



Research article

Polyphenols and Brazilian red propolis incorporated into a total-etching adhesive system help in maintaining bonding durability



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ABSTRACT

Objectives: The aim of this study was to evaluate the degree of conversion and bond strength of a commercial dental adhesive modified by the incorporation of quercetin, resveratrol (RES), and Brazilian red propolis (BRP).
Methods: BRP markers were identified using ultra-performance liquid chromatography coupled with a diode array detector, and the antioxidant activity (AAO) of the three substances was analyzed. Single Bond 2 adhesive (3M ESPE) was modified by adding BRP, quercetin, and RES, separately, at 20 µg/mL, 250 µg/mL, and 500 µg/mL, respectively. The degree of conversion (DC) was measured using near-infrared spectroscopy 24 h after photopolymerization. Measurements of the resin-dentin microtensile bond strength (µTBS) were carried out after 1 day and 1 year. Student's t test and ANOVA with Tukey's test were used for data analysis ($\alpha = 0.05$).

Results: The markers daidzein, liquiritigenin, pinobanksin, isoliquiritigenin, formononetin, pinocembrin, and biochanin A were found in the ethanolic extract of BRP. Quercetin, RES, and BRP showed high AAO. The DC of the tested adhesives remained adequate for this category of material, with a slight increase in the DC of adhesives with quercetin and BRP ($P > 0.05$). Comparisons between µTBS measurements made at 1 day and 1 year showed that, contrary to the control group, µTBS values for all modified adhesives were maintained after 1 year in distilled water ($P > 0.05$).

Conclusions: These findings suggest that quercetin, RES, or BRP might be useful in adhesive dentistry to help improve hybrid layer resistance.

Clinical significance: Dentin bonding agents with quercetin, RES, and BRP have potential to increase the longevity of composite restorations.

1. Introduction

Adhesive dentistry is based on bonding between resin-based materials and the dental hard tissue through an interface between the tooth and the

adhesive materials, the so-called hybrid layer [1]. Initially, the dentin is etched with acid to create spaces between the collagen fibrils, which will be filled by the adhesive. However, in some places, the resinous adhesive does not fully infiltrate into exposed collagen and the demineralized

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dentin at the bottom of the hybrid layer becomes vulnerable. This is the weakest part within the adhesive interface and is susceptible to the proteolytic activity of matrix metalloproteinases (MMPs) [1, 2]. Acid etching (low pH) of the dentin surface stimulates zymogens by activating proteolytic enzymes such as MMPs that can degrade the non-infiltrated exposed collagen in the hybrid layer, which compromises the longevity of adhesive restorations [3, 4, 5].

MMPs are zinc- and calcium-dependent endopeptidases that participate in the process of dental development, progression of caries, and degradation of collagen in acid-etched dentin [6]. MMP-2, -8, -9, -13, and -20 have been found in the organic matrix of dentin. They contribute to the organization and mineralization of the dentin matrix. MMP-2 and -9 are the most prevalent in dentin, with a much greater amount of MMP-2 than the others [7, 8, 9].

Synthetic or natural bioactive agents that inhibit endopeptidases of the collagen matrix have been incorporated into adhesive systems and acidic gel for dentin etching or applied as a solution for dentin pretreatment after acid etching in an effort to preserve the unprotected collagen at the hybrid layer and improve the durability of resin/dentin bonding [10, 11, 12].

Free radicals are unstable highly reactive chemical species produced by cells from different tissues. Increase in the production of these species without the effective action of endogenous and exogenous antioxidant systems generates a condition of oxidative stress, which potentially leads to destruction of structural proteins in tissues [13]. Antioxidant substances suppress oxidative stress and reduce the expression and activity of MMPs through the inhibition of free radicals [14, 15].

Some natural products, such as polyphenols and propolis, can play an important role in protecting unprotected collagen fibrils within the hybrid layer due to their high antioxidant potential [16]. Propolis has many pharmacologic activities, including antioxidant activity [16], antimicrobial and antifungal activity against *Streptococcus mutans*, *Lactobacillus acidophilus*, and *Candida albicans* [17], anti-inflammatory activity, and healing properties [18]. Brazilian red propolis (BRP) is rich in flavonoids, isoflavonoids, tannins, xanthenes, and guttiferones [16]. Due to its antioxidant action and antimicrobial activity against cariogenic bacteria, the addition of propolis to bonding agents could be relevant for prevention of enzymatic degradation of dentin collagen and secondary caries.

Polyphenols, natural inhibitors of MMPs [19], can stabilize the collagen chain by increasing the number of crosslinks of collagen fibrils and by decreasing the enzymatic biodegradation of collagen. The mechanism of action is complex, involving multifunctional activities, such as downregulation of endogenous protease expression, protease inactivation, and the prevention of free access of collagenase to active sites in collagen chains [10]. Quercetin (3,3',4',5,7-pentahydroxyflavone) is a major representative of the flavonoids, a subclass of flavonols, well known for their high antioxidant potential and antimicrobial and anti-inflammatory activities, among others [20]. The study by Park et al. [21] clearly showed inhibition of MMP-2 and MMP-9 by quercetin. Resveratrol (RES) is a natural polyphenolic compound, which acts functionally as a phytoalexin, identified in nature as *cis* and *trans* isomers [22]. With high antioxidant activity, RES has been shown to significantly reduce the expression of MMP-2 and MMP-9 in other sites of the human body [23, 24] and protect the collagen fibrils in the hybrid layer [12]. At low concentrations, RES has genoprotective action, reducing DNA damage induced by dentin adhesives [25]. Longer-lasting restorations reduce the need for replacements and as a result, the restorative cycle, which can result in increasingly complex treatments or even tooth loss, is reduced. Thus, it is possible to consider that the antioxidant potential and antimicrobial activity against cariogenic bacteria of quercetin, RES, and BRP, assist in maintaining the integrity of the adhesive interface by inhibiting MMPs in dentin and preventing secondary caries, promoting greater durability of composite resin restorations. The aim of this study was to evaluate the degree of conversion and bond strength of a commercial

dental adhesive modified by the incorporation of quercetin, RES, and BRP at different concentrations.

2. Materials and methods

Sixty-six sound unerupted human third molars, one per patient, were collected from adult patients of both genders aged from 18 to 46 years after written informed consent was obtained from all patients. All procedures followed in this study were in accordance with the ethical principles of the Declaration of Helsinki. The study protocol was reviewed and approved by the Research and Ethics Committee of the Federal University of Alagoas, Brazil. The teeth were stored in chloramine-T 0.5% solution at 4 °C before the experiment.

2.1. Chemicals

trans-Resveratrol (3,5,4'-trihydroxy-*trans*-stilbeno), quercetin dihydrate, ≥98% (HPLC) and chloramine-T were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Collection and elaboration of propolis extract

BRP was collected in the city of Marechal Deodoro, Alagoas, Brazil, in July 2013 (geographical coordinates 9° 44.555' S, 35° 52.080' O, 18.1 m above sea level). Access and transportation of BRP was previously authorized by regulatory agencies for the control of Brazilian Genetic Heritage and Biodiversity Conservation (protocol no. A88DA2B). The extraction of BRP was carried out by maceration in 80% ethanol to obtain an ethanolic extract of BRP (EEBRP).

Liquid-liquid extraction of this crude extract was performed to eliminate grease and waxes. The crude extract (8 g) was solubilized with absolute ethanol (35 mL) and then 15 mL of distilled water was added following of vigorous agitation and allowed to stand for a few minutes. This BRP crude extract was transferred to a separation funnel, and hexane (50 mL) was added to eliminate the grease and wax present in the crude extract. The hexane layer was removed with a separation funnel, and then ethyl acetate solvent (200 mL) was added in two liquid-liquid extraction steps to obtain an ethyl acetate extract enriched with the flavonoids and isoflavonoids from BRP, free of grease and wax. The ethyl acetate extract of Brazilian red propolis (EABRP) was subjected to distillation under reduced pressure in a rotary evaporator to obtain a solid mass (4.0 g), which was used in all the experiments in this study.

2.3. Identification of BRP markers

The identification and quantification of markers in the EABRP were performed as described by Porto et al. [17] using ultra-performance liquid chromatography coupled with a diode array detector (UPLC-DAD) from Shimadzu (Tokyo, Japan). The equipment consisted of the following modules: a high-pressure pump (model LC-20ADXR), degasser (model DGU-20A3R), auto-injector (model SIL-20AXR), oven chromatographic column, photo diode array detector (model EPDM-20A), a controller (model CBM-20A), and Shimadzu LabSolution software (Shimadzu, Tokyo, Japan).

The separation of flavonoids occurred using a reversed-phase column (C₁₈, 150 mm 4.6 mm; 5 μm), and a mobile phase that consisted of solvent A (Milli-Q water) and solvent B (acetonitrile), pumped at a flow rate of 0.3 mL/min. The initial elution gradient consisted of 70% water (A) and 30% acetonitrile (B) (v/v). The column was eluted by varying the percentage of (B) as follows: 0–2 min 30% B, 2–5 min 36% B, 5–8 min 46% B, 8–11 min 52% B, 11–14 min 52% B, 14–17 min 57% B, 17–20 min 62% B, 20–24 min 62% B, 24–28 min 68% B, 28–32 min 72% B, 32–36 min 90% B, 36–42 min 97% B, 42–50 min 100% B, 50–55 min 100% B; 55–57 min acetonitrile was reduced to 30% and this condition was maintained up to 60 min. This long method was developed in order to wash the column during the analysis with 100% acetonitrile, avoid

lack of accuracy and loss of precision during the entrapment assay, and avoid column fouling and excessive pressure buildup by irreversible retention of non-polar compounds (terpenes and guttiferones present in BRP extract). The injection volume was 2 μL . Analytical standards of flavonoids described as BRP markers (daidzein, liquiritigenin, pinobanskin, isoliquiritigenin, formononetin, pinocembrin, and biochanin A) and the EABRP were prepared in a stock solution of 10,000 $\mu\text{g}/\text{mL}$, using acetone as solvent and diluted to a concentration of 500 $\mu\text{g}/\text{mL}$. Calibration of the marker quantification method was carried out according to Nascimento et al. [26].

2.4. Antioxidant activity

Quantitative assessment of the antioxidant activity (AAO) of quercetin, RES, EEBRP, and EABRP were performed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. The method is based on the spectrophotometric measurement of the change in concentration of DPPH \bullet after reaction with an antioxidant. Ethanol was used as a blank. The inhibition of DPPH \bullet by the samples was monitored by measuring the decrease in absorbance of solutions with different concentrations. Quercetin, RES, EEBRP, and EABRP at an initial concentration of 1.0 mg/mL were diluted with ethanol to final concentrations of 0.05, 0.5, 1.0, 2.0, 4.0, 6.0, and 10.0 $\mu\text{g}/\text{mL}$. Then, 1.0 mL of 0.3 mM DPPH in ethanol was added to 2.5 mL of the three compounds, and the reaction was left to develop in the dark at room temperature (26 $^{\circ}\text{C}$) for 30 min. The absorbance readings were then performed with a spectrophotometer (Model UV-1700, Shimadzu, Kyoto, Japan) at 518 nm. The antioxidant activity of quercetin, RES, EEBRP, and EABRP was measured in triplicate, and the mean of the triplicates was recorded as representative of each compound.

2.5. Preparation of experimental adhesives

Initially, a mother solution of each product was prepared by adding 52 mg of quercetin, 50 mg of EABRP, or 50 mg of RES to 5 mL of ethanol and totally solubilized in ultrasound (UltraCleaner 700, Unique) for 1 min. Then, the following solutions were prepared by dilution of each mother solution in ethanol: solution 1 (0.5%), solution 2 (0.25%), and solution 3 (0.02%) of quercetin, RES, and EABRP. The adhesives were prepared by adding 25 μL of each solution 1, 2, and 3–240 mg of Single Bond 2 (3M ESPE, St. Paul, MN, USA) to obtain adhesives containing quercetin, RES, and EABRP at 500 $\mu\text{g}/\text{mL}$, 250 $\mu\text{g}/\text{mL}$, and 20 $\mu\text{g}/\text{mL}$. According to the manufacturer, the composition of Single Bond 2 adhesive is as follows: bisphenol A diglycidylmethacrylate (bis-GMA), 2-hydroxyethylmethacrylate (HEMA), a novel photoinitiator system and a methacrylate functional copolymer of polyacrylic and polyitaconic acids, water, ethanol, dl-camphorquinone, spherical silica particles (5 nm, 10% by wt). To test the effect of the solvent, 25 μL of pure ethanol was added to 240 mg of Single Bond 2 adhesive (BL). All experimental adhesives were kept in glass light-proof vials at 4 $^{\circ}\text{C}$.

2.6. Degree of conversion

The degree of conversion (DC) of the experimental adhesives was measured using Fourier transform infrared spectroscopy (FTIR; IRAffinity-1, Shimadzu, Kyoto, Japan). FTIR spectra ($n = 5$) were recorded in attenuated total reflectance mode from 128 scans at a wavenumber resolution of 4 cm^{-1} from uncured samples and 24 h after photoactivation of the adhesives with quercetin, RES, and BRP, pure Single Bond 2 (control) and Single Bond 2 + 25 μL ethanol (BL). Polymerized samples were made by placing 25 μL of adhesive on a polyester strip and left undisturbed for 30 s to evaporate the excess solvent. Then, the adhesive was covered with another polyester strip and light-cured for 20 s (LED, Emitec B, Schuster Com. Equip. Odontológicos Ltda., Santa Maria, RS, Brazil; 1150 mW cm^2 ; 420–480 nm wavelength range). The samples were kept dry in light-proof vials before FTIR analysis. To obtain spectra from uncured adhesives, 25 μL of adhesive was placed directly

into the equipment sample holder. DC was calculated by assessing the variation in the peak height ratio of the absorbance intensities of methacrylate carbon to the carbon (C=C) double bond peak at 1638 cm^{-1} by using the aromatic C=C double bond peak at 1608 cm^{-1} as an internal standard during polymerization of the uncured material using the following equation (Eq.1):

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

where R is the ratio between the absorbance peak at 1638 cm^{-1} and 1608 cm^{-1} .

2.7. Microtensile bond strength testing

The occlusal one-third of the tooth crown was removed using a water-cooled low-speed diamond saw (Buehler, Lake Bluff, IL, USA) to create a flat dentin surface. Then, a uniform smear layer was created by abrading the dentin surface with 600-grit silicon carbide (SiC) grinding paper for 60 s under water to standardize the bonding surface. The exposed dentin surfaces of all teeth were etched with 35% phosphoric acid gel (Scotch-bond Universal Etchant; 3M ESPE) for 15 s, then rinsed for 30 s with distilled water. Excess water was removed with absorbent paper and two coats of adhesive were applied immediately with gentle agitation using a fully saturated small brush (Microaplicador KG Brush - KG SORENSEN, Cotia, Brazil). After gently air drying to evaporate the solvent, the adhesive was light-cured for 10 s using a light-emitting diode curing device. A composite resin crown (Filtek Z350XT; 3M ESPE) was subsequently built with two incremental layers of 2 mm. Each increment was light-cured for 20 s with the same light source. The bonded teeth were stored in distilled water at 37 $^{\circ}\text{C}$.

Twenty-four hours after the bonding procedures, the teeth were sectioned longitudinally across the bonded interface in both the X and Y directions using a low-speed saw (IsoMet 1000 Precision Saw, Buehler, Lake Bluff, IL, USA) under water cooling to obtain bonded sticks with a cross-sectional area of $0.8 \pm 0.1 \text{ mm}^2$. Half of the sectioned sticks of each tooth were randomly selected for immediate testing. The remaining sticks were stored in distilled water at 37 $^{\circ}\text{C}$ for 1 year. For testing, each bonded stick ($n = 25$ per group) was attached to a testing jig with cyanoacrylate glue on a linear actuator-driven offset device for microtensile bond strength (μTBS) testing (OM 100, Odeme, Luzerna, SC, Brazil) and was stressed until failure.

2.8. Fracture pattern evaluation

The failure modes were evaluated at $60\times$ magnification (Stereo Zoom Leica S8 APO, Leica Microsystems; Wetzlar, Germany) and categorized into four groups as follows: cohesive failure in dentin, when located exclusively within dentin; cohesive failure in resin, when located exclusively within resin; adhesive failure when failure occurred at the dentin/adhesive interface; or mixed failure when more than one mode of failure occurred simultaneously.

2.9. Scanning electron microscopy

2.9.1. Silver uptake

Two bonded sticks from each group sectioned as described for microtensile testing were coated with two layers of nail varnish applied to within 1 mm of the bonded interfaces. The specimens were rehydrated in distilled water for 10 min before immersion in tracer solution for 24 h. Silver nitrate was prepared according to the protocol described previously by Tay et al. [27]. The sticks were placed in silver nitrate in darkness for 24 h, rinsed thoroughly in distilled water, and immersed in a photo-developing solution for 8 h under fluorescent light to reduce silver ions to metallic silver grains within voids along the bonded interface. Specimens were wet polished using SiC paper with decreasing grit (600, 800, 1000, 1200, 1500) and with 1- and $1/4\text{-}\mu\text{m}$ diamond paste (Erios

Prod Odont Ltd., São Paulo, SP, Brazil) using a polishing cloth. The specimens were cleaned in an ultra-sonic bath containing distilled water for 15 min at each polishing step, then mounted on aluminum stubs, and dried with silica gel for 24 h at 37 °C before sputtering with gold. Resin-dentin interfaces were analyzed in a scanning electron microscope (Vega LM, TESCAN Orsay Holdin, Ljubljana, Czech Republic) operated in backscattered electron mode at an accelerating voltage of 20 kV.

2.9.2. Morphology of the hybrid layer and mode of failure

To observe the morphology of the hybrid layer, the samples were polished using ascending SiC papers from 800 to 1500 grit. A further final polishing procedure was performed using diamond pastes (Buheler-MetaDi, Buheler, Lake Bluff, IL, USA) from 1 µm to 0.25 µm. The specimens were cleaned in an ultra-sonic bath with distilled water for 15 min at each polishing step. Next, the samples were treated with 37% phosphoric acid for 1 min, rinsed, and deproteinized with 2.5% sodium hypochlorite for 4 min. The samples were dehydrated in increasing concentrations of ethanol (25%, 50%, 75%, and 95%) for 20 min each. This was followed by 100% ethanol three times for 20 min each, and then the samples were fixed on stubs with conductive tape for analysis under a scanning electron microscope (SEM; Vega LM, TESCAN Orsay Holdin, Ljubljana, Czech Republic). Five images were captured from each sample after analysis of the whole hybrid layer region.

Debonded sticks of each type of failure were stored in groups according to the failure mode. Then four sticks from each group were randomly selected and observed using scanning electron microscopy to illustrate the mode of failure.

2.10. Statistical analysis

A two-way factorial ANOVA and Tukey's post-hoc test for pairwise comparisons between the means were used to analyze the effects of materials (adhesives) and storage time (1 day and 1 year) on bond strength to dentin. Student's t test was used to compare the effect of storage time in water on the mean bond strength. SPSS version 21 (SPSS, Chicago, IL, USA) was used for all statistical analyses. $P < 0.05$ was considered significant.

3. Results

3.1. Identification of BRP markers

A representative UPLC-DAD chromatogram of the EABRP is shown in Figure 1. The compounds were identified by comparing their chromatographic behavior (retention times and peak purity at the maximum wavelengths) with the analytical standards of flavonoids. The following markers of BRP were identified and quantified in the chromatogram: daidzein (1), liquiritigenin (2), pinobanksin (3), isoliquiritigenin (4), formononetin (5), pinocebrin (6), and biochanin A (7). Chromatographic parameters, such as retention time, maximum wavelength, and concentration of the markers in the EABRP, are shown in Table 1.

3.2. Antioxidant activity

The results for the AAO of quercetin, RES, and BRP extracts showed high antioxidant activity in all the substances tested (Table 2). Quercetin had the highest percentage of AAO, followed by EABRP, EEBRP, and RES. EABRP had better antioxidant activity than EEBRP, ranging from 58.73% (0.05 µg/mL) to 87.16% (10 µg/mL).

3.3. Degree of conversion

The mean values and standard deviations for the DC of all modified adhesives and controls are shown in Figure 2. The addition of BRP and quercetin to the adhesives increased the DC with no significant difference from the control group ($P > 0.05$). The highest DCs were recorded with quercetin at 500 µg/mL (87.95%) and quercetin at 20 µg/mL (88.50). RES at 20 µg/mL had the lowest DC (74.18%) among all the samples, with a significant difference from quercetin at 500 µg/mL ($P = 0.0007$), quercetin at 20 µg/mL ($P = 0.0004$), and BRP at 500 µg/mL ($P = 0.0077$).

3.4. Microtensile bond strength testing

Means ± standard deviations of the bond strength (MPa) test are shown in Figure 3A. The factor adhesive as well as the factor storage time had a significant effect on bond strength ($P < 0.0001$). The means of the microtensile strength of the dentin/resin interface of all experimental adhesives were within the range of appropriate values for this type of material, and after 1 year, they were significantly higher than that of the control group. Considering each time of evaluation, the averages were correspondingly higher for adhesives with propolis, varying between 41.40 MPa and 45.73 MPa. The overall baseline mean values (1 day) varied between 29.14 (BL) and 45.73 (BRP at 500 µg/mL) MPa, and after 1 year in water, the recorded values were between 21.56 MPa (BL) and 42.38 MPa (BRP at 500 µg/mL). The µTBS results showed that the addition of quercetin and RES at 20 µg/mL, and quercetin at 250 µg/mL, did not affect immediate bond strength ($P > 0.05$). BRP, quercetin at 500 µg/mL, and RES at 250 and 500 µg/mL improved the immediate µTBS ($P < 0.05$). All experimental adhesives showed a similar behavior, with slightly lower µTBS values ($P > 0.05$) after 1 year in distilled water compared with baseline ($P > 0.05$).

The failure pattern distribution (%) for resin-dentin µTBS testing was assessed using a stereomicroscope (60× magnification) (Figure 3B). Mixed failure mode was predominant in all groups followed by adhesive failure (cohesive failure in the adhesive and/or in the hybrid layer), except in the control and BL groups, regardless of the concentration of quercetin, RES, and BRP used. SEM images in Figure 4 show details of the debonded interface after µTBS testing for all failure patterns.

Figure 5 shows SEM images of the hybrid layer morphology for all the adhesives used and presents a similar interaction between resin and dentin for all groups, with resin tags fully filling dentin tubules in the usual pattern of a hybrid layer after total-etch adhesives were applied.

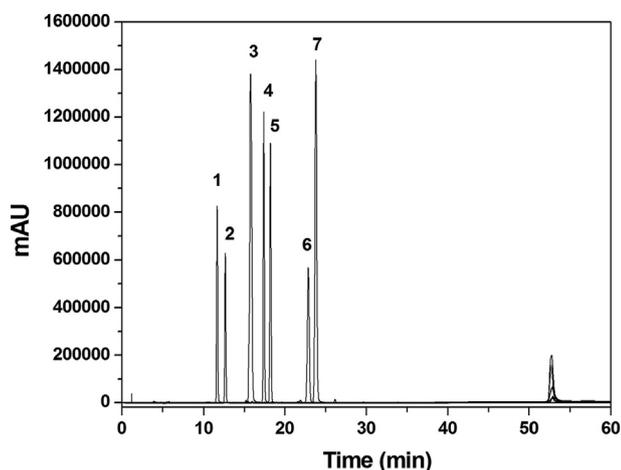


Figure 1. Chromatogram of the ethyl acetate extract of BRP at a concentration of 500 µg/mL. The following markers of BRP were identified in the UPLC-DAD profile at a wavelength of 205 nm: daidzein (1), liquiritigenin (2), pinobanksin (3), isoliquiritigenin (4), formononetin (5), pinocebrin (6), and biochanin A (7).

Table 1. Determination of the markers present in an ethyl acetate extract of Brazilian red propolis (500 µg/mL) using ultra-performance liquid chromatography coupled with a diode array detector.

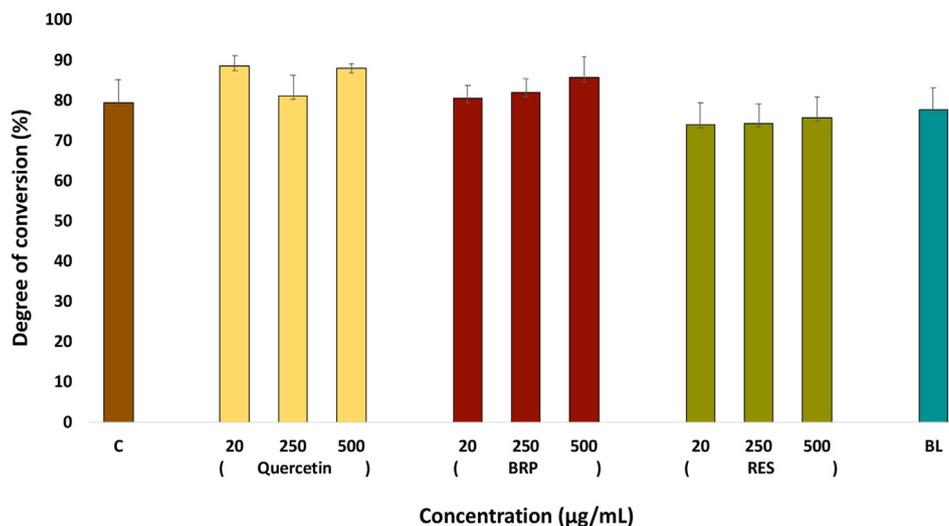
Marker	Formulas	RT (min)	λ (nm)	Concentration (µg/mL)	Concentration % (w/w)
Daidzein	C ₁₅ H ₁₀ O ₄	11.67	249	0.399	0.079
Liquiritigenin	C ₁₅ H ₁₂ O ₄	12.68	275	7.131	1.426
Pinobanksin	C ₁₅ H ₁₂ O ₅	15.78	289	0.385	0.077
Isoliquiritigenin	C ₁₅ H ₁₂ O ₄	17.41	366	5.123	1.024
Formononetin	C ₁₆ H ₁₂ O ₄	18.23	249	5.841	1.168
Pinocembrin	C ₁₅ H ₁₂ O ₄	22.88	289	1.335	0.267
Biochanin A	C ₁₆ H ₁₂ O ₅	23.81	260	0.315	0.063

RT, retention time (min); λ, maximum wavelength (nm).

Table 2. Antioxidant activity as a percentage (AAO%) for quercetin, RES, EEBRP, and EABRP.

Concentration	AAO (%), mean ± SD			
	Quercetin	RES	EABRP	EEBRP
0.05 µg/mL	64.78 ± 0.49	61.75 ± 0.76	58.73 ± 0.92	57.78 ± 0.933
0.5 µg/mL	69.25 ± 0.59	62.04 ± 0.85	59.60 ± 0.63	58.65 ± 0.87
1 µg/mL	73.22 ± 1.36	62.53 ± 1.27	64.64 ± 0.96	63.38 ± 0.13
2 µg/mL	76.68 ± 0.52	67.58 ± 1.05	70.58 ± 1.22	69.58 ± 0.59
4 µg/mL	89.25 ± 0.68	69.85 ± 0.79	79.56 ± 0.94	75.63 ± 0.42
6 µg/mL	93.27 ± 1.15	70.51 ± 1.02	80.34 ± 0.78	76.58 ± 0.76
10 µg/mL	96.68 ± 0.47	72.84 ± 0.93	87.16 ± 1.02	81.31 ± 1.01

SD, standard deviation; RES, resveratrol; EABRP, ethyl acetate extract of Brazilian red propolis; EEBRP, ethanolic extract of Brazilian red propolis.

**Figure 2.** Degree of conversion of experimental adhesives, BL, and the control group, expressed as a percentage (%). BRP, Brazilian red propolis; RES, resveratrol; BL, Single Bond 2 + 25 µL ethanol; C, control group (Single Bond 2).

3.5. Silver uptake

Typical nanoleakage patterns at the resin-dentin interfaces for each dental adhesive system are illustrated in Figure 6. Adhesives with BRP and RES at 500 µg/mL showed less diffusion of silver particles at the adhesive interface than those with quercetin, RES at 20 µg/mL, RES at 250 µg/mL, BL, and the control group. The control group (Single Bond 2), BL, and quercetin at 20 µg/mL showed the poorest seal, because many dentinal tubules were permeated with silver. In some parts, silver deposits appeared loosely distributed under the hybrid layer; multiple small deposits of silver were noted. Many tubules showed silver deposition between the resin tags and the tubule walls.

4. Discussion

This is the first study on incorporation of RES and propolis into dental adhesives, and few studies have been published on adding quercetin to an adhesive system [28, 29].

The composition of propolis is mainly determined by the phyto-geographic conditions around the hive, but it also varies seasonally within the same region [30]. UPLC-DAD has identified flavonoids and iso-flavonoids (liquiritigenin, pinobanksin, pinocembrin, isoliquiritigenin, daidzein, formononetin, and biochanin A) in the ethanolic extract of BRP used in this study. Polyphenols are described essentially as phenolic acids, stilbenes, flavonoids, lignans, and curcuminoids. In addition to the

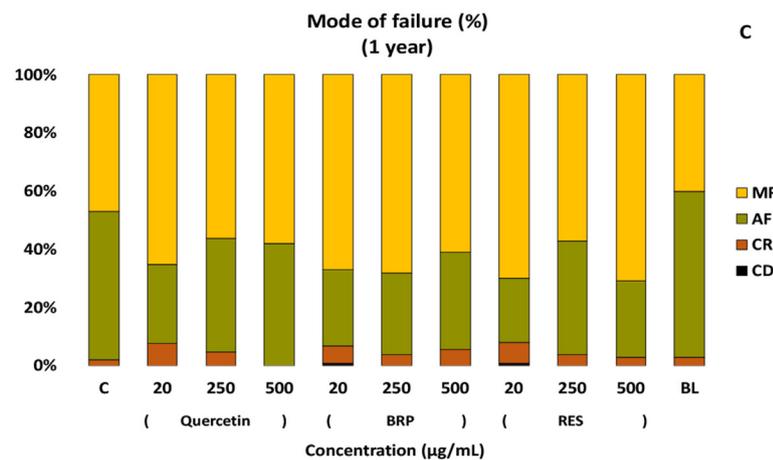
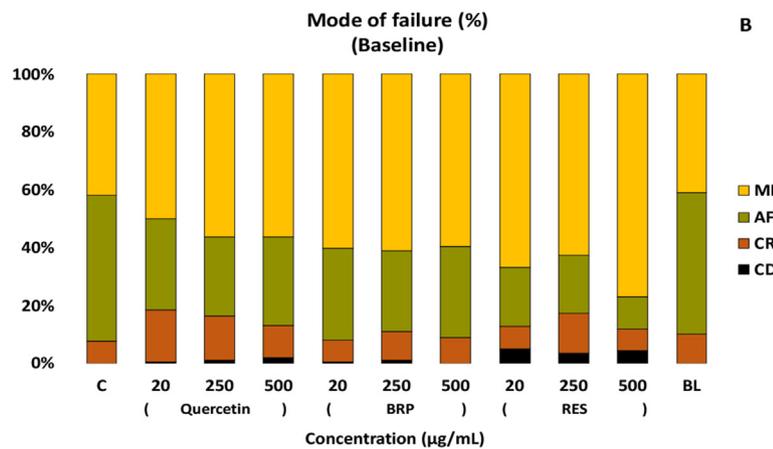
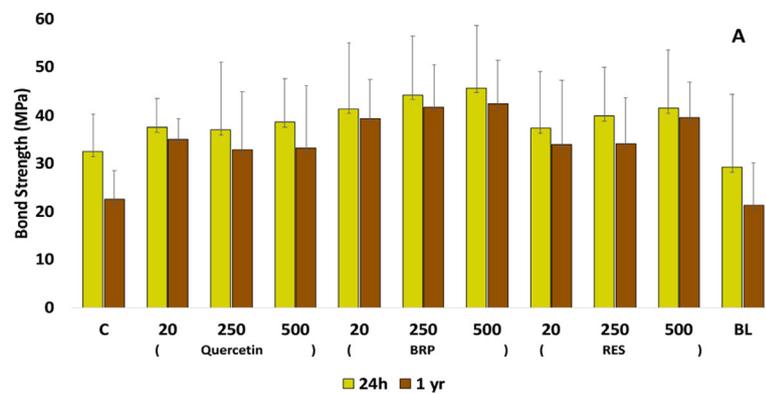


Figure 3. Mean values \pm standard deviation (SD) of dentin bond strength for all groups, tested as a function of storage time (24 h or 1 year) in distilled water. 3A: Compared with the control group, a significantly higher immediate bond strength (24 h) was recorded for Q500, R250, R500, and all BRP groups. After 1 year, all experimental adhesives exhibited a higher bond strength than the control group. Comparisons between the time of evaluation (1 day versus 1 year) showed a significantly lower microtensile bond strength only for the control and BL groups. 3B and 3C: There was a prevalence of mixed failure followed by adhesive failure for all experimental adhesive groups. In contrast, the control and BL groups showed more adhesive failures. BRP, Brazilian red propolis; RES, resveratrol; BL, Single Bond 2 + 25 μ L ethanol; C, control group (Single Bond 2).

ability of these molecules to act as antioxidants, they are able to interact with cell-signaling pathways, modulating gene expression in two different ways: (1) influencing the activity of transcription factors and (2) modulating microRNAs [31]. Interest in the study of phenolic compounds is mainly due to the antioxidant ability of these substances to scavenge free radicals [32]. Mechanical destruction and the presence of free radicals can also increase the processes of degradation and demineralization of the collagen matrix in dentin [33], affecting adhesive restorations.

Quercetin and resveratrol are polyphenols belonging to the flavonoids and stilbenes, respectively [12]. The structure of polyphenols, and in particular flavonoids, is ideal for scavenging free radicals. As antioxidants, flavonoids are more effective than ascorbic acid (vitamin C) and α -tocopherol (vitamin E). The antioxidant activity of flavonoids depends on their structure and can be determined in various ways: (1) reactivity as a donor agent of hydrogen atoms and electrons, (2) stability of the

flavonoyl radical produced; (3) reactivity toward other antioxidants, (4) ability to chelate transition metals, and (5) solubility and interaction with the membranes [34]. Polyphenols are able to stabilize the collagen chain, increasing the number of crosslinks of collagen fibrils and thus reducing collagen biodegradation [10]. Because BRP contain a high content of polyphenols, it also has the potential to neutralize the effect of metalloproteinases.

Our results confirmed the high antioxidant activity of quercetin, RES, and BRP used in this study through the DPPH \bullet assay. The process of partitioning the ethanol extract of propolis with hexane and then with ethyl acetate provides extracts with higher concentrations of benzophenones, flavonoids, and hydroxylated isoflavonoids [16, 30]. Thus, the higher concentration of flavonoids in EABRP may explain its superior capacity to scavenge the DPPH \bullet radical compared with EEBRP. Mendonça et al. [16] and Cabral et al. [35] demonstrated that the hexane fraction of ethanol propolis extract has a greater ability to scavenge

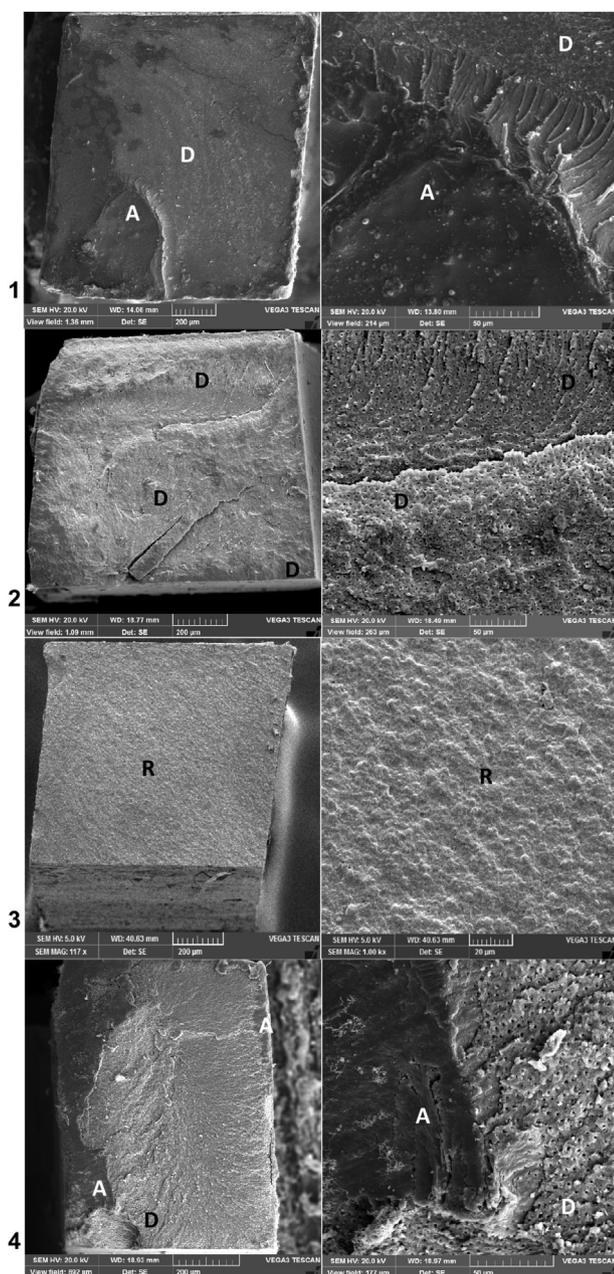


Figure 4. Scanning electron microscopy images illustrating each failure mode are presented in a sequence from left to right on each row. Row 1: adhesive failure. The biggest portion of the failures occurred in the adhesive/dentin interface. Rows 2 and 3: cohesive failure in dentin and cohesive failure in resin, respectively. Row 4: mixed failure (adhesive failure and cohesive in dentin failure). A, adhesive; D, dentin; R, composite resin.

DPPH free radical (74.4%) than ethanolic propolis extract (50.5%), which is in agreement with our results.

According to Lee et al. [36] and Essick et al. [37] quercetin, as an important antioxidant, inhibits the formation of reactive oxygen species (ROS), mediators of oxidative stress that act as activators of MMPs. In addition, quercetin suppresses the expression of extracellular matrix proteins MMP-9 and MMP-2 [29] through the kinase pathway regulated by extracellular signals (extracellular regulated kinase [ERK] and AKT, also known as protein kinase B [PKB]) [38]. In addition, quercetin has the potential to promote dentin collagen crosslinking by increasing the immediate bond strength and improving the adhesive interface [39, 40], which was confirmed by our results.

When proposing changes in dental bonding agents, biocompatibility is a fundamental factor. Quercetin has a stronger collagen crosslinking effect and is 100 times less cytotoxic than glutaraldehyde. Quercetin also induces dentinogenic differentiation of dental pulp cells, it has an antibacterial effect by inhibiting the metabolic activity of *S. mutans* [29], and inhibits the action of dentin MMPs [41]. These are important properties because quercetin added to dental bonding agents can increase the resistance of collagen fibrils at the hybrid layer, prevent secondary caries, and induce dental hard tissue formation after placing composite restorations.

RES has been shown to protect dentin collagen fibrils after enzymatic challenge with collagenase [12] and to significantly reduce the expression of MMP-2 [42] and MMP-9 [43] in places other than in dentin. The addition of RES can also contribute to the biocompatibility of adhesives. Dentin adhesives stimulate the production of ROS, DNA damage, and cell mutagenicity regardless of the composition of the adhesive. Oxidative stress is directly involved in the processes that trigger cell death by dentin bonding agents, and adhesives with RES could act as a cell protector. However, this effect of RES varies according to the composition of the adhesive. This variation may be associated with the interaction between RES and the chemical compounds in the dental adhesives [25].

These biological activities of RES are attributed to its simultaneous action on multiple molecular targets [44] through different antioxidant mechanisms, including inhibition of ROS production [45], scavenging free radicals, and stimulating the biosynthesis of endogenous antioxidants by for example, stimulating the activity of nuclear erythroid-related factor (Nrf2) [46]. RES suppresses the synthesis of prostaglandin-E by inhibiting the activity of the enzyme cyclooxygenase-2 (COX-2) and inhibits the transcription of the COX-2 gene, which can be particularly useful because the excess of COX-2 enzyme induces release of MMPs. RES also repressed HEMA-induced ROS production, so it can be assumed that RES exerts a protective effect by eliminating free radicals, increasing the quantity of antioxidant enzymes [25].

In addition to the cytoprotective effects of antioxidants, treatment of the dentin surface with antioxidants such as RES and quercetin has been shown to preserve adhesive microtensile strength [12, 25, 41, 47]. However, this is the first study on adding RES and BRP to dental adhesives, and it was not possible to compare our results on bond strength with other studies. Gotti et al. [28] showed that quercetin added to the same adhesive system used in this study significantly increased the microtensile strength values between 24 h (31.2 MPa) and 6 months (38.3 MPa).

Our results showed that with the addition of quercetin, RES, and BRP, bond strength was retained after 1 year of storage in distilled water. Thus, it is believed that adhesive systems containing quercetin, RES, and BRP may act to preserve dentin collagen, due to their potential to neutralize the effect of metalloproteinases without decreasing μ TBS, and as a functional dental material, could extend the clinical life of adhesive restorations. Although distilled water does not reproduce the oral environment accurately, it is a widely used storage medium [19, 29]. El-Deeb et al. [48] showed that the bond strength of specimens aged in distilled water was more intensely reduced in water than in artificial saliva due to different potential hydrolytic degradation. Thus, the polyphenols and propolis used in this study showed a promising approach to maintaining the bonding interface.

A true hybrid layer morphology with deep resin tags into the dentin tubules showed that the slight reduction in bond strength over time did not occur due to changes in the formation of the adhesive interface, but was more likely due to hydrolytic degradation of the polymer, which is common in all commercially available dental adhesives, and it seems to have been lower for all experimental adhesive groups than the control and BL groups.

Quercetin and RES are polyphenols abundantly found in nature [49, 50] and BRP is a compound rich in polyphenols, including quercetin [16]. Yang et al. [51] showed that quercetin crosslinks collagen by mean

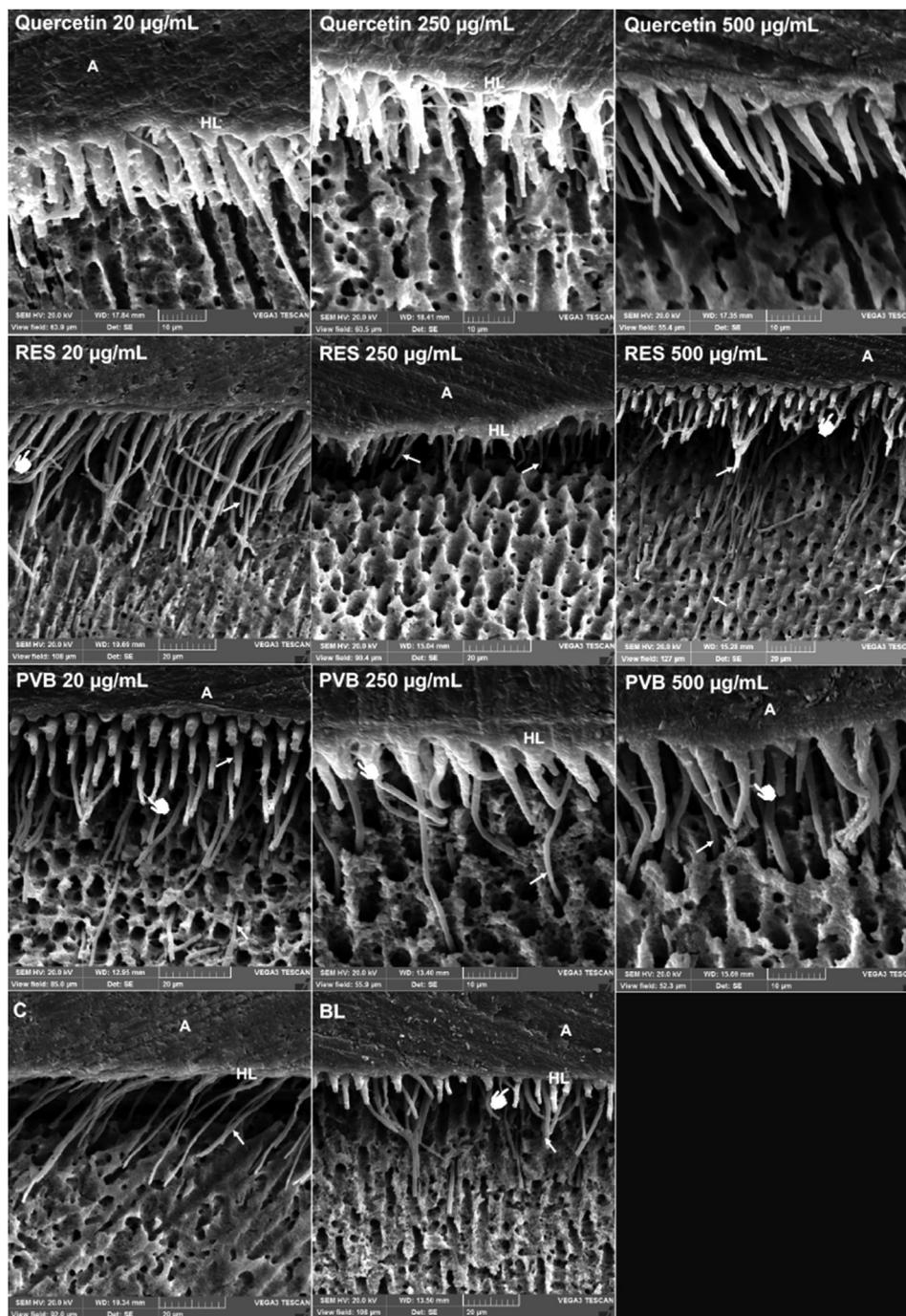


Figure 5. Representative scanning electron microscopy images of the bonding interface. All samples showed a well-built hybrid layer (HL) with the usual resin tags (white arrows) and some resin branches (white hands). A, adhesive; BRP, Brazilian red propolis; RES, resveratrol; BL, Single Bond 2 + 25 μ L ethanol; C, control group (Single Bond 2).

of four different types of forces: hydrogen bonds, van der Waals forces, electrostatic forces, and hydrophobic forces. Polyphenols have an amphiphilic feature showing a hydrophobic property provided by their planar aromatic nucleus and the hydrophilic characteristics given by their polar hydroxyl groups. The hydrophobic forces of the polyphenols drive them to the interfibrillar spaces, promoting the incorporation of the polyphenol into the collagen structure. The hydroxyl group associates with collagen proline residues through hydrogen bonds and in consequence, a secondary interaction occurs, which helps to protect the complex polyphenol/collagen fibrils [52]. Frazier et al. [53] suggested that the hydrophobic section in the aromatic rings could bind with the

hydrophobic section of other molecules and generate an attractive non-covalent interaction between aromatic rings (π - π stacking effect, Wan der Waals forces), whereas the hydrophilic portion is polarized and has the potential to react, favoring hydrogen bond creation.

In dentistry, an ideal adhesion to hard dental tissues is fundamental for the success of adhesive procedures. Thus, researchers have put much effort into both techniques and adhesive materials to improve the durability of the bonding interface. Some factors, such as the infiltration and the DC of the adhesive monomers in situ, are vital to establish long-lasting bonds. Dental adhesives with low DCs are associated with low values of bond strength and mechanical properties, high elution of

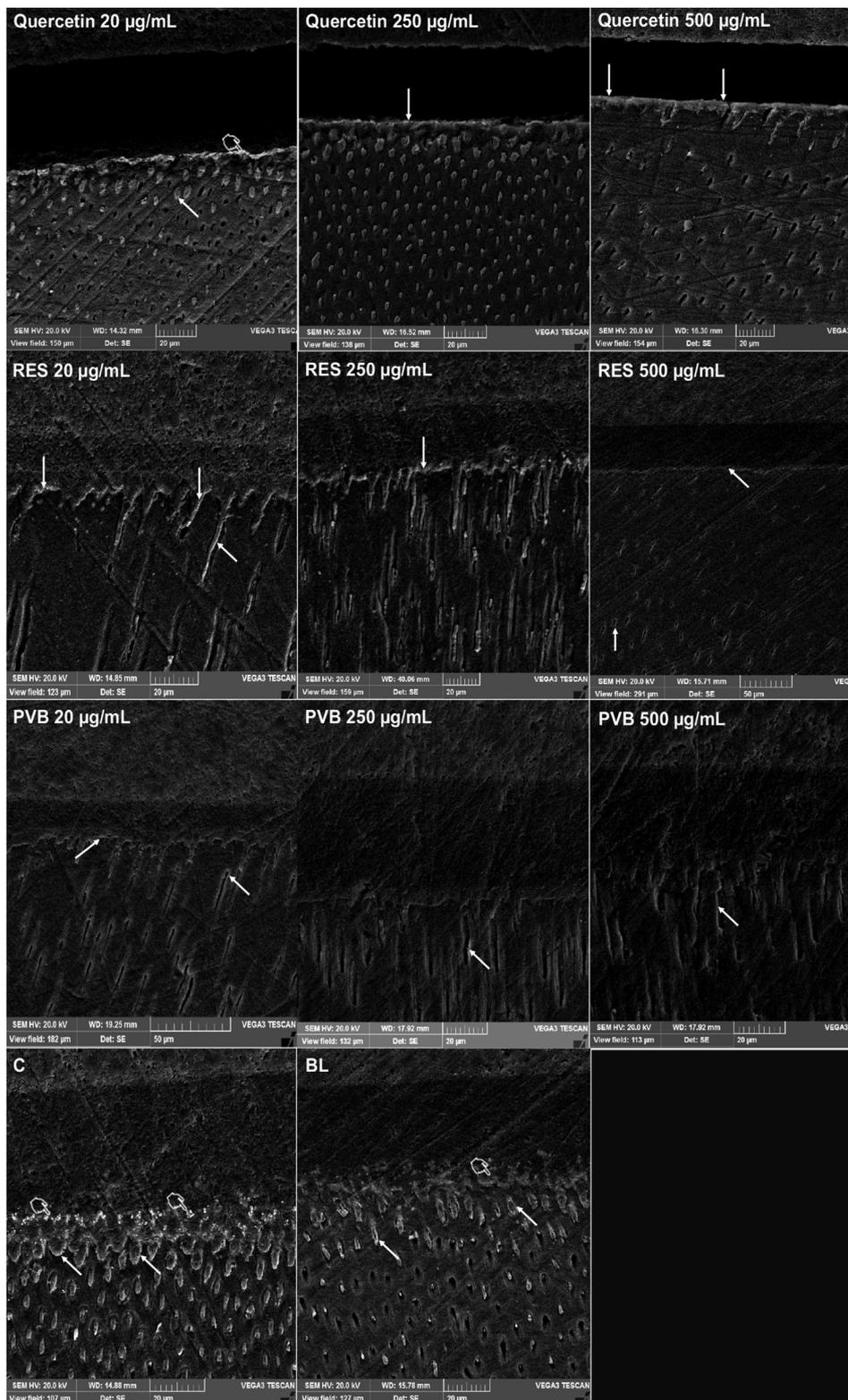


Figure 6. Representative scanning electron microscopy images of nanoleakage illustrate the typical pattern of silver nitrate deposition in the adhesive interface. BRP and RES 500 µg/mL adhesives showed markedly less penetration of silver particles at the adhesive interface than quercetin, RES 20 µg/mL, RES 250 µg/mL, BL, and control group. A thin layer of silver deposition was noted in the base of the hybrid layer of the quercetin, RES 20 µg/mL, and RES 250 µg/mL groups. The nanoleakage patterns of the control and BL groups showed silver accumulation at the base of the hybrid layer, and silver deposits (white hands) were observed underlying the hybrid layer. BRP, Brazilian red propolis; RES, resveratrol; BL, Single Bond 2 + 25 µL ethanol; C, control group (Single Bond 2).

unreacted monomers, greater cytotoxicity, and greater permeability of the hybrid layer. Thus, a high DC of adhesive systems is a crucial factor in the long-term durability of the hybrid layer [54, 55, 56, 57].

Nanoleakage is generally considered to be a relevant guide to estimate the ability of an adhesive system to seal dentin and create an effective bond [58]. The adhesives with BRP (all concentrations tested) and RES at 500 µg/mL showed the best immediate result in sealing

dentin, which was corroborated by the µTBS tests. Quercetin at 20 µg/mL, BL, and control groups showed the greatest uptake of silver.

In this study, all experimental adhesives showed adequate morphology of the hybrid layer with strong resin tags polymerized within dentin tubules. However, silver penetration in the entire thickness of the hybrid layer is not just the result of adsorption and deposition of silver into the pores. Silver particles can also be attached to collagen fibrils,

indicating that the adhesive system did not completely permeate the demineralized dentin, which can favor the subsequent hydrolyzation of the adhesive resin and degradation of collagen fibrils.

A high degree of conversion of C=C bonds in the methacrylate-based resinous materials is required to favor the mechanical properties and reduce polymer degradation, leading to successful adhesive procedures. The quality of the polymerization process can be directly related to the microtensile strength of the resin-dentin bond. The incorporation of BRP, RES, and quercetin did not produce significant changes in the DC of the experimental adhesives, although a slight increase in the DC of adhesives with propolis and quercetin was observed; the adhesives with RES showed a slight reduction compared with the control group.

Quercetin is a little studied material in dentistry. In agreement with our results, Yang et al. [29] showed that the DC of adhesives modified by the incorporation of quercetin at 100 and 500 µg/mL remained almost unchanged compared with the control group (without the addition of flavonoid). In contrast, Gotti et al. [28] showed that the addition of quercetin 5 wt% significantly reduced the DC of the Single Bond 2 adhesive. This concentration is much higher than that used in this study, and possibly this negative effect happened as a result of the high concentration of quercetin used.

Commercially available adhesive systems can be used as a parent material for initial testing of small modifications or addition of new compounds. As the formulation of adhesives is an industrial secret and making a new adhesive with a balanced composition takes a lot of time and study, we decided to use a commercial total-etch adhesive to formulate the adhesives with quercetin, RES, and BRP used in this research. The average values for bond strength and DC recorded in this research are in accordance with previous studies in which Single Bond 2 showed µTBS values between 31.52 MPa (1 day) and 26.15 MPa (1 year in distilled water) [59] and DC between 67.87% and 79.98% [57, 59, 60, 61].

In addition, quercetin, RES, and BRP have antibacterial activity [17, 22, 29, 49, 62]. Yang et al. [29] showed that a quercetin-doped adhesive at concentrations of 100, 500 and 1000 µg/mL was able to significantly reduce the number of live bacteria in *S. mutans* biofilm. The metabolic activity of *S. mutans* decreased with increasing quercetin concentration, and although the antibacterial activity of all groups was slightly reduced after thermocycling, quercetin at 500 and 1000 µg/mL presented effective bactericidal capacity.

The µTBS results showed that incorporating quercetin, RES, and BRP into a commercial adhesive can successfully preserve the resin/dentin bond strength after aging for up to 1 year in distilled water. A total-etch commercial adhesive was chosen for this research because it is a well-known bonding agent, and it was easier to add those compounds than use self-etch and universal adhesive systems.

The increasing demand for user-friendly adhesive systems has led to the development of self-etch and universal adhesive systems. The latter can be applied with any strategy of adhesion (total-etch or self-etch) and offer versatility of use with a variety of direct and indirect restorative materials. However, the available evidence does not support the claim that universal adhesives can be used with any adhesive strategy. Although they can chemically bond to various substrates such as tooth and direct and indirect restorative materials, the stability of this bond is material dependent and subject to hydrolytic degradation [63]. Clinical studies showed that the two adhesive strategies have similar retention rates. Self-etch mode tends to result in marginal staining and marginal deterioration compared with etch-and-rinse and selective-etch application modes [64, 65, 66]. Vinagre et al. [67] showed that the etch-and-rinse systems perform significantly better than self-etch systems regarding marginal adaptation, and it was the most influential parameter regarding the acceptability of restorations. Although this was an in vitro study, in vivo studies could provide more accurate results once clinical trials identify the differences regarding the operator who performed the restorations, patient features, properties of the materials, numbers of restorations, and study design all acting simultaneously. Additional

studies should be carried out to compare total-etch adhesives modified with quercetin, RES, and BRP with self-etch adhesives.

Therefore, the addition of RES, quercetin, and BRP to a bonding agent can guide research on the development of versatile adhesives with potential to act as antioxidant, antimicrobial, and MMP inhibitor. But only clinical trials with such adhesives can show if the biochemical and/or functional characteristics produced by different combinations of compounds with specific attributes of bonding agents will fulfill the ideal requirements for bonding restorative materials to dental tissue. Although considerable progress has been made in the development of dental adhesives, many challenges remain: (1) the long-term release of substances; (2) polymer degradation ratio; (3) local effects and action on adjacent tissues; and (4) avoiding fast inactivation.

5. Conclusions

The incorporation of quercetin, RES, or BRP at 20, 250, and 500 µg/mL concentrations did not negatively affect the DC, improved immediate bond strength, and was shown to maintain the bond strength after aging for up to 1 year in distilled water. These findings suggest that quercetin, RES, or BRP might be useful in adhesive dentistry to help improve hybrid layer longevity.

Declarations

Author contribution statement

Isabel Cristina Celerino de Moraes Porto, Ticiano Gomes do Nascimento: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Arthur Bezerra de Barros Rocha, Iverson Iago Soares Ferreira, Bruna Muritiba de Barros, Eryck Canabarra Ávila, Matheus Corrêa da Silva, Marcos Paulo Santana de Oliveira, Teresa de Lisieux Guedes Ferreira Lôbo, José Marcos dos Santos Oliveira: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Jeniffer McLaine Duarte de Freitas, Johnatan Duarte de Freitas: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declarations of interests statement

The authors declare the following conflict of interests: We, the authors have a patent in process related to this work.

Additional information

No additional information is available for this paper.

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