Dental Materials xxx (xxxx) xxx

Contents lists available at ScienceDirect

Dental Materials

journal homepage: www.elsevier.com/locate/dental

Strategies for flavonoid application on etched dentin: Bond stability, enzymatic activity, and biofilm inhibition

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ARTICLE INFO

Keywords:
Dentin
Demineralization
Flavonoids
Matrix metalloproteinase inhibitors
Anti-bacterial agents

ABSTRACT

Objective: This study evaluated the effects of different flavonoid application strategies either as dentin primers or incorporated into a universal adhesive system (SFA) on microtensile bond strength (μTBS), matrix metalloproteinase (MMP) inhibition, biofilm formation, and degree of conversion.
Materials and methods: Baicalein, kaempferol, and naringin were tested at 20 mM, either incorporated into a commercial adhesive or dissolved in 50 % ethanol and applied as primers, forming six experimental groups. Three controls were used: Negative (commercial adhesive), Positive (0.2 % chlorhexidine), and Ethanol (50 %). Dentin specimens were analyzed for μTBS (n = 10), dentin–adhesive interface morphology (DAM) (n = 3), and in situ zymography (n = 3). *Streptococcus mutans* biofilm was grown on adhesive surfaces to assess bacterial viability, and FTIR spectroscopy evaluated the degree of conversion. μTBS data were analyzed using generalized linear models; other data were analyzed by one-way ANOVA followed by Bonferroni or Tukey tests (α = 0.05).
Results: All flavonoid-treated groups showed significantly higher μTBS than the Negative Control after one year. Adhesives with incorporated flavonoids also outperformed the Positive Control. No adverse effects were observed on DAM, bacterial viability, or degree of conversion. All SFA strategies reduced MMP activity, with complete inhibition seen only in the Baicalein primer group.
Relevance: The use of flavonoids, either as a dentin primer or incorporated into adhesives, offers clinicians a simple and effective strategy to enhance the longevity of adhesive restorations by stabilizing the hybrid layer and reducing collagen degradation without altering application protocols or compromising material performance.

1. Introduction

Achieving durable bond stability between dentin and resin remains a major challenge in adhesive dentistry. The long-term success of this interface relies on the formation of a stable and homogeneous hybrid layer [1]. Despite significant advances in adhesive materials and clinical techniques, bond degradation over time continues to be a concern, particularly when bonding procedures involve acid-etched dentin [2]. This degradation is largely attributed to the activation of dentin matrix metalloproteinases (MMPs), which are produced by odontoblasts and remain latent within the mineralized matrix until triggered [3], as well as to the hydrolytic breakdown of adhesive resin polymers [4]. Both processes contribute substantially to the failure of resin-based restorations [5,6].

To address this, various strategies have been proposed to prevent or reduce dentin degradation [7–10]. Among them is the use of synthetic or natural agents, particularly those derived from plants and citrus fruits that promote collagen biomodification by enhancing resistance to enzymatic hydrolysis and inhibiting endogenous protease activity [7, 11]. Among these natural compounds, flavonoids have emerged as

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https://doi.org/10.1016/j.dental.2025.09.005
Received 17 May 2025; Received in revised form 11 August 2025; Accepted 8 September 2025

promising candidates. When applied as a dentin pretreatment solution [7] or incorporated into restorative materials such as phosphoric acid, adhesive systems, or composite resins [10,12], flavonoids have demonstrated superior performance in maintaining bond stability over time. However, despite the increasing interest in their dental applications, there is still a lack of studies directly comparing different modes of application of the same flavonoid compounds.

Flavonoids are known for their diverse biological properties, including antioxidant, anti-inflammatory, anti-mutagenic, and anti-carcinogenic effects [13]. They also exhibit significant enzyme inhibitory potential, particularly against enzymes such as xanthine oxidase, cyclooxygenase, lipoxygenase, and phosphoinositide 3-kinase [13]. Notably, some flavonoids have demonstrated greater efficacy in preserving the hybrid layer compared to synthetic agents like chlorhexidine digluconate [7]. In addition to their enzymatic inhibition, flavonoids also possess antibacterial properties [14], which may reduce biofilm accumulation on the surface of restorative materials [15,16]. In a previous study, kaempferol and naringin were evaluated as experimental dentin primers [7], however, their incorporation into universal adhesive systems remains largely unexplored. Furthermore, baicalein a flavonoid from a distinct subclass with a unique molecular structure may exhibit different behavior and bonding potential, as well the ion quelation and collagen cross-linker. Despite its promising bioactivity, baicalein has received limited attention in the context of adhesive dentistry.

Therefore, this study aimed to investigate the effects of three flavonoids baicalein, kaempferol, and naringin applied to demineralized dentin either as experimental primers or incorporated into a universal adhesive system, both at a concentration of 20 mM. The null hypotheses tested were: (1) no significant differences in dentin microtensile bond strength (μTBS) would be observed among the groups, regardless of flavonoid application strategy or aging; (2) the application strategy would not influence the morphology of the bonding interface; (3) enzymatic activity within the adhesive interface would not differ among groups; (4) no differences in antibacterial activity against *Streptococcus mutans* biofilm would be detected; and (5) the incorporation of flavonoids into the universal adhesive would not affect its degree of conversion.

2. Materials and methods

2.1. Teeth selection and preparation of the experimental primers and adhesives

One hundred and forty-four sound human third molars were obtained after the approval by the Ethics Committee in Research of the institution (CAAE #19855119.1.0000.5418). All teeth were cleaned by hand-scaling with a periodontal curette (SS White Duflex, Juiz de Fora, MG, Brazil), and polished with a paste of pumice and water. Afterwards, they were stored in aqueous solution of 0.5 % Chloramine-T (Merck KGaA, Darmstadt, Germany) at 4 °C for no longer than three months.

Based on a previous study conducted by our research group, which tested different concentrations for these flavonoids, 20 mM was selected as it provided the most favorable results regarding bond stability and interfacial adhesive morphology after one year of aging [7]. Thus, Baicalein, Kaempferol and Naringin (Merck KGaA, Darmstadt, Germany) were diluted at 20 mM concentration in 50 % ethanol and used as a primer (dentin pre-treatment) or incorporate at the same concentration in a commercial universal adhesive (Clearfil Universal Bond Quick, Kuraray Noritake Dental Inc., Tokyo Japan), which corresponded to the strategies of flavonoid applications used in this study. For the experimental *primers*, the compounds were diluted and homogenized with the aid of a magnetic stirrer and kept in light-proof containers, with their lids closed to prevent solvent evaporation. The commercial adhesive containing flavonoids were homogenized in a centrifuge at 3000 rpm for 5 min to ensure adequate dissolution of the powder within the adhesive.

Chlorhexidine digluconate (CHX) at 0.2 % concentration in aqueous solution and 50 % ethanol solution were also used as "control primers" ("Positive and Solvent Controls"). Negative Control consisted in the application of the universal adhesive resin without a previous priming step and flavonoids. The pH of these solutions and the adhesives was determined (Table 1) using a digital pH meter (MS Tecnopon Equipment's, Piracicaba, SP, Brazil).

2.2. Dentin microtensile bond strength test (μTBS)

The microtensile bond strength test followed the methodology described in the guidelines of the Academy of Dental Materials, which were published in Dental Materials [17]. The occlusal enamel of each tooth was removed with a diamond saw (Buehler Ltd., Lake Bluff, IL, USA) using a precision cutting machine, and dentin was exposed at medium depth in relation to the pulp, approximately 2 mm above the cementoenamel junction. Dentin surfaces were polished with silicon carbide paper (600-grit) for 5 s under water-cooling to create a flat surface and standardize the smear layer.

Ninety teeth were randomly assigned to nine groups (n = 10), according to the strategy of flavonoid application. The groups were six experimental (3 primers containing flavonoids and 3 commercial adhesives modified with the addition of flavonoids) and 3 Controls: the "Negative Control" (the commercial adhesive) and two primer Controls (0.2 % CHX/the "Positive Control" and 50 % ethanol solution).

For all groups (experimental and Controls) dentin was etched with 35 % phosphoric acid (Ultra-Etch, Ultradent Products, Inc., South Jordan, UT, USA) for 15 s and rinsed with water for 15 s by an oil-free air/water spray. Dentin was kept moist before application of 15 μL of each primer (experimental and Controls) to the etched dentin surfaces for 60 s, and the primer excess was removed with absorbent paper. Afterwards, the universal adhesive was applied for 15 s using light brushing motion, followed by air blowing for 3 s, and light-cured for 10 s (1474.2 mW/cm^2), according to the manufacturer's instructions. The light-curing unit used was Valo (Ultradent Products Inc., South Jordan, UT, USA), which has a tip with an internal diameter of 9.40 mm. The distance between the light tip and the surface sample was 1 mm.

A resin composite (Charisma Diamond, Heraeus Kulzer, Hanau, Germany/ Shade: A1) block of approximately 4 mm height was built-up on bonded dentin, using two consecutive layers of 2-mm-thick composite. Each composite layer was light-cured for 20 s using the same curing unit (Valo). The light-curing unit was verified by a spectroradiometer (MARC-PS, BlueLight Analytics Inc., Halifax, NS, Canada) to ensure the delivery of a radiant exposure of at least 16.8 J/cm^2.

Restored teeth were kept at relative humidity, at 37 °C, for 24 h. Afterwards, teeth were sectioned in lingual-buccal and mesial-distal directions to obtain at least 8–16 stick-shaped specimens per tooth with a cross-section area of approximately 1.0 mm^2. Half of the specimens were tested after 24 h of storage in simulated of body fluid solution (16.070 g NaCl, 0.710 g $NaHCO_3$, 0.450 g KCl, 0.462 g $K_2HPO_4{\cdot}3\ H_2O$, 0.622 g $MgCl_2{\cdot}6\ H_2O$, 0.584 g $CaCl_2$, 0.144 g Na_2SO_4) [18] at 37°C, while the other half was tested after 12 months of storage under the same conditions. The simulated of body fluid solution was replaced weekly.

Table 1
Potential of hydrogen (pH) of experimental *primers* and adhesives containing flavonoids (20 mM).

Primer	Group	pH
	Control (50 % ethanol)	7.3
	Baicalein	7.4
	Kaempferol	7.1
	Naringin	7.7
Adhesive	Control (no addition)	2.3
	Baicalein	2.4
	Kaempferol	2.2
	Naringin	2.3

Specimens were tested on a microtensile device that was attached to a universal testing machine (EZ Test, Shimadzu, Kyoto, Japan). Each specimen was fixed to the device with cyanoacrylate-based glue (Super Bonder Gel, Henkel/Loctite, Diadema, SP, Brazil) and tested at 1.0 mm/min speed, until failure. The cross-section area of the specimens was measured using a digital caliper (Mitutoyo Co., Kanagawa, Japan), and bond strength means were calculated from the average of the specimens per tooth, according to the evaluation time.

2.3. Failure mode analysis by scanning electron microscopy (SEM)

The surfaces involved in the fracture of each specimen after the microtensile bond strength test were analyzed by SEM regarding failure pattern classification. The fractured specimens were fixed on metallic stubs with carbon tape, keeping the areas involved in the fractures facing upwards. Then, the specimens were sputter-coated with gold (MED 010, BAL-TEC AG, Balzers, Liechtenstein) and observed under SEM (JSM 5600LV, Jeol, Tokyo, Japan), using 100x and 400x magnifications. Failure modes were classified according to the structures involved [19]: Type I - cohesive failure within the resin composite; Type II - adhesive failure between resin composite and the bonding agent; Type III - adhesive failure between dentin and the bonding agent; Type IV - mixed failure (dentin, bonding agent, and resin composite can be observed in the same fractured surface); Type V - cohesive failure within the bonding agent layer; Type VI - cohesive failure within the hybrid layer; and Type VII - cohesive failure within dentin. In order for a specimen not to be considered mixed, 70 % or more of a single specific failure mode should be present on the evaluated surface. Descriptive analysis was used to report failure modes with their respective percentages of occurrence.

2.4. Adhesive-dentin bonding interface morphology analysis scanning electron microscopy (SEM)

Twenty-seven human third molars (n = 3) were restored following the same methodology described for the microtensile bond strength test. The teeth were sectioned in slices at 2 mm and were embedded in epoxy resin. After 48 h, the samples were polished with grit SIC papers (#600; #1200; #2000 and #4000) and immersed in 37 % phosphoric acid solution for 10 s and 5 % sodium hypochloride for 5 min to expose the resin tags [20,21]. Then, the samples were dehydrated by an increasing concentration of ethanol (25, 50, 70, 90 % and absolute ethanol) for 10 min each and dried overnight. Afterwards, specimens were sputter-coated with gold (MED 010, BAL-TEC AG, Balzers, Liechtenstein) and analyzed by SEM (JSM 5600LV, Jeol, Tokyo, Japan) at x1000 magnification [20,21].

2.5. In situ zymography of the hybrid layer

Twenty-seven sound human third molars (n = 3) were prepared and restored following the same methodology described for the microtensile bond strength test, except for the use of a single 1 mm-thick layer of a flowable bulk-fill composite (SureFill SDR flow+, Dentsply-Sirona, Milford, DE, USA) instead of regular resin composite. The 6 experimental groups (primers and adhesives) and 3 Controls were tested. Teeth were vertically sectioned to expose the resin-dentin bonding interface, glued to glass slides, and ground down to obtain approximately 30 μm-thick specimens. *In situ* zymography was performed on three slices from the central region of each tooth with self-quenched fluorescein-conjugated gelatin as the MMPs substrate (E-12055, Molecular Probes, Eugene, OR, USA).

The substrate was prepared from 1 mL stock solution of DQ-gelatin (DQ-gelatin, E12055; Molecular Probes, Eugene, OR, USA). A fluorescein-conjugated gelatin was added to the dilution buffer (NaCl 150 mM, CaCl2 5 mM, Tris-HCl 50 mM, pH 8.0) in a proportion of 1:1:8 [22]. Twenty μL of the fluorescent-gelatin mixture was placed on top of each specimen. Samples were then covered by a coverslip, protected from light, and incubated in humidifying chambers at 37 °C for 24 h. The hydrolysis of the quenched fluorescein-conjugated gelatin was analyzed by confocal laser scanning microscopy Zeiss LSM 780 (excitation, 488 nm; and emission, 530 nm). Images were acquired and analyzed using ZEN software (Zeiss, Heidelberg, Baden-Württemberg, Germany).

2.6. Inhibition of S. mutans biofilm formation

The antibiofilm activity test was performed using *S. mutans* (Strain AU159) obtained from the Laboratory of Microbiology and Immunology of the institution. The bacterial culture was stored at −70 °C in BHI (brain heart infusion medium, Difco Laboratories, Detroit, OR, USA) containing 20 vol% glycerol.

Twenty-four resin composite discs (n = 6) were prepared using a microhybrid resin composite (Herculite XRV Ultra, Kerr Corporation, Orange, CA, USA) with a standard stainless-steel mold (2 mm thickness x 8 mm diameter). The resin composite was covered with a polyester strip and a glass microscope slide and pressed to remove the excess, and then was light-cured for 20 s. Discs were sterilized in UV light for 1 min each side [23]. Then, discs were divided according to the adhesive group of adhesives incorporated with flavonoids, and 20 μL of the adhesive was applied on the entire surface of the discs and light-activated for 10 s on each side [18]. An adhesive without flavonoid incorporation was used as a control.

Overnight cultures of *S. mutans* were grown in BHI supplemented with 1 % (w/v) sucrose at 37 °C and 5 % CO_2, measured regarding optical density (OD600 $\geq$ 0.900), and used as biofilm-grown inoculum. The discs were vertically placed in 24-well plates and inoculated with an *S. mutans* culture of approximately 2×10^6 CFU/mL (colony-forming units per mL). After 24 h, the discs were randomly divided into 4 groups and dip-washed three times in sterile saline solution (0.9 % NaCl). Additionally, one sterile disk per plate was incubated with sterile medium and used as a control.

After incubation, the biofilm was removed from the discs by scraping and subjecting them to an ultrasound bath (20 s pulse; output 7 W). The biofilm was then serially diluted in 96-well plates and plated in BHI agar (unknown concentration to 10^{-8}) in order to count the viable cells (CFU/mL) and, consequently, evaluate the antibacterial effectiveness of the adhesives incorporated with flavonoids.

2.7. Degree of conversion and polymerization rate

The degree of conversion of adhesive systems was evaluated in triplicate as described by Ely et al. [24]. In brief, each solution (3 mL) was directly dispensed onto the emission plate of a Fourier-transform infrared spectrophotometer (Alpha-P; Bruker Optics, Ettlingen, German) equipped with an ATR crystal with a 45-degree mirror angle (PIKE Technologies; Madison, WI, USA) with the light-curing unit positioned at a standardized distance of 1 mm. Before FTIR acquisition, a gentle air stream was applied to the sample surface for 10 s from a distance of 10 mm to promote ethanol evaporation from the adhesive system, following commonly adopted protocols for solvent removal. Spectra from FTIR analysis were recorded before and immediately after the photoactivation of each sample obtained in a range of 1800–1500 cm^{-1}, with 32 scans at 4 cm^{-1} resolution in transmission mode and 2.8 mm/s mirror speed. The degree of conversion was calculated based on the intensity of the C═C stretching vibrations (peak height) at 1635 cm-1 and using the symmetric ring stretching at 1608 cm^{-1} from the polymerized and non-polymerized samples as an internal standard. The coefficient of determination was greater than 0.98 for all curves. Data were plotted, and curve fitting was applied using logistic non-linear regression.

2.8. Data analysis

Data from the dentin microtensile bond strength test were submitted to normal distribution and homoscedasticity tests (Shapiro-Wilk and Levene tests) and did not present a normal distribution. Results were analyzed by generalized linear models ($\alpha = 0.05$). Conversely, data from the antibacterial activity and degree of conversion tests passed the Shapiro-Wilk normality test, as well as Levene's test for equality of variances. Results were then analyzed by a one-way ANOVA, followed by Bonferroni and Tukey test ($\alpha = 0.05$). These analyses were performed using the IBM SPSS software (IBM SPSS Statistics for Windows, Version 21.0, IBM Corp., Armonk, NY, USA).

Regarding failure mode, the percentages of frequency of each type of failure were reported. The SEM analysis of the morphology of the adhesive-dentin interface considered the hybrid layer and resin tags formation, and the visual differences between experimental groups and Controls. For *in situ* zymography analysis, fluorescence intensity within the hybridization zone and dentin tubules. The general appearance of the samples from the respective repetitions of each group was used to characterize trends of the experimental groups and Controls.

3. Results

3.1. Dentin microtensile bond strength test (μTBS)

Bond strength means (± standard deviation) are presented in Table 2. Generalized linear model analysis showed "treatment" ($p = 0.008$), "evaluation time" ($p = 0.000$), "flavonoids application strategy" ($p = 0.072$), and some interactions between each two factors significantly influenced bond strength results.

At 24 h, Baicalein used as a primer presented the highest dentin bond strength. However, when the flavonoids were incorporated into the adhesive, they did not show differences among them ($p = 0.863$). Furthermore, the application mode of each flavonoid did not influence the bond strength results at 24 h. All groups with flavonoids and in both application modes (primer or adhesive) showed a significant difference from the Negative Control (commercial universal adhesive). Naringin incorporated into the adhesive showed higher dentin bond strength than that obtained for the Positive Control, while Baicalein used in both application modes differed from the 50 % and the Positive Control.

At one year, all experimental groups and Controls presented a lower μTBS means compared to 24 h results ($p = 0.006$), regardless of application mode (primer or adhesive). Also, all flavonoid groups showed a significant difference from the Negative Control. Baicalein incorporated into the adhesive showed higher μTBS means compared to its use as a *primer*, while the incorporation mode did not influence the bond strength results to the other two flavonoids. Different from 24 h, all the flavonoids incorporated into an adhesive showed a difference from the Positive Control, while only Baicalein showed a difference from the 50 % alcohol.

3.2. Failure mode analysis

Representative images for each failure are depicted in Fig. 1(A), and the percentages of occurrence of failure modes for each tested group are presented in Fig. 1(B). At 24 h evaluation, there was a wide distribution between types of failures for all groups. Adhesive failures (types II, III, V, and VI) were predominant for baicalein, regardless of application mode, kaempferol, and naringin added into the adhesive (~50 %-70 %). Cohesive failures within resin composite (Type I) were predominant for control groups (~20 %-39 %), as well as kaempferol, regardless of application mode (~34 %). After one year of storage, all groups showed an increase (~19 %-29 %) in the prevalence of adhesive failures, except for baicalein used as a *primer*, which showed a reduction in adhesive failures (21 %) and an increase in mixed failures (Type IV), as well as kaempferol applied as a *primer*.

Table 2
Dentin μTBS means (± standard deviation), comparing the experimental groups with the Controls at different evaluation times (in MPa).

Treatments	24 hours			One year		
	Baicalein	Kaempferol	Naringin	Baicalein	Kaempferol	Naringin
Primer	78.6 (10.1) aA ▼▲◉	68.0 (9.9) aB ▼	66.5 (10.3) aB ▼	48.9 (3.6) bA* ▼	48.8 (4.0) aA* ▼	48.5 (3.9) aA* ▼
Adhesive	73.1 (9.7) aA ▼▲◉	66.2 (8.0) aA ▼	69.0 (9.7) aA ▼▲	55.7 (4.2) aA* ▼▲◉	53.7 (5.6) aA* ▼▲	52.21 (5.0) aA* ▼▲
(-) Control	51.1 (10.4) a			38.9 (9.6) a*		
(+) Control	54.7 (6.6) a			41.8 (7.3) a*		
(50% ethanol) Control	57.5 (10.2) a			43.8 (6.3) a*		

Means followed by different letters indicate significant difference ($p < 0.05$). Upper case letters compare the three flavonoids incorporated into primers or adhesives, within the same evaluation time. Lower case letters compare treatments (primers and adhesives) for the same type of flavonoid and evaluation time. Also, lower case letters compare Controls within the same evaluation time.
*: Differs from 24 h within same treatment, evaluation time, and type of flavonoid.
▼: Differs from the "Negative Control" within the same evaluation time.
▲: Differs from the "Positive Control" within the same evaluation time.
◉: Differs from the "Solvent Control" for the same evaluation time.

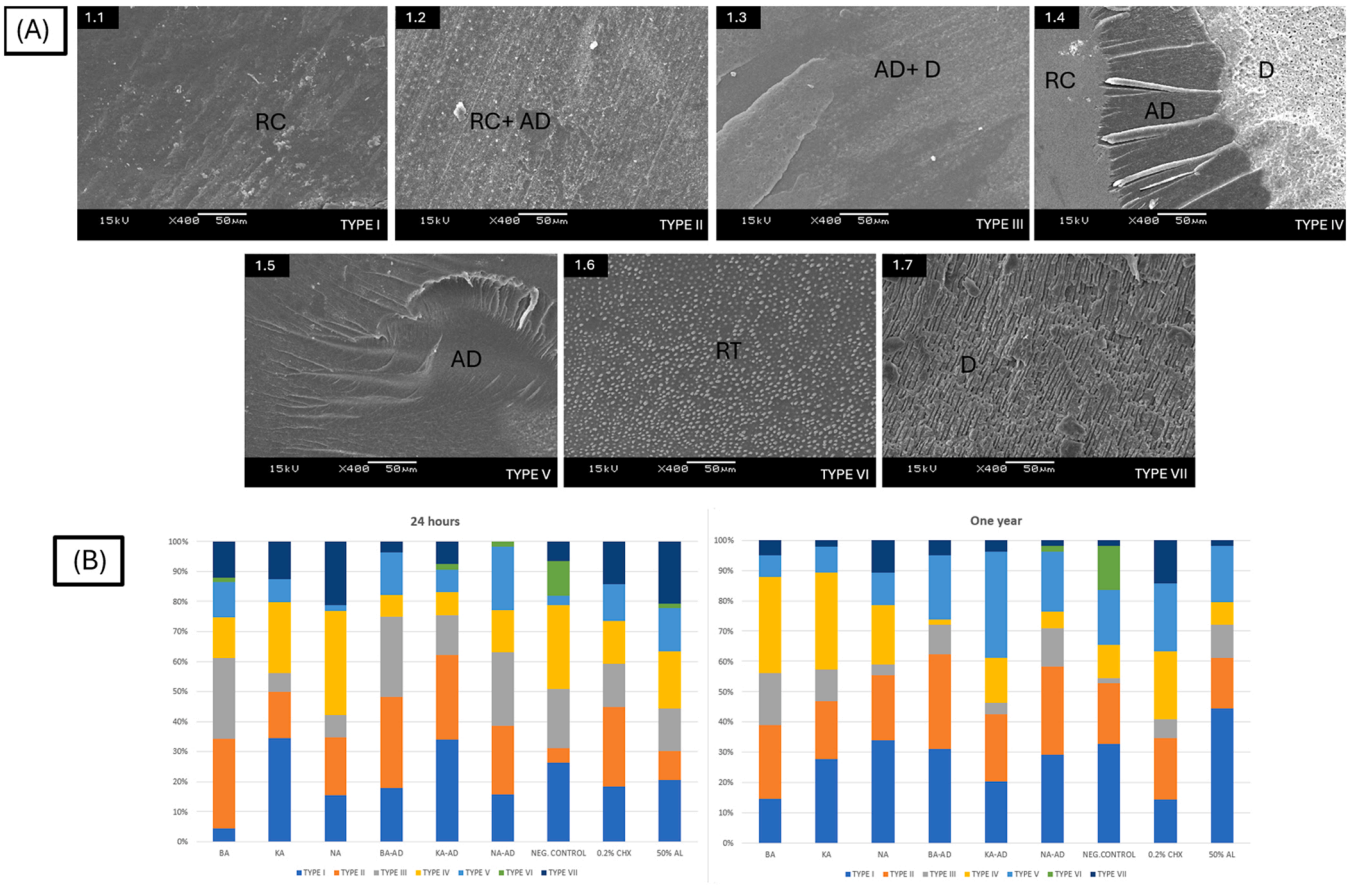

Fig. 1. (A). Scanning electron microscopy images of each failure mode at 400x magnification. (1.1) type I - cohesive failure of resin composite; (1.2) type II - adhesive failure between resin composite and bonding agent; (1.3) type III - adhesive failure between dentin surface and bonding agent; (1.4) type IV - mixed failure; (1.5) type V - cohesive failure of the bonding agent; (1.6) type VI - cohesive failure in the hybrid layer; (1.7) type VII - cohesive failure of dentin. RC - resin composite; AD - adhesive layer; RT - resin tags; D - dentin. (B). Distribution of failure mode at 24 h and at one year, according to each experimental group and Controls.

3.3. Adhesive-dentin bonding interface morphology analysis scanning electron microscopy (SEM)

Representative SEM images of dentin bonding interface morphology are depicted in Fig. 2. Resin tags and hybrid layer formations were observed in all groups, despite the application mode of each flavonoid. Slight differences were observed among groups regarding the number and length of resin tags. However, this feature depends on the dentin cutting direction.

3.4. In situ zymography of the hybrid layer

Micrographs of the *in situ* zymography analysis for each flavonoid application mode are shown in Fig. 3. This Figure comprises four sets of micrographs, each one containing an image of differential interference contrast (DIC), showing the optical density of the resin-dentin interface, merged with an image acquired in the green channel, showing fluorescence in dentin tubules and within the hybrid layer. The fluorescence intensity emitted by the hydrolyzed fluorescein-conjugated gelatin, which indicates endogenous proteolytic activity, was quantitatively analyzed using the adhesive without flavonoid as a Negative Control (commercial adhesive) and a solution of 0.2 % CHX used as a Positive Control.

The Negative Control (commercial adhesive) resulted in intense gelatinolytic activity at the hybrid layer and within dentin tubules, demonstrated by the high fluorescence emitted in the micrographs (Figure 3.7). Baicalein applied as a primer and the Positive Control (0.2 % CHX) shows a consistent inhibition of proteolytic activity in the hybrid layer and within the dentin region, with reductions of 64.03 % and 64.67 %, respectively, compared to the negative control. (Figures 3.1 and 3.8, respectively). In comparison to the Negative Control, the samples that were used with flavonoids (Figures 3.1, 3.2, and 3.3) also led to enzyme inhibition at the hybrid layer and within dentin (64.03 %, 29.42 %, 8.37 % respectively). While, in general, the flavonoids used as a primer showed an increased inhibition rate (34.43 %, 15.64 % and 0 % respectively) when compared to when applied into the adhesive (Figures 3.4, 3.5, and 3.6). The ethanol solution did not inhibit protease activity in the dentin matrix or hybrid layer. (Figure 3.9).

3.5. Inhibition of S. mutans biofilm formation

Analyses from the biofilm of *S. mutans* grown on top of the adhesive layer are described in Fig. 4. No statistical difference ($p > 0.05$) regarding the inhibition of *S. mutans* biofilm formation was observed between the Control (adhesive without flavonoids) and the adhesives containing flavonoids.

3.6. Degree of conversion and polymerization rate

Degree of conversion results are described in Fig. 5. No statistical difference ($p > 0.05$) regarding the degree of conversion was observed between the Control (adhesive without flavonoids) and the adhesives containing flavonoids.

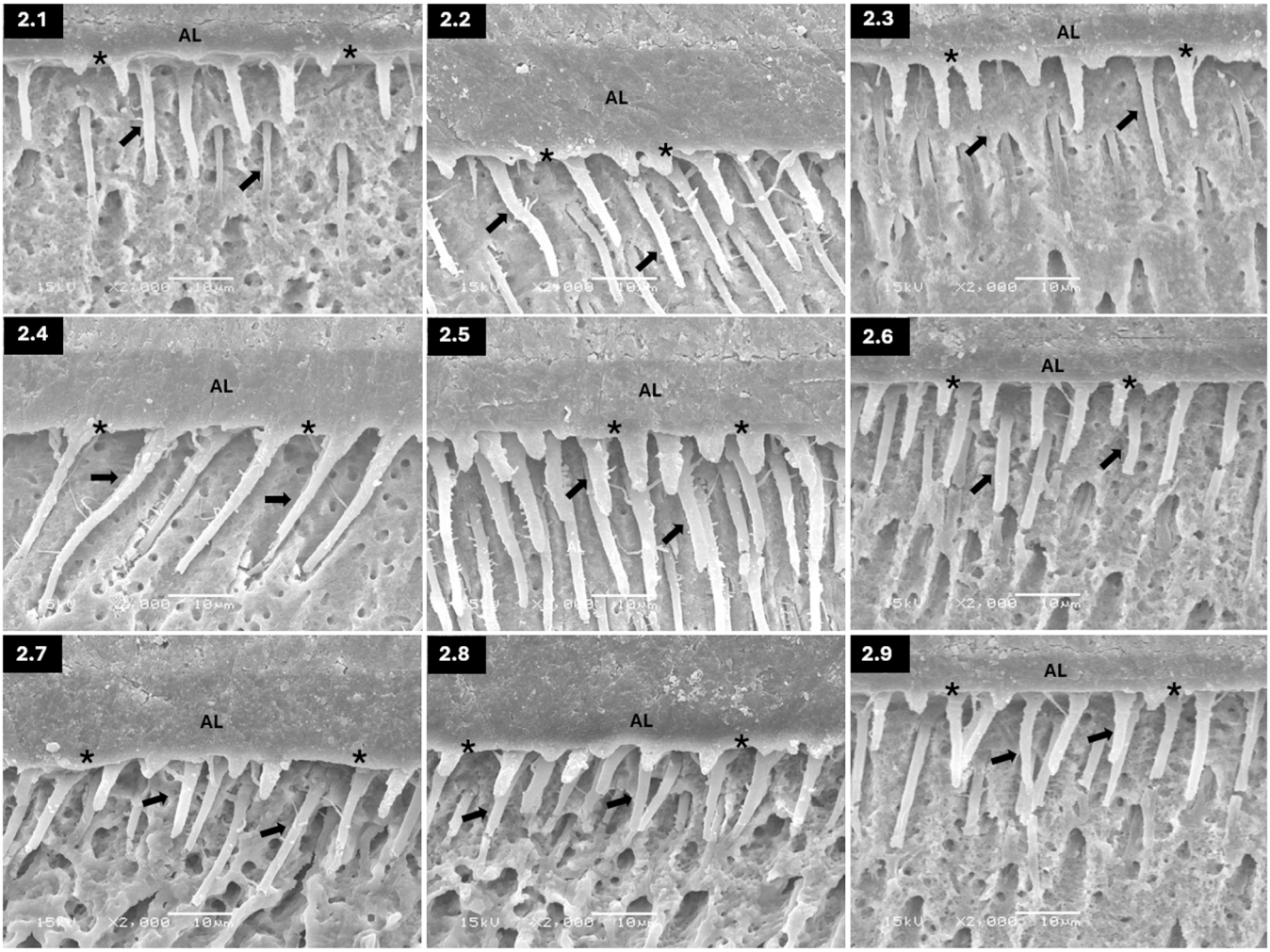

Fig. 2. Scanning electron microscopy images of the bonding interface according to each experimental group and Controls. 2.1: Baicalein; 2.2: Naringin; 2.3: Kaempferol (used as a *primer)*; 2.4: Baicalein; 2.5: Naringin; 2.6: Kaempferol (incorporated into the universal adhesive); 2.7: Negative Control (commercial adhesive); 2.8: Positive Control (0.2 % CHX) and 2.9: Solvent Control (50 % ethanol). (*) indicates the hybrid layers, and arrows (→) indicate resin tags. Abbreviation: AL: adhesive layer.

4. Discussion

The use of natural compounds, such as flavonoids, as a dentin bio-modification strategy has become increasingly favored over synthetic materials for reinforcing the dentin-resin interface. Historically, chlorhexidine digluconate (CHX) in varying concentrations has been the gold standard synthetic agent for enhancing hybrid layer longevity. However, several studies have highlighted a reduction in dentin bond strength values after one year of storage [25,26]. Although CHX can inhibit endogenous proteases and block proteolytic activity, its effectiveness declines after long-term storage (up to five years), even when it remains within the hybrid layer during this time [27]. In contrast, flavonoids outperform CHX in preserving collagen fibrils, acting as collagen crosslinkers to preserve the integrity of the hybrid layer over time. Moreover, flavonoids have been shown to significantly increase dentin bond strength values even after one year of storage [7].

Although substantial research has been conducted on flavonoids, the investigation of different subclasses remains ongoing, motivated by the search for novel bio-modifying agents with clinically relevant bioactive properties for enhancing the long-term performance of adhesive restorations. This is especially relevant in the context of dentin acid etching, which can compromise collagen stability. A notable strength of this study lies in its comparative design. Unlike previous investigations that assessed the effects of flavonoids either as pretreatment agents [7] or adhesive additives in isolation [10,12], our approach directly compares both application strategies using the same flavonoid compound under standardized conditions. This approach enables a more consistent evaluation of their influence on dentin bond strength and enzymatic activity. We observed that both the application strategy and aging time significantly influenced the microtensile bond strength (μTBS) values across the evaluated groups. Consequently, the first null hypothesis was rejected. As shown in Table 2, at the 24-hour evaluation, all flavonoid-treated groups exhibited significantly higher μTBS values compared to the negative control group, regardless of the application mode. After one year of storage, while all groups showed a decrease in μTBS compared to the 24-hour results, flavonoid groups still maintained superior bond strength compared to the negative control.

The efficacy of flavonoids in stabilizing collagen fibrils and their potential impact on dentin bonding can be attributed to their chemical structure [28], the type of interactions they engage in with dentin [29], and the chemical bonds they form, including covalent [30], hydrogen [31], ionic [32], and hydrophobic bonds [33]. Additionally, studies have emphasized the importance of the application strategy when incorporating flavonoids into demineralized dentin, either through adhesive incorporation or acid etching [34]. Interestingly, baicalein applied topically as a primer yielded the highest μTBS values at the 24-hour evaluation among the tested flavonoids. However, when incorporated into the adhesive, it did not significantly outperform the

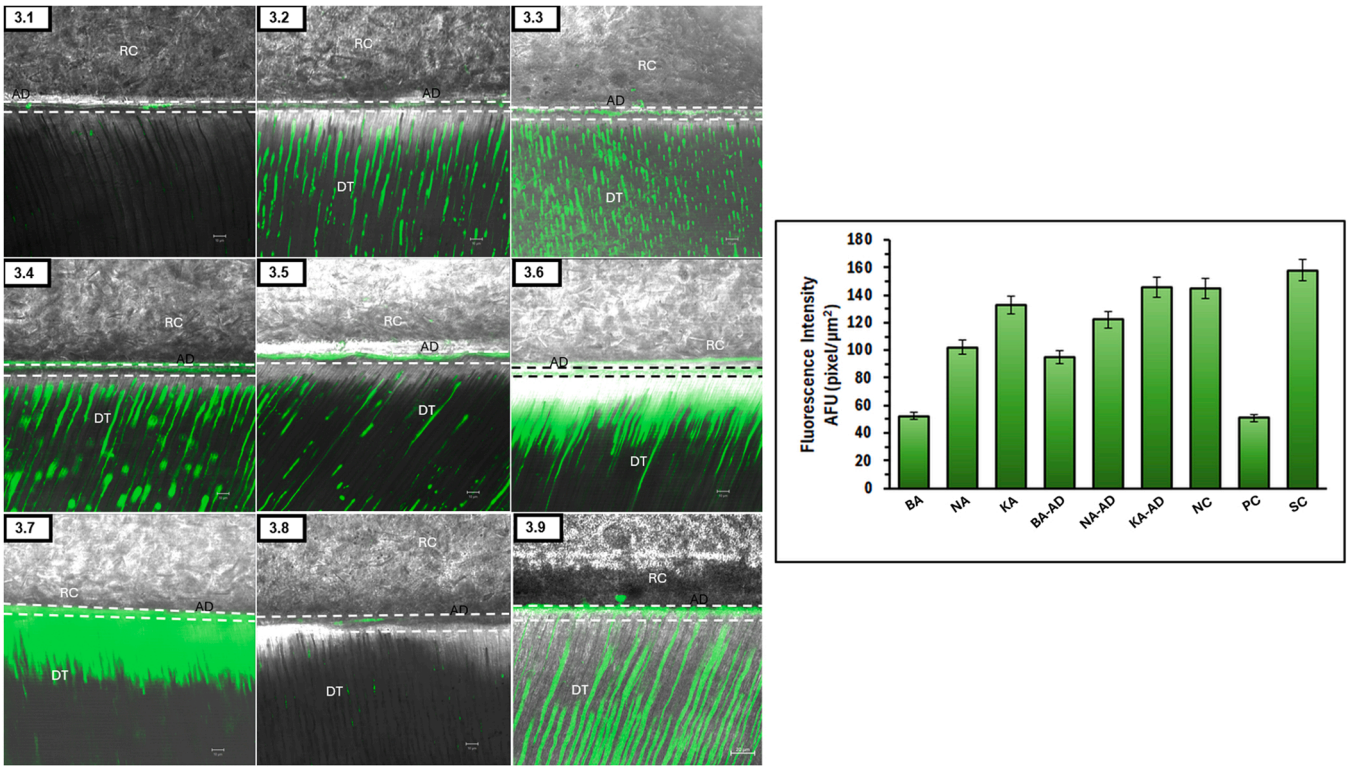

Fig. 3. Confocal laser scanning microscopy images of the resin-dentin bonded interfaces after 48-hour incubation with quenched fluorescein-labeled gelatin, used for in situ zymography to visualize gelatinolytic activity. The green fluorescent signal represents areas of gelatin cleavage, indicating the presence of proteolytic enzyme activity. Images are a merge of differential interference contrast (DIC) and emitted fluorescence signals at the green channel. 3.1: Baicalein (BA); 3.2: Naringin (NA); 3.3: Kaempferol (KA) (used as a *primer)*; 3.4: Baicalein (BA-AD); 3.5: Naringin (NA-AD); 3.6: Kaempferol (KA-AD) (incorporated into the universal adhesive); 3.7: Negative Control (NC); 3.8: Positive Control (PC - 0.2 % CHX) and 3.9: Solvent Control (SC - 50 % ethanol). RC - resin composite; AD - adhesive layer; DT – fluorescent signal within dentinal tubules. Dotted lines delineate the area of fluorescence within the hybrid layer. The bar graph shows the emitted fluorescence intensity in each experimental group.

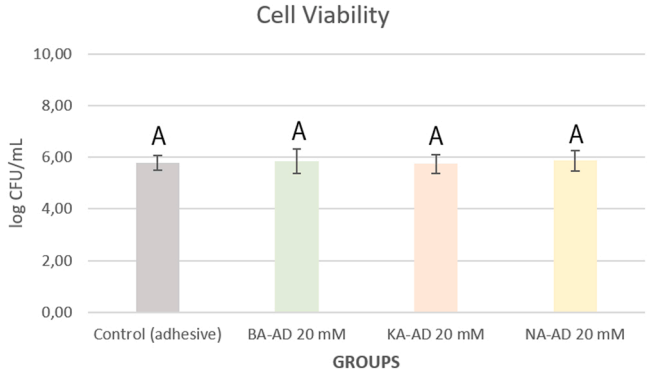

Fig. 4. Bacterial viability (log CFU mL^{-1}) after treatment of *S. Mutans* biofilm with the flavonoids incorporated into the universal adhesive. Different letters indicate a significant difference ($p < 0.05$). Control: commercial adhesive without flavonoid; BA-AD 20 mM: Baicalein incorporated into the adhesive bottle; KA-AD 20 mM: Kaempferol incorporated into the adhesive bottle; NA-AD 20 mM: Naringin incorporated into the adhesive bottle.

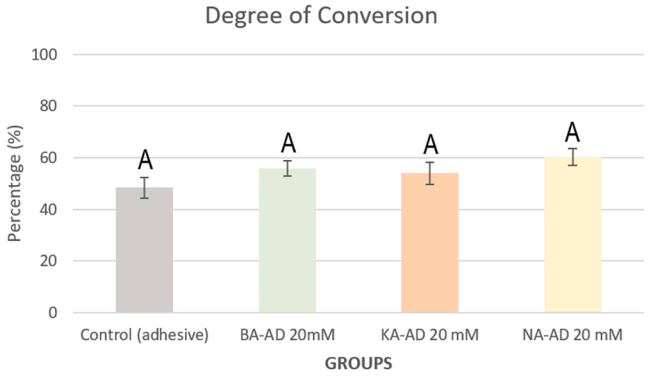

Fig. 5. Degree of conversion in percentage (%) of each group. Different letters indicate a significant difference ($p < 0.05$). Control: commercial adhesive without flavonoid; BA-AD 20 mM: Baicalein incorporated into the adhesive bottle; KA-AD 20 mM: Kaempferol incorporated into the adhesive bottle; NA-AD 20 mM: Naringin incorporated into the adhesive bottle.

other flavonoids. Conversely, after one year of storage, baicalein showed superior bond strength when incorporated into the adhesive, suggesting that different application strategies may influence a flavonoid's performance depending on the context. At this point, it is important to consider the dynamics of flavonoid availability and release over time. In the first 24 h, the adhesive matrix remains chemically stable, which likely limits immediate baicalein release, explaining the similarity in performance between application strategies at this stage. However, over time, resin matrices undergo degradation processes such as hydrolysis, water sorption, and enzymatic activity [35]. This gradual breakdown may facilitate the diffusion of incorporated compounds like baicalein, allowing interaction with demineralized dentin and contributing to bond strength preservation. Thus, the superior performance observed after one year in the adhesive group may be at least partly attributed to this time-dependent release mechanism.

The interaction of flavonoids with dentin can also be explained by several factors: I) their relatively small molecular size, which facilitates

their diffusion into the dentin tissue; II) the concentration of molecules available in the primer solution; III) the solubility index, which influences miscibility in the solvent used dilution; and IV) the number and type of reactive sites on each flavonoid molecule [36]. Moreover, the contact time with dentin differs depending on the application strategy: when applied topically, the contact predetermined, whereas adhesive incorporation may allow for prolonged interaction with demineralized dentin, potentially enhancing the compound's bioactive effects over time.

While baicalein exhibited differences in μTBS values based on application strategy after one year, kaempferol and naringin did not show similar variation. This difference may be attributed to their physicochemical characteristics, including larger molecular size, greater polarity, and lower enzymatic inhibitory activity, which could limit their diffusion through the adhesive matrix and reduce their effectiveness over time [13]. Naringin is a glycosylated flavonoid with a higher molecular weight and polarity, which may hinder its diffusion through the polymer and its ability to interact with dentin collagen after release [13]. Kaempferol, although structurally closer to baicalein, exhibits lower MMP-inhibitory activity, which reduces its protective effects even when released. As a result, these flavonoids may not provide additional benefits when incorporated into adhesives, explaining the absence of significant differences between application strategies following aging. Conversely, baicalein is completely insoluble in water, but the adhesive's chemical composition, which includes various solvents, may have facilitated its dissolution, explaining the observed differences in μTBS between application strategies. Kaempferol and naringin, which are slightly soluble in water, did not exhibit significantly altered solubility when dissolved in the 50 % alcoholic solution. It is known that alcohol enhances the reactivity of flavonoids, especially their polyphenolic interactions with collagen. And their hydrogen bonding potential [37], which may explain naringin's improved activity in alcohol-based solutions [38].

Another noteworthy finding after one year of storage is that all flavonoids incorporated into adhesives exhibited significantly higher bond strength than the CHX group. Despite CHX's well-documented ability to inhibit matrix metalloproteinases (MMPs) and other endogenous proteases, flavonoids have demonstrated both enzymatic inhibitory effects and superior bonding stability compared to the synthetic agent. This supports the concept that inhibiting latent dentinal proteases plays a crucial role in preventing or delaying enzymatic layer degradation, thereby maintaining long-term bond strength [4]. Therefore, the superior μTBS values observed with flavonoids may be directly associated with their capacity to inhibit endogenous protease activity. As shown in Fig. 3, significant differences in enzymatic activity were observed among the groups, leading to rejection of the third null hypothesis. The figures indicate that when flavonoids were applied as primers (Figures 3.1–3.3), they reduced enzymatic activity in the hybrid layer or even completely inhibited endogenous activity, particularly in the case of baicalein (Figure 3.1), which exhibited effects comparable to the CHX group (Figure 3.8). MMPs, such as MMP-2 and MMP-9, are key enzymes involved in the hydrolysis of dentin collagen fibers [3]. Previous studies have shown that flavonoids can inhibit these MMPs [39–43]. Baicalein contains hydroxyl groups (C6 and C7) capable of chelating transition metals such as Zn^{2+} [44], and given that MMPs require Zn^{2+}/Ca^{2+} as cofactors, this competitive reaction with metal ions may represent baicalein's primary inhibitory mechanism [11]. Furthermore, baicalein may alter the three-dimensional structure or molecular mobility of MMPs, thereby preventing collagenolysis and enabling hydrogen bonding with dentin collagen fibers, which protects collagen from degradation [11,45]. Although flavonoids incorporated into adhesives also reduced enzymatic activity, all groups showed a decrease in green fluorescence in the hybrid layer and dentinal tubules (Figures 3.4–3.6), which was similar to the 50 % alcohol group (Figure 3.9), and significantly lower than in the negative control group (Figure 3.7). It is worth noting that, although in situ zymography is a widely accepted technique for assessing enzymatic inhibition, it has certain limitations. For instance, it does not allow a precise distinction between the activities of specific enzymatic sites. Furthermore, local factors such as pH, natural inhibitors, and matrix composition may affect fluorescence intensity. Most importantly, the method provides only a static snapshot of enzymatic activity at a specific time point, limiting its capacity to monitor enzyme behavior dynamically over time within the same specimen.

Despite the observed differences in μTBS and enzymatic activity, no significant changes in failure modes were found among the groups at either evaluation time (Fig. 1B). After one year of storage, adhesive failures (Types II, III, V, and VI) predominated in all groups, except for baicalein applied as a primer, which showed a reduction (~21 %) in adhesive failures and a higher prevalence of mixed failures (Type IV). Failures within the hybrid layer (Type VI) were rare or absent in most groups after one year, suggesting that flavonoid treatments were effective in improving hybrid layer durability, compared to the negative control group, which showed a higher prevalence of hybrid layer failures (20 %).

Scanning electron microscope (SEM) images of the bonding interface revealed that all flavonoid application strategies resulted in the formation of a hybrid layer and resin infiltration into dentin, with no significant differences in bonding morphology between groups. Thus, the second null hypothesis was accepted. The figures 2.1–2.9 illustrate variations in the quantity and direction of resin tags infiltrating the dentin, likely influenced by tooth morphology and the cutting direction. Incorporation of materials into adhesives can alter their physical properties, such as increasing viscosity, which may hinder resin infiltration into demineralized dentin [46,47]. However, the flavonoid concentrations used in this study did not appear to interfere with the adhesive's infiltration capability. Groups with flavonoids incorporated into the adhesive bottle exhibited a greater quantity and length of resin tags compared to those treated with primers.

Although flavonoid incorporation into adhesives could potentially alter adhesive properties such as degree of conversion [48], this study found no significant effect on polymerization or bonding performance. The low concentration of flavonoids used may have been insufficient to significantly affect the degree of conversion, as confirmed by the acceptance of the fifth hypothesis. Further research is needed to assess the long-term effects of higher concentrations on adhesive performance.

One motivation for incorporating flavonoids into adhesives is their antibacterial potential, which could help prevent secondary caries and extend restoration longevity [47]. In this study, no significant antibacterial effect against *S. mutans* biofilm was observed in the flavonoid-containing adhesives, leading to acceptance of the fourth hypothesis. Although a 20 mM concentration was employed, the minimum inhibitory concentration or biofilm inhibitory concentrations ($MIC/MBIC_{50}$) values reported in the literature for baicalein [49], naringin [50], and kaempferol [51] generally exceed this level and reflect only partial biofilm inhibition. Notably, these values apply to flavonoids in solution rather than those embedded in a polymer matrix. Thus, incorporation into the cross-linked adhesive likely limited their release and surface availability, reducing interactions with bacterial cells. As a result, the effective concentration at the biofilm interface was likely below the inhibitory threshold, which may explain the absence of antibacterial activity. Additionally, the 24-hour biofilm assay used in this study did not capture potential long-term effects, as gradual degradation of the resin matrix in the oral environment could enable sustained flavonoid release and potentially enhance antimicrobial efficacy over time [52]. This study focused exclusively on S. *mutans* due to its established role as a primary cariogenic pathogen [53]. Ongoing investigations are expanding upon these findings by testing different flavonoid concentrations and evaluating multispecies biofilms. Future strategies to improve flavonoid bioavailability and sustained antibacterial action may involve the use of nanocarriers or surface-modified delivery systems to optimize release at the adhesive-biofilm interface [54,55].

In summary, flavonoids demonstrate multiple benefits, including enzymatic inhibition, collagen stabilization, and preservation of bond strength when used in both primer and adhesive formulations. While the concentrations employed in this study were sufficient to improve bonding without compromising adhesive performance, further research exploring optimized delivery systems may unlock their full bioactive potential in restorative dentistry.

5. Conclusion

Considering the limitations of this in vitro study, the results can be summarized as follows:

(1) Regardless of the application strategy, the use of flavonoids consistently improved dentin bond strength compared to the Negative Control group; however, when incorporated into the adhesive, they further enhanced the bond strength compared to the Positive Control group;

(2) Although baicalein completely inhibited enzymatic activity in the hybrid layer when applied as a primer, this effect did not significantly impact the μTBS results after one year of storage; all flavonoids, regardless of the application strategy, were able to reduce enzymatic activity in the hybrid layer;

(3) The addition of flavonoids to the dental adhesive did not cause any changes in pH or degree of conversion;

(4) Regarding antibacterial activity, the concentrations of flavonoids used were not effective in reducing biofilm formation on the adhesive surface.

Funding

This research was supported by São Paulo Research Foundation (FAPESP, # 2021/11972–0 and National Council for Scientific and Technological (CNPq, # 308654/2023–4), Brazil.

Declaration of Competing Interest

The authors declare that there is no conflict of interest related to this work

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