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Effect of proanthocyanidins-containing natural extracts on the resin-dentin bond strength and stiffness of dentin matrix.

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Abstract

Objectives: Aim: This study was conducted to evaluate the effect of proanthocyanidin-containing natural cross-linkers on the resin-dentin bond strength and dentin matrix stiffness.

Methods: Forty-five specimens were prepared from 9 recently extracted molars for microtensile bond strength (μTBS) testing. Specimens were divided according to the type of extract used for dentin pretreatment into 3 groups (n=15): Control group(CO), Grape seed extract (GSE), Cacao seed extract (CSE). The occlusal enamel of the teeth was removed to expose the superficial dentin. The exposed dentin surface was acid etched using 35% phosphoric acid, then the respective pre-treatment was applied using a microbrush for 1 minute and blot dried, afterwhich the etch and rinse adhesive was applied. Composite was then built onto the pre-treated dentin surface in 2 increments (2mm each) and the teeth were sectioned using an isomet saw into beams (1x1x8mm) and their micro-tensile bond strength was evaluated using a universal testing machine. For dentin stiffness evaluation, nine sound molars were used to obtain 45 dentin beams (1x1x6.5mm). The beams were then fully demineralized using a 10% phosphoric acid solution and rinsed for 10 minutes with distilled water. The beams were then divided into 3 groups (n=15) and treated with the respective extract solution for 1 minute. To evaluate the dentin stiffness the beams were subjected to 3-point loading.

Results: Microtensile bond strength results revealed insignificant difference between the tested groups. Concerning dentin matrix stiffness; grape seed extract recorded significantly higher mean value compared to cacao seed extract and control.

Conclusion: Treating demineralized dentin for 1 minute using grape seed extract improved the stiffness of dentin matrix, but was insufficient to improve the resin-dentin microtensile bond strength.

Keywords: Proanthocyanidins – dentin matrix stiffness – collagen cross-linking

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Introduction

Dentin bonding has always represented a challenge due to the complexity of the dentinal tissue. Dentin forms the main bulk of the tooth, thus a strong resin-dentin adhesive joint is necessary for the success of adhesive restorations. Improving mechanical properties of the resin-dentin interface has recently become one of the main aims of research in dentistry.(1)

The hybrid layer, is the most vulnerable part in the resin-dentin interface where stress is mostly concentrated and is the site where failure mostly occurs. Tissue engineering and biomimetic approaches have been recently applied in an attempt to improve the intrinsic properties of the dentinal substrate through mechanically enhancing the hybrid layer. Intrinsic collagen cross-links provide the tensile properties of collagen molecules.(2)

Extrinsic collagen cross-linkers have the ability to induce the formation of additional inter and intramolecular cross-links.(3) These cross-links are the foundation of the strength, viscoelasticity and stability of the dentinal collagen matrix. The number and type of cross-links also influences the thermal stability of collagen and its ability to resist biodegradation.(4) Improving the dentin matrix stability also indirectly influences the ability to use more hydrophobic adhesives without the risk of collapsing collagen matrix after drying and during adhesive application.(4, 5) Collagen cross-linkers have been previously demonstrated to be able to improve the ultimate tensile strength and the stiffness of demineralized dentin matrix.(6)

Chemical cross-linkers have been previously suggested for dentin biomodification where gluteraldehyde has been used as an effective collagen cross-linker. Yet, its cytotoxicity was a major concern that shifted research towards safer natural cross-linkers that are more biocompatible and less toxic.(7)

Proanthocyanidins (PA) are natural polyphenolic compounds that are widely present in vegetables, fruits, seeds and flowers. Proanthocyanidins are potent antioxidant cross-linking agents with vast biological activities. Grape seed extract and cacao seed extract contain the highest concentrations of proanthocyanidins among other extracts.(8) They have been previously shown to be able to improve the mechanical properties of demineralized dentin.(6)

Although, the exact mechanism of cross-linking is not completely understood, yet, multiple theories explain the interactions of PAs with proteins. These include covalent, hydrogen bonding, ionic and hydrophobic interactions. Hydrogen bonds between the protein amide carbonyl and phenolic hydroxyl groups are suggested to be the crucial forces that stabilize the PA cross-linked collagen fibrils. This stabilization is reflected on an increase in collagen matrix stiffness.(9)

Grape seed extract and cacao extract are among the most tested natural cross-linkers in terms of resin-dentin bond strength and durability.(2, 10) Yet, up to the extent of our knowledge, literature lacks sufficient data that investigate dentin matrix stiffness as an adjunct to bond strength testing.

Hence, this study was conducted to investigate the effect of grape seed extract and cacao extract on the 24 hour microtensile bond strength and dentin matrix stiffness. The first null hypothesis tested was that the pretreatment of acid etched dentin with the extracts provided no significant difference in microtensile bond strength among groups. The second hypothesis tested was that there was no difference among the tested groups in terms of dentin matrix stiffness.

Materials and Methods

1. Materials

The extracts used in this study, their composition, solvent, scientific names, manufacturers and lot numbers are shown in Table 1.

Table 1: Extracts used in the study, their composition, solvents, scientific names, manufacturers and lot numbers.

Extract	Composition	Solvent	Scientific name	Manufacturer	Lot no.
Grape seed	6.5% grape seed extract (w/v)[10, 28]	Hot distilled water	*Vitis Vinifera*	Swansons Health Producs, Inc. [33](Fargo, ND, USA)	B 224472
Cacao seed	6.5% Cacao extract (w/v)[10]	50% ethanol / 50% distilled water	*Theobroma Cacao*	Swansons Health Producs, Inc. (Fargo, ND, USA)	B 226185

2. Methods

2.1. Specimens grouping for microtensile bond strength testing

Nine molars were used to obtain a total of 45 specimens for micro-tensile bond strength testing. Specimens were divided into 3 groups (n=15) according to the dentin pre-treatment applied (after acid etching and before bonding):

Table 2: Materials used in the study, their description, composition, and lot numbers.

Material	Description	Composition	Lot no.
Filtek™ Z250 XT (3M ESPE, St.Paul, MN, USA)	Nano Hybrid Universal Restorative composite Shade A3	-**Resin:** Bis-GMA, UDMA, Bis-EMA, PEGDMA and TEGDMA -**Fillers :** Surface-modified zirconia/silica with a median particle size 3 microns or less - **Filler loading :** 82% by weight , 68% by volume	N882895
Adper™ Single Bond 2 (3M ESPE, St.Paul, MN, USA)	Total-etch, visible light activated bonding agent	-**Resin:** Bis-GMA, HEMA, dimethacrylates, ethanol, water, photoinitiator system, methacrylate functional copolymer. -**Filler:** 10% by weight 5 nanometer diameter spherical silica particles	N886065
Scotchbond™Etchant (3M ESPE, St.Paul, MN, USA)	Acid-etchant	35% phosphoric acid by weight	N881090
Ethanol (Gomhoureyya Chemicals, Cairo,EG)	Ethyl Alchohol C_2H_5OH	99% Ethyl alchohol	M1911025
Phosphoric acid (Gomhoureyya Chemicals, Cairo,EG)	H_3PO_4	100% Phosphoric acid	M1905021
Methanol (LabChem Chemicals, Cairo,EG)	CH_3OH	80% Methyl alchohol	M1812016

- **Group 1:***(Control group)* acid etched dentin bonded with a 2-step etch and rinse adhesive with no pre-treatment to dentin
- **Group 2:***(GSE)* acid etched dentin pre-treated with grape seed extract solution
- **Group 3:***(CSE)* acid etched dentin pre-treated with Cacao seed extract solution

2.2. Specimens' preparation for micro-tensile bond strength testing

Nine freshly extracted sound permanent molars were obtained from the Oral Surgery department, Badr university in Cairo. The teeth were cleaned from debris and kept in 0.1% Thymol and used within 3 months from extraction.[11]

Teeth were embedded in cylindrical moulds with self-cured acrylic resin 2mm below the cemento-enamel junction for easier handling of teeth. [12] The occlusal tooth enamel was cut horizontally using medium grit diamond burs (SSwhite, Lakewood, NJ, USA.) under extensive water cooling, until the last remnant of occlusal enamel was removed, reaching superficial dentin and obtaining a flat dentin surface. The teeth were randomly assigned to their respective group so that, every group included 3 teeth.[11]

The exposed dentin surface was acid etched for 15 seconds using 35% phosphoric acid and rinsed for 15 seconds using a three-way air/water syringe. After rinsing, each specimen was gently air-dried for 15 seconds at a distance of 10 cm.[11]

The etched dentin was pre-treated with the respective treatment solution by a single application of a fully-saturated microbrush. The pre-treatment solution was left on the dentin surface for 1 minute,[13, 14] after which dentin was blot dried using filter paper.[11]

The dentin adhesive was then applied by a dentin microbrush on the demineralized dentin surface for 10 seconds. The adhesive was air-dried using a 3-way syringe for solvent evaporation then, light cured for 10 seconds using a LED light curing unit with an output intensity of 850MW/Cm^2. Four mm of resin composite were built on top of the bonded dentin surface in 2 increments, 2mm each where, each increment was light cured for 20 seconds.

2.3. Specimens' testing for microtensile bond strength

After storing in distilled water for 24 hours, the teeth were longitudinally sectioned into beams (1x1x8) using a sharp diamond saw (Isomet, Buehler Ltd, Lake Bluff, IL, USA). Beams were attached to a metal fixture and loaded in a tensile mode on a universal testing machine (3345 Series, Instron, IL, USA) using a 500N load cell at a cross-head speed of 1mm/min. Calculation of the bond strength was done using the following equation[15]: $\mu TBS = (F/A)$, Where F: Force , A: Area.

2.4. Failure mode assessment

Failure mode pattern was examined using a stereomicroscope (SZX7, Olympus Corporation, Tokyo, Japan) under magnification of 40X and was classified as:

Adhesive(A) when failure occurred at resin/dentin interface.

Cohesive in dentin(CD) when failure occurred in dentin only.

Cohesive in composite(CC) when failure occurred in resin composite only.

Mixed(M) when the failure was both adhesive and cohesive.

2.5. Specimens' grouping for dentin matrix stiffness test

Nine teeth were used to obtain 45 specimens (dentin beams) for measuring demineralized dentin stiffness. Specimens were divided into 3 groups(n=15) according to the dentin treatment after demineralization into:

Group 1:*(Control group)* acid demineralized dentin beams with no treatment.

Group 2:*(GSE)* acid demineralized dentin beams treated with grape seed extract solution.

Group 3:*(CSE)* acid demineralized dentin beams treated with cacao seed extract solution.

2.6. Specimens' preparation for dentin matrix stiffness test

The occlusal enamel of each tooth was horizontally removed to expose the superficial dentin using an Isomet low-speed precision saw under copious water cooling. The teeth were horizontally cut again 1mm below the dentin surface to obtain a 1mm thick disc. Dentin beams (6.5x1x1mm) were then obtained via sagittal cuts through the dentin disc using the Isomet saw.

The beams were then completely demineralized by immersion into 10% wt phosphoric acid for 5 hours and rinsed with distilled water for 10 minutes to remove remnants of the acid. [7, 16]

Fifteen beams (n=15) were randomly assigned for each group, the beams were immersed for 1 minute in their respective treatment then rinsed and tested.[1] The control group was not subjected to any treatment.

2.7. Testing procedure for dentin matrix stiffness test

The treated dentin beams were subjected to a 3-point bending test. Each dentin beam was supported by two rods with a span of 2.5 mm.[17, 18] The test was performed using the universal testing machine with a load cell of 500N and at a crosshead speed of 1 mm/min up to the maximum displacement.[1] The load-displacement curve was converted into a stress-strain curve and the elastic modulus (E) was determined at the steepest most linear portion of the curve with the following formula:[14] $E = mL^3/4bd^3$ Where m=slope (N/mm); L=support span(mm); d=thickness of beam (mm); b= width of beam(mm).

2.8. Statistical analysis

The data obtained were subjected to statistical analysis using IBM® SPSS® Statistics Version 23 for Windows®. Data were explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests. Microtensile bond strength (MPa) results showed a parametric distribution. Data were presented as mean and standard deviation (SD) values. One-way ANOVA was used to study the effect of the type of extract followed by Duncan post-hoc test for pairwise comparisons. For dentin stiffness results, one-way ANOVA was used to compare between types of extracts followed by Duncan post-hoc test for pairwise comparisons. The level of significance was set at $p \leq 0.05$.

Results

3.1. Micro-tensile bond strength test results

Mean and standard deviation (SD) values for μTBS (MPa) using different extracts are presented in table 3.

Table 3: Mean and standard deviation (SD) values for μTBS (MPa) using different extracts

	Control		GSE		CSE		p-value
	Mean	SD	Mean	SD	Mean	SD	
24 hours	24.7	4.5	31.1	6.6	27.2	6.2	0.122 NS

NS= Not significant

After 24 hours, there was no significant difference in μTBS mean values reported for the different tested groups (p=0.131).

3.2. Failure mode analysis results

Failure mode analysis of the tested groups is presented in table 4 as percentages and was classified as (A)Adhesive, (CD)Cohesive in dentin, (CC)Cohesive in composite and (M)Mixed.

Mixed failures were predominant in all groups. Grape seed extract showed a higher percentage of mixed failures compared to the control and cacao seed extract groups.

Table 4: Failure mode distribution pattern (%) among types of extracts and storage periods (A)Adhesive, (CD)Cohesive in dentin (CC)Cohesive in composite, and (M)Mixed.

	A	CD	CC	M
Control	27%	12%	0%	61%
Grape seed extract	14%	0%	13%	73%
Cacao seed Extract	27%	6%	0%	67%

3.3. Dentin matrix stiffness test results

Mean and standard deviation (SD) values for the modulus of elasticity (MPa) of different groups are presented in table 5.

Table 5: Mean and standard deviation (SD) values for the modulus of elasticity (MPa) of different groups

	Control		CSE		GSE		p-value
	Mean	SD	Mean	SD	Mean	SD	
Modulus of elasticity (MPa)	2.4^{b}	0.4	4.8^{b}	1.32	7.42^{a}	1.63	0.000*

Means with the different superscripts letter within each row are significantly different at p<0.05
**= Significant*

Grape seed extract group showed a higher elastic modulus compared to all groups ($p \leq 0.05$). Control and cacao seed extract showed an insignificant difference between each other ($p > 0.05$).

Discussion

The mechanical strength of the hybrid layer is an essential determinant of the resin-dentin bond strength. Thus, dentin biomodification with collagen cross-linkers was suggested as a method of improving the biological and mechanical properties of dentin.[19] Natural cross-linkers were proposed as an alternative safe and biocompatible approach for biomodification of dentin rather than other chemical cross-linkers as gluteraldehyde that were reportedly toxic. [20]

The selection of the type and concentrations of the extracts used in the current study was based on previous studies. Grape seed extract and cacao seed extracts are two of the richest in PAs concentrations compared to other natural extracts[21, 22]. The extracts concentration used in the study (6.5%) was the most commonly used and most effective concentration in literature.[10, 23] Their effect on resin-dentin bond strength was tested in previous studies.[1, 10, 24]

The µTBS testing method was chosen as it is currently the most recommended approach to test resin-dentin bond strength. [11]

The selection of 1-minute pre-treatment was based on previous studies which was selected to be clinically relevant. [12, 23, 25-27]

Increasing the degree of cross-linking in dentin directly affects the modulus of elasticity.[7, 14] The increase in stiffness of etched dentin can lead to improving the strength of the resin-dentin bond.[10]

Thus, measuring the effect of the cross-linking extracts on both micro-tensile bond strength and dentin stiffness was performed to understand the extent of the effect of collagen stiffness on the bond strength.

The 3-point loading approach was chosen to evaluate the modulus of elasticity. One of the main advantages of this method is that it requires no gripping on such small delicate specimens.[6] The stiffness of dentin was measured after 1 minute (clinically relevant time) as an adjunct to justify the bond strength results.[13, 14]

In the current study, regarding the effect of extract type on micro-tensile bond strength, statistical analysis revealed insignificant difference between all the tested groups (extract groups and no-treatment control group) after 24 hours. Thus, the first null hypothesis was accepted. These results are in agreement with Hass et al.(2016)[23] and Mazzoni et al.(2018)[26] who found that natural extracts had no significant effect on the 24 hours resin-dentin bond strength after 1 minute pre-treatment.

It seems that the 1-minute pre-treatment time used in the study was insufficient to provide a significant change in 24 hours bond strength which was consistent with studies that suggested that the degree of cross-linking using natural extracts is directly proportional to the time of treatment application.[1, 6]

Bedran-Russo et.al,(2008) suggested that cross-linking in natural extracts such as grape seed is dependant on the duration of treatment unlike other synthetic cross-linkers as gluteraldehyde.[6] This is dependant on the mechanism of cross-linking for every extract or chemical used. Gluteraldehyde reacts primarily with the residues of the amino groups within the biological tissue and therefore the reaction was restricted to the availability of the amino groups within the tissue and reached a plateau afterwards, which means that its full potential of cross-linking was reached shortly after treatment. However, proanthocyanidins in grape seed extract cross-links collagen by multiple mechanisms such as: hydrogen, ionic and covalent bonding.[28] Thus cross-linking continues to happen and increase over time. This was supported by Castellan et al.(2010)[1] who confirmed that the effect of natural PAs on dentin cross-linking was dependant on the duration of treatment.

On the other hand, a different study by Castellan et al.(2013)[10]showed that grape seed and cacao extract both provided higher 24 hours bond strength results compared to the control group but the extracts were applied to dentin for 10 minutes. This further supports the results of this study that the short application time was insufficient to affect the 24 hours bond strength. Other studies measured the effects on dentin after 30 mins, 1 hour, and 4 hours[6, 10, 28-30].Although the longer treatment periods provided higher and more significant bond strengths, but none of these time frames were clinically relevant.

Among the factors that can affect cross-linking, is the source of the natural cross-linker used. A previous study concluded that dentin biomodification effect is dependant on the source of the polyphenol and this is due to the different mechanism of interaction of every polyphenol type with collagen proteins.[31] Similarly, there is a potential correlation between molecular weights and their ability to induce non-enzymatic dentin cross-links. This may be as a result of the presence of more OH molecules in larger oligomers that are capable of forming more bonds[31].

In the current study, the modulus of elasticity of demineralized dentin beams was calculated after immersion for 1 minute inside the cross-linking extracts. Grape seed extract showed a significantly higher elastic modulus compared to all other groups. Hence, the second null hypothesis was rejected. This was in line with the failure mode analysis results where grape seed extract group showed no cohesive failures in dentin confirming the increase in the mechanical properties of the treated dentin.[6, 14]

Cacao seed extract provided no significant difference in demineralized dentin stiffness compared to the control. Thus, it could be assumed that the concentration of condensed tannins in cacao seed extract was insufficient to provide adequate dentin biomodification compared to the high concentration of condensed tannins in grape seed extract. Castellan et al.(2013)[10] attributed the higher bond strength to the concentration of PAs present inside the extract where GSE contained 95% PAs while CSE contained only 45%. According to castellan, the low concentration could slow down or even decrease their ability to interact with collagen. In addition, cacao extract has also been described in literature as a potential collagen biomodification agent that requires a longer application time due to its structural complexity.[10, 32]

Based on the results obtained in this study, it could be highlighted that grape seed extract has the ability to improve the stiffness of demineralized dentin in a clinically relevant time. However, the improvement in the modulus of elasticity was insufficient to alter the 24-hour resin-dentin bond strength. The 1-minute treatment time used in the current study probably was insufficient for cacao seed extract to provide enough stiffness to the demineralized dentin matrix.

Nevertheless, further researches to investigate the effect of different concentrations and application times on both the stiffness and bond strength are still required.

Conclusions

1. Pre-treatment of acid-etched dentin with the tested natural cross-linkers for 1 minute has no effect on 24 hour microtensile bond strength.

2. Treatment of demineralized dentin with grape seed extract for 1 minute significantly increased dentin matrix stiffness.

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