynthesis.

INTRODUCTION

HHDP and DHHDP groups are representative acyl groups characteristic of ellagitannins. Many ellagitannins with complicated structures have been isolated from natural sources, and their various biological activities have also been reported. Previously, it was proposed that HHDP group was biosynthesized by simple oxidative coupling of two galloyl esters, and that further oxidation afforded DHHDP group (Fig. 1, full line). However, recent studies indicated that the DHHDP group undergoes redox disproportionation to afford HHDP group in several plants (Fig. 1, broken line).[1] In this study, oxidation mechanism of galloyl ester derivatives was investigated in detail to reveal the biosynthetic mechanism of HHDP groups.

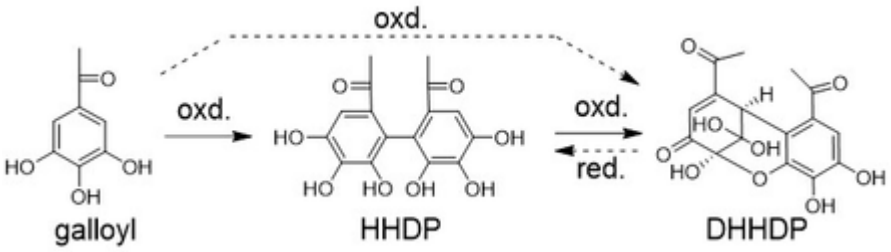

Figure 1. Hypothesis of ellagitannin biosynthesis. oxd.: oxidation, red.: reduction

MATERIALS & METHODS

Chebulinic acid was prepared from *Terminalia arborea*. $CuCl_2$ was added to a CH_3CN-H_2O (3:7) solution of chebulinic acid. After stirring for 2 h at room temperature, dithiothreitol was added to the reaction mixture. After stirring for another 40 min at 85 °C, the reaction products was separated using column chromatography such as Diaion HP20SS and Sephadex LH-20. Reaction products of other galloyl ester derivatives were also investigated in a similar manner.

RESULTS & DISCUSSION

Oxidation of methyl gallate (**1**) with $CuCl_2$ in aqueous solution afforded a DHHDP-type dimer (**2**) by intermolecular coupling. The structure was confirmed by conversion to a phenazine derivative (**4**), as well as NMR analysis of the reaction mixture. In addition, **2** underwent redox disproportionation to give an HHDP-type dimer (**3**) during chromatographic separation. In contrast, oxidation of **3** did not afford **2**. (Fig. 2). Furthermore, oxidation of 1,4-butanediol digallate (**5**) with $CuCl_2$ afforded a product (**6**) with a DHHDP group via intramolecular coupling, and the structure was also confirmed by derivatization and NMR analysis (Fig. 3).[2]

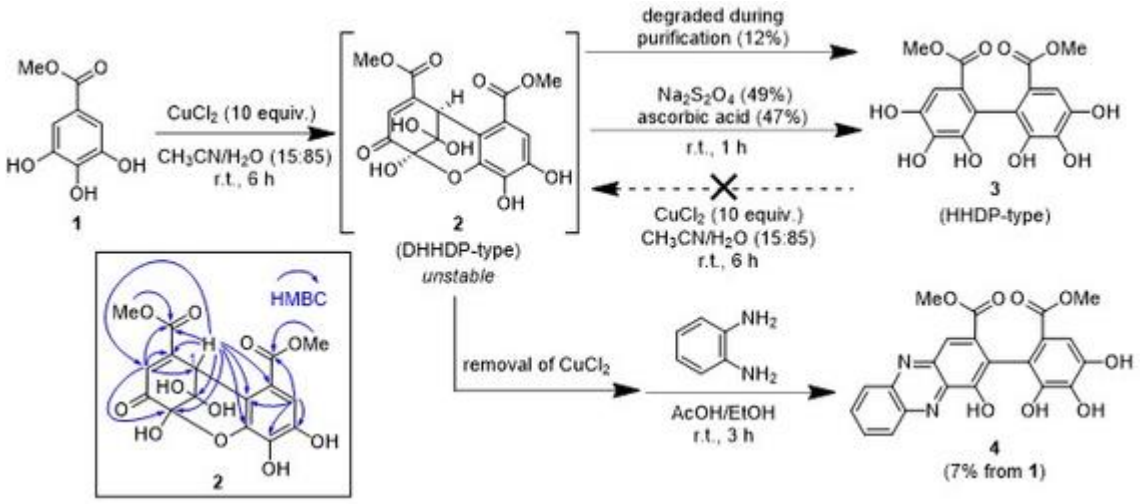

Figure 2. Oxidation of methyl gallate (**1**) with $CuCl_2$ in an aqueous medium.

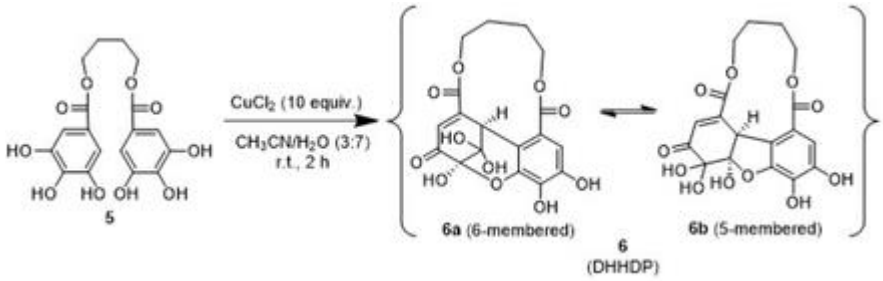

Figure 3. Oxidation of 1,4-butanediol digallate (**5**) with $CuCl_2$ in an aqueous medium.

Chebulinic acid (**7**), an ellagitannin with 1,3,6-trigalloyl-2,4-chebuloyl esters of $^1C_4$ glucose, was oxidized with $CuCl_2$ to afford products bearing a 3,6-(*R*)-DHHDP (**8**) and 1,6-(*S*)-DHHDP (**9**) esters (Fig. 4). Furthermore, reduction of the reaction mixture with dithiothreitol afforded chebulagic acid (**10**) with a 3,6-(*R*)-HHDP ester, along with a 1,6-(*S*)-HHDP isomer (**11**). These results suggested that **10** is oxidatively biosynthesized from **7** via a DHHDP intermediate **8**.

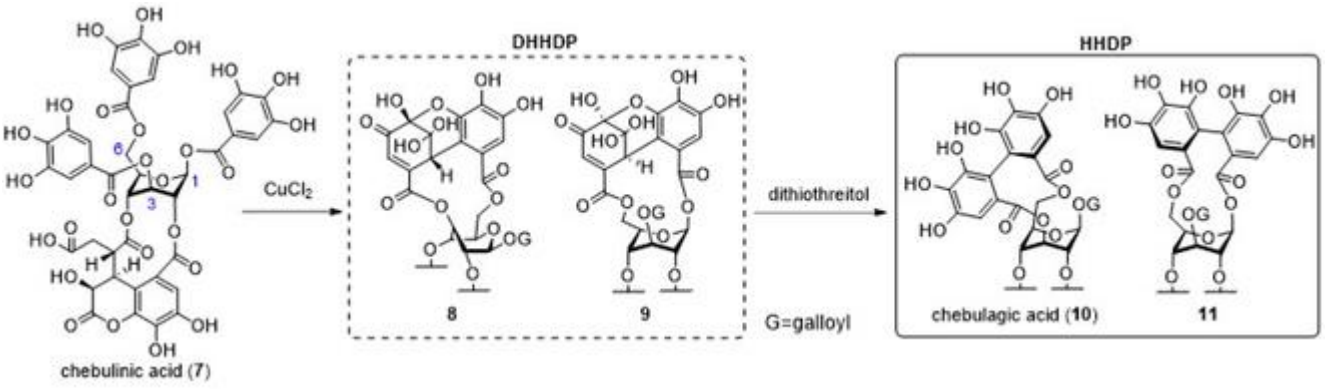

Figure 4. Oxidation products of chebulinic acid (**7**) with $CuCl_2$ (**8**, **9**) and their reduction products (**10**, **11**).

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P1.24

Photochemical cyclization of stilbenes isolated from Norway spruce root bark

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MAIN CONCLUSION

This study is focused on the natural compounds extracted from the bark of Norway spruce roots. Stilbenes were extracted and the photochemical stabilities of them were studied. It showed that stilbenes are unstable under fluorescent and UV light. The trans-stilbenes rapidly isomerized to cis-stilbenes upon UV irradiation. Under extended exposure cis-stilbenes cyclized to phenanthrene structures. This is the first time that phenanthrene derivatives are found from stilbenes of Norway spruce.

INTRODUCTION

Polyphenolic stilbenes compounds exist in the bark of Norway spruce, and provide protection against ultraviolet(UV)light and fungal pathogens or other microorganisms. Stilbenes have potential antioxidative properties against pathogens. Thus stilbenes as secondary metabolites are important natural compounds in the tree defense mechanism[1].

Stilbenes may exist as the trans and cis stereoisomeric forms, but due to the higher thermodynamical stability of the trans isomers, they exist naturally as trans isomers. Välimaa[2] recently reported that stilbenes from spruce bark showed increased radical scavenging activity after UVA-modification. This indicates that the stilbenes have potential biological properties. However there is still some lack of information on the structure of certain stilbene compounds which are formed after photoisomerization. Resveratrol as one of the derivatives photoisomerize. The trans-resveratrol is fully photoisomerized to cis-resveratrol at UV light 366nm. This result was also obtained by Montsko et al [3] and Francioso et al [4] when analyzing the structure of the photoisomerization compound. Both groups suggested possible cyclisation reaction based on the UV and MS spectra data results. However the characterization of ultimate compounds after UV irradiation still need to be done by using some other analytical methods like NMR. This could be more meaningful in future studies because stilbenes showed potential biological properties against fungal pathogens or microorganisms under UV light conditions.

In this study, we mainly focused on other stilbenes derivatives namely astringin and isorhapontin and their aglucones piceatannol and isorhapontigenin. We isolated stilbenes and did stability studies. We characterized the new compounds by spectroscopic techniques(UV, MS, NMR). This article is the first report in detail about astringin, isorhapontin, piceatannol and isorhapontigenen which are extracted Norway spruce bark.

MATERIALS & METHODS

1.Bark materials: Norway spruce root samples from Finnish forest.

2.Extraction of bark material

Stilbenes were sequentially extracted of the powdered bark. Hydrophobic constituents were removed with hexane and hydrophilic constituents were then collected using Accelerated Solvent Extraction. The ASE sequential extractions were carried by n-hexane and ethanol: water(95:5 V/V) . The crude extracts were purified using an XAD-7HP polymer resin filled column.

3.HPLC-DAD analyses

The HPLC. A Model 1100 series liquid chromatography system with a quaternary pump, degasser, autosampler, and column oven was used for separation of extracted stilbenes. Agilent 1100 series diode array detector coupled to Hewlett-Packard Chem Station was used for analysis.

4. UV stability study

An UV lamp with 366 nm wavelength (UV-A radiation) were used.

5. Structural elucidation

High resolution mass spectrometry ESI-TOF-MS in the negative ion mode and 500MHz and 300MHz NMR spectrometry were applied.

RESULTS & DISCUSSION

1.Stability of stilbenes:

1.1 The stability of stilbenes in solution: Stilbene derivatives were dissolved in methanol, and the solutions were monitored for 2 weeks under light protected and light unprotected conditions. In these experiments trans-piceid, trans-resveratrol and trans-astringin were unstable under light unprotected conditions. Whereas trans-isorhapontigenin and trans-isorhapontin were stable under same conditions. This might be due to the methoxy substituent in the isorhapontin structure and its aglucone being sterically hindered from isomerization.

1.2 Stability assessment of stilbenes in solid crude extract: according to our experiments, the stilbenes in the solid crude extract are stable under light unprotected conditions. This experimental result is in agreement with the other studies.

1.3 UV stability of stilbenes: stilbenes solutions were highly sensitive to UV irradiation. Trans-stilbenes were isomerized to cis-stilbenes after 10–30 min of UV exposure. With extended exposure time, cis-stilbenes started to form other new compounds. According to the GC-MS analysis the new compounds had lost 2 Daltons from the trans- or cis-stilbenes.

2. Structural elucidation of astringin and isorhapontin:

1H-NMR and 2D NMR spectroscopy clearly showed the absence of alkene H and 13C signals found in the trans- and cis-stilbenes. Instead the new signals appeared in the aromatic region and showed couplings characteristics of aromatic protons. According to our further analysis, we finally confirmed that trans-astringin and trans-isorhapontin firstly undergo photoisomerization to cis-astringin and cis-isorhapontin and are then cyclized to phenanthrene molecules by the loss of two hydrogens. Finally this study confirmed that the new compounds that had lost two Dalton mass units are phenanthrene molecules. This is the first time photochemical transformations of trans-astringin and trans-isorhapontin of Norway spruce are reported.

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P1.25

Unveiling the iron-tannin complexes behind medieval iron gall inks

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MAIN CONCLUSION

UV-Vis spectroscopy assays show that pentagalloylglucose (PGG)–Fe complex has higher binding constant compared with gallic acid–Fe complex.

Preliminary MALDI-TOF mass spectrometry studies show high intensity signals following the iron characteristic isotopic pattern.

INTRODUCTION

Until the beginning of the 20th century, iron gall inks were commonly used for writing or drawing. Hand-written documents, manuscripts, music scores and painting sketches form a fundamental part of our cultural heritage and were created using iron gall inks [1]. Unfortunately, many historical documents are at risk of total loss due to degradation of the writing support caused by the corrosion of these inks [2].

Medieval iron gall ink recipes typically contain three basic ingredients: a phenolic extract (*Quercus infectoria* galls), $FeSO_4$, and gum arabic (GA) [3]. The addition of Fe to a phenolic extract leads to the formation of a complex-aggregate overtime. GA helps to maintain these aggregates in suspension, preventing it from precipitate to fast.

Our knowledge on the molecular structures of the chemical compounds present in these inks is very limited and this gap prevents us to devise informed strategies for preserving the world written heritage.

It was believed that gallic acid was the main chemical compound found in the gall extract and in the iron gall inks produced. However, a recently published work shows that two gallotannins: pentagalloylglucose and hexagalloylglucose, are the main compounds of 4 of the 5 Iberian recipes reproduced [3].

The goal of this work is to characterize iron-tannin complexes present in iron gall inks. This will be accomplished through UV-Vis spectroscopy [4] studies on isolated tannins from galls by Preparative HPLC-DAD. The iron-complexes were prepared using $FeSO_4$ and GA in distilled water.

MALDI mass spectrometry was used to study the nature of iron complexes, collected as precipitates of acid gallic-Fe and PGG-Fe prepared according to Ponce et al, 2016 [5].

MATERIALS & METHODS

Gallic acid and $FeSO_4.7H_2O$ were purchased from Sigma-Aldrich, Germany. Gallnuts (*Quercus infectoria*) and gum arabic (*A. Senegal*) were purchased from Kremer Pigmente, Germany. Formic acid, acetic acid and acetonitrile (ACN) were obtained from ChemLab, Belgium.

A gall extract was prepared by dissolving grounded (stone mortar) galls in distilled water and stirring for 30 min at room temperature. The extract was centrifuged at 12000 rpm for 15 min and freeze-dried.

Gallotannins were isolated using low chromatography with Toyopearl HW-40(S) and Thermo Preparative HPLC-DAD [3].

UV-Vis assays were performed using a Thermo UV-Vis spectrophotometer and PMMA 2.5mL cells.

MALDI-TOF-MS assays were performed in a Bruker ultrafleXtreme mass spectrometer in the range m/z 140–1500 with approximately 1500 laser shots. The samples were mixed (1:1) with three different matrices. CID tandem mass spectra (TOF/TOF-MS) were acquired with argon as collision gas.

RESULTS & DISCUSSION

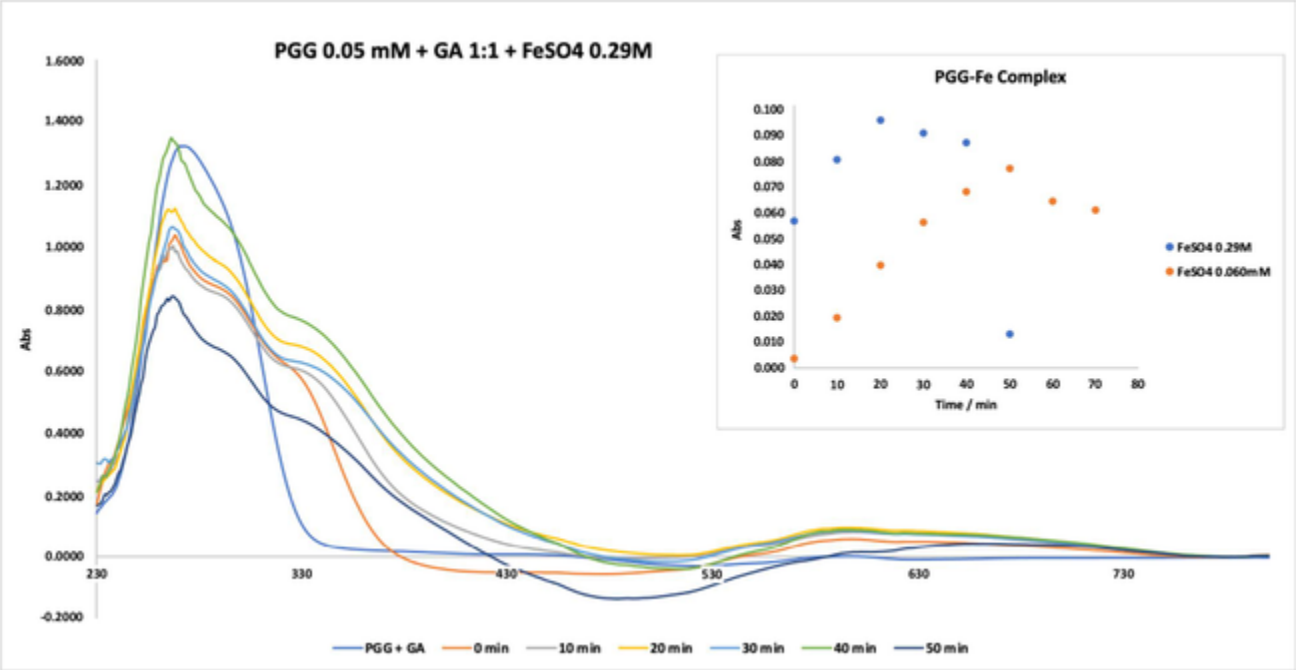

Figure 1. Kinetic absorbance spectrum of PGG-Fe complex. Inset: maximum wavelength kinetic for $FeSO_4$ 0.29M and 0.060mM.

The complexation of Fe (II) with gallic acid and gallotannins isolated from gallnuts was studied by UV-Vis spectrophotometry. Several assays were carried out to reach optimal conditions of concentration and reaction time.

As expected, the increase of $FeSO_4$ concentration leads to faster reactions. However, it also leads to turbidity, despite the addition of GA. As it can be observed in Figure 1, the addition of 0.29M $FeSO_4$ to a PGG 0.05mM + GA 1:1 (w/w), leads to the formation of two absorption bands around 300 and 338 nm. Figure 1 inset shows that the complex PGG-Fe reaches its maximum in 20 minutes for $FeSO_4$ 0.29M, following a decrease of absorbance indicating precipitation; while for $FeSO_4$ 0.060mM, the maximum is reached after 50 minutes, and no yellow absorbance bands are reported.

The results showed binding constants of 265.52 and 3969.50 M^{-1} for gallic acid and PGG, respectively [4].

MALDI-TOF-MS assays were performed with three different matrices, all showing good signal intensities: α-cyano-4-hydroxy-cinnamic acid (CHCA) in 50% ACN, 2,5-dihydroxybenzoic acid (DHB) in 70% ACN and trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) in 50% MeOH; in four different preparations: 1- 0.1% TFA, 2- no added medium, 3- 0.1% NH_3, 4- 1% NH_3. Fe-complex samples were suspended in water and in MeOH, and analyzed both in positive and in negative mode. Preliminary studies show the iron characteristic isotopic pattern in several signals detected. Further studies will be conducted for accurate complex identification.

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P2.1

Production of urolithins from ellagic acid using human intestinal bacteria and activation of sirtuin-related genes by urolithins

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MAIN CONCLUSION

Urolithin A (UA) and Urolithin B (UB) are a major microbiota-derived metabolite in humans that is produced from ellagitannins and ellagic acid. We found three kinds of intestinal bacteria which has a different dehydroxylation activity for urolithins. Then, we succeeded to produce UA or UB from ellagic acid (EA) by each co-culture system using these three bacteria. Furthermore, we have found that urolithins (URO) augmented sirtuin-related gene expression.

INTRODUCTION

Pomegranate is a well-known fruit with many health benefits such as anti-aging action. It contains ellagitannins and their hydrolysis product, EA and they were reported to have various biological activities including antioxidant, antiviral and antitumor activities.

Seven URO were previously identified as urinary and intestinal microbial metabolites in rats after oral administration of geraniin.[1)] Recently, novel URO derivatives was identified in human feces and urine after the intake of a pomegranate extract.[2)] Moreover, three kinds of URO metabotypes had been described for human according to the ability of producing each metabolites. UA (3,8-dihydroxy-6H-dibenzo[b,d]pyran-6-one) and UB (3-hydroxy-6H-dibenzo[b,d]pyran-6-one) are reported as one of major metabolites produced by gut-microbiota after consumption of pomegranate, ellagitannin, or EA. UA was reported that showed a lot of beneficial biological activities for human health, such as anti-inflammatory effects , skin anti aging effect, and mitophagy-inducing effect.[3)] Likewise, UB also has the biological activities such as anti-inflammatory, prevention of atherosclerosis and antitumor. We tried to isolate URO-producing bacteria from human feces and then we had already reported about identification of UA producing bacteria and production of UA from EA *in vitro*. In the recent research we identified novel bacteria which is responsible for producing UB. We also established a method of screening a functional food which augments sirtuin-related gene expression[4)]. Here we would like to report the results of the investigation about URO and its derivatives.

MATERIALS & METHODS

A diet intervention trial with thirty healthy men and women was undertaken to study about URO metabolism from EA. The fecal samples of URO producers, the participants who detected URO in there urine, were obtained and used for the isolation of URO producing strain

For the screening of UB producing strain, all cultivation experiments were performed in ABB medium (oxoid) with 1 g/L of UC using Hungate tubes. Cultures were carried out under an anaerobic condition with a gas phase of $N_2/H_2/CO_2$ (80/10/10) at 37ºC. After cultivation, the amounts of URO in the culture were determined by HPLC.

The fermentation test of URO was investivated by co-cultivation with three kinds of bacteria. Cultivation experiments were performed in the same manner as above except that EA is used as a starting material.

In the screening for augmentation of sirtuin-related gene expression, we investigated the activities which augments EGFP expression under the control of SIRT1 promoter.

RESULTS & DISCUSSION

The metabolites of EA by human feces were analyzed after cultivating in ABB medium. There are several URO derivatives depend on the number and position of hydroxy group. These were detected in the medium and a major metabolite was UA. We tried to isolate the bacteria which has dehydroxylation activities for URO. As a result, a gut bacterium which has a novel dehydroxylation activity were isolated when UC was used as a substrate. 16S rRNA gene sequence analysis showed to be most closely related to *Slackia heliotrinireducens* (SH). This bacteria were able to convert UC to isourolithin A (IUA) by pure culture but not to produce UA. Furtherly substrate specificity of dehydroxylation was evaluated by co-cultivation with another two kinds of bacteria having different dehydroxylation activity (*Gordonibacter pamelaeae* (GP) and *Clostridium bolteae* (CB)). GP and CB is responsible for producing UC from EA and UA from UC, respectively. CB is also able to convert IUA into UB. Among them, IUA was produced from EA when SH and GP was co-cultured and UB was produced from UC when SH and CB was co-cultured. These results showed that SH specifically catalyzed the dehydroxylation at 8-carbon position of URO. A co-culture system was investigated for production of UB from EA in one pot. Consequently, UB was produced in a cultured broth by co-cultivation of the above three kinds of gut bacteria when EA was used as a starting material. This study provides an industrial production method of UB and would expand the possibility for an production process of gut metabolites.

We also evaluated the effect for sirtuin-related gene expression. Sirtuin is mammalian homologs of the yeast *Sir2* gene and has anti-aging activity relating to DNA repairs and anti-inflammatory. Here we have found that URO augmented the expression of anti-aging genes including SIRT1 and SIRT3. In consequence, urolithins is promising candidate of a novel food ingredient with the anti-aging effects.

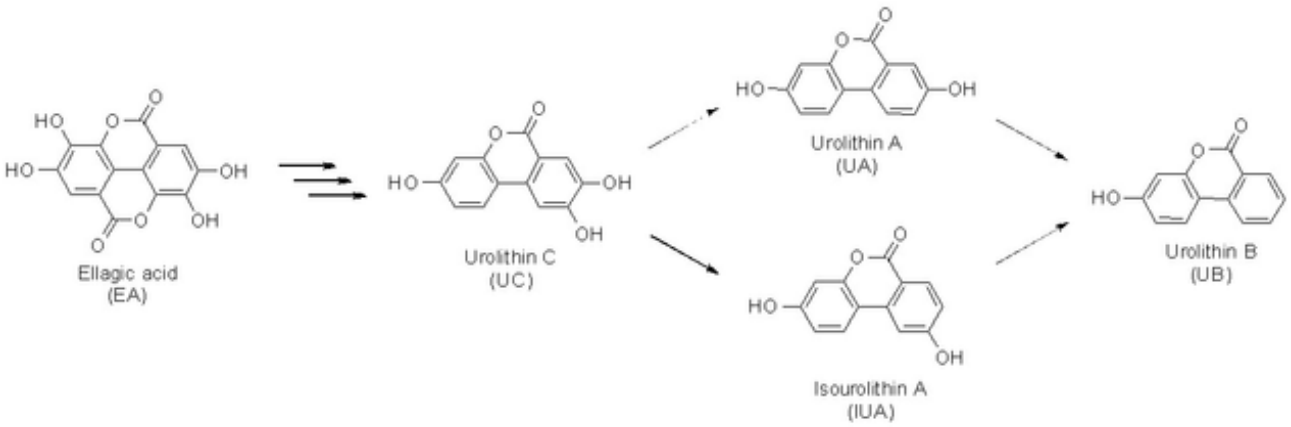

Figure 1 Biosynthetic pathway for UA and UB production by a co-culture system

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P2.2

Quantification of trans-ε-viniferin and its glucuro-conjugated metabolites in rat plasma after oral administration

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MAIN CONCLUSION

In vivo pharmacokinetic study was performed on rats that received orally 20 mg/kg bw of ε-viniferin, and glucuronides metabolites were specifically quantified. Results of plasma quantification showed very high glucuronidation of the native form with a maximum concentration of about 2 nmol/mL against 3 pmol/mL for the native form, highlighting an extensive metabolism of this stilbene.

INTRODUCTION

ε-Viniferin is a resveratrol dimer found in food, mainly in red wine (0.2 to 4.3 mg/mL). Several studies have revealed interesting biological activities of this phytoalexin, including antioxidant, anti-inflammatory and anti-obesity activities. For instance, ε-viniferin decreased lipid accumulation and expression of genes involved in adipogenesis according to *in vitro* studies on adipocytes cell line. Moreover, *in vivo* ε-viniferin administration decreased body weight of mice fed with high-fat diet.

While this stilbene showed interesting properties *in vivo*, only a few studies aimed at determining its pharmacokinetic parameters have been published. Among them, Kim *et al.*, (2017) calculated a bioavailability of 0.771% after oral administration in mice [1]. This poor bioavailability could be explained by low intestinal absorption of ε-viniferin and high metabolism. Indeed, Courtois *et al.*, (2018) highlighted high hepatic metabolism especially onto glucuronides forms after intra-peritoneal administration on rats. In this study, they quantified ε-viniferin-glucuronides in equivalent of the native form [2].

The specific quantification of metabolites requires having pure compounds as standards. Several techniques can be used to produce glucuronides derivatives, such as hemisynthesis. Courtois *et al.*, (2017) [3] developed a protocol to (i) produce the four regioisomers of ε-viniferin-glucuronide by hemisynthesis and (ii) separated them by chromatographic techniques. The purification of ε-viniferin-glucuronides, and more precisely the predominant form present in rat plasm is an essential step to allow the specific quantification of this molecule.

This study aimed to determine the pharmacokinetic parameters of ε-viniferin and its main glucuronide metabolite in rat plasma after oral administration (20 mg/kg bw). The obtain pure molecules allowed us to validate the quantification method in plasma matrix and specifically quantify ε-viniferin-glucuronide in rat plasma at different times.

MATERIALS & METHODS

Hemisynthesis: ε-Viniferin-glucuronides were produced by hemisynthesis with acetobromo-glucuronic acid methyl-ester and carbonate potassium. After optimizing the production yield, the 4 regioisomers were separated by semi-preparative HPLC and purities have been obtained by UPLC-DAD-MS.

Method validation: the plasma extraction and quantification protocols were validated with blank plasma from rat which did not receive ε-viniferin. For both native and glucuronide forms, method validation was done in terms of limits of quantification, selectivity, carry-over, accuracy, intra-day precision, matrix effect, and extraction recovery. The quantification was performed using a HR-LCMS.

***In vivo* study**: five Wistar rats have received 20 mg/kg bw of ε-viniferin by gavage. Blood samples were collected from the tail vein at different times and analyzed with the optimized and validated protocol of extraction and quantification.

RESULTS & DISCUSSION

The optimization of the yield of the glucuronidation reaction and the optimization of the purification protocol by semi-preparative-HPLC allowed us to obtain the 4 regioisomers of ε-viniferin-glucuronide. The ε-viniferin-glucuronide MG2 [3] was used for method validation since it is the most predominant in rats (Courtois et al., 2017 and 2018). After optimizing the extraction method, the quantification protocol was validated. The limits of quantification were 0.5 and 25 ng/mL for ε-viniferin and ε-viniferin-glucuronide, respectively. The use of blank plasma to establish the calibration curves allowed us to free ourselves from the matrix effect measured for ε-viniferin-glucuronide.

The extraction and quantification protocol was applied to the pharmacokinetic study of ε-viniferin. After administration of the native form (20 mg/kg bw) to Wistar rats by gavage, the plasmas collected at different times were analyzed. The native form reached a maximum concentration at one hour after administration at 28.7 pmol/mL. For ε-viniferin-glucuronide, two peaks of concentration were visible, at 20 min and 100 min after administration, with concentrations around 1 and 2 nmol/mL.

To our knowledge, this is the first time that the glucuronide forms of ε-viniferin have been specifically quantified in plasma after oral administration of the native form. Besides, results indicated strong glucuronidation. Comparing with intra-peritoneal administration [2] the ratio ε-viniferin-glucuronide / ε-viniferin was higher after oral administration, suggesting the presence of significant intestinal metabolism, in addition to hepatic metabolism. Finally, although this strong glucuronidation was partly responsible for the very low bioavailability of this stilbene, the study of the biological activities of the glucuronide forms could be useful to understand the biological properties of the native form determined *in vivo*.

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P2.3

Capability of mannoproteins isolated from *Saccharomyces cerevisiae* to interact with wine polyphenolic compounds

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MAIN CONCLUSION

Interactions between Mannoproteins (MPs) obtained from *Saccharomyces cerevisiae* using an enzymatic procedure and a flavanol extract obtained from grape seeds were performed at pH 3 and pH 4. Results show the preferential interaction of low MW mannoproteins (*circa* 85 kDa) with galloylated flavanols at pH 3 and an enhancement of the flavanol-MPs interactions, observed in a larger MW range (85-740 kDa), at pH 4.

INTRODUCTION

Global climate change is exerting an important influence on the grape composition. As a consequence, some sensory properties of wine are being altered, among them, the wine pH, which is being progressively increased and that implies an important challenge for winemaking. Some studies have reported that the addition of mannoproteins may have positive effects on colour stability and astringency of wine due to their capability to interact with wine polyphenols [1]; but the effect of pH on these interactions is still unclear.

Mannoproteins (MPs) are glycoproteins from the cell wall of oenological yeasts such as *Saccharomyces cerevisiae*, composed of one major glycosidic moiety (50−95%) covalently linked to one minor protein moiety. They are naturally released into the wine during alcoholic fermentation when yeast is actively growing or during aging when cell wall breaks down in the process known as autolysis. Also, commercial mannoproteins can be added during winemaking and/or ageing. Different procedures for MP extraction from yeast cell walls have been studied, including chemical, enzymatic, and physical methods. Extraction with enzymes containing β-1,3-glucanase activity is one of the most successful practices to obtain MPs for commercial purposes.

On the other hand, flavan-3-ols are a wide family of polyphenolic compounds present in red wines as monomers, oligomers or polymers of four main flavan-3-ols, namely catechin, epicatechin, gallocatechin and epigallocatechin. Some studies have been described the interaction between mannoproteins and flavanols; but, as far as we know, no studies on pH effect in the interaction has been conducted so far. The aim of this study was to isolate and purify MPs directly from the cell wall of *Saccharomyces cerevisiae*, characterize them and assess the effect of pH in the interaction with wine flavanols.

MATERIALS & METHODS

Saccharomyces cerevisiae was provided by Lallemand BIO S.L. The strain was growth in an adequate medium and then Zymoliase 20T was used to hydrolyze the cell wall. MPs were obtained after purification using ethanol, temperature (70 ºC) and dialysis (6–8 KDa MW cut-off). MPs were analyzed by SDS-PAGE and HRSEC-RID to determine the MW, following the method described by Manjón *et al.* [2]. The flavanol extract was obtained from *Vitis vinifera* L. Tempranillo seeds and its composition was determined by HPLC–DAD–MS following the methodology described by García-Estévez *et al.* [3]. The interaction between the flavanol extract and the MPs was performed at pH=3 and pH=4, in a ratio 1:15. After interaction, the samples were centrifuged and the supernatant were

analyzed by HRSEC-RID and HPLC-DAD-MS to determine MPs and flavanol compositions, respectively. All analyses were performed in triplicate.

RESULTS & DISCUSSION

The extract of mannoproteins was analyzed by SDS-PAGE and HRSEC-RID and the flavanol extract by HPLC-DAD-MS. Three main mannoprotein fractions were identified in the extract: F1 (3%) with a MW of 738,8 kDa; F2 (15%) with a MW of 182,4 kDa and F3 (67%) with a MW of 84,8 kDa. The flavanol extract is composed by monomers to pentamers both galloylated and non-galloylated. After interaction, a decrease of mannoproteins was observed at both pHs values, although some differences could be observed. The main decrease at pH 3 was observed for the mannoprotein fraction F3; while at pH4 all mannoprotein fractions decreased in a similar range. Regarding flavanols, we observed an important decrease of galloylated flavanols at pH3 and in general a global decrease of all families of flavanols at pH 4.

These results point to a preferential interaction of F3 mannoprotein fraction with galloylated flavanols at pH 3 and an enhancement of the interactions between flavanols and mannoproteins at higher pH values.

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P2.4

Curcumin conjugates are incompletely hydrolyzed by β-glucuronidase: Detection of complex conjugates in plasma

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MAIN CONCLUSION

b-Glucuronidase incompletely hydrolyzes complex sulfate-containing conjugates that appear to be major metabolites, resulting in an underestimation of the total plasma concentration of curcumin.

INTRODUCTION

Quantification of plasma concentration of polyphenols and their metabolites is required to evaluate the overall exposure and bioavailability, and to determine whether plasma concentrations are consistent with those required for activity in bioassays performed in vitro and in animal models [1]. The diphenol curcumin from turmeric is rapidly metabolized into phase II conjugates following oral administration, resulting in negligible plasma concentration of the free compound [2]. To simplify analysis, total plasma concentration of curcumin is often quantified after treatment with b-glucuronidase to hydrolyze curcumin-glucuronide, the most abundant conjugate in vivo [3]. Here we report the discrepancy in the amount of curcumin released from plasma samples treated with *Helix pomatia*-derived b-glucuronidase versus sulfatase enzyme preparations.

MATERIALS & METHODS

Human study: Six healthy volunteers received 1 serving (2 capsules, 180 mg curcumin) of Theracurmin. Blood was collected 2 h after administration of the supplement. Animal studies: One C57BL/6J female mouse was treated with a 1:1-mixture of d_0/d_6-curcumin (100 mg/kg) in DMSO by intraperitoneal (i.p.) injection. Blood was collected by cardiac puncture 30 min after treatment. Male C57BL/6J mice were administered curcuminoids in DMSO by i.p. injection (100 mg/kg) or by oral gavage (500 mg/kg). Blood was harvested by cardiac puncture after 20 min for i.p. injection or after 30 min for gavage. The efficiency of b-glucuronidase and sulfatase from *Helix pomatia* to hydrolyze curcumin conjugates was compared and metabolites from biological samples were analyzed by LC-MS.

RESULTS & DISCUSSION

Both b-glucuronidase and sulfatase completely hydrolyzed curcumin-glucuronide (Fig. 1-B, E). Unexpectedly, b-glucuronidase hydrolysis was incomplete, affording a large amount of curcumin-sulfate whereas sulfatase hydrolyzed both glucuronide and sulfate conjugates (Fig. 1-B, C, E, F). With sulfatase, the concentration of free curcumin was doubled in human and increased in mouse plasma compared to b-glucuronidase treatment (Fig. 1-A, D). Incomplete hydrolysis by b-glucuronidase suggested the presence of mixed glucuronide-sulfate conjugates.

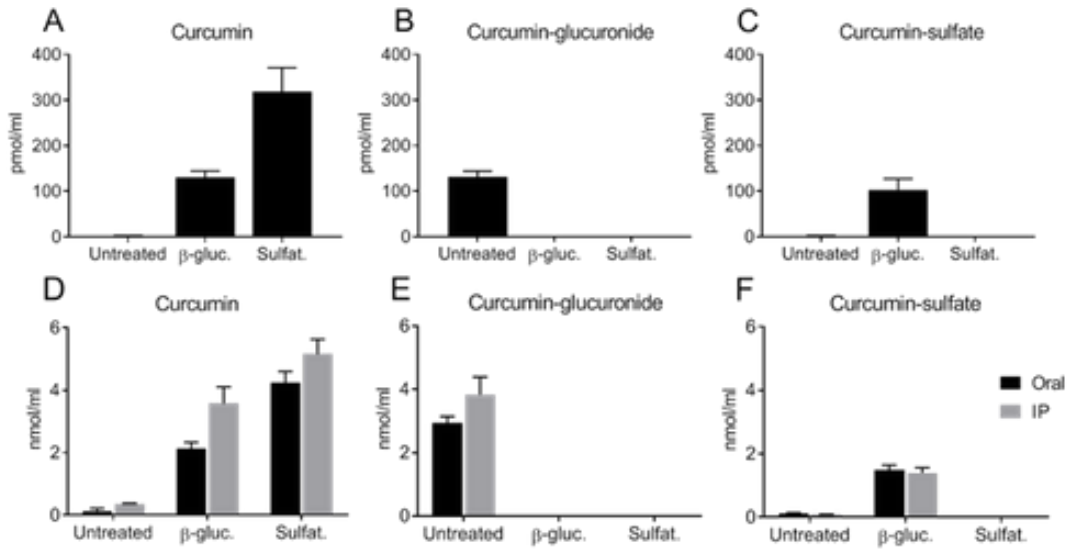

Figure 1. Plasma or serum concentration of curcumin (A, D), curcumin-glucuronide (B, E), and curcumin-sulfate (C, F) before (untreated) and after hydrolysis with b-glucuronidase (b-gluc.) or sulfatase (Sulfat.). Turmeric was administered to human volunteers (A–C), and plasma was obtained after 2 h. Mice (D–F) received turmeric extract by oral gavage or i.p. injection and serum was obtained after 30 or 20 min, respectively. Error bars indicate SEM.

The isotope-based approach facilitated detection of additional novel curcumin metabolites, namely curcumin-disulfate, curcumin-sulfate-glucuronide, and curcumin-sulfate-diglucuronide conjugates in mouse plasma. All conjugates showed the M/M+6 isotopic pattern, and peak abundances were markedly reduced upon hydrolysis. Hydrolysis of the higher-order sulfate mixed conjugates likely accounts for additional curcumin released by treatment of plasma with sulfatase compared to b-glucuronidase.

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P2.5

Polyphenols from Colombian *Passiflora ligularis* Juss (granadilla) inhibit, *in vitro* and *in vivo*, inflammatory agents

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MAIN CONCLUSION

Colombian *P. ligularis* (granadilla) polyphenol extracts have the ability to prevent loss of barrier function in Caco-2 cells, inhibit inflammatory action, and reduce weight gain in rats. When granadilla extracts were supplemented in the diet of Wistar rats food consumption remained the same, but extracts reduced weight gain. Results from this study illustrate a promising mode of inhibition of high-calorie induce weight gain and inflammation.

INTRODUCTION

Several non-communicable diseases are associated with chronic inflammation [1]. Chronic inflammation is a causally-linked to pathological states like obesity and metabolic syndrome and is a public health problem worldwide [2]. Identification of dietary approaches to reduce chronic inflammation is urgent in order to ameliorate inflammatory complications and to reduce disease risk [2]. Chronic inflammation affects the intestinal barrier and inflammation-induced barrier dysfunction is associated with obesity, inflammatory bowel disease, cardiovascular disease, and colon cancer [3]. The relationship between overweight, obesity and inflammation is due to the endocrine function of adipose tissue [4]. In this stage, the tissue increases the size and is infiltrated by macrophages secreting pro-inflammatory cytokines like interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α) giving rise to low-grade chronic inflammation [4].

Fruit polyphenols are a potential anti-inflammatory dietary alternative [3,4]. Polyphenols have shown to inhibit chronic intestinal inflammation in mice [4]. Colombian fruits could be beneficial as nutritional sources of polyphenol-rich foods. Therefore it is essential to evaluate polyphenols in Colombian fruits aiming for the long-term health of multiple communities.

MATERIALS & METHODS

The present work studied *in vitro* and *in vitro* biological activity of polyphenols from Colombian *Passiflora ligularis* Juss (granadilla). The previous was based on the ability of polyphenols to prevent disruption of barrier integrity of Caco-2 (colorectal adenocarcinoma) cells stimulated by an inflammatory cocktail (IC), containing IL-1β, TNF-α, IFN-γ, and pro-inflammatory lipopolysaccharide (LPS), and on the inhibitory effect of polyphenols on low-grade chronic inflammation associated with a high-fat diet in rats.

Caco-2 cells were treated with the IC and different concentrations of polyphenol extracts; transepithelial/endothelial electrical resistance (TEER%) percentage was monitored. To induce weight-gain, rats were fed a high-calorie chow diet and water-supplemented with 2.0, 2.5, and 3.0 g/L polyphenol extracts (n = 16) for 42 days. Weight gain and inflammatory markers IL-6 and TNF-α were evaluated at the completion of treatments.

RESULTS & DISCUSSION

Caco-2 cells were exposed to pro-inflammatory cytokines to induce barrier dysfunction. The inflammatory cocktail (IC) reduced barrier function, determined by transepithelial electrical resistance (TEER), by approximately half of the initial value at 36 h. Over the same time course, Caco-2 cells increased TEER by 9%. Cells, without exposure to IC, maintained barrier function for up to 48 h.

At 60 h, TEER was reduced to 41.7% of the initial values. Here, Caco-2 cells treated with granadilla had TEER value of 72.7%.

The average initial body weight in Wistar rats was 350 g. At the end of the diet adaptation period (14 days), bodyweight increased 17.7%. Water containing 2.0, 2.5, and 3.0 g/L polyphenol-rich extract was offered at will during the supplementation period of 28 days.

Serum TNF-α was statistically reduced in animals supplemented with 2.5 g/L of polyphenols from *P. ligularis* (granadilla) compared to both the lower-dose and control groups (p=0.03). For animals treated with granadilla polyphenol-extracts at a concentration of 2.5 g/L (1.75 mg/kg BW/day), TNF-α concentration was lowered by more than 2%.

Rats treated with the 3.0 g/L dose showed decreased IL-6 levels by about 6%, but this change was not statistically significant. Lu et al. reported that IL-6 levels decreased by the action of green tea polyphenol-supplementation using a 5.0 g/L dose in rats fed a high-fat diet. The highest granadilla polyphenol extract dose used in the present investigation was 3.0 g/L, and supplementation with higher polyphenol contents could suggest statistically significant inhibition of IL-6.

Our results are consistent with those of prior studies reporting decreased IL-6 and TNF-α following supplementation of rats with 200 mg/kg/day green tea. These authors attributed their results to the capacity of polyphenols to protect animals from fatty liver disease, as the cytokines IL-6 and TNF-α manifest during disease progression and with the appearance of fibrotic lesions.

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P2.6

Screening novel bioactivities and bark chemistry of Finnish willows

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MAIN CONCLUSION

Hot water extracts of bark from 16 different *Salix* clones showed potential as industrial feed stock for producing antiviral, antibacterial, and antioxidant preparations.

INTRODUCTION

Novel bioeconomy approaches call for more and faster production of lignocellulosic biomass and its better-tailored use for higher added-value. Willows have high growth potential: annual dry mass production of circa 10 – 13 tons ha^{-1} has been reported in about 5 year's rotations in Finland. Willows also show excellent coppice vigor, which makes them promising species for short rotation cultivation to produce raw material for different applications, including sustainable ingredients with biological activities.

Earlier studies have shown that bark of *Salix* species (Salicaceae family) is rich in extractives, including diverse bioactive phenolic compounds. However, we lack knowledge about the bark chemical properties of willow species and/or clones well adapted in the Finnish climate. This project focuses on the bark extractives of Finnish willows by screening their antioxidative, antiviral, antibacterial and antifungal properties.

MATERIALS & METHODS

Material of the study consisted of 16 willow clones (Figure 1). Of them, 12 clones were examples of two common and widely distributed native willow species, dark-leaved willow (*S. myrsinifolia* Salisb.) and tea-leaved willow (*S. phylicifolia* L. [1]), as well as their natural and artificial hybrids, and four clones were commercial willow varieties from the Swedish willow breeding program. Hot water extractions of bark specimens were carried out. The extracts were examined for their content of total phenolics. A set of biological activity assays was used to screen antiviral, antibacterial, antifungal, and antioxidative properties of the extracts.

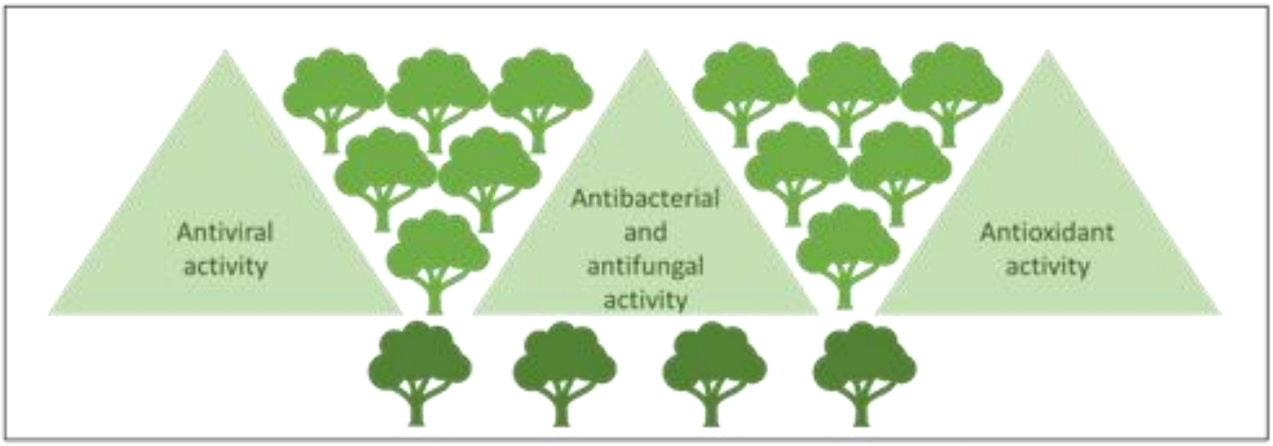

Figure 1. Graphical abstract. Potential of *Salix* bark as industrial feedstock for antiviral, antibacterial, antifungal, and antioxidant hot-water extracts – analysis of 16 clones.

RESULTS & DISCUSSION

Clonal variation existed in antioxidant properties of bark extracts between the studied 16 *Salix* clones. High antiviral activity against a non-enveloped enterovirus, coxsackievirus A9 was found, with no marked differences in efficacy between the clones. All the clones studied also showed positive antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, whereas only insignificant antifungal (*Aspergillus brasiliensis*) or yeasticidal (*Candida albicans*) efficacy was detected. Bark of all native willow clones of Finnish origin showed high bioactivity potential, thus allowing selection of the most suitable clonal feed stocks for short rotation coppice plantations adaptable in the Finnish climate.

Biochemicals obtainable from tree bark-biomass and side-products of biorefinery approaches have potential for various applications (e.g., health-promotion, cosmetics, pharmaceuticals, packaging, coatings, other functional materials, and surfaces). Willow, as a short rotation coppice species with fast growth rate and high yield on e.g., marginal lands, presents a promising alternative for the currently most common commercial tree species in Finland. Comprehensive and optimized utilization of willow lignocellulosic biomass will promote sustainability and carbon-neutrality but requires further research e.g., techno economic assessment of the production chain.

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P2.7

Differences in Chemical Composition and Antioxidant Potential Between Herb and Root Ethanol Extracts of *Rumex alpinus* L. 1753. (Polygonaceae)

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MAIN CONCLUSION

In this study we analyzed the phenolic profiles of *R. alpinus* L. 1753 herb and root ethanol extracts by quantitative HPLC-MS/MS and qualitative HPLC-DAD-MS methods, and evaluated their antioxidant potential in a series of *in vitro* spectrophotometric assays (FRAP, DPPH, NO, LP, $OH^{\cdot}$, $O_2^{-\bullet}$). Based on obtained results, *R. alpinus* ethanol extracts are rich source of polyphenols, with different distribution of flavonols, flavan-3-ol (monomers and dimers), and anthraquinones throughout the plant.

INTRODUCTION

Rumex alpinus L. species, native to European high mountain regions, belongs to *Rumex* subgenus, whose members have been used extensively in traditional medicine worldwide for treatment of different disorders due to their laxative, diuretic, antipyretic and antibiotic properties. Even more, leaves of these plants can be used in diet. Considering plethora of their possible applications, these plants should be thoroughly investigated both phytochemically and biologically. However, such scientific data for *R. alpinus* are scarce. In order to rate the antioxidant potential of prepared herb and root extracts of collected Munk's rhubarb plant material, different *in vitro* assays based on electron transfer (FRAP, DPPH) or hydrogen transfer ability (LP) or scavenging capacity towards $OH^{\bullet}$, NO, and $O_2^{-\bullet}$, were conducted. Our work also included quantitative analysis of 25 phenolics by LC/MS/MS, and qualitative LC/DAD/MS analysis of herb, root, but also of leaves, stems and inflorescence extracts.

MATERIALS & METHODS

Plant material of *R. alpinus* L. 1753 were collected at Stara Planina (34 T FP 2 30) and Kopaonik (34 T DN 3 89) in Serbia. Extracts were prepared by the maceration of air dried and powdered plant material (aboveground parts i.e. herb, and underground parts i.e. root) with 80% ethanol (1g:10mL) for 72h under constant stirring at RT. After filtration and vacuum drying (<45°C) yield of dry extracts were 6.3-9.0% for herb, and 22.1-26.4% for roots. Phenolic profile analysis both *quantitative LC-MS/MS* [1] and *qualitative LC-DAD-MS* [2] were performed on Agilent Technologies 1200 Series HPLC with DAD, coupled with Agilent 6410A Triple Quad tandem mass spectrometer with electrospray ion source. The *antioxidant* activity was evaluated by measuring ferric reducing ability of the extracts and their radical scavenging capacity towards DPPH, OH, NO and O_2^{-}, and inhibition of lipid peroxidation, in a series of *in vitro* spectrophotometric methods, described in details in our previous work [3,4].

RESULTS & DISCUSSION

In above ground parts quercetin derivatives (-3-*O*-rhamnoside, 4.3±2.7 mg/g d.e.; -3-*O*-galactoside, 11.3±10.5 mg/g d.e.; -3-*O*-rutinoside, 2.1±0.3 mg/g d.e.; -3-*O*-glucoside 1.4 mg/g d.e.; and 3-*O*-glucuronide) were dominant, with significant amount of anthraquinones (emodin, 1.1±0.4 mg/g d.e.;

and chrysophanol, 0.71±0.1 mg/g d.e.), flavan-3-ols (epicatechin, 0.25±0.4 mg/g d.e. and catechin, 0.20 mg/g d.e.), and naphthol-musizin derivatives. Root extracts were rich in epicatechin (6.4±1.4 mg/g d.e.), catechin (1.1±0.8 mg/g d.e.), emodin (5.9±0.8 mg/g d.e.) and chrysophanol (2.5±0.1 mg/g d.e.), but also in musizin aglicon and flavanol-3-*O*-gallate derivatives. Comparison of HPLC-DAD chromatograms of extracts prepared form different plant parts revealed that flavonols (350 nm) are present in aboveground parts, especially in leave extracts. Flavan-3-ol monomers and dimers (280 nm), and anthraquinones (430 nm) although present in the whole plant, mostly accumulate in roots.

Based on the results of antioxidant evaluation, herb extracts showed better activity in DPPH (IC_{50} 3.6 µg/mL vs. IC_{50} 4.21 µg/mL), $OH^{\bullet}$ (IC_{50} 229 µg/mL vs. IC_{50} 305 µg/mL), and $O_2^{-\bullet}$ (IC_{50} 19.4 µg/mL vs. IC_{50} 35.1 µg/mL), while root extracts were better in NO (IC_{50} 43.3 µg/mL vs. IC_{50} 87.9 µg/mL) and LP (IC_{50} 14.7 µg/mL vs. IC_{50} 30.1 µg/mL) assays. In FRAP assay results were similar (116 vs. 127 mg Ascorbic acid eq/ g d.e.).

Table (left): Concentrations of phenolics found in *R. alpinus* inflorescence, stems, leaves, herb and roots ethanol extracts (expressed as mg of phenolics per gram of dry extract). LoQ–limit of quantitation.

	Locality_ Stara Planina_46					Locality_ Kopaonik_53				
	inflorescence	stem	leave	**herb**	**root**	inflorescence	stem	leave	**herb**	**root**
Compounds	mg/g d.e.									
Quinic acid	0.038	0.012	0.055	0.030	0.060	0.098	0.011	0.053	0.035	0.035
Gallic acid	0.769	0.008	0.151	0.202	0.523	0.067	0.007	0.071	0.019	0.554
Protocatechuic acid	0.569	0.194	0.132	0.365	0.034	0.094	0.050	0.255	0.073	0.069
Catechin	1.265	0.116	0.078	0.130	1.700	0.000	0.000	0.052	0.001	0.485
5-*O*-Caffeoylquinic acid	0.009	0.033	0.036	0.044	0.000	0.011	0.021	0.115	0.027	0.000
p-hydroxybenzoic acid	0.026	0.035	0.061	0.090	<LoQ	0.144	0.087	0.166	0.088	0.025
Epicatechin	1.057	0.038	0.109	0.568	5.349	0.079	<LoQ	0.105	0.039	7.411
Caffeic acid	0.007	0.011	0.042	0.031	0.055	0.010	0.000	0.033	0.009	0.059
Vanillic acid	0.065	0.109	0.054	0.083	0.000	0.195	0.048	0.159	0.090	0.000
Syringic acid	0.068	0.056	0.076	0.108	0.037	0.097	0.064	0.078	0.098	0.031
p-Coumaric acid	0.035	0.039	0.064	0.055	0.037	0.165	0.040	0.110	0.050	0.059
Ferulic acid	0.026	0.067	0.099	0.071	0.087	0.198	0.047	0.219	0.091	0.109
Sinapic acid	0.000	0.000	0.016	0.012	0.000	0.000	0.000	0.083	0.006	0.000
Hyperoside	2.495	0.806	8.726	3.748	<LoQ	5.747	0.949	58.036	18.707	0.049
Rutin	1.788	0.493	1.383	1.892	0.004	2.820	0.589	10.115	2.379	0.030
Quercetin-3-*O*-glucoside	1.608	0.400	2.465	2.908	0.007	1.350	0.328	9.048	<LoQ	0.021
Quercitrin	2.470	1.247	9.763	6.237	0.014	0.059	0.104	3.311	2.357	0.014
Kaempherol-3-*O*-glucoside	0.039	0.019	0.099	0.128	<LoQ	0.101	0.046	1.047	0.208	0.002
Quercetin	0.143	<LoQ	0.142	0.110	<LoQ	0.231	<LoQ	0.429	0.159	<LoQ
Naringenin	0.013	0.006	0.013	0.012	0.026	0.013	0.005	0.004	0.004	0.014
Kaempferol	0.028	<LoQ	0.048	0.027	<LoQ	0.099	0.011	0.143	0.033	<LoQ
Aloe-emodin				0.019	0.131				0.029	0.142
Rhein				0.002	0.001				0.003	0.003
Emodin				1.404	5.323				0.882	6.495
Chrysophanol				0.722	2.643				0.705	2.454

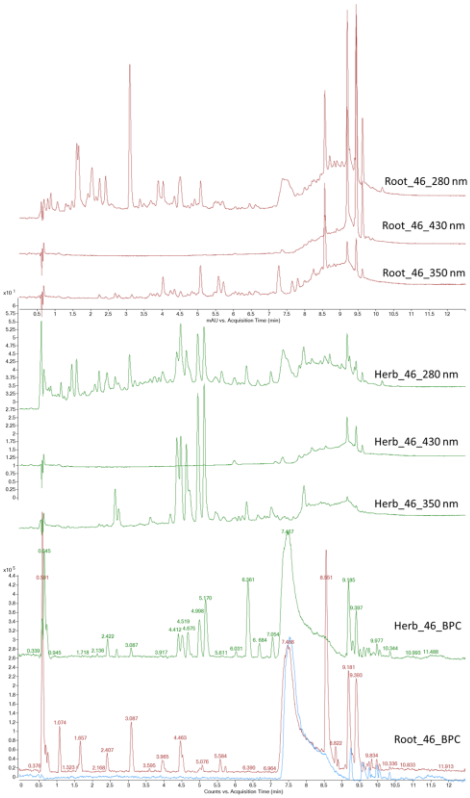

Figure (right): Selected chromatograms of herb_46 and root_46 ethanol extracts - HPLC-DAD chromatograms (280 nm, 430 nm, 350 nm) and ESI BPC (MS2Scan) -negative ionization mode.

In general, our results indicate a high potential for application of *R. alpinus*, as a source of biologically active compounds, especially due to this plant`s large size and its cultivation possibilities.

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P2.8

Bassia longifolia bark extract exhibits antimicrobial activity

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MAIN CONCLUSION

In vitro antimicrobial activity testing of *Bassia longifolia* methanolic bark extract shows antibacterial activity against gram-positive *Staphylococcus aureus* strains, both methicillin sensitive *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA), as well as anti-biofilm activity on those bacterial strains. The analytical investigation of this extract reveals that present polyphenols consist mainly of tannins, known for their antimicrobial potential.

INTRODUCTION

The bark of *Bassia longifolia* Koenig (=*Madhuca longifolia* Koenig, Sapotaceae) is known to be applied externally as paste in the treatment of cuts and wounds and, as such, represents a part of the phytomedicine in Nepal [1, 2]. This experiments were undertaken to explore the *in vitro* antimicrobial activity of the methanolic bark extract and its phytochemical composition. After a primary antimicrobial screening on several types of microbes, additional anti-biofilm activity testing was performed on *S. aureus* experimental models, both methicillin sensitive (MSSA) and methicillin resistant (MRSA) strains.

MATERIALS & METHODS

Total polyphenolic and tannin contents of methanolic bark extract were determined and expressed as catechin [3]. Standard laboratory microbial strains including *S. aureus* ATCC 6538, *Escherichia coli* ATCC 10536, *Candida albicans* ATCC 10231 and *Aspergillus brasiliensis* ATCC 16404, as well as clinical methicillin resistant *S. aureus* MFBF 10679 (MRSA), from stock-cultures of the *Collection of Microorganisms* (MFBF) of the Department of Microbiology, Faculty of Pharmacy and Biochemistry University of Zagreb were used. The **Agar well diffusion assay** was performed following European Pharmacopoeia guidelines (c = 20.00 mg mL^{-1}) [1]. **Minimal inhibitory concentrations (MICs)** were investigated following EUCAST guidelines [2]. MIC was defined as the lowest concentration of extract that allows no more than 20% growth of microbes in comparison with the extract-free control. The effect on **biofilm** formation of MSSA and MRSA was evaluated with a crystal violet assay [3].

RESULTS & DISCUSSION

The analytical investigation of the methanolic bark extract reveals that the present polyphenols consist mainly of tannins. A total polyphenolic content of 43.1% ± 1.4% was determined and tannins represent the majority with 42.2% ± 2.0%, both expressed as catechin. *In vitro* antimicrobial activity testing of this extract shows antibacterial activity against gram-positive *S. aureus* strains, both methicillin sensitive *S. aureus* ATCC 6538 (MSSA) and methicillin resistant *S. aureus* MFBF 10679 (MRSA). Measured zones of growth inhibition (ZI) in the Agar well diffusion assay for MSSA and MRSA were 16 ± 1 mm and 15 ± 0 mm, respectively. Minimal inhibitory concentration (MIC) in the Serial microdilution broth assay showed to be 208.17 ± 90.36 µg mL^{-1} for both strains. No antimicrobial activity was observed for other tested microbial strains in given experimental conditions (*E. coli* ATCC 10536, *C. albicans* ATCC 10231 and *A. brasiliensis* ATCC 16404). The anti-biofilm activity of *B. longifolia* methanolic extract on biofilm formation of MSSA and MRSA was expressed

as minimal biofilm-forming inhibition concentration (MBFIC). $MBFIC_{50}$ and $MBFIC_{90}$, calculated from linear regression of the log_{10} of extract concentration *vs*. % biofilm reduction, represent the lowest extract dilutions at which biofilm formation was inhibited by 50% and 90%, compared to the untreated control. The values for MSSA are as follows, $MBFIC_{50}$ = 1.38 µg mL^{-1} and $MBFIC_{90}$ = 1044.59 µg mL^{-1}, while values for MRSA showed to be higher, $MBFIC_{50}$ = 2.52 µg mL^{-1} and $MBFIC_{90}$ = 1138.43 µg mL^{-1}. Obtained antimicrobial activity data in this study are generally in agreement with tannins being well known for their antimicrobial activity. Shown *in vitro* antimicrobial activity of *B. longifolia* methanolic extracts gives impetus for further in depth antimicrobial and other bioactivity testing in the future, emphasizing in particular resistant *S. aureus* strains.

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P2.9

Uptake and anti-inflammatory properties of betalains in intestinal Caco-2 cells

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MAIN CONCLUSION

Increasing attention is being paid to the potential beneficial functions of the betalain group of phytochemicals. Our data demonstrate, that despite low detectable and therefore low available amount of betanin to intestinal cells, there is a strong inhibitory effect towards inflammation, emphasizing the potential importance of betalains.

INTRODUCTION

Betalains are a group of natural pigments abundantly present in beetroot, cactus pear, pitaya, and amaranth. Their radical scavenging and antioxidant properties have been frequently highlighted in the scientific literature as comparable to anthocyanins and resveratrol. Animal and human studies indicate potential health functions of betalains including amelioration of lipid peroxidation, anti-diabetic, anti-tumour, as well as hepato-, neuro-, and cardio-protective properties (Khan, 2016). Current knowledge on betalain availability is limited, indicating low systemic availability, therefore leaving a gap to align intake of betalain-rich food with associated health benefits. The aim of this study therefore was to explore the uptake and accumulation of betanin, as a major betalain, by small intestinal cells under different conditions, and investigate the molecular effects of betanin on intestinal inflammatory signalling. For uptake related experiments, differentiated Caco-2 cells, originating from human colon adenocarcinoma, were used as a commonly selected model to mimic small intestinal uptake properties.

MATERIALS & METHODS

Caco-2 cells were grown Dulbecco's Modified Eagle Medium (DMEM) under standard conditions. For intracellular accumulation experiments, cells were differentiated for up to 21 days. Cells at different differentiation stages were treated with betanin (5 mg mL^{-1}), added to DMEM medium, with incubation time tested as a contributor to betanin absorption. Neutral red test was conducted to confirm lack of cell toxicity exerted by betanin. Following incubation, the cells were harvested and betalains extracted using methanol and processed for HPLC-MS analysis. The impact of betanin on inflammatory response was examined via quantitative RT-PCR with target genes iNOS and COX-2. To this end, Caco-2 cells were stimulated with a cocktail of cytokines comprised of TNF-α, IL-1β, IFN-γ and LPS in the presence or absence of betanin (5-400 µg mL^{-1}) for 6 h.

RESULTS & DISCUSSION

The uptake experiment indicated less than 0.3% of applied betanin was accumulated intracellularly after 2-4 h of incubation, a time period that resembles the residence period of phytochemicals in the anterior segment of the human intestine. Overall, impacts of cell differentiation and incubation time were not markedly different, although the highest value of intracellular betanin was obtained from fully differentiated cells (i.e. 21 days) with 4 h incubation, indicating that betanin uptake is positively impacted by the development of polarised morphology and transport proteins on the Caco-2 monolayer surface (data not shown). There was no evidence, within available detection limit of this study, on the generation of metabolites during betanin uptake by Caco-2 cells.

A further aim was to examine whether betanin and beetroot extracts impact on cell signalling in Caco-2 cells, with a focus on inflammatory response. The results demonstrate a significant downregulation of pro-inflammatory targets iNOS and COX-2 on mRNA levels, of at least 25% at each betanin concentration for both targets, indicating strong anti-inflammatory properties (data not shown), in line with the literature. Conditions such as inflammatory bowel disease demonstrate increased iNOS activity with deleterious effects of nitric oxide as well as COX-2 and associated enhanced prostaglandin and thromboxane synthesis. Reduction of gut inflammatory response by betanin may therefore be a good candidate to alleviate intestinal inflammation-induced disease and cancer.

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P2.10

Health-promoting effects of lingonberry (*Vaccinium vitis-idaea* L.) in obesity: impact on lipid and glucose metabolism and low-grade inflammation

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MAIN CONCLUSION

Lingonberry supplementation significantly prevented high-fat diet-induced metabolic and inflammatory changes in a pre-clinical model of obesity shown as lower cholesterol, glucose, ALT and SAA levels. In addition, lingonberry had a wide-spectrum effect on liver transcriptome during high-fat diet, particularly on genes involved in lipid metabolism, inflammation, and diabetes. The results encourage evaluation of lingonberries as a part of healthy diet against obesity and its comorbidities.

INTRODUCTION

Obesity is an increasing health burden worldwide. According to the WHO statistics, over 30% of the world's adult population is estimated to be overweight or obese (1). Obesity induces low-grade inflammation in the body and that is a risk factor for comorbidities such as type 2 diabetes, cardiovascular diseases, and nonalcoholic fatty liver disease (NAFLD). Adipose tissue is an active tissue regulating metabolic, inflammatory, and various other pathophysiological processes. It is therefore no longer considered only as a passive energy storage. Adipose tissue releases several hormone-like factors called adipokines, and many of them have pro- or anti-inflammatory properties. In obese adipose tissue, immune cells secreting pro-inflammatory substances increase in number while those producing anti-inflammatory substances decrease. This imbalance contributes to the obesity induced low-grade inflammation and insulin resistance in the body (2).

Diet rich in vegetables and fruits has been shown to attenuate low-grade inflammation associated with obesity. Intervention studies have also shown that berries have beneficial effects in inflammation and cardiovascular diseases. Lingonberry (*Vaccinium vitis-idaea* L.) is grown in the Northern hemisphere, and its red berries are traditionally used in human diet in many forms. Lingonberry is rich in polyphenols and has high antioxidant activity (3). Lingonberry has been reported to have promising health-promoting effects and anti-inflammatory properties in experimental models (4–5). In the present study, we investigated the effects of lingonberry supplementation on low-grade inflammation and metabolic changes in a murine model of high-fat diet-induced obesity (6).

MATERIALS & METHODS

Thirty male C57BL/6N mice, 8 weeks of age, were divided into three groups (n=10/group) to receive low-fat (LF), high-fat (HF) and air-dried lingonberry powder -supplemented high-fat (HF+LGB) diet for 6 weeks. Both HF diets contained 46% of energy from fat and 36% from carbohydrate, while the LF diet had 10% of energy from fat and 72% from carbohydrate. Otherwise the diets were matched for protein, fiber, vitamin and trace element contents considering the composition of the lingonberry powder. At the end of the study, fasting glucose was measured and blood and tissue samples were collected for further analyses. Serum triglyceride and cholesterol levels, and alanine aminotransferase (ALT) activity were measured by fluorometric assays. Concentrations of leptin, resistin, adiponectin,

insulin, and serum amyloid A were measured by ELISA. RNA was extracted from the liver and subjected to RNA-seq and RT-qPCR.

RESULTS & DISCUSSION

Lingonberry supplementation totally prevented the HF diet-induced elevations in blood cholesterol and glucose levels and in ALT activity. Lingonberry supplementation also restrained the HF diet-induced increases in the inflammatory adipocytokine leptin (by 36%) and the acute phase reactant serum amyloid A (by 85%). In the genome-wide expression analysis, lingonberry supplementation prevented the HF diet-induced changes in the expression of 260 genes in the liver. Many of these are involved in lipid metabolism, inflammation and in the development of diabetes.

Food consumption (kcal/g body weight) did not differ between the HF and the HF+LGB groups, although energy intake in the LF diet group was lower, particularly during the first half of the study. The weight of mice in the HF diet group increased considerably during the study as compared to the mice in the LF control group. Lingonberry supplementation prevented by about 25% the HF diet-induced weight gain and visceral fat accumulation (measured as epididymal fat gain). Moreover, lingonberry supplementation prevented HF diet-induced liver weight gain, in which there was no difference between the LF and HF+LGB diet groups.

To our knowledge, this is the first study to demonstrate wide-spectrum health-promoting effects of air-dried lingonberry powder in a pre-clinical model of obesity. The results are remarkable also considering the rather short duration of the study. In conclusion, the present results show that lingonberry supplementation had a significant preventive effect on HF diet-induced low-grade inflammation, changes in lipid and glucose metabolism, and development of NAFLD in a murine model of obesity. The results encourage evaluation of lingonberries as a part of healthy diet against obesity and its comorbidities. Further studies are needed to reveal the detailed molecular mechanisms and the active constituents responsible for the observed beneficial effects.

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P2.11

Effect of the presence of mannoproteins on the interaction between flavanols, salivary proteins and oral epithelial cells

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MAIN CONCLUSION

Mannoproteins have shown ability to affect salivary-flavanol interactions. Saliva-tannin interactions appear to be much more important than cell-tannin interactions, and it seems clear that the presence of saliva prevents these last interactions. Different structural features of mannoproteins could not only determine the mechanism whereby MPs modulate the astringency elicited by tannins but could also be related to a preferential interaction with distinct procyanidins families.

INTRODUCTION

Astringency is a complex perceptual phenomenon involving several sensations that are perceived simultaneously, and the underlying molecular and physiological mechanisms have not yet been clarified. It has been proposed that this sensation involves multiple mechanisms occurring simultaneously such as the interaction between flavanols and salivary proteins leading to the aggregation and/or precipitation of salivary proteins or direct interactions between flavanols and oral epithelial cells [1].

Moreover, the astringency development can be affected by the presence of polysaccharides such as mannoproteins, and its use to modulate unbalanced astringency of red wines is a practice extensively spread in the wine industry. There are two main mechanisms proposed to explain the reduction of astringency provoked by the addition of polysaccharides: a) the formation of protein/polyphenol/polysaccharide ternary soluble aggregates [2] and b) the preferential interaction between the polyphenol and the polysaccharide, competing with protein aggregation [3]. Furthermore, a very recent study has reported a relationship between the compositional characteristics of mannoproteins and their differences in the mechanisms of action towards astringency modulation [4].

The main aim of this work was to study the effect of the presence of different mannoproteins showing structural differences on the interaction between flavanols and oral epithelial cells in presence and absence of salivary proteins.

MATERIALS & METHODS

Whole saliva was collected from seven healthy individuals (27-50 years) refereed as whole saliva (WS). Interaction assays were performed using TR146 cells, which have been shown to be suitable as an in vitro model of the human buccal epithelium, a flavanol extract obtained from grape seeds, whole saliva, and two different commercial mannoproteins that show important differences on both their protein and glicidic moieties.

After the interaction assays, the supernatant of the cells was removed and differences in the content of monomeric and oligomeric procyanidins and the salivary protein profile, before and after the interactions, were assessed by means of HPLC-DAD-MS. Moreover, the amount of procyanidins that remained bound to the epithelial cells was evaluated by DMACA assay.

RESULTS & DISCUSSION

Results have shown that tannins are able to directly bind oral cells, but saliva-tannin interactions appear to be much more important than cell-tannin interactions. The presence of saliva prevents cell-

tannin interactions mainly by a competitive mechanism, pointing out the prevalence of salivary proteins in the interaction with flavanols, when compared to TR146 cells.

The ability of mannoproteins to interact with both, salivary proteins and flavanols has also been revealed, although the characteristics of the interactions seems to depend on the structural features of the mannoproteins. Both mannoproteins have shown their ability to affect salivary-flavanol interactions, preventing the precipitation of saliva-tannin complexes. Moreover, the presence of mannoproteins also seems to inhibit the interaction between oral cells and flavanols and, again, that effect depends on the structure of the mannoprotein.

The mannoprotein with the lowest protein percentage on its structure seems to favor the formation of soluble aggregates, probably due to the formation of ternary soluble aggregates. However, the mannoprotein with the highest protein percentage has lower affinity for salivary proteins and seems to prevent flavanol-saliva interaction by a competitive mechanism.

Finally, results pointed out that mannoproteins with different compositional characteristics could exhibit a preferential interaction with some procyanidin families. This is the first time that the role of mannoproteins in the interaction between salivary proteins and flavanols has been studied in the context of an in vitro model of the oral cavity.

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P2.12

Protective effect of Manuka honey against inflammation and its related diseases

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MAIN CONCLUSION

Manuka honey acted as a natural agent for preventing oxidative stress and inflammatory-status as recently demonstrated in LPS-treated macrophages [1,2]. In addition, the effect of *in vitro* gastrointestinal digestion of Manuka honey, was also underlined in colon adenocarcinoma cell line [3]. All these evidence highlight the beneficial role of Manuka and its bioactive phenolic compounds against inflammation and its related diseases.

INTRODUCTION

Honey is a natural substance widely known for its therapeutic abilities: its content in flavonoids and phenolic acids plays a key role on human health, thanks to the high antioxidant and anti-inflammatory activities that it possesses. Manuka honey (MH), in particular, is a monofloral honey collected from the *Leptospermum scoparium* tree (Family: Myrtaceae) in New Zealand and Australia, extensively studied for its health beneficial effects. It is a complex mixture of various pharmacological and biologically active compounds with well-known nutritional and phytochemical properties. Different studies investigated the protective effect of Manuka honey against oxidative stress, underlying its role as anti-microbial and anti-cancer agent [1,2,4]. Nevertheless, only recently its anti-inflammatory properties were studied, paying particular attention to the molecular mechanisms involved [1]. According to this, collected evidence demonstrated the positive effect of MH treatment in LPS-treated RAW 264.7 macrophages by enhancing cellular viability, decreasing apoptosis, promoting wound healing and improving mitochondrial respiration and glycolytic activities in a dose-dependent manner [1]; at the same time this mechanism of action was induced by a reduction of ROS and nitrite levels, with a concomitant increment in antioxidant enzyme expression and activities, also reducing the expression of genes involved in inflammation [2]. Interestingly, these properties were also evaluated in colon-rectal adenocarcinoma cells, both for MH and the result of its *in vitro* gastric-intestinal digestion process, highlighting and increasing the knowledge of the effect of gastrointestinal digestion on the biological effect of honey against this common cancer condition [3].

MATERIALS & METHODS

Cellular viability was evaluated by MTT assay, while for determination of intracellular ROS generation CellROX® Oxidative Stress kit was used. Nitrite production was demonstrated by the Griess method and spectrophotometrically assays were performed for the determination of biomarkers of oxidative stress and antioxidant enzyme activities. RT-PCR and immunoblotting assays were applied for total RNA content and protein expression. Apoptosis was determined using the Tali™ Apoptosis Assay Kit–Annexin V Alexa Fluor® 488, wound healing was assayed as previously described [5]. Energetic metabolism was determined with XF-24 Extracellular Flux Analyzer. Finally, the in vitro digestion of MH was performed following the method described by Gil-Izquierdo et al. [6] and cell cycle analysis was performed with the Tali® Image-based cytometer.

RESULTS & DISCUSSION

In LPS-treated RAW macrophages, MH was able to enhance cellular viability, decrease apoptosis, promote wound healing and attenuate inflammation in a dose-dependent manner, by reducing the expression of caspase 3, p-p38 and p-Erk1/2 proteins. In addition, it improved mitochondrial respiration and glycolytic activities, stimulating the expression of p-AMPK, SIRT1 and PGC1α, counteracting in this way the deleterious effects of LPS. Pre-treatment with MH also inhibited LPS induced ROS and nitrite accumulation and increased the protection against the damage of cellular biomolecules such as DNA, proteins and lipids. LPS stimulation significantly suppressed both antioxidant enzyme activities and expressions, mainly focusing on Keap1-Nrf2 signaling pathway; these effects were significantly reversed in the presence of MH. The stimulatory effects in pro-inflammatory cytokines expression, such as TNF-α, IL-1β and IL-6, and other inflammatory mediators (like iNOS) after LPS treatment, was efficiently suppressed with MH pre-treatment. Moreover, MH also inhibited the expression of TLR4/NF-κB via IκB phosphorylation in LPS-stressed RAW 264.7 macrophages. Finally, in human colon cancer cells, MH and digested MH (DMH) at different concentrations possessed similar effects in inducing intracellular ROS production and in inhibiting the colon formation ability. In detail, MH induced a more marked apoptosis compared to DMH, while cell cycle was blocked in S phase by MH and in Sub G1 phase by DMH. Taken together, all the collected results clearly evidenced the key role of MH as natural agent for preventing oxidative stress and inflammatory-related diseases.

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P2.13

Chestnut (*Castanea sativa* Mill.) shells: A promising source of polyphenols as valuable compounds for cosmetic industry

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MAIN CONCLUSION

Overall, this study emphasized the bioactivity and safety of *C. sativa* shells extract, pointing out a promising application as a novel cosmetic ingredient.

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INTRODUCTION

The chestnut farming and processing industry is growing worldwide, contributing to the increase of chestnut by-products. *Castanea sativa* Mill. is the main chestnut species in Europe, accounting for 6.5% of the global production [1]. During the industrial chestnut processing, high amounts of by-products are generated, mostly shells. Chestnut shells are underexploited agro-residues and outstanding sources of valuable compounds, including polyphenols, particularly phenolic acids (namely gallic and ellagic acids) and tannins (condensed and hydrolysable), vitamin E and amino acids [2]. Such compounds may display antiviral and antimicrobial activity, besides chelating iron and scavenging radicals, regulating gene expression, enhancing endothelial function or inhibiting enzymes (such as matrix metalloproteinases) [2]. Nevertheless, several industries have attempted to create greener formulations using sustainable raw materials obtained from agro-industrial by-products, normally discarded as waste. The antioxidant and bioactive compounds present in this agro-residue may be explored as food supplements, for instance, to prevent oxidative stress which is strongly associated to aging and certain chronic diseases (e.g., Parkinson or Alzheimer's diseases), or even as anti-wrinkle agents in cosmetics [1,2]. This work focused on alternatives for the valorization of chestnut shells, particularly on the sustainable recovery of polyphenols through an environmentally friendly technique, aiming a cosmetic application. Subcritical Water Extraction (SWE) has arisen as an efficient technique and economically attractive for industrial processes. To the best of our knowledge, up to now, the applicability of SWE to extract bioactive compounds from chestnut shells has not been investigated.

MATERIALS & METHODS

The main purpose of this study was to optimize the extraction of chestnut shells' bioactive compounds by SWE through Response Surface Methodology (RSM). Shells were dehydrated (Excalibur Food Dehydrator, USA) at 41 °C for 24 h, grounded in a miller (Ultra Centrifugal Mill ZM 200, Retsch, Germany) to a particle size of 1 mm and stored in the dark at room temperature. A central composite design (CCD) was conducted to analyse the effects of time (6-30 min) and temperature (51–249 ºC) on antioxidant and antiradical activities (ABTS, DPPH and FRAP assays) and Total Phenolic Content (TPC). Further, the phenolic composition was also screened for the optimal extract to explore the

individual phenolic compounds responsible for the antioxidant activity as well as *in vitro* radicals scavenging capacity and intestinal cell effects (Caco-2 and HT29-MTX).

RESULTS & DISCUSSION

The extraction yield ranged from 6.70% to 9.19%. The optimal extraction conditions were attained at the binomial 220 °C/30 min and exhibited a high TPC (366.3 mg GAE/g DW) and antioxidant/antiradical activities (ABTS: 965.5 mg AAE/g DW; DPPH: 899.9 mg TE/g DW; FRAP: 8769.9 mg FSE/g DW). The experimental values for the four responses were similar (p>0.05) to the respective predicted ones, allowing the validation of the experimental design and demonstrating the effectiveness and good adequacy of the RSM. Regarding the phenolic profile, five compounds were accurately identified in the optimal extract, including ellagic acid (2.86 mg/g DW), protocatechuic acid (2.83 mg/g DW), pyrogallol (2.39 mg/g DW), methyl gallate (0.77 mg/g DW) and gallic acid (0.54 mg/g DW). These results are in line with other chestnut shells extracts obtained by a green extraction technology, namely ultrasound-assisted extraction (UAE) [2]. A remarkable scavenging efficiency was observed for HOCl (IC_{50}=0.79 µg/mL) and $O_2^{\cdot-}$ (IC_{50}=12.92 µg/mL). The antioxidant and radicals scavenging activities of the extract may be due to the phenolic profile, particularly the presence of ellagic acid, gallic acid, pyrogallol and protocatechuic acid, whose quenching power and antioxidant capacity have been described by several authors [1,2]. Considering the potential use of this extract in the cosmetic field, the screening of the extracts' effects on skin cell lines is a primary step in the evaluation of a new potential cosmetic ingredient. The optimal extract did not lead to a considerable decrease of fibroblasts (HFF-1) and keratinocytes (HaCaT) viability up to 1000 and 100 µg/mL. These results are in accordance with Lameirão *et al.* that evaluated the UAE extract on intestinal cell lines [2].

Keywords: chestnut shells; subcritical water extraction; polyphenols; bioactivity.

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P2.14

In vitro and *in vivo* bioassay-guided fractionation of olive mill wastewaters for effective biocontrol of *Verticillium dahliae* in tomato plants and *Phytophthora capsici* in pepper plants

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MAIN CONCLUSION

Preparative fractionation of OMW by an integrated pressure-driven ultrafiltration/solid-phase extraction procedure allowed recovery of two fractions, one enriched in hydroxytyrosol and tyrosol glycosides (HP-LMF) and the other, in oleacein and luteolin-7-O-glucoside (HP-LMF) that could be used as an alternative to synthetic fungicides in reduction of disease caused by *V. dahliae* in tomato plants. HP-LMF was effective, also, in reduction of disease caused by *V. dahliae* in pepper plants.

INTRODUCTION

Olive mill wastewaters (OMW) are a main by-product of 3-phase olive oil production, which is known for its high antimicrobial activity. This activity has been related directly to some phenolic compounds and secoiridoids present in OMW. Keynote of understanding and therefore taking advantage of this compounds is their separation and identification. In this communication, we offer an attempt to identify the compounds responsible for the antifungal activity of OMW against two of the most widespread crop pathogens, *Verticillum dahliae* and *Phytophthora capsici*.

MATERIALS & METHODS

OMW was preparatively fractionated in 3 fractions using an integrated pressure-driven tangential-flow ultrafiltration (UF) and solid-phase extraction (SPE) procedure. A macromolecular fraction (MF) was obtained by UF from 11 L of OMW and diafiltration with water on a 10 kDa membrane [1]. The permeate of the UF was further fractionated in a high polarity low molecular mass (HP-LMF) fraction and in a middle polarity low molecular mass (MP-LMF) fractions by SPE on a XAD7HP adsorbent. All these fractions were characterized by RP/NP-HPLC and tested *in vitro* for inhibition of *V. dahliae* conidia germination according to Carreras et al. [2]. The most active fractions were then evaluated for their *in vivo* effects against *V. dahliae* in tomato plants and *P. capsici* in pepper plants at controlled growing conditions.

RESULTS & DISCUSSION

The 10 kDa UF membrane showed a very good filtration flux of 30 to 48 L/hm^{2}bar and allowed recovery of 7.9 g of dry matter of macromolecules. The separation of dipole molecules from very polar and ionic species by SPE was also very good, but the adsorbent progressively lost its retention capacity, that means that further purification of phenols in this way was technically unfeasible. Even though the SPE allowed recovery of 18.6 g of purified HP-LM phenols and 24.3 g of purified MP-LM phenols.

The chemical analysis of some of the main phenols in OMW, MMF, HP-LMF and MP-LMF is shown in Table 1.

Table 1. Content (mg/g of dry matter) of the main phenols identified in the studied OMW and its fractions.

Compound	OMW	MF	HP-LMF	MP-LMF
Hydroxytyrosol (3,4-DHPE)	6.5_a	0.8_b	28.3_c	20.5_d
Hydroxytyrosol glucoside (3,4-DHPE-glu)	8.4_a	0.5_b	46.7_c	7.0_d
Tyrosol glucoside (4-HPE-glu)	18.9_a	1.2_b	91.9_c	26.3_d
Luteolin-7-O-glucoside (Lut-7-O-glu)	28.2_a	1.9_b	32.9_c	77.7_d
Oleacein	21.1_a	1.7_b	9.1_c	176.3_d
Oleuropein	2.5_a	0.1_b	4.1_c	12.5_d

a, b, c, - same letter in the same row indicates absence of significant differences ($p < 0.05$)

The results show that HP-LMF was enriched mainly in 4-HPE-glu and 3,4-DHPE-glu and MP-LMF, in oleacein and Lut-7-O-glu. The MF was composed mainly by polysaccharides (data not shown) and rests of low mass phenols that remained in it because of incomplete purification.

The *in vitro* test of inhibition of *V. dahliae* conidia germination showed that HP-LMF and MP-LMF had higher anti-*V. dahliae* effects at minor effective doses with values of 2.54 and 0.77 mg/mL, respectively, for 50% (ED50) inhibition. These two fractions reduced also disease effects caused by *V. dahliae* in tomato plants similarly to the control, methyl thiophanate, and suggest that these fractions are good alternative to the use of synthetic fungicides in reduction of disease caused by *V. dahliae* in tomato plants. These fractions were also tested *in vivo* against *Phytophthora capsici* in pepper plants, but in this case, only HP-LMF showed ability to reduce disease symptoms.

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P2.15

Agrifood waste as a source to obtain natural bioactive compounds

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MAIN CONCLUSION

The aim of this study was to explore the use of wastes products from the food industry to obtain extracts enriched in phenolic compounds. Extracts obtained from the peel of three varieties of red-coloured potatoes and of the grape pomace from Tempranillo variety were characterized and quantified. Twenty-two compounds were described including anthocyanins, flavonols and phenolic acids, being pelargonidin and malvidin the most abundant compound in potato's peel and grape pomace respectively.

INTRODUCTION

Nowadays, the continuous increasing in the production of organic wastes, especially agricultural wastes, is turning into an important environmental problem. During the last decade, a vast number of investigations show that these wastes could provide a useful and inexpensive source of high-value compounds for pharmaceutical, medical or food industry uses(1). Besides, this waste is a good source of phenolic compounds and particularly, grape pomaces and peels from red-coloured potato are rich in anthocyanins, which can be used as additives, colorants or preservatives, in the food industry.

MATERIALS & METHODS

Grape pomace from Tempranillo variety and potato's peels from three red-coloured varieties (Mozart, Manitou and Rudolph) were tested. Peels and seeds from grape pomace were separated and all the raw material was freeze-dried. Then, they were ground, and the powder was immersed in a methanol-HCl 0.5 N solution (95:5), placed in an ultrasound bath for 1 hour, centrifuged and filtered. This process was repeated twice more. Finally, the extracts were combined and concentrated using a rotary evaporator and were analysed by reverse phase high performance liquid chromatography with double detection by diode spectrophotometry and mass spectrometry (HPLC-DAD-MS). Compounds were identified by their retention time, UV–vis and mass spectra, comparison with our data library and standards when available and/or literature data (2–5). The compounds were quantified from the areas of their chromatographic peaks recorded at 330 nm, 360 nm and 520 nm.

RESULTS & DISCUSSION

The obtained results from potato peel showed a similar anthocyanin profile in all the varieties tested. Four major peaks were tentatively identified as pelargonidin acylated glycosides derivatives (caffeoyl, coumaroyl and feruloyl). Although some minor differences in the total anthocyanins yield could be established, being Mozart and Manitou the varieties which have better recoveries (around 23 mg anthocyanin/100 g dry peel). In the same way, the profile of phenolic acids, principally hydroxycinnamic acids, was quite similar among all the samples, being the most abundant caffeoylquinic acid derivatives (reaching the amount of 180 mg/100 g dry peel in the case of Mozart variety).

On the other side, grape pomace peels contain malvidin, petunidin and delphinidin derivatives, being the malvidin derivatives the major compounds (51% of total anthocyanins). As flavonols, quercetin, myricetin, kaempherol and isorhamnetin were identified, although quercetin was the most abundant ones (57% of total flavonols). Moreover, seeds presented a profile of flavonols similar than

of the peels, being again quercetin and myricetin the major flavonols (64% and 17% of total quantified flavonols respectively).

It was proved that agri-food by-products were rich in anthocyanins and other phenolic compounds such as flavonols and phenolic acids, so they can be considered as a useful and cheap source of bioactive compounds, contributing in this way to the waste reduction. However, further research is needed in order to find a sustainable extraction process and evaluate the stability and safety of the extracts to be incorporated in food formulations as an alternative to synthetic additives.

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P2.16

Comparison of *In vitro* assays to determine inhibition of α-amylase enzyme activity of anthocyanins

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MAIN CONCLUSION

The comparison of two enzyme assays to determine α-amylase inhibition demonstrated that the direct microplate-based assay is superior to determine α-amylase inhibitory properties in anthocyanin-rich samples. The direct assay has shown to be easy, fast and reproducible to perform, and should therefore be recommended, in particular for high-throughput applications.

INTRODUCTION

Intake of anthocyanins, a subgroup of polyphenols, which is abundantly found in many fruits and vegetables, has been associated as beneficial towards prevention and alleviation of metabolic disorders such as diabetes, hypertension and coronary heart disease (Konczak & Zhang, 2004). In particular, attenuation of postprandial glycaemia by delaying the digestion of dietary starch is a proposed mechanism that contributes to the antidiabetic potential of polyphenols including anthocyanins. The enzymatic hydrolysis of starch is aided by salivary and pancreatic α-amylases. Several methods have been applied for measuring α-amylase inhibition of polyphenols that use different substrates, including starch, amylose, amylopectin, and some chemically modified derivatives of polymers and malto-oligosaccharides of varying chain length linked to a chromophore, such as 4-nitrophenyl or 2-chloro-4-nitrophenyl. The most commonly used assay for measuring α-amylase inhibition involves DNS (3, 5-Dinitrosalicylic acid) reagent for detection of reducing sugars. The free carbonyl group in reducing sugars participate in an oxidation-reduction reaction with DNS and yield yellow to orange colour in the solution. However, the reducing potential of anthocyanins enable them to interfere with the colour development and therefore have impact on the assay results (Nyambe-Silavwe et al., 2015). There is a rationale to explore use of alternative assays to determine inhibition of α-amylase activity. The aim of the study therefore was to test performance of a direct colorimetric assay that utilizes a synthetic substrate in comparison with the DNS assay.

MATERIALS & METHODS

The DNS assay was conducted under previously described conditions using amylose as a substrate and porcine pancreatic amylase (PPA) enzyme (Nyambe-Silavwe et al. 2015), with absorbance readings recorded at 540 nm. The direct assay was based on a previously described method (Kalita, Holm et al. 2018) involving PPA and the substrate 2-chloro-4-nitrophenyl-α-d-maltotrioside. Change of absorbance at 405 nm was recorded over a 10 min period followed by a wavelength scan to confirm reaction product formation. Enzyme inhibition was calculated in comparison to samples without enzyme inhibitor presence. Acarbose, a well-known α-amylase inhibitor as well as anthocyanin-rich blueberry extract were tested in both assays over a range of concentrations.

RESULTS & DISCUSSION

The synthetic α-amylase inhibitor acarbose is a commonly used anti-diabetic drug (Glucobay, Precose), and was employed as a positive control for enzyme inhibition assays. Results from the DNS assay indicated a dose-dependent inhibition of α-amylase covering a concentration range from 1-100 µM. A 50% enzyme inhibition was achieved at a concentration of 58.3 µM. These results are in line with other literature reporting on acarbose inhibition using DNS assay, although the magnitude of

inhibition is depending on different factors, such as enzyme concentration or type of substrate. In the case of substrate, lower IC_{50} values have for example been reported for amylose in comparison with amylopectin (Nyambe-Silavwe et al., 2015). In contrast to DNS data, the results from the direct assay showed an IC_{50} of 5.5 µM for acarbose, indicating that the direct assay is superior to demonstrate inhibitory properties at lower inhibitor concentrations. Indeed, when inhibitory effects of blueberry extract were tested in both assays (up to 1896 µg/mL polyphenols), inhibition was only detectable using the direct assay with IC_{50} of 260 µg/mL. In fact, the DNS assay did not show enzyme inhibition at any of the concentrations tested. The increased detectability of α-amylase inhibitory properties with the direct assay could be related to different substrate properties. Given that the direct assay is easy to perform, fast and reproducible, it is advantageous, in particular for high throughput screening.

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P2.17

Polyphenols in six less cultivated fruit and berry species cultivated in Estonia

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MAIN CONCLUSION

The less known fruit and berry species like gooseberry (*Ribes uva-crispa*), Japanese quince (*Chaenomeles japonica*), Chokeberry (*Aronia melanocarpa*), Black elder (*Sambucus nigra*), European cranberrybush (*Viburnum opulus* var. *edule*), edible blue honeysuckle (*Lonicera caerulea* var. *edulis*) can be considered as a valuable source of polyphenolic substances with high potential for using in product development of foods and beverages or as natural antioxidants and colorants in various produce.

INTRODUCTION

The interest in fruit and berry species for niche and healthy products is increasing constantly. Fruits and berries are appreciated as a significant source of bioactive compounds with positive health-related effects. Polyphenols are a group of bioactive substances naturally abundant in fruits and berries contributing to their antioxidant activity. The less cultivated fruit and berry species when compared to traditional black currant (*Ribes nigrum* L.) or red raspberry (*Rubus idaeus* L.), could be considered as a valuable source of polyphenols for food industry and consumption. The gooseberry (*Ribes uva-crispa*) is an important source of polyphenolis, ascorbic acid, vitamin E and minerals [1]. Japanese quince (*Chaenomeles japonica*) is appreciated by its attractive aroma, increased organic acids and fiber content, polyphenols and vitamin C, creating a high potential for food industry and processing [2]. Chokeberry (*Aronia melanocarpa*) is valuable source of phenolic phytochemicals such as procyanidins and anthocyanins [3]. Black elder (*Sambucus nigra*) contains also components with high biological activity, mostly polyphenols and anthocyanins, flavonols, phenolic acids and proanthocyanidins [4]. European cranberrybush (*Viburnum opulus* var. *edule*) has high content of polyphenolic substances, mostly hydroxycinnamic acids in the fruits [5]. Blue edible honeysuckle (*Lonicera caerulea* var. *edulis*) has high content of polyphenols, mostly anthocyanins (cyanidin-3-O-glucoside) and flavan-3-ols in fruits [6]. All these prementioned species are good sources of polyphenolic compounds, but the contents have not been compared and investigated in Estonian climatic conditions. This article gives a short overview of total polyphenolic and anthocyanin content of six different fruit and berry species that are gaining popularity for cultivation and production in Estonia.

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MATERIALS & METHODS

TP and TA of 6 species were analyzed in 2019 at Polli Horticultural Research Centre, Estonia. Briefly, 1 g of homogenous sample (3x) was weighed into 20mL UltraTurrax® tube, added 50% etOH+1% HCl (v/v) solution. The mixture was milled 3 min by IKA®UltraTurrax® Tube Drive (IKA®-Werke GmbH & Co. KG, Germany), treated 30 min in ultrasonic bath (Branson 1800, Emerson, USA). The tubes were shaken 30 min 45 rpm by multi-rotator (Multi RS-60, Biosan Sia, Latvia), centrifuged 5 min at 15°C at 1000 rpm (Sigma Laborzentrifugen GmbH, Germany). The supernatants were pipetted into 1.5 mL tubes, centrifuged 10 min at 13×1000 rpm by Eppendorf MiniSpin (Eppendorf AG, Germany). Aliquots were pipetted into the vials, TP and TA were

quantified (UHPLC-DAD ACE Excel3 C18-PFP 100×2.1mm, Shimadzu Nexera X2, Japan) at 280 and 520nm. TP were expressed as mg chlorogenic acid eqv. 100 g^{-1} fresh weight (fw), TA as mg cyaniding-3-glucoside eqv. 100 g^{-1} fw. Average ± standard deviations were calculated per species.

RESULTS & DISCUSSION

TP ranged the most in chokeberry cultivars followed by edible blue honeysuckle, European cranberrybush, black elder, Japanese quince and being the lowest in gooseberry cultivars (Table 1). TA varied depending on cultivar in following descending order chokeberry < black elder < edible blue honeysuckle < gooseberry < European cranberry bush. As the fruit flesh of Japanese quince is pale yellow, therefore no anthocyanins were detected in it.

Table 1. Species, number of cultivars (cvs) and their minimum (min), average (avg) and maximum (max) contents of total polyphenols (TP) and anthocyanins (TA) in 2019 at Polli Horticultural Research Centre.

Spieces	No. of cvs	TP, mg 100g^{-1} fw			TA, mg 100g^{-1} fw		
		Min	Avg ± stdev	Max	Min	Avg ± stdev	Max
Black elder	4	375.1	434.6 ± 45.4	471.9	264.5	309.5 ± 38.3	346.4
Japanese quince	5	109.3	162.5 ± 49.2	226.2	n.d.	n.d.	n.d.
European cranberrybush	7	531.0	630.2 ± 71.0	751.2	25.8	37.0 ± 7.9	51.7
Chokeberry	7	747.5	917.5 ± 314.6	1625.4	471.8	558.8 ± 151.9	898.2
Edible blue honeysuckle	27	262.0	648.1 ± 144.7	980.9	173.9	441.3 ± 110.0	695.7
Gooseberry	11	11.7	55.6 ± 62.8	206.1	13.1	54.5 ± 55.8	150.9

Notes: fw – fresh weigh; n.d. – not detected; ± stdev – standard deviation.

The other authors have also presented high contents of TP and TA when concerning the polyphenols and anthocyanins in these prementioned fruit and berry species [1-6].

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P2.18

Microwave-assisted extraction of kiwiberry leaves for cosmetic purposes: Phenolic composition and bioactivity screening

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MAIN CONCLUSION

The results obtained supported that MAE is an effective technique to recover phenolic compounds from KB leaves extracts. The hydroalcoholic extract revealed the best results, achieving the highest antioxidant and free radical scavenging activity. Thus, KB leaves extracted by MAE can be classified as non-toxic, being suitable for cosmetic formulations. Further investigations, such as *in-vitro* skin and ocular irritation assays and *in-vivo* studies, are needed to understand this potential.

INTRODUCTION

Kiwiberry (KB) is a small grape-sized fruit, characterized by hairless skin and a pleasant aroma and flavor. Worldwide, the production of KB is increasing mainly due to the biological properties (such as antioxidant) associated. The most recent data stated that in 2015/16 the worldwide production was almost 1600 tons [1]. In Portugal, the national production achieved a record value of 120 tons in 2016 [1]. Nevertheless, during KB production different by-products are generated, such as fruits without caliber to be commercialized, pomace, or leaves [2]. The leaves remotion during production increases the fruits solar exposure [2]. Previous studies demonstrated that KB leaves are a source of polyphenolic compounds, such as neochlorogenic, chlorogenic, and cryptochlorogenic acids, as well as catechin and flavonols. These compounds are well known by their antioxidant, anti-inflammatory, and antimicrobial properties [2,3].

Microwave-assisted extraction (MAE) is an environmentally friendly technique that is considered one of the best methods to extract phenolic compounds due to its high extraction rate, short extraction time, and better product quality [4]. The main advantage of this technique is the facility of the solvent to penetrate inside the cell walls, which leads to a high phenolic compounds' recovery.

The aim of this work was to extract bioactive compounds from KB leaves by MAE, using green and sustainable solvents (water and ethanol), in order to valorize this by-product for cosmetic fields. With this purpose, phenolic compounds, antioxidant activity and in-vitro radical scavenging capacity were evaluated. Moreover, the *in-vitro* effects on keratinocytes and fibroblasts cell lines were screened.

MATERIALS & METHODS

Leaves were collected from ten different KB plants in October 2019 and dehydrated at 41°C for 24 h and grounded in a miller. Then, 300 mg of dried powdered leaves were extracted with 10 mL of solvent (water, ethanol and both (50%, *v/v*)) in a microwave with power fixed at 300 W and was performed at selected temperatures (72–94°C) during 1 to 5 min, with constant medium stirring.

The total phenolic and flavonoid contents (TPC and TFC, respectively) were measured spectrophotometrically. In addition, the phenolic profile was determined in HPLC-DAD/ESI-MS. The *in-vitro* antiradical and antioxidant activities were performed using DPPH free radical scavenging, ABTS radical scavenging activity and ferric reducing antioxidant power (FRAP) assays. The capacity to scavenge superoxide radical ($O_2^{\bullet -}$), hypochlorous acid (HOCl), nitric oxide ($^{\bullet}NO$),

and peroxyl radical ($ROO^{\bullet}$) were also evaluated. Lastly, the cell viability effects were assessed on keratinocytes (HaCaT) and fibroblasts cells (HFF-1).

RESULTS & DISCUSSION

The results revealed that the alcoholic extract contained the highest TPC and TFC (629.48 mg GAE/g dw and 136.81 mg CAE/g dw, respectively). Oppositely, the hydroalcoholic extract achieved the highest antiradical and antioxidant activities (IC_{50} = 95.22 μg/mL and 131.50 μg/mL for DPPH and ABTS assays, respectively; and FRAP = 3079.94 μmol FSE/g dw) and an excellent scavenging capacity against reactive oxygen and nitrogen species (IC_{50} = 29.10 μg/mL for $O_2^{\bullet-}$; IC_{50} = 1.87 μg/mL for HOCl and IC_{50} = 1.18 μg/mL for $^{\bullet}NO$). In a general way, the results obtained for radical scavenging activities are in line with the ones achieved for DPPH, ABTS and FRAP assays, with the hydroalcoholic extract demonstrating the best results.

The phenolic composition was similar for all extracts, being phenolic acids, flavonoids (derivatives of flavan-3-ol and flavonols) the most representative groups. Caffeoylquinic acids, apigenin-rutinoside, and quercetin-pentoside-hexoside are present in all samples. However, caffeoylquinic acids, quercetin-*O*-deoxyhexoside-rutinoside and quercetin-*O*-rutinoside/quercetin-*O*-galactoside-*O*-rhamnoside/quercetin-*O*-rhamnosyl-galactoside were detected in higher amounts in the alcoholic extract (35.33%, 18.46 and 11.06%, respectively), while proanthocyanidins were prevalent in the hydroalcoholic extract (12.61%).

To evaluate the KB leaves extracts effects on cell viability, an MTT assay was performed, and a range of concentrations was tested (0.1 – 1000 μg/mL). The results revealed that the alcoholic extract led to a decrease of the HaCat viability to 52% after exposure to the highest concentration (1000 μg/mL), while the aqueous and hydroalcoholic extracts did not affect the cells viability. Concerning HFF-1, the viability was maintained up to 60% for all tested concentrations. These values are probably related with the extraction technique employed, since microwaves are able to penetrate the sample and interact with the polar compounds.

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P2.19

Biological and physicochemical properties of *Solanum tuberosum* L. var. Vitelotte anthocyanins rich extract and its impact on membrane, albumin and cancer cells

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MAIN CONCLUSION

The study extract (VE) has broad biological activity, effectively protects the lipid membrane against free radicals and displays anti-proliferative activity in colon cancer cell lines. VE is able to interact mainly with membrane surfaces, by its interference with phospholipid polar heads, causing the rigifying effect, additionally binds to human albumin. Using the HPLC/LC–MS analysis malvidin 3-*O*-p-coumaroyl-rutinoide-5-*O*-glucoside was identified as the most predominant anthocyanin.

INTRODUCTION

There is a group of plants, including berries and red-purple vegetables, which is rich in phenols in particular anthocyanins. Acylated anthocyanins are proved to be the main compounds of red and purple flesh potatoes. The consumption of anthocyanin-rich food products, is associated with the modification of the expression of numerous genes which are involved in the processes of lipid metabolism, inflammation, and energy homeostasis in both liver and fat tissues. Moreover, on the basis of epidemiological *in vivo* studies and articles, it is stated that the consumption of food which contains high levels of flavonoids, might correlate with a decrease in the total mortality risk, as well as reduce the risk of developing certain diseases, e.g. dyslipidemia, hypertension, insulin intolerance, cardiovascular diseases, cancer and diabetes [1]. Furthermore, the nutritional and health-promoting properties of colored potatoes and the increasing interest in relation to their toxicological safety and antioxidant effects both indicate that this potatoes have great potential that enables them to be used in both pharmacological and nutraceutical industry. Therefore, there is a need to conduct extensive research, including the level of cell membranes and the impact of their biomolecules on membrane functioning. Although information regarding how anthocyanin interacts with and is able to protect biological membranes against the processes of peroxidation, as well as regarding the importance of its interaction with biomolecules at the level of the body, namely transport proteins, is still insufficient. We hope that our study is a great contribution in this direction. The aim of our study was to determine antioxidant activity of purple potatoes *Solanum tuberosum* L. var. Vitelotte and its interaction with a membrane and human serum albumin (HSA). In addition, the cytotoxic effects caused by pro-apoptotic properties of VE on two human cancer cells lines (HT-29 and Caco-2) were studied.

MATERIALS & METHODS

Identification and quantitative analysis of the components present in Vitelotte extract were performed using HPLC and UPLC–qTOF-MS/MS methods. A detailed description of these methods was presented in our previous article [2].

The antioxidant activity of the extract was carried out using the fluorimetric method. In this method, lipid oxidation was induced by AAPH compound. The exact description of this method was included in the previous work [2].

Cytotoxicity of VE was investigated in relation to colon cancer - HT-29 and Caco-2 and normal porcine epithelial intestinal cells employing MTT assay.

The effects of compounds present in the hydrophilic and hydrophobic regions of the membrane were determined using the fluorometric method applying MC540 and DPH fluorescence probes.

Determining of a binding between VE and HSA was carried out as a result of the process of albumin fluorescence quenching and was performed following the instructions described by Strugała et al. [2].

RESULTS & DISCUSSION

The analysis showed that the extract contains 357.5 mg of DM/g of total phenolic. Phenolic compounds in the VE belong to anthocyanins and phenolic acids. The dominant compounds are as follows: malvidin 3-*O*-p-coumaroyl-rutinoide-5-*O*-glucoside (22.2%) and chlorogenic acid (57.7%). The results are similar with these obtained by Bontempo et al. [3] who identified as dominant component exactly the same anthocyanin in Vitelotte variety.

Antioxidant activity was assessed referring to the extract ability to inhibit oxidation of phosphatidylcholine liposomes. Based on the kinetics plots, the percentage of oxidation inhibition for VE after 30 min was calculated. The results showed that VE (IC_{50}= 5.53±0.37 µg/mL) protects the lipid membrane against free radicals about 4 times more effectively than commonly used ascorbic acid (IC_{50}= 22.80±2.19 µg/mL) [2].

The results showed that whereas VE is able to decrease the viability of both cancer cell lines (HT-29 and CaCo-2) at the concentrations: 150, 200 and 250 µg/mL, it was essentially non-toxic against normal cells in this concentrations. The VE at the same concentrations decreased the viability of Caco-2 cells by, respectively, 37%, 49% and 78% and HT-29 by, respectively, 30%, 58%, and 74% compared to the control.

Furthermore, fluorescence intensity of the MC540 probe in bilayer changes under the influence of VE, suggests the significant stiffening effect of lipids in the polar region. However, the changes in the fluorescence anisotropy value of the DPH probe, indicate a slight reduction in the fluidity of the hydrophobic region of the membrane.

The results showed that the VE could interact with HSA and quench its intrinsic fluorescence by a static mechanism, the binding process is spontaneous and hydrogen bonding interactions and Van der Waals forces play major roles during the interactions between VE and HSA.

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P2.20

In vitro antibacterial activity against Helicobacter pylori of oligomeric and highly polymerised procyanidin-rich fractions from grape seed extract

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MAIN CONCLUSION

The whole GSE and the OPC-rich and PPC-rich fractions had high activity against *H. pylori*. Nevertheless, the PPC-rich fraction had the highest activity against *H. pylori*, due to the highest content of total procyanidins of this fraction.

INTRODUCTION

Helicobacter pylori (*H. pylori*) affects approximately 50% of the world´s population, sometimes causing chronic active gastritis, which can progress to peptic ulcer and gastric cancer. Resistance to antibiotics is increasing and people demand new natural antimicrobials effective against *H. pylori*, also being an option for the 20% of patients with symptoms for whom antibiotic treatment is ineffective.

Grape seed extracts (GSE) are among the most studied plant-derived products known for their high antibacterial activities. Some publications show that GSE can contribute to the inhibition of the growth of relevant human pathogens such as *H. pylori* [1,2]. They relate this activity with their procyanidin content and, particularly, with the oligomeric procyanidin (OPC) fraction. The aim of this study was to evaluate the antibacterial activity against *H. pylori* of two procyanidin fractions, one enriched in OPC and another, enriched in polymeric procyanidins (PPC), both obtained from a GSE by preparative ultrafiltration and solid-phase extraction processes.

MATERIALS & METHODS

PPC-rich and OPC-rich fractions were obtained by a 10 kDa ultrafiltration membrane and preparative solid-phase extraction (SPE) on a XAD7HP/XAD16 adsorbent resins [3]. Chemical characterization of GSE, PPC and OPC-rich fractions was based on the determination of total phenolic content (TPh) by Folin-Ciocalteu assay, total procyanidin content (TPC), by acid butanol assay, total carbohydrate content (TCH) by GC-FID-MS and total catechins, total OPC and total PPC by NP-HPLC-PAD in a semi-quantitative way [1]. Antibacterial activity of GSE and its fractions against *H. pylori* strains was evaluated following the procedure described in [4].

RESULTS & DISCUSSION

The composition of GSE, OPC-rich and PPC-rich fractions is shown in Table 1.

Table 1. Total phenolic, total procyanidin, and total carbohydrate contents of GSE, PPC-rich and OPC-rich fractions, expressed in g/100 g of dry matter.

Analytical parameters	GSE	PPC	OPC
Total phenolic (TPh)	25.1 ± 0.5	34.9 ± 0.5	49.5 ± 1.3
Total procyanidin (TPC)	8.5 ± 0.3	14.6 ± 0.5	12.9 ± 0.4
Total carbohydrate (TCH)	10.5 ± 0.2	3.6 ± 0.2	0.36 ± 0.02

TPh content is expressed as equivalents of gallic acid; TPC content is expressed as equivalents of cyanidin; TCH is a sum of all monomer and dimer saccharides, and polyols obtained by GC-FID.

The TPh content indicates that both PPC and OPC fractions contained almost 1.4 and 2-fold higher amounts of phenolic compounds than the GSE. On the other hand, the TPC showed that higher enrichment of procianydins was achieved in the PPC-rich fraction (1.7-fold) than the OPC-rich fraction (1.5-fold), but in both cases, it was lower than 2-folds. This difference could be explained by the high sensitivity of the Folin–Ciocalteu assay to other grape seed constituents, such as reducing sugars, that were present at higher amounts in the GSE (10.5 g/100 g).

Results from NP-HPLC show that major components of the GSE were PPC (84%), whereas catechins and OPC were at lower proportion (6 and 9%, respectively). Separation of the macromolecular fraction allowed enrichment of PPC up to 96% of the total flavan-3-ol content, converting this fraction of highly purified PPCs. Purification of the low molecular mass components by SPE allowed the recovery of a fraction enriched in catechins and OPCs up to 58% of its total flavan-3-ol content. Nevertheless, 42% of PPC remained present in this fraction.

The antibacterial activity of the GSE and its OPC and PPC fractions against 6 *H. pylori* strains is presented in Table 2.

Table 2. Effects of GSE, OPC-rich and PPC-rich fractions at 2 mg/mL on the viable counts of different H. pylori strains. Results are expressed as log CFU/mL ± SD (n = 3).

Strains	GSE		PPC		OPC	
	log CFU/mL reduction	MIC (mg/mL)	log CFU/mL reduction	MIC (mg/mL)	log CFU/mL reduction	MIC (mg/mL)
Hp44	2.87	0.075	2.19	0.075	1.88	0.25
Hp48	5.79	0.5	3.07	0.05	1.64	0.25
Hp53	5.28	0.075	3.56	0.05	2.60	0.05
Hp58	4.04	1.5	4.89	0.05	3.49	0.1
Hp59	3.79	0.5	4.35	0.1	1.24	0.5
Hp61	3.24	0.075	4.29	0.1	3.20	1.5

MIC: minimal inhibitory concentration, log CFU/mL values in the same row marked with different letters indicate significant differences by ANOVA post hoc LSD Tukey test (p≤0.05).

The results show that the whole GSE and the corresponding fractions enriched in OPC and PPC had high activity against *H. pylori*. Reduction of log CFU/mL was from 2.87 to 5.79, depending of the *H. pylori* strain. MIC values of GSE were from 0.075 to 1.5 mg/mL. The PPC-rich fraction had the highest antibacterial activity, showing a log reduction of CFU/mL from 2.19 to 4.89 and MIC values from 0.075 to 0.1 mg/mL. In contrast, the activity of OPC-rich fraction was between 1.24 and 3.20 log CFU/mL, depending of the growth of the different *H. pylori* strains, with MIC values higher than those of the PPC-rich fraction. Further studies should attempt to clarify which of the two factors (larger polymer size or higher procyanidin content) is most relevant in the antibacterial activity against *H. pylori*.

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P2.21

Bio-accessibility of bioactive compounds in blueberry smoothie enriched with pea protein: an *in-vitro* gastrointestinal digestion

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MAIN CONCLUSION

This study reveals that conjugation blueberry polyphenols with pea protein increase the bio-accessibility of blueberry's phenolic compounds and protects them from degradation during GI transit.

INTRODUCTION

With the growing interest in plant-based diets, consumers are shifting towards mixing fruits and vegetables with plant-based protein powder such as pea, hemp, soy, and brown rice. In recent years, this trend has increased in popularity through smoothies and shaking drinks, providing a good source of nutrients and incorporating additional protein in the diet (Couteau et al., 2001). Blueberries contain high amounts of polyphenols (Kuntz et al., 2015), with their consumption linked to decreased CVD (Basu et al., 2010) and type-2 diabetes risk (Wedick et al., 2012). However, evidence shows that polyphenols in fruits and vegetables conjugate with proteins, leading to changes in both compounds' structural, functional, and nutritional properties. It shows that a reduction in polyphenol antioxidant activity is caused by a decrease in free polyphenols due to the formation of a stable protein-polyphenol complex. In contrast, protein-polyphenol interactions may stabilize or even enhance polyphenols' antioxidant capacity in conditions like high temperatures or pH, protecting them from any autooxidation reactions. Ribnicky et al. (2014) studied the effects of soy proteins on polyphenols' bioavailability in an animal model. The study reported beneficial effects on the bio-accessibility of polyphenols when complexed with soy proteins. The majority of the studies have assessed the interaction of phenolic compounds with milk proteins, and there is limited evidence evaluating the interaction of plant proteins with phenolic compounds. This research's main objective is to use an upper gastrointestinal model to digest a type of plant protein-polyphenolic mixtures under specific digestive conditions. Using an in-vitro method of digestion makes it possible to collect a real digested sample (Minekus, 2015), more than just a physical mixture for chemical analysis, to elucidate how polyphenol bio-accessibility is hindered, improved or unaltered when co-delivered with proteins.

MATERIALS & METHODS

TIM-1 in vitro digestions

The model consists of four compartments, including the stomach, duodenum, jejunum, and ileum. Each compartment has an inner silicon tubing surrounded by water and encased in a glass exterior. Peristaltic valves determine the transport rate of the digestate between the different compartments. Peristaltic movement is simulated by the squeezing action of the silicon tubes as dictated by the pumping of the surrounding water.

Sample collection

Two hemodialyzers (Sureflux-07L, NIPRO Trading, Shanghai, China) were used for the dialysis and filtration of the bio-accessible components from the intestinal compartments of TIM-1. The jejunal and ileal dialysates and ileal efflux (reflecting the undigested and unabsorbed contents passing to the colon) were collected at 30, 60, 90, 120, 180, 240, and 300 min after meal introduction to TIM-1. The collected dialysates were cooled in ice, weighed and stored at -30°C for subsequent analysis.

RESULTS & DISCUSSION

The result showed that conjugated polyphenols to the pea protein-rich matrix protect it from degradation during GI transit (Fig 1). TIM-1 experiments showed that more significant polyphenols bio-accessibility could be obtained when blueberry was mixed with pea protein (Fig 2). The TIM-1 data showed that PP serves as a carrier for blueberry polyphenols and allowing polyphenols to be released for intestinal absorption. As expected, the most bio-accessibility was in the jejunum. This finding is consistent with previous TIM-1 studies showing higher bio-accessibility of blueberry anthocyanins sorbet to soy flour than unbound anthocyanins in blueberry juice.

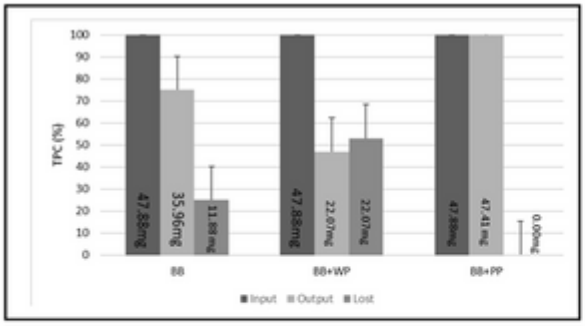

Fig. 1. TPC values (%) in BB, BB+WP, and BB+PP in the input, output, and lost amount (values are mean ± standard error). BB= blueberry; BB+WP= blueberry and whey protein; BB+PP= blueberry and pea protein.

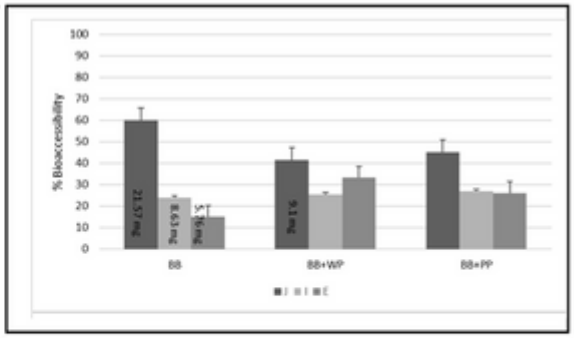

Fig. 2. Bio-accessibility values (%) of TPC in BB, BB+WP, BB+PP in the jejunum, ileum, and efflux (values are mean ± standard error). J=jejunum; I=ileum; E=efflux.

Understanding polyphenol and protein properties when formed into a complex may improve the process condition and parameters for the food containing both protein and polyphenol. An optimal ratio may apply to protein smoothie preparation that yields maximum health benefits for consumers. However, bioactive compounds bounded to the pea protein matrix being more accessible than whey protein or pure blueberry; their bioavailability should be investigated.

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P2.22

*Rol*B oncogene increased synthesis of phenolic compounds and bioactivity of *Dionaea muscipula* J. Ellis

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MAIN CONCLUSION

Transformation of *Dionaea muscipula* J. Ellis with *rol*B oncogene derived from wild *Rhizobium rhizogenes* bacteria conducts to creation of teratomas (transformed shoots) culture with increased synthesis of phenolic compounds. Such type of plants' transformation is a useful biotechnological tool to increase productivity of secondary metabolites (including 1,4-naphtoquinones) and enhances biologically active properties of transformed plants' tissue.

INTRODUCTION

Carnivorous plant *Dionaea muscipula* J. Ellis (Venus Flytrap) belonging to family Droseraceae is a medical plant, because of the ability to synthesize broad range of phenolic compounds like: flavonoids, phenolic acids and naphthoquinones. The major 1,4-naphtoquinone produced in Venus Flytrap tissue is plumbagin, having strong antibacterial, antifungal and cytotoxic activity. Because of the increasing demand for plant material rich in biologically active chemicals carnivorous plants may be a promising source of phenolic compounds for industry. Since obtaining of *D. muscipula* plants from natural habitats is impossible, effective protocols for *in vitro* propagation was developed.

One of the possible ways to enhance plant secondary metabolites production is transformation with wild strains of *Rhizobium rhizogenes* bacteria from family Rhizobiaceae. These microorganisms can pass T-DNA (transfer DNA) and incorporate the set of *rol* oncogenes into the plant genome. *Rol* oncogenes can trigger the complex and pleiotropic effect manifested by changes in plant's secondary metabolites synthesis. *R. rhizogenes*-mediated transformation usually conducts to hairy roots/teratomas (transformed shoots) creation with increased biomass accumulation, characterized by stable genotype.

This study presents how the transformation of Venus Flytrap plants with *rol*B oncogene incorporated in genomic DNA in a single copy affects synthesis of phenolic compounds in comparison to non-transformed plants. Moreover, obtained plants were tested for radical scavenging activity and their bactericidal properties against antibiotic-resistant human-pathogenic bacteria.

MATERIALS & METHODS

D. muscipula cultivated *in vitro* was transformed with *R. rhizogenes* (ATCC 15834). Using PCR and Southern Blotting analysis presence of *rol*B oncogene incorporated in single copy into the plant DNA was confirmed [1]. For the estimation of total phenolic content the spectrophotometric method with Folin-Ciocialteu reagent was used. Content of plumbagin and selected phenolic acids: gallic, chlorogenic, p-coumaric and ferulic acid was analyzed by DAD-HPLC. Radical scavenging activity of extract derived from plants' tissue was examined using method with DPPH radical, while bactericidal tests were performed with MBC (minimal bactericidal concentration) method against two pathogens: *Staphylococcus aureus* ATCC 25923 (Gram-positive) and *Escherichia coli* ATCC 25922 (Gram-negative). All analyses were done on transformed and non-transformated plants (control). One-way analysis of variance (ANOVA) was used to determine significant differences between means (Tukey test at $p < 0.05$ level).

RESULTS & DISCUSSION

Obtained results showed that after transformation with *rol*B gene Venus Flytrap created teratomas with increased biomass production compared to non-transformated plants. Such phenomenon is probably connected with changes in phytohormones metabolism, because transformation with *R. rhizogenes* bacteria may affect auxin biosynthesis. Transformed shoots accumulated higher total phenolic content. Moreover, analysis with DAD-HPLC technique reveled that level of plumbagin and gallic, chlorogenic, p-coumaric as well as ferulic acid significantly increased compared to non-transformated plants' tissue. Similar findings were reported in other plant species after transformation with wild strains of *R. rhizogenes* bacteria. Some authors postulate, that *rol*B genes included in plant genome act as endogenous elicitor in secondary metabolites synthesis [2].

Transformation also affected biological activity of plant-derived extract. Analysis with DPPH radical exhibits that radical scavenging activity of transformed tissue increased significantly compared to control plants. Also, bactericidal properties of Venus Flytrap teratomas increased. The MBC value decreased 33% for both examined pathogens: *S. aureus* and *E. coli*, although usually Gram-negative bacteria are less sensitive to plant secondary metabolites, because they have a lipopolysaccharide membrane surrounding the cell wall. We postulate that increased antioxidant properties of transformed plants are connected with enhanced phenolic acids synthesis. Changes in antibacterial activity were dependent on accelerated plumbagin accumulation in plants' tissue. 1,4-naphtoquinones derived from carnivorous plants may act as a cytotoxic agents and can be used in combat with antibiotic-resistant bacteria.

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P2.23

Protein Precipitation Capacity of Chemically Well-Defined Proanthocyanidin Oligomers and Polymers

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MAIN CONCLUSION

We successfully isolated chemically well-defined proanthocyanidin (PA) fractions from nine different PA-rich plant species, analyzed the fractions by UHPLC-MS/MS and measured their protein precipitation capacities (PPC) as a function of concentration to obtain EC_{50}-values. The acquired PA fractions differed in retention time, mean degree of polymerization (mDP) and prodelphinidin share (PD-%). Main factors affecting PPC were the mDP and PD-%. The effect of galloylation was less obvious.

INTRODUCTION

Proanthocyanidins are bioactive specialized plant metabolites, which characteristically precipitate proteins in aqueous solutions. Their complex formation with proteins has been linked to their many benefits in animal feeds e.g. they have been shown to reduce methane emissions, increase the protein intake from the feed and they have been proven to have anthelmintic properties. The diversity of different PA structures found in plants cause challenges for the isolation, analysis and structure-activity studies of PAs. To overcome these challenges, in this work, we isolated chemically well-defined PA fractions from nine different plant species. Then we studied the PA composition and PPC in a range of PA-concentrations.

MATERIALS & METHODS

Plant material from nine different PA-rich plant species was extracted with acetone-water (8/2; v/v). Extracts were pre-fractioned by Sephadex LH-20 column chromatography before the final fractionation by a semi-preparative HPLC method developed by Leppä et al. [1] Based on the UHPLC-MS/MS analysis the collected fractions were combined into five chromatically equally sized samples; altogether 45 PA fractions were obtained. The combined fractions were quantitatively analyzed by UPLC-DAD-QqQ-MS/MS to obtain mDP, PD-% and information of possible galloyl groups attached to the PA oligomer or polymer.

The PPC of the PA-fractions was determined by using 200 µM BSA in citrate buffer at pH 5. Equal amounts of 200 µM protein solution and tannin solution in six different tannin concentrations were mixed and the absorbance of the formed haze was measured by a well-plate reader at 414 nm.

RESULTS & DISCUSSION

The semipreparative fractionation resulted in chromatographically well-defined and structurally varying PA fractions. The PA fractions differed by retention time, PD-%, mDP and degree of galloylation. Chromatographically, the overall trend was that when moving towards later retention times, the mDP of the fractions increased, and for the PA oligomers containing galloyl groups the degree of galloylation decreased. Also, the PD-% varied along the retention time for all fractions.

With the quantitative MS/MS data, we could calculate the mean molar concentrations of the PA fractions used in the PPC tests and thus obtain molar concentrations for the EC_{50}-values. Overall, increased tannin concentration had positive effect on the PPC. The results yielded structure-activity patterns reported also in earlier studies [2]; the higher the mDP and the PD-% of the PA, the better its PPC was. While mDP correlated better than the PD-% with the PPC EC_{50}-values, high PD-%

seemed to be a pre-requisite for most effective protein precipitators: high mDP with low PD-% resulted in lower PPC than medium mDP with high PD-%. The effect of galloyl groups on the PPC was not that noticeable as the samples containing galloyl groups all had low mDPs.

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P2.24

Sugarcane polyphenols as non-antibiotic growth promoters in animal feeds

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MAIN CONCLUSION

The focus of this poster is on previously conducted pre-clinical and clinical studies both in vitro and in vivo, with broiler hens. Here, Polygain™ has demonstrated potential in the treatment and prevention of Coccidiosis by inhibiting the establishment of sporocysts. Polygain™ Significantly Reduces Infection Load In Vitro. Polygain™ offers a natural and effective alternative to commonly used growth promoting antibiotics.

INTRODUCTION

Polygain™ is a bioavailable, regulatory approved, natural sugarcane extract rich in antioxidant, anti-inflammatory and cell signalling properties. The patented supplement Polygain™, has been trialled in commercial settings with various animals. This includes: Ruminants, Horses, Poultry, Pigs, Finfish and Crustaceans.

The growing prevalence of microbial resistance has consumers calling for more antibiotic-free produce and farming practices. Unchecked, antibiotic resistance is expected to account for 10 million deaths each year by 2050 1. Polygain™ is a polyphenol-enriched sugarcane extract which may effectively be utilised as an alternative to growth promoting antibiotics. Genetic selection for economically desirable traits in addition to consistent over-supply of feed predisposes birds to chronic gastrointestinal inflammation. This inflammation inhibits nutrient absorption and enables opportunistic pathogens, including species of *Eimeria*, to become established. The ensuing Coccidiosis is believed to affect up to 92% of broilers in a single population at any given time. It is characterized by diarrhoea, permanent damage to the gastrointestinal tract and premature mortality[2]. It is estimated that $3 billion is lost globally as a direct result of *Eimeria sp*. infections each year, with a further $6 billion lost to secondary bacterial infections and sub-sequential necrotic enteritis[3]

MATERIALS & METHODS

In vitro study:

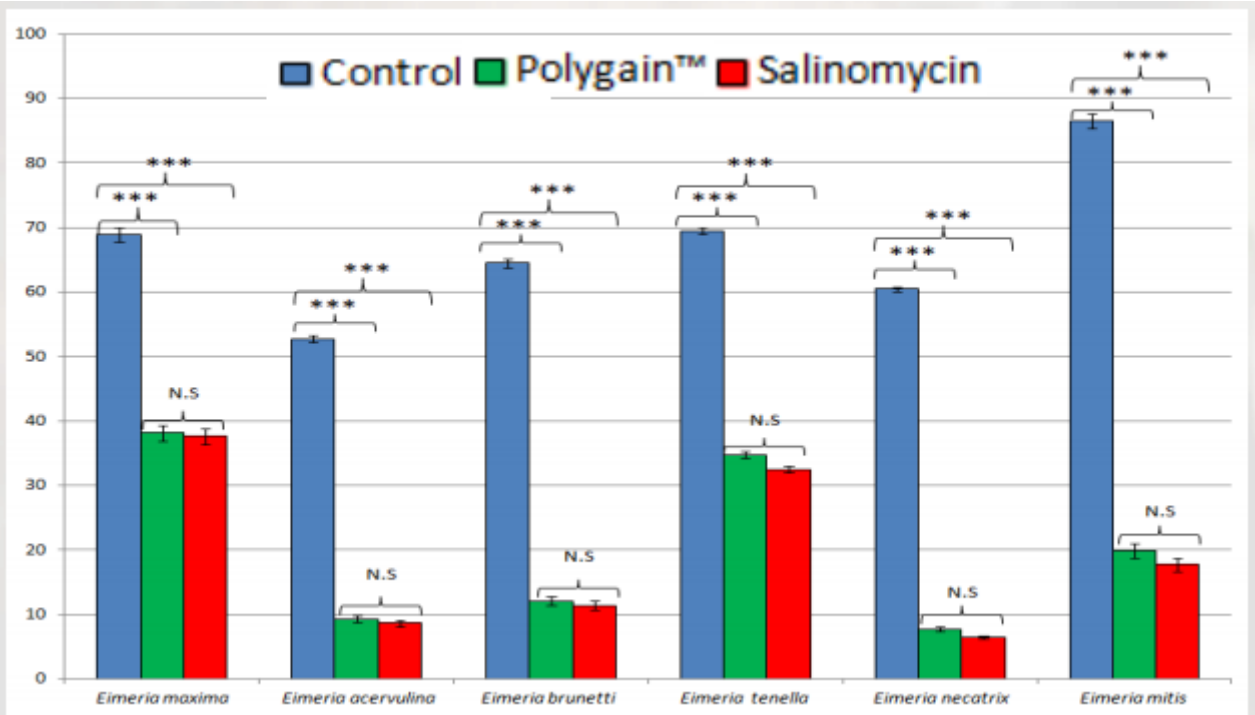
All 6 Coccidia species were incubated in RPMI nutrient media for 14 hours at 37 oC before the total number of Eimeria sporozoites were recorded. ϒ The 3 experimental groups included a control of 2000µl RPMI nutrient media, 20µl Polygain™ with 1980µl RPMI nutrient media and 20µl Salinomycin also with 1980µl RPMI nutrient media.

In vivo study

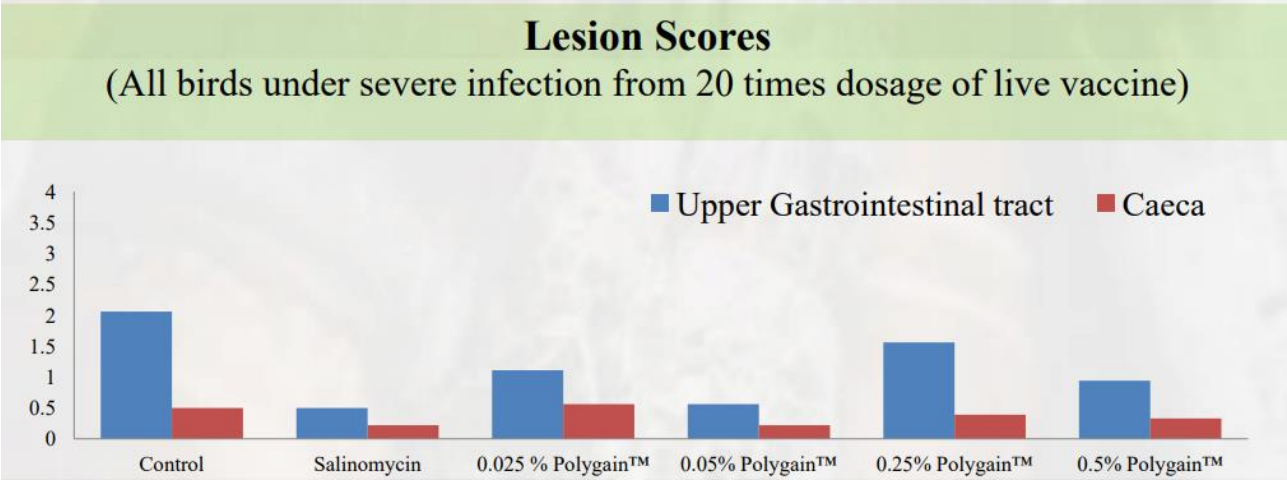
From hatching, 4 treatment groups were provided with a different dose of Polygain™. The 5 th group received Salinomycin, while hens in the control group received no supplementation. On day 14, hens were inoculated with 3 common strains of Eimeria sp. ─ On day 21, 18 hens from each group were culled for lesion scoring and caecal collection. ─ On day 35, the FCR, growth rate and weight of each hen was recorded

RESULTS & DISCUSSION

In vitro study. The In Vitro study illustrated the effectiveness of Polygain™ against six of the most prevalent species of Coccidia.



In vivo study. Polygain™ offers a natural and effective alternative to commonly used growth promoting antibiotics.



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P2.25

The bioactive & bioavailability properties of polyphenol- rich extract from sugarcane (*Saccharum officinarum*)

Barry Kitchen[1,2], Matthew Flavel[1,2], Roya Afshari[1], Mia Bettio[1], Stefanie Prendegast[1], Xin Yang[1]

[1]The Product Makers, Melbourne, Australia. [2]La Trobe University, Bundoora, Australia

MAIN CONCLUSION

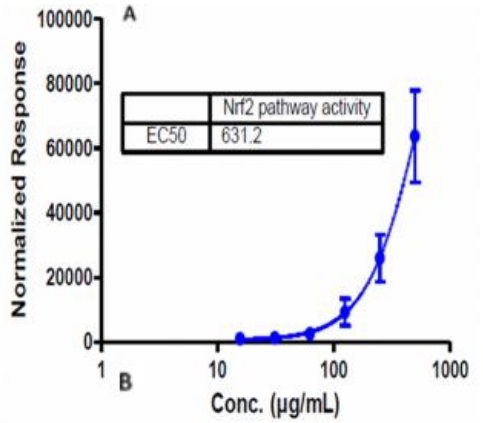
TPM's Enriched Sugar cane extracts have numerous beneficial properties with many potential applications in dietary supplements and in human health and wellbeing.

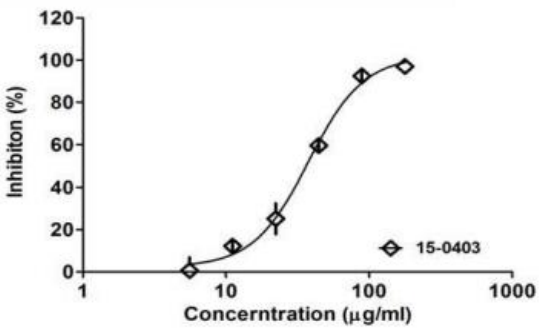
INTRODUCTION

Sugarcane (Saccharum officinarum) is a tall perennial grass indigenous to tropical South East Asia, but now grown in over 90 countries globally principally to produce raw sugar (1). Post-harvest processing of sugarcane results in several by-products such as molasses, with well over 60 million tonnes produced annually. Sugarcane and its by-product, molasses is known to be rich in unique phytochemicals including polyphenols and flavonoids which deliver powerful antioxidant, anti-inflammatory, anti-microbial, anti-viral, anti-proliferating, and immunomodulation properties (2-6). Here we summarize the bioactive properties of our Polyphenol Rich Sugarcane Extract (PRSE), essentially free of sugars, from this abundant crop. We have unlocked the goodness of the Sugarcane plant, deserving its elevation to the status as a Super Food, beyond its original use as a source of raw and white refined sugar.

MATERIALS & METHODS

PRSE and Anti-oxidant , Anti-inflammatory and Antiproliferative properties Analyte Concentration, µg/g dry weight PolynolTM Chlorogenic acid 74.29 Caffeic acid 7.54 Syringic acid 107.57 Vanillin 2.13 Homoorientin 0.58 Orientin 4.50 Sinapic acid 1.73 Vitexin 1.62 Swertisin 5.25 Diosmin 227.00 Apigenin 0.01 Tricin 0.40 Diosmetin 0.16 Total polyphenols 432.78 Table 1: Polyphenol Content of PRSE Table 2: Oxygen radical absorbance capacity (ORAC) of PRSE ♣Activates Nrf2, Master Redox Switch, generates indigenous anti-oxidant compounds ♣Modulation of Oxidative stress ♣Inhibits pro-inflammatory cytokines, TNF-α, NF-Kβ ♣Inhibits COX2, LOX-5, PLA2, PGE2 Arachidonic acid inflammatory pathway ♣Anti-proliferative effects via alteration in cytokines, VEGF-1 and NF-κB expression.

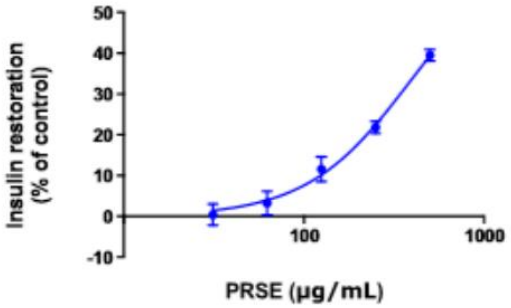




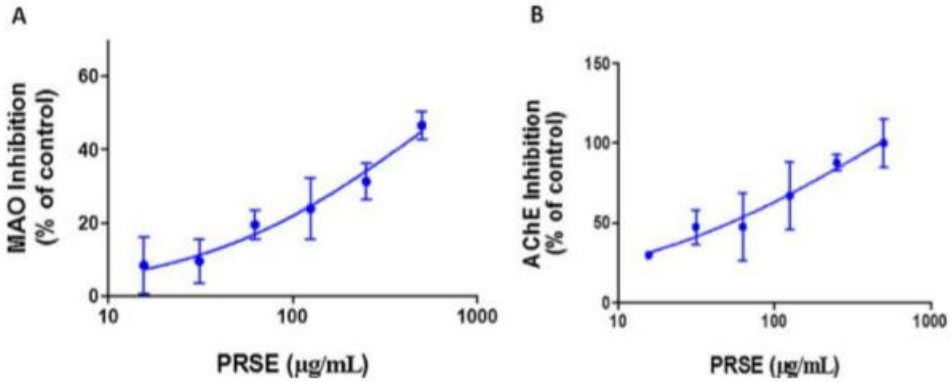
RESULTS & DISCUSSION

PRSE and Skin Protection and Health •Provides UV protection and control of Melanin metabolism, dark spot reduction •Inhibits collagenase and Elastase, key enzymes in skin cells regarding wrinkles •Inhibits MMP-1, a biomarker produced by UV stress, which causes excessive breakdown of connective tissue, increasing wrinkles and aging •Balances of hydrating minerals

PRSE and Anti diabetes and digestive health •Reduces the glycemic response/glacaemic index of ingredients and food •Activates insulin production/secration from dysfunctional pancreatic β-cells •Modulates intestinal transporters, GLUT-2, GLUT-4, SGLT-1



PRSE and Anti-aging and Cognitive Health •Upregulates neuronal genes relevant to cognitive health and anti-aging •Inhibits Monoamine Oxidase (MAO), possible elevating effects on serotonin and dopamine levels •Inhibits acetylcholinesterase (AChE)



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P2.26

Hydrophobicity and *logP* of hydrolysable tannins

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MAIN CONCLUSION

The partition coefficients (*logP*) of 47 purified and characterized HTs were measured to determine which HT stuctural features have the largest effect to their hydrophobicity. The most notable structural features affecting the hydrophobicity of HTs were the number of free galloyl groups, acyclic vs cyclic polyol, substitution of the anomeric position of glucose and $^{4}C_{1}$ versus $^{1}C_{4}$ conformation of the glucopyranose core.

INTRODUCTION

Hydrolysable tannins (HT) are plant secondary metabolites, which have been shown to possess pharmacological and nutritional benefits [1]. Different activities and properties, such as how well individual HTs interact with lipid bilayers, dictate the direction where they are transported in biological systems once ingested and how efficiently the transfer is done. The structural features of the HTs, such as hydrophobicity, can be useful predictors for these activities. Hydrophobicity of a compound can be determined experimentally based on the amount it partitions to either organic solvents or water. The most common solvent pair used when determining hydrophobicity is that of *n*-octanol/water.

MATERIALS & METHODS

Hydrolysable tannins (HT) are plant secondary metabolites, which have been shown to possess pharmacological and nutritional benefits [1]. Different activities and properties, such as how well individual HTs interact with lipid bilayers, dictate the direction where they are transported in biological systems once ingested and how efficiently the transfer is done. The structural features of the HTs, such as hydrophobicity, can be useful predictors for these activities. Hydrophobicity of a compound can be determined experimentally based on the amount it partitions to either organic solvents or water. The most common solvent pair used when determining hydrophobicity is that of *n*-octanol/water.

RESULTS & DISCUSSION

Upon the first inspection of the measured *logP* values of the 47 HTs (Figure 1) it was broadly determined that galloyl glucoses (GGs) and gallotannins (GTs) were more hydrophobic than similarly sized ellagitannins (ETs). However with a closer inspection there were more specific structural features that had an impact on the hydrophobicity of HTs and this was especially true within ellagitannins.

The hydrophobicities of GGs increased based on their galloylation degree all the way from mono- to pentagalloylglucose where all the five possible positions of the polyol glucose core have been galloylated. Increased galloylation beyond pentagalloylglucose via meta-depside bonds forms GTs and their hydrophobicity decreased from that of pentagalloylglucose at least until octagalloylglucose.

C-glycosidic ETs had generally much lower hydrophobicities than the similarly sized ETs with a glucopyranose core but within c-glycosidic ETs α-anomers proved to be more hydrophobic than the corresponding β-anomers. Within cyclic ETs the ones with more free galloyl groups and less HHDP and/or DHHDP groups had higher hydrophobicities. Additionally, ETs with the polyol glucose in $^{1}C_{4}$ conformation were more hydrophobic than the ones with $^{4}C_{1}$ conformation. This expanded

knowledge on the hydrophobicities of HTs can be used to predict the hydrophobicity of HTs not measured in this study as well as to estimate their possible affinity to interact with lipid bilayers.

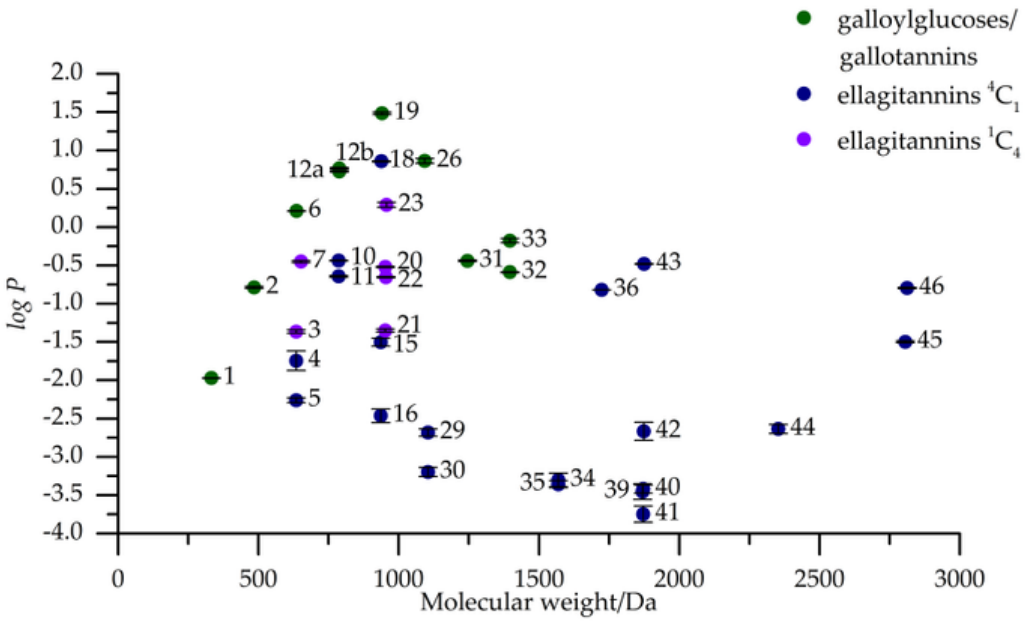

Figure 1. *LogP* values of 47 HTs plotted against their molecular weights. Galloylglucoses/gallotannins, $^4C_1$ glucose core ellagitannins and $^1C_4$ glucose core ellagitannins in separate series. For the numbering of HTs see Figures 1-2 and Table A1 in [2].

The study was funded by the Academy of Finland (grant number 310549 to Maarit Karonen)

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P2.27

UPLC-PDA-Q/TOF-MS profiling of phenolic compounds and anti-neurodegenerative potential of Hippophaë rhamnoides L. berries

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MAIN CONCLUSION

Flavonols constituted 98% of phenolic compounds in sea buckthorn berries. Fruits moderately and strongly inhibited the activity of AChE and BuChE, respectively, as an anti-neurodegenerative potential. The results provide information to determine identity and purity, check the origin and perform quality control of sea buckthorn as well as to target the selection of cultivars for industrial crops. The sea buckthorn cultivars may be a component of functional products rich in flavonols.

INTRODUCTION

Sea buckthorn (*Hippophaë rhamnoides* L.) belongs to the Elaeagnaceae olive family and is a fruiting and deciduous shrub. In some countries, inter alia, Germany, Finland, Czech Republic, Ukraine, Estonia, Belarus, Latvia, Russia, China, Japan, Slovakia, Chile, and Canada, sea buckthorn is used on an industrial scale. Application of sea buckthorn for therapeutic purposes is derived from Tibetan and Mongolian medicine and ancient Greece, but healthful properties of compounds from this plant are currently being researched. Studies conducted on cardiac patients proved that flavonoids from sea buckthorn contributed to lowering total cholesterol, triacylglycerides and the low-density lipoprotein (LDL) fraction and increasing the high-density lipoprotein (HDL) fraction. The cardioprotective effect of sea buckthorn intake can be attributed to its content of flavonoids and ß-sitosterol. Flavonol extracts from sea buckthorn berries reduced levels of serum glucose, serum triglyceride and serum cholesterol in mice, and effects on glycometabolism may be associated with glycogenesis [1].

Currently Poland is becoming one of the major countries in Europe interested in growing sea buckthorn on an industrial scale. So far, analysis of phenolic compounds in reference to anticholinergic activity in sea buckthorn has not been carried out. In this context, the first aim of this paper was detailed identification and quantification of phenolic compounds from berries of selected *H. rhamnoides* cultivars grown in Poland. The second objective was to assess the anticholinergic activity of berries and to determine the correlations between the composition and pro-health potential, as well as to establish the activity of the phenolic fraction in relation to the $ABTS^{\cdot+}$ reagent by on-line HPLC-PDA analysis.

MATERIALS & METHODS

Plant material:

Six sea buckthorn (Hippophae rhamnoides L.) cultivars: Botaniczeskaja-Lubitelskaja, Luczistaja, Moskwiczka, Podarok Sadu, Józef, and Aromatnaja were analyzed. Mature raw fruits were collected between July and August 2018 from orchard located in Dąbrowice (51°56′N 20°06′E) of Research Institute of Horticulture (Skierniewice, Poland).

Methods:

1. Identification and quantification of phenolic compounds by UPLC-PDA-Q/TOF-MS and UPLC-PDA methods according to Tkacz et al. (2020) [2]
2. Determination of anticholinergic activity as AChE and BuChE inhibition according to Tkacz et al. (2020) [2]
3. Antioxidant on-line profiling by HPLC-PDA coupled with post-column derivatization with $ABTS^{\cdot+}$ reagent according to Tkacz et al. (2020) [2].

RESULTS & DISCUSSION

In all sea buckthorn cultivars, flavonols were present in the following order of concentration: monoglycosides (from 61% to 68% of total flavonols) > diglycosides (from 24% to 30%) > triglycosides (from 3.9% to 6.1%). Isorhamnetin derivatives ranged from 66% to 72% of total flavonols. The percentage of quercetin derivatives ranged from 25% (Moskwiczka) to 32% (Botaniczeskaja-Lubitelskaja). It is worth mentioning that isorhamnetin-3-*O*-rutinoside of natural origin promotes apoptosis of human myelogenous erythroleukaemia cells. Other studies have shown that isorhamnetin-3-O-rutinoside may affect the control of adipose tissue mass because it inhibits adipogenesis in 3 T3-L1 adipocytes [3]. This study also proves high concentration of: isorhamnetin-3-O-glucoside, quercetin-3-O-glucoside, isorhamnetin-3-O-glucoside-7-O- rhamnoside and quercetin-3-O-rutinoside. Pearson's correlations between acetylcholinesterase (AChE) and butylcholinesterase (BuChE) inhibition and phenolic acid content were low (r = 0.388 and 0.355), and high for flavonols (r = 0.851 and 0.614). The PCA biplot showed the highest correlation between anticholinergic activity and all-trans-ß-cryptoxanthin, quercetin-3-O-glucoside, isorhamnetin-3-O-(2-rhamnosyl)glucoside, kaempferol-3-O-hexoside-7-O-rhamnoside, isorhamnetin-3-O-(6-rhamnosyl)hexoside, isorhamnetin-3-O-rutinoside, and isorhamnetin-3-O-glucoside concentrations. The post-column derivatization with ABTS·+ reagent proved that phenolic acids were stronger free radical scavengers than flavonols contained in tested *H. rhamnoides* fruits.

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P2.28

Phenolic profile and biological activities of *Chaenomeles* microencapsulated powders

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MAIN CONCLUSION

As well as the drying method, also the biopolimer used affected the phenolic profile and biological activities in the obtained powders. Among the drying techniques applied, freeze-drying (FD) resulted in the highest retention of polyphenols, while among the carrier agents maltodextrin was found to be the best biopolymer for obtaining high-quality powder. Also the use of FD and maltodextrin ensured that powders with the lowest content of undesirable 5-hydroxymethylfurfural (HMF) were obtained.

INTRODUCTION

The production of food powders is growing day by day. The global fruit powder market in 2017 was valued at $13.52 billion and a compound annual growth rate (CAGR) of 7.4% is expected to be reached by 2025. Northern Europe and Asia are the two most important markets for fruit powders, while the growing interest in functional beverages has strengthened the position of the European market. The increase in consumer interest in forgotten or unpopular fruits, observed over the past two decades, has made Chaenomeles the object of interest for the food industry. Japanese quince (JQ) fruit is an example of a raw material with high processing potential, but at the same time the high content of acids disqualifies it from direct consumption. JQ fruit can be used as a rich source of polyphenols, the most largest fraction being proanthocyanidins (57.06 g/kg dw, of which 70% is polymeric procyanidins), which is reflected in the significant antioxidant activity measured by the ORAC test (68.19 mmol Trolox/100 g dw) [1], and it exhibits a number of health-promoting activities [2]. Therefore, these fruits are a valuable raw material for which powder production can be an innovative method of application for food, increasing their final nutritional value and pro-health qualities. Fruit powders can be used in the bakery and confectionery industry, dairy products, beverages, snacks, and others. Above all, beverages dominate the market owing to the high application of fruit powders in smoothies, fruit drinks, energy drinks, and carbonated drinks.

MATERIALS & METHODS

Juice obtained from JQ fruit was mixed with 15% (w/w) inulin, maltodextrin and mix of those two (inulin: maltodextrin 2:1 and 1:2 w/w, respectively). All samples were subjected to different drying techniques: freeze drying, spray drying, and vacuum drying at 50, 70 and 90°C for respectively, 72, 48 and 24 h. Identification and quantification of polyphenols by the UPLC–PDA–ESI–QTOF–MS method was performed as described by [3]. Analysis of polymeric procyanidins was performed by phloroglucinolysis method as described by [3]. The analysis of HMF was performed using the Acquity UPLC system (Waters Corp., Milford, USA) according to [4]. All incubations were done in triplicate. Results were expressed as mg per kg of dw. Antioxidant activities (ABTS, FRAP, ORAC) and the inhibition of α-glucosidase, pancreatic lipase and acetylcholinesterase were measured as reported by [4]. All samples were assayed in triplicate and the result was expressed as IC_{50} (mg of dried sample per mL of enzyme).

RESULTS & DISCUSSION

The total phenolic content (TPC) in JQ juice powders obtained using different carrier agents and drying methods ranged from 1359.9 to 133.2 mg/100 g dw for, respectively, maltodextrin (MA) after freeze-drying (FD) and IN:maltodextrin (MA) 2:1 after vacuum drying (VD) at 90°C. The main group (over 80% of all phenolics) comprised flavan-3-ols. Procyanidin trimers, procyanidin B2 and C1 (17, 14 and 11% of TPC, respectively) were predominant compounds in the flavan-3-ol group. Polymers of procyanidins account for 17% and phenolic acids account for 12% of the TPC. The selected drying methods had a significant impact on the phenolic content ($p>0.05$). Comparing the influence of the drying method on the average content of phenolic compounds in JQ juice powders, they can be ranked by decreasing content: FD>SD>VD 50°C>VD 70°C>VD 90°C. The highest TPC content was found in powders after FD, and VD at 90°C in relation to FD caused their reduction by as much as 87%. The HMF content in JQ juice powders ranged from 0.1 (MA and IN:MA 2:1 after FD) to 315.6 mg/100 g dw (IN after VD at 90°C). The use of VD at 90 °C resulted in a 223-fold increase in HMF concentration compared to FD. The highest antioxidant capacity, both ABTS and FRAP, was shown by MA powder after FD. In turn, the highest oxygen radical absorption capacity (ORAC) was shown by IN and MA powder (1:2) after VD at 50°C, while the lowest was observed for IN after VD at 50°C. Powders obtained by SD and VD at 70°C had the lowest antioxidant capacity, while after FD and VD at 50 and 90°C they had the highest values of antioxidant capacity. IC_{50} for α-glucosidase ranged from 13.8 (MA after FD) to 21.8 mg/mL (MA after VD at 90°C). There was no effect of the carrier agents on the ability to inhibit α-glucosidase. Of all drying methods used, SD significantly reduced the ability to inhibit acetylcholinesterase compared to other methods – almost two times lower average activity compared to powders subjected to FD.

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P2.29

Profiling of polyphenols by LC-MS-ESI-QTOF, characteristics of nutritional compounds and in vitro effect on α-amylase, α-glucosidase, lipase activities of *Prunus avium* and *P. cerasus* leaves and fruits

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MAIN CONCLUSION

Presented results showed that sour cherry leaves are valuable natural source of polyphenolic, amino acids and mineral compounds. Leaves are promising sources for creating of new products with beneficial effects on industrial.

INTRODUCTION

In recent decades numerous studies have demonstrated that fruits, berries, vegetables, and herbs and their roots and bark have been known as a good source of various phytonutrients, dominantly vitamins, polyphenols, minerals, amino acids, fiber, etc. (Vincente et al., 2014). However, researchers knowing the great potential of plants still focus on new possibilities to find some sources of phytonutrient compounds not only from conventional but often from unconventional sources (Teleszko & Wojdyło, 2015; Wojdyło et al., 2021; Zdunić et al., 2020). Leaves of fruits and berry are recognized, in the word as "superfoods" due to the high content of bioactive natural products and the health benefits deriving from their consumption (Ferlemi & Lamari, 2016).

Sweet cherries (*Prunus avium* L.) and sour cherries (*Prunus cerasus* L.) are among the most important fruits, widely growing crops, and consumed fresh (mainly sweet cherries) or processed (mainly sour cherries) in many countries worldwide.

Most biological activities of these fruits were attributed to a wide array of phytonutrients such as vitamins, organic acids, fatty acids, mineral composition, pectins and amino acids(Kelley et al., 2018), beside their attractive appearance and taste. Besides, still little is known about the chemical composition, isoprenoids, triterpenes or amino acids, or even sugars and organic acids of sweet and sour cherry leaves. To our knowledge, still little information is available on the influence of these compounds on inhibition of some hyperglycemic, obesity, cholinesterase, or anti-inflammatory enzyme activity.

With regard to this topic, the main objective present work was to comprehensively study polyphenolics by LC-MS-ESI-QTOF, amino acids and triterpenic detailed analysis of sweet and sour cherry fruits and leaves and to evaluate antioxidant capacity, and in vitro biological activities against enzymes related to obesity (pancreatic lipase) and diabetes (α-amylase, α-glucosidase) effects.

MATERIALS & METHODS

Three sweet (Rivan, Techlovan, Summit cvs/) and sour (Łutówka, Turgieniewka, Topaz cvs.) cherry leaves and fruit cultivars cultivated in the Research Station for Cultivar Testing in Zybiszów near Wroclaw (Poland) were used in this study. Analysis of polyphenols, triterpene and amino acids of fruits and leaves was carried out on an LC-MS/PDA-QTOF system (Waters, USA) and Acquity UPLC system (Waters, USA) cooperated with a photodiode (PDA) detector. Polymeric procyanidins, amino acid, organic acids, sugars were determined by Wojdyło et al. (2020) using HPLC-PDA-FL (Waters Co.; Milford, CT, USA) and HPLC-ELSD (PL-ELS 1000; Merck; Hitachi, Japan), respectively. Pancreatic lipase, α-glucosidase, and α-amylase inhibitory activity levels were determined using the method proposed previously by Wojdyło et al. (2020). The FRAP, ABTS ORAC assays were determined using the method proposed previously by Wojdyło et al. (2020).

RESULTS & DISCUSSION

The fraction determined the chemical content and biological properties more strongly than the cultivars. A total of 27 phenolic compounds were identified by LC-MS-ESI-QTOF, and they included flavanols > phenolic acids > flavonols and flavanols >> anthocyanins for fruits. Higher total phenolic content was found in leaves(854.5-2955.6 mg/100 g dw) than fruits (288.4-871.7mg/100 g dw). Pearson's correlation and PCA showed that phenolic compounds, minerals and amino acids were the main contributors to the in vitro enzymatic activity. Leaves were the most active in ORAC capacity, and present high potential for inhibition of some enzymes including α-amylase. Fruits characterized by a high content of anthocyanins, triterpene compounds, organic acid and sugars were associated with ABTS and FRAP capacity and inhibition of α-amylase, α-glucosidase and pancreatic lipase.

The obtained results indicate that cherry leaves, especially sour, possess significant potential for further research and possible utilization as a component of various pharmaceutical products. Up to now, leaves were a waste product, but their utilization to generate a value-added product would provide economic and health benefits. Therefore, the sour cherry leaves may be used as a potential source of valuable ingredients in herbal teas, additive for functional foods, medicinal products, dietary supplements, and other products, which could open opportunities in novel applications. The results also increase the interest and potential use of leaves of sweet and sour cherries as a consolidated source of pharmaceutical agents. In the future, it will be valuable to analyze new formulations based on the sour cherry leaf fraction in in vivo studies.

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P3.1

Prospecting for bioactives with group-specific and molecular networking MS/MS approaches

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MAIN CONCLUSION

We were able to use rapid bioactivity tools to screen 250 plant species in a day for protein precipitation and oxidative activities. Two different types of MS/MS approaches enabled us to prospect for both the bioactive compound groups and individual compounds in the most bioactive species. The group-specific polyphenol fingerprinting tool was superior with large polyphenols while the feature based molecular networking tool was more visual for the overall prospecting of individual bioactives.

INTRODUCTION

Exotic plant species in botanical gardens are potential sources for many types of bioactive compounds. We tested during a plant screening course, whether it is possible to rapidly detect species with potential compounds for protein precipitation and oxidative activities. We wanted to further check, if the positive hits could be rapidly screened by two UHPLC-MS/MS instruments. We were especially interested to test how a group-specific MS/MS method with Waters Xevo triple quadrupole MS compares to the high-resolution MS/MS method with Thermo QExactive Orbitrap MS. The quantitative triple quadrupole data flow followed our exiting lab protocols, while the high-resolution data flow was converted to correspond the requirements of the Global Natural Products Social Molecular Networking mass spectrometry ecosystem (GNPS) to create a feature-based model of the molecular network (FBMN).

MATERIALS & METHODS

Plant material was crushed and extracted with acetone/water (80:20, *v*:*v*) in a ball mill. Acetone was removed in vacuo and the water phase was mixed separately with a BSA solution and a pH 10 buffer. These tests revealed plants with best potential to protein precipitation and oxidative activity. Bioactive plants were re-collected using different sampling types to emphasize the effects of alkaline and enzymatic oxidation.[1] Freeze-dried samples were extracted quantitatively with acetone/water (80:20, *v*:*v*), extracts were concentrated to water-phase and freeze-dried. Before the UHPLC-MS/MS analyses samples were filtered using either PTFE (the control samples and the oxidative activity samples) or nylon filters (the protein precipitation capacity samples). The two similar UPLC systems (Acquity UPLC®, Waters Corp., USA) were combined with a triple-quadrupole MS (Xevo TQ™, Waters Corp., USA) and with a hybrid quadrupole Orbitrap™ MS (Q Exactive™, Thermo Fisher Scientific GmbH, Germany).

RESULTS & DISCUSSION

All the samples that were tested positive with rapid screening tests, were successfully analysed by UHPLC-MS/MS tools to reveal the compound groups and compounds responsible for the observed activities. As expected, the MRM-based group-specific tool was more efficient rapidly revealing the compound groups responsible for the activities, together with the compound peaks with most significant activity. In contrast, the feature-based molecular network was better in revealing all the

individual compounds that were affected by the bioactivity-based treatments. In addition, the FBMN connected the smaller metabolites to each other based on their structural similarities and made the evaluation of studied bioactivity and its impact to the metabolites easy. However, FBMN was unable to detect the largest polyphenols such as polymeric proantocyanidins.[2] In practice this means that both the MS/MS methodologies are needed, if all different types of polyphenols are to be revealed as bioactives in the tested plant species. Both enzymatic and alkaline oxidation methods functioned well to reveal the oxidatively active polyphenols. Surprisingly, nylon filters worked well in removing the tannin-like compounds from water solutions, thus well mimicking the tannin-protein interactions. This fact should be taken into account in all studies where nylon is used to filter polyphenol-rich plant extracts.

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P3.2

Effect of climate change on the polyphenolic composition of the main varieties of grape from La Rioja (Spain) and new oenological strategies to correct these effects on the quality of red wine

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MAIN CONCLUSION

Based on the analysis of the results from the first year of experiments and previous experiences of the research group, the concentrations and evolution tendencies of the different compounds monitored were obtained. From these preliminary results the effect of two of the oenological techniques employed were found to affect significantly to the concentration of three of the main anthocyanin derivatives studied.

INTRODUCTION

Even though grapes grow all around the planet those aimed for high quality wine production occur in areas with very specific climate conditions. Moreover, each individual variety of grape requires conditions that are even more particular. Thus, any change on the climate or meteorological patterns can affect the chemistry of the grape and the final quality of wine. Recent studies have explored the impact of climate change on the worldwide production of grape [1], and some highlight an advance on the vintage date associated with a temperature increase, this being for instance, 18-21 days in the South of France from 1940 to 2000 [2].

Regarding the chemical composition of wine, anthocyanins and condensed tannins are the main type of polyphenolic compounds found in red wine. Anthocyanins are the main pigments of red grapes whereas tannins play an important role on the astringency and bitterness of wine. During wine making process, both types of compounds undergo chemical transformations leading to more stable polymeric pigments. The mechanisms of these reactions and the structures of these derivative pigments have been a recurrent topic of study. With the advances on the analytical techniques, some of these structures have been elucidated, discovering products of the direct and indirect condensation of anthocyanins and tannins. Additionally, anthocyanins have been found to react with other minor components present in wine, yielding a new class of compounds called pyranoanthocyanins and producing changes in the hue of the wine [3].

A better knowledge of the formation kinetics and evolution of these pigments during wine production will allow the producer to adapt the oenological practice to control and optimise the final quality of red wine. This work is aimed towards the study of climate influence on the polyphenolic composition of must and wine from *Tempranillo* grape cultivar, the main variety of grape in La Rioja (Spain), and its evolution during wine production.

MATERIALS & METHODS

Over two years small-scale experiments were performed in a winery in La Rioja with grapes of *Tempranillo* cultivar from two geographical areas with different climate conditions. Samples of the must/wine were taken during the process, from the start of the alcoholic fermentation (AF) until the end of the malolactic fermentation (MLF). In addition, different oenological techniques were

employed, like the addition of tannins, microoxigenation, ionic exchange and mixture of grape varieties (*Graciano* cultivar). For all samples, 13 anthocyanins were monitored by HPLC-DAD and 23 anthocyanin derivatives and 29 tannins by HPLC-DAD-MS/MS.

The separation conditions used for the analysis had been previously optimized and employed in our group [4]. Malvidin-3-*O*-glucoside and (+)-catechin were used for calibration purposes, and the results of the quantification of the compounds was obtained in equivalents of malvidin-3-*O*-glc for anthocyanins and anthocyanin derivatives and (+)-catechin for tannins.

RESULTS & DISCUSSION

The concentration of 13 anthocyanins, 23 anthocyanin derivatives and 29 tannins was obtained in equivalents of the corresponding standard for each of the samples from each experiment performed in the winery. An evolution curve was drawn for each compound and the main tendencies and differences between experiments were analysed. The following results correspond mainly to the experiments performed on the first year of the project. Even though they are preliminary, all the tendencies mentioned below are in agreement with previous work of our research group.

Anthocyanins are extracted from grape to must quickly during the first days of maceration and AF and they reach a maximum level at day 4-5. Formation of anthocyanin derivatives starts quickly after anthocyanin extraction from grape and maximum levels are reached during AF, a few days after apex of anthocyanins. During the last few days of AF a decreasing tendency is observed for all compounds, and when MLF starts a new increasing tendency occurs. The final part of MLF produces again a decreasing tendency for all derivatives. In general, extraction of tannins from grape occurs more slowly than that of anthocyanins. Their concentration increases during AF, and they remain stable or even show an increasing tendency towards the end of AF. During MLF all monomers remain quite stable.

For the analysis of the influence of the oenological techniques employed on the concentration of the main compounds monitored, an experimental design was performed with the data from the end of the FML. The inclusion of a different grape cultivar and the ionic exchange step prior to AF resulted significant for two of the main anthocyanin derivatives studied, the first causing an increase on the concentration of Mv-3-glc-acetaldehyde, and the latter a decrease on the concentration of Mv-3-glc-4-vinylphenol. Additionally, the interaction between the two mentioned variables positively affected the concentration of Mv-3-(6-*p*-coum)-glc *trans*.

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P3.3

Characterization of Finnish apple ciders by means of polyphenol profiles

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MAIN CONCLUSION

- The contents and composition of phenlic compounds generally decreased during the fermentation process.
- The profiles of phenolic compounds were mainly affected by the apple cultivars.

INTRODUCTION

Cider is typically defined as a fermented, alcoholic beverage which is made from apple products, with a range of alcohol content from 1.2% to 8.5% v/v.(Laaksonen, Kuldjärv, Paalme, Virkki, & Yang, 2017) As one of the fastest growing beverages, apple cider production has played an important and promising role in the fruit and beverage industry in the European countries, mainly in the United Kingdom, France, and the Northern European countries. Currently, there are no breeding programs for speciality cider apples in Finland, and no existed data concerning the potential of local Finnish apple cultivars into cider production. Thus, there is an increasing interest among cider producers to develop the local cultivars into cider apples for improving the overall quality of the apple cider products.

The polyphenols in apple ciders and their corresponding juices can be classified into four major sub-classes, including hydroxycinnamic acids, flavan-3-ols, dihydrochalcones, and flavonols. The polyphenolic profiles play an important role in defining the final quality of apple ciders, as they contribute to the color stability, astringency, sourness, and bitterness. Apple cultivars, maturity of apple fruits, processing methods, and yeast strains have been reported to affect the chemical compositional changes during the cider production period. Therefore, the study aimed to investigate the phenolic profiles of apple juices together with those ciders made from the selected eleven Finnish apple cultivars and two yeast strains.

MATERIALS & METHODS

Saccharomyces cerevisiae and *Schizosaccharomyces pombe* yeasts and eleven Finnish apple cultivars (Natural Resources Institute Finland) were selected to investigate the potential of Finnish local apple cultivars for cider making. The cider fermentation procedure was carried out in laboratory-scale fermentation according to our previous study.(He et al., 2021) The yeast strain was added into the juices and reach the level at 10^7 CFU/mL for inoculation. Phenolic compounds were extracted from the juice and cider samples with ethyl acetate. The identification of phenolic compounds were carried out by using a Bruker UHPLC-DAD-ESI-Q-TOF system and the quantification was performed on a Shimadzu UHPLC-DAD system. The distribution of phenolic compounds among juice and cider samples were studied with multivariate models, including principal component analysis (PCA) and partial least squares regression discrimination analysis (PLS-DA).

RESULTS & DISCUSSION

Altogether 34 phenolic compounds were tentatively identified, including 14 hydroxycinnamic acid derivatives, 7 flavan-3-ols, 9 flavonols, and 4 dihydrochalcones. The variations of individual phenolic compounds were also cultivar dependent based on the obtained results, which can be due to the

varietal and genetic interactions in the current study. The major polyphenolic compounds in all the studied juice and cider samples were hydroxycinnamic acids, accounting for around 80% of total phenolic contents on average, followed by flavan-3-ols (~10%), flavonols (~5%), and dihydrochalcones (~5%) in the study.

Apple juices contained higher amounts of flavonol glycosides and dihydrochalcones compared to the ciders whereas the processing resulted in increased amount of free hydroxycinnamic acids. Apple juices were highly associated with isorhamnetin-3-*O*-rhamnoside (Iso_rha), quercetin-3-*O*-rhamnoside (Qu_rha), quercetin pentoside I, (Qu_pen I) phloretin-2'-*O*-glucoside (Ph_glu) and phloretin-2'-*O*-xyloglucoside (Ph_xyglu) based on the PLS models. According to the obtained data, the phenolic changes during fermentation process were mainly dependent on the apple cultivars. Certain cultivars separated from the other cultivars and showed a strong positive correlation with hydroxycinnamic acids and dihydrochalcones, mainly as caffeic acid (CA), p-coumaric acid (pA), coumaroylquinic acid IV (ClqA_IV), phloretin-2'-O-glucoside (Ph_glu), and phloretin-2'-O-xyloglucoside (Ph_xyglu), in the study PCA model. Yeast strains also influenced the profiles of phenolic compounds based on the obtained data but the result was not that significant.

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P3.4

Epicuticular polyphenols as potential chemotaxonomic markers for common Finnish tree species

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MAIN CONCLUSION

A variety of plant metabolites, such as polyphenols and terpenoids, were found to serve as good chemotaxonomic markers for the studied tree species. With these markers, it was possible to identify the species from a single leaf bud with a new 6-min UHPLC-MS fingerprinting tool.

INTRODUCTION

Previously, epicuticular flavonoids were used as chemotaxonomic markers for Finnish birch species [1]. In this study, the aim was to explore the suitability of polyphenols in general as chemotaxonomic markers for a wider selection of common Finnish tree species. Firstly, suitable marker compounds were discovered from each species using high-resolution LC-ESI-Q-Orbitrap MS data. Secondly, the marker compounds were used to create simple and rapid species-specific fingerprinting tools with LC-ESI-QqQ MS. Finally, a single leaf bud was extracted from several replicates of each species, and the created chemotaxonomic tool was evaluated for its accuracy and robustness in plant species identification.

MATERIALS & METHODS

The study included leaf bud samples from 15 plant species collected in Turku, Finland. Samples were collected from 4–10 individuals per species. The freeze-dried leaf buds were extracted with a solution of ethanol and water (95/5, *v/v*) for 10 min. The MS analysis was performed with a high-resolution LC-ESI-Q-Orbitrap MS and the data was processed with MZmine 2, and a feature-based molecular network was built using the Global Natural Products Social Molecular Networking (GNPS) platform. The data was used to identify characteristic ions for each of the studied plant species. Using these ions, Selected Ion Recording (SIR) methods were created for each species using an UHPLC coupled with a Xevo QqQ mass spectrometer.

RESULTS & DISCUSSION

The studied 15 species were chemically diverse. The extracts contained both water-soluble and lipophilic compounds. In some of the species, the compounds covered the whole retention time range, while in some species the chemical diversity was rather poor. Consequently, the feature-based molecular network resulted in a varying number of potential marker compounds for the species. A total of 91 marker candidates were tested with the triple quadrupole mass spectrometer, and the cone voltage was optimized for each marker candidate. The final fingerprinting tool utilised 1–4 SIR channels per species with overlapping retention time windows. With the developed method, it was possible to identify the species in less than 6 min.

The markers belonged into many different plant metabolite subclasses. Polyphenols were suitable markers for *Acer platanoides,* for instance, as one of the markers was identified as a gallotannin. Another example is *Salix phylicifolia*, where a flavonoid, dihydromyricetin, was used as one of the markers. However, the most water-soluble flavonoids eluted early without proper separation due to the LC conditions and ethanol in the extraction solvent. Therefore, the more lipophilic compounds of the extracts were favoured in the process of finding suitable markers. In fact, even in the case of two

birch species, a hydroxycoumarin derivative and a triterpenoid were successfully used as marker compounds instead of the leaf surface flavonoids [2].

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P3.5

Biotransformation of 5-*O*-caffeoylquinic acid by gut bacteria: an interesting oxidative pathway.

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MAIN CONCLUSION

Contrarily to *Bacteroides fragilis* and *Bifidobacterium longum*, *Lactobacillus reuteri*, a gut bacterium, has shown the capacity to biotransform 5-*O*-caffeoylquinic acid (5-CQA), *via* different pathways, among them an oxidative one that has not been described so far.

INTRODUCTION

Polyphenols are known for their broad range of positive bioactivities in human. These molecules have shown the ability to lower oxidative stress involved in many pathologies and positively modulate redox cellular signalling pathways. However, given the pivotal role of microbiota to generate bioavailable and bioactive metabolites [1] and considering their superiority in terms of presence in systemic system compared to parent polyphenols, further insights are focused nowadays on their gut-derived metabolites as promising approach to prevent and attenuate neurodegenerative diseases [2].

We have investigated the biotransformation of 5-*O*-caffeoylquinic acid, known as chlorogenic acid, by three gut-bacteria species: *Lactobacillus reuteri (Firmicutes)*, *Bacteroides fragilis (Bacteroidetes)* and *Bifidobacterium longum (Actinobacterium)*, belonging to the dominant bacterial phyla of the gut microbiota. The biotransformed extracts were analysed by LC-MS/MS and the data were subject to MzMine 2.53 and GNPS treatment for subsequent molecular networking analysis. It is interesting to note that gut microbiota polyphenol biotransformation such as hydrolysis, cleavage and reduction are well known but there is a lack of information on possible oxidative pathways [3]. Hence, an electrochemical strategy was adopted to generate oxidized compounds of chlorogenic acid (5-CQA) and caffeic acid (CA), a gut-derivative of 5-CQA, in order to characterize and compare their mass profiles to the extract ones.

MATERIALS & METHODS

Bacteria were grown in BHI broth (Brain Heart Infusion) under anaerobic conditions using an anaerobic chamber. Resting cells biotransformation studies were carried out, when bacteria reached the stationary phase, by adding the solution of 5-CQA (1 mM) in phosphate buffer (pH 5.8) on biomass of each bacterial culture. They were incubated under anaerobic condition using sealed jars and anaerobic bags. After 24 h of incubation the extraction process was performed on pellet and on supernatant using ethyl acetate. After evaporation of organic solvents, the resulting supernatant and pellet extracts were analyzed by LC-MS/MS.

Additionally, electrooxidation of 5-CQA 2mM and CA 2mM were performed in phosphate buffer 0.1 M at pH = 5.8. A chronoamperometry technique i = f (t) was conducted at a fixed potential during 20 min using a platinum grid working electrode, in the absence of oxygen. The UV-Vis spectra were simultaneously recorded over a spectral range of 200 to 600 nm.

RESULTS & DISCUSSION

Some metabolites were annotated into extracts obtained after biotransformation of 5-CQA by *L. reuteri*, of which caffeic acid (CA) and 3-hydroxybenzoic acid (3-HBA). Interestingly, our study highlighted the formation of caffeic acid quinone generated by an oxidative pathway, which are unexpected in anaerobic gut environment. Mortele et al., in 2019 have already identified caffeic acid quinone in fecal biotransformation studies [4]. However, to our knowledge, the oxidative biotransformation reactions of cinnammate esters by gut microbiota has not been discussed so far. This finding highlighted the particular enzymatic machinery of *L. reuteri* strain.

Compounds arising from the electrooxidation of chlorogenic acid at pH 5.8 were not found in the extracts. However, the electrolyzed solution of caffeic acid, has also shown the presence of caffeic acid quinone, although it was not the major compound, as its stability is probably low. So far, we have no explanation why the stability of the caffeic acid quinone seems higher in the biotransformed extracts. Moreover, we did not find metabolites after bioconversion by *B. fragilis* and *B. longum*, suggesting that they are not able to biotransform 5-CQA.

Therefore, in this project, we aim to give more information on the oxidized derivatives issuing from the biotransformation of 5-CQA, which may show a higher activity that the native compounds.

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P3.6

Cranberry proanthocyanidins enhance chemotherapy-induced esophageal adenocarcinoma cell death

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MAIN CONCLUSION

Cranberry proanthocyanidin (C-PAC) pre-treatment and pre/post-treatment significantly decreased cell viability at 48, 72, and 96 hr. Both OE33 and OE19 cells failed to recover from C-PAC and chemo drug (carboplatin and paclitaxel) treatment indicating a durable effect. Western blot and RNA-seq analysis showed C-PAC treatment increased DNA damage response, induced apoptosis, inhibited cell cycle, and altered key epithelial-mesenchymal transition protein expression.

INTRODUCTION

Esophageal adenocarcinoma (EAC) is devastating cancer characterized by rising incidence and poor prognosis [1]. The five-year survival rate is 20% due to late-stage diagnosis and ineffective treatment [1]. It is estimated that only 15% of EAC patients have a complete histopathological response to standard chemotherapy [2]. Thus, new strategies are urgently needed to improve therapeutic efficacy and patient survival. Our recent analysis of prechemotherapy endoscopic biopsies of EAC patients revealed a differential response to neoadjuvant therapy. The differential response was reflected by two-year mortality of 9.1% in therapy responsive patients (Complete responders, no residual tumor), 22.2% in strong responders (improved TNM), 25.0% in intermediate responders (stable TNM), 42.9% in poor responders (worse TNM) and 77.8% in non-responders (worse TNM and metastasis). Transcriptomic analysis revealed differential response to therapy is linked to immune defense, inflammation (IL-4, IFNs), cell adhesion, signal transduction, and angiogenesis regulation. Similar pathways were mitigated by cranberry proanthocyanidins (C-PAC) in a rat surgical model for EAC. Herein, we investigated C-PAC pretreatment alone or in combination with Paclitaxel and Carboplatin (chemo drug) on EAC cell viability (Calcein-AM), gene expression (RNA-seq), and select protein levels (Western blot).

MATERIALS & METHODS

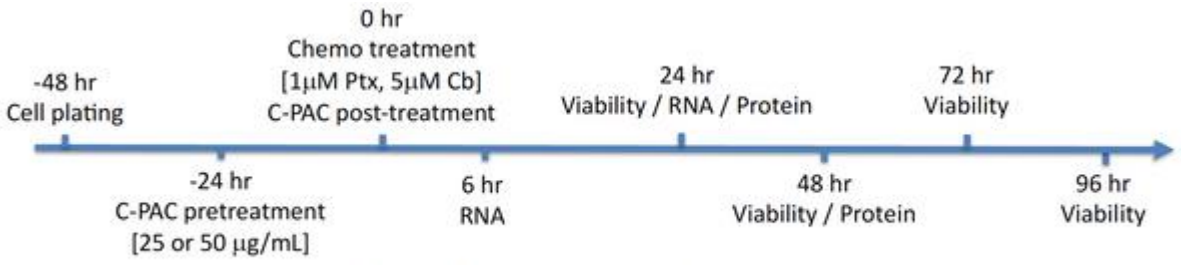

Figure 1. Experimental procedures.

The experimental procedure is outlined in Figure 1. In short, OE33 and OE19 cells were seeded in 96-well plates 48 hr prior to treatment initiation. 24 hr after plating, cells were either pre-treated with two concentrations of C-PAC or vehicle, followed 24 hr later with paclitaxel (Ptx) and carboplatin (Cb) treatment alone or in combination with C-PAC. Viability was measured using Calcein-AM at 24, 48, 72, and 96 hr post-treatment. Two-way ANOVA was performed followed by Tukey's multiple

pairwise comparisons test to calculate significance. At 6 hr and 24 hr post-treatment, RNA was isolated from triplicate samples of each treatment condition and paired-end sequencing was performed by BGI Americas (San Jose, CA) using the Illumina HiSeq 4000 system. RNA-seq analysis was performed using Qiagen CLC Genomics Workbench (Qiagen, Germany) and downstream analysis was performed using GSEA (Broad Institute, Cambridge, MA) and MetaCore (Clarivate, Philadelphia, PA).

RESULTS & DISCUSSION

C-PAC pre-treatment and co-treatment enhance chemotherapy-induced EAC cell death, with the greatest synergistic effects in chemo-resistant OE33 cells. C-PAC pretreatment followed by chemo drugs decreased cell viability by an additional 50% and 17% compared to chemo drugs alone in OE33 cells and OE19 cells, respectively. Greater reductions were noted with pretreatment of C-PAC, followed by co-treatment with chemo drugs (64.9% in OE33 and 49.4% in OE19 cells). Western blot analysis revealed that C-PAC enhanced chemo drug-induced EAC cell death via increased DNA damage, apoptosis, ECM degradation, and decreased epithelial-mesenchymal transition as evidenced by induction of phosphorylated H2AX, cleaved Caspase-3 and ZO-1, as well as inhibition of BCL_{XL}, MMP9, Vimentin, and Snail. C-PAC also mitigated elevated levels of P53, a gene with a well-documented role in EAC progression and therapeutic resistance. Gene pathway analysis showed top pathways altered by C-PAC treatment include S-phase in cell cycle, mitosis, and mismatch repair in both OE33 and OE19 cells. Additionally, OE33 cells also include unique pathway alterations, such as changes in cytoplasmic microtubules and DNA excision repair pathways. Further analysis will include comparing transcriptomics result with signaling networks differentially expressed in patients stratified by treatment responsiveness. Results suggest that C-PAC pretreatment may offer a non-toxic intervention strategy for enhancing chemotherapeutic efficacy.

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P3.7

Metabolomic approach of Arbosana olive (Olea europaea L.) leaves dryied by different technologies to identify polyphenols related to antioxidant capacity

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MAIN CONCLUSION

Olive leaves contain phenolic compounds that can be of interest for the food industry, due to their antioxidant capacity. The aim of the present work was to use a metabolomic approach to find the compounds highly correlated with the total antioxidant capacity. 'Arbosana' olive leaves were studied to assess the effectiveness of different drying methods (vacuum, ovendrying, freezedrying and airdrying) on the preservation of the phenolic compounds and the antioxidant capacity in those olive leaves.

INTRODUCTION

During olive harvesting, olive leaves are discarded entailing a problem for producers. Olive leaves contain phenolic compounds that can be of interest for the food industry, due to their antioxidant capacity. Nowadays, more than 3.3 million tons of olive oil are annually produced worldwide and Spain is the main producer reaching the 37% of total production in 2017/2018 season. Even if almost the 80% of this activity is located in Andalusia (south Spain), olive oil industry is also important in Navarre (north Spain), where 4036 tonnes of olive oil were produced. *Olea europaea* L. tree is typically cultivated in the Spanish Ebro River Valley region where specific cultivars grow; for example, 'Arbequina', 'Arbosana' and 'Empeltre' are native of the Ebro River Valley and 'Arroniz' is autochthonous of Navarre [1]. Nevertheless, olive oil industry generates a large number of wastes. Up to 5% of the total olive weight that enters in an olive mill is discarded as olive leaves and the 75% as olive pomace, so only the 20% is transformed into olive oil [2]. The high organic content and phytotoxicity of these residues entails an environmental problem in the olive oil production areas [3] and thus, an issue for producers.

Specifically, olive leaves, which are separated from olives before olive oil production, have been traditionally consumed as a remedy in Mediterranean countries for their antioxidant, anti-hypertensive, antiatherogenic, anti-inflammatory, hypoglycaemic and hypocholesterolemic properties. These health benefits have been related to the phenolic compounds of olive leaves among which hydroxytyosol and oleuropein stand out. For this reason, extracts of these compounds could be used in food products as bioactive compounds given them health benefits [4] or as preservatives, due to their antioxidant and antimicrobial properties. In consequence, the extraction of phenolic compounds from olive leaves could be a way to valorise this residue.

MATERIALS & METHODS

Four drying methods were applied to olive leaves: vacuum-drying at 40°C, oven-drying at 105°C without vacuum, freeze-drying and air-drying performed spreading leaves into a closed area with natural air at a room temperature between 20–25 °C.

Total antioxidant capacity (TAC) of the olive leaves extract was assessed using DPPH radical scavenging activity method. The results were expressed as μmol Trolox equivalents/g dry weight.

For LC-ORBITRAP analysis, dried olive leaves were ground and sieved. They were mixed with methanol/water at room temperature (2 extractions) and both supernatants were mixed and carried out in a final volume of 10 ml. The olive leaf extract was then evaporated under N2 and redissolved

in mobile phase for injection in the liquid chromatographic system. LC-ORBITRAP analysis was performed using a C18 reverse phase column using full scan data dependent mode to perform further data analysis. Metabolomic analysis was performed using Compound Discoverer Software.

RESULTS & DISCUSSION

Arbosana's leaves extracts were injected in ORBITRAP system and data were processed with Compound Discoverer trying to identify phenolic compounds present in each of the extract. A total of 500 compounds were found after filtering and analysing initial data.

CAT and phenolic profile compounds were further analysed by Principal Component Analysis and correlation analysis using Phyton programming.

In the correlation analysis of the different free polyphenols with antioxidant capacity, 44 polyphenols were found to be highly correlated (Pearson's r > 0.8) either positively or negatively. When we separated by positive correlation, we obtained that the antioxidant capacity is highly correlated with 4 of the polyphenols and negatively correlated with 40.

Polyphenols that showed positive correlation with TAC were: dihydroaffeic acid 3-O-glucuronide, epimedokoreanoside I, chlorogenic acid and 3,4,5,2',4',6'-hexahydroxychalcone 2'-glucoside.

Scatter plot analysis reveals that not only is there a strong correlation between these variables, but that the oven-drying system forms a separate cluster compared to the other drying systems.

In the PCA analysis, it can be observed how air-drying and vacuum-drying are quite close in the 2 principal components plot, whereas oven-drying and freeze drying constitute two separate clusters.

This means that the drying method significantly influences the final polyphenol profile of the dried olive leaf. Metabolomic approach allows not only to get to a deeper knowledge of the type of phenolic compounds present in this type of dry olive oil, but also to select the best drying method according to the final TAC properties of the dried material.

ACKNOWLEDGEMENTS

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P3.8

Cranberry proanthocyanidins mitigate bile-induced injury in primary normal esophageal cell lines isolated from patients with esophageal adenocarcinoma

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MAIN CONCLUSION

Pretreatment with cranberry proanthocyanidins (C-PAC) protect against bile-induced injury through upregulation of the detoxification enzyme GSTT2 in primary normal esophageal cells.

INTRODUCTION

Persistent and symptomatic reflux of gastric and duodenal contents, known as gastroesophageal reflux disease (GERD), is the strongest risk factor for EAC development [1-3]. GERD and esophagitis occur at similar rates among Blacks and Caucasians; yet, progression to EAC is significantly elevated among Caucasians [4]. Unique protective factors in the epithelium of Blacks may contribute to this disparity. Our research team recently reported that the detoxification enzyme *GSTT2* is higher in the esophageal squamous epithelium of Blacks compared to Caucasians with potential linkages to previously identified genomic variants in the GSTT2 locus (a 37 kb deletion and a 17 bp promoter duplication among Caucasians) [5]. Thus, the current study seeks to evaluate whether primary esophageal cell cultures isolated from Black or Caucasian cohorts can serve as discerning and relevant model systems to investigate risk factors linked to EAC progression, assess efficacy of mitigating agents and differential responses linked to race. We have shown that cranberry proanthocyanidins (C-PAC) mitigate DNA damage associated with reflux through upregulation of GSTT2 in a rat surgical model of reflux-induced EAC, but whether effects are recapitulated in humans or differentially based on race remains unknown [5].

MATERIALS & METHODS

Herein we isolated normal primary esophageal epithelial cells from Black and Caucasian patients and assessed GSTT2 genotype, GSTT2 protein levels and cellular viability following exposure of the cultures to a bile acid cocktail (BAC) [0.2mM] with and without C-PAC [50µg/ml] treatment.

RESULTS & DISCUSSION

Constitutive levels of GSTT2 were 1.7-fold higher in Blacks than Caucasians, with 71% of Blacks identified as high expressors compared to 33% of Caucasians. Pretreatment (48h) of primary cultures with C-PAC induced GSTT2 levels in all but one Black-derived culture which already expressed high basal levels. GSTT2 induction in normal epithelial cultures by C-PAC was greatest in cells with constitutively low GSTT2 expression; however, upon BAC challenge C-PAC effectively mitigated BAC-induced reductions in GSTT2 levels and subsequent loss of normal cell viability regardless of basal GSTT2 expression or race. C-PAC treatment pre- plus post-BAC imparted no additional benefit over pretreatment alone in preserving viability but did further increase GSTT2 levels. We have begun developing primary normal cell cultures form patients who have progressed to EAC. Data will be presented from nano LC-MS/MS proteomic profiling of normal cell cultures from progressors treated with vehicle, BAC, C-PAC and BAC+C-PAC. Taken together these data support that C-PAC may be used as an efficacious non-toxic agent serving to promote epithelial fitness and resiliency against the biologic and molecular sequelae linked to bile acid-induced esophageal injury and progression to EAC.

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P3.9

Untargeted metabolomic LC-MS fingerprinting of apple cultivars for the identification of biomarkers related to resistance to rosy apple aphid

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MAIN CONCLUSION

The isomerisations of hydroxycinnamic acids are the key metabolic reactions of the phenylpropanoid pathway that establish the tolerance of apple cultivars to rosy apple aphid (RAA), the isomers of caffeoylquinic and p-coumaroylquinic acids being the particular phenolic compounds involved.

Untargeted metabolomic LC-MS fingerprinting of apple cultivars is proved to be a useful less time consuming approach to identify biomarkers than the profiling approaches.

INTRODUCTION

Phenolic compounds may play important roles in plant responses to biotic-stress and in resistance mechanisms. In a previous work, the correlation between the phenolic composition of apple cultivars and their tolerance to RAA, *Dysaphis plantaginea*, a key insect pest of *Malus domestica* (Borkh.) was demonstrated [1]. The study was performed on a full-sib family of apple cultivars derived from a crossing between the local cider apple cultivar ‘Meana’ and the resistant cultivar ‘Florina’, using a profiling approach. Concentrations of forty individual phenolic compounds (flavan-3-ols, hydroxycinnamic acids, dihydrochalcones and flavonols) were determined by UHPLC-DAD in the parent cultivars and 155 ‘Meana’ x ‘Florina’ descendants; as well as their resistance to RAA by aphid inoculation. Pattern recognition techniques disclosed that the caffeoylquinic acid isomers, i.e. 4-CQA, 1-CQA and 3-CQA, and 4-p-coumaroylquinic acid (4-pCoQA) were related to the resistance of apple cultivars to RAA through the phenylpropanoid pathway; the isomerisations being the key metabolic reactions for a cultivar to be resistant or susceptible to RAA. This profiling approach was time consuming since it involved the identification and quantitation (including peak integration and external standard calibration) of the forty phenolics studied. Moreover, only the determined compounds could be considered as possible biomarkers; indeed the presence of other compounds related to RAA resistance could not be disclosed. In the present work, an untargeted metabolomic approach is proposed to identify biomarkers related to the resistance of apple cultivars to RAA. This is a fingerprinting approach, which is less time consuming since the identification and quantitation steps required in target analysis for profiling are not performed, and compound identification is only carried out for the actual biomarkers disclosed by the multivariate data analysis. Furthermore, unknown biomarkers can be discovered.

MATERIALS & METHODS

Apple juice samples were analysed by UHPLC-DAD-ESI(+)-QToF/MS using an Waters ACQUITY UPLC™ system, equipped with binary solvent delivery pump, autosampler, column compartment,

DAD detector and Waters SYNAPTTM G2 HDMS spectrometer. A Phenomenex Kinetex™ C18 column (2.1×100mm, 1.7µm) was used at 40 °C. Mobile phases: 0.1%(v/v) acetic acid(aq.) (A) and 0.1%(v/v) acetic acid in methanol (B). Elution conditions: 0–1.6min, 2%B isocratic; 1.6–2.11min, 0–8%Bgradient; 2.11–8.8min, 8%B isocratic; 8.8–9.8min, 8–10%B gradient; 9.8–17min, 10%B isocratic; 17–22min, 10–20%B gradient; 22–23.4min, 20–23%B gradient; 23.4–28.4min, 23–35%B gradient; 28.4–30.4min, 35–51%B gradient; 30.4–31.4min, 51–100%B gradient; 31.4–32.4min, 100 % B isocratic; and system re-equilibration. Flow rate was 0.35mL/min; injection volume, 5µL; and autosampler temperature, 4°C. UV-visible spectra were recorded from 210–500nm. The ESI(+)-QToF-MS^E conditions were previously reported [2].

RESULTS & DISCUSSION

UHPLC-ESI(+)-QTOF/MS fingerprinting of the same ‘Meana’x‘Florina’ descendants was carried out. The proposed untargeted metabolic approach consisted in: *(i)* data acquisition (retention time (RT), m/z, MS signal intensity); *(ii)* conversion of MassLynx raw files into CDF files, grouping them according to sample categories (resistant (R) and susceptible (S)); *(iii)* processing of NetCDF files by Metlin R-package XCMS, in order to convert the 3D LC-MS data (RT, m/z, intensity) into a table of time-aligned detected features for each sample (LC-MS artefacts were filtered by R-package CAMERA); and *(iv)* univariate and multivariate data analysis.

Apple fingerprints and data of their resistance to RAA were submitted to pattern recognition techniques: Principal Component Analysis (PCA), Hierarchical Cluster Analysis (HCA), Partial Least Square-Discriminant Analysis (PLS-DA) and Orthogonal PLS-DA (OPLS-DA). Dataset containing 1983 variables were pareto-scaled and logarithmic transformed. No outliers or extreme samples were detected by PCA. The bidimensional plot of the sample scores in the spaces defined by the two first principal components, as well as HCA, showed two clusters of the samples according to the established classes (R and S). PLS-DA achieved a binary classification model with recognition and prediction abilities in cross-validation of 100% of hits for both classes. The S-plots of the cross-validated OPLS-DA model obtained was used to select the relevant features for the differentiation of the cultivars according to their resistance to RAA. All these techniques revealed that the main features were due to 4-CQA, present in higher concentrations in resistant cultivars; and to 5-pCoQA, in higher concentrations in susceptible cultivars, in accordance with previous study [1], and confirmed that the isomerisations of hydroxycinnamic acids are the key metabolic reactions of the phenylpropanoid pathway for a cultivar to be resistant or susceptible to RAA.

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P3.10

Effect of temperature and developmental stage on the content of anthocyanins and phenolic acids in potato cultivars

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MAIN CONCLUSION

The main factors affecting the content of phenolic compounds in potato tubers were the cultivar and the stage of tuber development. Tubers collected at the final stages of maturity were less rich in phenolic compounds than the tubers collected at 60 or 70 days after planting.

The temperatures studied did not affect the content of anthocyanins. However, the content of phenolic acids tend to be higher in the cultivars 'Blue Congo' and 'Blaue Veltlin' when grown at the lower temperature, 13 °C.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the third most important crop for human consumption and hence plays an important role in food security. There are thousands of potato cultivars, which differ in size, flesh/skin colour, texture, shape as well as sensory characteristics. However, the white and yellow-flesh potato cultivars are the most cultivated worldwide.

Potato is an important crop for human nutrition. It is a source of carbohydrates, proteins, minerals, vitamins and phenolic compounds. The main phenolic compound in potatoes is chlorogenic acid while anthocyanins are present only in red-blue flesh cultivars [1]. The potato anthocyanins are glycosylated derivatives of cyanindin, delphinidin, pelargonidin, petunidin and malvidin, and the most common sugar moieties are glucose and rutinose. They are typically acylated with p-coumaric, ferrulic and caffeic acids.

Temperature is the main factor limiting the production of potato. Temperatures below 10 °C and above 30 °C inhibit the tuber growth, while temperatures between 18 and 20 °C produce optimum tuber yields. The production of phenolic compounds in plants increases when plants are exposed to low non-freezing temperatures [2] but decreases when exposed to heat stress [3].

The effect of developmental stage of the potato tubers on their nutritional value has not been widely studied. However, these studies - focused on the interaction of cultivar and stage of tuber development – have shown that the highest content of phenolic compounds was found in immature tubers [1]. Thus, the effect of temperature as one more variable was included in the study to identify the main factors regulating the production of phenolic compounds in potatoes.

MATERIALS & METHODS

Four purple potato cultivars ('Blue Congo', 'Blaue Veltlin', 'Blaue Schweden', and 'Synkeä Sakari') and one yellow cultivar ('Van Gogh') were studied. The potatoes were grown at two controlled temperatures (13 and 18 °C). The harvesting of potato tubers started 60 days after planting (DAP) and continue every ten days up to 120 DAP. The harvested tubers were lyophilized, ground and stored at -80 °C.

Extraction of the phenolic compounds from the lyophilized potato powder was performed with 70 % aqueous methanol containing 0.122 M/L of HCl.

Phenolic compounds were determined by HPLC-DAD and UPLC-ESI-MS with a gradient elution of acetonitrile in aqueous formic acid (10%). Anthocyanins were detected at 520 nm and phenolic acids at 320 nm. The identification of anthocyanins was performed by MS analysis (full scan) in a range of m/z 250–1000.

RESULTS & DISCUSSION

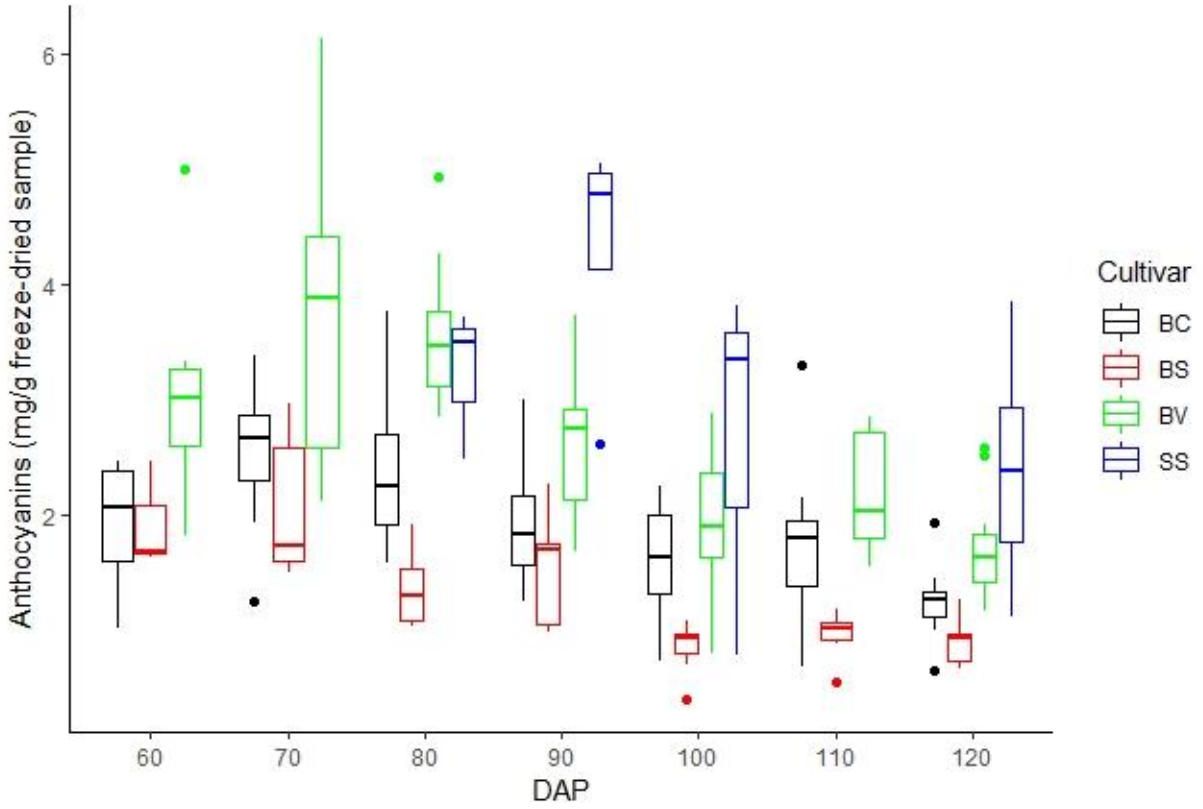

Figure 1. Changes in the content of anthocyanins during tuber development

The purple potato cultivars showed higher amount of polyphenols than the yellow cultivar at all developmental stages. The content of anthocyanins and phenolic acids was highest at 60 and 70 days after planting and then significantly decreased from 46% to 67% during maturation (Figure 1). Payyavula R. et al. also found that the content of anthocyanins and phenolic acids decreased during tuber maturation [1]. Pearson correlation showed that the content of anthocyanins and phenolic acids had a positive correlation ($r = 0.80$; $p < 0.001$). Irrespective of the temperature, 'Synkeä Sakari' was the cultivar with the highest content of anthocyanins at the final stage of maturity (2.4 mg/g freeze-dried sample) and 'Blaue Veltlin' the cultivar with the highest content of phenolic acids (5.5 mg/g freeze-dried sample). Petunidin-3-*p*-coumaroylrutinoside-5-glucoside was the main anthocyanin in 'Blue Congo', 'Blaue Schweden', and 'Synkeä Sakari' while malvidin-3-*p*-coumaroylrutinoside-5-glucoside was the main anthocyanin in 'Blaue Veltlin'. Principal component analysis revealed that 'Blue Congo' and 'Blaue Schweden' had similar anthocyanin profiles. This is because 'Blaue Schweden' was obtained by breeding techniques using 'Blue Congo' as the parent cultivar.

The potato tubers of 'Blue Congo' and 'Blaue Veltlin' grown at 13 °C had higher amount of phenolic acids than the tubers grown at 18 °C (13 and 18 % more, respectively). This shows that the cultivar-temperature interaction is a factor affecting the content of phenolic acids. However, the content of anthocyanins was not affected by the studied temperatures.

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P3.11

Diarylheptanoids – strong antioxidants in alder bark growing in Latvia: chemical profiling, isolation and their application potential

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MAIN CONCLUSION

The results indicate that crude extracts and especially oregonin are strong natural antioxidants, which could be applied in food systems and cosmetic products, as well in others health beneficial products. Oregonin containing extract was registered in Latvia as food supplement.

INTRODUCTION

Diarylheptanoids (1,7-diphenylheptane skeleton) are characteristic chemical compounds of Alnus species, even more it is reported that they in combination with principal component analysis could be used as chemotaxonomic markers at the species level [1]. Alders have a long history of use in folk medicine, they have been used as a remedy for fever, haemorrhages, burn injuries, diarrhoea, and alcoholism. Alder bark is known herbal product with broad spectrum of activities, including improving digestion, state of circulatory health, antiflammatory activity, normalizing state of microflora of alimentary canal etc., without anyone negative influence on heart- vascular, nervous and blood creative systems. Diarylheptanoids isolated from alders have drawn attention due to their physiological activities, especially their anticancer and antioxidative activities [2]. The structural – functional similarity of diarylheptanoids extracted from alder bark and curcuminoids – plant polyphenols isolated from Indian spice turmeric open possibility for similar application in the area of functional food and in perspective in drug formulation. Recently, the diarylheptanoid rich extract of grey alder bark appeared at Latvian market in the form of food supplement. In this aspect actualizes the problem of chemical control over diarylheptanoid content and chemical composition in alder bark extracts, which are used in various herbal products.

MATERIALS & METHODS

The oregonin and curcumin analytical standards were purchased from Sigma Aldrich , LC-MS hypergrade acetonitrile from Merck and formic acid (HiperSOLV Chromanorm) from VWR chemicals. Bark removed by debarking of a 27 years' age trees harvested in Talsu municipality of Latvia. The bark was air dried up to the water content of 9%, then ground by a knife type mill equipped with a 2.00 mm sieve. The accelerated solvent extraction and microwave assisted extraction were applied. Acquity UPLC system with UV detector coupled with Synapt G2-Si was used. The content of extractable polyphenolics was determined by Folin – Ciocalteu. Radical scavenging activity towards DPPH• and ABTS•+ radicals was expressed as IC50. The antioxidant activity was expressed as Trolox equivalent on one gram of a sample.

RESULTS & DISCUSSION

The profiling of secondary metabolites in the extracts of grey and black alder bark growing in Latvia showed that their chemical composition is rich with the polyphenolic compounds- diarylheptanoids. Total amount of polyphenols in these diarylheptanoid containing extracts varies from 0.5 up to 0.7 GSE · g on dry extract depending on species, extraction method and conditions applied. Together with high polyphenol content, it was estimated that diarylheptanoids are mostly presented by one dominant compound – oregonin (from 45% up to 81% on dry extract). Totally ten different

diarylheptanoids in extracts were identified: 1,7-Bis-(3,4-dihydroxyphenyl)-heptane-5-O-β-D-glucopyranoside, 1,7-bis-(3,4-dihydroxyphenyl)-3-hydroxyheptane-5-O-β-D-xylopyranoside, oregonin, hirsutanolol, aceroside VII, (4-Hydroxyphenyl)-7-(3,4- dihydroxyphenyl)-heptan-3- one-5-O-pentoside, rubranosoide A, rubranol xyloside, 1,7-Bis-(4-dihydroxyphenyl)-3-heptanol 3-O-D-Glucopyranosyl-(1→3)-D-xylopyranoside, hirsutenon). More than 70% of black alder EtOAc extract (or ~ 8% from o.d. bark) was identified as linear diarylheptanoids. More than 85% of these DAH are presented by oregonin, that make these extracts prospective raw materials for obtaining of the pure oregonin for application in medicine. Isolation of oregonin was performed using flash chromatography, from black alder bark. Purified oregonin contained 90% oregonin, 5% 1,7-bis-(3,4-dihydroxyphenyl)-3-hydroxyheptane-5-O-β-D-xylopyranoside and 4% hirsutanolol. The quality is assessed by comparison with the oregonin standard purchased from Sigma. The purified oregonin showed high antioxidant activity in ABTS•+ and DPPH•tests: IC_{50} value was 3.1mg·L^{-1} test 4.5 mg·L^{-1} respectively. Synthetic antioxidant trolox taken for comparison show in both tests IC_{50} values ~ 4.0 mg·L^{-1}. The diarylheptanoid extracts antioxidant activity was 10.7 mg·L^{-1}(grey alder) and 9.5 mg·L^{-1} (black alder) in DPPH and 6.5 and 5.8 in ABTS test.

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P3.12

Extraction and identification of polyphenols from spruce bark using HPLC-DAD-ESI-MS/MS

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MAIN CONCLUSION

The results indicated that Fe_3O_4-CA exhibits better efficiency than bare Fe_3O_4 and 1h of shaking was optimal (4h shaking degraded the original polyphenol composition, and 15 min was not efficient). MNPs show the potential in extracting viable polyphenol from bark (hot water extraction) followed by different modified Fe_3O_4 separation, and possibly continuous (cycling) processes.

Acknowledgment:

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INTRODUCTION

Spruce is a widely used raw material in the pulp and paper industry where it contributes significantly to considerable amounts of biomass waste generated. As with most woody biomass used for pulp and paper, the removed bark from spruce ends up being a low-value by-product. In spruce, the bark constitutes 10-15% total weight of tree stems, is detrimental in the pulping process, as it has to be removed before processing. Once removed, the bark is generally used as an energy source. Extraction of commercially viable compounds from the bark before burning is an interesting value-added option. Bark is heterogenous both morphologically and chemically, as it contains cellulose, hemicelluloses, pectins, lignin and various extractives in different proportions. Spruce bark is a renewable source of biologically active compounds. More than 60 antioxidant compounds can be isolated from spruce bark including piceid astringin, quercetin and resveratrol [1]. These polyphenolic compounds showed anti-inflammatory, anti-microbial, anti-tumor, antioxidants and antiaging properties, and therefore, are potential ingredients for cosmetics, food and pharmaceuticals.

The present study describes the extraction of polyphenols from spruce bark using hot water extraction followed by the separation of polyphenols from the debarking water using magnetic beads through adsorption and desorption methodology. In the experimental section, the efficiency of magnetic iron oxide nanoparticles (MNPs - bare Fe_3O_4, modified Fe_3O_4), concentration and shaking time were tested.

MATERIALS & METHODS

Magnetic nanoparticles synthesis: MNPs were synthesized by coprecipitation from an aqueous solution of Fe^{2+} and Fe^{3+} salts in a two-step process. First, the pH was raised to 3 and after 30 min to pH 11 by using ammonia solution (25%). The synthesized particles were washed several times with water, collected by magnet and re-dispersed in water in a form of water-based suspension with a concentration of 10 g/L (bare Fe_3O_4). Additionally, citric acid (CA) was adsorbed on a bare Fe_3O_4 to modify their surface charge (Fe_3O_4-CA).

Bark polyphenol extraction: 500mg of samples (38 to 850 µm) were taken in 20mL of water and ultrasonicated at 80°C for 30min followed by filtration. Different concentrations (0.5, 2.5 g/L) of Fe_3O_4 and Fe_3O_4-CA were added into the filtrate at different shaking times (15min, 1h and 4h). Then, MNPs were collected by a magnet and debarking water was then separated and analyzed; 5mL of methanol was added to the collected MNPs shaked and the separated methanol was analyzed.

RESULTS & DISCUSSION

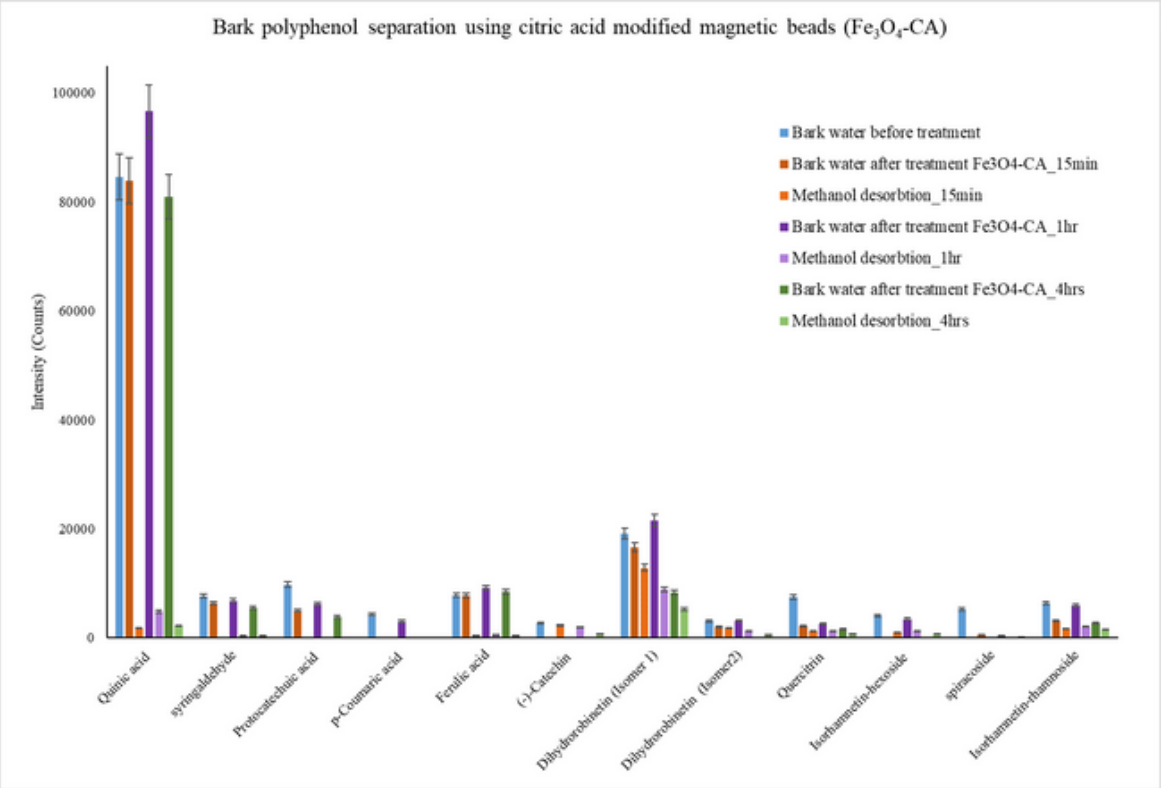

Figure1. Bark polyphenol separation using citric acid modified magnetic beads (Fe3O4-CA).

Identification and determination of phenolic compounds in the methanol fraction after desorption from the Fe_3O_4 and Fe_3O_4-CA were analyzed using LCMS-QTOF MS/MS. Based on the fragmentation pattern reported in the literature [2], quinic acid, syringaldehyde, p-coumaric acid, ferulic acid, dihydrorobinetin, isorhamnetin-hexoside, and spiraeoside were identified with Fe_3O_4.

The efficiency of Fe_3O_4 and Fe_3O_4-CA with different shaking time (15min, 1h, and 4h) was tested and the results for Fe_3O_4-CA (2.5 g/L) is presented in Fig.1, where individual polyphenol concentrations are shown for bark water before and after Fe_3O_4-CA treatment, and in methanol desorption samples. The results demonstrated that 15min shaking time is not sufficient to separate the polyphenols from the debarking water, whereas 4 h of shaking time degraded the polyphenols, as exhibited by the lower MS intensity counts. On the other hand, 1 h shaking time showed a better result compared to either 15 min or 4 h. Further experiments were also carried out with different Fe_3O_4-CA concentrations (0.5 and 2.5g/L) and the results indicated that an increase in Fe_3O_4-CA concentration enhances the extraction yield of polyphenols.

Therefore, the efficiency of bare Fe_3O_4 and Fe_3O_4-CA for polyphenols separation was compared at the higher concentration (2.5 g/L &1 h). Fe_3O_4-CA extract contains additional polyphenols such as catechin, quercitrin, and isorhamnetin-rhamnoside. No such compounds can be found with bare Fe_3O_4 extraction. However, comparing the methanol desorption results with the debarking water before and after magnetic nanoparticles (Fe_3O_4 and Fe_3O_4-CA), it is evident that only a small quantity of the polyphenols was removed. Therefore, the follow-up investigation will focus on either increasing the concentration of Fe_3O_4-CA, different modification methods for Fe_3O_4, or the possibility of several MNP extraction cycles.

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P3.13

Comparing aryltetralin lignans production by adventitious roots from three *Linum* species

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MAIN CONCLUSION

The results of this study indicate that *L. dolomiticum* ARc, described for the first time, is the ATLs best producer among the three species investigated. Moreover, the two elicitors used were effective in enhancing secondary metabolism of these tissues, but the effect of each molecule could be different in relation to the species tested.

INTRODUCTION

Plant secondary metabolism represents a network of chemical signals which connect the plant with its environment. Among the secondary metabolites, lignans are plant phenols deriving from phenylpropanoid pathway and play an important role in plant defense, conferring protection against herbivores and microorganisms, and also showing antibacterial, antiviral, and antifungal properties. Moreover, they display a wide range of applications as components of food, textiles, and medicine. These compounds have attracted the attention for their potent antiviral and antineoplastic properties. In particular, the aryltetralin-lignans (ATLs) have received much attention since the discovery of podophyllotoxin, the most representative compounds of this class, well-known for its anticancer properties and used as lead compound for the semi-synthesis of the anticancer drugs (etoposide, teniposide) [1]. It is currently produced largely from *Podophyllum hexandrum* and *P. peltatum*, slow-growing endangered species. Although several plant species are known to synthetize podophyllotoxin and its derivatives, *Linum* species seem to be particularly promising because they can accumulate high amounts of these compounds and can be easily grown in fields or cultivated as cell cultures [2].

In this work, a biotechnological approach was used, based on in vitro tissue cultures, to enhance lignans production. In particular, adventitious roots cultures (ARc) from *L. mucronatum*, *L. flavum* and *L. dolomiticum* were obtained and different elicitation strategies were applied to improve ATLs production. Total phenolic content, total flavonoid content and antioxidant activity were also assessed. Additionally, structural investigations by HPLC and NMR analysis were performed to compare the production of podophyllotoxin (PTOX), 6-methoxypodophyllotoxin (MPTOX) and 6-methoxypodophyllotoxin 4-β-D glucoside (MPTOX-Glc) among the different species.

MATERIALS & METHODS

0.8 g of ARc of the three *Linum* species were grown into 20 ml of MS liquid medium and were left for 21 days at 23 °C on an orbital shaker (110 rpm) in dark condition. Then, two elicitors were added in the final concentration of 100µM methyl jasmonate and 1 µM and 10 µM coronatine and then the roots were harvested after four days. They were weighted and lyophilized, then the lignans were extracted by means of methanol 80% (v/v). The extract was used for total phenolics and total flavonoids determination, DPPH radical scavenging activity and chromatographic analysis to quantify ATLs content. HPLC separation was performed using a Synergi polar RP 80 Å (250mm x 4.60mm, 4µm, Phenomenex) with a gradient system consisting of water and acetonitrile. 6-methoxypodophyllotoxin and 6-methoxypodophyllotoxin 4-β-D glucoside were purified using TLC preparative and identified with NMR analysis. The results were subjected to statistical analysis by means of R.

RESULTS & DISCUSSION

The presence of ATLs was previously reported in different tissue cultures of *Linum* species belonging to the *Sillinum* section, in particular in cell and hairy roots cultures. In this study the ARc of *L. mucronatum* and *L. dolomiticum* were developed and described for the first time. Moreover, the elicitors methyl jasmonate and coronatine, never used on ARc of these species, were able to improve ATLs content. These elicitors caused a significant inhibition of growth for *L. flavum* and *mucronatum*, but do not affect the growth of *L. dolomiticum*. The elicitors have a strong effect to increase the total phenolic content in the three species, whereas enhance the content of flavonoids and antioxidant activity only in *L. flavum* and *L. mucronatum* ARc. Two ATLs (MPTOX and MPTOX-Glc) were identified using ^{1}H-NMR and quantified in the control and elicitor treated samples in addition to PTOX. The results showed that *L. flavum* and *L. dolomiticum* ARc produced, after elicitation, a good amount of PTOX, as compared to *L. flavum* hairy roots previous studied. On the contrary, PTOX was not detected in *L. mucronatum* ARc. However, the most abundant compounds present in the ARc of the three *Linum* species were MPTOX and MPTOX-Glc. For these molecules, *L. dolomiticum* was the highest productive species, after treatment with methyl jasmonate (for MPTOX) and coronatine (for MPTOX-Glc). *L. mucronatum* ARc is less suitable to yield ATLs, producing more antioxidant compounds such as phenols and flavonoids, whereas *L. dolomiticum* and *L. flavum* ARc seemed to be more performing. Overall, ARc represents a very good system to be used for large-scale production of secondary metabolites because of their biosynthetic ability, great stability and they are simpler to obtain and safer than hairy roots.

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P3.14

Are Dirigent like Domains from Bacteria Belonging to the DIR protein Family?

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MAIN CONCLUSION

In 2020, the occurrence of Dirigent Protein Like (DPL) domains has been recognized in 42 bacteria[1]. Our new analysis on Interpro database reveals 49 bacterial species each carrying a gene encoding a DPL. Sequence comparison with plants DPs, analysis of the 1D, 2D and 3D structures of proteins as well as localization and organization of cluster inside the genomes reveal candidates for the identification of proteins involved in the synthesis of new bioactive molecules of pharmaceutical interest.

INTRODUCTION

The first DP *Fi*DIR1, discovered in *Forsythia intermedia* has been found to direct the stereoselective biosynthesis of (+)-pinoresinol ((+)-PINO) from coniferyl alcohol radical monomers[2]. The initial radical forming reaction is catalyzed by oxidative enzymes. *In vitro*, the coupling reaction results in a heterogenous mixture of dimeric compounds (fig 1). When a DP is added to the reaction, one stereoisomer of one compound is highly enriched. As DPs have no radical forming activity on their own, in the absence of oxidase, no reaction will occur. Therefore, DPs are a class of proteins which dictate the stereochemistry of a compound the synthesis of which is initiated by other enzymes.

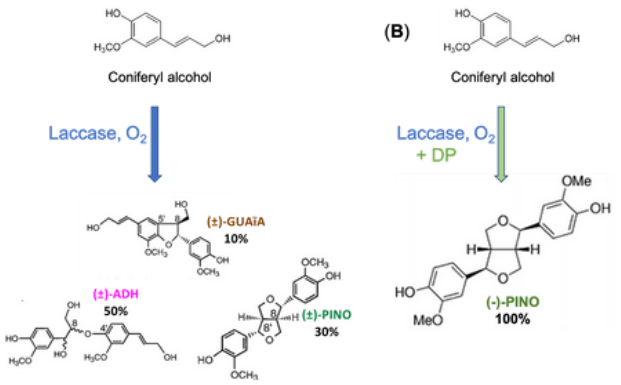

Figure 1: Bimolecular phenoxy radical coupling products from Coniferyl alcohol. (**A**) Dimeric lignans formed via "random" coupling. (±)-Guaïa: (±)-erthro/threo-guaiacylglycerol 8-O-4'-coniferyl alcool ethers, (±)-ADH: (±)-dehydorconoferyl amcphol, (±)-PINO: (±)-pinoresinol. (**B**) Stereoselective coupling to give (-)-pinoresinol.

Since the initial discovery, few enantiocomplementary DPs have been identified from plants and a little substrate/product (S/P) pairing have been distinguished: 1- proteins forming (+) or (-)-PINO are belonging to the lignan-forming DPs. 2 - proteins promoting the coupling of hemigossypol to (+)-gossypol ((+)-GOSSY) (*Gh*DIR4 and others from cotton) are belonging to aromatic diterpenoid-forming DPs. 3 -proteins involved in the conversion of pisatine into (–)- or (+)-medicarpins (e.g., PTS1 from *Glycyrrhiza echinate* or *Pisum sativum*) are belonging to aromatic terpenoid-forming DPs. From these studies, a common biochemical mechanism in quinone methide-stabilizing function in DPs was proposed[3]. Despite their huge representation in nature and their high potential in terms of biotechnology and chemistry, to date there has been no lighter shed on other DP function in plants mostly because of difficulties in the identification of the S/P couple.

Nowadays, the use of microorganisms in the search for secondary metabolites to enrich pharmaceutical industries with new therapeutic molecules is a hot spot. With the vast majority (>99%) of DP candidates still waiting for the discovery of their biochemical functions, a new bioinformatic analysis focusing on DPs domains newly identified in bacteria was done.

MATERIALS & METHODS

By combining informations from several database, InterPro classify proteins into families and predicting domains. Reactualisation of the analysis from 2020[1] of the DP domain signature permits to identify 7 new more bacterial DPL.

ClustalW and ClustalO proteins sequence alignment were performed between the 49 DPLs and *At*DIR6 to determine identity and similarity percentages or between 49 DPLs and 7 Plant DPs to shed light conserved (inspired by Pfam 03018, the HMM PD domain signature) or divergent residues and to make the phylogeny tree.

Bacterial peptide signal sequences were predicted by SignalIP 5.0. Cysteine (C) forming disulfide bond were predicted by DIANNA.

2D structure prediction was done by PredictProtein and 3D one by Swiss model with AtDIR6 or PTS1 sequences as query.

Ensembl/EMBL genome visualization interface permitted the identification of surrounding genes at DPL loci.

Table 1. List of DPLs obtained from Bioinformatic analysis.

	Species	Family	Gram genre	DPL Accession number	a.a. sequence size	Identity % to AtDIR6 (clustalW)	Similarité % to AtDIR6 (clustalW)	SignalP Signal type and probability score P	Signal peptide size in a.a.	S-S bridges prediction / probability Score
1	Streptomyces sp. Root1304	Streptomycetaceae	+	A0A0T1SRD5	166	21,90%	30,20%	Signal peptide (Sec/SPI) P=0,66	29	(14-98) / 0.13054
2	Streptomyces sp. PanSC19	Streptomycetaceae	+	A0A3N1Q171	167	21,50%	26,90%	Signal peptide (Sec/SPI) P=0,82	30	(12-14)/ 0.0262 & (85-99) / 0.0128
3	Streptomyces sp. 62	Streptomycetaceae	+	A0A309LXQ3	167	21,50%	32,50%	Signal peptide (Sec/SPI) P=0,80	30	(85-99) / 0.01227
4	Sphaerobacter thermophilus DSM 20745	Sphaerobacteraceae	+	D1C1V3	173	20,70%	34,50%	Signal peptide (Sec/SPI) P=0,89	36	(155-169) 0.0119
5	Kutzneria albida DSM 43870	Pseudonocardiaceae	+	W5WKJ3	164	20,50%	28,30%	Signal peptide (Tat/SPI) P=0,97	34	Only one Cys
6	Streptomyces vietnamensis GIMV4.0001	Streptomycetaceae	+	A0A085I9B2	160	20,10%	28,60%	Signal peptide (Sec/SPI) P=0,97	32	(13-101) / 0.99768
7	Streptomyces viridochromogenes	Streptomycetaceae	+	A0A0L8K704	166	19,50%	27,40%	Signal peptide (Sec/SPI) P=0,98	29	(84-98) / 0.01164
8	Cystobacter fuscus DSM 52655	Archangiaceae	-	A0A250WWY0	154	19,50%	39,40%	Lipoproteine signal peptide (Sec/SPII) P= 0,88	27	(81-92) / 0.99973
9	Streptomyces bottropensis ATCC 25435	Streptomycetaceae	+	M3E484	161	19,00%	30%	Signal peptide (Sec/SPI) P=0,99	29	(17-100) / 0.02139
10	Nocardioides iriomotensis NBRC 105384	Nocardioidaceae	+	A0A4Q5J010	152	18.80%	31,90%	Signal peptide (Sec/SPI) P=0,95	24	(81-92) / 0.18914
11	Methylomicrobium alcaliphilum	Methylococcaceae	-	G4T3L1	147	18,70%	31,00%	Lipoproteine signal peptide (Sec/SPII) P= 0,97	19	(27-87) / 0.94541
12	Archangium violaceum Cb vi76	Archangiaceae	-	A0A0845GS8	148	18,60%	32,20%	Lipoproteine signal peptide (Sec/SPII) P= 0,94	21	(75-86) / 0.99927
13	Chloroflexi bacterium GWC2_73_18		-	A0A1F8LMI0	155	18,10%	30,60%	Signal peptide (Sec/SPI) P=0,98	25	(82-93)/ 0.01037
14	Nocardia mexicana DSM 44952	Nocardiaceae	+	A0A370GIR3	161	17,90%	29.9%	Lipoproteine signal peptide (Sec/SPII) P= 0,95	35	(87-101) / 0.011
15	Gammaproteobacteria bacterium HGW		-	A0A2N1ZX47	146	17,70%	29,60%	Lipoproteine signal peptide (Sec/SPII) P= 0,99	18	(12-19)/0.11475 & (75-86)/0.01037
16	Cystobacter fuscus DSM 2262	Archangiaceae	-	S9NWQ1	137	17,60%	27,10%	No prediction	ND	(64-75) / 0.99977
17	Archangium gephyra	Archangiaceae	-	A0A0L8K704	151	17,30%	27,10%	Lipoproteine signal peptide(Sec/SPII) P= 0,99	19	(78-89) / 0.99976
18	Cystobacter ferrugineus str. Cbfe23	Archangiaceae	-	A0A1L9BSJ6	137	17,30%	26,90%	No prediction	Non	(64-75)/ 0.99977
19	Streptomyces sp. SA15	Streptomycetaceae	+	A0A2A2YWR6	206	17,00%	32,70%	Signal peptide (Sec/SPI) P=0,64	30	(106-118) / 0.01551
20	Stigmatella erecta	Archangiaceae	-	A0A150JK20	162	16,80%	25,20%	Signal peptide (Sec/SPI) P=0,48	ND	(89-100) 0.99972
21	Streptomyces alboflavus str. MDJK44	Streptomycetaceae	+	A0A1Z1WCN3	163	16,80%	31,80%	Signal peptide (Sec/SPI) P=0,87	27	Only one Cys
22	Streptomyces sp. NWU339	Streptomycetaceae	+	A0A2U2Z012	205	16,50%	32,20%	Signal peptide (Sec/SPI) P=0,89	30	(105-112) / 0.99952
23	Streptomyces formicae KY5	Streptomycetaceae	+	A0A291Q451	163	16,20%	27,60%	Signal peptide (Sec/SPI) P=0,63	33	(13-72) / 0.01115
24	Streptomyces sp. CB02400	Streptomycetaceae	+	A0A1Q5L121	205	16,10%	27,70%	Peptide signal (Sec/SPI) P=0,52	30	(105-117) / 0.01269
25	Streptomyces caelestis	Streptomycetaceae	+	A0A0M8QLY2	205	16,10%	27%	Lipoproteine signal peptide(Sec/SPII) P= 0,51	30	(105-117) / 0.01276
26	Pseudonocardiales bacterium YIM PH 21723	Pseudonocardiaceae	+	A0A2W6QT35	168	16,00%	28,30%	Signal peptide (Sec/SPI) P=0,76	33	(96-109) / 0.01038
27	Methylomicrobium kenyense (AMO17)	Methylococcaceae /cystobacteraceae	-	A0A543V9V5	149	15,80%	26,50%	Lipoproteine signal peptide (Sec/SPII) P= 0,97	ND	(5-16) / 0.01037, (76-148) / 0.10677 / (87-123) /0.0151
28	Streptomyces griseoruber DSM 40281	Streptomycetaceae	+	A0A1015V50	161	15,80%	25,40%	Signal peptide (Sec/SPI) P=0,96	29	(89-100) / 0.84116
29	Chloroflexi bacterium		-	A0A3A0ARB1	201	15,70%	27,30%	Lipoproteine signal peptide (Sec/SPII) P= 0,96	27	(21-112) / 0.01103
30	Thiogranum longum DSM 19610	Ectothiorhodospiraceae	-	A0A4R1H9Y2	148	15,70%	25,30%	Lipoproteine signal peptide (Sec/SPII) P= 0,94	21	(3-87) 0.01044 / (11-13) 0.01037 / (20-76) 0.99835
31	Streptomyces fungicidicus str. TXX3120	Streptomycetaceae	+	A0A494UTW6	205	15,40%	27,80%	No prediction	ND	(18-105) / 0.01038
32	Streptomyces toyocaensis str. NRRL 15009	Streptomycetaceae	+	A0A081KHD4	205	15,20%	29%	Lipoproteine signal peptide(Sec/SPII) P= 0,66	30	(105-117) / 0.8796
33	Streptomyces showdoensis ATCC 15227	Streptomycetaceae	+	A0A2P2GJ53	167	15,10%	21,10%	Signal peptide (Sec/SPI) P=0,87	29	(86-99) / 0.9521
34	Enterobacter ludwigii P101	Enterobacteriaceae	-	W0BV16	154	14,90%	22%	Signal peptide (Sec/SPI) P=0,77	19	(83-94) / 0.24511
35	Stigmatella aurantiaca	Archangiaceae	-	A0A1H7HD66	162	14,90%	24,60%	Lipoproteine signal peptide (Sec/SPII) P= 0,5	35	(19-30)/0.99661 & (89-100)/0.99972
36	Modestobacter sp. DSM 44400	Geodermatophilaceae	+	A0A1H3XGG2	123	14,80%	25,40%	No prediction	ND	(41-52) / 0.01041
37	Streptomyces radiopugnans	Streptomycetaceae	+	A0A1H9OCJ1	205	14,80%	27%	Signal peptide (Sec/SPI) P=0,55	32	(105-117) / 0.01319
38	Methylomicrobium buryatense 5GB1C	Methylococcaceae	-	A0A4P9UY52	131	14,80%	26,10%	No prediction	ND	(4-60) / 0.01179 & (7-71) / 0.01322
39	Vitiosangium sp. GDMCC 1.1324	Archangiaceae	-	A0A2T4VJB7	146	14,60%	25,00%	Lipoproteine signal peptide (Sec/SPII) P= 0,72	ND	(74-85) / 0.99977
40	Archangium sp. Cb G35	Archangiaceae	-	A0A1Q3H6L3	145	14,50%	22,90%	Lipoproteine signal peptide (Sec/SPII) P= 0,6	ND	(72-83) / 0.99927
41	Cellulomonas aerilata	Pseudonocardiaceae	+	A0A512DF30	174	14,40%	24,50%	Signal peptide (Sec/SPI) P=0,85	24	(99-115) / 0.0145
42	Streptomyces sp. NRRL S-4	Streptomycetaceae	+	A0A0N1K8E7	203	14,30%	25,10%	Signal peptide (Sec/SPI) P=0,79	30	(105-117) / 0.01052
43	Streptomyces silvensis ATCC 53525	Streptomycetaceae	+	A0A0W7K1T4	166	13,90%	26%	Signal peptide (Sec/SPI) P=0,71	33	(13-75) / 0.01037
44	Streptomyces sp. CNZ306	Streptomycetaceae	+	A0A2M9AZV5	258	13,80%	27,30%	No prediction	ND	(158-170) / 0.01045
45	Streptomyces dysideae sp. RV15	Streptomycetaceae	+	A0A124IFP4	187	13,20%	22,60%	Signal peptide (Sec/SPI) P=0,65	36	(112-124) / 0.06384
46	Streptomyces regalis	Streptomycetaceae	+	A0A0K3UWY4	122	12,50%	17,90%	No prediction	ND	(47-59) / 0.01173
47	Streptomyces sp. 13-12-16	Streptomycetaceae	+	A0A1X4I170	205	12,40%	24,10%	Signal peptide (Sec/SPI) P=0,58	30	(105-117) / 0.01039
48	Streptoalloteichus hindustanus	Pseudonocardiaceae	+	A0A1M5GN54	161	12,10%	22,20%	Signal peptide (Sec/SPI) P=0,90	31	(87-101) / 0.01037
49	Hyalangium minutum	Archangiaceae	-	A0A085VSV8	96	11,70%	20,30%	No prediction	ND	(23-34) / 0.95615

RESULTS & DISCUSSION

Soil bacteria are of major interest industrially and fundamentally since they are at the origin of the production of many bioactive molecules, as antibiotics. To continue the initial work performed by Dabravolski[1], we first updated datas obtained from the Interpro data base. We increased the candidate number to 49 sequences predicted to code bacterial DPL (Tab 1).

ClustalW protein sequence alignment was performed on the 49 DPLs and *At*DIR6, the best characterized plant DP to date[4]. DPLs have 12−22% amino acid (aa) sequence identity to *At*DIR6 (Tab 1) a score close from that obtained when comparing PINO- and GOSSY-forming plant DPs (24% identity between *At*DIR6 and *Gh*DIR4). Most of these DPLs have a predicted signal peptide sequence suggesting that they are secreted. The DP HMM profile Pfam03018 identified highly conserved aa amongst the DPL family members. But a divergence from aa found in active site PINO-forming DPs suggests functions other than lignan formation (Fig 2).

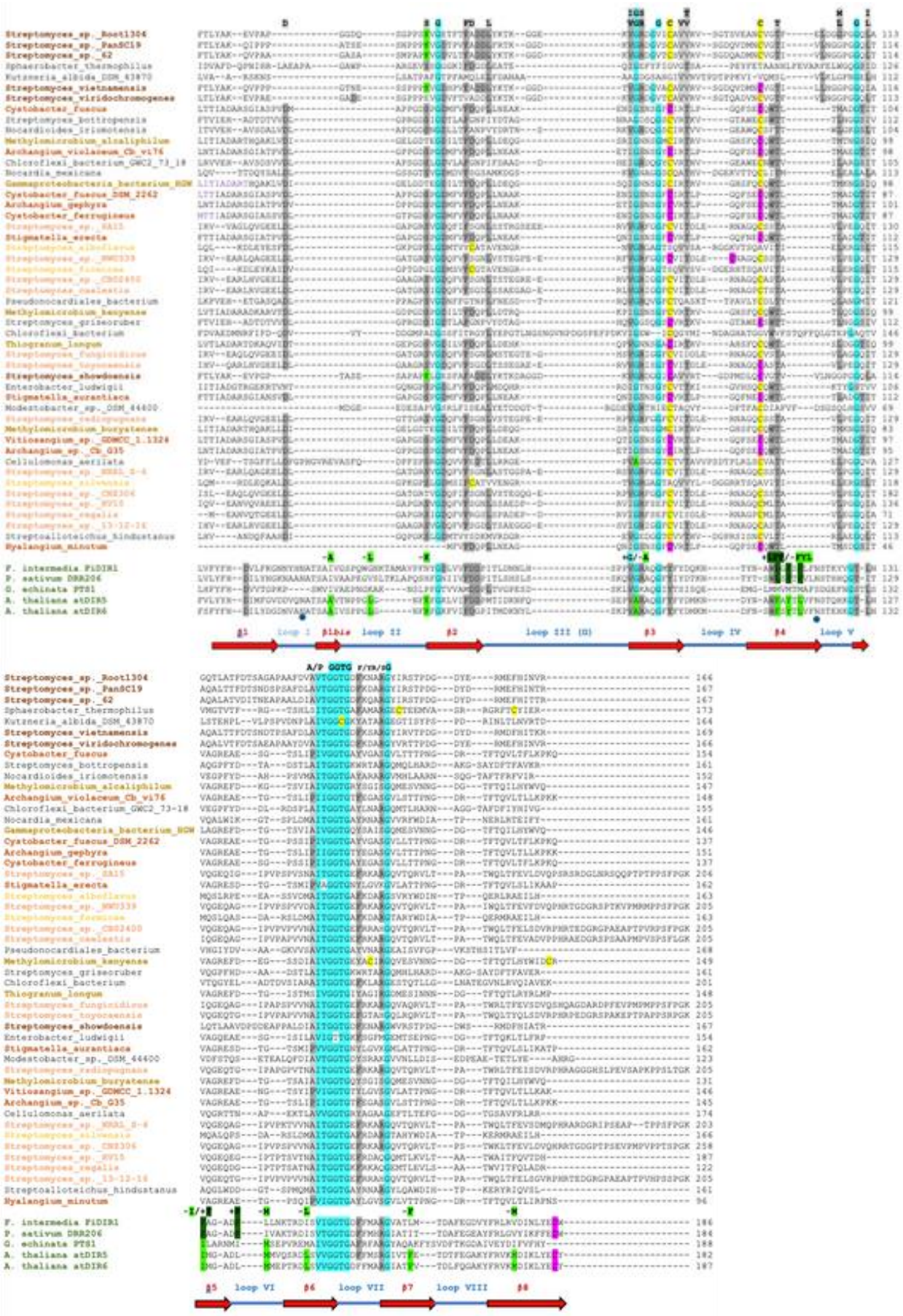

Figure 2: Annotated representation of the 49 DPLs and 5 plant DPs sequences alignment by ClustalO. X: Highly conserved residue inside the Pfam Dirigent family (PF03018), X: Highly conserved residue inside DPLs, X: Conserved residue inside DPLs. C: Cystein, C: Cystein with S-S bridge predicted, X: Residue from the peptide signal prediction. X: Residues involved in (+)-PINO synthesis in PINO-forming DPs, X: Residues involved in (-)-PINO synthesis in PINO-forming DPs. Blue lane: loop, red arrow: beta sheet. DPL Family I, DPL Family II, DPL Family III, DPL Family IV, DPL Family V, Not belonging DPL family, Plants DPs sample.

The phylogenetic analysis indicates 5 independent clusters apart from plants DPs (Fig 3) suggesting families regrouping proteins on substrate specificity.

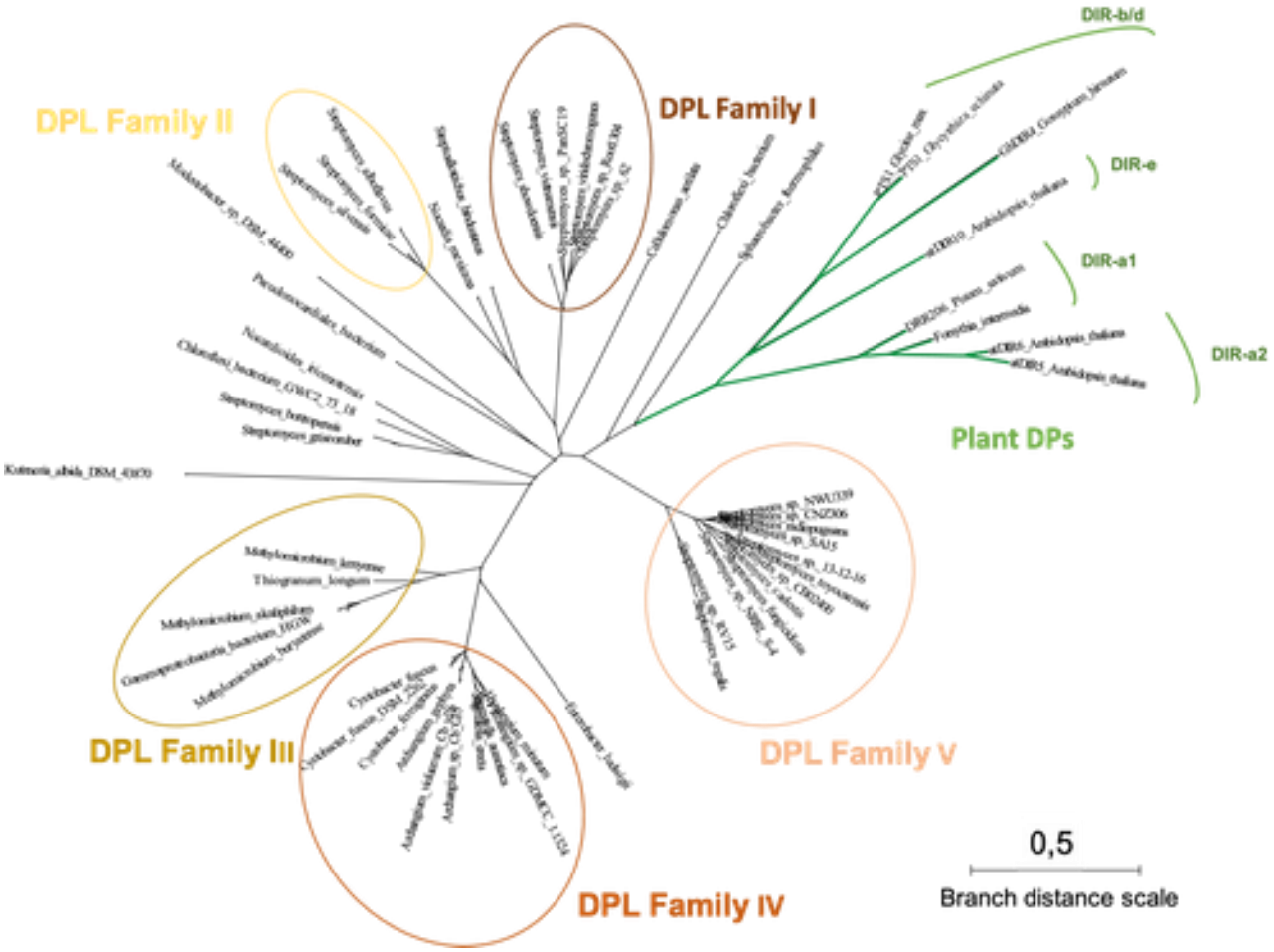

Figure 3: Phylogenic tree based on 49 DPLs and 8 plant DPs sequence alignment via Seaview software. The neighbour-joining algorithm was used with a Poisson-based distance calculation.

From 2D prediction and 3D modelling, the structure organization appears well conserved between DPLs and DPs since 6-8 central β barrels can be modeled using the 8 β strands of *At*DIR6 or PTS1 as query, the 3D structure of which is known[3,5] (Tab 2).

	DPL from species	Template AtDIR6		Template PTS1		4D structure	Number of β strand predicted	
		GMQE	Q-mean	GMQE	Q-mean		AtDIR6	PTS1
1	*Streptomyces sp. Root1304*	0.25	-4.70	0.32	-3.56	Monomeric	6	8
2	*Streptomyces sp. PanSC19*	0.39	-4.30	0.39	-4.30	Trimeric	8	8
3	*Streptomyces sp. 62*	0.31	-3.10	0.33	-3.03	Monomeric	7	7
4	*Sphaerobacter thermophilus strain DSM 20745*	0.22	-6.01	0.09	-5.35	Trimeric	7	4
5	*Kutzneria albida DSM 43870*	0.22	-5.66	0.28	-5.43	Monomeric	6	7
6	*Streptomyces vietnamensis GIMV4.0001*	0.25	-3.63	0.31	-2.91	Monomeric	6	7
7	*Streptomyces viridochromogenes*	0.36	-2.32	0.33	-2.59	Monomeric	8	7
8	*Cystobacter fuscus DSM 52655*	0.21	0.13	0.28	-2.58	Monomeric	6	7
9	*Streptomyces bottropensis ATCC 25435*	0.36	-1.39	0.35	-1.42	Monomeric	8	8
10	*Nocardioides iriomotensis NBRC 105384*	0.24	-2.59	0.27	-3.25	Monomeric	7	7
11	*Methylomicrobium alcaliphilum*	0.20	-0.38	0.28	-1.47	Monomeric	6	7
12	*Archangium violaceum Cb vi76*	0.22	-0.69	0.30	-2.28	Monomeric	6	7
13	*Chloroflexi bacterium GWC2_73_18*	0.23	-2.57	0.29	-2.43	Monomeric	7	7
14	*Nocardia mexicana DSM 44952*	0.23	-2.56	0.55	-0.75	Monomeric/ Trimeric	6	8
15	*Gammaproteobacteria bacterium HGW*	0.21	-1.14	0.29	-1.87	Monomeric	6	7
16	*Cystobacter fuscus DSM 2262*	0.26	-0.62	0.26	-0.23	Monomeric	6	6
17	*Archangium gephyra*	0.21	0.05	0.29	-1.42	Monomeric	6	7
18	*Cystobacter ferrugineus str. Cbfe23*	0.26	0.06	0.50	-2.07	Monomeric/ Trimeric	6	8
19	*Streptomyces sp. SA15*	0.18	-1.62	0.27	-2.24	Monomeric	6	8
20	*Stigmatella erecta*	0.18	-0.58	0.24	-1.50	Monomeric	6	7
21	*Streptomyces albaflavus str. MDJK44*	0.21	-2.21	0.27	-2.15	Monomeric	6	7
22	*Streptomyces sp. NWU339*	0.17	-2.35	0.25	-2.42	Monomeric	7	8
23	*Streptomyces formicae KYS*	0.21	-1.38	0.21	-2.84	Monomeric	6	6
24	*Streptomyces sp. CB02400*	0.16	-2.17	0.25	-2.70	Monomeric	6	8
25	*Streptomyces coelestis*	0.16	-2.03	0.25	-3.43	Monomeric	6	8
26	*Pseudonocardiales bacterium YIM PH 21723*	0.22	-1.85	0.22	-3.02	Monomeric	6	6
27	*Methylomicrobium kenyense (AMO1?)*	0.23	-0.17	0.27	-2.04	Monomeric	6	7
28	*Streptomyces griseoruber str. DSM 40281*	0.24	-1.49	0.34	-0.83	Monomeric	7	8
29	*Chloroflexi bacterium*	0.07	-1.58	0.09	-2.59	Monomeric	4	5
30	*Thiogranum longum str. DSM 19610*	0.23	-0.25	0.30	-1.33	Monomeric	6	7
31	*Streptomyces fungicidicus str. TXX3120*	0.17	-2.31	0.25	-1.93	Monomeric	7	8
32	*Streptomyces toyocaensis str. NRRL 15009*	0.20	-2.25	0.25	-2.29	Monomeric	7	8
33	*Streptomyces showdoensis ATCC 15227*	0.32	-2.78	0.33	-2.52	Monomeric	7	7
34	*Enterobacter ludwigii str. P101*	0.17	-1.30	0.17	-0.98	Monomeric	6	6
35	*Stigmatella aurantiaca*	0.18	-0.50	0.20	0.21	Monomeric	6	7
36	*Modestobacter sp. DSM 44400*	0.40	-4.07	0.37	-2.36	Monomeric	6	6
37	*Streptomyces radiopugnans*	0.17	-1.62	0.23	-2.41	Monomeric	6	7
38	*Methylomicrobium buryatense str. 5GB1C / 4,99 Mpb, 4152 cds*	0.26	-0.54	0.35	-1.01	Monomeric	6	7
39	*Vitiosangium sp. GDMCC 1.1324*	0.23	0.19	0.30	-1.57	Monomeric	6	7
40	*Archangium sp. Cb G35*	0.23	-0.12	0.31	-1.20	Monomeric	6	7
41	*Cellulomonas aerilata*	0.19	-1.66	0.21	-1.79	Monomeric	6	6
42	*Streptomyces sp. NRRL S-4*	0.16	-1.54	0.21	-2.51	Monomeric	6	7
43	*Streptomyces silvensis ATCC 53525*	0.23	-3.17	0.21	-1.63	Monomeric	6	6
44	*Streptomyces sp. CNZ306*	0.12	-1.13	0.19	-2.43	Monomeric	6	8
45	*Streptomyces dysideae sp. RV15*	0.18	-3.23	0.29	-3.59	Monomeric	6	8
46	*Streptomyces regalis*	0.39	-3.30	0.40	-2.43	Monomeric	6	6
47	*Streptomyces sp. 13-12-16*	0.16	-2.45	0.20	-3.21	Monomeric	6	7
48	*Streptoalloteichus hindustanus*	0.23	-1.58	0.25	-2.34	Monomeric	6	6
49	*Hyalangium minutum*	0.43	0.24	0.44	-0.75	Monomeric	6	6

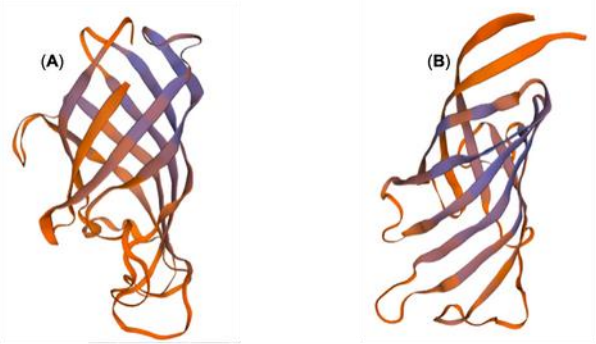

Table 2: Summary table of modelling and two examples of 3D prediction model realized on Swiss-model with AtDIR6 or PTS1 as templates. (A) *S. Viridochromogense* 9xjB5L 3D prediction model (template AtDIR6) and (B) *S. SA15* NUgJb prediction model (template PTS1).

Additionally, two C are found in the putative active site pockets (in *β3 and β4*[5], Fig 2) possibly forming disulfur-bridges predicted for 28% of the DPL sequences. Such a proposed organization differs that found in plant DPs in which C likely stabilizing the barrel structure are localized at the N- and C-extremities of the sequences[5].

Interestingly, for at least 17 DPLs, genomic organization analysis reveals that a DPL coding gene clusters with genes the products of which are involved in already known Biosynthetic pathways. These hits are primary candidates for cloning and further studies.

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P4.1

Exploring extractable and non-extractable polyphenols in banana flower and banana pseudo-stem. Effect of harvest year

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MAIN CONCLUSION

The detailed analysis of phenolic compounds in DBF (dried banana flower) and DBPS (dried banana pseudo-stem) showed that DBF may be used as an additional source of phenolic compounds, particularly NEPA, although the establishment of accepted content ranges between harvest years may be needed for their use as functional ingredients.

INTRODUCTION

Banana flower (DBF) and banana pseudo-stem (DBPS) are by-products from banana fruit harvest and processing in several industries. The interest in the potential biological activities of these banana residues has been renewed in recent times. Studies on polyphenols have been traditionally focused on the so-called extractable polyphenols (EPP), although increasing evidence is showing the relevance of non-extractable polyphenols (NEPP) or macromolecular antioxidants, which are high molecular weight polyphenols or small ones associated with macromolecules such as protein or dietary fibre (Pérez-Jiménez *et al*., 2013).

Nevertheless, studies on the phytochemical characterization of DBF and DBPS are still scarce and focused on extractable polyphenols (EPP). Thus, Bashkar *et al*. (2012) is the only found study with these two banana by-products from *Musa sp. elakki bale*, where HPLC analysis for EPP profile was performed. Also, Schmidt *et al*. (2015) have reported some characterization of banana male flower and bracts from *Musa cavendish* in which EPP content by spectrophotometry was measured. Recently, increasing evidence is showing the biological relevance of NEPP (Pérez-Jiménez *et al*., 2013) and, therefore, there is an interest to identify the presence of these compound on more vegetal materials.

The present study aimed to characterize EPP and NEPP profile in DBF and DBPS by HPLC-MS analysis, including the effect of harvest time, to advance towards their use as functional ingredients.

MATERIALS & METHODS

DBPS and DBF were supplied by local producers in 2017 and 2018 for both materials. EPP determination was performed following the protocol by Pérez-Jiménez et *al*. (2008). NEPP were measured in the residues of EPP extractions as the sum of hydrolysable polyphenols (HPP) and non-extractble proanthocyanidins (NEPA). HPP was determined by the protocol established by Arranz *et al*. (2009) and Hartzfeld *et al*. (2002), based on acid hydrolysis with temperature, while determining the butanolysis procedure was applied (Pérez-Jiménez *et al*. 2009). The EPP and HPP fractions from DBF, as well as HPP fraction of DBPS, were concentrated (6:1) For separation, HPLC with DAD and an ESI-QTOF mass analyzer (Agilent G6530A) was used. Peak identity was established by comparison with the retention times of commercial standards when available and the molecular formula proposed were compared with previously reported phenolic compounds in banana and other vegetal materials.

RESULTS & DISCUSSION

The identity of individual phenolic compounds in DBF and DBPS was performed for the first time by HPLC-MS. Detailed HPLC-ESI-QTOF MS analysis showed that in both EPP and HPP fractions, phenolic acids were the most relevant constituents, although some flavanols were also identified in the EPP fraction. Due to the total concentration and profile of individual phenolic compounds in DBF, this product, as an additional source of phenolic compounds in the context of the whole diet, might have health benefits. Indeed, protocatechuic acid was detected in DBF at a concentration higher than that observed in most common foods, according to the Phenol-Explorer database (Neveu *et al*., 2010). Although the potential effects would be mostly derived from the combination of all phenolic compounds present in this product. Also, regarding the high NEPA content detected in DBF, it should be remarked that other products rich in these phenolic compound fractions, such as grape pomace, have shown several health-related properties, for instance regarding fasting insulin (Martínez-Maqueda *et al*., 2018). Significant differences between harvest year were detected for some individual phenolic compounds and some phenolic fractions. Nevertheless, for most detected phenolic compounds, identity could not be assigned, since they corresponded to structures not present in common databases or in literature on banana. This indicates the need for further structural analysis in DBF and DBPS to suggest structures for some of these unidentified compounds.

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P4.2

Snailase is a powerful tool for the enzymatic hydrolysis of flavonoids

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MAIN CONCLUSION

The enzyme mix snailase is a promising tool for the enzymatic hydrolysis of various flavonoids. The highest yields were obtained by hydrolysis with snailase of flavonoids present in leaves (*R. pseudoacacia*, *F. sylvatica*, *Q. robur*, *M. domestica*) and flowers (*B. ferulifolia*, *P. hybrid*a) compared to other enzymes (cellulase, cellobiase, β-glucosidase and pectinase) as well as acidic hydrolysis.

INTRODUCTION

Flavonoids are one of the most studied secondary plant metabolites with an estimated number of about 10.000 different compounds [1]. Many flavonoid subgroups are known, to name a view, flavones, flavonols, isoflavones, chalcones, aurones or anthocyanins. Flavonoids are mostly accumulated as glycosides and can be mono or poly glycosylated on different positions. Glycosylation at various positions of a flavonoid can lead to more complexity and peak interferences during chromatographic analysis. Hence, for many purposes, glycose moieties are removed, which can be performed chemically with strong acids at high temperatures [2]. However, the optimal acidic hydrolysis conditions depend on the type of flavonoid and a standardized protocol to hydrolyze all classes of flavonoids simultaneously is not described. Therefore, it is less suitable for studies, which focus on the analysis of various types of flavonoids from different raw materials. On contrary, removal of sugar moieties can also be carried out enzymatically by hydrolases [3], but the activity of the enzyme can be influenced by co-extracted inhibitors, such as tannins or other parameters, for instance pH value as well as temperature, which has to be considered.

To evaluate the efficiency of protocols for sugar moiety removal from different flavonoid classes, we produced methanolic extracts of the leaves of *Quercus robur*, *Fagus sylvatica*, *Robinia pseudoacacia* and *Malus domestica* as well as of the flowers of *Bidens ferulifolia* and *Petunia hybrida* and investigated the yields of the flavonoid aglycones after enzymatic and acidic hydrolysis. The study comprises four individual enzymes (cellulase, cellobiase, β-glucosidase and pectinase), the enzyme mix ‘snailase’ and chemical hydrolysis by hydrochloric acid (HCl).

MATERIALS & METHODS

The raw material was shock-frozen with liquid N_2, grinded homogenously with a mortar and 0.30 g were extracted with 3 mL MeOH for 2h. Extractions were performed in triplicates, filtrated and stored at -20°C in the dark until analysis.

For enzymatic hydrolysis, 5–20 U enzyme (cellobiase, cellulose, β-glucosidase, pectinase) or 5 mg snailase, were used in a 100 or 200 µL assay with 10 vol% plant extract. The hydrolysis was carried out for 25 min at pH (4.5–6.0) and temperature (37–52°C) depending on the enzyme. Acidic hydrolysis was performed with 1–2 M HCl at 70–100°C for 1–2 h with a ratio of 1:5 extract:HCl. The preparation of the enzymatic reaction solution and HPLC analysis was based on literature [3]. Quantification of quercetin and dihydrokaempferol was performed with the respective standard substance. Acacetin, myricetin, phloretin and okanin are expressed as quercetin equivalents for preliminary comparison. Yields are presented as µg flavonoid /g plant material.

RESULTS & DISCUSSION

The preliminary yields of the predominant flavonoids of each investigated plant material after enzymatic and acidic deglycosylation are presented in the figure below.

The hydrolysis of methanolic extracts by snailase yielded higher amounts of acacetin (Figure 1A, 39%), myricetin (Figure 1B, 27%) and okanin (Figure 1E, 13%) compared with other tested protocols. Quercetin yields (Figure 1C) are comparable between enzymatic hydrolysis by snailase and cellobiase. In addition, both enzymes yielded similar amounts of phloretin and acidic hydrolysis led to slightly less amounts of the dihydrochalcone (Figure 1D). Furthermore, similar yields of dihydrokaempferol were obtained with snailase, cellubiase, cellulose and pectinase (Figure 1F).

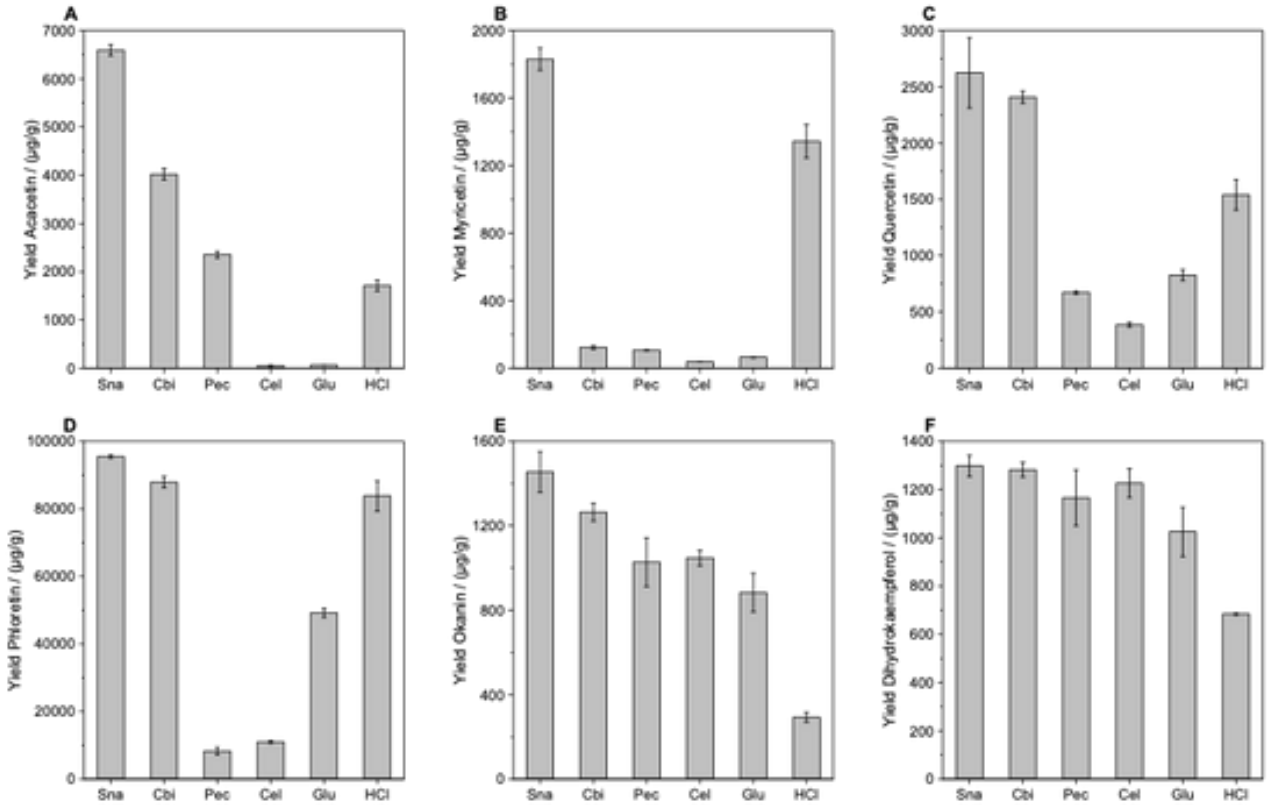

Figure 1. Yields (µg/g) of predominant flavonoids by enzymatic and acidic hydrolysis. **A**: Yield of acacetin in *R. pseudoacacia*. **B**: Yield of myricetin in *F. sylvatica.* **C**: Yield of quercetin in *Q. robur.* **D**: Yield of phloretin in *M. domestica.* **E**: Yield of okanin *B. ferulifolia.* **F**: Yield of dihdrokaempferol in *P. hybrid.* **Sna**: Snailase (5 mg) at 37°C pH 5.5 for A-F; **Cbi**: Cellobiase (5 U) at 37°C pH 4.5 for A-F; **Pec**: Pectinase (20 U) at 40°C pH 4.5 for A-F, **Cel**: Cellulase (20 U) at 52°C pH 6.0 for A-F; **Glu**: β-Glucosidase (20 U) at 37°C pH 5.0 for A, C and pH 5.5 for B, D, E, F; **HCl**: Acidic hydrolysis by HCl for A (1 M/100°C/2 h), B (1 M/70°C/1 h), C (1 M/70°C/2 h), D and F (1 M/100°C/1 h), E (1 M/100°C/2 h) (n = 3 ± SD).

We gratefully acknowledge funding by the Vienna Science and Technology Fund (WWTF) under grant number ESR17-027.

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P4.3

Isolation and purification of betalains from red beetroot (*Beta vulgaris* L.) using automated flash chromatography

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MAIN CONCLUSION

The flash chromatography method employed in this study successfully purified betanin and vulgaxanthin I from red beetroot extract. The purity of betanin and vulgaxanthin I was 95% and 84% according to the HPLC peak area analysis with 4.87 ± 2.12 and 3.02 ± 2.89 mg/g purification yield respectively.

INTRODUCTION

Betalains are water-soluble nitrogen-containing pigments which are formed by the conjugation of glycosyl derivatives or amino acid derivatives with betalamic acid. This combination leads to formation of two different types of betalains, violet coloured betacyanins and yellow coloured betaxanthins. Red beetroot, prickly pear and dragon fruit are the most popular dietary sources of betacyanins and betaxanthins (Gandía-Herrero et al., 2012). Betalains have gained increasing attention in recent years not only due to their high tinctorial properties but also their promising bioactive properties.

Purification of individual betalains has been reported using different techniques, such as column chromatography and preparative HPLC, including from complex food matrices (Gonçalves et al., 2012). However, the purification yield and efficiency of target compounds are not adequate for further applications such as to evaluate their bioactivity. Flash chromatography is a simple and robust column based technique for compound purification and can be applied to both reversed and normal phase separation. Further, use of high flow rate with relatively low pressure provides good separation within short time under chromatographic conditions (Stevens and Hill, 2009). This method is more frequently applied to purification of synthetic compounds, however, there is an increasing interest in utilizing flash chromatography for natural compound purification approaches.

The development of a purification method for betalains which can be effectively scaled up is important to industrial applications as well as for analytical purposes. Therefore, the present study aimed to develop a simple and effective method for purification of betanin and vulgaxanthin I from red beetroot using reversed-phase flash chromatography.

MATERIALS & METHODS

Red beetroot (10 g) was extracted with 500 mL of 30% (v/v) ethanol by homogenization and sonication. The extracts were filtered and narrowed down to 20% of initial volume using Genevac centrifugal evaporator. Concentrated extracts of red beetroot (15 mL) were submitted to a reversed-phase flash chromatography procedure using a KP-C18-HS Biotage SNAP cartridge mounted on a fully automated Bitoage Isorela system. Eluted fractions were stored separately at -20 °C until further use. Identification of the collected fractions was done with HPLC/MS and accurate mass methods.

RESULTS & DISCUSSION

The major betalains present in the purified fractions were betanin and vulgaxanthin I with purification yields of 4.87 mg/g and 3.02 mg/g, respectively, based on the HPLC-MS analysis. After initial identification, purity of betanin and vulgaxanthin I was analyzed using HPLC-PDA and monitored at

536 nm, and 486 nm which are characteristic λ_{max} for betacyanins and betaxanthins, respectively. A higher percentage purity (>95%) was observed for betanin while vulgaxanthin I showed 84% purity, when using the peak area of PDA chromatograms. The structural data for ESI-MS: m/z for betanin was 551 $[M+H]^+$ whereas vulgaxanthin I was 340 $[M+H]^+$ which is in alignment with previous studies conducted by Gonçalves et al. (2012). Further research is aiming to apply the methodology to other betalains and determine in vitro biological properties of different betalains.

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P4.4

Salicis cortex: influences of sex and harvest season on polyphenolic content in four *Salix* species

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MAIN CONCLUSION

In this study polyphenolic patterns of willow bark extracts were evaluated in terms of interspecific and sex-related effects during the growth period 2018.

Analysed extracts of the observed dioecious species revealed interspecific, but no intersexual differences in composition throughout the considered timeframe. Therefore sex-related influences on phenolic pattern could be neglected, while impacts of interspecific and seasonal volatility on extract composition were evident.

INTRODUCTION

The ESCOP (European Scientific Cooperative on Phytotherapy) monographs willow bark as herbal drug used for the treatment of lower back pain, fever associated with common cold and mild rheumatic conditions[1]. These effects cannot be attributed to salicylic alcohol derivatives solely, but other polyphenols such as flavonoids and proanthocyanidins (PAs) are also considered to contribute to the pharmacological effects[2]. The European Pharmacopoeia (Ph. Eur.) suggests three dioecious species for the collection of the drug Salicis cortex: *Salix fragilis* L., *S. daphnoides* Vill. and *S. purpurea* L.. Besides these, any *Salix* species can be used as long as a minimum content of salicylic alcohol derivatives of 1.5%, calculated as Salicin referred to dried drug, is complied[3]. The monography does not define a harvest season, disregarding potential quality changes during the growth period.

To study the seasonal fluctuation of proanthocyanidins, flavonoids and other phenolic compounds, sprouts of the current year were collected at four dates spread over one growing season (May, June, July, September 2018) based on prior studies[4]. The individuals included in this evaluation are all located at the Ecological-Botanical Gardens of the University of Bayreuth (ÖBG Bayreuth), Germany.

To investigate possible intraspecific sex-dependent differences and similarities, four species were selected. *Salix fragilis* L. *and S. cinerea* L. were represented by four male and four female individuals while *S. purpurea* L. (5♀/3♂) and *S. caprea* L. (3♀/4♂) were not equally represented (one female *S. caprea* was excluded). Phenolic contents were analysed by RP-chromatography applying an UPLC®-PDA-method[4].

MATERIALS & METHODS

Samples were collected according to Ph. Eur. 10 at ÖBG Bayreuth, cut and dried in desiccators over silica gel, ground (Retsch® MM400) and stored at -10°C until further use. For analysis, methanolic extracts (≈ 50 mg/ml, 30 min ultrasonic bath) were filtered (0.2 µm) and analysed by UPLC®-PDA. Chromatography was performed on a Waters ACQUITY UPLC® consisting of ACQUITY H-Class QSM, FTN and PDA detector (column: Phenomenex® Luna Omega C18; eluent A: 1% formic acid; eluent B: acetonitrile + 1% formic acid). For peaks having an area > 50k units in maxplot (240–400 nm), UV-maxima were extracted and dispensated according to **Tab. 1**.

Table 1. Dispensation matrix polyphenols.

class	class 1 (flavan-3-ols)	class 2 (salicylic alcohol derivatives)	class 3 (phenylpropanes)	class 4 (flavonoids/ chalcones)	class 5 (flavanones)
maximum (absolute)	220 – 253 nm	264 – 283 nm	220 – 253/ 254 – 263 nm	220 – 253/ 320 – 339 nm	284 – 293 nm
maximum (local)	274 – 293 nm	220 – 253 nm	300 – 319 nm	320 – 339/ 220 – 253 nm	220 – 253 nm

RESULTS & DISCUSSION

Regarding the relative composition of polyphenols within one species at one specific date, no significant differences could be observed. **Fig. 1** shows exemplarily the percentual proportions of evaluated classes of polyphenolic constituents for *S. caprea* in July 2018. *S. caprea* showed speaking likeness, whereas class 1 was the dominating fraction accounting for a proportion of 71 ± 8% peak area (mean ± SD). Intraspecific diversity in other species was in comparison much higher, e.g. individuals of *S. purpurea* varied for class 1 between 0–21% (class 2: 11–24%; class 3: 0–3%; class 4: 18–61%; class 5: 2–39%)

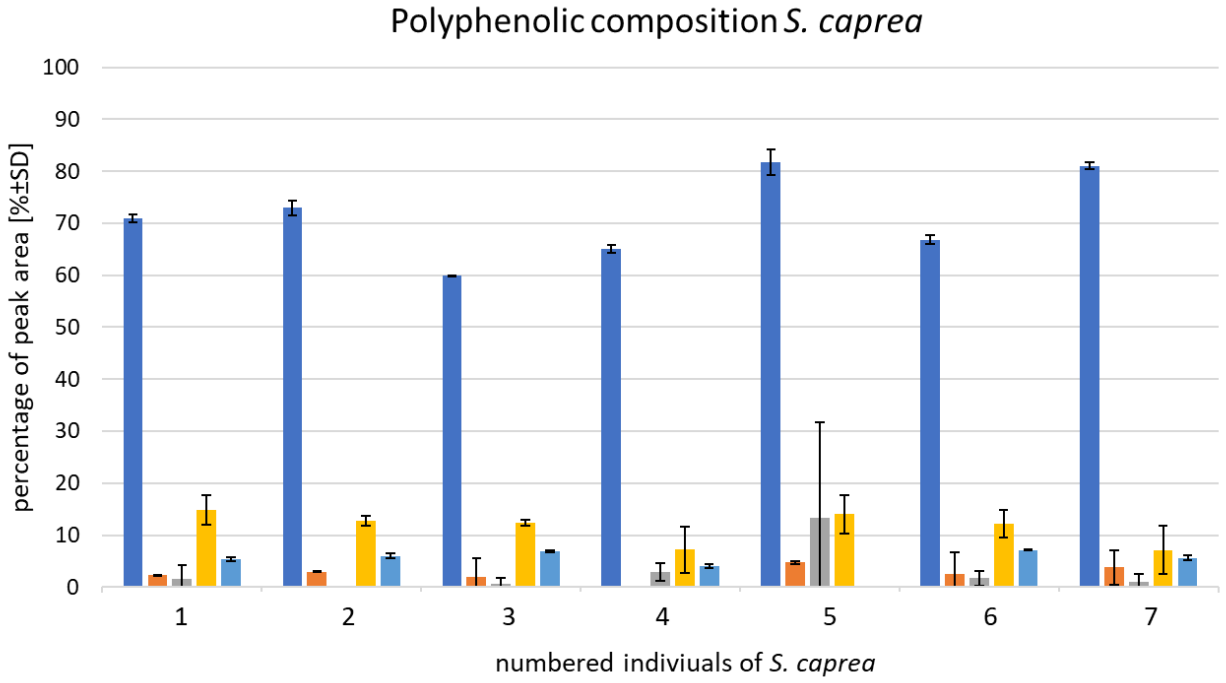

Figure 1. Polyphenolic composition of seven included *S. caprea* in percent of area (July 2018); class 1 (blue), class 2 (orange), class 3 (grey), class 4 (yellow), class 5 (light blue).

When percentages are averaged intersexual differences within one species are negligible, while interspecific distinctions were evident. For all four species, a dominant class of constituents could be defined: *S. caprea* preferred flavan-3-ols, *S. cinerea* phenylpropanoids while in *S. fragilis* and *S. purpurea* flavonoids and flavanones were prevailing. **Fig. 2** shows averaged proportions for both sexes of evaluated dioecious species in May 2018.

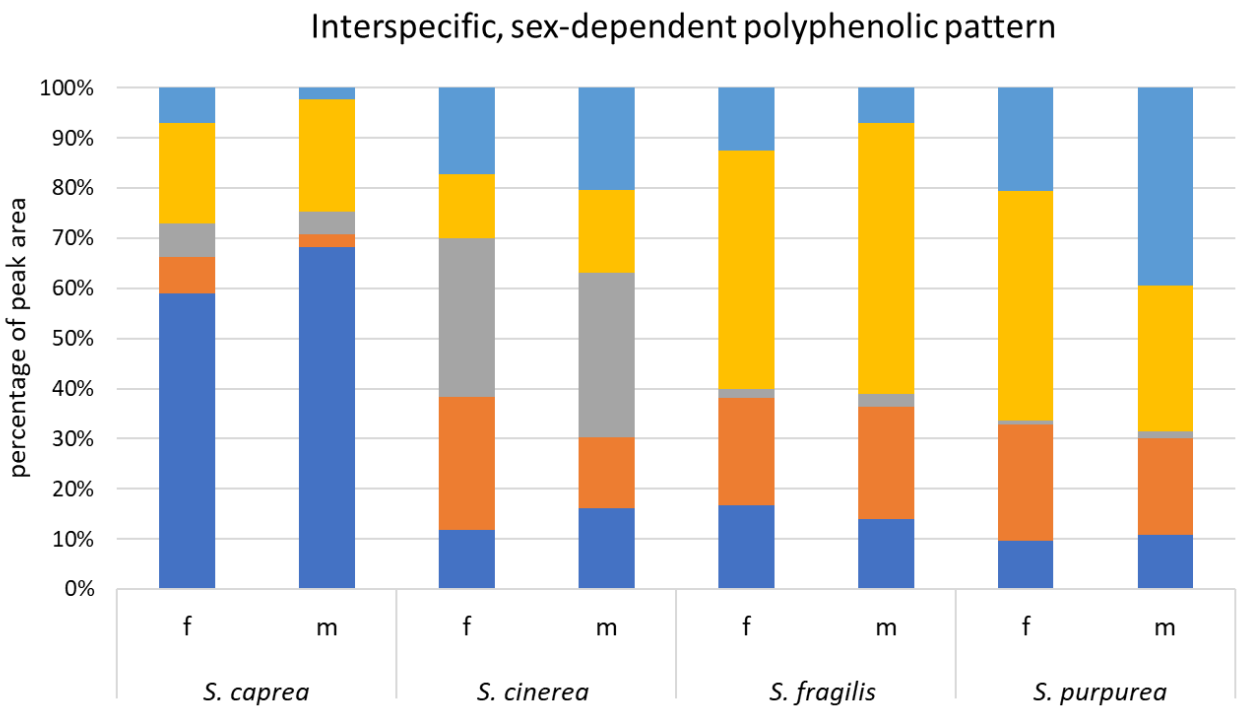

Figure 2. Polyphenolic composition by species and sex (f= female, m= male; species below); class 1 (blue), class 2 (orange), class 3 (grey), class 4 (yellow), class 5 (light blue); May 2018

Seasonal fluctuation effects the phenolic pattern varyingly strong. Percentages of *S. caprea* became apparent as very stable throughout the growing season, while other species, such as *S. purpurea* seem to be more affected by growing season as influencing variable.

Future studies have to show, if changes in composition throughout the growing season comply with a rule every year. Similar to this study, samples will be analysed for the growing season 2019 to compare the obtained data with the results presented here.

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P4.5

Procyanidin variation in leaves and stems of wild and cultivated *Vaccinium* species

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MAIN CONCLUSION

Results from this study indicate that leaves and stems of different wild and cultivated *Vaccinium* species are suitable for valorization as sources of natural procyanidins al well as to be valuable raw material for the manufacture of herbal supplements.

Acknowledgements: „This work was supported by a grant of Ministery of Research and Innovation, CNCS - UEFISCDI, project number PN-III-P1-1.1-PD-2016-1060, within PNCDI III".

INTRODUCTION

Leaves and stems of *Vaccinium* plants are used as food and dietary supplements due to their nutritional value and richness in antioxidant polyphenols. The *in vitro* and *in vivo* biological activities of phenolic compounds from natural sources involve application as antioxidants, antibacterial and anticarcinogenic agents, amendments in bioremediation, allelochemicals, and plants growth regulators (Bujor et al., 2015). In plants, several factors may influence the quality and quantity of phenolic compounds, including the parts of the plant to be used, the harvest period, the stage of growth, the environmental growing conditions, the species and the cultivars. As shown in the recent studies carried out by O.-C. Bujor, different contents of procyanidins in aerial parts of wild *Vaccinium* species were found. (Bujor et al., 2016; Bujor et al., 2018).

The aim of this study was to assess the variations of the procyanidins in leaves and stems of wild bilberry (*Vaccinium* myrtillus L.), wild lingonberry (*Vaccinium vitis-idaea* L.) and cultivated blueberry (*Vaccinium corymbosum* L., Blueray and Coville varieties). A HPLC method based on the thioacidolysis of the oligomeric procyanidins was used for analysis of the degree of polymerization and flavanol unit constitution.

MATERIALS & METHODS

Analysis of procyanidins by HPLC following thioacidolysis was applied on crude materials (powders) of leaves and stems of wild bilberry (*V. myrtillus* L.), wild lingonberry (*V. vitis-idea* L.) and cultivated blueberry (*V. corymbosum* L., Blueray and Coville varieties).

Procyanidin analysis was performed by HPLC after thioacidolysis using a method adapted from Le Bourvellec et al. (2011). Procyanidins were characterized by their subunit compo-sition and their average degree of polymerization (mDP). HPLC analyses were performed using an An Agilent Technologies 1200 chromatograph equipped with an UV-DAD detector. Separations were achieved using a (250 mm x 4 mm i.d.) Licrocart (Licrospher PR-18 5 µm) column (Merck, Darmstadt, Germany) operated at 30 °C. Phenolic compounds were identified by comparison of their retention time, their UV-visible spectra with those of standards and literature.

RESULTS & DISCUSSION

In lingonberry, thioacidolysis revealed low degrees of polymerization (2.5 in stems and 3.1 in leaves) and (-)-epicatechin as the main flavan-3-ol unit. Procyanidins contain (+)-catechin and (-)-epicatechin as both extension and terminal units. In leaves, the flavanol monomers were detected as

(+)-catechin and (-)-epicatechin, the former being highly preponderant. By contrast, almost similar amounts of flavanol monomers were quantified in stems.

In bilberry and blueberry varieties, mDP ranging from 3.1 to 7.6 suggests the presence of small-size oligomers. Epicatechin was the only constituting unit of flavanol oligomers and the rest as extension units.

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P4.6

Influence of choice of solvents and extraction techniques on the recovery of phenolic phytochemicals linked to the antioxidant and enzyme inhibition potential of *Clerodendrum glandulosum* Lindl.

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MAIN CONCLUSION

This study exhibited the competency of different solvents on the recovery of polyphenolic phytochemicals and based on the results, it is confirmed that polar solvent (95% methanol) found to be the best solvent for extraction of phenolic antioxidant bioactive phytoconstituents like verbascoside from this species. On the other hand, soxhlet extraction technique using 95% methanol as a solvent has proven to be the best technique to recover maximum polyphenols from *C. glandulosum*.

INTRODUCTION

Polyphenols are the major class of phytochemicals present in the medicinal plants and renowned for their wide range of biological functions including antioxidant, anti-inflammatory, anti-bacterial, anti-diabetic, anti-fungal and anti-atherosclerotic etc. However, pharmacological activity of polyphenolic herbal extracts may vary with the selection of solvents and extraction techniques.

Clerodendrum glandulosum Lindl. (CG), a common perennial herb of North-Eastern (NE) India with 33 related species, is a member of the Lamiaceae family. It has traditionally been used to treat metabolic disorders such as obesity, diabetes, hypertension, and hypercholesterolemia, as well as helminth infections, diarrhoea, indigestion, asthma, bronchitis, and fever. CG has been shown to have cardioprotective, anti-hyperlipidemic, anti-hypertensive, hepatoprotective, antioxidant, and antihelminthic properties in several investigations. The polyphenol rich extract obtained from the leaves of CG have been exhibited significant anti-hyperlipidemic and antioxidant effects in the animal models. The leaves of CG contains many important phytochemicals where verbascoside is reported to be the principle compound, which is a phenolic molecule having diverse biological potential.

However, there is no comprehensive studies have been performed on the optimization of solvents for extraction and suitable extraction techniques for maximum recovery of phenolic phytochemicals. Therefore, in this study a comparative picture of extraction efficiency of different solvents and extraction techniques to achieve optimum bioactivity and optimum recovery of phytochemicals have been drawn.

MATERIALS & METHODS

The powdered leaves of *Clerodendrum glandulosum* (10 gm) were extracted with different solvents viz; ethyl-acetate (EA), methanol (ME), 95% methanol (MH), ethanol (ET), 95% ethanol (EH), acetone (AC) separately by cold maceration technique.

Similarly powdered leaves (10 gm) was extracted with 95% methanol by employing various extraction techniques including microwave assisted extraction (MAE), ultrasound assisted extraction (UAE), soxhlet extraction and heat reflux extraction. Percentage yield of all the extracts was estimated.

All the extracts have been subjected to estimate total phenolic (TPC) and flavonoid contents (TFC). Antioxidant capacity of the extracts was estimated by chemicals assays (DPPH, ABTS, FRAP and phosphomolybdenum assays). The enzyme inhibitory activities of the extracts against α-

glucosidase and pancreatic lipase were also studied. The concentration of principle compound, verbascoside has been quantified using HPLC-PDA technique.

RESULTS & DISCUSSION

It was observed that, polar solvent (95% methanol) gives highest yield (18.83 %) of extract compared to the other solvents used for extraction. Hydromethanolic (95% methanol) extract exhibited the highest TPC (171.44 ± 2.097 mg GAE g^{-1}) and TFC (152.52 ± 5.084 mg QE g^{-1}) whereas; lowest TPC (32.83±1.66 mg GAE g^{-1}) and TFC (90.35 ± 5.28 mg QE g^{-1}) were observed in ethyl acetate extract. From the results it is understood that the TPC and TFC content increases slightly with the addition of water in the alcohol or using a hydro-alcoholic solvent system. Likewise, the TPC and TFC hydromethanolic extract also showed highest antioxidant capacity in terms of DPPH free radical scavenging potential (164.06 ± 2.06 mg AAE g^{-1}) whereas, ethyl acetate extract showed the least effect (53.18 ± 4.60 mg AAE g^{-1}). Hydromethanolic extract also exhibited highest ABTS radical scavenging effect (100.18 ± 5.79 mg AAE g^{-1}) compared to all other solvent extracts. Significantly higher enzyme inhibition capacity has been exhibited by hydromethanolic extract. HPLC analysis indicates that hydromethanolic extract contains the highest concentration of verbascoside (154.45 ± 0.28 mg g^{-1}). EA and AC contains considerably lower amount of verbascoside than the alcoholic solvents.

On the other hand, out of all the extraction techniques performed, soxhlet extraction yields maximum (19.03%) crude extract as well as showed highest TPC (245.33 ± 1.44 mg GAE g^{-1}) and TFC (189.52 ± 3.42 mg QE g^{-1}) compared to others. HPLC analysis showed extraction with soxhlet recovers maximum phenolic compound verbascoside (191.21 ± 0.22 mg g^{-1}) and antioxidant phytochemicals. Likewise, it has shown highest antioxidant capacity and substantial enzyme inhibition potential. However the enzyme inhibition capacity of extract obtained from soxhlet extraction technique and cold maceration was not found significantly different.

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P5.1

Exudate flavonoid diversification of *Primula auricula* L. populations in an ecological context

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MAIN CONCLUSION

Both farinose and efarinose *P. auricula* populations were shown to secrete varying amounts of *Primula*-type flavonoids (flavones **1**–**6**) in differing proportions along altitudinal gradients. Gradual distribution of the traits "farina" and "exudate composition" also in mixed populations points rather to a phenotype-habitat correlation in an ecological context, coinciding with lack of genetic population divergence.

INTRODUCTION

Different phenotypic expressions of farinose and efarinose flavonoid exudates on aerial parts of *P. auricula* L. s. l. are used for the classification of two different subspecies: Farinose *P. auricula* subsp. *auricula* (Aur), and efarinose subsp. *balbisii* (Bal) [1; 2]. Mainly unsubstituted flavone (**1**; Figure 1) and a variety of other flavonoids with uncommon substitution patterns ("*Primula*-type flavonoids"), constitute these glandular hair exudates on leaves and inflorescences [3]. Particularly **1** is produced in great amounts and might aid to freezing tolerance or to UV-protection, but other ecological functions remain unknown [3]. Ongoing investigations on seasonal changes of exudate composition reveal farina production mainly in autumn and winter, suggesting its protective function of young tissues against harsh weather conditions (temperature, light intensity). Our study aimed at testing the stability of farina production along altitudinal gradients in a variety of populations, in view of ecological versus taxonomic significance. Thus, variation of relative amounts, and chemical composition of secreted material per leaf area was analysed and correlated to elevation (montane (M) vs. alpine (A) populations) and subspecific classification, using phenotypic expression of the exudate (Aur (farina present), Bal (farina absent); intermediate (Int): little farina) as the determining character [1].

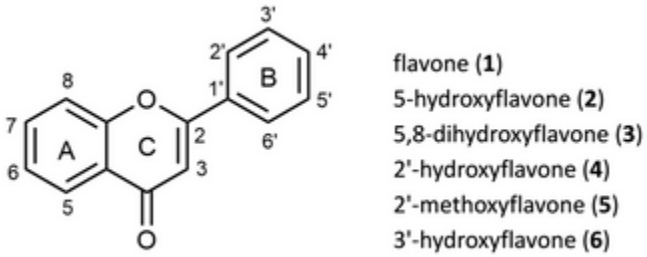

Figure 1. Basic structure of flavones.
A-ring substituted flavones; B-ring substituted flavones.

MATERIALS & METHODS

Plant material was collected at two sites in eastern Austria: Schneeberg (S) and Leopoldsteiner See (L), along altitudinal transections, determined following [1], and deposited in the Herbarium WU. Air-dried leaves were rinsed briefly with acetone, the evaporated extract dissolved in methanol at a concentration of 2 mg mL^{-1} and analysed by HPLC as described in [3], with a LC column (Hypersil 3 BDS-C18 100 × 4.0 mm, 3 µm particle size, Agilent), detection wavelength 300 nm and eluted with

a gradient of methanol (A) in aqueous buffer (B) starting with 55% A in B for 0–5 min, 78% A in B from 5–9 min and 98% A in B from 9–12 min. Surface areas of leaves were determined by digitization using a flat-bed scanner and pixel analyses using ImageJ. Statistical analyses (i. a. Analyses of variance (ANOVA)) were performed by employing STATGRAPHICS 18® (Version 18.1.06; Statgraphic technologies).

RESULTS & DISCUSSION

Comparison of exudate amounts per leaf area revealed significant differences between meta-populations: Individuals from L secreted considerably higher amounts and a larger variety of substances than those of S. Populations of Aur occur in A habitats, and those of Bal in M habitats, but Int forms were detected in mixed populations (Fig. 2c). The exudates were mainly composed of flavone (**1**), accompanied by **2**–**6** (Fig. 1), in varying amounts and proportions. The proportions change significantly with elevation: **1** and **2**–**3** increase, while **4**–**6** decrease, coinciding with subspecies alignment. This pattern of chemical diversification is more evident in location L (Fig. 2b). Int individuals of both S and L show intermediate relative amounts and a high proportional variability suggesting a gradual character of the traits "farina" and "exudate composition".

Although there is some correlation between presence of farina and exudate composition in the proposed subspecies, the gradual distribution also in mixed populations points rather to phenotype-habitat correlations. This is further backed by recent population genetic studies on the same collections which showed a lack of genomic diversification (Paun, pers. comm.). Increased formation of (**1**) at higher altitudes and during cold periods is functional, pointing to its role as ecological character.

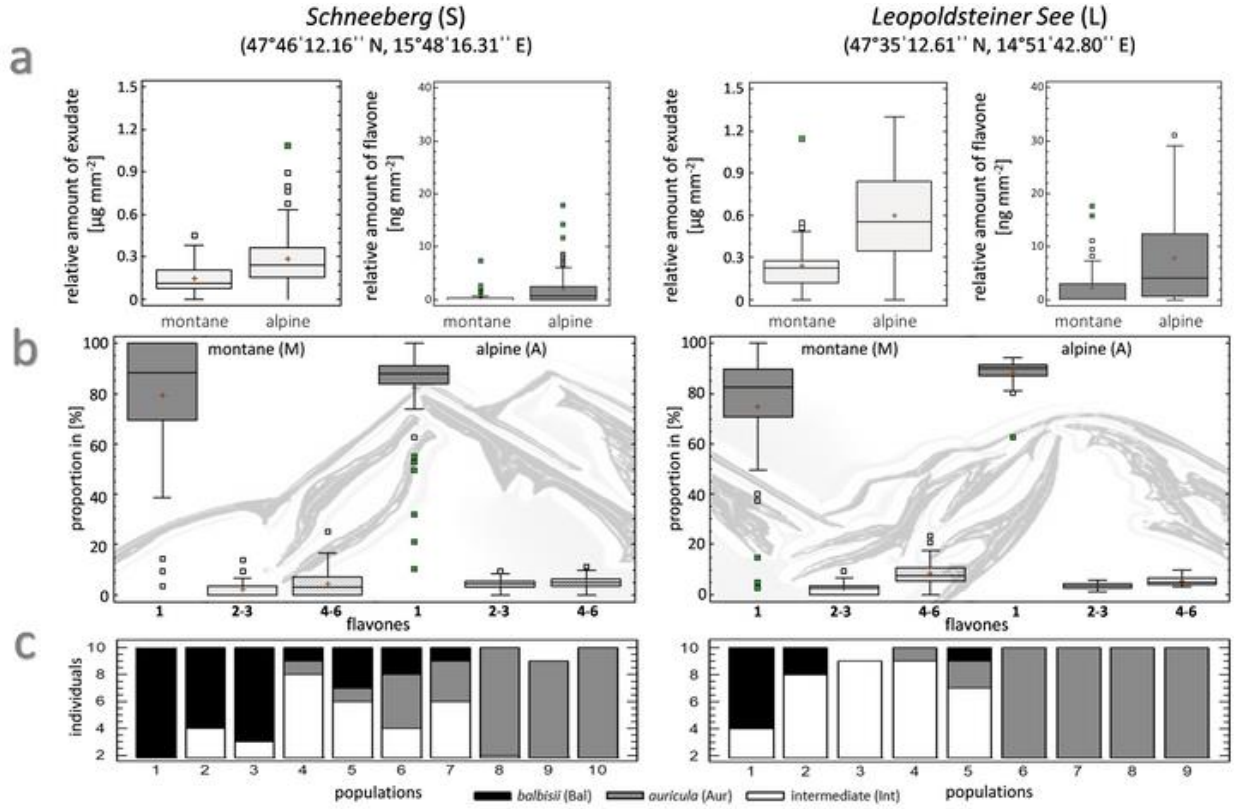

Figure 2. a ANOVAs of relative amounts of exudate and **1** per surface of leaves. **b** ANOVAs of proportions of flavones (**1**, **2**–**3** and **4**–**6**) in M and A populations. **c** distribution of subspecies along altitudinal gradients.

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P5.2

A transfer to a new host plant and a change in a polyphenol content can affect the metabolism of *Lymantria mathura* larvae

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MAIN CONCLUSION

Our results suggest that a previous habituation to a specific host plant influences insect herbivores and their metabolism. A generalist herbivore was able to digest new 3-caffeoylquinic acid and apigenin glycoside-rich diet, but slight differences in the larvae's frass profiles and capability to digest and egest polyphenol compounds depending on the previous host were detected.

INTRODUCTION

Polyphenols play an important role in anti-herbivore protection and insect host-choice. They have pronounced effects on herbivore development, survival, or fecundity. Caterpillars of *Lymantria mathura* can use various host plants as their food source. Some of these plants are known to have a high and diverse polyphenol content, making this insect species a favorable model for testing the effects of various polyphenols on herbivores.

Here we examined how the larvae metabolize diet based on leaves from hosts with different polyphenol profiles and if a transfer to a new host influences the larval digestion process of polyphenol compounds. In this study we focused especially on *Acer palmatum* eating larvae, because this host species seemed to cause difficulties in the digestion for those larvae, which had previously fed on a different host.

This study helps us to understand herbivores' capability to adapt to new hosts based on the plant's polyphenol profile and allows us to predict possible host shifts of i.e. invasive herbivore species.

MATERIALS & METHODS

In the first phase of the feeding experiment (F1), three host plants (*Acer palmatum*, *Carpinus cordata* and *Quercus crispula*) were fed to the *Lymantria* larvae. Part of the *Acer*-fed larvae continued feeding on their original host as a control and one third of *Carpinus* and *Quercus*-fed larvae were transferred to *Acer* leaves in the second phase (F2) and continued feeding on *Acer* at the third phase (F3) of the experiment.

The frass produced by the larvae was weighed at every stage of the feeding experiment to see, how effectively the larvae ate the host plant. The polyphenol composition of three hosts and frass was analyzed with UPLC-DAD-MS/MS. Polyphenol profiles of the leaves and frass at different phases were compared to examine, how the polyphenol composition changes in the larval digestive system and how the former host plant affects metabolism.

RESULTS & DISCUSSION

38 main polyphenol compounds were identified on the basis of their retention time, UV spectrum, *m/z* value and fragmentation. The main polyphenol compounds of *Acer palmatum* were 3-caffeoylquinic acid (3-CQA), and two apigenin glycosides. Compounds with lower concentrations included other cinnamic acid derivatives, flavonoid glycosides and proanthocyanidins. The most abundant compounds of *Carpinus cordata* were geraniin and 5-CQA, and *Quercus crispula*'s vescalagin, castalagin and vescavaloninic acid.

At some cases generalist larvae have been reported to avoid apigenin and apigenin glucoside rich diet in food preference experiments [1]. Also caffeoylquinic acid (CQA) is known to function as a

defensive compound against a broad range of herbivores and to cause insect's growth deterrence. This is probably due to the CQAs ability to oxidize into harmful o-quinones [2]. This can be one factor to explain the difficulties in adjusting to *Acer* host, but the detailed reasons why this happens after a habituation to another host remains unresolved.

The main compounds of *Acer* were detected also in the larvae's frass but the amounts were lower due to the larvae's ability to degrade or isomerize the compounds. The relative amount of 3-CQA decreased in the frass while the amounts of 4- and 5-CQA isomers increased. This is a known pattern, which depends on the pH of the herbivores' gut [2]. The concentration of the main compounds was highest on *Quercus*-fed larvae's F2 and F3 frass compared to *Acer*- and *Carpinus*-fed larvae, which can tell about the *Quercus*-larvae's better ability to egest undigested or harmful material.

Our results show, that a herbivore's original host's polyphenol profile can have an effect on the adaptation to a new diet, but the significance of different compounds needs further analysis.

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P5.3

Chemical composition and biosynthesis of poplar bud resin in *Populus trichocarpa* and *Populus balsamifera*

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MAIN CONCLUSION

The leaf bud resin from *Populus trichocarpa* and *Populus balsamifera* was analyzed for flavonoid and phenolic content, in parallel with a transcriptomic analysis of leaf buds. The transcriptomes revealed new resin-specific enzymes, including uncharacterized flavonoid modifying enzymes such as *O*-methyltransferases (OMTs). These enzymes are predicted to contribute to the biosynthesis of methylated lipophilic flavonoids and chalcone aglycones which form a major component of poplar leaf bud resin.

INTRODUCTION

Trees in the genus *Populus* (poplars, cottonwoods, and aspens) are found throughout the Northern Hemisphere, often acting as keystone species. Extensive genomic research has made *Populus* important model tree for molecular biology. However, the diversity of phenolic compounds found in *Populus* also makes it an attractive genus for studies of phenolic biochemistry and chemical ecology. In particular, poplars produce sticky bud resin, which consists of many unique hydrophobic phenylpropanoids and flavonoids. The chemical composition of poplar leaf bud resin can vary significantly between different poplar species [1]. Poplar bud extracts have been widely used in traditional medicine, and the extracts have been shown to contain phenolics, which exhibit biological activity that protects honey bees against pathogens [2].

Despite a wealth of chemical knowledge, the biosynthesis of these resin compounds has not been previously investigated. The objective of this research was to study the biosynthesis of phenolic metabolites in the bud resin of two different poplar species black cottonwood (*P. trichocarpa*) and balsam poplar (*P. balsamifera*). Our study is focused on biosynthesis and biochemical modifications of specialized metabolites, in particular methylated flavonoids and dihydrochalcones present in poplar bud resin extracts. To identify resin-specific enzymes which methylate flavonoid or dihydrochalcone aglycones during bud development, RNA sequencing was used to study the gene expression of leaf buds producing resin. The pattern of gene expression, as well as phylogenetic analysis, were used to identify candidate enzymes, which will be studied and characterized as recombinant proteins. Finding a connection between the chemical composition and the enzymes that can cause modifications of phenolic metabolites in bud resin will reveal novel enzyme activities that connect gene expression to chemical phenotype.

MATERIALS & METHODS

Dormant cuttings of *P. trichocarpa* and *P. balsamifera* were placed in water in a growth chamber in controlled light (16 hours of light) and temperature conditions (22°C) to induce bud break. Leaf buds were sampled over the course of bud break. The major phenolics in poplar bud resin extracts were identified using ultra performance liquid chromatography (UPLC) coupled to a diode array detector (DAD) and single-quadrupole mass detector. Transcriptomics was used as the main tool for finding candidate genes and enzymes involved in the biosynthesis of poplar bud resin compounds. RNA sequencing analyses from resin-synthesizing tissues were used to identify which genes are expressed in the leaf buds. Phylogenetic analysis comparing sequence similarity to known and characterized OMTs from other species, together with the expression pattern and level of gene expression were used to select candidate enzymes for OMTs related to bud resin biosynthesis.

RESULTS & DISCUSSION

Chemical analysis of the studied poplar species revealed that the leaf bud resin in both species shared common phenolic compounds. The main resin flavonoids were identified to be dihydrochalcone and flavanone aglycones. In particular, 2',6',4-trihydroxy-4'-methoxydihydrochalcone and 2',6'-dihydroxy-4'-methoxydihydrochalcone were identified as major phenolics in methanolic resin extracts. The high relative abundance of monomethylated flavonoids indicated the existence of flavonoid-specific OMTs, which are responsible for modifying these bud resin-specific compounds.

Following phylogenetic analysis and in silico expression profiling, we looked for potential flavonoid-specific OMTs expressed in both *P. trichocarpa* and *P. balsamifera* leaf buds. We are targeting different clades of OMTs that are predicted to use different classes of flavonoids as substrates, based on previously characterized genes in other species. We also identified potential candidate genes for OMTs predicted to be responsible for modification of dihydrochalcones, via comparison with OMTs shown to methylate chalcones. The top candidates also possess relatively high expression in both target poplar species, as well as expression profiles that match qPCR analysis of general flavonoid genes at different stages of bud break. These candidate enzymes will be produced as recombinant proteins and purified, and sequentially tested and characterized using enzyme assays with different flavonoid substrates.

Characterization of these resin-specific OMTs will lay a foundation for studying diverse enzymes involved in resin production, as well as enable investigations of the seasonal dynamics for the synthesis of leaf bud resin in *Populus*. Developing the tools to dissect the mechanisms of flavonoid resin secretion will ultimately allow us to test the importance of leaf bud resin as a mechanism for adaptation to environmental stress.

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P5.4

Leaf proanthocyanidins act as in planta antioxidants and protect poplar trees against the effects of oxidative stress from drought and UV-B

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MAIN CONCLUSION

We tested if leaf PAs can function as in vivo antioxidants using transgenic high-PA or low-PA transgenic poplars. Elevated PA content led to greater protection from drought, UV-B, or methyl viologen, seen in reduced leaf necrosis, lower hydrogen peroxide content, and lower malondialdehyde levels. Poplar plants with reduced PAs showed a greater susceptibility to oxidative stresses and the opposite patterns. This work shows is the first demonstration of the in planta antioxidant function of PAs.

INTRODUCTION

Proanthocyanidins (PAs) are polymeric the most widely distributed secondary plant metabolite, and particularly abundant in trees and woody plants. Also knowns as condensed tannins, they bind and precipitate protein. PAs are major end products of the flavonoid pathway and consist of polymeric flavan-3-ols. They are commonly associated with defense against pathogens and vertebrate herbivores, but have the potential for many other ecological and physiological functions. In poplar, a model tree that can be genetically transformed, a variety of biotic and abiotic stressors including UV-B and nitrogen stress induce PA synthesis.

Like many flavonoids and phenolics, extracts containing PAs show substantial antioxidant activity. However, it is not known if this antioxidant potential contributes to defense against oxidative stress. Environmental or abiotic stresses such as drought, temperature stress, salinity, or UV-B cause a rise of reactive oxygen species (ROS) in plants; often this is related to disruption of photosynthetic electron transport, which leads to the creation of superoxide. Plants have evolved multiple mechanisms for protecting against excess ROS, including enzymes such as superoxide dismutase, catalase, and ascorbate peroxidase. Furthermore, cellular constituents such as ascorbate and carotenoids also act as ROS scavengers.

Phenolic compounds, especially those with ortho-hydroxyl groups, are potent antioxidants depending on the oxidative conditions. Some flavonoids have been shown to functions as physiological antioxidants under plant stress. The role of PAs as *in vivo* antioxidants during environmental stress has not previously been tested. However, the in vitro antioxidant capacity of PAs is well established (1). Our work tests the hypothesis that in woody plants, which accumulate large amounts of PAs in leaves and vegetative tissues, PAs contribute to protection against oxidative stress caused by drought and UV-B exposure.

MATERIALS & METHODS

We tested the function of PAs by using high- and low-PA transgenic poplar plants, which overexpress CT-regulating MYB transcription factors MYB134 and MYB115. These plants have been previously characterized in detail (2), which established that PAs are the primary target of these factors, and that other flavonoids have not been affected significantly. In some experiments, we also used RNAi knock-down plants, which accumulate less PA than controls. For drought experiments, 3-month-old saplings were exposed to reduced water availability for two weeks. Chlorophyll fluorescence was measured over the drought and recovery period. At the end of the experiment, leaves were harvested

and assayed for H_2O_2, a common ROS species, and malondialdehyde (MDA), an indicator of lipid oxidation. In addition, experiments were carried out in environmental sunlight simulation chambers with and without UV-B exposure, and the same parameters were measured as for drought.

RESULTS & DISCUSSION

High-CT MYB plants showed reduced oxidative stress after a two-week drought period according to several indicators. The high-CT plants showed less photosystem damage (as measured by chlorophyll fluorescence, *Fq'/Fm'*)) and had less necrosis compared to wild-type controls. They also accumulated lower concentrations of H_2O_2 and MDA, considered a marker for ROS and oxidative damage to biomolecules. The same protective effect of CTs was observed in both MYB134 and MYB115 transgenic lines. When we tested MYB134RNAi (low CT) plants, we found the expected inverse effect: low CT plants were more susceptible to the oxidative damage caused by drought compared to controls. Therefore, we conclude that CTs can contribute to limiting the effects of oxidative damage during drought.

UV-B exposure causes oxidative stress by different mechanisms. Following two weeks of UV, we found no impact on photosystem II by chlorophyll fluorescence (*Fv/Fm* or *Fq'/Fm'*) in high-CT transgenics, even though controls were impacted. Likewise, high-CT plants accumulated significantly less H_2O_2 and MDA than control leaves. Overall, high-CT plants were less affected by UV-B exposure than controls.

Based on the consistent protective effects of CTs against oxidative stress by two distinct stresses, we conclude that CTs contribute to plant health as physiological antioxidants. The mechanisms behind this effect this will require further investigation. CTs are localized to the vacuole, but superoxide and other ROS are typically produced in chloroplasts by damage to photosystems. Superoxide is rapidly dismutated by the enzyme superoxide dismutase to H_2O_2, which can be removed by catalase or other systems. H_2O_2 is relatively stable and moves readily across membranes, including the tonoplast. We therefore predict that vacuolar CTs provide a strategy to limit the damage of oxidative stress in plants. This is particularly important in trees, which can accumulate very large amounts of tannin in leaves.

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P5.5

Combined effects of ozone stress with drought or salt stress on selected parameters of the antioxidant machinery in city trees

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MAIN CONCLUSION

Abiotic stress factors, such as drought and increased salt concentrations, cause changes in the antioxidant machinery of urban trees, which could have a negative environmental impact on the cities' climate during hot summers.

INTRODUCTION

Ozone is a serious health problem in many cities during hot summer days. The influence of cities' trees on both production and absorption of ozone depends on the species, but also on the physiological status of the tree, such as the degree of drought or salt stress, which are common in urban environments. Exposure to abiotic stress such as drought causes many reactions in plants and secondary metabolites are involved in protective functions as a response. Flavonoids and enzymes, are known to be involved in scavenging reactive oxygen species in plant cells and volatile organic compounds (VOC) in the intercellular space. By linking the environmental conditions to the physiological stress response and this to the VOC production and ozone absorption, a better understanding and quantification of the physiological mechanisms can be achieved. Exposure to abiotic stress such as drought causes many reactions in plants. Under drought conditions, secondary metabolites are involved in protective functions as a response [1]. Environmental stress disturbs the delicate balance of generation and scavenging of reactive oxygen species (ROS), which cause extensive damage to protein, DNA and lipids, thereby affecting normal cellular functions [2]. Several enzymatic and non-enzymatic antioxidants are part of the antioxidant machinery and known to be involved in scavenging of ROS in plant cells and VOC in the intercellular space [2]. To analyse physiological changes in urban trees as a result of stress, we focused on the antioxidant machinery of the plant and analysed *in vivo* antioxidant capacity, total phenolic content and peroxidase activity in 4 different species at two different stress conditions in the presence or absence of ozone stress.

MATERIALS & METHODS

Two year old seedlings of four different species, *Q.robur*, *C.betulus*, *F.sylvatica* and *B.pendula*, were planted in 7 L pots in March 2019. The substrate consisted of one third soil used by the city gardeners for city trees in Vienna and two thirds of quartz sand to improve drainage. The seedlings were then fertilized, hydrated and illuminated according to Peron et al. [3].

To study the effect of ozone exposure the trees were separated into two groups, and one group fumigated with 100 ppb O3 (DS_OS) inside the enclosure for 1 h each day after the daily measurement of BVOCs. Measurements of peroxidase activity, and total phenol content in was performed as recently described [3]. Cellular antioxidant activity was determined with *Saccharomyces cerevisiae* ZIM 2155 as a model system following the procedures described in Slatnar et al. [4], which estimates intracellular oxidation by fluorometric measurements using the ROS-sensitive dye 2',7'-dichlorofluorescin (H2DCF).

RESULTS & DISCUSSION

An influence of combined stress on the phenolic content could be observed. The majority of the species showed a distinct stress response pattern which was characterized by a decrease of total phenol content by salt stress, whereas drought stressed trees had a slight to marked increase in total phenol content. A general strong effect of the ozone treatment was not observed, but for *Q.robur* there was a continuous increase of TPC from drought. In all species, the stress had a negative effect on the antioxidant capacity. For *Q.robur* and *B.pendula*, the effect was higher in drought stressed plants than those subjected to salt stress, in which the effect was less visible. In *C.betulus* and *F.sylvatica* we saw a negative effect with both stress types and it was higher in salt stressed plants.

Peroxidase activity *per se* is quite different in species, but stress response patterns were similar, and we observed an increase of peroxidase activity under drought stress, with the highest effect in *C.betulus*. There was, however, no difference between ozone treated and no ozone treated trees in the stress response. Strongest effects were found for peroxidase activity in salt stressed *Q.robur* and *C.betulus*, probably also in the total phenolic content.

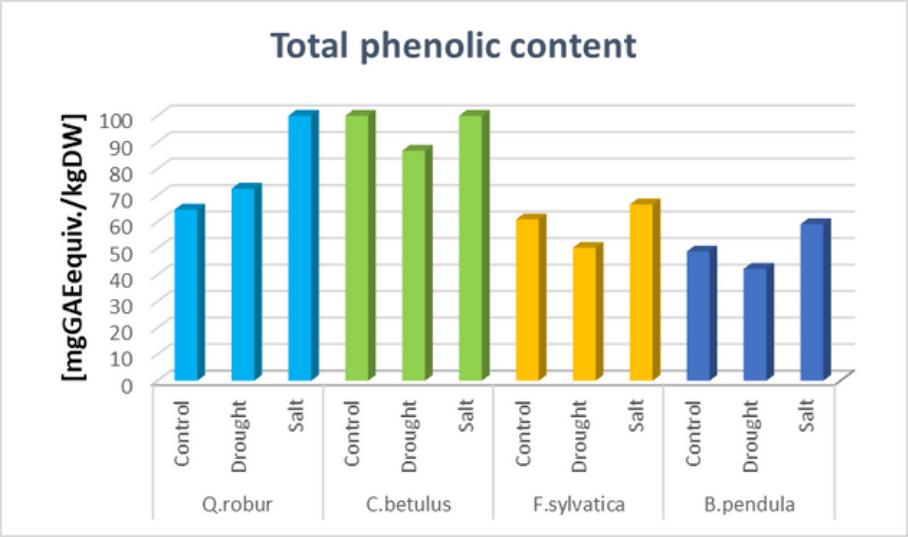

Figure. Comparison of total phenolic content in four city tree species cultivated under different stress conditions.

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P5.6

Altered polyphenol metabolism associated with cut carrot blackening

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MAIN CONCLUSION

The data presented here reveal a shift from primary to secondary metabolism in the blackened carrot tissues. This is accompanied by significant changes in the carbohydrate content and composition of the cell walls in the blackened regions. Since the aging process appears to an important factor contributing to the blackening, these data suggest that subsequent wounding and storage exacerbate cell death responses in secondary metabolism that is absent from carrots harvested earlier in the season.

INTRODUCTION

Vegetables such as carrots are a rich source of phenolic compounds, particularly chlorogenic acids (1). Orange carrots are also rich in α and β-carotene, while lutein is predominant in yellow carrots, lycopene in red carrots, anthocyanins in purple carrots and black carrots are rich in anthocyanins and phenolic acids (2,3). The content and composition of phenolic compounds in carrot roots is likely to be influenced by a range of parameters, including genetic background, growing and storage conditions. Carrots grow underground. For industry purposes, the carrots are stored until harvest by covering the plants in plastic tarpaulin and straw throughout the year. Once harvested, the carrots are cut into batons and placed in bags for sale. Unfortunately, cut carrots can develop blackened regions within hours to several days after processing (Figure 1, B). However, the development of blackening is unpredictable although the probability of its occurrence increases in carrots harvested towards the end of the annual harvest period. The aim of the following studies was therefore to investigate the molecular and metabolic changes that occur in blackened carrots, with a view to determining the underpinning biological mechanisms and finding an appropriate solution to the problem for the produce industry.

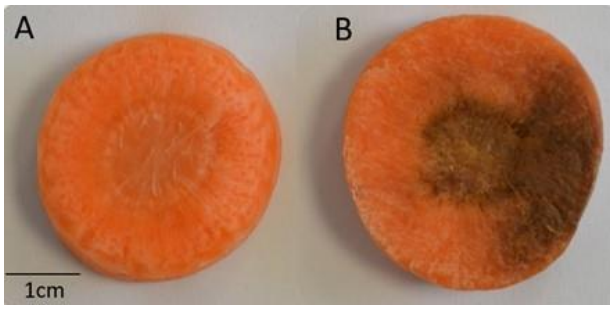

Figure 1: Photographs of an orange cut carrot and a blackened cut carrot. Orange cut carrot (A) and blackened cut carrot (B) were of the Nairobi variety, provided by Kettle Produce Ltd.

MATERIALS & METHODS

Samples of cut carrots with and without blackening were provided directly from the production line by Kettle Produce Ltd (Scotland). Fresh samples were analysed within 24 hours of receipt or frozen at -80^{O}C until analysis. Metabolic profiles of orange and blackened cut carrots were performed using gas chromatography and mass spectrometry (GCMS) as well as high-performance liquid chromatography (HPLC) coupled to MS at the James Hutton Institute (Scotland). Some metabolites had several peaks, which were isomers either present in the carrot tissue or formed as a result of derivatisation reactions

during GC/MS. These isomers are numbered lowest retention time first (Table 1). Lignin was extracted from carrot alcohol-insoluble residue using an acetyl bromide assay at the Luke institute (Finland). Using immunolabelling with a range of monoclonal antibodies, sections of orange and black carrot regions were viewed under a fluorescence microscope to detect targeted cell wall components.

RESULTS & DISCUSSION

The metabolomics analysis revealed large changes in primary and secondary metabolites between the black and orange regions of the carrots. For example, the levels of amino acids and soluble sugars were generally decreased in blackened carrots, while the abundance of fatty acids and phenolic compounds, particularly chlorogenic acid were increased in the blackened carrots. The lack of available soluble sugars in blackened carrots suggests that vital carbohydrate reserves essential for ATP production are running out.

Analysis under a fluorescence microscope revealed that the black regions of the carrots had high levels of autofluorescence. Studies were therefore performed to determine whether the changes in autofluorescence were related to alterations in cell wall composition. The immunofluorescence microscopy analysis of cell wall polysaccharides revealed that several components such as xyloglucan, HG-pectin and RG-pectin were decreased in the blackened carrot tissues. Lignin content was also measured and found at higher levels in the blackened carrot regions. Degradation of pectin and xyloglucan is likely caused by the same factors that trigger carrot blackening, which then activates the plant's own defensive response in the form of a flux of secondary metabolite synthesis, increasing defence and lignification of cell walls. This is supported by data shown in the metabolomics analysis.

Table 1. Metabolites identified in carrot root. The result column states the significant or non-significant differences seen in blackened carrots when compared to orange carrots.

Grouping	Metabolites	Result
Phenolics	Chlorogenic acid	Increased
	Dicaffeoylquinic acid 1	
	Cinnamic acid 1	No difference
	Cinnamic acid 2	
	Cinnamic acid 3	
	Caffeoylquinic acid 1	
	Caffeoylquinic acid 2	
	Caffeic acid	
	5-Caffeoylquinic acid 1	
	5-Caffeoylquinic acid 2	
	5-Caffeoylquinic acid 3	
	Dicaffeoylquinic acid 2	
Organic acids	Threonic acid	Increased
	Galactaric acid	
	Dihydroxypropanoic acid	Decreased
	Fumaric acid	
	Malic acid	
	Quinic acid	
	Oxalic acid	
	Citric acid	
	Succinic acid	No difference
Fatty Acids	Tetradecanoic	Increased
	n-pentadecanoic	
	Hexadecenoic	
	Hexadecanoic	
	Heptadecanoic	
	α-linolenic	
	Octadecenoic	
	2-OH Hexadecanoic	
	n-octadecanoic	
	Eicosanoic	
	Heneicosanoic	
	Docosanoic	
	n-tetracosanoic	
	br-pentadecanoic	
	Octadecenoic	
	Tricosanoic	
	2-OH Tetracosanoic	
	Pentacosanoic	
	Linoleic	No difference

Grouping	Metabolites	Result
Amino Acids	γ-Aminobutyric acid	Increased
	Valine	Decreased
	Leucine	
	Isoleucine	
	Proline	
	Glycine	
	Serine	
	Threonine	
	Methionine	
	Oxoproline	
	Aspartic acid	
	Glutamic acid	
	Phenylalanine	
	Asparagine	
	Tyrosine	
	Histidine	
	Tryptophan	
	β-Alanine	No difference
	Lysine	
Carbohydrates	Mannose	Increased
	Galactose	
	Glycerol	
	Mannitol	
	Fructose 1	Decreased
	Glucose 1	
	Glucose 2	
	Sucrose	
	Inositol	
	Fructose 2	No difference
Amines/ Polyamines	Allantoin	Increased
	Putrescine	
	Ethanolamine	
	Piperidinecarboxylic acid	No difference
Fatty Alcohols	Octacosanol	No difference
	Hexacosanol	
	Tetracosanol	

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P5.7

Phenolic compounds profile of *Dionaea muscipula* J. Ellis leaves and traps after UV-A treatment

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MAIN CONCLUSION

The varied response of flavonoids in different organs of *Dionaea muscipula* after UV-A radiation treatment indicate that adaptative response to UV-A radiation occured in the plants. However, the exact cause and mechanism of this regulation is unknown. Further research requires measurements of intermediates content and analysis of intermediary enzymes activity.

INTRODUCTION

Venus flytrap (*Dionaea muscipula* J. Ellis) is a carnivorous plant, which natural habitat is restricted only to North and South Carolina of the United State. This habitat is characterised by moist, poor in nutrients soils and lack of canopy. In such conditions Venus flytrap is exposed to increased level of solar radiation, including UV-A radiation. Due to high content of phenolic compounds, Venus flytrap has been used in pharmacology and medicine from ages. Over the years it turned out that the plant is rich in phenolic compounds, such as napthoquinones, cinnamic acid derivatives and flavonoids, among others: ellagic acid, caffeic acid, salicylic acid, myricetin and hyperoside. In addition to the well-known antioxidant activity of aforementioned flavonoids, these compounds offer other beneficial effects. It has been shown that ellagic acid has anti-inflammatory activity in acute or chronic model of ulcerative colitis. Caffeic acid can act as a protective agent in an Aβ25-35-induced Alzheimer's disease model. Salicylic acid is known for its dermatological application, due to its keratolytic activity. Myricetin damages reverse transcriptase of HIV-1 and acts as its inhibitor. Hyperoside improves cardiovascular function and has neuroprotective properties. UV-A radiation stress has a potential to alter metabolic pathways of flavonoids biosynthesis, therefore the composition and the amount of phenolic compounds accumulated by the plant can change after UV-A radiation treatment. This alteration is associated with changes of redox state of photosynthetic apparatus, what may lead to enhanced performance of oxidative pentose-phosphate pathway, which provides precursors for shikimic acid biosynthesis, and thus for flavonoids biosynthesis. The aim of this research was to investigate how UV-A radiation affects phenolic compounds composition and content in leaves and traps of *Dionaea muscipula* both directly after UV-A irradiation and 24 h after irradiation.

MATERIALS & METHODS

Dionaea muscipula J. Ellis plants were cultivated in controlled conditions (30–40% humidity, temperature 23 ± 1 °C, light intensity 290 µmol m^{-2} s^{-1}, 16 h light/8 h dark) for 7 days. Subsequently plants were divided into three groups. One group was Control. Two remaining groups were exposed to UV-A radiation for 24 h (intensity: 50 µmols m–2 s–1) (Treated). After irradiation, Control and one group of exposed to UV-A radiation plants (Treated) were harvested, divided into organs (leaves, traps and roots) and lyophilised. The third group of plants was harvested 24 h after irradiation (Recovery), also divided into organs and lyophilised. Dry tissue was extracted with 80% methanol and centrifuged. Estimation of ellagic acid, caffeic acid, salicylic acid, myricetin and hyperoside

content in obtained extract was conducted on HPLC with stationary phase Agilent Zorbax SB-Phenyl and in separation gradient (eluent A: 0.1% TFA in acetonitrile; eluent B: 0.1% TFA in H_2O).

RESULTS & DISCUSSION

The content of phenolic compounds was significantly different in leaves and traps of examined plants, except myricetin and salicylic acid. Generally, traps accumulated more ellagic acid, caffeic acid and hyperoside than leaves. UV-A treatment did not change the content of myricetin, neither in leaves nor traps. Hyperoside content increased significantly in both organs after 24 h from irradiation. UV-A treatment caused increased accumulation of ellagic acid only in traps immediately after irradiation. In case of caffeic acid, the increase of its accumulation occurred in traps immediately after irradiation and in leaves after 24 h from irradiation. Salicylic acid content decreased in leaves after UV-A treatment and further after 24 h. In traps the decrease of salicylic acid content was observed after 24 h from irradiation.

Flavonoids are compounds which serve as UV filters in epidermis. They may be produced as a result of activation of specific UV-A/blue light fotoreceptors, such as cryptochrome 1 (CRY 1) or as a result of increased ROS pool in plant tissue and act as ROS scavengers. It is postulated that decrease in total flavonoids content may be a result of photooxidation. In our study, however, we observed that different flavonoids content fluctuated. Reaction also varied between different organs. The results indicate, that observed changes in flavonoids content was probably mostly photochemical response and decline in some flavonoids content was not a result of photooxidation. This photochemical response may be associated with selective activation and inactivation of leaf UV-A photoreceptors, what allows for precise and independent induction of various flavonoids.

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P5.8

The different substrate specificities of the *Zea mays* dihydroflavonol 4-reductase paralogs A1 and A1* are determined by few amino acids

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MAIN CONCLUSION

Two *DFR* paralogs *A1* and *A1** from maize show divergent substrate specificities; some but not all can be interchanged by the mutation of only a few amino acids.

INTRODUCTION

The dihydroflavonol 4-reductase (DFR) is an oxidoreductase (EC 1.1.1.219) that catalyzes the NADPH dependent stereospecific reduction of the keto group of dihydroflavonols in position 4 to the respective flavan 3,4-diols (leucoanthocyanidins), as well as the reverse reaction in the presence of $NADP^+$. DFRs can show substrate specificity with respect to the B-ring hydroxylation pattern of the dihydroflavonol substrates dihydrokaempferol (DHK), dihydroquercetin (DHQ) and dihydormyricetin (DHM). Several DFRs can convert dihydroflavonols irrespective of their B-ring hydroxylation pattern, whereas others show distinct substrate specificity. For example, DFRs of *Gerbera hybrida*, *Matthiola incana*, *Callistephus chinensis* or *Dianthus caryophyllus* can utilize all three dihydroflavonols as substrates. DFRs of e.g. the roseaceous species *Malus* x *domestica*, *Pyrus communis* and *Crataegus monogyna*, or the ornamental plants *Petunia hybrida*, *Cymbidium hybrida* and *Angelonia* x *angustifolia*, convert DHQ and DHM but do not reduce DHK effectively. A DHK preferring DFR is known from *Fragaria* sp. A 26 amino acid long region was found to be particularly relevant for substrate acceptance [1] [2].

In maize, beside the *A1* gene, a *DFR* paralog is expressed, which was named *A1** and shows amino acid differences in the substrate determining region. We heterologously expressed *A1* and *A1** in *E. coli* and characterized the recombinant enzyme. By use of site-directed mutagenesis we defined amino acids that are responsible for the divergent substrate specificities of wildtypes A1 and A1*.

MATERIALS & METHODS

A1 and *A1** were cloned into the bacterial expression vector pGEX-6P1. *In silico* analysis of the presumed substrate recognition site of DFRs enabled us to select specific amino acids that could be responsible for the respective substrate specificity. We used the Q5 Site-Directed Mutagenesis Kit (NewEngland Bioloabs, Vienna Austria) to generate *DFR* point mutants from the wildtype DFRs to evaluate the effects of these amino acid exchanges on their substrate specificity. Heterologous expression was performed in *E. coli*. The GST fusion proteins were purified from the cell extract using Sepharose 4B (GE Healthcare, Germany). The DFR was liberated from GST by PreScission protease according to the GST Gene Fusion System Manual (GE Healthcare, Germany). Kinetic data with recombinant maize DFRs were gained using the substrates DHK, DHQ and DHM as described previously.

RESULTS & DISCUSSION

Kinetic data for A1 indicate a slightly higher substrate specificity for DHQ than DHM, whereas DHK was not accepted. For A1*, DHK was the preferred substrate. DHQ was accepted to a minor extent, whereas DHM was not accepted (Table 1).

Table 1. Kinetic data of recombinant maize DFRs A1 and A1*.

	Substrate	Km (µmol/L)	kcat (s-1)	kcat/Km (L/mol*s)
A1	DHQ	0.6	4.3E-04	1093
A1	DHM	6.1	23.5E-04	675
A1*	DHK	4.2	7.5E-04	180

In general, most DFRs have an N or D in position 133, whereas in position 134 V or I are most common (Numbering according to *Vitis* DFR (P93799), of which the crystal structure 2c29 is available). Johnson et al. [1] suggested that mainly N133 plays an important role in DHK acceptance, but this was not confirmed by our DHK rejecting A1. There are other examples where DFRs with N133 do not accept DHK as substrate, like *Angelonia* (AHM27144, [3]) or *Cymbidium* (AAC17843, [2]). *In vitro* single point mutation of petunia DFR from D133 to N133 did not result in DHK acceptance of the recombinant enzyme [4]. On the other hand, DFRs with D133 as found in petunia, could also accept DHK as substrate (*Euphorbia*, AUV64092, [5]). We therefore operate under the assumption that substrate specificity is not solely determined by the amino acid in position 133, but rather that other(s) may be relevant too.

Interconversion of amino acid positions 133 and 134 between our A1 and A1* led to an A1 mutant, which accepted mainly DHK as substrate, whereas the A1* mutant accepted DHQ and DHM but did not accept DHK.

However, there are other amino acid differences within the 26 amino acid long substrate determining region, which also influenced substrate specificity, but not in such an explicit, directional way.

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P5.9

Synthesis of flavonol-bearing probes and proteomic analysis of Asteraceae petals via affinity-based protein profiling

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MAIN CONCLUSION

We developed a versatile workflow in order to identify uncharacterized enzymes from petal proteomes as candidates for unknown enzymes involved in flavonoid biosynthesis. These candidates will be investigated with biochemical methods in future studies.

INTRODUCTION

Flavonoids in petal tissues contribute to the yellow pigmentation of Asteraceae flower petals [1], and play another important role in the attraction of pollinators by the formation of a strongly UV absorbing inner circle of the flower, also known as honey guide. Responsible for these crucial functions are mainly higher hydroxylated flavonols such as quercetagetin (6-hydroxy quercetin) and gossypetin (8-hydroxy quercetin). The late stages of the biosynthesis of those flavonoids are still today not completely characterized, notably in terms of the enzymes, in particular hydroxylases, methyl- and glycosyl- transferases [2], involved in the corresponding anabolism.

MATERIALS & METHODS

We apply affinity-based protein profiling (ABPP) aimed at identifying protein candidates for novel uncharacterized enzymes taking part in the biosynthesis of flavonoids. The molecular probes were synthesized from commercially available flavonols. The petal proteins of three Asteraceae species, namely *Rudbeckia hirta, Tagetes erecta* and *Chrysanthemum segetum* were separated from soluble plant polyphenols by precipitation or ultracentrifugation. The ABPP technique incorporates the method previously presented by our group for covalent affinity labelling of proteins by taking advantage of the intrinsic electrophilic properties of catechol-containing compounds upon oxidative activation using sodium periodate [3]. We then applied a classical streptavidin-based pull-down on the probe-labelled (and hence biotinylated) petal proteins. The isolated proteins were quantitatively analysed by shotgun proteomics using LC/MS-MS techniques with a label-free quantification protocol.

RESULTS & DISCUSSION

We firstly designed a versatile scheme for the synthesis of different flavonol-bearing biotinylated chemoproteomic probes. Secondly, we optimized and generalized the oxidation-mediated covalent capture protocol in order to adapt it to different proteomic sources. This notably includes an *in situ* redox titration of the required minimum amount of sodium periodate, as well as an iterative extraction of the proteome. The analysis of the resulting complex data sets of significantly captured proteins was carried out according to two complementary strategies. First, a “top-down” strategy relying on

the development of bioinformatics tools enabled the comparison of sequences of uncharacterized enzymes with those of known related enzymes of the flavonoid biosynthesis pathways. Second, a "bottom-up" strategy consisted in the implementation of several (manual) approaches for the identification of a limited set of significantly captured candidates, which could then be further investigated on using classical biochemical methods. The comparison of the differentially captured proteins using similar flavonol probes, as well as the control catechol probe revealed interesting structural specificity aspects and enabled the identification of highly significant protein targets involved in flavonoid biosynthesis.

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P5.10

Experimental warming induces species-specific changes in phenolic chemistry of boreal tree seedlings

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MAIN CONCLUSION

We found variable responses to elevated temperature for birch, spruce and pine seedlings. The response of individual phenolics was highly plant part and species-specific. Increased biomass and decreased flavonoids were detected only for birch and pine. In all species foliage proanthocyanidins decreased under warming and chemistry in foliage was more affected than in stems. Our result underline that the effect of warming for boreal forests should not be extrapolated from single species responses.

INTRODUCTION

The main boreal tree species in Northern Europe are Scots pine (*Pinus sylvestris*), Norway spruce (*Picea abies*) and Silver and Downy birches (*Betula pendula* and *Betula pubescens*). Long-living trees are susceptible to various insects and pathogens during their lifespan, and at seedling phase especially pine and birch are vulnerable to mammal herbivores, such as voles (*Microtus agrestis* and *Myodes glareolus*) and moose (*Alces alces*). Accordingly, trees have diverse constitutive defensive chemistry against herbivores and pathogens e.g., phenolics and terpenoids in birches, and phenolics, terpenoids and alkaloids in conifers, respectively.

Both plant growth and concentration of defensive compounds (e.g. phenolics and alkaloids) have shown to react strongly to temperature elevation [1]. On the other hand, warming is expected to increase carbon sequestration and growth of boreal trees on the cost of concentrations of carbon-based defensive compounds such as phenolics, but concentration of nitrogen-based compounds, such as alkaloids, is expected to increase. Warming is also expected to increase damage risk by prevailing herbivore and pathogen species, but also by introducing new species.

Here, we compared the responses in phenolic chemistry and growth of silver birch, Norway spruce and Scots pine to warming in a multi-year experiment. We compared changes in phenolic chemistry to the biomass allocation, and alkaloid chemistry of conifers, to reveal possible trade-offs between defense and plant growth. The growth of birch, which is a deciduous fast-growing species, is expected to gain most of the climate warming, followed by pine and spruce. Therefore, our hypothesis is that birch seedlings will have more severe reduction of phenolics than slower growing coniferous seedlings, where simultaneously increased alkaloid concentrations are expected.

MATERIALS & METHODS

In our study, 1-year-old birch seedlings and 2-year-old spruce and pine seedlings were planted on outdoor experimental field located in Eastern Finland (Joensuu) in spring 2016. Modulated system was set-up to increase temperature by 2°C in heated plots compared to ambient control plots during growth season (May-August). The experiment lasted three years for birch (due to its overgrowth) and four for conifers, respectively. Experimental set-up included 6 replicates (48 seedlings in each) for ambient and elevated temperature for each study species. At the end of growing season, leaf/needle and stem samples of 5 individuals (10 in 2016) were harvested and pooled for chemical analyses. Small molecular weight phenolics were analysed with HPLC-DAD [2], proanthocyanidins with acid-butanol assay [3], and alkaloid chemistry of conifers with GC-MS [4]. Herbivore damage, nitrogen content, and above-ground biomass were measured for all sampled seedlings.

RESULTS & DISCUSSION

According to our hypothesis, the average above-ground biomass of birch seedlings was 51% greater under experimental warming treatment after 3 growth seasons than in ambient conditions. In pine seedlings, corresponding increase was 175% after 4 growing seasons, whereas in spruce seedlings, the average above-ground biomass did not differ between ambient and heated plots.

Our results show that there is a large variation in the defensive chemistry responses to warming between tree species. In all tree species: 1) foliage chemistry was affected more than stem chemistry, and 2) in the leaves/needles the nitrogen levels increased in the first growing season after planting. 3) The proanthocyanidin levels in leaves/needles decreased under warming in average 39, 23, and 14% for birch, pine and spruce, respectively, whereas 4) alkaloid concentrations increased as a response to warming in the first growing seasons for both pine and spruce. On the other hand, responses of individual small molecular weight phenolics concentration to warming varied strongly depending on species, plant compartment and growing season. In pine and birch foliage, concentration of flavonoids decreased in addition to proanthocyanidins, but change in concentration of total flavonoids was not found in spruce.

Decrease in foliage phenolics was severe and increase in biomass allocation remarkable for both birch and pine seedlings. In addition, although the growth of the spruce was not affected by warming treatment, phenolic and alkaloid chemistry was affected. Our results show that large differences appear for responses of allocation of resources for plant biomass and defense chemistry to warming especially between spruce and pine, which may also affect their herbivore resistance under a changing climate. Consequently, this should be taken into account in cultivation of different tree species in order to ensure the forest regeneration success of boreal forests.

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P5.11

Iron solubilization in mangrove sediments associated with leaf-derived polyphenols and benthic animals

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MAIN CONCLUSION

In mangrove ecosystems, leaf removal into the burrow by crabs means depositing the leaf-derived polyphenol to the sediments. The depositing of phenolic compounds related to leaf-removing crabs may accelerate the solubilization of insoluble iron (e.g. oxidized Fe) in mangrove sediment. According to the result of this study, the small size of sesamid crabs (Sarmatium sp., Parasesarma sp.) can have essential roles in iron solubilization in mangrove sediment.

INTRODUCTION

Removal of fallen leaves by sesamid crabs into their burrows are known to be an important trophic pathway in mangrove forests, and this process prevents the tidal export of organic matter from mangrove ecosystems to the sea (e.g. Ashton 2002; Chen and Ye 2008). Therefore, the leaf-removing crabs help to retain organic matter and nutrients within the mangrove forests. Among them, *Neosarmatium smithi* is known to be a leaf-removing crab in mangroves. In their habitat, a large amount of the leaf-litter is removed into the crab burrows.

Major mangrove species contain large amounts of phenolic compounds in the leaf tissue, and the phenolics can change the iron forms from insoluble to soluble forms. This mechanism is attributed to the properties of reduction and complexation with metals by polyphenols (Hinokidani et al., 2019). Therefore, removal of the leaves by crabs may improve iron bioavailability by promoting iron solubilization in mangrove sediments. Our previous study showed mangrove produced polyphenols still remain in the faecal materials of the crabs, and the feces can solubilize iron in mangrove sediment (Nakanishi et al., 2020). However, it has not demonstrated that how much the dissolved iron can be contained in mangrove sediment where the leaf-removing crabs inhabit.

In this study, to clarify the ecological roles of the leaf-removing crabs on iron solubilization in mangrove sediments, the amounts of dissolved iron and phenolic contents in each mangrove sediment that inhabit benthic animals include the leaf-removing crab, were examined vertically, and the relationship among the iron solubilization, phenolic substances, and interaction by leaf-eating benthic animals was demonstrated.

MATERIALS & METHODS

This study was carried out at the Urauchi mangrove estuary (St1 and St 2) and Komi mangrove forest (St 3 and St) in Iriomote Island, Okinawa, Japan. St 1, 2, and 3 are dominated by *Bruguiera gymnorrhiza*. St 4 were dominated by *Rhizophora stylosa* and *B. gymnorrhiza*.

Firstly, it was conducted field investigation at all study sites. The density of principal benthic animals in each study sites and whether which types of sesamid crabs carry the mangrove leaves to their burrow were investigated.

Sediment core samples (0 to 80 cm depth) were taken from 3 research plot (10m x 10m) at every 4 sites by using a core sampler, and the cores were cut into 8 pieces (10 cm each), respectively. Sediment samples (n = 96) were dried and passed through a 2 mm mesh sieve. Total phenolic content in sediments was measured by the Folin-Ciocalteu method, and the amount of dissolved iron in sediments was measured by ICP-AES following the method of a previous study (Hinokidani et al., 2019).

RESULTS & DISCUSSION

In St 1, crab burrows were mostly sized 30–50 or over 50 mm, and *Episesarma lafondi* were observed as dominant benthic animals. In St 2, the size of crab burrows mainly was less than 30 mm, and it was suggested that small sesamid crabs (*Perisesarma* sp. and *Sarmatium* sp.) were dominant benthic animals, and it was observed that the crabs consume leaf-litter (cutting leaf and currying it to burrows). In St 3, a detrivorous snail, *Terebralia palustris*, and small sesamid crabs (*Metopograpsus* sp. and *Perisesarma* sp.) were observed as dominant benthic animals. In St 4, crab burrows were mostly sized 30-50, and *E. lafondi* and *Neosarmatium smithi* were observed as dominant benthic animals.

As shown in Figure 1, total phenolic content (TPC) in the mangrove sediments was higher in site St 1 than in St 2, St3, and St4. The maximum amount was 41±5 mg/100g, observed in 50 to 60 cm sediment layer, at St 2. The surface layer was a low amount of phenolics compared with deeper sediments. As shown in Figure 2, the amount of D-Fe in the mangrove sediments was higher in site St 2 than in site St1, St 3, and St 4. The maximum amount was 29±10 mg/100g, observed in 40 to 50 cm sediment layer, at St 2. D-Fe in the surface layer of St 2 was low compared with deeper sediments. The D-Fe was 41 times higher in 40 to 50 cm layer than 0 to 10cm layer. These trends can be explained by leaf removal into the burrow by crabs feeding behaviour. At this point, small sesamid crabs may strongly affect iron solubilization in mangrove sediments.

Figure 3 shows the relationship between total phenolic content and the amount of dissolved iron in mangrove sediment samples (n = 32). A significant positive linear correlation was observed between phenolics and dissolved iron in mangrove sediments. The relation indicates when polyphenol was contained a higher amount in the sediment, dissolved iron was also a high amount in the sediment.

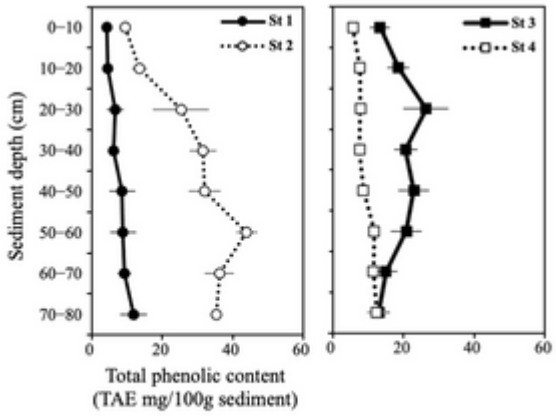

Fig. 1 Total phenolic content in each sediment layer collected from mangrove forest where sesamid crab inhabits.

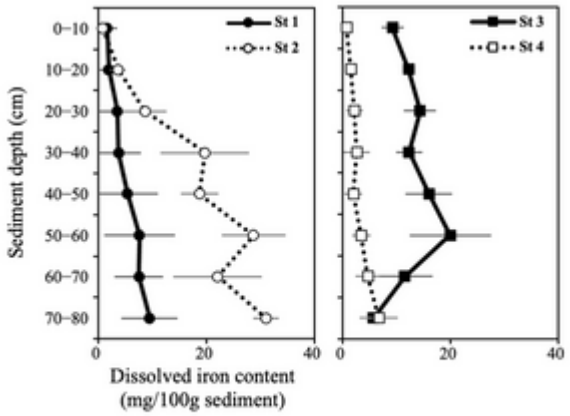

Fig. 2 Amount of dissolved iron in each sediment layer collected from mangrove forest where sesamid crab inhabits.

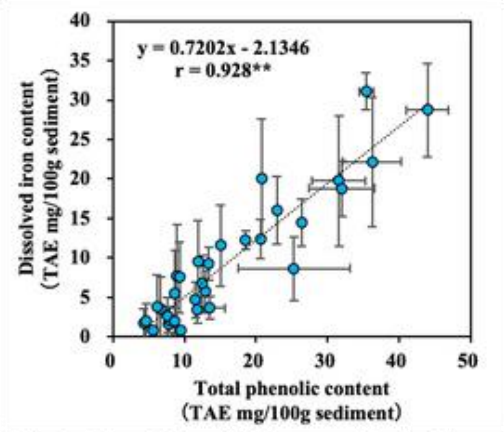

Fig. 3 The relationship between dissolved iron and polyphenol in mangrove sediment samples ($n = 32$). **: $p < 0.01$.

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P5.12

Insect and fungus specialists on aspen leaves have opposite relationships to condensed tannins

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MAIN CONCLUSION

Long-term balancing selection (BS) maintain multiple alleles of single genes in the gene pool. In *Populus tremula* BS was found for genes coding for cellular macromolecules and aromatic compound metabolic processes related to synthesis of polyphenols. In the TanAsp garden of tannin extreme aspen genotypes, two antagonists correlated oppositely with foliar condensed tannins and with each other supporting the potential for biological diverse stressors to act as long-term BS selection forces.

INTRODUCTION

European aspen canopies are inhabited by large communities of mainly specialist arthropods and microorganisms that coevolved to tolerate and even utilize leaf defence phenolics. The influence of natural variation in plant traits on canopy associated communities indicates the importance of genetic variation in wild trees and suggests that aspen can be a resource for the study of selection mechanisms behind natural resistance to consumers (Robinsson et al., 2012). Long-term balancing selective processes in aspen with multiple alleles of genes in the gene pool have been related to nucleic acid, cellular macromolecule, heterocyclic, and aromatic compound metabolic processes (Wang et al., 2020), that in turn (according to EMBL-EBI matches) relate to anabolic functions relevant for the evolution of the phenylpropanoid pathway (Weng and Chapple, 2010). To search for opposing or balancing selective biological forces, representations of phenolic compounds in tannin extreme field grown genotypes of *P. tremula* were surveyed for damage symptoms from consumers.

Pearson's correlation coefficients between foliar phenolic representations (green circle), antagonists including Venturia sp. (upper picture, courtesy Magdalena Kacprzyk) and Phyllocnistris labyrintella miners on aspen leaves (lover picture, orange circles), and between phenolics and antagonists (brown circle).

MATERIALS & METHODS

The TanAsp garden (Bandau et al., 2021) with ten aspen genotypes, representing extreme tannin expressions was used for sampling of leaves for analyses for total phenolic and condensed tannin concentrations using the methanol (Ossipova et al., 2001) and acid-butanol assays (Porter et al., 1986; Hagerman, 2002), respectively. Damage symptoms caused by leaf miners, galling insects and Venturia pathogens were scored according to Robinson et al. (2012).

RESULTS & DISCUSSION

Supporting previous studies of aspen phenolic compounds the phenolics included in this study were positively correlated (r: 0.17–0.53***) with each other, Venturia infection symptoms were negatively related to leaf tannins (r: -0.40**) confirming previous studies (Bandau et al., 2021), the presence of *Phyllocnistris labyrintella* leaf miners was positively related to foliar tannin contents (r: 0.28*); and the two antagonists were also negatively correlated with each other(r: $-0.14^{n.s.}$). Two additional antagonists (petiole galls and leaf galls) also showed opposite relationships to tannins although not with statistic significance (se figure).

Surveys *of P. tremula* in Sweden includes a wide array of specialist arthropod herbivores, and fungal pathogens confirming the status of aspen as an important keystone species for biodiversity. Genetic and environmental variation in the arthropod assemblage and community composition between populations of aspen have earlier been reported (Albrectsen et al., 2010; Robinson et al., 2012) suggesting regional variation in resistance to canopy arthropods, leaf-mining moths, and leaf rust fungus. Balancing selection works on several traits simultaneously (Wang et al., 2020) complicating studies of relationships between phytochemicals and antagonist selection impact. The opposite relationships demonstrated in this study by two abundant specialist antagonists relative to condensed tannin expressed in one common garden, suggested that biological stress could indeed over a long period of time provide opposing stress factors related to tannin expressions. Thus, while these results support the ideas behind balanced selection, the selection force at the landscape scale appears ordinarily relatively low; however, *Venturia* occasionally wipes out entire stands of young aspen and *P. labyrinthella* may occasionally reach outbreak levels in parts of Sweden.

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P6.1

Membrane assisted solid-liquid extraction for the recovery of polyphenolic fractions from grape pomace

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MAIN CONCLUSION

Acidified water (acetic acid 5% (v/v)) and acidified methanol (acetic acid 5% (v/v)) were used for two-step membrane assisted extraction. Methanolic extracts were richer in TPC. However, ME presented a higher TPC (2648 mg/kg GP), while acidified extract had the lowest TPC (238 mg/kg GP). Anthocyanins were present mainly in the ME: At pH 2.5, 61.2% were sulfite bleaching resistant, while at pH 3.5, 82.5% resisted bleaching. Oligomeric polyphenols were exclusively found in methanolic fractions.

INTRODUCTION

As a result of processing grapes for wine production, large amounts of grape pomace (GP) are generated, accounting to 20–25% of grapes weight [1]. GP is the main by-product originated as a consequence of wine-making. GP consists of seeds (around 5%) [2], grape skins (up to 50%) and, occasionally, residual stalks [1]. These residues are of particular interest considering their nature and composition, as GP can be a good source of high-value compounds. It has been reported that a tonne of GP can contain up to 9 kg phenolics [3]. Therefore, the development of alternative extraction-separation methods that do not present adverse effects on the molecules of interest is greatly desired.

The purpose of this work is to determine the viability of a membrane assisted solid-liquid extraction using a Zero Head Space Extractor (ZHE) as an alternative method for the recovery and fractionation according to size of phenolic compounds.

MATERIALS & METHODS

GP (*Vitis vinifera* Syrah variety) was provided by a locally-based vineyard situated 30 km north of Chihuahua, Chihuahua, Mexico. The skin and seeds were kept frozen until used.

The extraction was carried out as a two-stage solid-liquid process, using a commercial nitrocellulose membrane. Acidified water (acetic acid 5% (v/v)) was employed for the first stage, and acidified methanol (acetic acid 5% (v/v)) for the second.

Extraction took place in a ZHE. A 50 g sample was place in the cylindrical chamber. The membrane was place inside the ZHE and, after the trapped air was removed, the pressurized solvent (500 mL at 50 lb/in^2) was dispensed and the ZHE was agitated end-over-end.

Once the fractions were obtained, Total Polyphenolic Content (TPC), Total Anthocyanin Content (TAC), Monomeric Anthocyanin Content (MOC), and sulfite bleaching resistant pigments were measured. Moreover, in order to determine the presence of proanthocyanidins (PA), acid butanol assay was carried out.

RESULTS & DISCUSSION

Preliminary studies (data not shown) were performed to determine the best solvent system for the extraction. Three main compounds were identified: Malvidin-3,O-glucoside, acylated malvidin-3-O-glucoside and malvidin-3-O-coumaroyl-glucoside. The hydro-alcoholic mixture of 80–20% methanol/water-acetic acid 5% (v/v) was selected, as it presented the higher PA, TAC and TPC.

After the two-step membrane assisted extraction, the following fractions were obtained: “aqueous extract” (AE) and “methanolic extract” (ME). An additional methanolic fraction was obtained from the “cake” (the spent solid residues left from the membrane assisted extraction) and referred to as CE.

Out of the three fractions, methanolic extracts were richer in TPC. However, ME was the one with the higher TPC (2648 mg/kg GP), while acidified water extract had the lowest TPC (238 mg/kg GP).

Anthocyanins were present mainly in the ME. At pH 2.5, 61.2% of pigments were sulfite bleaching resistant pigments, and at pH 3.5, 82.5% of pigments resisted bleaching.

After the acid butanol index assay was carried out to both AE and ME, only the ME resulted positive for acid butanol assay, indicating the presence of proanthocyanidins (PA). CE was also tested for PA, with all the extracts testing positive for proanthocyanidins. Therefore, the acid butanol assay confirmed the existence of oligomeric polyphenols exclusively in both methanolic fractions.

As of now, it is concluded, based on the preliminary results, that the Zero Head Space Extractor (ZHE) can be used as an alternative method for the extraction of polyphenolic compounds.

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P6.2

Exploring the colour and bioactivity of anthocyanin related structures towards skin healthcare – bridging food and therapeutics

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MAIN CONCLUSION

Anthocyanins and related structures were found to reduce the biofilm production, exhibited UV-filter capacity, attenuated the formation of reactive oxygen species in skin cells and restrained the activity of skin-aging related enzymes, in the absence of cytotoxic effects towards human skin fibroblasts and keratinocytes. These pigments might be useful for incorporation in topical formulations in the context of skin health maintenance and protection against skin aging related ailments.

INTRODUCTION

The physiological balance of human skin is commonly compromised by a combination of internal and external factors, including intrinsic aging, exposure to UV radiation and skin pathogens[1]. In this regard, the use of compounds derived from natural sources represents one of the possible strategies to prevent and treat skin related damages and disorders. Among the different existing classes of compounds, anthocyanins have long provided strong evidence of their wide-ranging bioactivity repertoire, allied to their visually attractive and varied colors. The purpose of this study was to assess the potential protective and skin health promoting effects of a group of anthocyanins and derivatives, including their antimicrobial activity, UV-filter behavior, inhibitory capacity towards ROS production within skin cells and modulation of the activity of skin-aging related enzymes. Cyanidin- and malvidin-3-*O*-glucosides were isolated from an extract of blackberries and from a young red wine, respectively, and their corresponding deoxyanthocyanins, luteolinidin and deoxymalvidin, (which lack the substitution at the C-3 position, making them less sensitive to water addition at C2) and pyranoanthocyanins (known for their higher structural and color stability in a wider pH range) were further obtained by chemical modification of the two native anthocyanins, resulting in a set of compounds with distinct chromatic and structural properties. Given their physical-chemical characteristics, the stability of anthocyanins is commonly affected by factors such as pH and temperature variations, which might compromise their applicability. The inclusion of these structural derivatives in this study represents an alternative to overcome this issue.

MATERIALS & METHODS

Anthocyanins were extracted from blackberries and red wine and their further reaction with pyruvic acid and acetone resulted in the formation of carboxy and methylpyrano derivatives[2]. Deoxyanthocyanins were obtained by acid aldol consensation[3]. Inhibition of biofilm formation by *P. aeruginosa* and *S. aureus* was assessed by crystal violet assay. Standard MTT assay was used to evaluate the cytotoxicity of the compounds towards human skin cells. Production of reactive oxygen species in both cell lines, after incubation with the test compounds, was determined according to the DCFDA assay protocol. The absorption spectrum of each compound, diluted in ethanol, was measured from 290 to 320 nm and *in vitro* solar protection factors were calculated according to Mansur equation. Enzymatic inhibition assays (collagenase, hyaluronidase and tyrosinase) were carried out and consisted on monitoring spectrophotometrically the enzymatic activity in the presence and absence of the test compounds.

RESULTS & DISCUSSION

Treatment with some of the tested compounds, including luteolinidin, cyanidin-3-*O*-glucoside and the carboxypyrano derivatives of both anthocyanins resulted in a decrease of biofilm biomass which, although more accentuated in *S. aureus*, was observed in both strains.

Over a period of 48 h of incubation, none of the compounds showed significant effects on the cellular viability of neither of the two cell lines, up to 100 μM.

Concerning the ROS production, cyanidin-3-*O*-glucoside related structures exhibited a more notorious effect, with cyanidin-3-*O*-glucoside and its carboxypyrano derivative being particular effective. This antioxidant effect appears to be more prominent in dermal fibroblasts, where a reduction level around 50 % was observed.

Overall, the *in vitro* SPF values of anthocyanins and their structural derivatives ranged between around 14 to 30, with luteolinidin exhibiting the highest SPF, supporting the UV filter activity and further application of these compounds as additives in UV-protective formulations.

From the conducted enzymatic assays, certain compounds displayed a significant inhibitory effect. Tyrosinase activity for instance, was considerably lower in the presence of both deoxyanthocyanins and native anthocyanins, with inhibition rates ranging from 40 to 60%. In the case of hyaluronidase, the great majority of the compounds (at 50 μM) was able to interfere with the activity of the enzyme, exhibiting moderate inhibition rates, ranging from 20 to 40%. In the particular case of carboxypyranocyanidin-3-*O*-glucoside and luteolinidin, the inhibition rate increased to 61.2% and 52.0%, respectively, when tested at 100 μM. With respect to collagenase activity, both carboxypyrano derivatives stood out from the remaining tested compounds, resulting in an inhibition rate of 40%.

Overall, results herein presented highlight the skin-health promoting effects of this group of compounds, evidencing their potential utility for cosmeceutical purposes.

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P6.3

Valorisation of food wastes to obtain polyphenolic rich extracts and extract fractions

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MAIN CONCLUSION

Berry press residues can serve as a rich source of various health-beneficial phytochemicals, which can be retrieved using environmentally friendly extraction methods. *Vaccinium* spp. pomace extracts demonstrated anti-inflammatory properties in an LPS-stimulated THP-1 cell inflammation model and inhibited COX-2 activity. Thus, Vaccinium spp. berry pomace extracts could serve as a valuable source of anti-inflammatory compounds.

INTRODUCTION

Berries (*Vaccinium* spp.) of Northern Europe are consumed fresh or processed into various consumer products. Bluberries, American cranberries are widely cultivated in Northern Europe and USA, while bilberries, lingonberries and bog cranberries are gathered in the wild, in forest and peat bogs. Both, cultivated and wild berries are used for production of jams, marmelades, dried and frozen, but the main use of these valuable berries is juice production. The residues, which are left as a waste after juice processing, are used inefficiently mainly due to lack of processing methods and valorisation. Berry pomace consists of berry skins, which are highly pigmented, and seeds. Berry press residues contain high levels of valuable phytochemicals - polyphenolics, lipids, vitamins as well as residual carbohydrates, protein and fibre. Valorisation of this waste material could help in the developement of bioeconomics where biomass is used to produce functional ingredients and products with high-added value. Several approaches exist for retrieval of these phytochemical - solvent extraction, enzyme degradation of cell wall materials, ultrasonic assisted extraction, super critical fluid extraction and others. Conisdering the concerns of environment and demand for cleaner products, solvent-free extractions are preferred, however, the technologies must be developed further to ensure high purity and high quality extracts. The main group of phytochemicals found in press residues is polyphenolics (flavanoids, anthocyanins, flavanols) which are polar compounds and have good solubility in water-ethanol based solvents. Polyphenolics are known for their radical scavengin properties, but several groups of polyphenolics have specific effect on human health. In this study, extraction, purification, fractionation of berry press residue polyphenolics has been studied and the effects of the prepared extracts has been evaluated considering potential applications.

MATERIALS & METHODS

The extraction method for berry press residues was optimized using RSM approach to achieve the highest possible extraction yields. Extract contents were evaluated using Folin-Ciocalteu method, pH differential method. Prepared extracts were tested using various methods for determination of antioxidative potential (DPPH, ABTS, FRAP, CUPRAC, ORAC). The prepared extracts were further purified using sorbtion chromatography (KP-SIL, XAD, LH-20). Anthocyanin profiles were determined using UPLC-PDA, identification of individual procyanidins was done using FT-ICR-HRMS. THP-1 monocytes were pre-incubated with prepared extracts following LPS stimulation. NF-κB nuclear translocation was assessed by flow cytometry. TNF-α, MMP-9, IL-23, IL-10, IL-1β, CCL22, and IL-8 secretion was analyzed in cell culture supernatant using Luminex assay and ELISA. The expression of IL-6, TNF-α, IL-10, IL-23, and TGF-β1 was assessed by qPCR, and COX-2 activity was determined by a fluorometric inhibition assay.

RESULTS & DISCUSSION

Extracts prepared from *Vaccinium* species press residues were found to contain high levels of polyphenolics (up to 30%), especially anthocyanins (up to 60%). Fractionation of procyanidins was succesfully carried out allowing to obtain 80% purity. Using FT-ICR-HRMS 61 different procyanidins were identified with degree of polymerisation of up to five. Procyanidins were split into high molecular (>5) and low molecular (<5) weight procyanidins. Using antioxidant potential evaluation methods it was concluded that low molecular weight fraction was up to 7 times more effective than the high molecular weight fraction in scavenging free-radicals.

The cytotoxicity of the berry pomace extracts was examined and it was found that the extracts do not considerably alter the viability of THP-1 cells at concentrations of 1 mg/mL and below. Our data showed that bilberry, blueberry, American cranberry, and bog cranberry pomace extracts inhibited LPS-induced NF-κB nuclear translocation to the level of residual NF-κB nuclear translocation in unstimulated THP-1 cells. Unexpectedly, blueberry pomace extract showed inhibitory effects, even at the lowest concentration tested (0.04 mg/mL). The finding that bilberry and blueberry extracts inhibited NF-κB nuclear translocation was in line with previous studies that used an LPS-induced human monocytic U937-3xκB-Luc cell model [1]. It is of interest that lingonberry pomace extract did not show a significant inhibitory effect on NF-κB translocation in LPS-stimulated THP-1 cells. The inhibition of anti-inflammatory cytokine IL-10 secretion by American cranberry, bog cranberry, and lingonberry pomace extracts was significantly different from LPS-stimulated THP-1 cells; however, the level of IL-10 was low in general and the meaning of this effect is ambiguous. In addition, lingonberry pomace extract inhibited MMP-9 secretion. MMP-9 expression is reported to be associated with the development of cardiovascular diseases [2].

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P6.4

Can plant polyphenol inspired surface modifications improve tissue integration of titanium implants?

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MAIN CONCLUSION

Despite polyphenols have been reported to suppress microbial growth and act anti-inflammatory, coatings made from tannic acid and pyrogallol could not confirm this effect *in vitro*. Challenging the coatings with *S. epidermidis, S. aureus,* and *C. albicans* showed that microbial colonization could not be prevented. The anti-oxidant properties of the coating were correlated to radical scavenging *in vitro*. However, LPS/IL-1b induced inflammation could not be prevented.

INTRODUCTION

Bacteria associated infections are still a widespread phenomenon faced in modern implant technology. Routine administration of anti-microbial agents, does not always sufficiently prevent infections, and can evoke the progression of antimicrobial resistances. In the “race to the surface”, the success of any implant is highly dependent on the host tissue integration.[1] Our research thus aimed to influence the relation between bacterial colonization and tissue integration through anti-inflammatory and antimicrobial surface modifications.

To achieve this goal, the utilization of the antioxidant and antimicrobial properties of naturally derived polyphenols was investigated.[2] We specifically focused on tannic acid (TA) and pyrogallol (PG), which are able to form surface-independent coatings. They also show the ability to continuously build-up and form surface coatings beyond monolayers. Since polyphenols typically show a higher microbial inhibitory concentration compared to classic antibiotics, an increase in coating thickness was required to increase the release of potential active molecules from the coated surfaces.

To stimulate wound healing processes, the anti-oxidant properties of polyphenolic coatings was investigated for their potential to reduce intracellular oxidative stress. Thereby, inflammation may be modulated and tissue repair processes improved.

Special focus during wound healing around implants is also the foreign body response. Changing the physico-chemical properties of implant surfaces particularly affects the initial adsorption of biomolecules forming a conditioning film on the implant surface. This conditioning film determines the recognition of the implant surface by the host immune and hemostatic system.

Thus, the aim of this work was to investigate whether these different properties of polyphenolic molecules can be used to create multifunctional surfaces, which improve tissue integration of implant surfaces and prevent potential infections.

MATERIALS & METHODS

TA coatings were obtained using silicic acid crosslinking, whereas PG coatings were based on oxidative polymerization.[3]

Protein adsorption (200 mg/ml in PBS) was monitored under constant flow of 10 ml/min using a quartz-crystal microbalance with dissipation monitoring (QSense E4).

In vitro cell culture studies were conducted with primary human gingival fibroblasts (hGFs), which were cultured in DMEM at 37°C/5% CO_2. hGFs were seeded on coated surfaces at a cell density of $7x10^3$ cells/well in 96-well plates.

Innate immune response and coagulation was tested with human blood obtained from three healthy donors with their informed written consent.

Coatings were challenged with biofilm forming bacteria *S. epidermidis*, *S. aureus*, and the oral fungus *C. albicans*. 150 ml of bacteria suspension ($1x10^7$ CFU/ml) was incubated with the coated surfaces in TSB culture medium at 37°C/5% CO_2. *C. albicans* suspensions were diluted to OD = 0.3 ($2x10^7$ CFU/ml) in YPD and surfaces were incubated at 37°C.

RESULTS & DISCUSSION

Our *in-vitro* studies confirmed good biocompatibility of TA and PG coatings towards fibroblasts. The hydrophilic, negatively charged polyphenolic coatings generally increased the adsorption of blood and salivary proteins compared to bare Ti surfaces. TA coatings were consistently binding more protein mass than PG coatings. The binding appeared to be irreversible and affecting the subsequent exchange with high molecular mass biomolecules.

Evaluation of processes in the early foreign body response showed that despite complement and coagulation activation, leukocytes were not activated during incubation with coated surfaces. This altered their expression of inflammatory cytokines compared to the Ti control. Further, TA and PG coatings reduced H_2O_2 initiated intra-cellular oxidative stress, which may cope with unspecific ROS release by macrophages during inflammation. However, coatings did not prevent inflammation in hGFs stimulated by LPS/IL-1b. No effect on the NF-kB cell-signaling pathway was observed in our primary hGFs and inflammatory interleukins were continuously released. This result may in part be a cell specific effect as we noticed adverse reactions of hGFS to LPS compared to hOBs.

Furthermore, the obtained coatings were not able to prevent bacterial and fungal biofilm formation. Although TA and PG inhibited the growth of planktonic bacteria, biofilms formed after a period of 24 h.[4] These results were similarly obtained with *C. albicans*, which grew on coated surfaces similar to the Ti control group. It appears that with a released concentration of approximately 10 mM TA and PG the MIC preventing microbial growth was not reached in our experimental setup.

Finally, we tested whether coated surfaces reduce microbial adhesion. However, no significant anti-adhesive effects were observed under laminar flow condition, which could curb with surface colonization of microbes. Additionally, the low anti-adhesive effects were attenuated by adsorbed protein films.

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P6.5

Polymerization possibilities of polyphenols from the flavonoid group (Funding: National Science Centre, Poland, grant No. 2018/31/N/ST8/02565)

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MAIN CONCLUSION

As a result of the polymerization of flavonoids, compounds with better antioxidant and antimicrobial properties as well as higher thermal stability and resistance to oxidation can be obtained. These improved properties are due to the structure of the poly(flavonoids). The greater number of OH groups of the polymeric flavonoid determines a better ability to scavenge free radicals. The cross-linked structure is responsible for the greater thermal stability of polymeric polyphenols.

INTRODUCTION

Flavonoids are natural compounds valued for their antioxidant, anti-aging and pharmacological properties. These valuable properties of flavonoids are determined by their chemical structure, and particular properties, such as the ability to reduce free radicals and antimicrobial properties, are closely related to the structural elements of these polyphenols. Total amount and configuration of hydroxy groups has important impact on mechanisms of antioxidant capacity of flavonoids. Structural elements of flavonoids such as catechol moiety, C2-C3 double bond, OH group number and their position provide pharmacological activities (antiviral, antibacterial, cytotoxic, anti-inflammatory, cardioprotective and other). Structural relationships of monomeric (SAR) flavonoids are quite well studied, however, there are only a few scientific reports on the relationship between the structure and properties of polymeric forms of these polyphenols. Among the polymeric polyphenols, the relationships of oligomeric flavan-3-ols were investigated. The free radical scavenging capacity of oligomeric or polymeric flavan-3-ols increased with increasing degree of polymerization. Oligomeric or polymeric forms of flavan-3-ols have also been investigated for pharmacological properties. It was found that dimeric flavan-3-ols played a unique, protective role in the human diet, and the larger forms of these compounds showed better resistance to acid hydrolysis in the stomach (Latos-Brozio, M.; Masek, A. 2019. Chemistry & Biodiversity 16: e1900426). The aim of study described in this manuscript is to compile and summarize the relationships between polymeric forms of flavonoids obtained by various polymerization methods and their properties. In the considerations, the literature data on photopolymerization and polymerization with a cross-linking compound of quercetin, rutin, catechin and naringenin were analysed.

MATERIALS & METHODS

Flavonoids representing different subgroups of these compounds were selected for consideration: quercetin (5,7,3′, 4′-flavon-3-ol), rutin (Quercetin-3-rutinoside), catechin (flavan-3-ol) and naringenin (4', 5,7-Trihydroxyflavanone).

The polymeric forms of the above flavonoids were obtained as a result of two types of polymerization: photopolymerization and polymerization with a cross-linking agent.

Photopolymerization: Solutions of quercetin, rutin and naringenin, prepared in phosphate buffers at pH 5, 6, 7, 8, were irradiated with blue light (440-490nm) for 4h (Latos-Brozio, M.; et al. 2020. ISBN: 978-83-7934-425-3, p. 17-25). Catechin was dissolved in buffers at pH 6, 7, 8, and then irradiated with blue light from 0.5 to 216h [1-2]. The polymerization progress was monitored by UV-Vis spectroscopy.

Polymerization with cross-linker: It was performed using L-α lecithin as a surfactant, cyclohexane as the organic phase and glycerol diglycidyl ether (GDE) as a crosslinking agent [3-4].

RESULTS & DISCUSSION

As a result of photopolymerization, Liang et al. [1] obtained a polymeric form of catechin with Mw 578.14 g/mol. Latos-Brózio et al. [2] modified the reaction proposed by Liang. In the first step they received proanthocyanidins, which were characterized by greater antioxidant activity (H_2O_2 scavenging capacity) than catechin. In the second step, the polymeric catechin complex compound was obtained. On the basis of FTIR spectroscopy, groups specific for polymeric flavonoids have been identified. Poly(catechin) was characterized by excellent thermal stability.

The photopolymerization of quercetin, rutin and naringenin did not allow synthesizing their polymers. Unlike catechin, the structure of those flavonoids is based on a carbon skeleton with a ketone group in position 4, in the C ring. The presence of a ketone group may hinder the opening of flavonoid rings and further polymerization (Latos-Brozio, M.; et al. 2020. ISBN: 978-83-7934-425-3, p. 17-25).

Sahiner obtained polymeric forms of quercetin and rutin with a cross-linking compound [3]. Poly(quercetin) had a higher oxidation temperature and poly(rutin) was characterized by a much higher enthalpy of oxidation than monomer. Both poly(flavonoids) exhibited higher ability to reduce multivalent metal ions [4]. Polymeric forms of catechin and naringenin were also synthesized with a cross-linker (Latos-Brozio, M.; Masek, A.; Piotrowska, M. 2021. Biomolecules 11: 50 and Materials 14: 2142). The polymeric structure of catechin and naringenin was confirmed by FTIR. Analysis of the antioxidant activity showed that the polymeric catechin and naringenin had better activity for reducing ABTS free radicals. DSC analysis confirmed the higher resistance of both poly(flavonoids) to oxidation. Poly(catechin) presented antibacterial activity against *Staphylococcus aureus*, stronger than catechin. Polymeric naringenin had activity against *Candida albicans*, while the monomer showed no activity.

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P6.6

Extraction of grape polyphenols during maceration and by organic solvents in relation to vineyard relief

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MAIN CONCLUSION

Concentration of proanthocyanidins (PAs) was higher in skins of Merlot grapes grown in alluvial plains. Higher galloylation of skin and seed PAs was found in grapes from terraces.

Wines from terraces contained more PAs, anthocyanins, total polyphenols and showed higher galloylation of PAs than wines from alluvial plains.

Only part of polyphenols was extracted into red wines during maceration. Grape pomace represents a reliable source of polyphenols.

INTRODUCTION

Grape proanthocyanidins (PAs) are mainly found in skins and seeds. During maceration in winemaking process, a certain part of skin and seed polyphenols is extracted into the wine, which affects the sensory characteristics of the wine, such as colour, astringency and bitterness. The content and structural characteristics of PAs between skins and seeds are different. Seed PAs are composed of the flavan-3-ols catechin, epicatechin, and epicatechin gallate, and skin PAs of catechin, epicatechin, gallocatechin, epigallocatechin, and epicatechin gallate [1]. The content and structural characteristics of PAs in grapes are also influenced by environmental factors such as geological setting and soil properties. In a relatively small-scale area in the winegrowing region of Vipava Valley in Slovenia, two distinctly different soil types are present: dry, skeletic Eutric Cambisol on terraced slopes and silty-loamy Eutric Fluvisol at alluvial plains. Flysch soils on terraces contain less soil organic matter and up to 80% coarse skeleton; both contribute to a lower water holding capacity as compared to dense, loamy and predominantly non-skeletal soils in alluvial plains. Consequently, the water deficit is more frequent in terraced vineyards [2]. The aim of this study was to investigate i) concentrations of total polyphenols (TP), total anthocyanins (TA) and low and high molecular weight PAs (LMWP and HMWP) in grapes (seeds and skins extracted separately in organic solvents) and in wines from terraces and alluvial plains and ii) structural characteristics of PAs (mean degree of polymerisation (mDP), percentage of galloylation (%G) and percentage of prodelphinidins (%P) in grape skins and seeds and in wines in relation to vineyard relief.

MATERIALS & METHODS

In 10 vineyards of flysch terraces (n=5) and alluvial plains (n=5) of Merlot variety in Vipava Valley (Slovenia) in the growing season 2019, ratio between leaf area and yield (Smart Index) was homogenised. Hundred matured grapes were randomly sampled (3 plots per vineyard) on 21 September, skins and seeds of frozen berries were separated, lyophilised for 5 days and extracted with Dionex ASE 350 extractor under 103 bars at 20–23 °C with acetone: water (80:20. v/v), followed by methanol: water (60:40. v/v). Solvents were evaporated under reduced pressure at 40 °C, samples were re-dissolved in water and lyophilised to obtain a crude extract. For microvinifications 5 kg of grapes per plot (3 plots per vineyard) were sampled. The content of polyphenols was determined spectrophotometrically in skin and seed extracts and in wines after one month as described [3], [4]. Structural characteristics of PAs in wines and in seed and skin extracts were analysed by UHPLC-DAD-MS/MS [3].

RESULTS & DISCUSSION

Content of PAs (mg/g DW) and % P was higher in grape skins from alluvial plains. On the other hand, % G was higher in skins and seeds from terraces, but in case of seeds the difference was not statistically

significant (Table 1). Higher content of PAs in skins of grapes grown on alluvial plains might be due to less matured grapes compared to terraced vineyards. Previously, the flowering and veraison dates were advanced on terraced hills and the delay in maturation together with the higher % G of skin and seed PAs from terraces could be due to the greater vine water stress, in agreement with other reports [3].

Content of TP, TA and LMWP expressed on berry fresh weight (mg/kg FW) was higher in grapes from terraced vineyards. Moreover, wines from terraces contained more TA, PAs and had higher % G compared to wines produced from alluvial plains (Table 2.). The smaller berry size ascertained on the vineyards on terraces compared to berries from alluvial plains may explain the higher PAs concentrations in FW berries and in wines. The mass of 100 berries from terraces and alluvial plains was 116.7 g and 150.6 g, respectively, representing a 23.8% lower berry mass on terraces. After 15 days of maceration, PAs were only partially extracted from grape skins and seeds. According to the approximation that 0.75 l of wine is obtained from 1 kg of grapes, only 26.4–27.7% LMWP and 17.3–21.5% HMWP were extracted into the wine (Table 2). The extractability of PAs during maceration depends on several factors, but it is never as high as when organic solvents are used. Therefore, grape pomace could be a good source of polyphenols that can be used for other purposes, which could contribute to sustainable food use.

Table 1: Concentrations of polyphenols (TP), anthocyanins (TA), proanthocyanidins (PAs) expressed in mg/g dry weight (DW) and structural characteristics of PAs after extraction in organic solvents, in relation to the vineyard relief.

	Skins (mg/g skins DW)			Seeds (mg/g seeds DW)		
	Alluviums	Terraces	Sign. F	Alluviums	Terraces	Sign. F
TP	48.41	40.14	0.003**	212.45	205.87	0.481ns
TA	24.07	23.98	0.956ns			
LMWP	20.04	17.17	0.09.	106.90	101.85	0.306ns
HMWP	56.30	45.97	0.04*	137.45	130.87	0.408ns
mDP	15.58	14.53	0.045*	5.71	5.74	0.902ns
%G	12.71	15.11	0.099.	45.68	46.88	0.152ns
%P	24.83	26.64	0.133ns			

Note: t-test (ns, not significant; ., 0.05<p<0.1 (marginally significant); *, p < 0.05; **, p < 0.01; ***, p < 0.001). HMWP/LMWP, high/low molecular weight proanthocyanidins; mDP, mean degree of polymerization; %G, percentage of galloylation; %P, percentage of prodelphinidins.

Table 2: Concentrations of polyphenols (TP), anthocyanins (TA), proanthocyanidins (PAs) in berries and in microvinificated wines in relation to vineyard relief.

	Grapes[a] (mg/kg berry FW)			Wine (mg/L)			Extractability[b] (%)	
	Alluviums	Terraces	Sign. F	Alluviums	Terraces	Sign. F	Alluviums	Terraces
TP	10800	12700	0.017*	1207	1428	0.001***	8.4	8.5
TA	2765	3470	0.01*	602	744	0.002**	16.4	16.1
LMWP	4134	4698	0.016*	1520	1648	0.368ns	27.7	26.4
HMWP	8781	9503	0.304ns	2023	2714	0.008**	17.3	21.5
mDP				4.3	3.9	0.093.		
%G				12.5	15.6	0.017*		
%P				23.7	19.4	0.01*		

Note: (t-test: ns, not significant; ., 0.05<p<0.1 (marginally significant); *, p < 0.05; **, p < 0.01; ***, p < 0.001). HMWP/LMWP, high/low molecular weight proanthocyanidins; mDP, mean degree of polymerization; %G, percentage of galloylation; %P, percentage of prodelphinidins.
[a] Skins + seeds extracted in organic solvents, expressed in mg/kg berry fresh weight (FW).
[b] Estimated extractability from grape to wine considering that from 1 kg of grapes is obtained 0.75 L of wine.

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P6.7

Optimization of alcohol extraction of polyphenols from distillery stillage

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MAIN CONCLUSION

Distillery stillage is a rich source of polyphenols of high antioxidant activity. Optimization of the extraction is important before reusing or utilizing stillage in the environment. The highest efficiency of polyphenol recovery was achieved using an 80% ethanol solution (70% efficiency; 204.15 mg GAE/L). Optimizing the extraction from distillery stillage performed in this work allows the production of high-value extracts without pretreatment, using solvents available in any distillery.

INTRODUCTION

Distillery stillage is a by-product of ethanol production. From 1 L of the spirit produced, 9 to 14 L of distillery stillage are generated. This stillage may cause serious environmental problems because it contains high concentrations of nitrogen and difficult-to-degrade organic compounds, and it has a low pH, a high temperature, and a dark brown colour (Fito et al. 2019). Although this by-product is currently used as fertilizer, animal feed, and raw material for biogas production, these methods of utilization do not solve problems with its management (Bustamante et al. 2009). Thus, it is important to manage stillage by utilizing it to produce value-added compounds. One possibility for valorising stillage is extracting polyphenols.

Many factors influence the content of bioactive phenolics in distillery stillage. It mainly depends on the type and variety of raw materials used during alcoholic fermentation, as well as the storage conditions and the method of processing (Librán et al. 2013). Additionally, an important element is a method of extracting polyphenols. These bioactive compounds differ in terms of structure and their interactions with other components are not fully known, and this is a very important aspect when choosing to determine the conditions of the extraction process. Despite the development of new extraction techniques, classical extraction is environmentally friendly and is characterized by reduced energy demand and the use of non-toxic solvents, with high recovery of the substance (Chandrasekar et al. 2015).

Extraction effectiveness depends on the type and concentration of the extractant used. However, there is a lack of data on the optimization of polyphenol recovery from distillery by-products. Therefore, the objective of this study was to determine the effectiveness of recovering polyphenols from distillery stillage by extracting them with ethanol at concentrations of 70%, 80%, and 90%.

MATERIALS & METHODS

The experiments were carried out with the use of distillery stillage collected during the production of concentrated crude ethyl alcohol from cereals (a company in north-east Poland). The method of the determination of total polyphenols used Folin-Ciocalteu reagent. A sample of the supernatant (0.25 mL) was mixed with 0.125 mL of Folin-Ciocalteu reagent and 0.5 mL of 14% Na_2CO_3 solution. The mixture was incubated for 30 min in dark at room temperature. The absorbance was measured at λ=765 nm regarding the blank. A standard curve was prepared using gallic acid (0.1–0.5 mg/mL), and the polyphenol content in the distillery residues was expressed as gallic acid equivalents (GAE) in mg/L of distillery stillage. Conventional extractions of polyphenols were carried out using ethanol at various concentrations (70%, 80%, and 90%). The mixture was shaken for 30 minutes at room temperature.

RESULTS & DISCUSSION

The concentration of polyphenols in the distillery stillage was 291.64mgGAE/L. The extraction of polyphenolic compounds from the stillage shown that the 80% ethanol solution proved to be the most effective extractant (70% efficiency; 204.15mgGAE/L of stillage in the extract). A 9% lower extraction efficiency compared to an 80% solution was demonstrated by a 90% ethanol solution. On the other hand, the 70% alcohol solution turned out to be the least effective extractant; a 12% lower efficiency of polyphenol recovery was obtained compared to an 80% ethanol solution. Table 1 shows the concentrations of polyphenolic compounds.

Table 1. Polyphenol concentration in extracts from stillage depending on the concentration of the ethanol solution.

Sample	Concentration of polyphenol compounds (mg GAE/L)
extraction with 70% ethanol solution	175.57
extraction with 80% methanol solution	204.15
extraction with 90% ethanol solution	183.74

Librán et al. (2013) identified the most efficient conditions for the extraction of phenolic compounds from grape pomace. The highest recovery of phenolic compounds (80% efficiency) was recorded during 2 hours of extraction in a 75% liquid mixture of ethanol at pH 2. The content of polyphenols in waste depends on many factors, including the quality of the raw material tested, as well as the place of origin and the type of raw materials. Many researchers have noted that during transport, residues are exposed to environmental factors such as temperature, humidity, and environmental pollution which affects their content of polyphenols.

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P6.8

Capability of yeast mannoproteins to modify phenolic compound-salivary protein aggregation

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MAIN CONCLUSION

Mannoprotein composition and flavanol structure are both relevant in the way by which MPs could modulate the harsh astringency. MPs with elevated carbohydrate content could stabilize soluble ternary aggregates formed with salivary proteins and non-galloylated flavanol oligomers. However, MPs with higher protein percentage mostly could precipitate non-galloylated flavanols with lower degree of polymerization, which partially prevents the formation of insoluble flavanol-salivary protein aggregates

INTRODUCTION

The main accepted theory that explains the astringent sensation perceived in the oral cavity during red wine tasting is the precipitation of salivary proteins by phenolic compounds. Among these compounds, the condensed tannins or flavanols are the most responsible for the wine astringency development. Regarding their chemical structure, they can be found as monomers, oligomers, or polymers depending on their polymerization degree, and they can be attached or not to gallic acid, being galloylated or non-galloylated flavanols, respectively [1].

As a consequence of the global climate change, the phenolic maturity of grapes cannot be completely achieved, leading to wines with unbalanced astringency. To resolve it, potential strategies are being currently used like the addition of yeast mannoproteins (MPs) to wines [2]. MPs from yeast represent around 35% of total wine polysaccharides and are characterized by being highly glycosylated and composed of one major glycosidic moiety covalently linked to one minor protein moiety (5–50%) [3].

The effect of two yeast MPs showing important structural differences (M10 or M30) on the interaction of flavanols with salivary proteins has been studied in this work to propose a relationship between MPs structure and the molecular mechanisms by which they could modulate astringency.

MATERIALS & METHODS

Selected species. Commercial MPs from *S. cerevisiae* cell walls were chosen by their structural characteristics, especially by their differences in the protein content, ~10% in M10 and ~30% in M30. The salivary proteins (S) were obtained from the whole saliva of 8 volunteers (20–50 years old). The flavanol extract (E) was obtained from *Vitis vinifera* L. Tempranillo seeds and its composition was determined by HPLC–DAD–MS following the methodology described by García-Estévez [4].

Interaction assays. The size of the complexes formed between the previous components was assessed by Dynamic Light Scattering (DLS). Three different systems were studied: S-MP, E-MP and S-E-MP. The intensity of the scattered light was detected at 633 nm, angle of 173º and 25ºC. Likewise, all binary and ternary combinations were analyzed by HPLC–DAD–MS, following the methods described by García-Estévez [4] and Ramos-Pineda [5].

RESULTS & DISCUSSION

DLS and HPLC–DAD–MS techniques were employed to study the interaction mechanisms of flavanols with salivary proteins in presence of MPs (M10 or M30), previously selected by their properties as effective modulators of wine astringency, as well as by their different structural characteristics.

The DLS results confirmed the presence of both binary and ternary aggregates with different sizes, in which different molecules are involved depending on the MP studied: the amount of flavanols that bound to MP was higher in M30 than in M10; on the contrary, M10 bound with higher affinity to salivary proteins than M30. The chromatographic analysis indicated the two MPs reduced the precipitation of the different types of salivary proteins but did not reduce the precipitation of flavanols in the same way. In ESM10 ternary interaction, high amount of flavanols remained in solution, possibly in form of ternary aggregates, which would be more soluble than their corresponding binary aggregates. However, in the ESM30 system, the amount of flavanols in solution was significant smaller than that found in the binary systems, reaching a percentage of precipitation slightly lower than the sum of the precipitation percentages found in the binary interactions. According to this, M30 might remove flavanols from the solution by precipitation and, consequently, avoid its interaction with salivary proteins.

Moreover, our data pointed out that both MPs preferentially bound to non-galloylated flavanols –unlike what happens with salivary proteins–. Regarding these flavanols, the data showed each MP act in a different way: M10 would easily form soluble ternary complexes when the interaction involves non-galloylated oligomers, whereas M30 could precipitate non-galloylated flavanols with lower degree of polymerization, which partially prevents the formation of insoluble S–E aggregates. Therefore, not only the MP composition but also the flavanol structure establishes the MP action mechanism.

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P6.9

Effect of mannoproteins obtained from different oenological yeast on pigment and color stability of red wine

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MAIN CONCLUSION

Mannoproteins obtained from the cell wall of different oenological yeasts showed important structural differences, *i.e.* different molecular weight and protein content. Moreover, the effect of their addition on wine pigment and color stability is different depending on their origin. Mannoproteins isolated from *Torulaspora delbruekii* are postulated as the most appropriate candidates to stabilize the wine pigment composition.

INTRODUCTION

Global climate change is exerting an important influence on the phenolic maturity of grapes. As a consequence, some sensory properties of wine are being altered. Some studies have reported that the addition of mannoproteins (MPs) can have positive effects on wine color and astringency [1, 2]. However, it has been pointed out that different mannoproteins can exert different effects on wine organoleptic properties, mainly with regards to color, since controversial results have been reported [1]. MPs from cell walls of oenological yeasts are glycoproteins composed of one major glycosidic moiety covalently linked to one minor protein moiety. They are naturally released into the wine during alcoholic fermentation when yeast is actively growing or when cell wall breaks down in the process known as autolysis [3]. Over the last decades, the role of non-*Saccharomyces* yeasts in winemaking has been re-evaluated, with a view to improving the quality of wines [4]. Therefore, wines could present a great diversity of cell wall MPs released from different yeasts besides the principal oenological yeast, *Saccharomyces cerevisiae*.

Hence, the aim of this work was to isolate, purify and characterize MPs from the cell wall of *Saccharomyces* and non-*Saccharomyces* yeast and evaluate their effect on wine color and pigment composition.

MATERIALS & METHODS

Four commercial yeast strains (Lallemand Inc.) were used: *S. cerevisiae* (*S.cer*), *Lachancea thermotolerans* (*L.the*), *Metschnikowia pulcherrima* (*M.pul*) and *Torulaspora delbrueckii* (*T.del*). Cells were mechanically lysed and a purification process was performed to obtain a MP-rich extract by the application of temperature (2h, 70ºC) and dialysis (12-14 KDa MW cut-off). The MP-rich extracts were characterized: total protein content was determined by Lowry method and analyzed by SDS-PAGE. The MW of the MPs was determined by HRSEC-RID following the method described by Manjón, E. [2]. To evaluate the effect on wine pigment and color stability, a control wine was compared to the same wine but added, separately, with 500 mg/L of each extract. Wine color and pigment composition were determined after two different treatments: cold treatment (4ºC, darkness, 4 and 11 days) and room temperature (22ºC, darkness, 7 and 18 days), by the evaluation of CIELAB color parameters and HPLC-DAD-MS.

RESULTS & DISCUSSION

MP-rich extract from four different yeast were obtained. *M. pulcherrima* showed the highest yield, which was almost 4-times higher than for the others yeast assayed. According to the Lowry data, these MP-rich extracts showed differences in their protein content, being *S.cer* mannoprotein extract

the one showing the highest protein percentage (48,6%), whereas *M.pul* showed the lowest one (10,0%). Likewise, the protein profile determined by SDS-PAGE was also different. HRSEC-RID revealed significant differences among mannoprotein molecular weights: MP-rich extracts of *S.cer* and *T.del* contained the largest mannoproteins and the smallest ones were released from *L.the*. Furthermore, the chromatographic profile of the MPs was different, indicating that there were important structural differences in the MPs obtained from the different yeasts.

During the period of study color stability at room temperature was almost not affected by the presence of mannoproteins. The wine added with *S.cer* extract was the one which showed the most important differences with regard to control wine, but all the mannoprotein assayed lead to wines with color differences $\Delta E_{ab}<1$ when compared to control wine.

After cold treatment, wine hue was slightly lower for the wines added with mannoproteins, being the wines added with *L.the* or *T.del* the ones that showed higher differences when short or long cold treatment was applied, respectively.

The effect of the presence of these mannoproteins on wine pigment composition was also evaluated after both type of treatments. After room temperature storage, wines added with mannoproteins showed slightly lower pigment contents than control wine, although these differences do not seem to be enough to affect wine color. On the contrary, after cold treatment, wines added with *T.del* showed significant higher pigment contents than control wine, mainly for anthocyanin coumaroyl derivatives, which may explain the lower hue values obtained from those wines.

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P6.10

Comparing the lignin degrading abilities of lower and higher termites

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MAIN CONCLUSION

Overall, the results show that the lower termites, which feed mostly on fresh wood, prefer to degrade the polysaccharides and leave the lignin mostly intact. On the other hand, the higher termites more effectively degraded the major interunit linkages in lignin.

INTRODUCTION

Degrading lignin, the heterogeneous plant cell wall polyphenol derived from hydroxycinnamyl alcohols, is an arduous task due to its diverse interunit linkages produced via radical coupling during lignification. Within a day, termites can degrade lignocellulosic material efficiently, digesting >74% of cellulose, >65% of hemicelluloses, and leave a modified lignin-rich residue as their feces. Termites are conventionally grouped into lower and higher termites with the presence (lower) or absence (higher) of flagellated protistan symbionts in their hindgut as a distinguishing feature. The anaerobic nature of their gut is believed to resist degradation of lignin. However, several studies have shown that both lower and higher termites are capable of depolymerizing lignin, and in some cases prefer to degrade the most recalcitrant condensed guaiacyl units [1]. In this study, we characterize and quantify the structural chemistry of the lignin-rich feces from three lower termites (*Cryptotermes* spp., *Incisitermes* spp., *Zootermopsis* spp.) and three higher termites (*Nasutitermes* spp., *Odontotermes* spp., *Trinervitermes* spp.) using two-dimensional (2D) NMR spectroscopy in conjunction with gas chromatography.

MATERIALS & METHODS

Each insect species had a diet and a feces sample. The samples were ball-milled using a Retsch PM-400 planetary ball-mill (Newtown, PA) with a 50 mL ZrO_2 jar with ten 10 mm ZrO_2 balls and milled at 300 and 200 rpm, respectively, and dissolved directly into an NMR tube using 500 µl of DMSO-d_6 and sonicated. NMR spectra were acquired on a Bruker-Biospin (Rheinstetten, Germany) AVANCE III HD^{TM} 500 MHz spectrometer fitted with a nitrogen-cooled 5 mm Prodigy^{TM} TCI gradient cryoprobe with inverse geometry. One-bond ^{1}H–^{13}C correlation spectra were acquired using Bruker pulse program hsqcetgpsisp2.2 and processed as previously described [2]. Methoxyl analysis was performed to determine the quantity of methoxyls in the lignin of each sample using an iodometric method combined with gas chromatography. Quantitative analyses were performed using a Varian (Agilent, Santa Clara, CA) model 3800 gas chromatograph interfaced to a Varian model 4000 ion trap mass detector, as previously described [1].

RESULTS & DISCUSSION

The 2D NMR analysis was performed on nonderivatized samples to visualize the structural changes in the lignin and polysaccharides between the diet and feces samples. The three higher termites showed visually dramatic depletions in lignin sidechains (i.e., β-aryl ethers, phenylcoumarans, and resinols) and anomeric polysaccharides with *Trinvervitermes* displaying a more significant loss in β-aryl ethers compared to *Nasutitermes* and *Odontotermes*. The three lower termites showed minor depletions in lignin sidechains and polysaccharides with *Incisitermes* and *Cryptotermes* displaying the most losses.

To quantify the losses in lignin sidechain linkages, the samples were analyzed for saccharide composition, Klason lignin, and methoxyl content analysis. The 2D NMR peaks that corresponded to the lignin sidechains were integrated based on the methoxyl peak. Normalizing the methoxyl peaks with the methoxyl contents, the percent depletion in lignin sidechains was calculated. From these data, the higher termites showed that, going from diet to feces, much of the β-aryl ethers are lost along with all phenylcoumarans and most of the resinols. Along with the lignin sidechains the higher termites displayed large losses in methoxyl contents, showing that these termites have the capability of cleaving all types of ethers as well as carbon-carbon bonds in lignin. On the other hand, the lower termites showed much lower losses in β-aryl ethers, phenylcoumarans, and resinols, and losses in methoxyls were much lower than those in the higher termites. Nevertheless, the lower termites do have the capability to degrade lignin to a certain degree, which is also seen in brown-rot fungi; this approach may allow the lower termite access to the more desirable polysaccharides. The evolutionary success of these higher termites suggests that their ability to degrade the lignin barrier allows for a more efficient digestion of more varied plant materials as compared to lower termites.

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P6.11

Anti-aging effects of constituents in Wine or Wine compression residue

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MAIN CONCLUSION

The 17 compounds of, flavonoids, pyranoanthocyanins, ethyl-bridged dimers of anthocyanins, dimers or trimer of anthocyanins, and its glycosides were isolated from Merlot, Cabernet Sauvignon, Muscat Bailey A and Black Queen wine, and wine compression residue. As for the ethyl-bridged dimers of anthocyanins and its glycosides which are substances peculiar to wine, high AGEs (Advanced glycation end-products) inhibitory activity was shown.

INTRODUCTION

The people have an interest for health, and cosmetics market and the health food market accomplish sudden growth. Also, the use of the natural system material in behalf of the synthetic pathway material by the naturalism point is demanded.

The search of these new materials becomes the important research theme. Therefore we search for an ingredient derived from nature for the purpose of the anti-aging. Quantity for approximately 3,000t a year egests the refuse of the wine and is discarded, and utilization of the wine compression residue is an important problem. Glycation which is a nonenzymatic reaction between reducing sugars and amino groups in proteins progresses in the food as well as body. In the case of body, it forms the AGEs (Advanced glycation end-products) which effect the protein including body tissue. Recently it's reported that forming and accumulating the AGEs are associated with one of the key to factor of the aging.

We searched for anti-glycation substances from wine compression residues of Chardonnay, Cabernet Sauvignon, and Merlot, and found that the type of anthocyan varied with the kind of grape. In this study, we focused on anti-glycation and searched for an anti-glycation substance that can be utilized as health food and cosmetics.

MATERIALS & METHODS

Materials

We used the Merlot, Cabernet Sauvignon, Muscat Bailey A and Black Queen wine, and their wine compression residue.

Isolation procedure of wine or wine compression residue

Wine removed the ethanol, and were isolated by silica gel column chromatography and gel filtration. Wine compression residues were extracted with 70% MeOH, then obtained MeOH extract. The MeOH extract was extracted with hexane, then with 1-butanol. These extracts were isolated by silica gel column chromatography and gel filtration.

As a means to evaluate anti-glycation, we conducted the AGE (advanced glycation end products) inhibition test [1].

RESULTS & DISCUSSION

Remarkable inhibitory active substance in wine or wine compression residue was separated by gel filtration and silica gel column chromatography.

The 17 compounds of, flavonoids, pyranoanthocyanins, ethyl-bridged dimers of anthocyanins, dimers or trimer of anthocyanins, and its glycosides were isolated from Merlot, Cabernet Sauvignon, Muscat Bailey A and Black Queen wine and their wine compression residue, and their structures were established on basis of MS and NMR spectroscopy. When we consider a structure-activity relationship, proanthocyanidin has high activity, and the content is approximately 200 mg per bottle of wine. Also the pyranoanthocyanin included in the wine and wine compression residue showed remarkable activity. Especially as for the ethyl-bridged dimers of anthocyanins and its glycosides which are substances peculiar to wine, high inhibitory activity was shown in Figure 1.

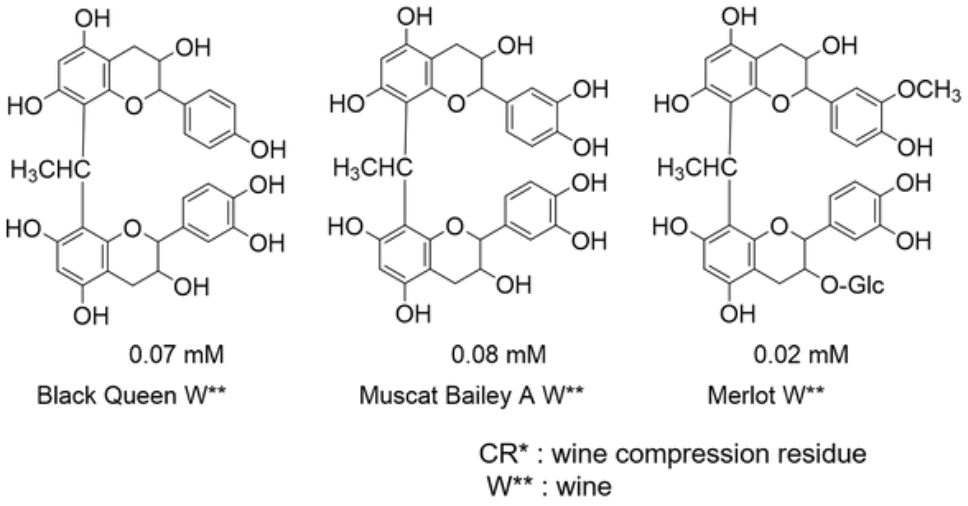

Fig.1 Structures of ethyl-bridged dimers of anthocyanins and its glycosides.

In conclusion, it might be used in general as food additives or cosmetics because remarkable AGEs inhibitory activity was found from the wine or wine compression residue.

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P6.12

Surfactant-mediated green extraction of polyphenols from red grape pomace

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MAIN CONCLUSION

The aqueous solutions of surfactants can significantly improve extraction of phenolics from grape pomace regarding to water and therefore non-ionic surfactants could be considered as promising agents for green extraction of polyphenols without usage of organic solvents.

INTRODUCTION

The winery waste is considered an environmental problem as well as low-cost and rich source of high value ingredients, including polyphenols. The waste exploatation and the maximization of polyphenols recovery from grape pomace have become subjects of great interest [1]. A surfactant-based extraction method represents novel approach to the effective solid-liquid extraction of polyphenols from the matrix, following green chemistry principles [2]. The aim of this study was to examine the extraction rate of polyphenols from red grape pomace by the application of selected non-ionic surfactants.

MATERIALS & METHODS

Cabernet Franc red grape pomace was provided by a local winery, dehydrated by lyophilization, grounded and sequentially extracted using aqueous solutions of non-ionic surfactants Brij S10, Brij S20, Brij O10, Brij O20, Tween 20, Tween 60, Tween 80, Tween 85, Triton X-100, Poloksamer 237 and Poloxamer 407. The surfactant concentrations were 0,5%, 1%, 2% and 3%. Water, ethanol and water:ethanol (1:1) were used as control solutions. All solutions were adjusted to pH 4.0 ± 0.05 and the extraction procedure was performed for 45 minutes with solvent-to-material ratio 100 mL/g at room temperature with constant stirring. The efficiency of the extraction process was determined in terms of total phenolic content, using Folin-Ciocalteu method [3]. The results were expressed as mg of gallic acid equivalents per gram of liophylized grape pomace (mg GAE/g). Triplicate measurements were taken and results are expressed as mean value ± standard deviation.

RESULTS & DISCUSSION

Among investigated control solutions, mixture of water and ethanol was the most efficient in the recovery of polyphenols (47.69 ± 2.13). Ethanol and water individually showed lower extraction efficacy, with the total phenolic content of 38.77 ± 1.49 and 25.36 ± 4.37, respectively.

Poloxamer 407 was more efficient in the recovery of polyphenols than Poloxamer 237 at all investigated concentrations. In comparison to Brij S10 and Brij O10 surfactants, Brij S20 and Brij O20 respectively led to higher yield of target components at all examined concentrations. Brij S20 and Brij O20 showed greater efficacy compared to Triton X-100 as well. Taking into account differences in chemical structures of the tested Poloxamer, Brij and Triton X-100 surfactants, the observed results could indicate that the length of oxyethylene chain as well as the size of hydrophobic part of surfactant molecule significantly affect the extraction efficiency.

The increase in the concentration of surfactants in solutions was followed by the increase in the extraction efficacy for both investigated Poloxamers. Moreover, solutions containing 3% of Poloxamer 407 and Brij S20 exhibited extraction rates comparable to the mixture of water and ethanol, with the total phenolic content of 48,95 ± 3,34 and 44.46 ± 2.96, respectively. Although

Triton X-100 and Tween surfactants did not reach the yield of pholyphenols obtained by the mixture of water and ethanol, they showed greater efficacy than water.

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P6.13

Polyphenols and urban mining. A green alternative for the recovery of valuable metals from scrap printed circuit boards

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MAIN CONCLUSION

The recovery of four metals (namely, silver, copper, copper, chromium and tin) from printed circuit board (PCB) leachates has been studied. The use of conventional reductants such as metallic iron or sodium borohydride was tested in this urban mining process, but no satisfactory results were achieved. However, by using an olive leaf extract with a high polyphenol content as a reduction adjuvant, a full recovery of the four metals initially present in the leachate was successfully accomplished.

INTRODUCTION

With the almost compulsive use of mobile phones, computers and other devices by society, as well as the global growth of industrialisation and increased competitiveness, equipments are becoming obsolete in less and less time, taking into account the decreasing lifetimes of products. These factors have led to a constant increase in the purchase of equipment and therefore the generation of electrical and electronic waste (EEW). Particularly, scrap PCBs are among the most valuable elements of EEW.

EEW constitutes a source of environmental pollution and possesses severe health problems [1]. Moreover, the sustainability of the electronics industries has also been affected, due to the scarcity of mineral resources. In fact, large amounts of precious metals such as Au, Ag, Pd and Cu are used in the manufacture of PCBs. These metals are present in scrap PCBs in much higher concentrations than those normally found in the electronics industry and, of course, in nature. Hence, they have given rise to the phenomenon known as "urban mining" [2], that mainly aims at recovering the precious metals contained in urban waste.

In this work, the recovery of four metals (namely, Ag, Cu, Cr and Sn) from PCB leachates has been studied. The major novelty is the fact that we have tried to take advantage of the reducing properties of the polyphenols contained in the olive leaf, in order to reduce the high cost and pollution caused by conventional methods.

MATERIALS & METHODS

Polyphenols were extracted from olive leaves under reflux for 30 minutes at temperature below 80ºC. The extract was allowed to cool down and finally filtered. The total polyphenols content in the olive leaf extract was determined according to the Folin-Ciocalteu method and expressed as gallic acid equivalents.

Ground PCBs were leached under reflux in HNO_3 (1:3) aqueous solution, for 2h at 60ºC. Then, the PCB leachate and the adequate volume of natural extract were placed in a reactor beaker and the solid reagents (Fe and/or $NaBH_4$) were added. Samples were magnetically stirred and then left to stand for approximately 12 hours. The metal content in the sample was determined before and after each experiment to calculate the percentage of metal recovered from each sample.

A factorial, composite, centred, orthogonal and rotatable experimental design was used, consisting of 23 experiments in a matrix with 3 operational variables (amounts of Fe, NaBH4 and olive leaf extract).

RESULTS & DISCUSSION

The experimental results show that none of the three reducing agents, if used separately, is able to achieve full recovery of any of the four metals present in the leachate. However, by using the statistical design of experiments, full recovery of all four metals is achieved by the combined use of the three reducing agents at the appropriate concentrations.

Statistical analysis of the experimental results shows that the factors that most influence the recovery of silver from the leachate are the concentration of polyphenolic extract and sodium borohydride. For the other three metals (Cu, Cr and Sn), the factors that most influence the recovery efficiency are the iron concentration and the polyphenolic extract concentration. Therefore, it can be stated that the latter represents a very promising alternative to the use of conventional reductants and, in particular, NaBH4, which is the least environmentally friendly of the three.

By using the statistical design of experiments, it is possible to optimise the recovery process of the four metals individually. Operating under the right conditions it is possible to remove 100% of all of them.

Finally, due to the problems implied by the presence of tin in the precipitate obtained (especially with regard to the formation of intermetallic compounds and/or intermediate phases when separating the metals by fusion), it is considered very convenient to continue the research trying to maximise the recovery of Ag, Cu and Cr while keeping Sn in solution, so that it does not interfere in the separation of the rest of the metals.

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P6.14

Polyphenols-mediated green synthesis of nZVI for the removal of dyes from water

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MAIN CONCLUSION

The use of nZVI prepared by environmentally benign procedures, green synthesis, is an excellent alternative for the decontamination of water in which dyes are present and provides iron nanoparticles stable over time. The results showed that, by carefully selecting the operational conditions, it was possible to eliminate over 90% of the dye initially present in the solution.

INTRODUCTION

In the last decade, many studies have concluded that bioactive components such as polyphenols, polysaccharides, proteins, vitamins, etc. extracted from plants can be used in a wide variety of fields. Among these biomolecules, polyphenols are the most active ones in the reduction of Fe (III) to Fe (0) when reacting with a Fe precursor in the synthesis of zero-valent ion nanoparticles (nZVI). They also have the advantage of being soluble in water and organic solvents.

On the other hand, the use of dyes has proliferated in various industries such as textiles, leather, food and cosmetics, and the waste from these industries is a major environmental damage [1] which pose a significant risk to aquatic ecosystems as their presence prevents access to visible light, thus handicapping the growth of aquatic flora and fauna. For this reason, it is of great interest to have procedures and technologies that avoid the harmful effects of an accidental spillage of dyes in an aqueous environment. So, the removal of different dyes to alleviate sewage pollution from industries is important area of basic and applied research.

Currently, the use of (nZVI) in the treatment and elimination of contaminants is one of its most promising applications [2-3]. On the one hand, iron is a cheap and non-toxic metal and therefore environmentally compatible, in addition the nanoparticles are of such small particle size (1-100 nm) that they can be efficiently transported by a water flow.

Much research is aimed at maximizing its adsorbent properties and applications in caring for the environment. On the other hand, the advantage that it is magnetic allows it to be easily recovered and reused.

MATERIALS & METHODS

In this work, polyphenols were obtained from tea waste by reflux extraction for 20 minutes at temperature below 80 ºC. The extract was allowed to cool down and finally filtered. The total polyphenols content in the tea waste extract was determined according to the Folin-Ciocalteu method and expressed as gallic acid equivalents [4]. Such an extract was added to an aqueous solution containing the dye and Fe (III) nitrate as a precursor of the nZVI.

Nanoparticles were generated in situ and after the addition of amounts of hydrogen peroxide, led to the removal of the dye (namely, methylene blue, methyl orange or orange G) by a hetero-catalytic Fenton process. The influence of variables, namely, the concentrations of polyphenols, Fe (III), and H_2O_2, on the dye removal efficiency was analysed using a statistical design of experiments consisting of 23 experiments in a matrix with 3 operational variables (amounts of Fe (III), of polyphenols and of H_2O_2) and response surface.

RESULTS & DISCUSSION

The use of polyphenols as a reducing agent allowed the synthesis of nZVI to be carried out satisfactorily. Through the use of electron microscopy (SEM / TEM), it was possible to verify that particles of fundamentally spherical morphology and nanometric size are obtained. On the other hand, the adsorption isotherms of N_2 at 77 K reveal that a high development of the surface and the porosity of the nanoparticles ($S_{BET} \approx 100 m^2 g^{-1}$). The X-ray diffraction (XRD) technique reveals the presence of crystalline forms of metallic iron, as well as various iron oxides and oxohydroxides. The latter is corroborated in view of the profiles of different thermal analyzes (DTP, TPR and TPO).

The statistical analysis of the experimental results shows that the three operative variables exert a synergistic effect on the elimination of dyes, being the concentrations of iron and hydrogen peroxide the two variables that exert a more marked effect on the elimination efficiency. However, the concentration of polyphenols exerts adjuvant effect on the process, significantly reducing the amount of both reagents necessary to achieve maximum elimination of dyes.

Using the FTIR technique, it has been possible to verify that this elimination occurs through an advanced heterofenton-like oxidation process, since the presence of adsorbed dye molecules on the surface of the iron nanoparticles cannot be detected.

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P6.15

Impact of green tea extraction in ternary deep eutectic solvent on chitosan-based films properties for food applications

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MAIN CONCLUSION

Ternary NADESs exhibit great potential to extracts polyphenolics compounds. CH biofilms (with and without ternary NADESs), produced by casting method, present compact, homogenous and transparent structure. Ternary NADES (ChCl/lactic acid/glycerol) exhibit great potential to be used as a plasticizer for polysaccharides biofilms. Through varying the composition/structure of these potentially biodegradable films, the morphology as well the water resistance proprieties of the films can be altered.

INTRODUCTION

The current demand for antioxidant active packaging, that is achieved by introducing antioxidants into food packaging material, is increasing due to both the unquestionable advantages compared to the addition of antioxidants directly to the food and the tendency to consume healthy and fresh products. When developing active packaging materials, research has been focused on natural and biodegradable polymers (such as polysaccharides like chitosan) containing polyphenol compounds (as bio preservative) in their formulation.

Due to their rare solvation properties, the natural deep eutectic solvents (NADES), are considered good solvents to be used as an extraction medium of bioactive products. Moreover, the search for the use of ecological solvents for this field has intensified due to their acceptable toxicity profile and chemical diversity. The use of these inexpensive, non-volatile, and nonaqueous biodegradable solvents, complying with the Green Chemistry principles, could potentially improve the stability of the polyphenolic compounds allowing to retard food spoilage for packaging material,

Catechins (components of green tea (GT)), a polyphenolic group known for its high value-added antioxidant properties, have been associated with health-promoting effects. In this sense, the incorporation of GT components for food application purposes and as active ingredient in packaging materials can be expected to improve food functionality and availability, while performing a dual role (antioxidant and antimicrobial agent) and possibly also having low-cost advantages.

MATERIALS & METHODS

Materials: The commercial Green Tea (GT) sample was kindly supplied by Gorreana.The Shrimp chitosan (Chit) commercial sample, with *M*w 150-250 kDa, and 78% degree of deacetylation, was delivered by Primex ((Siglufjordur, Iceland, ChitoClear®), batch TM5213). Acetic acid glacial (AA) from Merck. Choline chloride (ChCl) (Sigma-aldrich, *M*w 139.62g×mol-1, 98%), lactic acid (LA) (Sigma-aldrich *M*w 90.08g×mol-1, 90%), glycerol (Gly) (Hi-AR™ Himedia, *M*w 92.094g×mol-1, purity≥ 99.00 %). Distilled water was used in all experiments.

Methods: High Performance Liquid Chromatography (HPLC-DAD) and Mass Spectroscopy (HPLC-MS) were used for the identification of extraction products. Tensile strenght (TS) and elongation ate break (EB) tests were performed for mechanicals proprieties studies. Water Vapor Permeability (WVP) and Swelling Index (SI) were performed for understanding the films barrier proprieties. Scanning Electron Microscopy (SEM) was used to see the superficial of the films.

RESULTS & DISCUSSION

HPLC-DAD/HPLC-MS

SEM: Compact and homogenous microstructures were observed for Chit film without plasticizers. Outer surface of all films show no cracks or pores. Surface morphologies depending on NADESs type used.The presence of GT polyphenols leads to different surface structures so the surface becomes rough with the increase of GT content.

WVP/Thickness: Films prepared with Chit show higher thickness values than plasticized films. Films with NADES showed different WVP from the unplasticized film. Within films containing NADES/GT extraction (Table 2), the lowest value was displayed by Chit-NADES3-GT.

SI: Similar increase of water retention capacity evolution was observed. Despite the process being very fast for all the samples, it depends on the type of NADESs used.

MP: The mechanical properties (TS, E%) values of all films were in ranges of 27.42 to 52.01 MPa and 4.50 to 19.21 E%. The presence of 10% of NADES increases the TS values when compared with Chit film without additive. However, the increase of plasticizer concentration (from 10 to 30%) exerts a strong influence over TS values in all the formulation leading to a decrease in resistance. The results have shown that Chit films prepared with NADES-3 10% are the most resistant, however, the presence of GT in the NADES plasticizer decrease 39.53%.

Table 1. Identification of polyphenolics compounds in GT extraction.

	Retention Time (min)	Polyphenolic compound	Concentration (in Epicatechin equivalent) $x10^{-5}$
NADES3 -GT	12.58	EGC/GC	1.58±0.31
	20.27	C/EC	86.10±0.23
	21.10	EGCG	233.40±0.60
	30.35	ECG	117.00±2.00

Legend:(E)C-(Epi)Catchein, (E)GC-(Epi)Gallocatechin and EGCG-Epigallocatechin Gallate.

Table 2. WVP and thickness, **d**, values of investigated films with ternary NADES.

Films	*d* (mm)	WVP $(g^{-1}\cdot s^{-1}Pa^{-1})x10^{-10}$
Chit	0.085±0.006	3.12±0.03
Chit -NADES3-10%	0.067±0.002	2.79±0.06
Chit -NADES3-20%	0.073±0.004	3.30±0.05
Chit -NADES3-30%	0.084±0.001	3.67±0.18
Chit-NADES3-GT-10%	0.068±0.004	3.22±0.06
Chit-NADES3-GT-20%	0.077±0.001	3.30±0.08
Chit-NADES3-GT-30%	0.077±0.004	4.36±0.34

Acknowledgements

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PL history

The “Groupe Polyphénols" (International) today: beyond the hopes of its founders, 50 years ago!

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MAIN CONCLUSION

Founders of the GP would be proud of the evolution of their initiative 50 years ago. A better sharing of everyone's knowledge, conveyed by the newsletter. The last presidents of the GP, like their predecessors, brought together the "best" of each member, to make a learned society with such an intellectual heritage that we can be proud of it! Just a few key words to fuel the thinking of tomorrow's researchers: polyphenols and 1)hormesis 2)PAINS properties 3)NaDES (Natural Deep Eutectic Solvents)

INTRODUCTION

A handful of scientists, meeting for the first time (June 22, 1970) at the INRA in Narbonne, then on April 20, 1971, at the INRA in Avignon-Montfavet, under the leadership of Dr Michel Bourzeix, had the objective to "bring together" all those who were interested in polyphenols, to share their fundamental and practical discoveries in all fields (whether to get rid of them or to enrich them). The "constitutive" General Assembly of the Polyphenols Group (International) of Narbonne (May 1972) decided that the « Journées Internationales d’Étude sur les polyphénols » would bring together its members. In fact, all had the same need to know better this growing family of specific metabolites: biogenesis, extraction, structural analysis, physicochemical and pharmacological properties. The Group allowed this pooling through its “publications” which make it so rich!

The acts of the JIEP ("Bulletin de liaison") are published annually until 1980 (Vol. 1 to 10), then every 2 years, from 1982 (Vol. 11) to 1992 (Vol. 16). From 1994 (congress of Palma de Mallorca) to 2004 (Helsinki), the plenary lectures are published as "Polyphenols XX", and the notes and posters, as "Polyphenols Communications". It is still the case, today, for the latter, but from 2006 (Winnipeg, Canada), the congress is named "International Conference on Polyphenols" and the plenary conferences form one volume of a collection. edited by Wiley Blackwell: Recent Advances in Polyphenol Research (RAPR 1 to 7, for the ICP of Madison, Wis, USA, in 2018).

The quality and accuracy of its publications was the main concern of the GP founders: they had to be theoretical and practical link between all members, whether researchers, teachers or industrialists. From this point of view, the "family" spirit, instilled initially, allowed a very respectful and friendly frankness between them, which did not stop developing within the GP: what a pleasure to meet the "popes" of polyphenols, like their “acolytes”, at each congress!

MATERIALS & METHODS

A careful rereading of the lectures, notes or posters of the first of the bulletin, allows, even today, to rediscover this richness of the GP. The wealth of information available is colossal. They go well beyond the only themes concerned by polyphenols! For example, the contribution of the Nobel Prize, RL Synge[1], helps to understand the chemical properties of polyphenols at the origin of the interactions of tannins with proteins, and why they pose so many problems to the producers of beer, wine, tea …, but also discover the biosynthesis of proanthocyanidins during fruit ripening! Contributions from Prof. Edwin Haslam[2] who wondered until the end "where do the tannins go" will complete the information. If we could develop a “database” gathering all the contributions over 50 years (“big data”?), No doubt, then would we have a much more precise vision of the global properties of poyphenols. None of them are insignificant. This tool is a real treasure as there is only at the GP!

RESULTS & DISCUSSION

We will show this richness, taking the example of the contribution of Nimz, H.H., *^{13}C NMR spectra of lignins*. 1978, Bulletin de liaison, ed. GP. Vol. 8, Nancy, p.197. This is a true lesson in structural analysis! Due to the fact that ^{13}C has a narrower line width than ^{1}H (much greater relaxation time), and that their chemical shifts are much more sensitive to the environment (constitute the backbone of molecules), and to the substituents they bear, they give characteristic "figures" of the 5 main linkages encountered in lignins (ß-arylethers, phenylcoumarans, pinoresinols, ß-1-units and benzyl-ethers, see fig.), which are therefore deduced from the spectra obtained. Even if, since that date, the structure of Freudenberg's lignin (Science, 1965, 148, 595) has significantly evolved (presence of stilbenic precursors, ... in addition to coniferyl alcohol), this technique is still very current.

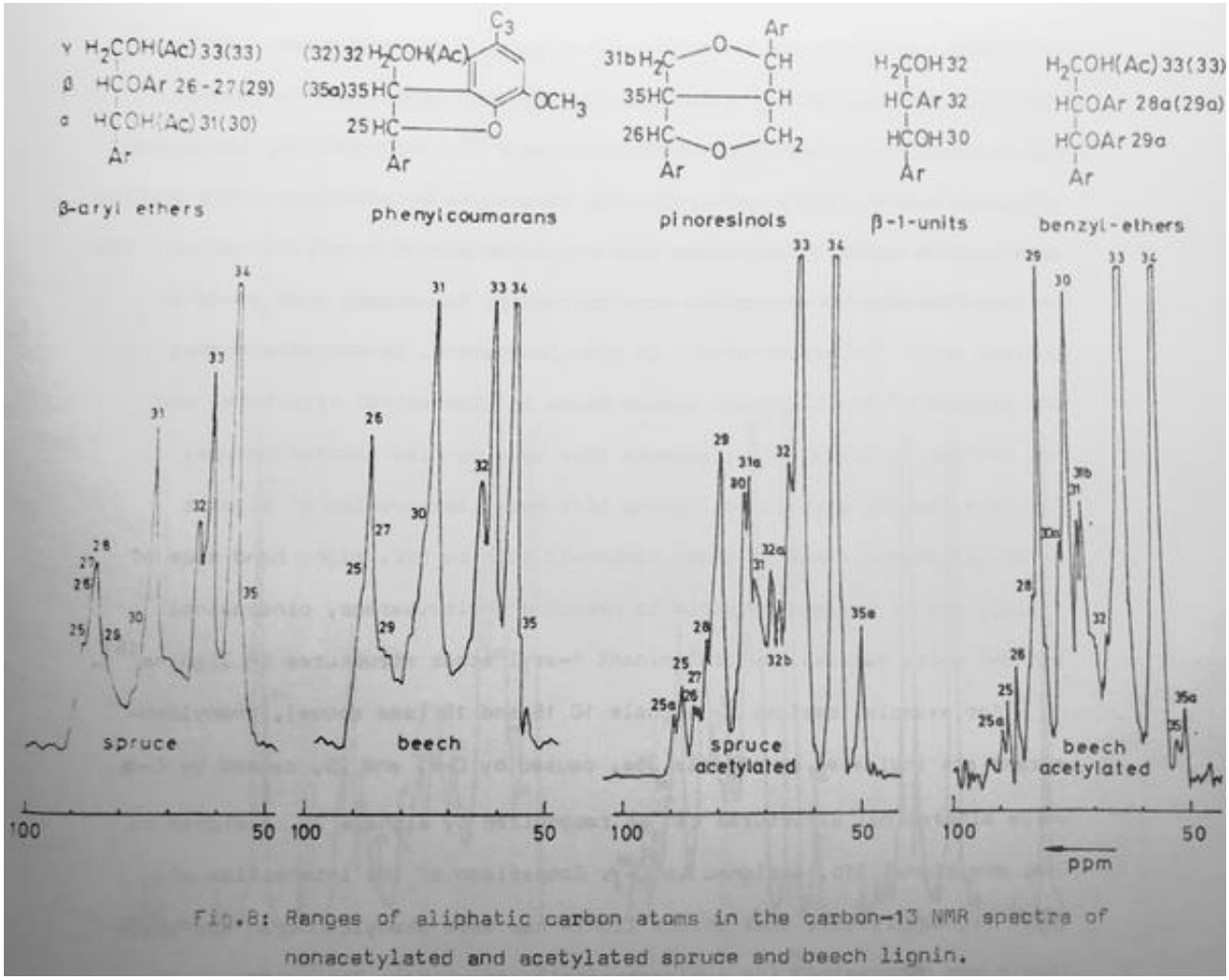

Fig.8: Ranges of aliphatic carbon atoms in the carbon-13 NMR spectra of nonacetylated and acetylated spruce and beech lignin.

Other results (Enzymatic glucosylation of pentahydroxyflavene, M. Girardin, Bulletin de liaison, ed. GP. Vol. 8, Nançy, p. 126; Enzymologie de biosynthèse de la lignin, G. Gross 1980, Bulletin de liaison, ed. GP. Vol. 10, Neufchatel, p. 105), will be commented on, in the light of the recent discovery (Natural Deep Eutectic Solvents = NaDES) by R. Verpoorte (Plant Physiol. 2011, p. 1701) which may impact strongly, in the future, the questions posed by the metabolism and solubility of glucosylated polyphenols, or not. Resveratrol and Hormesis, if there is time ...

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PL1.1

The Lignans – A Family of Biologically Active Polyphenolic Secondary Metabolites

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MAIN CONCLUSION

We show two, complementary approaches to mimic noncanonical oxidations using both photocatalysis and copper catalysis. The details of these discoveries will be described in the course of this presentation.

INTRODUCTION

Molecular oxygen (O_2) is an ideal reagent for chemical synthesis: it is innocuous, naturally occurring and energetic, and can provide a strong driving force for otherwise challenging transformations. The production of H_2O as the principal by-product of its reduction provides additional motivation for its use in green or sustainable chemistry.

Despite these attractive features, O_2 remains underutilized in fine and pharmaceutical synthesis due to issues of selectivity. O_2 is prone to radical reactions that can be difficult to control, and in complex settings, where there are often multiple sites of potential oxidation, issues of regio- and stereoselectivity often preclude its use.

MATERIALS & METHODS

To address these challenges, the Lumb Group draws inspiration from metalloenzymes, which activate and utilize O_2 with unparalleled levels of control. Several of our research programs attempt to mimic the active sites of these enzymes with simple catalyst components, with the aim of bringing O_2 into the coordination sphere of transition metals. We have also explored alternative reagent combinations to mimic biosynthetic oxidative cyclization reactions. Oxidative cyclization is often a defining feature of a natural product's biosynthesis, and it often installs the key structural features that afford biological activity. While it would it would often be most convenient to simply mimic these steps in the lab, they are often very difficult to recreate in the absence of the enzymes, which enforce conformational biases through secondary substrate/enzyme interactions.

RESULTS & DISCUSSION

In this talk, I will draw upon two recent examples from each of these complementary programs. The first portion of my talk will discuss a total synthesis of the tawainkadsurins and related lignan natural products that are derived from medicinal plants of Kadsura (Fig. 1).[1] These natural products are classified as dibenzocyclooctadienes (DBCODs), and they comprise a small family of medium-sized, 8-membered ring containing metabolites that have a variety of promising biological activities. Their intricate structures, which include a defining axially chiral biaryl ring, present a number of challenges for chemical synthesis, including ring systems that are installed in the late stages of biosynthesis by oxidative cyclization reactions. It appears that these transformations involve long-lived radicals that would result biosynthetically from an Fe=O mediated C-H abstraction. Because the lifetimes of most radicals are short relative to the rates of cyclization, these biosynthetic transformations raise many mechanistic questions about how these enzymes function.

Time permitting, I will also describe a recent synthesis of the benzyl tetrahydroisoquinoline alkaloid cularine (BnTHIQ).[2] In this case, I will focus on a different approach for mimicking the key oxidative cyclization that forms cularine's defining 7-membered dihydrobenzoxepine ring. Here, we discovered a surprising autoxidation of catechols that transiently affords an electrophilic *ortho*-quinone in a net redox neutral radical chain reaction. It is uncommon to see well-behaved autoxidation

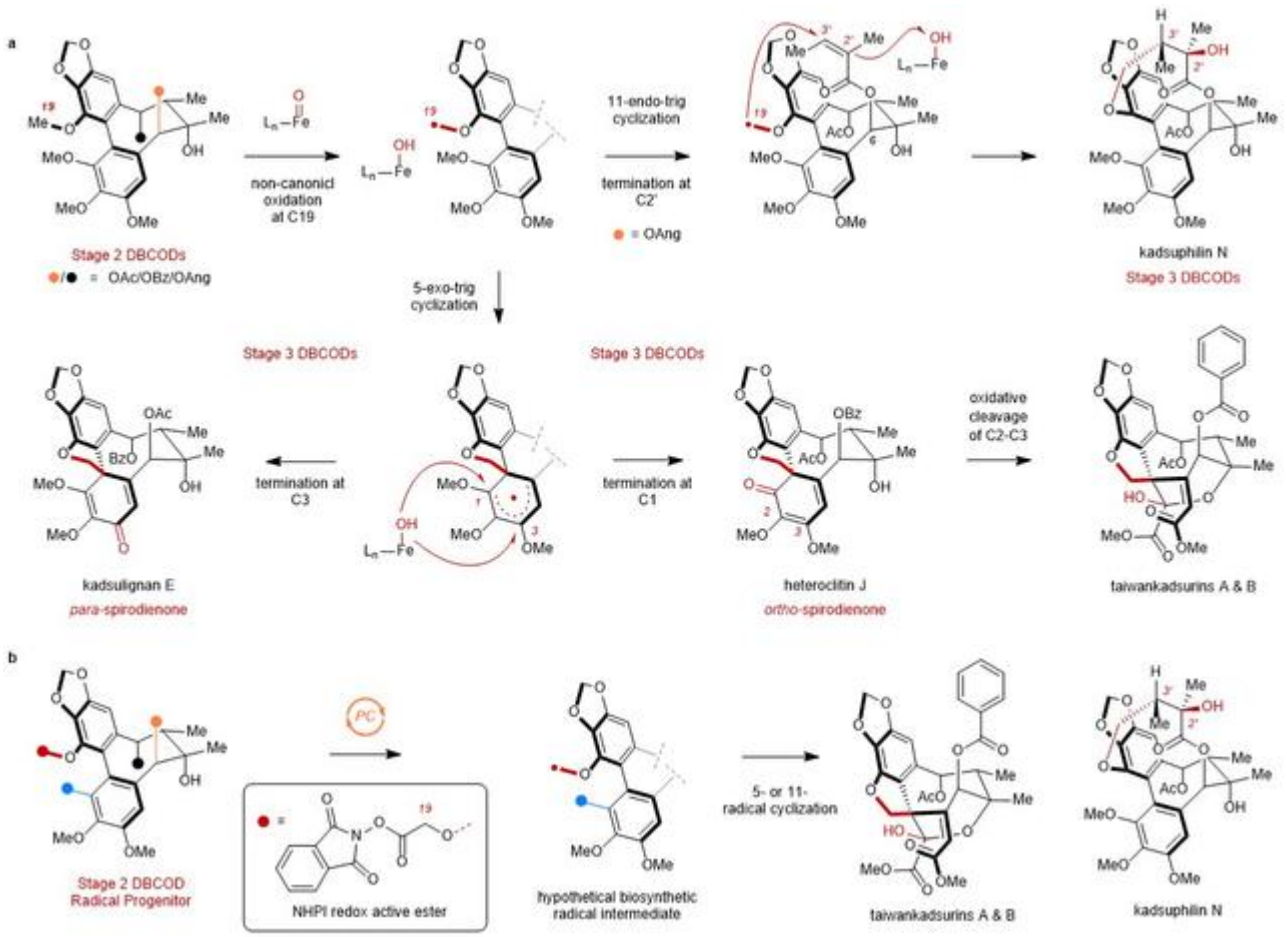

Figure 1. Summary of DBCOD biosynthesis and our approach for biomimicry.

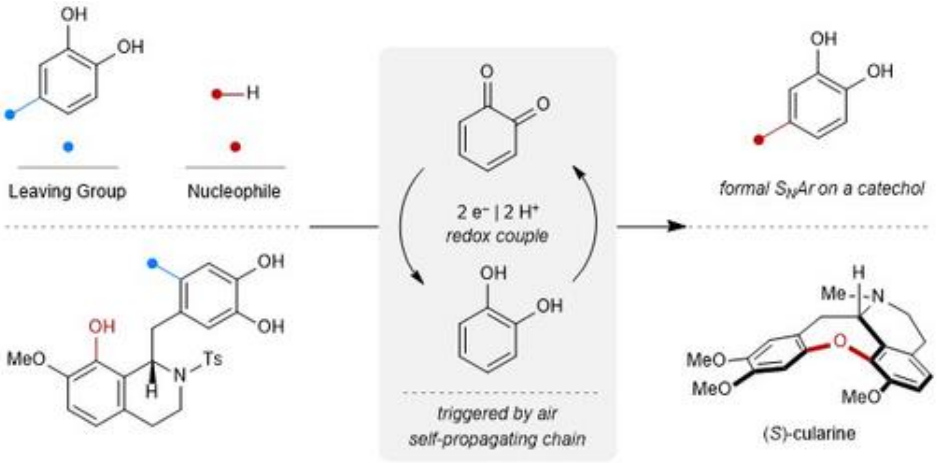

Figure 2. Application of the catechol-quinone redox couple to the synthesis of cularine.

of catechols, particularly in complex molecule settings, and the success of this reaction raises interesting possibilities for future reaction design. Our program in the BnTHIQs has hinged on our group's mimicry of the enzyme tyrosinase, and I will briefly discuss the evolution of this program, from simple phenolic oxidation to *ortho*-quinones, to the more advanced examples in total synthesis.

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PL1.2

Total synthesis of hybrid type polyphenols and confirmation of its absolute configuration

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MAIN CONCLUSION

Total synthesis of hybrid polyphenols, princepin (**1**), isoprincepin (**2**) and sophoraflavanone H (**3**) and the development for the confirmation methodology for the absolute configuration were accomplished.

INTRODUCTION

During the course of our synthetic investigation of polyphenol such as catechins and flavones, recently, we turned the our attention to total synthesis synthetic of hybrid type polyphenols by the applying our synthetic methodologies for the construction of monomer polyphenols. Initially, we expected that the simple combination of the synthetic method for the monomer polyphenols could readily produce these hybrid polyphenols. However, synthesis of hybrid polyphenols was impossible by a simple combination of monomer synthetic methodologies. Therefore, there are very few reports of the synthesis of hybrid polyphenols, in spite of the many reports of the synthesis of monomeric polyphenols. Additionally, the complete confirmation of the absolute configuration of hybrid polyphenols has not yet been accomplished, because each of the asymmetric centers was located in a remote position. Since observe the NOE correlation was also difficult the determination of the relative configuration had not yet been accomplished. Based on these background, we started total synthesis of hybrid polyphenols and. In this lecture, I will introduce total synthesis of princepin (**1**), isoprincepin (**2**) and sophoraflavanone H (**3**) and the development for the confirmation methodology for the absolute configuration.

MATERIALS & METHODS

Princepin (**1**) and isoprincepin (**2**) are hybrid sesquineolignans composed of asymmetric furofuran and 1,4-benzodioxane lignans, and were isolated from seeds of *Joannesia princeps*. Sophoraflavanone H (**3**), which was isolated from the root of the plant *Sophora moorcroftiana*, is a hybrid polyphenol composed from prenyl flavanone and 2,3-dihydrobenzofuran lignin moieties.

RESULTS & DISCUSSION

Utilizing our quinomethide-mediated construction for the furofuran and 1,4-benzodioxane rings, we have accomplished the diastero- and regiodivergent total synthesis of princepin (**1**) and isoprincepin (**2**) in the comprehensive manner. Employment with 4 diastereomers, we also established a CD and/or chiral HPLC analysis could be distinguished the each diastereomer for the confirmation of the configuration of hybrid type polyophenol **1** and **2**. Furthermore, confirmation of the absolute configuration of hybrid type polyophenol **3** was accomplished by comparison of the calculated and observed CD value.

We have achieved a stereo-controlled total synthesis of sophoraflavanone H in 14 steps from commercially available benzoic acid derivative. Our synthesis of dihydrobenzofuran skeleton was features an asymmetric Rh-mediated C-H insertion reaction. After regioselective incorporation of prenyl group by reverse prenylation and Claisen rearrangement, construct the flavanone ring was accomplished by employment the oxy-Michael reaction. In this cyclization reaction interesting selectivity was observed by the interaction between prenyl and *tert*-butyl ether.

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PL2.1

The potential of low molecular weight (poly)phenol metabolites for attenuating neuroinflammation and treatment of neurodegenerative diseases

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MAIN CONCLUSION

On overview of the current knowledge will be presented on low molecular weight (poly)phenol metabolites in circulation, their ability to cross the blood-brain barrier (BBB) and what is known related their potential role in attenuating neuroinflammation, one central hallmark common in neurodegenerative diseases. Current work concerning the possible mechanisms behind their role on modulating neuroinflammation will be presented.

INTRODUCTION

Age-associated pathophysiological changes such as neurodegenerative diseases comprise multifactorial diseases with increasing incidence and no existing cure. Furthermore, prevention and treatment of neurodegeneration, will require novel multi-targeted therapeutic strategies, targeting different disease key points. The possibility of altering the progression and development of these multifactorial diseases through diet is an emerging and attractive approach with accumulating supporting data. Studies with (poly)phenols arising from our diet (dietary (poly)phenols) have been shown their multipotent and pleiotropic ability to modulate several cellular and molecular pathways and in that sense, can emerge as an alternative, with potential to be further explored. However, the precise contribution of dietary (poly)phenols and circulating (poly)phenol metabolites to human health is still in the beginning of being elucidated. Absorption and blood concentrations of some (poly)phenols is quite low, which can hamper the research in terms of understanding their effects in specific biomarkers of disease.

The difficulty in demonstrating (poly)phenols true effects can also be justified by the uncertain and complex metabolic fate of dietary (poly)phenols. In fact, it is necessary to identify the bioavailable metabolites resulting from (poly)phenol ingestion through the diet. Low molecular weight (poly)phenol metabolites are produced from colonic metabolism. In the colon, bacterial enzymes from local microbiota promote the ring fission of flavonoids into low-molecular weight metabolites, those are in general accepted to comprise a considerable percentage of the total diversity of (poly)phenol metabolites. Due to their smaller size, they have the potential to overcome cellular barriers and reach target tissues, such as the brain.

RESULTS & DISCUSSION

Research on (poly)phenols in recent years, focusing on flavonoids, have been tackling the missing links for this potential future for (poly)phenol use in the prevention and treatment of neurodegenerative diseases. A large amount of evidence has come from *in vitro* research using single flavonoids, typically aglycones, at supra-physiological concentrations which have given valuable clues for further studies with relevant circulating metabolites. In fact, increasing evidence suggests that metabolism of (poly)phenols may actually increase their biological activity. It will be listed the most abundant circulating metabolites of dietary (poly)phenols, the low molecular weight (poly)phenol metabolites resulting from phase I and phase II metabolism and microbiota

transformations [1]. Their disposition within human organs and pathways that are affected after dietary ingestion by those compounds will be also presented.

The current knowledge of the low molecular weight (poly)phenol metabolites brain permeability and their role on neuroinflammation, a central hallmark in neurodegenerative diseases, are two topics on its infancy, still with research gaps hampering a comprehensive view of their impact to mitigate brain inflammation. The *in silico* data for passive permeation across the BBB, *in vitro* and *in vivo* evidences of transport of these metabolites in cellular models of the BBB based in brain endothelial cells will be discussed [2].

The anti-inflammatory effects of physiologically attainable (poly)phenol metabolites in healthy subjects is starting to be unveiled. The sustained release of pro-inflammatory mediators that characterize neuroinflammation is harmful to microglia cells, the brain immune cells. Therefore, having brain permeable compounds that can promote a shift in cells from inflammatory and neurotoxic phenotype to an anti-inflammatory and neuroprotective phenotype are crucial to efficiently repair the damage and restore the homeostasis, down-regulating inflammation. A comprehensive analysis of the currently described low-molecular weight (poly)phenol metabolites for their ability to attenuate the inflammatory processes in activated microglia was performed. The data gathered has identified the main active metabolites as well as highlighted their possible molecular targets. These low-molecular weight (poly)phenol metabolites produced by gut microbiota are well absorbed in the intestine and persist in the plasma for a substantial time meanwhile reaching the brain. Therefore, they could play a relevant role and alter our perspective of an effective prevention and co-treatment for NDs.

Acknowledgment

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PL2.2

Relevance of dietary flavonoids on the mitigation of metabolic disorders

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MAIN CONCLUSION

Extensive evidence supports the beneficial health benefits of select dietary flavonoids in the context of the pathologies associated to the consumption of high fat/high carbohydrate diets and associated obesity. The actions of flavonoids at the gastrointestinal (GI) tract appear to be highly relevant for their systemic beneficial effects.

INTRODUCTION

Select flavonoids have been shown to exert beneficial actions against obesity- and Western style diet-induced disruptions of normal metabolism and disease, including dyslipidemia, type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease. Several of these protective effects can be initiated at the gastrointestinal (GI) tract, were dietary flavonoids reach high concentrations. The GI tract plays a central role in the absorption, distribution, metabolism, and excretion of flavonoids. Additionally, flavonoids and/or their metabolites can modulate multiple events at the GI tract that can have both local and systemic impact (Figure 1) [1]. We have investigated different aspects of the potential beneficial actions of select flavonoids on the cross-talk between the GI tract and different organs, i.e. liver, adipose tissue and brain, particularly in the context of the consumption of diets high in fat and/or carbohydrate content that can promote dysmetabolism and/or obesity [1].

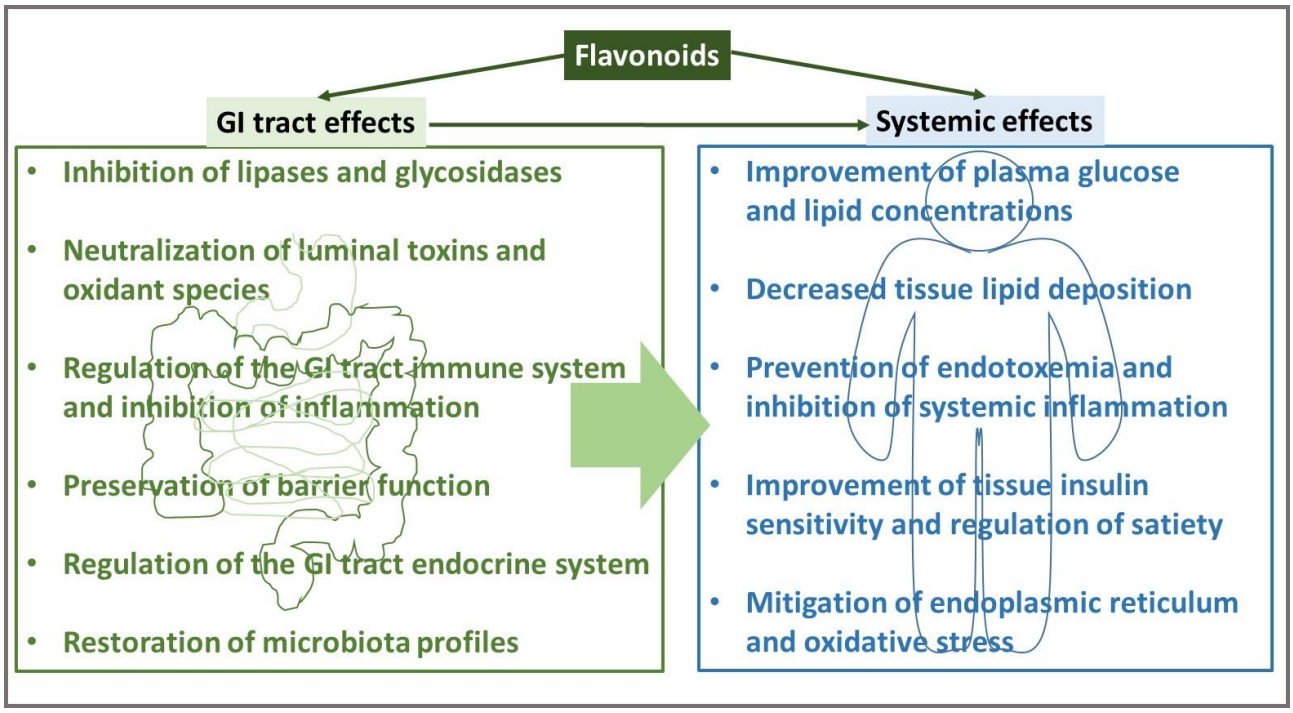

Figure 1. The GI tract-systemic crosstalk in the beneficial health effects of flavonoids.

RESULTS & DISCUSSION

Overnutrition and the associated obesity can cause intestinal permeabilization, inflammation and dysbiosis, which can negatively impact GI functions and promote alterations in gut microbiota profiles (dysbiosis). This can contribute to the development of insulin resistance and T2D, steatosis, NAFLD, different types of cancers, altered behavior and several other co-morbidities. We observed that the flavan-3-ol (-)-epicatechin (EC) mitigates in mice fed a high fat or a high fructose diet, the associated dysmetabolism, insulin resistance [2, 3], liver and adipose tissue lipid accumulation and endoplasmic reticulum/oxidative stress [2, 3], and neuroinflammation. At the GI tract, EC and/or its polymers (procyanidins, PCA) were able to: i) maintain the intestinal barrier integrity and prevent endotoxemia [4]; iii) regulate the synthesis/secretion of gut hormones (GLP-1 and GLP-2) that have GI trophic actions and modulate glucose/lipid metabolism [5], iv) inhibit inflammation and oxidative stress; and v) exert anti-colorectal cancer activity. Several of these effects can be in part due to the capacity of EC to inhibit NADPH oxidase, oxidant production and redox-regulated signaling cascades (NF-κB, JNK1/2, ERK1/2). Several of the effects/mechanisms described for EC were also observed for the structurally related anthocyanidins (AC) cyanidin and delphinidin. Supplementation with AC also reverted high fat diet-induced dysbiosis.

In vitro and *in vivo* (animal models and humans) studies support the potential capacity of EC, PCA and anthocyanins to mitigate obesity- and high fat/high carbohydrate diets-associated comorbidities. However, more preclinical and clinical studies are needed to assess the effects of flavonoids at the GI tract given the relevance of a normal gut function in sustaining overall health.

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PL3.1

Applications of MS-based metabolomics to investigate the biomarkers of the co-metabolic processing of apple polyphenols

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MAIN CONCLUSION

This work describes the fate of apple polyphenols in human biofluids and takes a small initial step in linking systems level metabolic processing of dietary polyphenols with microbiome architecture, a necessary move away from limited taxonomic descriptions or measurement of metabolic potential and towards improved understanding of microbiome metabolic function and nutrikinetics.

INTRODUCTION

Two nutritional clinical trials were designed to study apples metabolism in human: acute postprandial kinetics (AGER) and long-term intake (AVAG). In the AGER - cross-over blind trial, healthy volunteers were given 250 ml of cloudy apple juice or the same apple juice enriched with an apple extract powder containing apple polyphenols. In the AVAG - randomized, controlled, crossover intervention, forty mildly hypercholesterolemic volunteers consumed two whole apples or a sugar and energy-matched control beverage, daily for eight weeks. In both studies, the metabolome in plasma and urine samples was analyzed via untargeted metabolomics.

The aim of the acute AGER study was to identify the metabolic products of apple polyphenols and to follow their dose dependent appearance in plasma and excretion in urine after different intakes. A secondary aim was to explore the relations between microbiota and metabolites resulting statistically significant after enriched juice intake. The AVAG study on the other hand, allowed us to investigate the biomarkers of long-term apple intake in free-living population and explore how apples impact on the human plasma and urine metabolite profiles.

In brief, from the combination of these studies we have identified several specific blood and urine metabolic biomarkers of apple polyphenol intake and found evidence of phloretin glucuronide and phloretin glucuronide sulfate as promising biomarkers of apple intake considering the evaluation of several criteria such as specificity, plausibility, time–response and dose–response.

We suggest that properly designed metabolomic experiment can be used within multiomic dataset, to explore the correlations between metabolites modulated by the dietary intervention and faecal microbiota species at genus level. Supporting the discovery of putative associations of polyphenols metabolites with specific genera of faecal bacteria, which need validation in specifically designed mechanistic studies.

MATERIALS & METHODS

The AGER study was a cross-over, blind human trial, where 12 healthy volunteers consumed 250 mL of cloudy apple juice (CAJ), Crispy Pink apple variety, or 250 mL of the same juice enriched with 750 mg of an apple polyphenol extract (PAJ). Additional information in ref. 1.

The AVAG trial was a randomized, controlled, crossover, dietary intervention in 40, free-living, mildly hypercholesterolemic, otherwise healthy volunteers, were randomly allocated to one of two groups to incorporate daily, into their normal diet, either two whole apples (Renetta Canada) or a sugar and energy matched control beverage. Additional information in ref. 2.

Metabolomic data-sharing

Untargeted data in mzXML format and metadata are available from the MetaboLights repository (https://www.ebi.ac.uk/metabolights), for the AGER study with the persistent unique public identifier MTBLS473. The AVAG data are deposited in MetaboLights with the persistent unique public identifier MTBLS469,

RESULTS & DISCUSSION

The AGER study allowed to reliable identify the metabolic transformation products of apple polyphenols using an untargeted metabolomic approach. Additionally it allows following their nutrikinetics in plasma and urine after different intakes considering the dose-response relation. The observation of coherent trends for both plasma concentration and urine excretion profiles for the same metabolic pathways provide experimental evidence of the presence of two main nutrikinetics patterns. A fraction of the native polyphenols is quickly absorbed and metabolized in the upper gut with little or no contribution from the human colonic microbiota and is rapidly excreted in urine. This group of catabolites is characterized by an early absorption peak in plasma, which is followed by a rapid decrease (within five hours) in their concentration and a fast appearance in urine. A second group of catabolites do not reach their maximum concentrations in plasma within the five hours after juice ingestion and they are characterized by a delayed appearance in urine, suggesting a prolonged metabolism along the gut with a likely involvement of the gut microbiota.

In total, as many as 110 metabolites were significantly elevated following intake of PAJ, with large inter-individual variations. The combination of the data from these studies allowed to compare the metabolites of apple polyphenols in human biofluids after acute and long-term intake. Moreover, we observed that long and sustained consumption of apples triggers the gut microbiota to activate tyrosine, tryptophan and the indole metabolic pathway.

We have confirmed the strong involvement of the intestinal microbiota in the systems-level metabolism of complex plant polyphenols ingested with food and speculated that the late appearance of several small phenolic catabolites of apple polyphenols in blood and urine can be significantly correlated to the relative abundance of different, phylogenetically distantly related bacterial genera.

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PL3.2

Deciphering complex natural mixtures through metabolome mining of mass spectrometry data: the plant specialized metabolome as a case study

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MAIN CONCLUSION

This study shows how plant chemical diversity gets increasingly captured by linking untargeted mass spectrometry-based metabolomics acquisition methods to metabolome mining workflows. By showcasing examples from three different plant chemistry studies, it is shown how metabolomics data is transferred into information-rich molecular networks that enable us to answer key biological questions linking plant taxonomy and herbivore resistance phenotypes to plant specialized metabolome constituents.

INTRODUCTION

Plant biochemistry and the specialised metabolite arsenal play pivotal roles in growth and health, for example in defence against herbivores. By illuminating the plant chemical diversity, mass spectrometry-based metabolomics workflows are revolutionizing the natural products discovery field [1]. Technological advances in equipment result in increasingly information-dense data files from which relevant chemical information needs to be extracted. Through comparing many spectra to each other, metabolome mining strategies like molecular networking [2] and substructure pattern discovery [3] are now widely used to form molecular networks consisting of metabolite families. Furthermore, it is now also possible to combine this metabolite family grouping with *in silico* annotation tools to add structural information to the molecular network [4]. Here, we will highlight results of three case studies that investigate the role of plant chemical diversity in ecology, taxonomy, and herbivore resistance using computational metabolomics approaches.

MATERIALS & METHODS

High-resolution tandem mass spectrometry (LC-MS/MS) data sets were acquired from extracts of the Rhamnaceae family [5] and *Euphorbia* genus [6], as well as four accessions of the *Capsicum annuum* L. The MolNetEnhancer workflow [4] was applied in these three case studies to extract and link chemical knowledge to plant taxonomy and herbivore resistance phenotypes. The formed molecular families were annotated with chemical class information providing a high-level chemical overview, whereas substructure information was mapped onto the molecular networks to detect metabolite subfamilies and as aid in structural annotation.

RESULTS & DISCUSSION

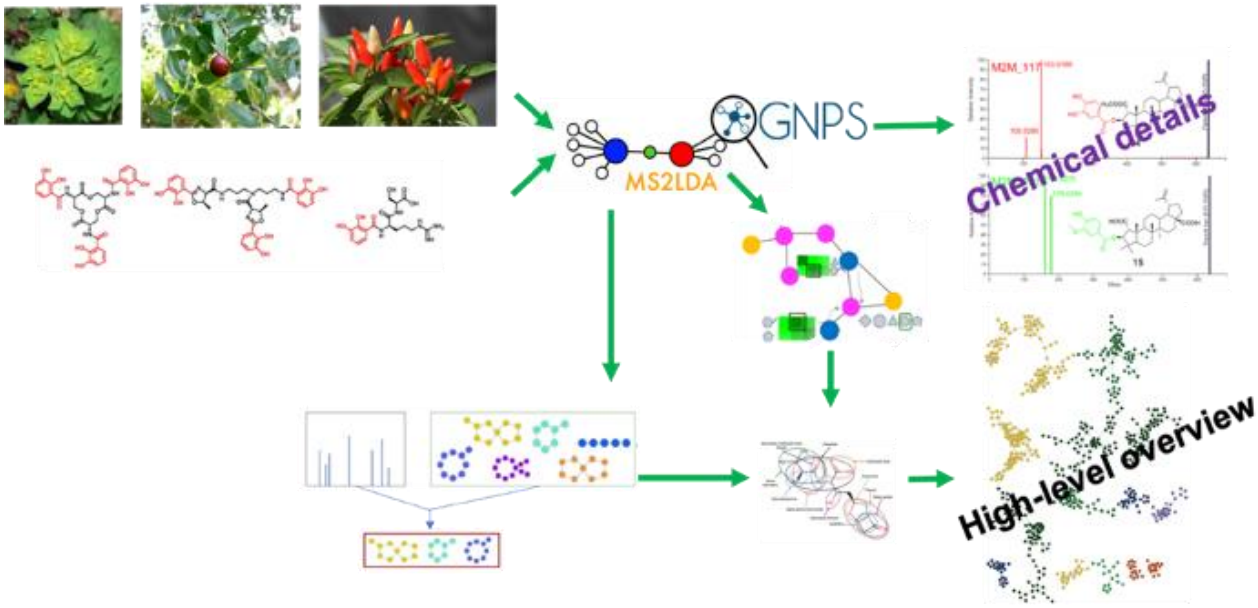

Figure 1. MolNetEnhancer workflow that combines the output of various metabolome mining and annotation workflows resulting in information-rich molecular networks. The workflow provides both a high-level chemical overview by grouping metabolites into main chemical compound classes and at the same time reports detailed chemical knowledge to identify molecular subfamilies.

A comprehensive metabolomics annotation workflow was used that combines molecular networking with *in-silico*annotation, substructure identification and metabolite classification, thus allowing for in- depth insight into the structurally diverse plant chemical space (Figure 1). Firstly, the chemical diversity of two major clades, Ziziphoid and Rhamnoid, of the Rhamnaceae plant family was compared by analysing an enhanced molecular network of 70 plant extracts. Combining comparative metabolomics with chemical class information led to major differences observed in flavonoid and triterpenoid contents [5]. Furthermore, subfamilies of flavan-3-ol and triterpene ester families were described. Secondly, the chemical diversity in the cosmopolitan plant genus *Euphorbia* is thought to increase its fitness by exploring increased biological space and toxicity. Here, the integrated metabolomics workflow facilitated the identification of different diterpene spectral fingerprints clustered within several molecular families from 43 plant extracts. Indeed, *Euphorbia* diterpenes may be possible evolutionary drivers of specialized metabolite diversity at a global scale involved in herbivore resistance. Thirdly, in extracts of pepper plants, comparative metabolomics resulted in differential metabolites correlated to leaf susceptibility towards herbivores and to developmental changes between vegetative and generative plants. Accession-related metabolite markers were identified as well as metabolites correlating to plant and leaf developmental stages. These analyses resulted in the following main findings in pepper plants: an increased presence of diterpenoids and terpene glycosides strongly correlated to herbivore fending, whereas isoflavones and sesquiterpenoids correlated with differences in developmental stage.

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PL4.1

Analysis of Proanthocyanidins in Food Ingredients by the 4-Dimethylaminocinnamaldehyde Reaction

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MAIN CONCLUSION

Many publications demonstrate that the acid catalyzed reaction of DMAC with PACs is a robust method for quantification. However, there are several pitfalls to accuracy and precision that need to be overcome to make the method more robust. More research on the mechanism, kinetics, stoichiometry and products of the acid catalyzed reaction of DMAC with PACs is required to overcome these pitfalls.

INTRODUCTION

Introduction: Proanthocyanidins are oligomeric flavans and flavan-3-ols found in fruits and botanicals that are used as ingredients in dietary supplements because of their putative health benefits, related to antioxidant, anti-inflammatory and antimicrobial activity. Accurate, precise and rapid methods for quantifying PACs are required to determine authenticity, standardization and efficacy of these food ingredients. Our group is working on improved methods based on the acid catalyzed reaction of 4-dimethylaminocinnamaldehyde (DMAC) with PACs to support research and development efforts for the food industry. This extended abstract, as well as our chapter in the next issue of Recent Advances in Polyphenol Research and Reed's presentation at the 2020 International Conference on Polyphenols summarizes this research in order illustrate the pitfalls of the DMAC method.

Background: The first publication on the use of the DMAC reaction was for colorimetric quantification of catechin in solution and not for PACs. Treuter (1989) subsequently developed the reaction for post column derivatization of catechin and PACs for colorimetric quantification by HPLC.[1] These original publications demonstrated that the DMAC reaction was more specific for flavan-3-ols and proanthocyanidins than other methods. The chromophore has maximum absorption (l_{max}) at 640 nm, which avoids the interference from anthocyanins. Therefore, the DMAC reaction has an advantage over the vanillin-HCl or butanol HCl methods. The acid catalyzed reaction of vanillin with flavan-3-ols and PACs produce a chromophore that has a l_{max}around 500nm and falls within the broad absorbance band of anthocyanins. The acid catalyzed autooxidation of PACs to form anthocyanidins in the butanol-HCl method obviously has problems with interference from anthocyanins.

MATERIALS & METHODS

Mechanism of reaction: The acid catalyzed reaction of phenolic aldehydes, such as vanillin and DMAC, with flavan-3-ols and PACs is driven by the generation of a strong electrophile at the carbonyl carbon of the phenolic aldehyde in acid which attacks the nucleophilic carbon at C8 and C6 of the A ring in flavan-3-ols and the C8/C6 terminal flavan-3-ol unit of PACs. The reaction generates a new carbon bond with the loss of ($^{-}$OH) and a conjugated ring system with a positive charge, which no doubt leads to the formation of the chromophore. The initial products rearrange and undergo subsequent oxidation to yield other products. Under the strong acid conditions of the currently used DMAC methods, the initial chromophore is unstable and begins to disappear after 5 to 20 minutes.

RESULTS & DISCUSSION

Reaction Products: 3 publication show a putative structure of a single substituted DMAC moiety at the C8 terminal flavan-3-ol with the positive charge delocalized to the 4-dimethylamino group. However, reaction of naturally occurring cinnamaldehydes with flavan-3-ols and PACs leads to the formation of stable pyrylium derived pigments that may be isolated and characterized by spectrophotometry, mass spectrometry and nuclear magnetic resonance.[2, 3] Our research indicates that DMAC also reacts with catechin, procyanidin B2, procyanidin A2 and PACs to form stable pyrylium cations that are detected by MS and suggest that these cations are not the chromophores that are detected 640 nm.

Accuracy and Precision: Publications demonstrate decent repeatability and precision but accuracy is a serious problem that needs to be addressed if the method is to be useful for standardizing and authenticating dietary ingredients. The problem with accuracy is caused by the relationship between PAC structural heterogeneity, kinetics and stoichiometry of the DMAC reaction, and choice of standards. Our research and others have shown that catechin, procyandin B2, procyandin A2, and PACs differ in the kinetics of chromophore formation so that the time at which absorbance is measured will influence the slope of the standard curve. Since the relationship between absorbance and concentration is linear, the reaction will yield precise results that are inaccurate and poorly repeatable among labs. In addition, DMAC reacts with the C8 terminal flavan-3-ol and therefore the stoichiometry of the reaction yields a much lower slope for the standard curve as PAC degree of polymerization increases. Using catechin or procyanidin dimers as standards will grossly underestimate PAC concentration. Our research indicates that accurate results may only be obtained by using PACs that have been isolated from the plant material of interest as standards for the DMAC reaction.

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PL4.2

Why should non-extractable polyphenols be systematically included in polyphenol analysis?

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MAIN CONCLUSION

NEPP are important quantitative and qualitative contributors to total polyphenol content in foods. Since focusing the characterization of polyphenol profile on EPP provides partial information on these bioactive constituents, the analysis of NEPP should be systematically included when assessing polyphenol profile of plant materials. Nevertheless, advances on a standardized methodology for NEPP are characterization are needed in order to perform such assays as routine analysis.

INTRODUCTION

Sustained scientific evidence has shown the relevance of plant polyphenols both from a technological and biological perspective. The study of these compounds is based on performing extractions, with different solvents or techniques from pant materials, analyzing the derived supernatants. Nevertheless, some decades ago it was shown that polyphenols present in these supernatants are indeed a fraction of these compounds (extractable polyphenols, EPP), since the residues from these extraction also contain polyphenols, named by different authors as non-extractable polyphenols (NEPP), insoluble polyphenols or bound polyphenols. NEPP include either high molecular weight structures, mostly polymeric proanthocyanidins, or small phenolic compounds, commonly phenolic acids. In both cases, they are present in the plant matrix as structures associated with other macromolecular structures, such as proteins or dietary fibre. For these reasons, the term macromolecular antioxidant has also been suggested for defining them. Overall, there is cumulative evidence on the partial bioavailability of NEPP after their intake and, therefore, or their associated biological effects. It important to highlight that, analyzed or not when characterizing a food, NEPP are ingested, contributing to total polyphenol intake and to the pool of potentially bioactive circulating metabolites. Thus, excluding them for analysis means a partial information on the dose of phenolic compounds consumed in an observational, a preclinical or a clinical study.

MATERIALS & METHODS

Several approaches were explored for releasing NEPP from plant matrix: enzymatic hydrolysis, acid hydrolysis, alkaline hydrolysis. Specific hydrolysis were needed to release the different classes of NEPP. Thus, for the release of non-extractable proanthocyanidins the best procedure was based on butanolysis, adapting Porter's method [1]. In the case of hydrolysable polyphenols (low molecular weigth NEPP), a procedure with methanol/H2SO4 was applied [2]. And a specific hydrolysis procedure for the release of non-extractable ellagitannins was performed. Once NEPP were released, different strategies for their analysis were applied. Spectrophotometry analysis provided overall information on the content of the different classes, with the limitations associated to such methods, while HPLC or HPLC-MS allowed to obtain information about the individual constituents of these fractions.

RESULTS & DISCUSSION

NEPP have been analyzed in a wide variety of samples and conditions and using different procedures for their release and characterization. This has allowed the observation of several facts:

- Acid hydrolysis is commonly the most efficient procedure for releasing NEPP, although for some foods or compounds alkaline hydrolysis or enzymatic hydrolysis may be valid options. Although some emerging technologies such as pressurized hot water have been evaluated for NEPP release, they are not as effective as chemical hydrolysis.

- Some analytical aspects in the characterization of NEPP remain to be solved, such as the selection of the most appropriate standard for spectrophotometric determinations or the elucidation of the original macromolecular structures where NEPP were present. At the same time, information on non-extractable proanthocyanidins is still very limited, since it is based on spectrophotometric determinations.

- NEPP are important contributors to total polyphenol content both in common foods and in byproducts such as fruit peels or grape pomace.

- NEPP analysis in the characterization of underexplored botanical sources may help to identify products with potential health-related properties. For instance, roselle (Hibiscus sabadariffa) and the byproduct derived from its decoction, or carob pod (Ceratonia siliqua) exhibit a relevant NEPP content.

- Variety or processing conditions affect NEPP content, so these compounds should be considered when performing quality analysis of batches. This is especially relevant in case a standardized NEPP concentration is to be provided.

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PL5.1

Colour bio-factories: production of anthocyanins in plant cell cultures

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MAIN CONCLUSION

Anthocyanins can be induced by MYB and bHLH proteins such that every tobacco cell produces high levels of cyanidin 3-rutinoside. Decoration of the anthocyanins can be modified by adding in new decorating enzyme activities to produce high levels of delphinidin-3-rutinoside and cyanidin 3-O-(coumaroyl) rutinoside can be engineered. For scale-up, productivity can be improved by limiting the efficacy of the transcriptional regulators, so that the cells grow more rapidly.

INTRODUCTION

Anthocyanins are the most widely distributed group of water-soluble pigments that colour the fruit and flowers of plants. More than 650 different anthocyanins have been identified, distinguished by methylation, glycosylation and acylation with both aliphatic and aromatic groups [1,2]. There is mounting evidence that consumption of anthocyanin-rich food promotes health, supported by many recent studies of anthocyanin-rich fruits such as blodd orange, blueberry, bilberry and cranberry [3-5], anthocyanin-rich corn [6] and fruit engineered to be rich in anthocyanins [7,8] .

The natural colours segment is one of the fastest growing markets of the food and cosmetic colourants industries, with a calculated global market volume for anthocyanins of $291.7 Million in 2014 and a forecast of $387.4 Million for 2021. Industrial production of anthocyanin pigments relies on extraction from whole plants, with the most common sources being waste grape skins from the wine industry, black carrots, red cabbage, sweet potato, the blue algae Spirulina (*Arthrospira platensis*), and Scandinavian berries [9-11].

Commercial use of plant cell cultures was considered early on for the sustainable production of high-value secondary metabolites, such as terpenoids, flavonoids and alkaloids, because production may be dissociated from low yields associated with natural harvests or the high costs associated with complex chemical synthesis [12-14]. Production of anthocyanins, through the use of plant cell cultures has been of industrial and academic interest for decades [15,16]; the most popular cell culture systems being: black carrot, grape berry skins and sweet potato [17,18].

MATERIALS & METHODS

In-vitro production systems allow continuous production independent of regional or seasonal influences, stable product quality under Good Manufacturing Practice (GMP) environments, sustainable manufacturing avoiding agrochemicals, and targeted optimization and automation of biomass production [13,14]. However, production of secondary metabolites in plants usually occurs in specialized differentiated cells. A feature of fast growing conventional plant cell cultures is that undifferentiated cells predominate. [18]. Additionally, the relatively high yields and low production costs, achievable in plant cell cultures, are often not matched by market prices, particularly prices in the natural colorants market. Exceptions are pharmaceutically-relevant plant secondary metabolites with limited natural availability and complex chemical synthesis, such as Paclitaxel in cell cultures of *Taxus brevifolia* [19] and the production of ginsenosides from ginseng (*Panax* spc.) hairy root cultures [20].

RESULTS & DISCUSSION

Anthocyanins are flavonoids and are synthesized via the phenylpropanoid pathway. Their synthesis in plant cells is controlled by a complex of R2R3MYB, bHLH and WD Repeat transcription factors

(the MBW complex) which is well conserved across all Angiosperms. Numerous reports have shown that expression of the R2R3MYB protein can induce anthocyanin biosynthesis in vegetative tissues of tobacco [21]. However, co-expression of both MYB and bHLH proteins under the control of a strong constitutive promoter can ensure that every tobacco cell produces high levels of cyanidin 3-rutinoside [22,23]. The nature of the anthocyanins that accumulate can be modified by adding in new decorating enzyme activities by expression of the encoding genes constitutively. In this way, tobacco cells producing high levels of delphinidin-3-rutinoside and cyanidin 3-O-(coumaroyl) rutinoside were engineered [23]. We found that use of regulatory genes to engineer high level anthocyanin production could be adopted in species other than tobacco to engineer high levels of more highly decorated anthocyanins with a range of colours.

Anthocyanins are synthesised in the cytoplasm but need to be stored under acidic conditions in the vacuole, for stability. Two contrasting models for anthocyanin transport to the vacuole have been proposed: 1) anthocyanin transporters in the membrane of the vacuole (the tonoplast) transport anthocyanins to the vacuole or 2) vesicle-mediated transport that is independent of tonoplast transporters, directs anthocyanins to the vacuole. The tobacco cells producing high levels of anthocyanins provide an excellent model system that shows that these two pathways are non-exclusive and that the same transport proteins that localise to the tonoplast also load vesicles with anthocyanins, while trafficking through the endomembrane system, from the site of their biosynthesis (the endoplasmic reticulum/cytosol) to their final destination (the vacuole).

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PL5.2

The puzzle of displaying orange: Substrate specificity of dihydroflavonol 4-reductase

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MAIN CONCLUSION

Orange and scarlet anthocyanins in flowers are pelargonidin derivatives, while red and blue are derived from cyanidin and delphinidin. The first dedicated enzyme of the anthocyanin branch of flavonoid biosynthesis, dihydroflavonol 4-reductase (DFR), shows substrate specificity. The most famous example is petunia, which does not carry orange flowers unless genetically engineered to do so. Here we swap the amino acids between two gerbera DFRs and pinpoint three important for substrate preference.

INTRODUCTION

Helsinki railway station

On a sunny morning in July 2015 I (THT) crossed the railway station in Helsinki on my way to the lab and saw the impossible. Bright orange petunia flowers were growing in boxes decorating the station. Petunia flowers are never orange. Did petunia breeders finally crack the pathway? I picked a flower, stuffed it in my backpack and in the lab threw it in the freezer.

Petunia cultivars carry different shades of color from red to blue and purple in the flowers. But never orange. This is because petunia anthocyanins do not include the orange pelargonidin derivatives. Anthocyanin biosynthesis splits into three main branches, leading to pelargonidins (orange), cyanidins (red) and delphinidins (blue). The branches differ in the number of hydroxyl groups in the B-ring of the molecule (one, two or three, respectively). It is easy to understand why roses are not blue, the plant does not have a gene for the second hydroxylase. But why are petunias not orange? The precursor for pelargonidin is the simplest of the three and the secret lies in the first enzyme dedicated for anthocyanin biosynthesis on the pathway, dihydroflavonol 4-reductase (DFR).

Meyer et al. [1] introduced the maize DFR encoding gene *A1* in petunia and opened the pathway to orange pelargonins. By crossing the resulting laboratory line with ornamental petunia, Oud et al. [2] produced stable, bright orange and showy derivatives. The breeding paper ends in a statement that these crosses will enter commercial breeding programs. And the market? Wishful thinking, regulation of GMOs in Europe was already in effect.

So, when I ran HPLC of the petunia flower from the railway station, I was thrilled to find pelargonidin. Either the breeders had finally found a mutation in petunia DFR that allows pelargonidin biosynthesis, or somehow the cultivars developed from the *A1* carrying GMO line had escaped to the market. Quick PCR proved the latter to be true [3]. This is how the petunia carnage began.

MATERIALS & METHODS

Coding sequences of the gerbera enzymes GDFR1-2 and GDFR1-3 were PCR amplified in a mixture of the two templates using very short extension time. The resulting fragment was recombined with the vector pDONR221 using Gateway BP clonase, transformed into *E. coli*, plated on LB-agar with selection and the colonies used for plasmid isolation. The plasmid library was recombined with the vector pEAQ-HT-DEST1 [4] using LR clonase, transformed into into *E. coli* and an expression library was prepared as plasmid DNA, to be transformed into agrobacterium C58C1(pGV2260) and plated to obtain single colonies. The agrobacterium colonies were sequenced and used to infiltrate

Nicotiana benthamiana leaves. After three days of expression, total leaf proteins were extracted and used for DFR enzymatic assay at pH 7.0 according to Porter et al. [5]. Concentration of NADPH in the assay was 1 mM and of the dihydroflavonols dihydrokaempferol (DHK), dihydroquercetin (DHQ) or dihydromyricetin (DHM) 0.1 mM.

RESULTS & DISCUSSION

DFR substrate specificity

Petunia is not the only plant species where DFR shows substrate specificity and avoids DHK, arabidopsis is among the other examples. Johnson et al. [6] made fusions with the petunia DFR (avoids DHK) and gerbera DFR (favors DHK) and found a region in the polypeptide that was responsible for substrate specificity (amino acids 132-157). Particularly position 134 was suggested to divide DFRs into DHK users and nonusers.

Our favorite plant gerbera

In gerbera, the cultivar 'Regina' carries flowers with pelargonidin pigmentation and is heterozygous for the DFR encoding gene (*GDFR1-1/GDFR1-2*). 'President' is a cyanidin cultivar and homozygous for a third allele, *GDFR1-3*.

All three gerbera DFR alleles were expressed in a heterologous plant system and tested for substrate preference. GDFR1-1 and GDFR1-2 showed strong preference for DHK, but GDFR1-3 used DHK, DHQ and DHM equally well.

GDFR1-3 differs from the two others in eight amino acid positions, only one of them (position 135) in the region found to be important. Exchanging this position has only a minor effect, the 252 remaining combinations should hold the answer.

Using PCR we generated mixtures of the coding sequences of *GDFR1-2* and *GDFR1-3*. Measuring the substrate preference for 37 different combinations of the amino acids, we could pinpoint three positions responsible for the differential substrate use.

The puzzle of making orange

Plants synthesize anthocyanins also in vegetative tissues, particularly when stressed. In the gerbera cultivar 'Regina' vegetative anthocyanins are cyanidin derivatives, as they are in strawberry and pelargonium leaves (both with pelargonins in fruits or flowers). Appears that pelargonin is attractive to some animals but does not function in protection against stresses. The antioxidant activity of flavonoids is strongest when the B-ring carries two adjacent hydroxyl groups, as in cyanidin. That may be the difficulty in making orange, it is a compromise.

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PL6.1

Advances in biobased thermosetting polymers

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MAIN CONCLUSION

The two oil shocks of 1973 and 1979 have evidenced the dependence of the world economy on oil supply, not only for energy but also for petrochemicals. Beyond the depletion of fossil resources, the consequences of their rapid and excessive consumption on global warming and environmental pollution are pushing modern societies to move towards a model of sustainable development. The transition towards this model relies on both the replacement of fossil energies by decarbonized energies, and of petrochemicals by molecules and macromolecules derived from renewable carbon according to the concepts of cascade biorefinery and recycling of resources. These emerging challenges have motivated research that started since the seventies.

Among petrochemicals, synthetic polymers have encountered unparalleled success over the past century and a half in the manufacture of many everyday plastic objects that we can no longer do without today. In this connection, polymers based on benzene or phenol monomers represent a challenging class of materials due to their unique properties, which on the one hand make them indispensable for certain applications, and on the other hand are very often associated with a potential impact on health and the environment. In particular, polycarbonates and epoxy resins made up with bisphenol A have recently been banned in several countries for the manufacturing of food contact materials (packaging and containers). For their substitution, the challenge is thus double: to ensure the availability of bioresources in terms of accessibility, volumes and quality, and to ensure that the biobased products have indeed a lower environmental and health impact than the products they substitute.

The presentation will review the chronology of the production of thermosets involving industrial lignins and tannins [1]. Thermosets, unlike thermoplastics, are the category of plastics that cannot be melted upon heating. Referred to as reaction polymers, they encompass phenol-formaldehyde (PF) resins, epoxy (EP) resins, polyurethanes (PU), polyester and vinyl ester resins. The manufacture, use and end of life of such polymers rise health and environmental concerns due to the toxicity of their constitutive building blocks, which may be found partly unreacted in the final products. For example, phenolic resins are prepared by the reaction of phenol with an aldehyde, most often formaldehyde, and epoxy resins are mainly based on the diglycidyl ether of bisphenol A.

First works on substitution started with the incorporation of lignin and tannin extracts in the synthesis of PF resins to partly replace phenol. Because of their polymeric structure, lignin and condensed tannins generally lack the required properties to be used as the only phenolic component in thermoset materials. Indeed, they could only be incorporated up to a maximum of 50% as a direct replacement for phenol in phenolic resins or for the enrichment of synthetic resins without altering the properties of the materials produced. Nevertheless, condensed tannins have been shown to be able to efficiently replace phenol in wood adhesives and rigid foams, either with a cross-linking agent or through self-condensation.

In order to overcome the limitations associated with their original polymeric structure, research developments have focused, over the last decade, on the depolymerization of condensed tannins and lignin to obtain monomeric building blocks that can be functionalized to achieve designed properties for use as prepolymers or hardeners. First developed for analytical purposes, the depolymerization of condensed tannins was more recently used to produce monomers with various functionalities, including the replacement of phenolic building blocks in polymer synthesis. In this respect, the use of furan-type nucleophiles as trapping reagents in the depolymerization reaction yields monomer

derivatives containing furan moieties, the linkages of which to the flavanol monomers are particularly stable under the alkaline conditions required for glycidylation [2]. Two examples of fully biobased epoxy resins based on phenolic building blocks will be given. Regarding lignin, most depolymerization processes yield indeed a mixture of rearrangement products that are difficult to exploit. However, strategies for lignin stabilization by reaction with aldehydes or metalloles have recently been proposed and patented [3,4], which allow its depolymerization into simple phenols with good yields. Along with depolymerization strategies, syntheses have been developed to produce mono-, bis- and tris-phenols that have been functionalized with epichlorohydrin to give the targeted epoxy prepolymers.

However, for the industrial development and marketing of biobased thermosetting polymers, these promising technologies face competition from petroleum-based materials, the cost of which is still largely underestimated.

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PL6.2

Advanced Polyphenol-Based Materials via Supramolecular Assembly

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MAIN CONCLUSION

The formation of defined networks of abundant polyphenols with transition metals is achieved via supramolecular assembly. These networks display a range of properties that can be engineered, including permeability, self-healing, shape persistence, injectability, adhesiveness, and mechanical properties. The ease and scalability of the assembly process, combined with the tunable properties of the materials make them of interest for biomedical, environmental, and advanced materials applications.

INTRODUCTION

Advanced materials with network structures prepared via supramolecular assembly are of widespread interest. In particular, the growing interest in supramolecular networks, including gels, driven by metal–ligand coordination stems from the opportunity to blend various properties (e.g., redox, optoelectronic, mechanical, biological) into a matrix and exploit the dynamic nature of coordination bonding to design advanced systems with tailored properties. This presentation will focus on the formation of a class of supramolecular networks, metal–phenolic networks (MPNs), that are assembled through the assembly of polyphenols and metal ions. Particular focus will be placed on the assembly mechanism of the networks and the role of the polyphenols and metal ions in determining the formation, structure and properties of the MPNs. The preparation of MPNs as macroscopic materials as well as the use of MPNs in nanoparticle formats for biological applications will also be presented.

MATERIALS & METHODS

Various polyphenols and metal ions can be used in the assembly of the network structures, details of which can be found elsewhere [1-3].

RESULTS & DISCUSSION

MPN materials can be formed as coatings, capsules, macroscopic materials and nanoparticles for a range of potential applications, spanning membranes and drug delivery vehicles. For example, MPN coatings can be prepared with tunable pore sizes, making them of interest for separations. Another example is the use of MPN-based materials as carrier systems to explore biological interactions. The flexibility of the approach and the suite of materials that can be incorporated into these systems offers a versatile approach for the assembly of a range of advanced materials.

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