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Effect of green tea and two mulberry leaf extracts on micro-tensile bond strength to dentin

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ABSTRACT

Objective: This study was conducted to compare the effect of green tea and two Mulbery leaf extracts on micro-tensile bond strength immediately and after thermocycling.
Material and methods: 42 freshly extracted molars were utilized in this study. Occlusal enamel was removed to expose mid coronal dentin and they were randomly divided into seven groups: G 1: Green tea water extract; G 2: Green tea alcohol extract; G 3: *Morus nigra* water extract; G 4: *Morus nigra* alcohol extract; G5: *Morus alba* water extract; G 6: *Morus alba* alcohol extract and Group 7: no pretreatment (control). Adhesive system was applied in etch and rinse mode, and resin composite were built. The blocks were sectioned and they were either tested for microtensile bond strength immediately or after thermocycling. Data were tabulated and statistically analyzed using parametric test.
Results: There was a statistically significant difference between tested groups in immediate micro-tensile strength values and after thermocycling. After thermocycling the micro-tensile bond values were decreased regardless of the treatment applied to dentin.
Conclusion: Green tea water extract and *Morus* species alcohol extracts has no adverse effect on micro-tensile strength. The application of natural extracts does not prevent loss of micro-tensile bond strength with thermocycling.

1. Introduction

The success of adhesive dentistry is to create an effective, durable bond. The preservation of integrity of collagen network is important for improving bond durability. Resin-infiltrated hybrid layer is the most important factor that determine bond integrity, stability and longevity [1,2]. The incomplete resin infiltration at the bottom of hybrid layer will result in incomplete collagen encapsulation and enhance the proteolytic degradation by the action of collagenolytic enzymes such as matrix metalloproteinase (MMPS) and cysteine cathepsins [3–5]. The stabilization of collagen with biocompatible cross-linking agents and the use of matrix metalloproteinase (MMPs) inhibitor to decrease enzymatic degradation may be clinically beneficial in improving bond strength to dentin [6].

Various synthetic cross-linkers have been evaluated such as formaldehyde and glutaraldehyde. However, every one of them has some disadvantage, such as mismatch mechanical properties, high toxicity and non-significant long-term stability. Proanthocyanidins (PAs), a plant flavonoid, has recently been use as natural cross-linking agents and they could inactivate more than 90% of MMPs [6,7]. They are plant metabolites naturally occurring in vegetables, fruits, barks and seeds. Bilberry, cranberry, apple, grape seed, black tea and green tea contain these flavonoids.

Matrix metalloproteinases (MMPs), also known as matrixins, are a group of zinc metallo-endopeptidases secreted by cells of connective tissue such as fibroblasts, osteoblasts and odontoblasts [8–10]. Matrixins consists of 28 members including the membrane type matrix metalloproteinases. They are physiologically inactive, and they are responsible for the matrix components turnover. Activation of these enzymes may result from pH fluctuation due to cariogenic challenges, acid etching or acidic dental adhesive monomer [11]. As a result, collagenolytic activities increase and finally the bond strength gradually

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decreases [4,9–11]. However, the collagenolytic activity of MMPs is inhibited by different endogenous tissue inhibitors of metalloproteinases (TIMPs) such as TIMPs (1–4).

Different attempts have been discussed to improve the bond durability as pretreatment with natural or synthetic MMP inhibitors before application of adhesive resin. Synthetic inhibitors include doxycycline, marimastat and trocade. Beside these products, chlorhexidine digluconate (CHX) and Ethylenediaminetetraacetic acid (EDTA) have been promoted and widely used as a synthetic inhibitor of MMPs based on their zinc and calcium chelator property, thus preventing the disintegration of the bonding interface [12–16].

Recently, the use of natural products for the inhibition of MMPs has gained increasing attention. Several compounds have shown promising results regarding matrix metalloproteinases inhibition such as alkaloids, flavonoids, and phenolic compounds from plant sources. Marine source compounds such as Fucoidan extracts from seaweeds *Claisiphon novaecaledoniae* **and** Ageladine A from the marine sponge have also shown profound inhibition [17] In addition, several compounds from plant origin are described as natural matrix metalloproteinase inhibitor such as A barbadensis Miller (Aloe vera) [18] curcumin from *Curcuma longa*, flavonoids from *Passiflora foetida* [19].

Green tea leaves (*Camellia sinensis*) belong to genus *Camellia*, family Theaceae. Green tea has been described as a natural MMPs inhibitor [16]. *Camellia sinensis* contains alkaloids, amino acids, polyphenols (catechins, flavonoids), polysaccharides, lipids, volatile acids, vitamins as well as inorganic elements [21–23]. The polyphenols named catechins are strongly related to MMP inhibition [20]. The health benefits of tea depends on various factors such as climatic conditions, plucking season, processing, extraction method, storage and drying [24]. Green tea extract has antioxidant, anticarcinogen, anti-inflammatory activity [25,26].

Morus nigra (black mulberry) and *Morus alba* (white mulberry) leaves belong to the genus *Morus* family Moraceae. 24 different species of *Morus* were discoverd [27]. *Morus* species demonstrate important biological activities such as antioxidant activity, neuroprotective effect [28], neutralized the edema, hemorrhage [29] and anti-inflammatory activity [30]. Mulberry is effective in treatment of obesity, liver and cardiovascular diseases, diabetes and block cancer progression by modulating several apoptotic pathways and matrix metalloproteinases (MMPs) [31,32]. The biological activity of *Morus* have been attributed to its flavonoids content such as quercetin 3-(-malonylglucoside), rutin, isoquercitin, cyanidin 3 rutinoside and cyanidin 3-glucoside [33,34]. Mulberry leaf has been described as a natural MMP-2 and MMP-9 due to its high polyphenols content [35]. The chemical composition of plant extracts is significantly affected by the type of solvent. Water and alcohol are considered as typical solvents for isolation of biologically active ingredients [6].

However, the need for highly selective and more potent inhibitors remains a mainstay necessitating the exploration of more potent natural products. The null hypothesis tested was that the tested MMP inhibitors would not have any effect on bond stabilization. The aim of this in-vitro study was to compare the bond strength of green tea extract and *Mulbery* extracts immediately and after thermocycling.

2. Material & methods

2.1. Experimental design

Two factors were evaluated: first, the pretreatment solutions applied after acid conditioning i.e.: Group 1:Green tea water extract; Group 2: Green tea alcohol extract; Group 3: *Morus nigra* leaves water extract; Group 4: *Morus nigra* leaves alcohol extract; Group 5: *Morus alba* leaves water extract; Group 6: *Morus alba* leaves alcohol extract and Group 7: no pretreatment (control). Second, time of micro-tensile bond strength testing namely: after 24 h water storage and after thermocycling. The quantitative outcome variable was bond strength value expressed in MPa.

2.2. Methods of leaves extraction

Samples of leaves of *Morus nigra*, *Morus alba* family (Moraceae) and *Camellia sinensis* family (Theaceae) grown in Egypt were collected during August 2017. Identification of the plants material were verified by Dr. Therese Labib senior head of specialist for plant identification, Orman Botanical Garden, Giza, Egypt.Three voucher specimens (No. MN-2 *Morus nigra*, MA-3 *Morus alba* and CS-4 *Camellia sinensis*) were deposited in the herbarium of Pharmacognosy Department, Faculty of Pharmacy, Ahram Canadian University. Samples of *Morus nigra*, *Morus alba* and *Camellia sinensis* leaves were dried naturally during one month and grounded in a blender before extraction.

For total alcohol extracts, each of air-dried powdered leaves of *Morus nigra* (2 gm.), *Morus alba* (2 gm) and *Camellia sinensis* (2 gm) were extracted with 70% (methanol: water) (50 ml × 2) at room temperature. The combined methanolic extract for each plant was evaporated to dryness under reduced pressure using rotatory evaporator (Buchi, G. Switzerland) to yield 0.4 gm, 0.43 gm and 0.58 gm respectively then each of them is dissolved in100 ml distill water by stirring for 1 h using magnetic stirrer (R. Espinar,S.L.) then filtered using Whatman (No.1) filter paper.

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

2.3. Tooth selection and preparation

42 freshly extracted intact human maxillary molar teeth, from an age of 20–40 years, extracted for therapeutic reasons, were selected in this in-vitro study. Immediately after extraction, they were washed, scrubbed and scaled carefully using hand scaler (Dentsply Ash instruments, Surrey, UK) to remove any remnants of periodontal ligaments, plaque and blood. Then they were examined using magnifying lens to exclude fractures, cracks, caries or other defects. The selected teeth were stored at room temperature in distilled water and utilized within two weeks of extraction.

A specially fabricated metal mold (15 mm diameter and 40 mm height), was used to fabricate acrylic resin blocks. Every prepared tooth was impressed vertically parallel to the long axis of the mold in a centralized position during setting of acrylic resin. Occlusal surfaces of the teeth were grinded until the dentino-enamel junction using low speed diamond saw *(Buehler, Lake Bluff, IL, USA)*. Then a line was drawn 2 mm below DEJ using a caliper. The teeth were cut horizontally and flattened under copious water coolant. The dentin walls were ground with 320–400/grit silicon carbide abrasive papers (Recife, PE, Brazil). The surfaces were verified for the absence of enamel and/or pulp chamber exposition with laboratory magnification lens at 4X magnification. They were finished with 600 grit silicon carbide paper for 20 s to produce a homogenous standardized smear layer.

2.4. Bonding procedure

Etching of exposed dentine surface was performed for 15 s using 37% orthophosphoric acid (N-Etch, Etching Gel, Ivoclar Vivadent), rinsed thoroughly withdistilled water and blotted dry. They were divided into seven groups (n = 6), according to the solution tested. Twenty microliters of tested solutions were applied to dentin for 60 s with a microbrush applicator under slight rubbing motion. The dentin was dried gently with absorbent paper to remove the excess prior to application of bonding agent leaving the dentin surface saturated with moisture. The etch-and-rinse adhesive system (Tetric N-Bond Universal

adhesive, Ivolcar Vivadent) was then applied according to manufacturer's instructions for 5 s in two layers with a micro brush followed by air blow and finally light cured for 10 s using light emitting diode curing unit (LED) (EliparTM S 10, 3M ESPE).

2.5. Resin composite restorative material application

Specially constructed two halves spilt Teflon round mold with a central square hole (5 mm × 5 mm in diameter and 4 mm in depth) was fabricated for resin composite build up. Nano filled visible light resin composite (Ivoclar, Vivadent, A3) was built up into two increments each 2 mm in thickness on dentin surface. Each increment was packed using Teflon tipped instrument then light cured using (LED) with an output of 700 mW/cm2 for 20 s. The output of the light-curing unit was checked with a radiometer (Newdent Equipamentos Ltda., Ribeirão Preto, SP, Brazil). The blocks were then stored at 37 °C for 24 h in distilled water.

2.6. Beam preparation

The composite specimen was serially sectioned, 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, Buehler Ltd., Lake Bluff, IL, USA*). Serial sectioning was done in a bucco-lingual direction then rotated 90° clockwise and sectioned in a mesio-distal direction. Four beam specimens were obtained per tooth with 0.9 ± 0.1 mm in cross section and 5.5 ± 1 mm in length so as to have 24 specimens for each group. A digital caliper (Mitutoyo, Tokyo, Japan) was used to check the thickness and length of all beam specimens. Subsequently, each beam was stored in distilled water at 37 °C in a tight-seal plastic cone labeled according to treatment applied. Then they were assigned into two subgroups (12 beam specimens per subgroup). The first subgroups were immediately tested for micro-tensile bond strength and the second ones were tested after thermocycling in three water baths with different temperatures for 1000 cycles. The specimens were immersed at 5 °C followed by 55 °C for 20 s each, with an intermediary bath 37 °C.

2.7. Micro-tensile bond strength test

For each tested subgroup, 12 beam specimens were tested. Geraldeli's jig was used to mount beams onto the universal testing machine (*Instron, MA, USA*). Each beam was aligned in the central groove of the jig and glued in place by its ends using cyanoacrylate-based glue (Zapit, DVA Inc, USA). Zapit accelerator was used to accelerate hardening of the glue (The jig was in turn mounted into the universal testing machine *(Instron, MA, USA)* with a load cell of 500 N. Tensile load was applied, at a cross-head speed of 0.5 mm/min, until bonding failure of the specimen occurred. Bond strength values were calculated in Mega Pascal *(Bluehill Lite software, Instron, MA, USA)*.

2.8. Statistical analysis

The mean and standard deviation values were calculated for each group in each test. Data were explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests and showed parametric (normal) distribution. One-way ANOVA followed by Tukey post hoc test was used to compare between more than two groups in non-related samples. Independent sample *t*-test was used to compare between two groups in non-related samples. The significance level was set at $P \leq 0.05$. Statistical analysis was performed with IBM® SPSS® Statistics Version 20 for Windows.

3. Results

The mean and standard deviation values (MPa) of studied groups are presented in Table 1 and Fig. 1. The results showed a statistically significant difference between tested materials in immediate micro-tensile bond values and after thermocycling ($p < 0.001$). Pretreatment with *Morus alba* water extract revealed lowest bond strength values. No statistically significance difference between green tea water extract and control group but with highest mean value for green tea water extract. The means of micro-tensile bond strength value of green tea water extract were significantly higher than those with green tea alcohol extract pretreatment. On the other hand, mean values for alcohol extract of *Morus alba* and *Morus nigra* were higher than their corresponding water extracts.

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

Variables	Tensile bond strength				*p-value*
	Before thermocycling		After thermocycling		
	Mean	SD	Mean	SD	
Control	28.38 aA	6.68	17.39 aB	1.71	**0.007***
GT/W	29.22 aA	6.29	18.97 aB	6.66	**0.037***
GT/A	16.70 bA	5.30	12.73 aA	6.63	**0.327ns**
MA/L/W	4.01 cA	1.92	2.64 bA	2.27	**0.333ns**
MA/L/A	26.68 abA	5.81	17.93 aB	4.82	**0.032***
MN/L/W	24.90 abA	6.74	17.83 aA	6.57	**0.132ns**
MN/L/A	26.68 abA	5.81	17.93 aB	4.82	**0.032***
p-value	< **0.001***		< **0.001***		

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

Regarding thermocycling, there was a statistically significant difference between all tested groups ($p < 0.001$). The micro-tensile bond values were decreased regardless of the treatment applied to dentin. However, pretreatment with *Morus alba* water extract revealed also the lowest bond strength values while the highest mean value was found in green tea water extract. A statistically significant difference was found in bond strength values between the immediate results and after thermocycling with all tested materials except with green tea alcohol extract, *Morus alba* water extract and *Morus nigra* water extract with p values 0.327, 0.333 and 0.132 respectively.

Data in Table 2 shows the results of Two-way ANOVA analysis for the effect of different variables on tensile bond strength. The results showed that different Groups and thermocycling had a statistically significant effect, while the interaction between the two variables had no statistically significant effect.

4. Discussion

Degradation of exposed collagen fibrils in the hybrid layer has been proved as a key factor in the deterioration of adhesive -dentin interface. Dentin matrix degradation has been attributed to MMP enzymes that slowly degrade the collagen fibrils [4,10,36]. Pretreatment of dentin with MMP inhibitors has been advocated to reduce collagen fibrils degradation, increase stability of resin dentin adhesive thus improving bond durability [37]. The present study conducted on leaf extracts derived from natural products that have been reported to possess anti-MMP potential. Among them, green tea, *Morus alba* and *Morus nigra* were selected because of their inhibitory effect against MMPs. *Morus nigra* and *Morus alba* were compared as they contain different concentration of phenolic compounds which have inhibitory activity on MMPs [38–40]. Amicro-tensilebond strength test was performed after 24 h of storage as it aimed to determine whether these natural extracts used would be really effective on immediate bond strength. In addition, thermocycling was done to evaluate bond durability in dynamic

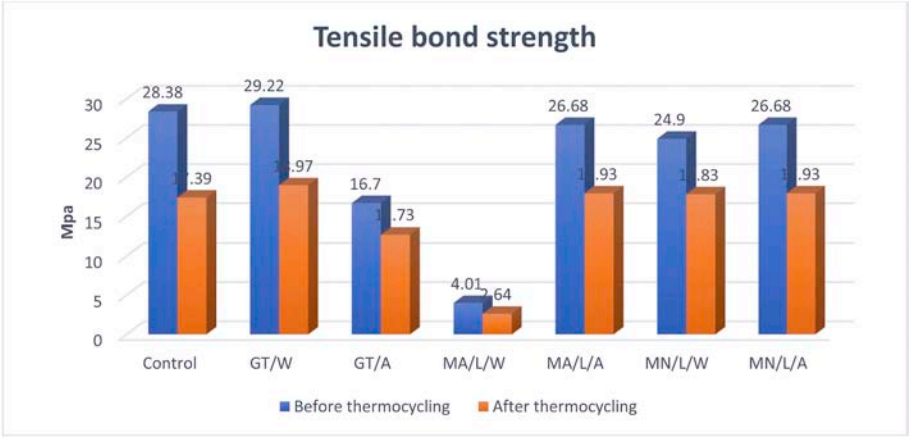

Fig. 1. Mean values of micro-tensile bond strength in different groups.

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	3320.160	6	553.360	18.662	.000*
Thermocycling	935.048	1	935.048	31.535	.000*
Groups × Thermocycling interaction	182.331	6	30.388	1.025	.419ns

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

conditions simulating a clinical setting.

Liquid extraction is commonly used for recovery of active ingredients. The chemical composition of plant extracts is influenced by extraction parameters such as the degree of fragmentation of plant material, extraction time, temperature, pH and the type of solvent [41]. Thus, it is important to select the appropriate method of extraction and solvent composition as they may influence the structure, composition, reactivity and cross-linking potency [6] In this study we used two methods of extraction, first by infusion using distilled water and the second by maceration using 70% alcohol followed by complete evaporation of alcohol under reduced pressure using rotator evaporator then the residue was dissolved in distilled water to avoid any interaction with different solvents from the adhesive system [6,42].

There are two possible options when incorporating natural MMPs inhibitors either as a primer or as an additive to adhesives. Proanthocyanidin (PA) incorporated directly into adhesives resulted low degree of double bond conversion caused by the radical scavenging ability of PA [43]. Moreover, the long-term performance of PA as a primer was significantly higher than that of PA-adhesive group [44]. Also, adding them to adhesive system may result in interfering with well-balanced monomer-solvent system. Liu et al., found that the incorporation of PA into the resin formulation affects monomer conversion and alters the polymerization kinetics. These findings support the use of MMPs inhibitors as a primer in this study [45].

In the present study, immediate micro-tensile bond strength provided values that served as a baseline for comparison with values measured after thermocycloing. Comparison of the immediate micro-tensile bond strength values showed a statistically significance difference between all groups. Green tea water extract revealed the greatest values, but it is not statistically significant than control group. This could be attributed to water content of the extract which could not be eliminated completely from the bonding interface. Prabhu et al., stated that the persistence of lytic changes at the interface could be attributed to the compromised moisture control rather than any ineffectiveness of anti-MMP agents [46].

The micro-tensile bond strength evaluation suggested that green tea water extract preconditioning retained highest bond strength values. This could be attributed to Epigallocatechin gallate (EGCG) that possess inhibitory effect against matrix metalloproteinases and act a cross-linking agent thus improving the mechanical and enzymatic resistance of collagen matrix [47]. Sun et al., 2018, reported increased the thermal degradation temperature of dentin collagen and improved the hydrophobicity of demineralized dentin [48].

Additionally, because green tea polyphenols contain EGCG, it has been classified as a proanthocyanidin which has the ability to bind to proline-rich proteins, such as collagen, and facilitate the enzyme proline hydroxylase activity essential for collagen biosynthesis [49]. Liu et al., attributed the stabilization of the bonding interface to the anti-proteolytic activity of proanthocyanidine [50]. The cross-linking mechanism between PA and collagen has been attributed to the formation insoluble compounds as result of the hydrogen bonding between the protein amide carbonyl and phenolic hydroxyl groups in addition to hydrophobic and covalent bonds [51]. Water vapor permeability of collagen/PA films decreased as result of formation of a denser structure thus moisture permeation was prevented and the hydrophobicity of PA-modified collagen films was improved [52]. Green et al., stated that PA increased collagen resistance to degradation by collagenase activity by masking the cleavage site of collagen through the formation of hydrogen bond in multiple sites on collagen molecules [43]. Castellan et al., reported an immediate increased in the elastic modulus of dentin matrix with a reduction in its swelling ratio resulting in decreasing both the collagenase sorption and enzymatic degradation of PA-treated dentin [52]. In addition, inhibitory effect on production of matrix metalloproteinases (MMPs) 1,2,3,7,8 and 9 was demonstrated [6]. Epasinghe et al., revealed that PA could cross-link more than 90% of soluble MMP and 70–80% of cysteine cathepsins. These cross-links involve stable covalent bonds unlike the reversible electrostatic binding of chlorohexidine. In addition, vitamin C content of green tea plays an important role in formation and stabilizing collagen helical configuration. Also, vitamin C act as MMP inhibitors [53–55]. Also green tea contains methylxanthine alkaloid called caffeine which induces (MMP-2) and (MMP-9) down-regulation [56]. On the contrary, results from a previous study showed that application of a green tea decreased immediate bond strength values but kept it stable in the long-term [16].

Regarding *Morus*, micro-tensile bond strength values in this study showed that *Morus alba* and *Morous nigra* in alcohol water extracts exhibited higher bond strength values than water extracts. Protective effects of phytochemicals in mulberry is mainly due to phenolic compounds content and high antioxidant capacity [41]. Mulberry leaf extracts are rich in polyphenols (gallic acid, protocatechuic acid, catechin, gallocatechingallate, caffeic acid, epicatechin, rutin, and quercetin).

They are responsible for inhibit the activities of matrix metalloproteinases (MMPs) MMP-2 and MMP-9 [57]. Also, Mulberry leaf extract contain Proanthocyanidin (catechin, gallocatechingallate, gallic acid and epicatechin) acts as natural dentin biomodifier in adhesive dentistry [6]. The difference in mean value between two species could be attributed to the different in the percentage of total polyphenol content between the two species [39].

It is reasonable to predict that the extracting solvent interacts differently. One probable factor contributing for the high bond strength values obtained with *Morus* could be the use of methanol as extracting solvent with its highest extraction efficiency, among other solvents, of polyphenols from mulberry leaves [58]. Arabshahi-Delouee and Urooj, revealed that extracts form mulberry leaf obtained using alcohol extraction contain methanol, acetone and water exhibited 93, 85, and 71 mg total phenolics/g, respectively [59]. However, water extract of green tea results in significant effect than alcohol extract due to water is a better solvent for extraction of polyphenols from green tea leaves than methanol and solubility of caffeine in boiling water is higher than that in alcohol [60].

The results of this study showed a drop-in bond strength values after thermocycling in tested groups. Pretreatment with green tea extract retained highest bond strength value after thermocycling. Thermocycling results in decrease bond strength which may be attributed to diffusion of water through hybrid layer thus resulting in a degradation of demineralized collagen. Pashly et al., explained the cause of this weak durability to the activation of MMPs by weak acids such as lactic acid and etchants used in adhesive bonding systems [61]. Also, samples were subjected to cyclic loading produced marginal gaps and leakage in comparison to static aging [46] Xie et al., concluded that thermo-cycling of tiny beam specimens resulted in a significant decrease in bond strength as result of chemical degradation of interface [62]. Munck et al., revealed that thermo-cycling results in combined contraction/expansion stresses thus accelerating degradation [63].

To date, there is still no in-depth studies pertaining to the effect natural extracts on stability of dentin collagen matrices. Whether they can enhance resin dentin bonds under simulated pulpal pressure has not been studied. The investigation of failure pattern is an important tool that should be evaluated to identify the weakest area of dentin-composite interface. In addition, more in vivo studies are required to validate its use in clinical setting. The durability and long-term stability of the resin -bonded dentin is still questionable. This provides the justification for further studies to evaluate resin –dentin bond longevity.

5. Conclusions

Under the limitations of the present study it was concluded that green tea water extract and *Morus* species alcohol-solvent extracts as pretreatment agents to dentin, has no adverse effect on the immediate micro-tensile strength. However, application of natural extracts tested does not prevent loss of micro-tensile bond strength with thermocycling.

Declarations of interest

None.

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