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EFFECT OF MATRIX METALLOPROTEINASE INHIBITOR FROM MULBERRY FRUIT EXTRACT ON THE MICROTENSILE BOND STRENGTH STABILITY: AN IN VITRO STUDY

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ABSTRACT

Objectives: The present in vitro study aimed to analyze the effect of two Mulberry fruit extracts on microtensile bond strength of an etch & rinse adhesive immediately and after thermocycling.

Materials and Methods: Flat dentin surfaces of 30 sound human molars were bonded with an etch and rinse adhesive. Dentin surfaces were left untreated (control group) or were pretreated with either *Morus alba* water extract, *Morus alba* alcohol extract, *Morus nigra* water extract or *Morus nigra* alcohol extract. Nano-hybrid resin composite were incrementally built-up. The tooth/composite specimen was serially sectioned in order to produce beams of 0.9±0.1 mm in thickness. Each group was subdivided into two subgroups whether the teeth/composite specimens subjected to thermocycling or not. Each beam was individually fractured by a micro tensile testing machine. The data recorded in MPa, were tabulated and statistically analyzed.

Results : Specimens treated with *Morus alba* and *Morus nigra* water extracts yielded significantly higher mean bond strengths than alcohol extracts in immediate micro-tensile bond values and after thermocycling. In addition ,*Morus alba* water extract revealed no statistically significant difference immediately and after thermocycling.

Conclusion : Dentin pretreatment with Mulbery water extracts has no adverse effect on the immediate microtensile bond strength and it was able to maintain bond stability of adhesive to dentin.

KEY WORDS: Mulberry, Microtensile bond strength, Matrix metalloproteinases

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INTRODUCTION

The main objective of adhesive dentistry is to improve the bond durability between tooth structure and the restorative material. Matrix metalloproteinases (MMPs) and cysteine cathepsins contribute to the enzymatic degradation of the collagen matrix[1,2]. MMPs are present in dentin matrices as inactive proenzymes, but promote the degradation of collagen fibril and extracellular matrix when activated[3]. The low-pH environment caused by acid etching or acidic monomers can modulate the activity of MMPs and the interaction with cysteine cathepsins[4,5]. The degradation occurs in consequent stages: first water is absorbed by the polymer then resin components are leached from the hybrid layer; and finally, the exposed collagen fibrils are degraded by (MMPs) and/or by cysteine cathepsins [6-8]. MMP-8 is capable of converting type I into fragments of 3/4- and 1/4-length [9].

The understanding of the mechanism collagen degradation has encouraged the evaluation of substances that may inhibit the function of both MMPs and cysteine cathepsins. Different strategies have aimed to preserve the collagen fibrils using different matrix metalloproteinase inhibitors as a pretreatment or by admixing it to primers[10-14]. The application of chlorhexidine digluconate (CHX) has been found to be valuable in the preservation of the bond strength over time[15,16] . Recent researches have highlighted the toxic effect of CHX on both odontoblast-like cells[17], and stem cells [18]. Therefore, it is relevant to find an alternative MMP inhibitor to enhance bonding durability. Various compounds from natural sources have shown promising inhibition of matrix metalloproteinases especially some alkaloids, flavonoids and phenolic compounds from plant sources.

The anthocyanin group of polyphenols demonstrated beneficial pharmacological activities as anti-inflammatory, antioxidant and chemoprotective properties[19]. It was found to reduce the expression of MMP and stimulating the expression of matrix metalloproteinase-2 inhibitor (TIMP-2) [20]. Anthocyanins responsible for red, purple, and blue colors of vegetables and some tropical fruits such as berries, grapes which have high anthocyanins content [21].

Mulberry fruits are valuable horticultural sources of health benefits compounds. They belong to the genus *Morus*, family Moraceae which is composed of 10–16 species of deciduous trees and cultivated in many temperate world regions[22]. *Morus nigra known as* black mulberry and *Morus alba* known as white mulberry fruits*are* rich in many bioactive components such as alkaloids, flavonoids and carotenoids, fats, vitamins, and minerals [23].

Mulberry exhibit many biological activities such as prevention and treatment of human cancer, cardiovascular disease and modulates several apoptotic pathways and matrix metalloproteinases (MMPs) [24,25]. *In addition, Morus alba* root bark extract exhibits antibacterial activity against oral pathogens (*Streptococcus mutans*) causing dental caries[26]. While *Morus alba* L. fruit exhibits hypolipidemic and antioxidant effects[27-29]. The bioactivity of mulberry fruits was linked to the presence of phenolic compounds such as anthocyanins. Anthocyanins compounds isolated from *Morus nigra* and *Morus alba* fruits are cyanidin 3-O-rutinoside and cyanidin 3-O-glucoside which reduced extracellular matrix proteinases including matrixmetalloproteinase-2 (MMP-2)[30,31]. Moreover, it contains flavonoid compounds such as kaempferol, rutin, scopolitin and quercetin in addition to other essential organic acids and vitamins such as vitamin C [23].

However, to date, the efficacy of fruit extracts MMP inhibitors in dentistry has not been completely investigated. Thus, the aim of the present study was to determine the microtensile bond strength (TBS) of an etch and rinse adhesive after pretreatment with different Mulberry fruit extracts immediately and after thermocycling. This study tested the null

hypothesis that: A) micro TBS is not affected by pretreatment extracts and B) aging of resin-dentin bonds is not adversely affected by thermocycling.

MATERIAL AND METHODS

Preparation of total alcohol and water fruits extracts

Two grams of air-dried powdered fruits of both *Morus nigra* and *Morus alba* were extracted using methanol (50ml × 2) at room temperature. The combined extracts were evaporated in rotary evaporator (Buchi, G. Switzerland) to dryness under reduced pressure to yield 0.47 gm and 0.53 gm respectively. The exctracted powder samples were dissolved in100 ml distill water by stirring for 1 hr using Magnetic stirrer (R. Espinar, S.L.) then filtered using Whatman (No.1) filter paper.

For water extracts, each of 2 gm air-dried powdered fruits of both *Morus nigra* and *Morus alba* were topped with 100ml boiled distilled water and incubated for 2 hours at room temperature then filtered using Whatman (No.1) filter paper.

Sample preparation

Thirty non-carious human molars were collected for this study.All collected teeth were extracted for therapeutic reasons from patients of age group (35-45 years). Selected teeth were free of caries and showed no hypoplastic defects or cracks. The selected teeth were thoroughly cleaned from calculus, tissue deposits, polished with pumice and rotating brush at conventional speed. The teeth were stored in saline solution at room temperature and were used within four weeks after extraction.

A specially fabricated cylindrical, split Teflon mould of 19mm height, 22mm external diameter and 17mm internal diameter was used for the fabrication of acrylic resin blocks. Each tooth was vertically embedded into self-curing acrylic resin (Acrostone Dental Factor, England) up to the level of the cervical line with their occlusal surface being parallel to the acrylic resin base.

Grouping

The teeth were allocated randomly to five groups of 6 teeth each, according to the dentin surface pretreatments, namely Group 1: water extract of *Morus alba* fruit; Group 2: alcohol extract of *Morus alba* fruit; Group 3: water extract of *Morus nigra* fruit; Group 4: alcohol extract of *Morus nigra* fruit and Group 5: no pretreatment (control). Each group was further subdivided into two subgroups according to time of bond strength testing whether immediately after storage in water for 24 hours or after thermocycling.

Teeth preparation

Occlusal enamel of teeth was removed perpendicular to tooth long axis and parallel to acrylic resin base to obtain flat mid coronal dentin surface at a standardized depth at approximately 4mm above cementoenamel junction using a slow speed diamond saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA). Exposed dentin surface was ground with rotary grinding milling machine using #180-grit silicon carbide paper (Gamberini s.r.1, Via Della Bastia, Caslecchio Di Reno, Italy) under continuous water coolant to create a uniform thickness of smear layer.

Bonding procedure

Etch and rinse adhesive system was applied to dentin surfaces following the manufacturer's instructions. Etching of exposed dentine was performed for 15 seconds using Scotch bond™ universal Etchant (3M ESPE, USA). Fruit extracts were then applied on etched dentin for 60 seconds with microbrush under a slight rubbing motion. The excess was removed using an absorbing paper, leaving dentin with a glistening, moist aspect. The adhesive system was then applied with a microbrush in two consecutive coats followed by air blow

for five seconds and light cured for 10 seconds with LED light curing unit Light (Elipar LED Curing Light; 3M ESPE) at an output intensity of 700 mW/cm^2.

Resin Composite restorative material application

A specially constructed two halves spilt Teflon mold with a central square hole (5mm X 5mm in diameter and 4mm in depth) was constructed for composite build up. Nano filled resin composite (**Z 350**, 3M ESPE, USA) was built up into two increments each 2mm in thickness. Each increment was inserted using Teflon tipped instrument and photoactivated for 20 seconds with the same LED light curing unit and output intensity. The specimen was stored in distilled water at 37 °C for 24 h to ensure sufficient polymerization.

Beam preparation

After 24 hours storage in water at 37°C , mounting the tooth/composite block in the gripping attachment was done. The tooth/composite specimen was sectioned into sticks , using a 0.3-mm thick diamond coated disc (Buehler, IL, USA), at 2050 rpm; 8.8 mm/min feeding rate under copious coolant, mounted in an automated diamond saw (*Isomet 4000, BuehlerLtd., Lake Bluff, IL, USA*). Sectioning was done first in a bucco-lingual direction then mesio-distally by rotation 90° clockwise. Resultant beam was 0.9±0.1 mm in thickness and 3.5±1 mm in length. The four central sticks from each specimen were selected and their thickness was checked using a caliper. Beams were stored in distilled water at room temperature in a tight-seal plastic cone.

Micro-tensile bond strength measurement

Each beam was held on the universal testing machine (Instron, MA, USA) through cementation in the groove of the Geraldeli's jig by its ends using cyanoacrylate-based glue (Zapit, DVA Inc, USA). Applying the tensile load, at a cross-head speed of 0.5 mm/min, was done till the bonded beams failed. The strength of microtensile bond was calculated in Mega Pascal (MPa) (Bluehill Lite software, Instron, MA, USA).

Thermo-cycling

The specimens were subjected to 1000 thermal cycles in three water baths for 20 seconds with temperature of 5°C followed by 55°C for 20 seconds each, with an intermediary bath 37°C.

Statistical analysis

Statistical analysis was performed with IBM® SPSS® Statistics Version 20 for Windows .The bond strength values were statistically analyzed for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests and showed parametric (normal) distribution. For comparison between more than two groups in non-related samples One way ANOVA followed by Tukey post hoc test was used. In non-related samples independent sample t-test was used. The significance level was set at $P \leq 0.05$.

RESULTS

The mean micro TBS for each group was computed (Table1 ,Figure 1). There was a statistically significant difference between immediate micro TBS of different groups. The highest mean value was found in water extracts of both *Morus nigra* and *Morus alba* followed by control group while the least mean value was found in alcohol extracts. The micro TBS of the etch and rinse adhesive decreased after thermocycling with non-statistically significance difference. The highest mean value was found in water extracts of both *Morus alba* and *Morusnigra*. In addition, a statistically significant decrease was found between immediate bond strength values and after thermo cycling in all groups except in *Morus alba* extracts in which the initial high bond strength microTBS value declined only slightly but not significantly after thermocycling. The results of Two way ANOVA showed that different tested groups and thermocycling had a statistically significant effect .However, the interaction between the two variables had no statistically significant effect (Table 2).

TABLE (1): The mean, standard deviation (SD) values of tensile bond strength of different groups.

Variables	Tensile bond strength				
	Before thermocycling		After thermocycling		*p-value*
	Mean	SD	Mean	SD	
Control	28.38 ^{abA}	6.68	17.39 ^{aB}	1.71	**0.007***
MA/W	29.30 ^{abA}	7.31	20.55 ^{aA}	8.85	**0.127ns**
MA /A	17.39 ^{bA}	1.63	10.26 ^{aA}	8.28	**0.096ns**
MN /W	35.03 ^{aA}	5.24	20.60 ^{aB}	5.97	**0.004***
MN /A	19.72 ^{bA}	8.82	18.05 ^{aA}	7.84	**0.760ns**
p-value	**0.002***		**0.166ns**		

Means with different small letters in the same column indicate statistically significant difference, means with different capital letters in the same row indicate statistically significant difference. *; significant (p<0.05) ns; non-significant (p>0.05)

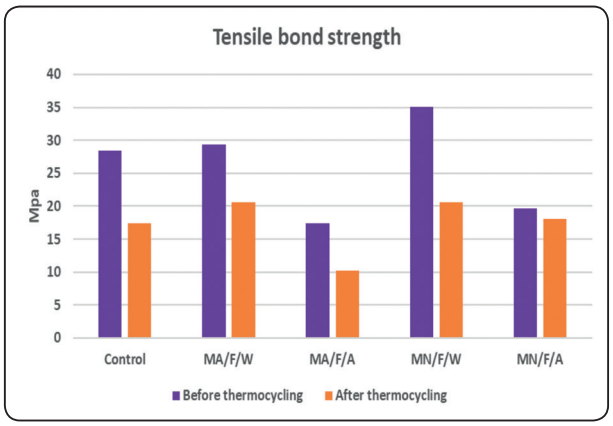

Fig. (1) Bar chart representing tensile bond strength

DISCUSSION

The understanding that dentin matrix metalloproteinases and cysteine cathepsins are both involved in the hybrid layer degradation process has driven the search for natural products that deactivate these enzymes. Thus, this study aimed at assessing the effect of pretreatment with Mulberry fruit water and alcohol extracts on both immediate bond strength and after thermocycling. The null hypothesis that the micro TBS is not affected by pretreatment with fruit extracts was corroborated with alcohol extracts. However, pretreatment with all water extracts prevented micro TBS loss of etch and rinse adhesive significantly.

The set up of the present study was done using mid coronal flat dentin surfaces as the bond was shown to be directly affected by dentin location and the cavity configuration [32,33]. Also ,mid coronal flat dentin surfaces allowed for the use of multiple beams per tooth. Etch and rinse adhesive system was selected due to the significant levels of MMP-2 and MMP-9 activity in comparison with self-etching adhesives[34]. The tested solutions were applied in a separate step with 60 seconds application time, which seems realizable under clinical conditions. In addition, the addition of MMP inhibitors into adhesives might affect the degree of conversion and E-modulus of products[35]. Long term water storage and thermocycling are the most commonly used artificial ageing technique. The bond durability might become more apparent with thermocycling[13]. The ISO TR 11450 standard indicates that 500 cycles with alternate temperatures of 5°C and 55°C

TABLE (2) Results of Two-way ANOVA for the effect of different variables on Tensile bond strength.

Source of variation	Type III sum of Squares	df	Mean Square	*F* - value	*P* - value
Groups	1191.383	4	297.846	6.580	.000*
Thermocycling	922.477	1	922.477	20.378	.000*
Groups x Thermocycling interaction	224.843	4	56.211	1.242	.309ns

df:degrees of freedom=(n-1),*Significant atP≤0.05

is a suitable regime for accelerated aging [36]. Gala and Darvell indicated that 10,000 cycles would be equivalent to one year of in vivo function [37]. So, 1000 thermal cycles would be equal to 1.2 months aging.

Natural products have always been a rich source of biologically active compounds. Some of these products are not used for this purpose in dentistry, but are widely used in other medical fields. The present study was conducted on natural products that have been reported in literature to have anti-MMP potential. Among them, we selected Mulberry fruits because of it contains high contents polyphenols[38].

Various extraction conditions are reported, however there is no standard method for extraction [39,40]. The most widely used solvents for extracting phenolic compounds are water, alcohols and their water mixtures, with acid or not [41,42]. However, methanol and water were the most efficient solvents for the extraction due to the better solvation of antioxidant compounds as a result of interactions between the polar sites of the antioxidant molecules and the solvent. In addition, methanol and water, are also proton donors[43]. However, the chemical nature of phenolic compounds, method of extraction employed, storage time and conditions affect the efficiency of the extraction methods [44]. This may explain the results obtained in this study, where a significant difference between water and methanol extracts in both *Morus* species was detected. The mean micro TBS value of *Morus nigra and Morus alba* water extract is greater than its alcohol extract due to total anthocyanin pigment of water extract is higher than of its alcohol extract which is expressed as mg of cyanidin-3-O-glucoside[45,46].

For both *Morus* extracts, pretreatment with water extracts were able to maintain immediate dentin bond strength. Pretreatment with *Morus Nigra* resulted in significant increase in micro TBS value. Several factors may have accounted for the observed results which is mainly related to the reduction of enzymatic dentin degradation. *Morus nigra* and *Morus alba* fruits are rich in anthocyanins[47], which are water-soluble pigments and present in our plant-based diet with little or no known toxicity[48]. Anthocyanins compounds isolated from *Morus nigra* and *Morus alba* fruits are cyanidin 3-O-rutinoside and cyanidin 3-O-glucoside which reduced extracellular matrix (ECM) proteinases including matrixmetalloproteinase-2 (MMP-2) [31,47]. Moreover, *Morus nigra* and *Morus alba* fruits rich in phenolic compounds such as catichin, epicatichin and gallic acid [49,50], which make inhibition of matrixmetalloproteinase-2 (MMP-2)[51]. Moreover, Catechin, epicatichin and gallic acid have been classified as a proanthocyanidin which acts as natural dentin biomodifier[52].

The mean micro TBS of *Morus nigra* extracts is greater than that of *Morus alba* extracts due to the content of anthocyanidins compounds (cyanidin-***3-O***-glucoside, cyanidin-***3-O***-rutinoside) in *Morus nigra*is higher than that present in *Morus alba* extract [53]. In addition, the total phenolic contents catichin,epicatichin and galic acid of *Morus nigra* fruit extract is higher than that was present in *Morus alba* fruits [54]. Ercisli and Orhan (2007) concluded that total phenolic and flavonoids content are affected by both plant geno type and cultivation[50].

As mean micro TBS were affected by thermocycling, the second null hypothesis that aging of resin- dentin would not affected by the thermocycling is rejected. It was anticipated that micro TBS of all tested groups declined after thermocycling. This could be contributed to water diffusion into the adhesive interface which could be expediated especially after the teeth had already been sectioned into sticks [55]. However, *Morus* water extracts was able to reduce the decline in dentin bond strength after thermocycling as compared with other groups but not prevented. This could be attributed to proanthocyanidin content that functions as dentin collagen matrix stabilizer thus increasing the resistance to biodegradation[56,57]. In addition, the hydrophobicity of proanthocyanidin modified collagen films improved due to cross-linking

between proanthocyanidin and collagen which in turn prevented moisture permeation[58]. Moreover, Vitamin C content increases the level of messenger RNA of collagen I and III and act as MMP inhibitors. This vitamin C plays an important role stabilizing the helical configuration of collagen because it is a cofactor for the amino acids hydroxyproline and hydroxylysine [59,60].

The application of *Morus* water extracts was found to be beneficial to the bond strength of the etch-and-rinse adhesive and play a crucial role in bond durability. This provides the justification for further researches that are designed to investigate the effects of natural extracts on the longevity of resin-dentin bonds with longer thermocycling regimes to stimulate longer in vivo service periods of restorations.

CONCLUSION

With the limitations of this study, it may be concluded that pretreatment with Mulbery water extracts has no adverse effect on the immediate microtensile bond strength and it was able to maintain bond stability of etch and rinse adhesive to dentin.

Conflict of Interest

The authors of this manuscript certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

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