



Characterization and Anti-Caries Effect of an Experimental Adhesive Containing Natural Antimicrobial Agents

Rachel Cavalcanti Ribeiro^a / Eduardo Moreira Silva^b / Carina Mattos de Carvalho^c /
Maria Elisa da Silva Nunes Gomes Miranda^d / Maristela Barbosa Portela^e / Cristiane Mariote Amaral^f

Purpose: To characterize experimental adhesives containing natural antimicrobial agents (proanthocyanidins, apigenin, tt-farnesol) in the primer and to evaluate their anti-caries effect.

Materials and Methods: Natural agents were incorporated in the primer of an experimental adhesive: 4.5% proanthocyanidins (PA), 1 mM apigenin (API), 1 mM apigenin + 5 mM tt-farnesol (API + FAR), and primer without antimicrobial agent (control). Microtensile bond strength (μ TBS) to dentin and nanoleakage were measured immediately ($n = 7$) and after 1 year of storage ($n = 7$). Water sorption (WS), solubility (SO), and degree of conversion (DC%) of the adhesives were measured. The hardness loss of enamel ($n = 6$) and dentin ($n = 6$) at the restorative margin was evaluated after biofilm formation.

Results: DC%, nanoleakage, and immediate μ TBS were similar for all groups. After 1 year, API + FAR showed higher nanoleakage and lower μ TBS than the other groups, which were similar. WS and SO of API + FAR were lower than in the other groups. PA, API, and API + FAR presented less hardness loss than did the control group. At enamel, PA and API presented less hardness loss than the control and API + FAR groups at distances 50 μ m and 100 μ m; the hardness loss of enamel was similar for all groups 150 μ m from the margin.

Conclusion: The addition of proanthocyanidins and apigenin to the adhesives decreased the hardness loss of dentin and enamel submitted to biofilm formation, without jeopardizing the physical properties of the adhesives. The combination of apigenin + tt-farnesol decreased the hardness loss of dentin but not of enamel, and decreased the μ TBS after 1 year of storage.

Keywords: adhesion, adhesive systems, apigenin, proanthocyanidins.

J Adhes Dent 2021; 23: 527–537.
doi: 10.3290/j.jad.b2287719

Submitted for publication: 16.06.20; accepted for publication: 22.05.21

^a Dentist, Analytical Laboratory of Restorative Biomaterials, School of Dentistry, Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil. Performed the experiments in partial fulfillment of the requirements for a degree, wrote the manuscript.

^b Titular Professor, Analytical Laboratory of Restorative Biomaterials, School of Dentistry, Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil. Experimental design, statistical analysis, proofread the manuscript.

^c Undergraduate Student, Analytical Laboratory of Restorative Biomaterials, School of Dentistry, Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil. Performed the WS and SO evaluations, assisted in μ TBS testing.

^d Postdoctoral Student, Analytical Laboratory of Restorative Biomaterials, School of Dentistry, Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil. Collaborated in tests, statistical analysis, wrote the manuscript.

^e Adjunct Professor, Analytical Laboratory of Restorative Biomaterials, School of Dentistry, Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil. Experimental design, performed antibacterial evaluation.

^f Associate Professor, Analytical Laboratory of Restorative Biomaterials, School of Dentistry, Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil. Project coordinator, idea, experimental design, statistical analysis, proofread the manuscript.

Correspondence: Dr. Cristiane Mariote Amaral, Universidade Federal Fluminense, Faculdade de Odontologia, Rua Mário Santos Braga, nº 30, Campus Valonguinho, Centro, Niterói, RJ, Brazil CEP 24020-140. Tel: +55-21-2629-9832; e-mail: cristianemariote@id.uff.br

Resin composites are widely used as direct filling materials for tooth restorations. However, secondary caries is the main cause of failure of these restorations,^{1,2} due to the deposition of biofilm and formation of gaps at the restoration margins.^{11,27} Thus, the control of biofilm formation without changing the ecological balance is essential for the prevention of secondary caries and to increase the longevity of the restoration.^{2,18,24,26}

Bacterial resistance to antimicrobials represents a worldwide problem. Therefore, new strategies and new drugs are constantly being explored. Natural products are a little-explored source of non-toxic anti-biofilm agents, which can be used in combination with fluoride as an alternative to broad-spectrum antimicrobials, such as chlorhexidine.^{23,42,47} *Streptococcus mutans* is a factor influencing caries development, as a result of its virulence associated with its ability to produce extracellular polysaccharides (EPS) and weak acids from sucrose, in addition to its ability to adapt to large fluctuations in pH, oxygen tension, and nutrient availability.² The EPS matrix acts as a framework for the biofilm, provides

binding sites, is a source of sugar, and creates an acidic environment. The EPS matrix consists mainly of glucans synthesized by microbial glycosyltransferases (Gtfs). Thus, a fundamental means of controlling the formation of biofilm would be to inhibit the function of these enzymes.^{16,22,25,35}

In recent years, the anti-biofilm activity of polyphenols has been demonstrated.^{16,22,34,40,43,47} Grape seed extract (GSE) and cranberries are rich in these compounds, which consist mainly of the free monomeric flavanols such as proanthocyanidins.^{16,35,47} GSE has been shown to biomodify dentin matrices, increasing and stabilizing their mechanical properties due to their effects on the biochemistry of collagen and interactions with proteoglycan.^{13,14,17,29} Proanthocyanidins can also improve the preventive and reparative abilities of dentin,^{4,7} because they positively affect the demineralization-reminereralization processes in artificial caries lesions,^{34,40} in root dentin,^{38,46} and in enamel.¹ Proanthocyanidins has the ability to suppress the formation and growth of *S. mutans* biofilms without exhibiting bactericidal effects.^{16,22,47} Proanthocyanidins bind to proteins and form protein-polyphenol complexes that can affect enzymatic activity by irreversibly binding to the glucan-binding domain.^{1,16,21,22,40,47}

Propolis is a non-toxic and relatively unexplored resinous substance produced by honeybees (*Apis mellifera*). Two compounds derived from propolis, apigenin and tt-farnesol, are potential anti-caries agents that do not exert a significant effect on oral microbiota viability^{2,6,23-25} and do not influence dentin bonding.² The incorporation of both into a dental restorative material could be beneficial, especially in areas with higher biofilm accumulation, such as interproximal and cervical regions.² The way by which apigenin and tt-farnesol affect the cariogenicity of *S. mutans* is based on the synthesis of glucan, the inhibition of EPS accumulation, the production of acids, and the inhibition of biofilm formation.^{2,6,23-25}

The incorporation of proanthocyanidins, apigenin, and tt-farnesol into an adhesive could help prevent secondary caries. Thus, the aim of this study was to characterize an experimental adhesive containing natural antimicrobial substances (proanthocyanidins, apigenin, tt-farnesol) in the primer and to evaluate its effect on the prevention of secondary caries in enamel and dentin. The hypotheses tested were: 1. adhesives containing natural antimicrobials affect the degree of conversion, water sorption, solubility, bond strength to dentin, and nanoleakage (immediate and after one year); and 2. adhesives containing natural antimicrobials exert an anti-caries effect in enamel and dentin.

MATERIALS AND METHODS

For this in vitro study, an experimental two-bottle etch-and-rinse adhesive was used, containing the following natural antimicrobial agents in the primer: 4.5% proanthocyanidins,⁹ apigenin at 1 mM² and the combination of apigenin at 1 mM + tt-farnesol at 5 mM.² Thus, 4 groups were formed: no added antimicrobial agents (control), addition of 4.5% proanthocyanidins (PA), addition of apigenin 1 mM (API), and addition of apigenin 1 mM + tt-farnesol 5 mM

(API + FAR). The composite, Filtek Z250 (3M Oral Care; St Paul, MN, USA), was used as the restorative material. The composition of these materials is shown in Table 1. The compounds of adhesives were mixed using a dual centrifuge (150.1 FVZ Speed Mixer DAC, Flack Tek; Herrliberg, Germany) for 2 min at 1300 rpm.

The powdered grape seed extract containing 95% proanthocyanidins was dissolved in ethanol at 30% (w/w). After magnetic agitation (Quimis, Diadema; SP, Brazil) for 30 min and 72 h of storage in a closed container, the solution was filtered (paper filter n. 6c) and used as solvent in group PA, until reaching a concentration of 4.5% of proanthocyanidins in the primer.⁹

Adhesive Characterization

pH assessment of the primers

Three ml of each primer was dispensed into clean glass vials and agitated for 30 s. The pH was measured in triplicate using a pH meter (Seven Compact pH/ION, Mettler Toledo; Columbus, OH, USA) at room temperature, and the mean of these three values was used as the pH for each material.

Microtensile bond strength to dentin (μ TBS) and nanoleakage evaluation

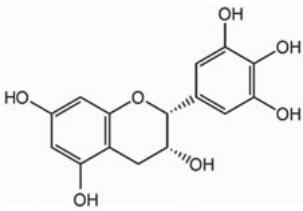
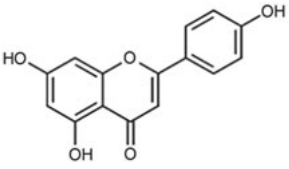
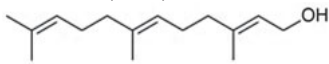
Fifty-six extracted, caries-free, human third molars (Ethics Committee Approval HUAP CAAE: 17552319600005243) were disinfected in a 0.5% chloramine-T aqueous solution (Vetec Química Fina; Rio de Janeiro, RJ, Brazil) for 7 days, then stored in distilled water and used within six months after extraction. The μ TBS measurement was performed as previously described.⁸ The occlusal dentin of the teeth was exposed using a cutting machine (IsoMet 1000, Buehler; Lake Bluff, IL, USA) and the peripheral enamel removed using a diamond bur #4138 (KG Sorensen; Cotia, SP, Brazil). The dentin smear layer was standardized using #400 and #600-grit SiC papers (Arotec; Cotia, SP, Brazil) in a polishing machine (DPU 10, Struers; Ballerup, Denmark) for 1 min each. The teeth were divided into 4 groups ($n = 7$) according to the antibacterial agent used (control, PA, API, and API + FAR) and two storage time intervals: immediate and after 1 year of storage.

Dentin surfaces were conditioned with 37% phosphoric acid for 15 s, rinsed with water for 30 s, and dried with absorbent paper. The primer was actively applied for 10 s, followed by a gentle air stream for 5 s at a distance of 15 cm. The adhesive was applied and light cured for 20 s at an irradiance of 650 mW/cm² (Radii-Cal SDI; Bayswater, Victoria, Australia). Five 1-mm-thick increments of microhybrid resin composite were horizontally added to the bonded surfaces and individually light cured for 40 s at an irradiance of 650 mW/cm².

After storage in distilled water at 37°C for 24 h, the teeth were longitudinally sectioned in both the mesio-distal and buccal-lingual directions across the bonded interfaces, yielding sticks with a cross-sectional area of approximately 1 mm². Each tooth provided 15 to 22 sticks. Two sticks obtained from each tooth were set aside for the nanoleakage test.

After each storage time (24 h or 1 year), the cross-sectional area of each stick's adhesive interfaces were mea-

Table 1 Materials used in this study

Material	Composition (% in weight)	Manufacturer
Experimental adhesive	Primer: 35% ethanol, 40% HEMA, 20% PMGDM, 4% water, 0.5% camphorquinone, 0.5% EDMAB Adhesive: 70% bis-GMA, 29% HEMA, 0.5% camphorquinone, 0.5% EDMAB	
Filtek Z250	Bis-EMA, bis-GMA, TEG-DMA, UDMA, zirconia/silica with 0.01 to 3.50 µm and 60% in loading particles	3M Oral Care; St Paul, MN, USA
Grape seed extract	95% proanthocyanidin (4.5 w/w%) ⁸ 	Active Pharmaceutica; Palhoça, SC, Brazil.
Apigenin	Apigenin (1mM) ² 	Sigma-Aldrich; St Louis, MO, USA
tt-farnesol	Tt-farnesol (5mM) ² 	Sigma-Aldrich
<p>HEMA: 2-hydroxyethyl methacrylate; PMGDM: pyromellitic dianhydride glycerol dimethacrylate; TEG-DMA: triethylene glycol dimethacrylate, EDMAB: ethyl 4-dimethylaminobenzoate; bis-EMA: bisphenol A diglycidyl methacrylate; bis-GMA: bisphenol A-glycidyl methacrylate; UDMA: urethane dimethacrylate.</p>		

sured using a digital caliper, then the specimens were individually fixed to a microtensile bond-strength device (ODMT03d, Odeme Biotechnology; Joaçaba, SC, Brazil) using cyanoacrylate glue (Superbonder Gel 3M; São Paulo, SP, Brazil) and loaded under tension in a universal testing machine (EMIC DL 2000; São José dos Pinhais, SP, Brazil) at a cross-head speed of 1.0 mm/min until failure occurred. The μ TBS (MPa) was obtained by dividing the load at failure (N) by the cross-sectional area of the stick (mm²). Each failed stick was evaluated using a stereomicroscope (SZ40; Olympus, Tokyo, Japan) at 40X magnification, and the failure mode was classified as: adhesive (debonding at the adhesive interface), cohesive (debonding occurring mainly within dentin or resin composite), or mixed (mixture of adhesive and cohesive failure within the same fractured surface). Additionally, representative fractured sticks exhibiting different failure modes and with μ TBS close to the mean of each group were observed using confocal laser microscopy (LEXT OLS4001, Olympus; Tokyo, Japan) operating in scanning mode XYZ fast scan, using a MPLAPONLEXT 50 lens at 1.4X zoom. The images were taken at 1024x1024 image size (pixels).

After storing (immediate or 1 year), two sticks of each tooth were prepared for the nanoleakage test as previously

described.^{33,41} The sticks received two layers of nail varnish up to 1 mm from the bonding interface on both sides and were individually immersed in 50 wt% ammoniacal silver nitrate solution (pH = 7.0) in a dark environment for 24 h. Each stick was thoroughly rinsed in running water and then immersed in a photodeveloping solution (Kodak; Rochester, NY, USA) under fluorescent light for 8 h, to reduce the silver ions into metallic silver grains at the bonding interface. Afterwards, the surfaces were wet polished using 600-grit, 1200-grit, and 4000-grit silicon carbide paper, ultrasonically cleaned in water for 10 min (Ultrassom 750 USC, Quimis; Rio de Janeiro, Brazil), and dried for 48 h in a desiccator with blue silica gel at 37°C.

The adhesive/dentin interfaces were observed using scanning electron microscopy (SEM) (Phenom ProX, Phenom-World BV; Eindhoven, the Netherlands), at an accelerating voltage of 15 kV, backscattered mode, and using a charge reduction sample holder (low vacuum environment). Three images were registered for each stick, essentially evaluating the entire adhesive interface: two images from both ends (right and left sides) and one central image (magnification: 2000X).^{33,41} In these three images, the amount of silver nitrate uptake in the hybrid layer was registered as a percent-

age of the total area observed, using an energy-dispersive x-ray spectroscopy (EDS) detector (Phenom ProX, Phenom-World BV; Eindhoven, the Netherlands). The percentage of Ag of each image was recorded. The mean percentage of Ag of the three images (right, left and central) was calculated and considered as the experimental unit ($n = 7$).

Degree of conversion (DC%) of the primers

Increments of each experimental primer were inserted into a Teflon mold (0.785 mm^3) positioned on the ATR crystal of an FT-IR spectrometer (Alpha-P/Platinum ATR Module, Bruker Optics; Ettlingen, Germany), and the spectra between 1500 and 1800 cm^{-1} were recorded with the spectrometer operating with 40 scans and a resolution of 4 cm^{-1} .

Afterwards, the increments were light cured for 40 s at an irradiance of 650 mW/cm^2 , and the spectra were recorded exactly as was performed for the unpolymerized increments. Five samples of each primer of the adhesives were evaluated ($n = 5$). The DC% was calculated as the ratio of the integrated area of absorption bands of the aliphatic C=C bond (1638 cm^{-1}) to that of aromatic C=C bond (1608 cm^{-1}), used as an internal standard, obtained from the cured and uncured films, using the following equation:

$$\text{DC\%} = 100 \times [1 - (R_{\text{polymerized}} / R_{\text{unpolymerized}})]$$

where R = integrated area at 1638 cm^{-1} / integrated area at 1608 cm^{-1} .

Water sorption (WS) and solubility (SO) of the primers

Antimicrobial substances were added to the adhesive primers. Then, only WS and SO of the primer of each group was evaluated. These methodologies were based on the ISO 4049 Standard (2000);²⁰ however, the period of immersion was prolonged until a constant mass was achieved.

Before building up the specimens, the solvent of each primer was evaporated. The primers (3 ml) were dispensed into a container (5.0 cm in diameter and 1.0 cm deep) on an analytical balance with a precision of 0.01 mg, which was protected from ambient light to prevent premature polymerization. The initial mass was recorded and the specimens remained on the analytical balance until they reached mass equilibrium. Then, disk-shaped specimens were prepared using an aluminum mold (6.0 mm in diameter and 1.0 mm thick). A micropipette was used to dispense the adhesive phases directly into the mold. After filling the mold completely, all visible air bubbles were carefully removed with a hypodermic needle. Then a polyester strip and glass slide were placed on top of the mold and the disks were light cured at an irradiance of 650 mW/cm^2 for 40 s. The disks were removed from the mold and light cured from the bottom for 40 s. The top and bottom surfaces of all disks were manually polished using 4000-grit SiC abrasive paper to eliminate any surface irregularities. Twenty disks were produced and assigned to four groups ($n = 5$).

Immediately after polymerization, the disks were placed in a desiccator containing dehydrated silica gel, and transferred to an oven (Q316B15, Quimis) at $37 \pm 1^\circ\text{C}$. The disks were weighed daily using an analytical balance with 0.01 mg

precision until a constant mass was attained (m_1) for three consecutive days with a mass variation under 0.01 mg. The thickness and diameter of each disk were measured at four points using a digital caliper (MPI/E-101, Mitutoyo; Tokyo, Japan), and the volume (V) was calculated in mm^3 . The specimens were then individually placed in sealed glass vials containing 10 ml of distilled water at 37°C . The immersion medium was renewed every seven days to prevent the proliferation of fungi and bacteria. The disks were repeatedly weighed at 24-h intervals until a constant mass was attained (m_2) for three consecutive days with a mass variation of less than 0.01 mg. Before weighing, the specimens were gently wiped with soft, absorbent paper. The specimens were again put into a desiccator containing fresh silica gel, maintained in a pre-conditioning oven at 37°C , and weighed daily until a constant mass was obtained (m_3). WS and SO in mg/mm^3 were calculated using the following formulae:

$$\text{WS} = (m_2 - m_3) / V$$

$$\text{SO} = (m_1 - m_3) / V$$

where m_1 is the disk mass (mg) after drying, m_2 is the disk mass (mg) at equilibrium uptake (maximum sorption), m_3 is the mass (mg) of the re-dried disk and V was the disk volume (mm^3).

Assessment of Caries Inhibition

Cavity preparation

Twenty-four extracted, caries-free, human third molars (Ethics Committee Approval HUAP CAEE 17552319600005243) were disinfected in a 0.5% chloramine-T aqueous solution for 7 days, then stored in distilled water. The roots were removed at the cemento-enamel junction using high-speed diamond burs. The crowns were sectioned in the vestibular-lingual direction forming 2 fragments for each tooth. The proximal enamel surface of one fragment of each tooth was ground flat with sandpaper disks in a polishing machine (DPU 10, Struers), while the other fragment was polished until the dentin was exposed (#320, #600, and #1200-grit SiC). In this way, 24 fragments of the enamel surface and 24 fragments of the dentin surface were obtained. Standardized cavities (2 mm x 2 mm x 2 mm depth) were prepared at the center of each fragment using #2292 diamond burs (KG Sorensen; Barueri, SP, Brazil) in a high-speed handpiece fixed in a standardization machine. After that, the prepared specimens were randomly divided into four groups for enamel ($n = 6$) and dentin ($n = 6$), according to the experimental primers tested: control, PA, API, and API + FAR.

In all groups, the enamel or dentin was conditioned with 37% phosphoric acid for 15 s, rinsed with distilled water for 30 s, and blot dried with absorbent paper. The primer was actively applied for 10 s, followed by air drying for 5 s at a 15-cm distance. The adhesive was applied and light cured for 20 s. The composite Filtek Z250 (3M Oral Care; St Paul, MN, USA) was inserted in 2 increments, each of which was light cured for 20 s each. The restorations were polished with #2500 and #4000-grit SiC papers (Arotec; Cotia, SP, Brazil) in a polishing machine.

Table 2 Means and standard deviation of μ TBS (MPa), after 24 h and 1 year of storage, and DC%

Groups	μ TBS		DC%*
	Immediate	1 year	
Control	31.9 (7.7) ^{Aa}	27.4 (2.9) ^{Aa}	73.4 (10.0)
PA	27.3 (5.0) ^{Aa}	24.9 (4.7) ^{ABa}	71.4 (5.7)
API	25.6 (4.5) ^{Aa}	31.6 (2.5) ^{Aa}	72.9 (7.7)
API + FAR	30.6 (4.0) ^{Aa}	18.7 (4.6) ^{Bb}	67.8 (6.8)

Means followed by different superscript letters (uppercase in columns; lowercase in rows) are statistically significantly different (Tukey's HSD test, $\alpha = 0.05$).
* ANOVA showed no significant difference among groups ($p = 0.6643$).

Initial microhardness of enamel and dentin surfaces

Initial Knoop microhardness (50 g, 10 s, HMV-2000, Shimadzu; Tokyo, Japan) of enamel and dentin surfaces was measured at 50 μ m, 100 μ m and 150 μ m from the restoration margin, with 5 indentations in each line (50 μ m from each other) (SMH control). After that, half of the restoration and enamel or dentin margins were covered by two layers of nail varnish to maintain a sound reference surface.³⁰

Biofilm formation

The biofilm model chosen for the experiment was composed of *S. mutans*. This microorganism plays the main role in the onset and progression of enamel caries and it is commonly found in the oral cavity.³⁰

The specimens were sterilized with ethylene oxide and fixed at the bottom of 24-well plates (24 Zellkultur Festplatte F, TPP; Trasadingen, Switzerland) with the surface of the restorations facing upwards. *S. mutans* isolate from the American Type Culture Collection (ATCC 25175, Fundação Oswaldo Cruz; Rio de Janeiro, RJ, Brazil) was cultured in brain heart infusion (BHI) broth (Difco; Sparks, NV, USA) supplemented with 2% sucrose at 37°C under anaerobic conditions for 24 h. Afterwards, the bacterial suspension was adjusted to 0.5 in accordance with the McFarland scale at 550 nm using a UV/Vis spectrophotometer (Beckman CoulterDU 530, LifeScience; San Diego, CA, USA). Then, the suspension was diluted 1:100 and 10 μ l of this suspension was added to each well, containing a specimen with 2 ml of BHI broth supplemented with 2% sucrose. The 24-well plates were kept for 48 h at 37°C under microaerophilic conditions. During the experiment, the growth medium was changed every 24 h.

Final hardness of dentin and enamel

After the biofilm formation period, samples were removed from the plates and the nail varnish on the reference surfaces was carefully removed with acetone-soaked cotton wool. The final surface microhardness evaluation (SMH₁) of enamel and dentin was performed 50 μ m away from the initial indentations.

The percentage of hardness change for enamel and dentin was calculated as follows: %hardness = 100 (SMH₁-SMH_{control})/SMH_{control}

Statistical Analysis

The data were analyzed using Statgraphics Centurion XVI software (STATPOINT Technologies; Warrenton, VA, USA). The normal distribution of errors and the homogeneity of variances were checked using the Shapiro-Wilk and Levene tests, respectively. Based on these preliminary analyses, DC%, WS, and SO data were analyzed using ANOVA. Nanoleakage, μ TBS, and microhardness were analyzed using two-way ANOVA. Tukey's HSD test was used for multiple comparisons when a statistical difference was detected. All analyses were performed at a level of significance of $\alpha = 0.05$.

RESULTS

The pH values of each primer were: control: 4.25; PA: 4.10; API: 3.93; API + FAR: 4.29. The means and standard deviation of μ TBS and DC% are summarized in Table 2. For the μ TBS test, two-way ANOVA showed that the factors "antibacterial agent" ($p = 0.0136$) and "storage time" ($p = 0.0080$), as well as the interaction between antibacterial agent and storage time ($p = 0.0005$), were statistically significant. In the immediate evaluation, μ TBS was similar for all groups. API + FAR presented a significant decrease in μ TBS after 1-year storage when compared to the immediate evaluation. In the other groups (CONTROL, PA and API), μ TBS did not decrease after storage. When groups were compared after 1 year of storage, API + FAR had significantly lower μ TBS compared to API and control, but with no difference from PA. PA and API presented μ TBS similar to that of the control group. Failure mode analysis after the μ TBS test is shown in Fig 1. The failure mode was predominantly adhesive in all groups.

Regarding the DC% data, ANOVA showed no significant difference among groups ($p = 0.6643$). The incorporation of antimicrobials did not affect the DC% of the experimental adhesives (Table 2).

Results of nanoleakage testing are summarized in Table 3. Two-way ANOVA showed that the factor storage time ($p = 0.0000$) was statistically significant. The antibacterial agent factor ($p = 0.1919$) and the interaction between antibacterial agent and storage time ($p = 0.0876$) were not significant. At the immediate evaluation, nanoleakage was similar for all groups. PA showed no increase in nanoleak-

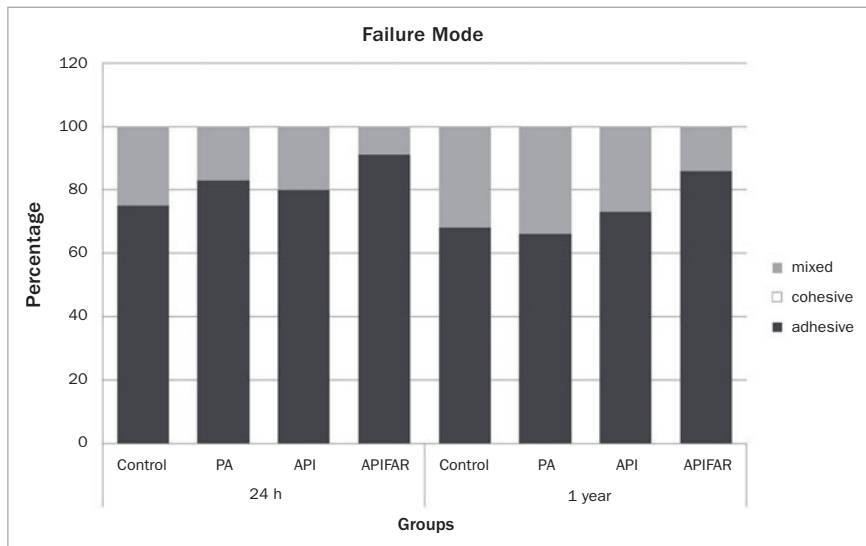


Fig 1 Failure mode (%) of each group after different periods of storage in distilled water.

Table 3 Means and standard deviation of nanoleakage (% silver) after 24 h and 1 year of storage

Groups	Nanoleakage (silver%)	
	Immediate (24 h)	1 year
Control	0.9 (1.2) ^{Aa}	5.3 (3.0) ^{ABb}
PA	1.1 (1.8) ^{Aa}	2.6 (4.4) ^{Aa}
API	0.9 (1.5) ^{Aa}	5.0 (2.9) ^{ABb}
API + FAR	0.8 (0.3) ^{Aa}	5.4 (4.0) ^{Bb}

Means followed by different superscript letters (uppercase in columns; lowercase in rows) are statistically significantly different (Tukey's HSD test, $\alpha = 0.05$).

age after 1 year of storage when compared to the immediate evaluation. In all other groups (control, API, API + FAR), nanoleakage increased after 1 year of storage when compared to the immediate evaluation. After 1 year of storage, PA presented significantly less nanoleakage when compared to API + FAR, but with no difference to control and API. API and API + FAR showed nanoleakage similar to that of the control group. In Fig 2, representative images of nanoleakage are presented.

With regard to WS and SO (Table 4), ANOVA showed a significant difference between the groups ($p = 0.0000$ for water sorption; $p = 0.0000$ for solubility). WS and SO of PA and API did not differ when compared to the control. However, API + FAR showed the lowest WS and the lowest SO when compared to the other groups.

The results of the hardness loss of dentin and enamel are detailed in Tables 5 and 6, respectively. For dentin, two-way ANOVA showed that the factors antimicrobial agent

($p = 0.0000$) and distance ($p = 0.0000$) were significant. The interaction between antimicrobial agent and distance was not significant ($p = 0.1081$). Control specimens showed a significantly greater hardness loss when compared to PA, API and API + FAR at all distances evaluated (50 μm , 100 μm and 150 μm from the margin). In all distances evaluated, PA, API and API + FAR presented inhibition of demineralization (lower hardness loss). At 150 μm from the restoration margin, API showed significantly lower hardness loss when compared to API + FAR and control groups, but this did not differ from PA. When the distances from the restoration margin were compared, all groups presented a significant increase in hardness loss at 100 μm when compared to 50 μm from the margin. With an increase in distance from the margin to 150 μm , no increase in hardness loss was observed for any of the groups when compared to 100 μm from the margin.

Considering the enamel margins, two-way ANOVA showed that the factors antimicrobial agent ($p = 0.0000$) and distance ($p = 0.0000$) were significant. The interaction between antimicrobial agent and distance was not significant ($p = 0.2943$). PA and API presented significantly less hardness loss when compared to control and API + FAR at 50 μm from the margin. At 100 μm from the margins, PA showed significantly less hardness loss when compared to the control and API + FAR groups, but with no difference from API; API and API + FAR did not differ from the control. No significant differences between groups were observed at 150 μm from the margins, showing no effect of antibacterial agents at this distance. When comparing the distances from the margin, control and API + FAR presented similar hardness loss at all distances. PA exhibited significantly greater hardness loss at 150 μm when compared to 50 μm and 100 μm . API presented significantly greater hardness loss at 150 μm when compared with 50 μm , but did not at 100 μm .

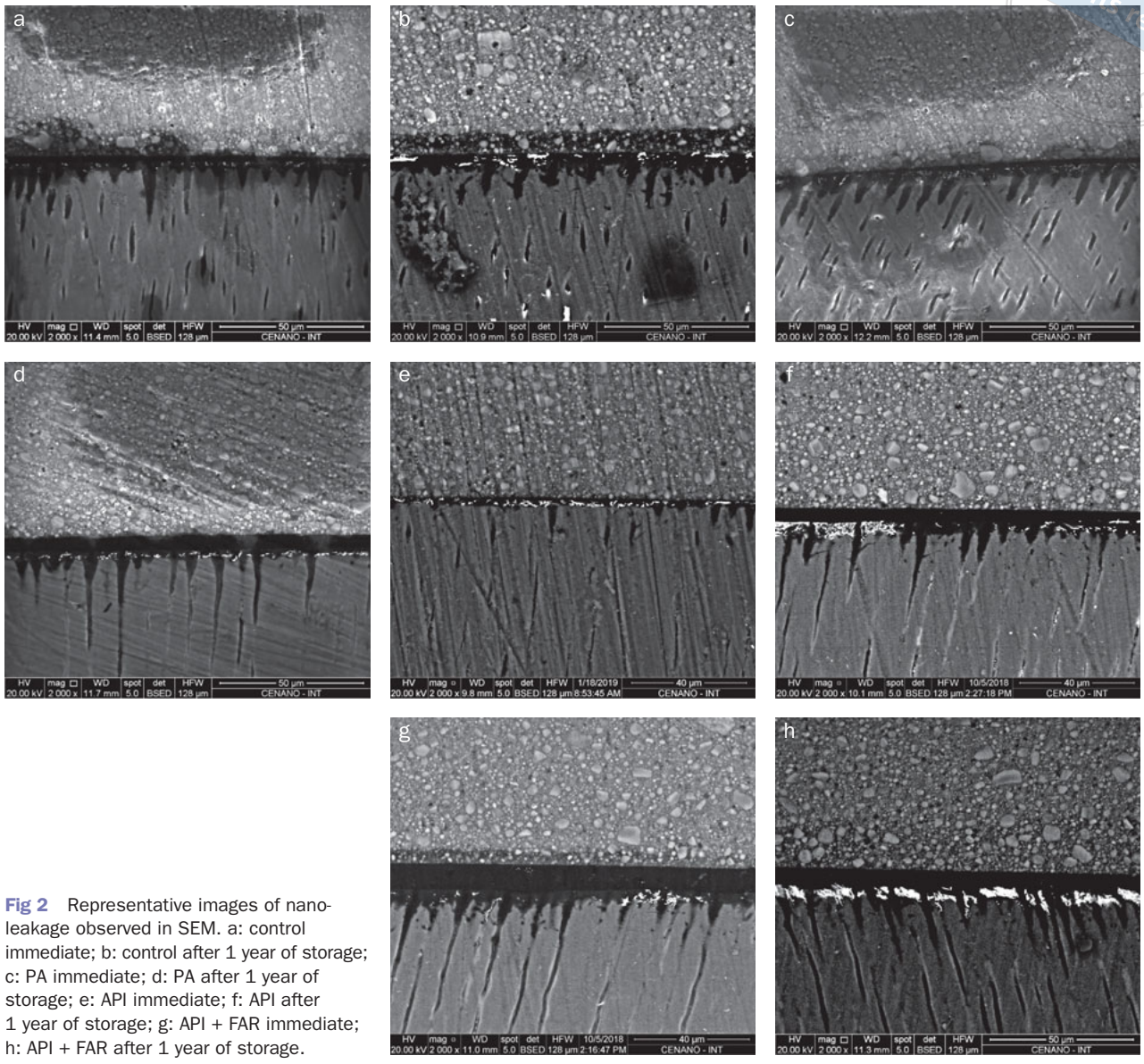


Fig 2 Representative images of nano-leakage observed in SEM. a: control immediate; b: control after 1 year of storage; c: PA immediate; d: PA after 1 year of storage; e: API immediate; f: API after 1 year of storage; g: API + FAR immediate; h: API + FAR after 1 year of storage.

DISCUSSION

In this study, antimicrobial substances were added to an experimental adhesive in order to analyze the real effects of these substances, eliminating the influence of possible unknown ingredients present in commercial adhesives.⁴⁰ Moreover, commercial adhesives do not report the exact percentage of each substance. Therefore, an experimental adhesive with a simple formulation and with the necessary characteristics to produce a good interaction with the dental substrate was chosen. HEMA was chosen for the primer because it is a hydrophilic functional monomer commonly used in commercial dentin-bonding agents. Additionally, its great penetration ability makes this water-soluble monomer an excellent adhesion-promoting agent, leading to an increase

in immediate bond strengths.^{19,36} PMGDM is a functional monomer that has been used in commercial⁴⁴ and in experimental adhesives^{45,31} or modified resin composites.^{10,32} This monomer was chosen to compose the primer, because it offers a tetramethacrylate and two COOH groups with adhesion-promotion potential through ionic interactions with the mineral content of the tooth.³² Experimental primers with PMGDM and HEMA were used in previous studies.^{31,45} For the adhesive blend, bis-GMA and TEG-DMA was chosen. Bis-GMA is a high-molecular-weight aromatic monomer that improves the mechanical properties of the adhesives;³⁷ TEG-DMA, a hydrophobic low-molecular-weight monomer, was used as a cross-linking agent. The higher flexibility of TEG-DMA compensates for the rigidity of bis-GMA, and admixture will result in resins with higher conversion rates.³

Table 4 Means and standard deviations of the WS and SO tests ($\mu\text{g}/\text{mm}^3$)

Groups	WS	SO
Control	193.8 (9.8) ^A	91.0 (8.1) ^A
PA	198.0 (8.0) ^A	84.3 (4.5) ^A
API	191.6 (13.5) ^A	91.2 (17.6) ^A
API + FAR	131.0 (9.1) ^B	60.5 (14.3) ^B

Means followed by different superscript letters (in columns) are statistically significantly different (Tukey's HSD test, $\alpha = 0.05$).

Table 5 Percentage of hardness loss of dentin at different distances from restoration margin after biofilm formation

Groups	Hardness loss in dentin (%)		
	50 μm	100 μm	150 μm
Control	30.9 (4.2) ^{Aa}	63.2 (3.9) ^{Ab}	78.0 (6.3) ^{Ab}
PA	7.8 (3.1) ^{Ba}	31.6 (12.5) ^{Bb}	45.0 (15.3) ^{BCb}
API	14.0 (6.5) ^{Ba}	23.1 (11.6) ^{Bab}	34.2 (18.0) ^{Cb}
API + FAR	10.5 (5.7) ^{Ba}	36.9 (13.9) ^{Bb}	56.9 (17.9) ^{Bb}

Means followed by different superscript letters (uppercase in columns, lowercase in rows) are statistically significantly different (Tukey's HSD test, $\alpha = 0.05$).

Table 6 Percentage of hardness loss (SD) for enamel adjacent to restorations after biofilm formation at different distances

Groups	Hardness loss in enamel(%)		
	50 μm	100 μm	150 μm
Control	25.8 (15.1) ^{Aa}	25.3 (8.3) ^{ABa}	37.0 (16.4) ^{Aa}
PA	3.3 (2.6) ^{Ba}	9.4 (3.6) ^{Ca}	32.9 (16.8) ^{Ab}
API	5.2 (3.8) ^{Ba}	16.3 (1.0) ^{BCab}	31.6 (4.7) ^{Ab}
API + FAR	33.1 (1.0) ^{Aa}	39.4 (2.1) ^{Aa}	45.8 (3.2) ^{Aa}

Means followed by different superscript letters (uppercase in columns, lowercase in rows) are statistically significantly different (Tukey's HSD test, $\alpha = 0.05$).

The natural antimicrobial agents proanthocyanidin, apigenin, and tt-farnesol were selected because previous studies have shown that these substances act against *S. mutans* biofilm through the inhibition of glucan synthesis.^{2,6,16,21-25,47} However, no other study evaluated the use of these substances incorporated into adhesive in terms of their ability to prevent recurrent caries using the model of restorations submitted to *S. mutans* biofilm.

In the present study, the substances incorporated into the experimental adhesive did not affect the DC% (67.8% and 73.4%), which is consistent with findings on commercial adhesives.¹⁵ It is likely that the antimicrobial powders at low concentrations do not chemically react with the experimental adhesive formulation, thus not interfering with the propagation of the polymerization reaction and remaining trapped between the linear chains after formation of the

adhesive polymer network. These results are in accordance with the study by Dias et al,⁹ who showed no differences among DC% of an adhesive containing proanthocyanidins at concentrations of 1%, 2%, 4.5% and 6%, in which the proanthocyanidins were diluted in acetone and filtered with filter paper.⁹ In contrast, Green et al¹⁷ demonstrated a reduction of DC% when proanthocyanidins were incorporated into the adhesive, as they observed a more porous hybrid layer. Liu et al²⁹ observed that the incorporation of 5% or 10% PA caused a reduction of the adhesive's DC%, while a concentration of 2.5% did not. However, in the study by Green et al,¹⁷ proanthocyanidins were not diluted and filtered as in the present study. In the study by Liu et al,²⁹ the proanthocyanidins were diluted in the solvent, but not filtered. In addition, differences in the monomer composition of the adhesives may cause differences in DC%.

The incorporation of antibacterial agents did not affect either the immediate μ TBS or that after 1 year of storage, except for API + FAR. Results ranged from 27.3 to 31.9 MPa and were compatible with values of commercially available adhesives.⁸ In a previous study,⁹ different concentrations of proanthocyanidins (1%, 2%, 4.5% and 6%) were shown to not affect immediate and long-term μ TBS, while the concentration of 4.5% showed greater μ TBS after 1 year when compared to the control group. Leme-Krauss et al²⁶ found superior adhesive properties for primers with proanthocyanidins both immediately and after 1 year of aging. Castellan et al⁷ investigated the long-term effect of proanthocyanidins on the μ TBS of demineralized dentin, and showed that treatment with proanthocyanidins resulted in greater μ TBS both after 24 h and 1 year of storage.

However, Epasinghe et al¹³ showed that the incorporation of 3% proanthocyanidins in an experimental adhesive decreased the dentin μ TBS. The addition of 3% proanthocyanidins reduced the degree of polymerization, which resulted in lower μ TBS and more adhesive failures. This might have occurred because the proanthocyanidins were not diluted and not filtered before adding them to an adhesive. On the other hand, the incorporation of proanthocyanidins at lower concentrations (1 or 2%) provided a reduction of nanoleakage and did not decrease the immediate μ TBS.¹³ API + FAR did not maintain μ TBS after 1 year of storage and presented lower μ TBS when compared to the other groups. The same pattern was observed with the results of nanoleakage.

In this study, nanoleakage was quantitatively evaluated by EDS. Three images of each stick were taken to (semi-)quantify the Ag infiltration in the hybrid layer at 2000X magnification.^{33,41} Thus, with greater magnification and three images, a smaller area of dentin subjacent to the hybrid layer, which can take up Ag, was evaluated. Thus, it can be assumed that most Ag infiltration into the hybrid layer was detected.

The lowest μ TBS and greater nanoleakage of API + FAR might be explained by the lipid nature and higher molecular weight of tt-farnesol (222.37 g/mol),²⁸ which might have interfered with the penetration of the adhesive into the dentin tubules. Poor penetration can create a hybrid layer that is less resistant and more susceptible to degradation over time.⁵ Andre et al² showed that the addition of apigenin (1 mM), tt-farnesol (5 mM), or a combination of the two, to adhesive systems did not influence the immediate μ TBS. However, after 1 year of storage, the results were material dependent: the μ TBS for Clearfil S3 Bond Plus (Kuraray Noritake; Tokyo, Japan) was not reduced by the incorporation of the substances, whereas the OptiBond (Kerr; Orange, CA, USA) S groups all experienced a reduction in μ TBS.²

The WS and SO of adhesives were evaluated because these characteristics involve a series of phenomena that may influence a material's clinical behavior. The polymer chains undergo relaxation, due to swelling caused by the absorbed water, which reduces the frictional forces between the polymeric side chains, causing plasticization of the adhesive polymer network. Unreacted monomers trapped within the network of adhesive polymers can be released into the surrounding medium, creating spaces in the struc-

ture of the material. These phenomena reflect a degradation mechanism of the material, which could weaken the network of adhesive polymers and the hybrid layer.⁸ In terms of WS and SO, no statistically significant differences were found between control, PA, and API, with values varying from 191.6 to 193.8 $\mu\text{g}/\text{mm}^3$ for WS and from 84.3 to 91.2 $\mu\text{g}/\text{mm}^3$ for SO. Theoretically, similar DC% would indicate that the adhesives had a similar number of unreacted monomers that could be leached from the polymer networks, thus influencing their solubility.⁸ It can be speculated that natural agents were tightly confined within the polymer network and were not dislodged by the absorbed water; therefore, there was no interference in the solubility of PA and API when compared to the control group. A previous study also showed that proanthocyanidins incorporated in adhesives did not affect the WS and SO.⁹ The lowest WS (131.0 $\mu\text{g}/\text{mm}^3$) and SO (60.5 $\mu\text{g}/\text{mm}^3$) obtained for API + FAR could be due to the lipid and hydrophobic nature of tt-farnesol, which, when trapped in the primer polymer matrix, is not soluble in water and was not leached.

Although the development of primary or recurrent caries depends on a complex microbial community, and biological cycling may occur with demineralization/remineralization phases in oral environments, a bacterial monoculture subjected to cariogenic challenge without demineralization/remineralization cycles was used in the present study. Nevertheless, this experiment model can be considered appropriate to the aim of the research, considering the need to better understand the results obtained with natural antimicrobial agents added to an experimental adhesive for the first time. Furthermore, the exclusive use of a *S. mutans* monoculture biofilm has been considered as the first screening for antibacterial measures of experimental materials, such as composite,³⁰ glass-ionomer cement³⁹ and adhesive.^{2,9} Additionally, the use of *S. mutans* biofilm models can be considered quite versatile because several analyses can be done, such as the measurement of the antimicrobial activity of the tested substances,⁹ influence on the virulence factors of the bacteria forming the biofilm,² and any anti-caries effect through the measurement of dental tissue hardness,³⁰ as used in this study.

In the present study, all adhesives containing the antimicrobial agents showed a reduction in hardness loss of dentin when compared to the control group. Even at the greatest distance from the margin (150 μm), PA, API, and API + FAR showed lower demineralization than did the control group. This indicates that all of the antimicrobial agents incorporated into the adhesive inhibited demineralization of the dentin exposed to the biofilm, which is very important for clinical practice. A previous study² found a decrease in biofilm dry weight with the addition of apigenin to the commercial adhesives Clearfil S3 Bond Plus (CS3, Kuraray Noritake) and OptiBond S (OPT, Kerr), with tt-farnesol in OPT and with the combination of the two agents in CS3, when compared to the adhesives lacking these substances. The amount of insoluble polysaccharides decreased with the addition of apigenin to CS3 and with the addition of tt-farnesol to OPT. Intracellular polysaccharides decreased with the

addition of apigenin or apigenin + tt-farnesol to CS3. These results showed that the effects of antimicrobial agents also depend on the adhesive used, the composition of the antimicrobial agents, and how the addition of agents may cause changes in the adhesives. Other studies showed positive results with use of GSE in dentin.^{21,38,46} In a previous study, the antibacterial effects of adhesives containing proanthocyanins also was demonstrated.⁹ However, only disks of adhesives were evaluated in this study.⁹ In another study, immersion in a 6.5% proanthocyanidin solution for 30 min reduced the progression of artificial carious root lesions produced by the demineralization-remineralization process when compared to the control group.³⁸ Dentin pretreatment with proanthocyanidins inhibited the development of secondary caries immediately adjacent to the adhesive-dentin interface.²¹ The protective effect of proanthocyanidins against the development of secondary caries in dentin was clearly illustrated by the presence of a zone of inhibition.²¹ Three possible mechanisms by which GSE may inhibit secondary caries in dentin have been described: tissue stabilization, interfacial sealing, and antimicrobial activity. GSE can also positively affect the remineralization process by deposition of minerals and by interaction with the organic portion through the proanthocyanidin-collagen interaction, stabilizing the exposed collagen matrix.⁴⁶

In enamel, PA and API presented significantly less hardness loss at 50 μm and 100 μm from the margin when compared to the control group. API + FAR did not differ when compared to the control group. All groups were similar at 150 μm from the margin, showing that the inhibition of recurrent caries is limited by distance from the margin. The best results in the prevention of enamel demineralization were observed for PA and API. A previous study²¹ showed no inhibition of secondary caries in enamel when pretreatment with GSE, chlorhexidine digluconate, or carbodiimide was performed. On the other hand, an *in vivo* (rats) study showed that topical treatments with proanthocyanidins resulted in smooth surface lesions that were 40%-45% less severe when compared to the control group.²² The immersion in a 12.5% GSE solution also significantly increased the microhardness of artificial carious lesions in enamel when compared to the control group.³⁴ It is believed that GSE can induce crosslinking through four mechanisms: covalent interaction, ionic interaction, hydrogen bonding interaction, and hydrophobic interaction. It is also possible that the remineralization of enamel is due to mineral precipitation, especially in the interior, since most of its structure is composed of inorganic matter.³⁴

Koo et al²⁴ showed that the severity of smooth surface caries was significantly lower in groups treated with apigenin, tt-farnesol, apigenin+tt-farnesol, fluoride (F), and chlorhexidine when compared to a control group. The combination of apigenin + tt-farnesol was more effective when compared to any compound alone.²⁴ This potentialized effect of apigenin with tt-farnesol was not observed in the present study, likely because the substances were added to the primer and not directly applied to the dental surface. In another study,²⁵ biofilms treated with apigenin and/or

tt-farnesol in combination with fluoride displayed less biomass and fewer insoluble glucans and iodophilic polysaccharides than did those treated with natural antimicrobial agents alone. The combination of the natural agents with fluoride was also highly effective in preventing caries development in rats, especially the combination of apigenin + tt-farnesol + fluoride.²⁵

Thus, proanthocyanidins, apigenin, and tt-farnesol incorporated into adhesives have the potential to inhibit the development of recurrent caries in enamel and dentin (except apigenin +tt-farnesol in enamel), which is very important in clinical practice. However, clinical studies are necessary to elucidate their efficacy.

CONCLUSION

The incorporation of proanthocyanidins and apigenin in an adhesive can prevent recurrent caries around the restoration margins in dentin and enamel, without jeopardizing the physical properties of the adhesive. The association of apigenin+tt-farnesol decreased the hardness loss of dentin, but it also decreased dentin μTBS after 1 year of storage, and was not effective in preventing recurrent caries in enamel.

ACKNOWLEDGMENTS

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (CAPES), Finance Code 001.

REFERENCES

1. Abraham S, Ghonmode WN, Saujanya KP, Jaju N, Tambe VH, Yawalikar PP. Effect of grape seed extracts on bond strength of bleached enamel using fifth and seventh generation bonding agents. *J Int Oral Health* 2013; 5:101–107.
2. Andre CB, Rosalen PL, Galvao LCC, Fronza BM, Ambrosano GMB, Ferracane JL, Giannini M. Modulation of *Streptococcus mutans* virulence by dental adhesives containing anti-caries agents. *Dent Mater* 2017;33: 1084–1092.
3. Asmussen E, Peutzfeldt A. Influence of selected components on cross-link density in polymer structures. *Eur J Oral Sci* 2001;109:282–285.
4. Benjamin S, Sharma R, Thomas SS, Nainan MT. Grape seed extract as a potential remineralizing agent: a comparative *in vitro* study. *J Contemp Dent Pract* 2012;13:425–430.
5. Betancourt DE, Baldion PA, Castellanos JE. Resin-dentin bonding interface: mechanisms of degradation and strategies for stabilization of the hybrid layer. *Int J Biomater* 2019;35268342.
6. Cao L, Zhang ZZ, Xu SB, Ma M, Wei X. Farnesol inhibits development of caries by augmenting oxygen sensitivity and suppressing virulence-associated gene expression in *Streptococcus mutans*. *J Biomed Res* 2017; 31:333–343.
7. Castellan CS, Bedran-Russo AK, Antunes A, Pereira PN. Effect of dentin biomodification using naturally derived collagen cross-linkers: one-year bond strength study. *Int J Dent* 2013;2013:918010.
8. da Silva EM, de Sa Rodrigues CU, de Oliveira Matos MP, de Carvalho TR, dos Santos GB, Amaral CM. Experimental etch-and-rinse adhesive systems containing MMP-inhibitors: Physicochemical characterization and resin-dentin bonding stability. *J Dent* 2015;43:1491–1497.
9. Dias PG, da Silva EM, Carvalho CM, Miranda M, Portela MB, Amaral CM. Characterization and antibacterial effect of an experimental adhesive containing different concentrations of proanthocyanidin. *J Adhes Dent* 2020; 22:139–147.
10. Dickens SH, Flaim GM, Floyd CJ. Effects of adhesive, base and diluent monomers on water sorption and conversion of experimental resins. *Dent Mater* 2010;26:675–681.

11. Diercke K, Lussi A, Kersten T, Seemann R. Isolated development of inner (wall) caries like lesions in a bacterial-based in vitro model. *Clin Oral Invest* 2009;13:439–444.
12. Eltahlah D, Lynch CD, Chadwick BL, Blum IR, Wilson NHF. An update on the reasons for placement and replacement of direct restorations. *J Dent* 2018;72:1–7.
13. Epasinghe DJ, Yiu CK, Burrow MF, Hiraishi N, Tay FR. The inhibitory effect of proanthocyanidin on soluble and collagen-bound proteases. *J Dent* 2013; 41:832–839.
14. Epasinghe DJ, Yiu CK, Burrow MF, Tay FR, King NM. Effect of proanthocyanidin incorporation into dental adhesive resin on resin-dentine bond strength. *J Dent* 2012;40:173–180.
15. Faria-e-Silva AL, Lima AF, Moraes RR, Piva E, Martins LR. Degree of conversion of etch-and-rinse and self-etch adhesives light-cured using QTH or LED. *Oper Dent* 2010;35:649–654.
16. Furiga A, Roques C, Badet C. Preventive effects of an original combination of grape seed polyphenols with amine fluoride on dental biofilm formation and oxidative damage by oral bacteria. *J Appl Microbiol* 2014; 116:761–771.
17. Green B, Yao X, Ganguly A, Xu C, Dusevich V, Walker MP, Wang Y. Grape seed proanthocyanidins increase collagen biodegradation resistance in the dentin/adhesive interface when included in an adhesive. *J Dent* 2010; 38:908–915.
18. Han Q, Li B, Zhou X, Ge Y, Wang S, Li M, Ren B, Wang H, Zhang K, Xu HHK, Peng X, Feng M, Weir MD, Chen Y, Cheng L. Anti-caries effects of dental adhesives containing quaternary ammonium methacrylates with different chain lengths. *Materials (Basel)* 2017;10:643.
19. Hasegawa T, Manabe A, Itoh K, Wakumoto S. Investigation of self-etching dentin primers. *Dent Mater* 1989;5:408–410.
20. ISO 4049. Dentistry – Polymer-based filling, restorative and luting materials. 2000(E).
21. Kim GE, Leme-Kraus AA, Phansalkar R, Viana G, Wu C, Chen SN, Pauli GF, Bedran-Russo A. Effect of bioactive primers on bacterial-induced secondary caries at the tooth-resin interface. *Oper Dent* 2017;42:196–202.
22. Koo H, Duarte S, Murata RM, Scott-Anne K, Gregoire S, Watson GE, Singh AP, Vorsa N. Influence of cranberry proanthocyanidins on formation of biofilms by *Streptococcus mutans* on saliva-coated apatitic surface and on dental caries development in vivo. *Caries Res* 2010;44:116–126.
23. Koo H, Jeon JG. Naturally occurring molecules as alternative therapeutic agents against cariogenic biofilms. *Adv Dent Res* 2009;21:63–68.
24. Koo H, Pearson SK, Scott-Anne K, Abranches J, Cury JA, Rosalen PL, Park YK, Marquis RE, Bowen WH. Effects of apigenin and tt-farnesol on glucosyltransferase activity, biofilm viability and caries development in rats. *Oral Microbiol Immunol* 2002;17:337–343.
25. Koo H, Schobel B, Scott-Anne K, Watson G, Bowen WH, Cury JA, Rosalen PL, Park YK. Apigenin and tt-farnesol with fluoride effects on *S. mutans* biofilms and dental caries. *J Dent Res* 2005;84:1016–1020.
26. Leme-Kraus AA, Aydin B, Vidal CM, Phansalkar RM, Nam JW, McAlpine J, Pauli GF, Chen S, Bedran-Russo AK. Biostability of the proanthocyanidins-dentin complex and adhesion studies. *J Dent Res* 2017;96:406–412.
27. Li Y, Carrera C, Chen R, Li J, Lenton P, Rudney JD, Jones RS, Aparicio C, Fok A. Degradation in the dentin-composite interface subjected to multi-species biofilm challenges. *Acta Biomater* 2014;10:375–383.
28. Lide, DR. *Handbook of Chemistry and Physics*. Boca Raton, FL: CRC Press, 2005.
29. Liu Y, Dusevich V, Wang Y. Addition of grape seed extract renders phosphoric acid a collagen-stabilizing etchant. *J Dent Res* 2014;93:821–827.
30. Maia AC, Mangabeira A, Vieira R, Neves AA, Lopes RT, Pires TM, Viana GM, Cabral LM, Cavalcante LM, Portela MB. Experimental composites containing quaternary ammonium methacrylates reduce demineralization at enamel restoration margins after cariogenic challenge. *Dent Mater* 2019; 35:e175–e183.
31. Melo MA, Cheng L, Weir MD, Hsia RC, Rodrigues LK, Xu HH. Novel dental adhesive containing antibacterial agents and calcium phosphate nanoparticles. *J Biomed Mater Res B Appl Biomater* 2013;101:620–629.
32. Milward PJ, Adusei GO, Lynch CD. Improving some selected properties of dental polyacid-modified composite resins. *Dent Mater* 2011;27:997–1002.
33. Miranda MEDSNG, Silva EMD, Oliveira MF, Simmer FS, Santos GBD, Amaral CM. Resin-dentin bond stability of etch-and-rinse adhesive systems with different concentrations of MMP inhibitor GM1489. *J Appl Oral Sci* 2020;28:e20190499.
34. Mirkarimi M, Eskandarion S, Bargrzan M, Delazar A, Kharazifard MJ. Remineralization of artificial caries in primary teeth by grape seed extract: an in vitro study. *J Dent Res Dent Clin Dent Prospects* 2013;7:206–210.
35. Munoz-Gonzalez I, Thurnheer T, Bartolome B, Moreno-Arribas MV. Red wine and oenological extracts display antimicrobial effects in an oral bacteria biofilm model. *J Agric Food Chem* 2014;62:4731–4737.
36. Nakaoki Y, Nikaido T, Pereira PN, Inokoshi S, Tagami J. Dimensional changes of demineralized dentin treated with HEMA primers. *Dent Mater* 2000;16:441–446.
37. Oguri M, Yoshida Y, Yoshihara K, Miyauchi T, Nakamura Y, Shimoda S, Hanabusa M, Momoi Y, Van Meerbeek B. Effects of functional monomers and photo-initiators on the degree of conversion of a dental adhesive. *Acta Biomater* 2012;8:1928–1934.
38. Pavan S, Xie Q, Hara AT, Bedran-Russo AK. Biomimetic approach for root caries prevention using a proanthocyanidin-rich agent. *Caries Res* 2011; 45:443–447.
39. Porter GC, Tompkins GR, Schwass DR, Li KC, Waddell JN, Meledandri CJ. Anti-biofilm activity of silver nanoparticle-containing glass ionomer cements. *Dent Mater* 2020;36:1096–1107.
40. Silva AP, Goncalves RS, Borges AF, Bedran-Russo AK, Shinohara MS. Effectiveness of plant-derived proanthocyanidins on demineralization on enamel and dentin under artificial cariogenic challenge. *J Appl Oral Sci* 2015;23:302–309.
41. Simmer FS, da Silva EM, Bezerra R, Miranda M, Noronha Filho JD, Amaral CM. Bond stability of conventional adhesive system with MMP inhibitors to superficial and deep dentin. *J Mech Behav Biomed Mater* 2019;100: 103402.
42. Supuran CT. Nanoparticles for controlled release of anti-biofilm agents WO2014130994 (A1): a patent evaluation. *Expert Opin Ther Pat* 2015; 25:945–948.
43. Swadas M, Dave B, Vyas SM, Shah N. Evaluation and comparison of the antibacterial activity against streptococcus mutans of grape seed extract at different concentrations with chlorhexidine gluconate: an in vitro study. *Int J Clin Pediatr Dent* 2016;9:181–185.
44. Van Landuyt KL, Snauwaert J, De Munck J, Peumans M, Yoshida Y, Poitevin A, Coutinho E, Suzuki K, Lambrechts P, Van Meerbeek B. Systematic review of the chemical composition of contemporary dental adhesives. *Biomaterials* 2007;28:3757–3785.
45. Velazquez E, Vaidyanathan J, Vaidyanathan TK, Houpt M, Shey Z, Von Hagen S. Effect of primer solvent and curing mode on dentin shear bond strength and interface morphology. *Quintessence Int* 2003;34:548–555.
46. Xie Q, Bedran-Russo AK, Wu CD. In vitro remineralization effects of grape seed extract on artificial root caries. *J Dent* 2008;36:900–906.
47. Zhao W, Xie Q, Bedran-Russo AK, Pan S, Ling J, Wu CD. The preventive effect of grape seed extract on artificial enamel caries progression in a microbial biofilm-induced caries model. *J Dent* 2014;42:1010–1018.

Clinical relevance: The incorporation of proanthocyanidins, apigenin, or apigenin+tt farnesol into the primer of an adhesive can prevent recurrent caries at dentin margins; however, proanthocyanidins and apigenin are preferred for enamel margins.