ABSTRACT

Objectives: This study aimed to evaluate *Emblica officinalis* (Indian gooseberry or amla) as an acid etchant and matrix metalloproteinase (MMP) inhibitor, and to compare its effect on the microshear bond strength of composite resin with orthophosphoric acid (OPA) and 2% chlorhexidine (CHX) as an acid etchant and MMP inhibitor, respectively.

Materials and Methods: The etching effect and MMP-inhibiting action of amla on dentin samples were confirmed by scanning electron microscopy (SEM) and gelatin zymography, respectively. Dentinal slabs (3 mm thick) from 80 extracted human molars were divided into 10 and 20 samples to form 2 control groups and 3 experimental groups. Groups 1, 2, and 4 were etched with OPA and groups 3 and 5 with amla juice. An MMP inhibitor was then applied: CHX for group 2 and amla extract for groups 4 and 5. Groups 1 and 3 received no MMP inhibitor. All specimens received a standardized bonding protocol and composite resin build-up, and were subjected to microshear bond strength testing. The force at which the fracture occurred was recorded and statistically analyzed.

Results: Amla juice had a similar etching effect as a self-etch adhesive in SEM and 100% amla extract was found to inhibit MMP-9 by gelatin zymography. The microshear bond strength values of amla were lower than those obtained for OPA and CHX, but the difference was not statistically significant.

Conclusions: Amla has a promising role as an acid etchant and MMP inhibitor, but further studies are necessary to substantiate its efficacy.

Keywords: Amla; Chlorhexidine; Gelatin zymography; Matrix metalloproteinases; Microshear bond strength; Scanning electron microscopy

INTRODUCTION

The durability of bonds formed by bonding agents to enamel and dentin is critical for the clinical success of bonded resin composite restorations [1]. The resin-dentin bonds created
Author Contributions
Conceptualization: Sangeetha R; Data curation: Sangeetha R; Formal analysis: Sangeetha R, Annapoorna BM; Funding acquisition: Sangeetha R; Investigation: Sangeetha R; Methodology: Sangeetha R; Project administration: Annapoorna BM; Resources: Sangeetha R; Software: Sangeetha R; Supervision: Annapoorna BM; Writing - original draft: Sangeetha R; Writing - review & editing: Sangeetha R, Annapoorna BM.

ORCID iDs
Divya Sangeetha Rajkumar https://orcid.org/0000-0002-7099-6494
Annapoorna Ballagere Mariswamy https://orcid.org/0000-0002-9621-9685

with contemporary hydrophilic dentin bonding systems deteriorate over time [2]. Hydrolytic degradation of the hybrid layer occurs due to the degradation of collagen matrices, leading to a loss of bond strength [3]. With total-etch adhesives, incomplete infiltration of monomers within the acid-etched dentin results in denuded collagen fibrils along the bottom of the hybrid layer [4]. These fibrils undergo auto-degradation by the slow action of host-derived matrix metalloproteinases (MMPs) [5].

Human dentin contains MMP-2, MMP-8, MMP-9, and MMP-20, which are members of a class of zinc and calcium-dependent endopeptidases. After dentin is bonded with resin, these MMPs slowly become activated, causing thinning and disappearance of collagen fibrils below the hybrid layer that were not infiltrated by the adhesive resin [5]. Various in vitro attempts have been made to arrest the release of MMPs with the help of MMP inhibitors and collagen-stabilizing agents such as proanthocyanidine/grape seed extract, riboflavin, green tea extract (epigallocatechin-3-gallate), chlorhexidine (CHX), and glutaraldehyde [4,6-8]. These are non-specific MMP inhibitors and cross-linking agents. Specific synthetic inhibitors such as galardin, batimatstat, and carbodiimide have been proven to be effective cross-linking agents [1,9].

Emblica officinalis (commonly known as Indian gooseberry or amla) is a rich food source of vitamin C, minerals, amino acids, and various phenolic compounds [10]. Amla juice is highly acidic (pH 2.85), with a pH comparable to that of self-etching primer. This observation raises the possibility of using this readily available and multifunctional Indian fruit as a source of natural etchant for restoration purposes in dentistry. The effect of amla extract as a potent MMP inhibitor on soft tissue samples has already been demonstrated in many studies [11,12].

In the literature, although many agents have been tried as etchants and MMP inhibitors separately, studies have not yet speculated on the use of a single agent with both functions. Thus, this study aimed to assess the effect of amla juice as an acid etchant by scanning electron microscopy (SEM) and as an MMP inhibitor by gelatin zymography, followed by evaluation of its effect on the microshear bond strength of composite resin. The null hypothesis was that there would be no statistically significant difference in the microshear bond strength values between:

1) Orthophosphoric acid (OPA) (Scotchbond Universal Etchant, 3M ESPE; St. Paul, MN, USA) and amla juice as an acid etchant (groups 1 and 3)
2) CHX (Consepsis; Ultradent Inc., South Jordan, UT, USA) and amla extract as MMP inhibitors with OPA as an etchant (groups 2 and 4)
3) OPA etchant with CHX as an MMP inhibitor (group 2) and amla juice etchant with amla extract as an MMP inhibitor (group 5)
4) Amla etchant as an MMP inhibitor (group 5) and amla etchant or amla MMP inhibitor (group 3 or 4)

MATERIALS AND METHODS

The study design was approved by the Institutional Ethical Committee meeting conducted by JSS Dental College and Hospital, Mysuru under the protocol number JSS/DCH/IEC/MD-46/2016-17 (2). In total, 86 freshly extracted intact human molar teeth were collected. Debris was cleaned using a rubber cup and pumice slurry in a micro-motor handpiece at slow speed, and the teeth were stored in distilled water containing 0.2% thymol antiseptic solution (Thymol; Sigma-Aldrich; St. Louis, MO, USA) for 48 hours at 37°C immediately after
Teeth with carious and non-carious lesions, restorations, fractures/crack lines, discoloration, fluorosis, hypoplasia, and developmental defects were excluded from the study. Of these teeth, 80 samples were used for microshear bond strength analysis, 5 samples for SEM, and 1 for gelatin zymography.

**Extraction and preparation of pure amla juice and amla extract**

For pure amla juice, fresh amla fruits were washed with distilled water, cut into small pieces, macerated in a mechanical grinder, and then filtered using a clean muslin cloth. No preservatives were added to the obtained juice. For amla extract preparation, 100 mL of fresh extract of amla was mixed with 0.3 g of sodium benzoate as a preservative. This procedure was performed in NKCA Pharmacy Ltd., Ayurvedic Centre, Mysore, India.

**SEM study for assessing the etching effect on dentin**

To assess teeth samples under the microscope, the roots of 2 teeth were sheared off from the crown 2–3 mm below the cemento-enamel junction. The occlusal one-third of each crown was then ground using a water-cooled, slow-speed diamond disc to expose the dentin surface, resulting in the formation of dentin discs. A uniform smear layer was created by abrading the dentin surface with 600-grit silicon-carbide grinding paper under water for 30 seconds.

The dentin surface of 1 disc was treated with OPA while the other was treated with pure amla juice as an acid etchant. Both etchants were applied using applicator tips for 15 seconds, rinsed with water spray for 10 seconds, and air-dried for 5 seconds. The specimens were then examined under a SEM (Zeiss, EVO LS-15; Oberkochen, Germany) at ×5,000 magnification to analyze the etching effect.

**Gelatin zymography to assess MMP activity**

After grinding away enamel and cementum, pulpal soft tissue was removed from a tooth sample. The sample was then washed with phosphate-buffered saline to remove any red blood cells and other contaminants. Pieces of dried dentin were reduced to a fine powder using a mortar and pestle. The obtained pulverized powder was divided into 4 equal parts and subjected to the following treatment protocols:

- Untreated mineralized dentin powder served as a negative control.
- Dentin powder demineralized with 1% OPA for 10 minutes at 4°C served as the positive control.
- Dentin powder demineralized with 1% OPA for 10 minutes at 4°C and then incubated with 50% and 100% amla extract for 30 minutes served as the study groups.

All the samples were centrifuged for 20 minutes at 12,000 rpm at 4°C. The supernatant obtained was discarded and the remaining sample was collected for further protein extraction.

1) Dentin protein extraction: 25 mg of the amla-treated sample amla was mixed with 300 μL of ice-cold lysis buffer, ground with a mortar, and maintained in constant agitation at 4°C for 2 hours. It was then centrifuged for 20 minutes at 12,000 rpm at 4°C in a microcentrifuge. The tubes were gently removed from the centrifuge and the supernatant was subjected to total protein estimation using the Bradford assay. Samples were prepared in a standard non-reducing loading buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis with the gelatin substrate embedded in the separating gel.

2) Zymographic analysis: Following electrophoresis, sodium dodecyl sulfate was removed from the gel by incubation in un-buffered Triton X-100. The gel was rinsed for 5–10 minutes in the incubation buffer at 37°C with agitation. A fresh incubation buffer was added and incubated for 24 hours at 37°C. The gel was stained using Coomassie Brilliant...
Blue staining solution (Brilliant Blue R Concentrate; Sigma-Aldrich) for 30 minutes to 1 hour and finally rinsed with water to remove the excess staining solution. The gel was incubated with a destaining solution until the bands were visible.

3) Setting the effective concentration of amla extract with MMP activity: Areas of enzyme (MMPs) activity appeared as white bands against a dark blue background band. The concentration at which the amla extract (either 50% or 100%) showed an effective decrease in the intensity and thickness of the white band was considered the effective concentration of amla extract as an MMP inhibitor and used in further procedures.

**SEM study for assessing structural changes caused by an MMP inhibitor on dentin**

According to the above-described protocol to assess teeth samples under the microscope, 3 teeth were prepared to evaluate the structural changes caused when an MMP inhibitor was used on dentin. Two dentinal discs were etched with OPA for 15 seconds, rinsed with water spray for 10 seconds, and air-dried for 5 seconds. After etching, one of the dentinal discs was treated with 2% CHX, while the other was treated with the effective concentration of amla extract (determined based on gelatin zymography). Both MMP inhibitors were applied for 60 seconds using an applicator tip, washed off, and air dried. For the third dentinal disc, pure amla juice was applied as an etchant for 15 seconds, rinsed with water spray for 10 seconds, and air-dried for 5 seconds followed by the effective concentration of amla extract as an MMP inhibitor for 60 seconds using an applicator tip, washed off, and air-dried. A representative SEM image of each sample was captured at ×5,000 magnification.

**Microshear bond strength of composite resin**

1) Specimen preparation: The roots of 80 selected teeth were cut 2 mm below the cemento-enamel junction and the occlusal surfaces were ground to expose the dentin in order to obtain dentinal slabs with a uniform thickness of 3 mm. The exposed dentinal surfaces were finished using 600-grit silicon-carbide papers to create a standardized smear layer. Slabs obtained were then mounted in cold cure acrylic resin (DPI-RR Cold cure; Dental Products India, Mumbai, India) using square rubber molds of standardized dimensions (25 × 25 mm). The 80 prepared specimens were then assigned into control and experimental groups, as shown in Figure 1. To standardize, acid etchants (either 37% OPA or pure amla juice) were applied on the dentin surface for 15 seconds using applicator tips, rinsed with water spray for 10 seconds, and then air-dried for 5 seconds. MMP inhibitors (either 2% CHX or 100% amla extract) were applied for 60 seconds using applicator tips, washed off, and air-dried. After preparing the samples according to the control and experimental groups, the bonding protocol described below was followed.

2) Bonding protocol: For all specimens, irrespective of their groups, a dentin adhesive (3M Single bond Universal adhesive; 3M ESPE) was gently rubbed using an applicator tip for 20 seconds, followed by gentle air-drying for 5 seconds and light-curing using a light-emitting diode light-curing unit (Woodpecker Med. Instrument; Guilin, China) for 10 seconds. Polyethylene tubes with an internal diameter of 2 mm and a height of 2 mm were firmly attached over the bonded surface of the dentin to expose a standardized dentinal surface area with a diameter of 2 mm. Nanocomposite resin (C1 body shade of 3M Filtek™ Z350XT universal restorative; St. Paul, MN, USA) was placed inside the polyethylene tube in 2 increments of 1 mm thickness, each increment was cured for 20 seconds at a radiant emittance of 400 mW/cm² at zero distance. The tubes were then removed with a sharp knife after composite build-up and were stored in distilled water for 2 weeks at 37°C.
3) Bond strength evaluation procedure: After being stored in distilled water, each acrylic resin block along with its bonded cylinders was fixed to the lower compartment of a universal testing machine (TEC-SOL India; Chennai, India) using tightening screws. A loop of orthodontic stainless steel wire (0.2 mm in diameter) was wrapped around the bonded cylinder as close to its base as possible and aligned with the load cell axis of the upper movable compartment of the testing machine. The specimens were then subjected to microshear bond strength testing with a load cell of 5 kN at a crosshead speed of 0.5 mm/min at the dentin-composite resin interface until fracture occurred, and the data were recorded using a computer software (TEC-SOL India). The operator performing the bond strength tests was blinded during the entire procedure. The microshear bond strength values (MPa) were calculated using the following formula: microshear bond strength = peak load at failure/bonded surface area.

Statistical analysis
The sample size was calculated using G*Power software version 3.1 (University of Düsseldorf, Düsseldorf, Germany) with an effect size of 0.7, an alpha error of 0.05, and a power of 0.95. The mean microshear bond strength values and standard deviations were determined for all groups. The normality of the data was checked using the Shapiro-Wilk test. One-way analysis of variance was used as a parametric test, followed by the post hoc Bonferroni test. A $p$ value of more than 0.05 indicated no statistical significance.

RESULTS
Comparison of the etching effect of amla juice with 37% OPA on dentin by SEM
The SEM images of 37% OPA on dentin showed complete removal of the smear layer and smear plug with open dentinal tubules. The peritubular and intertubular dentin was completely demineralized, with clearly visible collagen fibrils. In contrast, the amla juice-treated dentin showed a partially removed smear layer and smear plug, with areas of opened and occluded dentinal tubules. Collagen fibrils were invisible, with only partial demineralization of the peritubular and intertubular dentin (Figure 2).
Effective concentration at which amla extract acts as an MMP inhibitor by gelatin zymography

The gel revealed a thick white band in lane 2 (positive control) indicating MMP-9 gelatinolytic activity (molecular mass of 86 kDa). Thus, MMP-9 activity could be confirmed in the proteins extracted from demineralized dentin powder. The negative control group exhibited no enzyme activity. The lane treated with 50% amla extract showed a decrease in the intensity and thickness of the white band, indicating effective inhibition of MMP-9. However, the white band was faint, vague, and indistinct with 100% amla extract, indicating potent inhibition of MMP-9 (Figure 3). Therefore, 100% amla extract was considered to be the effective concentration as an MMP inhibitor.

Comparison of the effect of amla extract with 2% CHX as MMP inhibitors on dentin by SEM

SEM images of dentin treated with OPA followed by CHX as an MMP inhibitor showed complete removal of the smear layer and smear plug, fully opened dentinal tubules, significant demineralization of peritubular and intertubular dentin, visible collagen fibrils, and crystal-like deposits. The SEM images of dentin treated with OPA followed by 100% amla extract as an MMP inhibitor presented complete removal of the smear layer and smear plug, with fully opened dentinal tubules. Further, collapse of the peritubular dentin and partial demineralization of the intertubular dentin were noted. No collagen fibrils were evident.

Figure 2. Scanning electron microscopic images evaluating and comparing the etching effect of 37% OPA with pure amla juice on dentin samples at ×5,000 magnification. (A) Etched with 37% OPA. (B) Etched with pure amla juice. OPA, orthophosphoric acid.

Figure 3. Effective concentration at which amla extract acts as an matrix metalloproteinase inhibitor as determined using gelatin zymography.

Lane 1, untreated mineralized dentin powder (negative control); Lane 2, dentin powder demineralized with 1% OPA for 10 minutes at 4°C (positive control); Lane 3, dentin powder demineralized with 1% OPA for 10 minutes at 4°C and then incubated with 50% amla extract for 30 minutes; Lane 4, dentin powder demineralized with 1% OPA for 10 minutes at 4°C and then incubated with 100% amla extract for 30 minutes. OPA, orthophosphoric acid.
The SEM images of dentin treated with amla juice as an etchant and 100% amla extract as an MMP inhibitor showed only partial removal of the smear layer, presence of the smear plug, and partially occluded and open dentinal tubules. Complete collapse of the peritubular dentin and partial demineralization of the intertubular dentin were noted without collagen fibrils (Figure 4).

Comparison of microshear bond strength values between the control and experimental groups

In decreasing order, the values of mean microshear bond strength between the control and experimental groups were found to be 14.21 MPa (group 2), 13.44 MPa (group 5), 13.10 MPa (group 4), 12.15 MPa (group 1), and 11.79 MPa (group 3) (Figure 5).

**DISCUSSION**

One of the factors responsible for the deterioration of the hybrid layer is the hydrolysis and enzymatic degradation of the poorly resin-infiltrated collagen fibrils by the slow action of host-derived MMPs [2,5,13]. The application of either a natural or synthetic MMP inhibitor on dentin surfaces was found to prevent this degradation, thereby improving the bond strength [4,6-9,14]. Amla extract, which is a potent MMP inhibitor on soft tissue samples [11,12], was also found to be highly acidic, with a pH of 2.85 [10]. Thus the present study aimed to assess the effect of amla as an acid etchant and MMP inhibitor on hard tissue tooth samples.
In the analysis of etching characteristics using SEM, the samples were prepared following Green et al. [15]. Our SEM findings of 37% OPA on dentin were similar to the “funnel-shaped appearance” of dentinal tubules observed by Susin et al. [16]. However, amla juice etchant partly demineralized the dentin, and most of the dentinal tubules were occluded with a smear plug. Despite having a highly acidic pH of 2.85, the etching effect of amla juice was lower than that of OPA. However, the findings observed in Figure 2B are comparable to those of SEM images of dentin specimens treated with a self-etching primer having a similar pH [16].

In 2008, it was established that amla extract had a significant MMP-inhibiting property on human skin fibroblasts in a dose-dependent manner [11]. Herein, we used gelatin zymography to evaluate proteinase profiles. The protocol for gelatin zymography of the tooth sample was carried out according to Mazzoni et al. [17] 2010. The zymogram obtained in our study (Figure 3) showed a decrease in the MMP activity when treated with amla extract compared to the positive control. The 50% amla extract showed effective inhibition of MMP-9, while a further increase in enzyme inhibition was observed with a 100% concentration of the extract. Similar to amla, several natural extracts such as *Camellia sinensis*, chitosan, and marine sponges have also revealed selective MMP-9-inhibiting properties [18]. The MMP-inhibiting activity of amla could be associated with the high amounts of hydroxyl groups and polyphenols in its composition.

Since the application of MMP inhibitors influences the bond strength of composite resin [19-21], we evaluated the changes they caused on etched dentin using SEM. As an MMP inhibitor, 2% CHX showed SEM images with crystal-like deposits. Lapinska et al. [22] confirmed that the deposits were components of CHX (carbon, nitrogen, and chlorine) by dispersive spectroscopy. They hypothesized that the deposits increased the adhesive surface area, thereby providing additional retention of the hybrid layer responsible for a short- and long-term increase in the bond strength. Specimens exposed to 100% amla extract as an MMP inhibitor showed certain irregularities in the intertubular region. We speculated that these irregularities might represent specific chemical constituents of amla, collapsed peritubular dentin, or a chelated outcome of amla with the calcium present in dentin. However, a further analysis using dispersive spectroscopy would be necessary to evaluate the composition of these irregularities.

After determining the acid etching ability and MMP-inhibiting property of amla, its effect on the bond strength of composite resin was evaluated, following the protocol of Ahmed et al. [23]. The mean microshear bond strength value of group 3 (amla juice as an acid etchant) was lower than that of group 1 (37% OPA as an etchant), although the difference was not statistically significant. That could be due to the insufficient etching ability of amla (as seen in Figure 2B), resulting in reduced resin penetration and decreased bond strength. The first hypothesis is thus supported. However, the bond strength values of group 3 were similar to those of self-etched adhesives from previous studies [16,23].

CHX is an amphiphilic molecule that binds to various proteins by a cation-chelation mechanism. It inhibits the catalytic activity of MMPs by binding with Zn$^{2+}$ or Ca$^{2+}$. The substantivity of CHX is also advantageous, since CHX can therefore remain active on the tooth surface for a prolonged duration [24]. After acid etching, the intertubular dentin matrix is composed of 70% water and 30% organic matrix. Application of CHX to this demineralized dentin results in its diffusion into the unbound water and electrostatic binding with
carboxyl groups of the collagen matrix. Eight-fold higher absorption of CHX was found with demineralized dentin than with mineralized dentin [19]. All these factors could explain why group 2 showed the highest bond strength values. The bond strength values of group 2 obtained in our study are similar to those reported by Heshmat et al. [3] in 2018. Other studies have also reported that CHX as an MMP inhibitor did not influence the bond strength [20,21].

The bond strength values of groups 4 and 5 were lower and statistically non-significant compared to group 2 (Figure 5). Thus, the second and third hypotheses are supported. Figure 4B and 4C show that amla extract caused the collapse of collagen, peritubular dentin, and intertubular dentin, which could explain the lower bond strength values. Furthermore, unlike amla, CHX showed complete inhibition of MMP activity with no detectable bands in a previous study [22].

Amla had specific MMP-9 inhibiting activity and was retained in dentin for a longer duration when used together as an etchant and MMP inhibitor. Thus, the mean bond strength of group 5 was higher than that of group 3, and this difference was statistically significant, enabling the fourth null hypothesis to be rejected. Furthermore, the higher molecular compatibility between both the liquids (amla juice and amla extract) could contribute to significant changes within the dentin, thereby increasing the bond strength.

Further parameters to be assessed include the dentinal changes caused by different application times of amla etchant and the depth of penetration of amla etchant into dentin. The chemical composition of the dentin surface treated with amla and the molecular compatibility of OPA etchant and amla as an MMP inhibitor also require evaluation.

Although the microshear bond strength in group 5 was lower than in group 2, it was higher than in groups 3 and 4. Thus the dual nature of amla as an etchant and MMP inhibitor could be validated, underscoring the enormous potential of this unique extract for application to the resin-dentin interface.

CONCLUSIONS

On dentin, pure amla juice had an etching effect similar to that of a self-etching adhesive. Amla extract also showed selective MMP-9–inhibiting activity. When used both as an etchant and MMP inhibitor, amla increased the microshear bond strength of composite resin more effectively than when used separately. Within the limitations of this study, the dual nature of amla as an etchant and MMP inhibitor was validated, underscoring the enormous potential of this unique extract for application to the resin-dentin interface.

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