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Long-term stability of dentin matrix following treatment with various natural collagen cross-linkers

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Abstract

Objectives—Collagen disorganization is one of the main degradation patterns found in unsuccessful adhesive restorations. The hypothesis of this study was that pretreatment using natural collagen cross-linking agents rich in proanthocyanidin (PA) would improve mechanical properties and stability over time of the dentin collagen and, thus, confer a more resistant and lasting substrate for adhesive restorations.

Methods—PA-based extracts, from grape seed (GSE), cocoa seed (CSE), cranberry (CRE), cinnamon (CNE) and açai berry (ACE) were applied over the demineralized dentin. The apparent elastic modulus (E) of the treated dentin collagen was analyzed over a 12 months period. Specimens were immersed in the respective solution and E values were obtained by a micro-flexural test at baseline, 10, 30, 60, 120 and 240 min. Samples were stored in artificial saliva and re-tested after 3, 6 and 12 months. Data was analyzed using ANOVA and Tukey test.

Results—GSE and CSE extracts showed a time-dependant effect and were able to improve [240 min (MPa): GSE=108.96±56.08; CSE=59.21±24.87] and stabilize the E of the organic matrix [12 months (MPa): GSE=40.91±19.69; CSE= 42.11±13.46]. CRE and CNE extracts were able to maintain the E of collagen matrices constant over 12 months [CRE=11.17±7.22; CNE= 9.96±6.11; MPa]. ACE (2.64±1.22 MPa) and control groups immersed in neat distilled water (1.37±0.69 MPa) and ethanol water (0.95±0.33MPa) showed no effect over dentin organic matrix and enable their degradation and reduction of mechanical properties.

Significance—Some PA-based extracts were capable of improving and stabilizing collagen matrices through exogenous cross-links induction.

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Keywords

dentin; collagen; cross-linking; proanthocyanidin; stiffness

1.1 Introduction

Lack of marginal seal and decreased bond strength of adhesive restorations affects the longevity of restorations placed in enamel and dentin (Monticelli, Toledano et al., 2008). Current clinical evaluations attest that continuous degradation of the dentin-resin bond prevails for several bonding systems and can also lead to an increase in the loss rates of restorations for different adhesives systems (Hashimoto, Ohno et al., 2000; van Dijken, Sunnegardh-Gronberg et al., 2007). Adhesive/dentin interface can be porous and also behaves as a permeable membrane (Tay, Pashley et al., 2002) allowing elution of unreacted monomers (Hashimoto, Ohno et al., 2003), water sorption, polymer swelling, resin hydrolysis (Malacarne, Carvalho et al., 2006) and, also, enzymatic activity that degrades the exposed type I collagen fibrils located at the bottom of the hybrid layer (Pashley, Tay et al., 2004). The two main degradation patterns within the hybrid layer: loss of resin from interfibrillar spaces and disorganization of the collagen fibrils (Hashimoto, Ohno et al., 2000), suggest the need of novel therapies focused not only on the stability of the resin components of the interface but also on the dentin organic content involved in the restorative/repairative treatment.

The dentin organic content is mainly composed of type I collagen (~ 90%) (Veis and Schlueter, 1964). This triple-helix molecular structure consists of three polypeptide chains, two (α 1 and one (α 2), that are intertwined to one another and folded into a ropelike right-handed structure (Yamauchi, 2002). For its use as a biomaterial, or as a substrate for adhesive restorations in Dentistry, alterations of the mechanical properties varying the degree of inter or intra-molecular cross-links are possible (Stenzel, Miyata et al., 1974). Cross-links are bonds between the side chains of amino acids present in collagen molecules. These bonds increase the mechanical properties of collagen fibrils by preventing the long rod-like collagen molecules from sliding past each other under stress (Goh, Meakin et al., 2007).

Proanthocyanidin (PA) has been reported to stabilize and increase the cross-linkage of collagen based tissues, including dentin (Masquelier, 1981). A large number of studies have emerged over the past few years showing all the potential health benefits of PA from fruits (cranberry, grape and cocoa), leaves and bark (Cos, De Bruyne et al., 2004; Hess, Hess et al., 2008; Bertelli and Das, 2009). This compound is a polyphenolic natural product composed of flavan-3-ol subunits linked mainly through C4–C8 (or –C6) bonds (Kennedy and Taylor, 2003). Their structures depend on the nature of the flavan-3-ol starter and extension units, the position and stereochemistry, the degree of polymerization, and the presence or absence of modifications such as esterification of the 3-hydroxyl group. The use of a PA rich grape seed extract improved the mechanical properties, (apparent elastic modulus and ultimate tensile strength) of demineralized dentin (Bedran-Russo, Pereira et al., 2007; Bedran-Russo, Pashley et al., 2008) as well as of the dentin-resin bonded interface (Al-Ammar, Drummond et al., 2009). Other PA-rich agents, such as cocoa seed extract has also been shown to greatly decrease the enzymatic degradation, increase stiffness and decrease the swelling ratio of demineralized dentin (Castellan, Pereira et al., 2010; Castellan, Pereira et al., 2010). Hence, these natural extracts have reduced cytotoxicity when compared to synthetics agent effects (Han, Jaurequi et al., 2003).

A large variety of PA-based derivatives/extracts are available and therefore a better understanding of the ability of different sources of natural occurring collagen biomodifiers to interact with dentin organic matrix is essential to determine potential new restorative therapies that can impact the stability of collagen. In particular, the long-term effects of the PA-based agents on the mechanical properties can provide significant information of the ability of the biomodified dentin to maintain its strength and decrease its vulnerability to degradation by endogenous enzymes. This article aims to analyze the elastic response of dentin that can be achieved by biomodification of the organic matrix with natural agents (grape seed, cocoa, cranberry, açai berry and cinnamon) at various exposure time (10, 30, 60, 120 and 240 minutes) and up to 12 months storage in artificial saliva. The null hypothesis is that there will be no differences on the apparent elastic modulus of dentin organic matrix treated with different sources of PA-rich collagen biomodifiers.

1.2 Materials and Methods

1.2.1 Samples Preparation

The use of fifteen sound extracted human third molars was approved, by the Institutional Review Board Committee of the University of Illinois at Chicago (protocol # 2009 – 0198). The teeth were kept frozen for no longer than 6 months, thawed, cleaned of adhering soft tissues. The cusps were ground flat with # 320 grit silicon carbide abrasive paper (Carbimet Discs, Buehler, Lake Bluff, IL) under running water to create a flat occlusal surface and enable more accurate sectioning of samples. The root portion was sectioned 1 mm below the cement enamel junction and discarded.

Teeth were sectioned into 0.5 ± 0.1 mm thick disks in the mesio-distal direction using a slow speed diamond wafering blade (Buehler-Series 15LC Diamond) under constant water irrigation. The disks were further trimmed using a cylindrical diamond bur (# 557D, Brasseler, Savannah, GA) in a high-speed handpiece to a final rectangular shaped beam (0.5 ± 0.1 mm thick $\times$ 1.7 ± 0.1 mm wide $\times$ 7.0 ± 0.2 mm length). A dimple was made at one end of the surfaces for orientation to allow repeated measurements to be performed on the same surface. Specimens were immersed in 10 % phosphoric acid solution (LabChem, Pittsburgh, PA) for a period of 5 h for complete demineralization and thoroughly rinsed with distilled water for 10 min (Bedran-Russo, Pereira et al., 2007). Complete demineralization was verified by X-ray analysis (Bedran-Russo, Pereira et al., 2008).

1.2.2 Experimental design

The concentration (6.5%) and solvent systems (distilled water and ethano-water) used were selected according to previous studies (Castellan, Pereira et al., 2010). Ethyl alcohol was used without further purification. The five different extracts (grape seed, cocoa seed, açai berry, cinnamon and cranberry) were dissolved in their respective solvent system and the pH of the slightly acidic solutions was adjusted to 7.2 using NaOH. After pH adjustment, the solutions were filtered through paper filter n°6 (Whatman, London, England). Experimental groups are presented in Table 1. Neat solvent (distilled water) and solvent mixture (ethanol-water) were used as negative controls.

Specimens (n=10) were immersed in their respective solution for different application time evaluation and before each evaluation samples were profusely rinsed with distilled water. For a more descriptive time-dependency analysis, samples were tested before treatment for baseline measurements and after 10 min (t_{10}), 30 min (t_{30}), 60 min (t_{60}), 120 min (t_{120}) and 240 min (t_{240}) incubation periods. Stability was assessed immediately, 3 months, 6 months and 12 months following treatment. Samples were stored in artificial saliva containing 1.5 mm Calcium and 0.9 mm Phosphate in a buffer solution of 0.1 m Tris buffer at pH 7.0 (Hara,

Queiroz et al., 2004). The samples were kept at 37°C and the artificial saliva (AS) was changed every two weeks. Figure 1 illustrates the experimental design of this study.

1.2.3 Mechanical properties assessment

An aluminum alloy flexural fixture with 2.5 mm span was glued to the bottom of a glass Petri dish. Specimens were tested in 3-point bending while immersed in distilled water using a 1 N load cell mounted on a universal testing machine (EZ Graph, Shimadzu, Kyoto, Japan) at crosshead speed of 0.5 mm/min.

Displacement (D) during compression was displayed in millimeters and calculated at a maximum strain of 3% using the following formula (Bedran-Russo, Pashley et al., 2008):

$$D = \epsilon L^2 / 6T$$

Where ϵ is strain, L is support span, and T is thickness of the specimen. The apparent elastic modulus (E) of the specimens was expressed in MPa (Mega Pascal) and calculated using the following formula:

$$E = PL^3 / 4DbT$$

Where P is the maximum load, L is the support span, D is the displacement, b width of the specimen, and T is the thickness of the specimen.

Specimens were immersed in water for baseline measurements and then in their respective solutions for cumulative exposure time. All measurements were performed with specimens immersed in distilled water. The data were collected and statistically analyzed. For a three-way repeated measurements ANOVA (treatment vs. treatment time vs. aging period) the Box test of equality of the covariance matrices was statistically significant thus indicating that the dependent variable does not meet the sphericity assumption. So, it was necessary to perform two distinct two-way repeated measurements ANOVA (treatment vs. treatment time; treatment vs. aging period) and Tukey's post-hoc tests ($\alpha = 0.05$).

1.3 Results

The effect of treatment and exposure time data is illustrated in Figure 2. There was a statistically significant interaction between both factors (treatment vs. exposure time, $p < 0.001$) and also significant differences within each factor ($p < 0.001$ for both). A numerical and statistical descriptive table with mean values and standard deviations is presented in Table 2. While GSE, CSE and CNE increased the apparent elastic modulus of dentin matrices ($p < 0.001$), the other PA-based extracts did not significantly affect the mechanical properties of dentin matrix. GSE and CSE effect was time-dependant, so increased exposure time enhanced the apparent elastic modulus of demineralized dentin ($p < 0.001$, for all). CNE was able to increase E values after 240 min treatment when compared to its baseline results, but when comparing to both control groups it showed no significant statistical difference. CRE ($p = 0.126$), ACE ($p = 0.370$) and both control groups ($p > 0.05$ for both) did not have any kind of effect on mechanical properties of dentine matrices after the 240 min incubation period.

The effect of artificial saliva storage on the long-term properties is presented in Table 3. Statistically significant differences were observed for both factors, ageing period and treatment ($p < 0.001$) and their interaction ($p < 0.001$). GSE was significantly negatively affected by long storage periods ($p < 0.001$) when compared to CSE ($p = 0.120$). After 3

months in AS storage, no statistically significant differences were detected between GSE and CSE groups, but they presented higher apparent elastic modulus than all other groups, regardless of storage time ($p < 0.001$). The changes on the apparent elastic modulus for each treatment on different storage times are illustrated in Figure 3. GSE (Figure 3A) showed decreased elastic modulus values following 3 months evaluation ($p < 0.001$). CSE (Figure 3B), CRE (Figure 3D) and CNE (Figure 3E) were able to stabilize the elastic modulus of dentin ($p = 0.120$, $p = 0.946$, $p = 0.130$; respectively). Beside ACE inability to increase the modulus of elasticity of dentin matrices after 240 minutes of treatment, it could not stabilize the E values ($p = 0.003$) (Figure 3C) along the storage periods. Artificial saliva storage decreased the modulus of elasticity values of both control groups (DW and ETW, $p < 0.001$), as shown in Figure 3 F and G.

1.4 Discussion

The present study confirmed a remarkable ability of certain PA-based agents to strengthen the dentin matrix by increasing the apparent elastic modulus. The influence remained effective over time, as demonstrated by apparent elastic modulus values that remained up to 40 fold high when compared to control groups. The present study shows evidence of the potential long-term effect of GSE and CSE on demineralized dentin matrix, most likely due to induction of exogenous collagen cross-linking. The null hypothesis was partially accepted.

The relative stiffness of the collagen fibrils depends on the formation of endogenous and exogenous cross-links (Goh, Meakin et al., 2007). Endogenous cross-links happens between the telopeptide and adjacent helical domains of type I collagen molecules. Type I collagen has at least four cross-linking sites: one in each telopeptide (COOH-terminal and NH₂-terminal) and two other sites in the triplehelical domain at residues 87 and 930 (Takaluoma, Lantto et al., 2007). The interaction of PA and collagen results in the induction of exogenous cross-links primarily by hydrogen bonding between the protein amide carbonyl and the phenolic hydroxyl (Hagerman, A E and Klucher, 1986) and also covalent and hydrophobic bonds. The relatively large stability of these cross-links compared with other polyphenols (such as tannins) suggests structure specificity (Hagerman, A. E. and Butler, 1981), which although encouraging hydrogen binding also create hydrophobic pockets (Han, Jaurequi et al., 2003). Such microenvironments by virtue of decreasing the dielectric constant enhance the stability of such H-bonds. Hydrogen bonds that are not stabilized by adjacent hydrophobic bonds can be dissociated by treatment with aqueous buffers (Han, Jaurequi et al., 2003), as artificial saliva, over time. This could explain the decrease of GSE values after 3 months, and also, its subsequent stabilization. Alcohols on the other hand, by decreasing the dielectric constant of the media, also stimulate PA and collagen interactions (Han, Jaurequi et al., 2003). CSE using an ethanol/water solvent system resulted in a mild decrease on stiffness values.

PA belongs to a category known as condensed tannins, highly hydroxylated structures capable of forming insoluble complex with carbohydrates and proteins (Cao, Fu et al., 2007). PA has been shown to increase collagen synthesis and accelerate the conversion of soluble collagen to insoluble collagen during development (Han and Nimni, 2005). Grape seed, cocoa seed, açai berry, cinnamon and cranberry are PA sources described in literature with several beneficial effects for human health (Cos, De Bruyne et al., 2004). Our study is on agreement with other studies that showed the immediate effect of a PA based extract from grape and cocoa seed on the apparent elastic of demineralized dentin in a time-dependant way (Bedran-Russo, Pashley et al., 2008; Castellan, Pereira et al., 2010). The same substrate was used in previous studies to describe the immediate effect of both extracts on properties like enzymatic degradation and swelling ratio, and their ability to positively

affect the bond strength after 24 hours storage (Castellan, Pereira et al., 2010). The immediate interaction between CNE, CRE, ACE and dentin and also the long-term effect of any PA-based extract on dentin organic matrix is not found in literature. Unfortunately, the interaction between CNE, CRE, ACE with demineralized dentin collagen was very weak. Short and long-term effects were unnoticed for açai berry and extremely little for cranberry and cinnamon, which could be explained by chemical structural differences and/or PA content. Due to its complexity, PA from distinct sources can be difficult to analyze and it provides a phytochemical challenge (Shi, Yu et al., 2003).

Grape seed tannins consist of a very complex mixture of oligomers and polymers (Thompson, 1972). Cocoa and cinnamon, on the other hand, show a relative simple composition with low molecular oligomers (Thompson, 1972). Açai berry has a very high phenolic content, especially phenolic acids, catechins monomers and procyanidin oligomers (Prior, Lazarus et al., 2001). Therefore, it is reasonable to predict that this variation of chemical structure, stereochemistry pattern and overall composition affects general characteristics of these natural products and therefore its ability to interact with Type I collagen.

PA concentration is another reasonable explanation for stiffness differing values. GSE and CSE higher values could be explained by their high PA content (95% and 45%, respectively). Hence, the sourcing of start material and the manufacturing process has profound effects on the composition, potency and PA content of resulting extract. For example, raw cranberry, similar to grape and cocoa seed, contain higher amount of total phenols than other common fruits including blueberry, apples, red grapes and strawberry. But, it has two major classes of phenolics identified, and only 56% is proanthocyanidin (McKay and Blumberg, 2007). The antimicrobial, antiproliferative, antiradical and protective properties against oxidative stress on cell lines by two CRE commercial extracts has been shown to be strongly affected by PA concentration (Menghini, Leporini et al., 2011). So, the proanthocyanidin content is critical to determine the extract efficacy. The commercial CRE used in this study contained less than 1% of PA, which could explain its unexceptional effect. The milder effect of cinnamon extract, or the negligible effect of açai berry extract, could also be explained by their PA content, only 8% and 1% at best, respectively. The different results when comparing CRE and ACE, both with almost the same amount of PA, could be due to their, already mentioned, chemical structural differences. The structure of PAs as well as its concentration in the plant/extract are important factors determining their activity and role in diverse applications such as agricultural systems (Min, Barry et al., 2003), natural environment (Zucker, 1983) and biological systems.

Besides the hydrogen bonds between PA/collagen that are not stabilized and are dissociated by storage in saliva, matrix metalloproteinases (MMPs) are another possibility for the decrease of stiffness values over time. MMPs are a family of zinc-dependent proteolytic enzymes that are capable of degrading the dentin organic matrix after demineralization, which could explain the lower values of both control groups after long-term storage (Pashley, Tay et al., 2004). Enzymes with gelatinolytic (MMP-2 and MMP-20) activities are present within intact dentin matrix (Martin-De Las Heras, Valenzuela et al., 2000) and in carious dentin (Tjaderhane, Larjava et al., 1998). Some of these naturally occurring cross-linkers, especially GSE and CRE, has been reported to inhibit MMP activity (Matchett, MacKinnon et al., 2005; Matchett, MacKinnon et al., 2006) and also reduce proteolytic cleavage site within the collagen molecule by hiding or modifying the structure due to protein folding (Jayakrishnan and Jameela, 1996). Further studies should clarify the effect of specific PA-based agents on the endogenous and exogenous MMPs activity in dentin and long-term resin-dentin bond strength.

There is a great need in medicine and other health areas for biomaterials that are versatile and compatible with human tissues. Clinical needs include implantable devices and extracorporeal ones. The introduction of cross-links, restructuring and stabilizing the collagen, a natural occurring biopolymer, is an important aspect of the use of collagen as a biomaterial (Stenzel, Miyata et al., 1974).

1.5 Conclusions

Integrity and stability over time are both preconditions for collagen as a biomaterial or as an essential substrate for adhesive restorations. Proanthocyanidin (PA) rich extracts are important tools for tailoring collagen matrix with cross-linking bonds and keep them stable over long time periods. Their effect depends on their chemical structure, solvent system used, PA content and time of incubation.

Research highlights

- Natural collagen cross-linkers are capable of increasing the elastic modulus of demineralized dentin;
- Proanthocyanidin based extracts were able to maintain the stiffness of collagen matrices constant over 12 months;
- Exogenous collagen cross-links induction due to a natural biomimetic agent;

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Figure 1.

Illustration of experimental steps. A – samples cutting; B – dentin rectangular beams; C – dimensions of samples and dimple; D – phosphoric acid demineralization for 5 hours; E – immersion in solutions for time periods; F – schematization of micro-flexural test for apparent elastic modulus assessment.

Figure 2.

Influence of proanthocyanidin-based agents on the stiffness of demineralized dentin (MPa) in different incubation times (min). GSE- grape seed extract; CSE- cocoa seed extract; ACE- açai berry extract; CRE- cranberry extract; CNE- cinnamon extract; DW- distilled water; ETW- ethanol-water.

Figure 3.

Elastic modulus of cross-linked dentin for long-term periods. A-Elastic modulus of demineralized dentin treated with GSE (grape seed extract) storage for several time periods. B - Elastic modulus of demineralized dentin treated with CSE (cocoa seed extract) storage for several time periods. C - Elastic modulus of demineralized dentin treated with ACE (açai berry extract) storage for several time periods. D - Elastic modulus of demineralized dentin treated with CRE (cranberry extract) storage for several time periods. E - Elastic modulus of demineralized dentin treated with GSE (grape seed extract) storage for several time periods. E - Elastic modulus of demineralized dentin treated with CNE (cinnamon extract) storage for several time periods. F - Elastic modulus of demineralized dentin treated with DW (distilled water) storage for several time periods. G - Elastic modulus of demineralized dentin treated with ETW (ethanol-water) storage for several time periods.

NOTE: Different scales for each graph.

Table 1

Different natural extracts and their composition, manufacturer, solvent system, proanthocyanidin content and extraction method.

Group	Composition (w/v)	Manufacturer	Solvent system	PA content*	Extraction method*
GSE	6.5% grape seed extract	Vitis vinifera, Mega-Natural gold grape seed extract, Lot 13682503-01, Polyphenolics Madera, CA, USA	Distilled water	95%	Hot water
CSE	6.5% cocoa extract	Theobroma cacao, polyphenol extract, Lot CMIE-7LJKF-Foratero, Barry Callebaut, Lebbeke-Wieze, Belgium	Ethanol-water (50/50)	45%	30% acetone/water
CRE	6.5% cranberry extract	Vaccinium macrocarpon, NutriCr-an 90S 90%, Lot 080118, Degas Botanical Synergies, Carver, MA, USA	Distilled water	Maximum 0.95%	Water and spray dried
CNE	6.5% cinnamon extract	Cinnamomum cassia, cinnamon bark powdered extract, C-0154130, Draco Natural Products, San Jose, CA, USA	Distilled water	Maximum 8%	Controlled temperature water extraction process, spray dried
ACE	6.5% açai berry extract	Euterpe oleraceae, açai berry extract, Lot ACAXX02/0407SSantosflora Comércio de Ervas, SP, Brazil	Distilled water	Maximum 1%	Spray Dryer

* Information provided by the manufacturer.

GSE- grape seed extract; CSE- cocoa seed extract; CRE- cranberry extract; CNE- cinnamon extract; ACE- açai berry extract.

Table 2

Apparent elastic modulus (MPa) of demineralized dentin treated (standard deviation) with different cross-linkers in cumulative time periods.

Extract	Apparent elastic modulus (MPa)					
	Baseline	10 min	30 min	60 min	120 min	240 min
GSE	5.8 (2.0) Aa	36.1 (9.7) Aa,b	58.1 (21.7) Ab,c	70.5 (32.9) Ab,c	91.6 (52.0) Ac,d	109.0 (56.1) Ad
CSE	7.6 (1.5) Aa	20.9 (5.2) Ba,b	29.7 (10.4) Bb,c	33.2 (13.1) Bb,c	42.3 (16.3) Bc,d	59.2 (24.9) Bd
ACE	8.0 (2.3) Aa	7.4 (2.3) Ca	6.5 (2.3) Ca	5.5 (2.01) Ca	5.4 (2.2) Ca	5.4 (2.2) Ca
CRE	6.6 (3.1) Aa	6.5 (3.5) Ca	8.9 (4.9) Ca	8.4 (4.1) Ca	9.2 (5.3) Ca	12.1 (6.8) Ca
CNE	5.4 (2.1) Aa	7.2 (3.2) Ca,b	9.0 (4.1) Ca,b	9.8 (3.6) Ca,b	12.0 (4.3) Cb,c	16.2 (6.3) Cc
DW	6.5 (1.7) Aa	5.8 (1.6) Ca	6.3 (1.4) Ca	5.8 (1.2) Ca	6.1 (1.6) Ca	5.7 (1.4) Ca
ETW	5.1 (1.5) Aa	5.2 (1.7) Ca	5.0 (1.5) Ca	4.8 (1.6) Ca	4.8 (1.4) Ca	4.7 (1.3) Ca

Different upper case letters represent statistical difference ($p < 0.05$) among rows and different lower case letters among columns. GSE- grape seed extract; CSE- cocoa seed extract; ACE- açai berry extract; CRE- cranberry extract; CNE- cinnamon extract; DW- distilled water; ETW- ethanol-water.

Table 3

Apparent elastic modulus of demineralized (standard deviation) dentin following cross-linking treatments and storage in artificial saliva for 3, 6 and 12 months.

Extract	Apparent elastic modulus (MPa)			
	Immediately	3 months	6 months	12 months
GSE	129.0 (56.1) ^a	51.7 (21.9) ^a	46.5 (21.3) ^a	40.9 (19.7) ^a
CSE	59.2 (24.8) ^b	50.3 (17.5) ^a	53.4 (22.5) ^a	42.1 (13.5) ^a
ACE	5.4 (2.2) ^c	4.3 (1.0) ^b	4.3 (1.5) ^b	2.6 (1.2) ^b
CRE	12.1 (6.8) ^c	12.8 (6.7) ^b	12.7 (6.6) ^b	11.2 (7.2) ^b
CNE	16.2 (6.3) ^c	14.5 (5.4) ^b	13.4 (5.5) ^b	10.0 (6.1) ^b
DW	6.0 (1.4) ^c	3.0 (0.9) ^b	2.3 (0.7) ^b	1.4 (0.7) ^b
ETW	4.7 (1.3) ^c	1.9 (0.8) ^b	1.9 (0.7) ^b	0.9 (0.3) ^b

Statistical difference for each column is shown by different letters.

GSE- grape seed extract; CSE- cocoa seed extract; ACE- açai berry extract; CRE- cranberry extract; CNE- cinnamon extract; DW- distilled water; ETW- ethanol-water.