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ABSTRACT

Although proanthocyanidins (PACs) modify dentin, the effectiveness of different PAC sources and the correlation with their specific chemical composition are still unknown. This study describes the chemical profiling of natural PAC-rich extracts from 7 plants using ultra high pressure/performance liquid chromatography (UHPLC) to determine the overall composition of these extracts and, in parallel, comprehensively evaluate their effect on dentin properties. The total polyphenol content of the extracts was determined (as gallic acid equivalents) using Folin-Ciocalteu assays. Dentin biomodification was assessed by the modulus of elasticity, mass change, and resistance to enzymatic biodegradation. Extracts with a high polyphenol and PAC content from *Vitis vinifera*, *Theobroma cacao*, *Camellia sinensis*, and *Pinus massoniana* induced a significant increase in modulus of elasticity and mass. The UHPLC analysis showed the presence of multiple types of polyphenols, ranging from simple phenolic acids to oligomeric PACs and highly condensed tannins. Protective effect against enzymatic degradation was observed for all experimental groups; however, statistically significant differences were observed between plant extracts. The findings provide clear evidence that the dentin bioactivities of PACs are source dependent, resulting from a combination of concentration and specific chemical constitution of the complex PAC mixtures.

KEY WORDS: collagen, proanthocyanidins, modulus of elasticity, natural resources, cross-linking reagent, biodegradation.

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Dentin Biomodification Potential Depends on Polyphenol Source

INTRODUCTION

Dentin organic matrix is described as a macromolecular complex composed by ~90% of collagen, especially fibrillar type I (Goldberg *et al.*, 2011). Novel approaches in restorative dentistry emphasize the importance of increasing collagen cross-linking densities to enhance the dentin biomechanical properties and decrease the biodegradation rates (Bedran-Russo *et al.*, 2008, 2009, 2011; Al-Ammar *et al.*, 2009; He *et al.*, 2011).

The term *biomodification* is used to accurately define the range of agent-induced effects on dentin. Dentin biomodification strategies have mostly focused on synthetic and natural chemicals (Bedran-Russo *et al.*, 2008, 2011; Dos Santos *et al.*, 2011). The interactions between dentin and naturally occurring agents are an attractive, renewable, and multimechanistic alternative that has raised interest in many clinical applications (Al-Ammar *et al.*, 2009; Pavan *et al.*, 2011; Xie *et al.*, 2008). Specific sources for these agents are plant extracts rich in proanthocyanidins (PACs), also known as condensed tannins, which are based on flavan-3-ol core structures. Their oligomeric forms (OPACs) and polymers comprise increasing amounts of monomers such as (epi)catechins and their derivatives, including gallates (Li and Deinzer, 2006; Bedran-Russo *et al.*, 2014).

Changes in the type and concentration of PAC phytoconstituents can vary greatly among sources, extraction techniques, and geographic origin (Li and Deinzer, 2006). In general, variations in the individual compounds and distribution of PAC monomers, OPACs, and polymers will determine their potential health benefits. Specifically to their potential dental application, the correlation between the dentin biomechanics-biodegradability properties and the chemical extract profiles is crucial to generate the necessary evidence for the most suitable materials for clinical intervention.

The dentin interactions with polyphenol-rich extracts of *Vitis vinifera* (grape) and *Theobroma cacao* (cocoa) seeds have been reported (Castellan *et al.*, 2010, 2011; Bedran-Russo *et al.*, 2011), albeit with limited extract profiling. Additional potential sources of PACs were selected to expand the scope of the study and help identify the most suitable PAC sources before initiating isolation of bioactive phytoconstituents. The specific aim of the present study was to assess the total polyphenol content (TPC), perform a high-resolution chemical profiling of the different PAC sources, and correlate these findings with the targeted dentin bioactivities (*i.e.*, mechanical properties and matrix biodegradability). The hypothesis tested was that there is a relationship between dentin matrix-PAC interaction and the TPC and phytochemical composition of PAC-rich extract.

Figure 1. Schematic of the study design. **(A)** Dentin beams were obtained from coronal portion of the tooth (1.7 × 0.5 × 6.0 mm). **(B)** Phosphoric acid demineralization, dry weight recorded (M1), and baseline assessment of the modulus of elasticity. **(C)** Specimen biomodification by proanthocyanidin-rich plant extracts and modulus of elasticity assessment, dry weight recorded (M2), collagenase challenge for biodegradation assay, and dry weight recorded (M3).

MATERIALS & METHODS

Selection and Sourcing of PAC-Rich Extracts

The following plants, plant parts, and extraction conditions were selected: seeds of grapes, *Vitis vinifera* L. cf. (Polyphenolics MegaNatural Godl Grape Seed Extract, Madera, California, USA, No. 206112508-01/122112505-01); seeds of cacao, *Theobroma cacao* L. cf. (Barry Callebaut Ltd., NCE-PO804-WW41 [polyphenol extract], Meulan, France, No. 700900001); leaves of the tea plant, *Camellia sinensis* L. cf. (extract: Sunphenon 90D, Taiyo International Inc, Minnesota, USA, No. 103181); stem bark of true cinnamon, *Cinnamomum verum* J. Presl. (Oregon's Wild Harvest, Sandy, Oregon, USA, No. CIN-07011p-OMH01); stem bark of Chinese cinnamon, *Cinnamomum cassia* Nees. (Oregon's Wild Harvest, No. CSS-11180P-OHQ03); fruits (açai berries) of the palm tree *Euterpe precatoria* Mart. (South Jordan, Utah, USA, No. As414A), and the inner bark of *Pinus massoniana* Lamb. (Xi'an Chukang Biotechnology Co. Ltd., China, No. PB120212).

Chemical Characterization

Total Polyphenol Content

The Folin-Ciocalteu assay determined the TPC of the crude extracts, using gallic acid as a standard (see Appendix Figure). Gallic acid (Sigma Chemicals, Perth, Australia) was prepared in 6 two-fold dilutions, from 200 µg/mL to 6.25 µg/mL, to produce concentrations ranging from 30 to 0.98 µg/mL in the 96-well

plate. All extracts were dissolved in methanol to prepare stock solutions of 400 µg/mL and produce final concentrations in the reaction mixture ranging from 60 to 1.9 µg/mL.

The reaction mixtures were prepared in triplicates. Samples of each extract (30 µL) were added to 130 µL of water, followed by 30 µL of 1N Na₂CO₃ solution (ThermoFisher Scientific, Waltham, Massachusetts, USA) and 10 µL of 2N Folin-Ciocalteu's Phenol reagent (ThermoFisher Scientific). The gallic acid standard curve was prepared with 30 µL of gallic acid solution instead of reaction mixtures. The 96-well plates were agitated at 25°C for 60 min, and the absorbance peak was measured at 650 nm via a Spectramax Plus 384 Microplate Reader (Molecular Devices, Sunnyvale, California, USA). The percentage of TPC in the plant extracts was calculated as µg/well of gallic acid equivalents (GAEs), which was converted to µg GAE/µg of extract and overall percentage of GAE in the plant extracts (Katsube *et al.*, 2004).

Ultra High Performance Liquid Chromatograph

Ultra high pressure/performance liquid chromatography (UHPLC) profiles based on gradient elution were used to examine the overall polyphenol composition of the extracts. Water (HPLC grade), acetonitrile, and formic acid were obtained from Fisher Scientific (Fair Lawn, New Jersey, USA). Extract samples were prepared with 50% MeOH at a concentration of 10 mg/mL and filtered (filter: Acrodisc CR 13 mm, 0.45-µm PTFE membrane) before injection (5 µL). The mobile phase used was 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), with a gradient from 5% B to 90% B over 18 min and

2 min of reequilibration. The UHPLC data were acquired on a Shimadzu (Kyoto, Japan) UPLC system at 280 nm with a Diode Array Detector (Shimadzu, SPD-M20-A) via an ACQUITY BEH UPLC column (C18 1.7 μ m; 2.1 $\times$ 50 mm; Waters Inc., Milford, Massachusetts, USA).

Dentin Bioactivity Assays

Dentin Specimen Preparation

Twenty-four extracted sound human molars were selected following approval by the Institutional Review Board Committee of the University of Illinois at Chicago (protocol No. 2011-0312). A total of 120 dentin beams (1.7 $\times$ 0.5 $\times$ 6.0 mm) were obtained as previously described (Bedran-Russo *et al.*, 2009). Afterward, beams were fully demineralized in 10% H₃PO₄ (Ricca Chemical Company, Arlington, Texas, USA) for 5 hr at room temperature. Figure 1 depicts the schematic of the experimental design.

PAC Dentin Biomodification

All 7 plant extracts were dissolved in buffer solution (0.02 M HEPES), except for *C. verum*, *C. cassia*, and *E. precatoria*. These extracts were dissolved in an ethanol:buffer mixture (ratio 50:50), followed by centrifugation (9600 $\times$ g for 1 min at room temperature). All extracts were diluted to a final concentration of 6.5% and pH adjusted to 7.2 (Castellan *et al.*, 2011). The control group was established by immersion of specimens in 0.02 M HEPES buffer for the same exposure time. Dentin beams were randomly divided into 8 groups ($n = 15$) and kept in their respective solutions for 1 hr.

Modulus of Elasticity

The demineralized dentin beams were assessed at baseline and after 1 hr in their respective solution. Apparent modulus of elasticity was determined in a three-point bending flexural test with a 1-N load cell mounted on a universal testing machine (EZ Graph, Shimadzu) at crosshead speed of 0.5 mm/min (Castellan *et al.*, 2010). Data were expressed in MPa, and the fold increase in the modulus of elasticity was calculated as the ratio of the final value (after PAC dentin biomodification) to the initial values (baseline).

Mass Change

Demineralized dentin beams were weighed before (M_1) and after (M_2) dentin biomodification with an analytical balance (XS105 Dual Range, Mettler Toledo Inc., Columbus, Ohio, USA) with a precision of 0.01 mg. Specimens were initially dried in a vacuum desiccator containing anhydrous calcium sulfate for 24 hr at room temperature. Evaluation of mass change ($W_{mc}\%$) was determined as the percentage of gain or loss in mass for each specimen based on the following formula:

$$W_{mc}(\%) = ((M_2 \times 100) / M_1) - 100,$$

where M_1 is the demineralized dentin matrix mass before dentin biomodification and M_2 is the biomodified dentin matrix mass.

Biodegradation Rates

The specimens used above were rehydrated for 1 hr, and each dentin specimen was immersed in 1.5 mL of digestion medium containing bacterial collagenase (100 μ g/mL *Clostridium histolyticum*; Sigma-Aldrich, St. Louis, Missouri, USA) in 0.2 M ammonium bicarbonate buffer (pH 7.9) for 24 hr at 37°C (Bedran-Russo *et al.*, 2011). Following digestion, specimens were desiccated and individually weighed (M_3) as described above. The following formula was used to assess the percentage of enzymatic biodegradation rates (R):

$$R(\%) = 100 - ((M_3 \times 100) / M_2),$$

where M_2 is the biomodified dentin matrix mass and M_3 is the dentin mass after bacterial collagenase digestion.

Statistical Analysis

Descriptive analyses were carried out for TPC and UHPLC studies. Elasticity data analysis was performed via two-way analysis of variance, followed by *post hoc* Games-Howell test ($\alpha = 0.05$). The fold increase in the modulus of elasticity, mass change, and biodegradation activity was statistically analyzed via one-way analysis of variance and *post hoc* Games-Howell test ($\alpha = 0.05$). Bicorrelation between the TPC with respect to the dentin activity after PAC biomodification was evaluated for each method according to the Pearson correlation coefficient. Additionally, the same correlation analysis was used to assess the strength of the correlation between dentin assays.

RESULTS

Different TPC concentrations were observed among crude extracts (Table 1). The highest values were noted for *C. sinensis* (88% $\pm$ 10.5), *T. cacao* (84% $\pm$ 4.3), *V. vinifera* (79.6% $\pm$ 3.6), and *P. massoniana* (78.8% $\pm$ 4.6), whereas *C. verum* (50% $\pm$ 2.9) had an intermediate value and *C. cassia* (22.5% $\pm$ 4.6) and *E. precatoria* (13.8% $\pm$ 6.7) considerably less. UHPLC analysis (Fig. 2) revealed that the plant extracts contain many types of polyphenols, ranging from phenolic acids to OPACs and highly condensed tannins, corresponding to monomeric, intermediate- and higher order oligomeric ($n = 2$ to *ca.* 8) and polymeric ($n \geq ca.$ 9) PACs, respectively. From an overall perspective, *C. sinensis* extract is rich in monomeric PACs, such as epigallocatechin gallate (EGCG) and epicatechin gallate (EC), while the *T. cacao* contains a greater balance of both monomeric and oligomeric PACs. In comparison, *V. vinifera* and *P. massoniana* extracts are rich in both oligomeric and polymeric PACs.

Dentin bioactivity is shown in Table 1. There were statistically significant differences between the baseline and post-PAC dentin biomodification for all groups ($p < .001$). *V. vinifera*, *T. cacao*, *C. sinensis*, and *P. massoniana* extracts significantly increased elasticity between 11.2- and 15.8-fold ($p < .001$). The modulus of elasticity of the control group increased 1.5-fold and was not statistically different to *E. precatoria*-treated dentin ($p > .001$). The percentage of mass gain after PAC dentin biomodification was significantly higher for *V. vinifera*, *T. cacao*,

Table 1. Mean (SD) of Different Assays

Groups	TPC, %	Modulus of Elasticity			Mass Change, %	Biodegradation Rates, %
		Baseline, MPa	Treatment, MPa	Fold Increase		
HEPES buffer (control)	—	4.5 ^b (1.4)	6.6 ^a (2.2)	1.5 ^d (0.3)	-3.9 ^d (1.8)	77.6 ^f (15.6)
<i>Vitis vinifera</i>	79.6 (3.6)	4.7 ^b (1.1)	74.0 ^a (30.6)	15.8 ^a (4.8)	19.6 ^a (5.4)	10.0 ^c (1.5)
<i>Theobroma cacao</i>	84.0 (4.3)	4.3 ^b (1.0)	48.5 ^a (13.7)	11.4 ^a (3.3)	19.0 ^a (12.7)	13.0 ^d (2.3)
<i>Camellia sinensis</i>	88.0 (10.5)	4.3 ^b (1.0)	48.2 ^a (21.6)	11.2 ^a (4.2)	21.7 ^a (3.0)	17.7 ^e (4.5)
<i>Cinnamomum verum</i>	50.0 (2.9)	4.2 ^b (1.2)	27.1 ^a (8.2)	6.5 ^b (2.3)	3.4 ^b (3.1)	2.3 ^a (1.2)
<i>Cinnamomum cassia</i>	22.5 (4.6)	4.1 ^b (0.8)	9.6 ^a (2.8)	2.3 ^c (0.7)	-1.7 ^c (1.4)	6.2 ^b (3.2)
<i>Euterpe precatoria</i>	13.8 (6.7)	4.0 ^b (0.8)	6.3 ^a (1.5)	1.6 ^d (0.2)	-3.6 ^d (1.6)	24.3 ^{cde} (17.4)
<i>Pinus massoniana</i>	78.8 (4.6)	4.0 ^b (1.0)	55.1 ^a (14.9)	13.7 ^a (5.8)	20.6 ^a (7.9)	5.6 ^b (3.3)

Different uppercase letters indicate statistically significant differences comparing the modulus of elasticity before and after treatment ($p < .05$).

Different lowercase letters indicate statistically significant differences between groups ($p < .05$).

TPC, total polyphenol content.

Figure 2. A three-dimensional graphical representation of the UHPLC-UV profiles of different sources of proanthocyanidins observed at 280 nm. The x-axis is the chromatographic retention time in minutes, the y-axis is the absorbance intensity (mAU), and the z-axis represents the different plant extracts. The UHPLC profiles show that all the crude extracts differ in their polyphenol composition and that each crude extract exhibits a characteristic fingerprint of its polyphenol composition.

C. sinensis, and *P. massoniana* extracts (range, 19.0%- 21.7%; $p < .001$). All PAC-rich extracts exhibited protective effect against collagenase degradation when compared to the control group; however, individual differences were evident ($p < .001$). *C. verum* revealed the lowest biodegradation rates (2.3%), followed by *P. massoniana* (5.6%) and *C. cassia* (6.2%).

Pearson test (Fig. 3) indicates a high TPC correlation coefficient, which was statistically significant for both elasticity fold increase ($r = 0.932$, $p = .002$) and mass change ($r = 0.799$, $p = .031$); however, a negative correlation was found between TPC and biodegradation rates ($r = -0.161$, $p = .730$). In addition, the results revealed a positive correlation between fold increase in the modulus of elasticity and mass change ($r = 0.958$, $p < .001$). Both assays demonstrated a negative correlation with biodegradation rates ($r = -0.495$, $p > .001$; $r = -0.453$, $p > .001$, respectively).

DISCUSSION

Bioactive natural metabolites derived from various plant extracts have shown potential oral benefits (Shinada *et al.*, 2007; Macedo *et al.*, 2009; Bedran-Russo *et al.*, 2011; Pavan *et al.*, 2011; Narotzki *et al.*, 2012). To the best of our knowledge, this is the first study to systematically evaluate the dentin bioactivity of different and well-defined sources of PACs and correlate these findings with their phytoconstituent profiles. Notably, all investigated plant sources of PACs showed individual potential to improve the mechanical properties and/or reduce the matrix biodegradability; therefore, the hypothesis tested was confirmed.

The highest statistically significant means of elasticity and mass incorporation after dentin biomodification were observed for *V. vinifera*, *T. cacao*, *C. sinensis*, and *P. massoniana*. These extracts revealed the highest levels of TPC, and a high positive correlation was observed between elasticity and mass change. This indicates a potential correlation between the molecular weight of the PACs and their ability to induce nonenzymatic dentin collagen cross-links. This might be due to the presence of additional hydroxyl groups in the larger oligomers, capable of inducing more covalent and noncovalent bonds (Bedran-Russo *et al.*, 2014). In addition, the dentin activity is strongly related to the complexity of the PAC structures and the specific constitution of the bioactive compounds. For instance, the highest TPC in *C. sinensis* (88%) has mainly been attributed to the high prevalence of monomeric PACs (catechins), in particular EGCG (Moore *et al.*, 2009), and this was verified by the UHPLC profile (Fig. 2). New insight into the effects of EGCG in dentin biological activity demonstrated a protease inhibitor role due to reduced dentin matrix degradation (Kato *et al.*, 2012) and a relevant fold increase in the modulus of elasticity (6.5-fold) when assessed at lower concentration (0.65%) (unpublished observations).

The effects of *V. vinifera* extracts, one of the most bioactive extracts, on collagen dentin matrix have been widely studied, with known stability over time (Bedran-Russo *et al.*, 2011; Castellan *et al.*, 2011; Dos Santos *et al.*, 2011). This property has mainly been attributed to the TPC (79.6%) and the high ratio of polymeric PACs/tannins to monomers. Similar remarkable

performance was observed for *P. massoniana*, which contains a TPC of 78.8% and an overall composition closer to *V. vinifera* extract. Interestingly, *P. massoniana* has demonstrated potent activity against inflammatory skin diseases (Wu *et al.*, 2009) and tumor cell growth (Zhang *et al.*, 2012). Cocoa polyphenolic phytoconstituents have important activity on the skin-restructuring components, including type I collagen (Gasser *et al.*, 2008). *T. cacao* extract contains relatively low content in highly condensed PACs/tannins in comparison to the *V. vinifera* and *P. massoniana* extracts, and the TPC of *T. cacao* extract was between those of *C. sinensis* and *V. vinifera* extracts. However, the fold increase of the modulus of elasticity and mass change of *E. precatorea* treated dentin were not statistically different from the control group, and *C. cassia* and *C. verum* demonstrated intermediate values. As UHPLC profiles indicated that each plant contains different types of polyphenols (*e.g.*, relative and absolute concentrations, linkages, and specific monomeric constituents of oligomers), the results indicate that the effects of (O)PACs on the dentin matrix depend strongly on the exact chemical composition and the concentrations of these complex phytoconstituents.

Biodegradability is assessed by mass loss of solubilized dentin matrix after collagenase digestion. This may have important applications on dentin-resin interface durability and caries progression/remineralization. Although *C. verum* exhibited the highest protective effect against collagenase digestion, all extracts yielded significant decrease in dentin biodegradation. The weak negative correlation between TPC and biodegradation rates ($r = -0.161$) indicates that the protective mechanism of (O)PACs are different from those associated with mechanical strengthening of the dentin matrix. All extracts contain relatively high percentages of polyphenols, except for the extracts of *E. precatorea* and *C. cassia*. The observed differences in biodegradation are more likely due to the species-specific differences in the chemical structures of the (O)PACs (Lizarraga *et al.*, 2007; Urios *et al.*, 2007), including the type of monomers, the degree of polymerization/permutation, and/or the degree of galloylation of the catechin derivatives.

Although this study evaluated the TPC of each plant extract, these extracts comprise complex mixtures. Quantification of

Figure 3. Pearson correlation coefficient (r) between the total polyphenol content and dentin assays (fold increase in the modulus of elasticity, mass change, and biodegradation rates), as well as comparison among the dentin assays: (A) fold increase in the modulus of elasticity vs. total polyphenol content, (B) mass change vs. total polyphenol content, (C) biodegradation rates vs. total polyphenol content, (D) fold increase in the modulus of elasticity vs. mass change, (E) fold increase in the modulus of elasticity vs. biodegradation rates, and (F) biodegradation rates vs. mass change.

specific monomeric and oligomeric constituents is the goal of ongoing studies aimed at mapping the structure dependency of dentin bioactivity parameters. The present data confirm already that changes in the extraction process and bioassay protocol have notable effects on the bioactivity of (O)PAC sources. The outcome shows that the flavan-3-ol monomers, which are ubiquitous in all natural sources of OPACs, can modulate the biomechanical properties of dentin matrix. However, the OPAC-mediated dentin bioactivity is clearly source dependent and linked to the oligomeric and polymeric phenolic metabolome of

the individual source plants. In summary, the present study provides justification for a rationale use of standardized OPAC-rich intervention materials as well as the knowledge foundation to further characterize 4 high-potency OPAC-rich extracts as sources of bioactive phytoconstituents exhibiting desirable dentin biomodification ability for use as clinical agents.

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