Dual function of quercetin as an MMP inhibitor and crosslinker in preventing dentin erosion and abrasion: An in situ/in vivo study

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DOI: https://doi.org/10.1016/j.dental.2022.09.019

Dual function of quercetin as an MMP inhibitor and crosslinker in preventing dentin erosion and abrasion: An *in situ/in vivo* study

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Abstract

Objective. The aim of the present study was to evaluate the *in situ/in vivo* effect of quercetin on dentin erosion and abrasion.

Methods. Human dentin blocks (2 × 2 × 2 mm) were embedded and assigned to 6 groups: 75 μg/mL, 150 μg/mL and 300 μg/mL quercetin (Q75, Q150, Q300); 120 μg/mL chlorhexidine (CHX, positive control); and deionized water and ethanol (the negative controls). The specimens were treated with the respective solutions for 2 min and then subjected to *in situ/in vivo* erosive/abrasive challenge for 7 d as follows: *in vivo* erosion 4 times a day and then *in vivo* toothbrush abrasion after the first and last erosive challenges of each day. Dentin loss was assessed by profilometry. An additional dentin specimen was used to evaluate the penetration depth of quercetin into dentin by tracking the spatial distribution of its characteristic Raman peak. Moreover, dentin blocks (7 × 1.7 × 0.7 mm) were used to detect the impact of quercetin on dentin-derived matrix metalloproteinase (MMP) inhibition by *in situ* zymography, and the inhibition percentage (%) was calculated. Additionally, the potential collagen crosslinking interactions with quercetin were detected by Raman spectroscopy, and the crosslinking degree was determined with a ninhydrin assay. Fully demineralized dentin beams (0.5 × 0.5 × 10 mm) were used to evaluate the impact of quercetin on the mechanical properties of dentin collagen fibre by the ultimate micro-tensile strength test (μUTS). The data were analysed by one-way analysis of variance and Tukey's test ($\alpha = 0.05$).

Results. Compared to the negative controls, all treatment solutions significantly reduced dentin loss. The dentin loss of Q150 and Q300 was significantly less than that of CHX (all $P < 0.05$). The amount of quercetin decreased with increasing dentin depth, and the maximum penetration depth was approximately 25-30 μm. *In situ* zymography showed that quercetin significantly inhibited the activities of dentin-derived MMPs. The inhibitory percentages of Q75 and Q150 were significantly lower than that of CHX (all $P < 0.05$), but no significant difference was found between Q300 and CHX ($P = 0.58$). The collagen crosslinking interactions with quercetin primarily involved hydrogen bonding and the degree of crosslinking increased in a concentration-dependent manner. Statistically significant increases in μUTS values were observed for demineralized dentin beams after quercetin treatment compared with those of the control treatments (all $P < 0.05$).

Significance. This study provides the first direct evidence that quercetin could penetrate approximately 25-30 μm into dentin and further prevent dentin erosion and abrasion by inhibiting dentin-derived MMP activity as well as crosslinking collagen of the demineralized organic matrix.

Keywords: Erosion; Abrasion; Quercetin; Penetration capacity; Zymography; Tensile strength

1. Introduction

Dental erosion, defined as an irreversible loss of tooth enamel and dentin due to a chemical process without the involvement of microorganisms, has become one of the most common oral diseases in recent years [1,2]. Growing evidence indicates that the prevalence of dental erosion is increasing [3–5]. A recent review estimated that the global prevalence in permanent teeth ranges between 20% and 45% for children and adults[6]. Dental erosion can be caused by intrinsic (e.g., gastric reflux and excessive vomiting) and/or extrinsic (e.g., acidic foods and drinks and acid fumes at work) factors. Given the lack of obvious symptoms at earlier stages and the insufficient knowledge and attitude of patients towards the condition [7,8], in most cases, dental erosion is generally detected at late stages [9]. At late stages, dentin becomes increasingly exposed, and tooth hypersensitivity, loss of occlusal vertical height and the destruction of pulp may occur [1].

The histological features and mechanism of erosion in dentin are completely distinct from those of enamel [10]. When dentin is exposed to acid attack, the minerals dissolve rapidly which leads to the further presence of the demineralized organic matrix (DOM) on the surface [11,12]. The maintenance of DOM stability has been considered essential, as it acts as a barrier to prevent subsurface erosion. In addition, DOM serves as a scaffold during the remineralization process and provides sites for nucleation and mineralization [11,13]. After demineralization, however, inactive dentin-derived matrix metalloproteinases (MMPs), including mainly MMP-2, MMP-8, and MMP-9,

are exposed and activated in acidic environments, and these active enzymes are responsible for the disintegration of the exposed collagen fibrils, which eventually leads to DOM breakdown [12,14,15]. The application of MMP inhibitors and crosslinkers has been utilized to challenge this enzymatic degradation [12,16–18].

Our previous study reported that quercetin, a naturally occurring flavonol, slowed the progression of dentin erosion through preservation of the DOM [19]. Several studies have reported that quercetin has a potential role in modulating the expression or proteolytic activity of MMPs in various tumour cells [20–22]. In previous studies, quercetin has been shown to reduce the degradation of adhesive-dentin interfaces by inhibiting MMP activity [23,24]. Furthermore, quercetin possesses a molecular structure with phenolichydroxyl functional groups, suggesting its cross-linking properties. Quercetin can improve the mechanical properties of collagen by forming covalent and hydrogen bonds between collagen fibres to stabilize collagen molecules [12–14]. One could, therefore, hypothesise that the mechanism by which quercetin preserves DOM might be related to dentin-derived MMP inhibition and enhancement of mechanical properties. On the other hand, in a previous study, quercetin was applied only once before erosive challenges, and DOM was observed after 7 d of erosive cycling [12]. Because the permeation of the drug is highly related to the therapeutic effect, the relatively long-lasting effect of quercetin may be associated with its dentin penetration capacity. However, the permeation of quercetin into dentin has not been evaluated in the literature. Importantly, dental erosion is a multifactorial condition and

is typically accompanied by mechanical factors [25]. After demineralization, the DOM is usually mechanically fragile and provides insufficient protection against abrasive forces, such as toothbrushing [26]. Thus, whenever possible, abrasive challenges should be investigated in dental erosion experiments to simulate clinical conditions. However, the role of quercetin in preventing dentin erosion and abrasion remains unclear.

Thus, the aim of this study was to determine whether quercetin can inhibit dentin erosion and abrasion *in situ/in vivo* and to explore the potential mechanism of quercetin protection against dentin erosion and abrasion. The following null hypotheses were tested: (1) quercetin has no effect on the progression of dentin erosion and abrasion; (2) quercetin does not permeate into dentin; (3) quercetin has no effect on the activation of dentin-derived MMPs; and (4) quercetin has no effect on the mechanical properties of dentin collagen fibre.

2. Methods and materials

Ethical approval for the study was provided by the Institutional Review Board, School and Hospital of Stomatology, Fujian Medical University, China (FMUSS-19-046).

2.1 Dentin erosion/abrasion

Sixty dentin blocks (2 mm × 2 mm × 2 mm) were cut from mid-crown portions of the human third molar using a low-speed saw (Isomet, Buehler, Lake Bluff, IL, USA) with water cooling. The dentin blocks were then embedded in acrylic resin (Varidur,

Buheler, USA) using a silicon mould. The specimens were ground flat and polished using carborundum discs (#320, #600 and #1200; Buehler) under water cooling conditions and were then cleaned in an ultrasound bath for 5 min. The final dimensions of the specimens were as follows: top surface diameter, 4 mm; bottom surface diameter, 5 mm; and thickness, 2 mm. Both sides of each specimen were covered with two layers of nail varnish (Top Speed, Revlon, USA) as the reference area, and the middle (1 mm wide) was exposed as the treated area [12,27,28]. Then, the specimens were randomly assigned to 6 groups (n=10): 75 μg/mL, 150 μg/mL and 300 μg/mL quercetin (Q75, Q150, Q300); 120 μg/mL chlorhexidine (CHX, positive control); and deionized water and ethanol (the negative controls). Thereafter, the specimens were treated with the corresponding solutions with a microbrush for 2 min [29,30]. Fifteen volunteers (9 men and 6 women; average age: 25 ± 5 years) participated and were asked to follow the *in situ/in vivo* experimental protocol, which was modified from previous studies [12,27,28,31].

The intraoral appliance was fabricated with a 0.035-in-thick soft-tray sheet (Ultradent Products Inc., South Jordan, UT, USA) and a heat/vacuum tray-forming machine (Ultraform, Ultradent Products Inc). The intraoral appliance was made with 4 slots (located on the buccal surfaces of the central and lateral incisors) to allow for the storage of the dentin specimens. An opening was made on each slot so that the top surfaces of the specimens were exposed to the oral cavity [12,28,31]. The volunteers were asked to wear the intraoral appliance continuously for 24 h, removing it only at

mealtimes and storing it in artificial saliva. Over the following 7 d, erosive challenges were performed intraorally 4 times a day (at 08:00, 12:00, 16:00, and 20:00), and abrasive challenges were performed intraorally twice a day (30 min after the first and last erosive attacks). For each erosive challenge, the volunteers rinsed with 150 mL of freshly opened cola drink (pH = 2.3) (Coca-Cola, Coca-Cola China, China) for 5 min. In contrast to previous studies, abrasive challenges were performed intraorally. For each abrasive challenge, the volunteers brushed the buccal surface of the appliance in the upper front teeth area for 30 s with a manual brush (Colgate Plus, Colgate-Palmolive Company, USA), and approximately 0.09 g of dentifrice containing 1450 ppm fluoride was used (Colgate Cavity Protection, Colgate-Palmolive Company, USA) (relative dentin abrasivity (RDA) = 60.80) [32,33]. The volunteers were instructed to brush along the specimens parallel to the teeth to simulate the "scrub technique", which is the most widespread brushing technique [34]. All the volunteers were carefully trained to standardize the procedure. After the erosive and abrasive challenges, the varnishes were carefully removed [35], and the dentin loss (μm) of each specimen was measured by contact profilometry (SEF 680, Kosaka Laboratory, Japan) from a reference surface to the other surface. The average of 5 points with 0.2-mm intervals was obtained [12,31].

2.2 Raman spectra

To acquire the characteristic peaks of quercetin to distinguish quercetin and dentin phases, an additional dentin specimen and quercetin powder (≥ 98% purity, Sigma-Aldrich, USA) were used for spectral acquisition with a confocal Raman

spectrophotometer (inVia, Renishaw plc, UK). Before data collection, the spectra were Raman-shift-frequency calibrated with known lines of silicon [36]. For the sampling, the additional dentin specimen and quercetin powder were positioned upright on Tienta Spectra RIM slides (Tienta Sciences, USA), and the laser beam was focused onto the sample surface. The spectra were acquired at 785 nm (200 to 3200 cm-1) with approximately 2 mW of single-mode power and a resolution of 2.5 cm^{-1}; the acquisition time of each spectrum was 10 accumulations of 10 s each. Labspec software (version 4.12, Horiba Jobin Yvon, Edison, NJ, USA) was used to analyse the acquired Raman spectra. The raw spectral data were subjected to background and noise removal with dark count correction before the statistical analysis. The background was removed by the polynomial (degree 4) function, and noise reduction was performed using the denoise function. Baseline correction was undertaken by manual multiple-point correction and smoothening was performed using the Savitzky-Golay algorithm [37,38]. The corrected spectra were then normalized to the 1001 cm^{-1} peak [39].

2.3 The penetration depth of quercetin into dentin

To evaluate the penetration of quercetin into the dentin, 20 mL of the Q300 was deposited on the surface of the dentin specimen for 2 min. After the diffusion period, the dentin surface was cleaned with a dry cotton swab. Given that Raman peak intensity is directly related to the quercetin concentration [40,41], a characteristic Raman peak that could be easily distinguished was chosen to track the spatial distribution of quercetin in dentin.

The characteristic Raman peak was recorded at each image point (pixel) at a given confocal position. One point in the image corresponds to the intensity of the characteristic Raman peak. To investigate the distribution of quercetin on the dentin surface, a two-dimensional (2D) image was obtained by recording the characteristic Raman peak with a step size of 2 μm in the x–y plane. The 2D image size generated from dentin was 400 μm × 400 μm and contained 40,000 points. Then, an area on the 2D Raman image was randomly selected that allows a faster volume imaging speed. Three-dimensional (3D) Raman maps of quercetin-treated dentin were obtained with a step size of 2 μm using the x–y–z stage (40 μm × 40 μm × 40 μm, including 64,000 points) [42–47]. The images were generated using the volume viewer feature of WiRe 4.1 software (Renishaw Plc., UK).

2.4 *In situ* zymography

Dentin blocks (7 × 1.7 × 0.7 mm) were cut from human third molar crowns with low-speed saw (Isomet, Buehler, Lake Bluff, IL, USA) under water cooling. Thereafter, the dentin blocks were ground and polished with a series of water-cooled carborundum discs (320#, 600# and 1200#; Buehler) to standard dimensions. The dentin block was randomly assigned to 6 groups as mentioned above. Each dentin block was wet-polished, ultrasonically cleaned, placed on glass slides, and etched with 10% H_3PO_4 (Scothbond Etchant, 3M ESPE, Germany) for 10 s. Then, blocks were rinsed in deionized water for 20 s [48]. Subsequently, each etched dentin block was treated with 50 μL of the respective treatment solutions for 10 min.

Fluorogenic dye-quenched (DQ)-gelatine was used *in situ* to investigate whether quercetin directly inhibits dentin-derived MMPs. Briefly, quenched fluorescein-conjugated gelatine (E-12055, Molecular Probes, USA) was added to the etched dentin block at 37°C for 24 h with light protection. The amount of green fluorescence produced by the dissolved quenched fluorescein-conjugated gelatine mixture produced by MMPs was observed by confocal laser scanning microscopy (excitation/emission: 488/530 nm; CLSM) (Zeiss, LSM 780, Carl Zeiss, Germany). Then, 85-μm thick optical sections were acquired from different focal planes, and the stacked images were analysed, quantified, and processed with ZEN 2010 software (Carl Zeiss, Germany) [49]. The MMP inhibitory percentage was calculated using the following formula [50,51]:

Inhibitory percentage (%) = (1-average absorbance of treated group/average absorbance of control group) × 100%

2.5 Degree of crosslinking

The 1-mm-thick dentin slices were cut from human third molars' crowns using a low-speed diamond saw. Then the slices were pulverized in liquid nitrogen into a fine powder using a steel mortar/pestle. The dentin powder was filtered through a 20-μm strainer and completely demineralized with 10% phosphoric acid (pH = 1.0) (Panreac Química, Barcelona, Spain) for 5 h. The demineralized dentin powder was rinsed with deionized water by repeated centrifugation (4,000 g for 10 min at 4°C) and lyophilized using a vacuum lyophilizer (DF8606HT, Fevik, Germany). The demineralized dentin

powder was divided into 5 groups (n=3): (1) Q75; (2) Q150; (3) Q300; (4) deionized water; and (5) ethanol. A 1-mg quantity of demineralized dentin powder was immersed in 5 mL of treatment solution for 2 min at room temperature. Therefore, the treated dentin powder was extensively washed with deionized water by centrifugation and lyophilized.

The degree of crosslinking was determined using the ninhydrin assay and compared to the nontreated dentin powder [52,53]. Briefly, the treated and non-treated dentin powder was incubated in boiling water for 20 min with ninhydrin reagent (Sigma-Aldrich, UK) for 20 min [52,54], and then the optical absorbance was recorded with a Multiskan FC microplate reader (Thermo Fisher, USA) at 570 nm. The crosslinking degree was calculated according to a standard curve of the glycine concentration. The crosslinking degree was calculated as below [52]:

Degree of crossinking (%) = $(M_0\text{-}M_1)/M_0 \times 100\%$,

where M_0 is the amount of free amino groups in nontreated dentin powder, and M_1 is the amount of free amino groups in treated dentin powder.

2.6 Ultimate microtensile strength of collagen

Dentin beams (0.5 × 0.5 × 10 mm) were cut from human third molars' crowns and then randomly divided into 5 groups (n=12) as described in Section 2.6. Thereafter, the dentin beams were immersed in 0.5 M ethylenediaminetetraacetic acid (EDTA) at a pH of 7.4 for 3 d. X-rays were used to confirm the complete demineralization of dentin beams [55]. The beams were thoroughly rinsed with deionized water for 7 d to

remove any residual of EDTA. After the rinse periods, the beams were immersed in treatment solutions for 1 h at room temperature. Subsequently, the beams were thoroughly rinsed with deionized water to remove residual treatment solution [56].

To determine the ultimate microtensile strength (μUTS), each beam was glued with cyanoacrylate adhesive (Zappit, Dental Vestures of America, USA) to the two free-sliding components of a jig, which was mounted on a microtensile tester (T-61010K, Bisco, USA) and subjected to microtensile testing at a crosshead speed of 1 mm/min until rupture occurred [57]. The μUTS (MPa) was calculated according to the following formula [58]:

$$\mu UTS = p/(b \times d),$$

where p is the maximum load (N), b is the sample width (mm), and d is the sample thickness (mm).

2.7 Statistical analysis

The assumptions regarding the equality of the variances and normal distribution of errors were confirmed using the Levene test and the Kolmogorov-Smirnov test, respectively. For dentin loss, MMP inhibitory percentage, degree of crosslinking, and μUTS, the data were statistically analysed using one-way analysis of variance (ANOVA) and Tukey's test. The data were analysed using the Statistical Package for the Social Sciences (SPSS) statistical software package (SPSS 19.0 for MAC, SPSS, Chicago, IL, USA), and all statistical analyses were performed at a significance level of 0.05.

3. Results

3.1 *In situ/in vivo* erosion and abrasion

As shown in Table 1, the specimens treated with different concentrations of quercetin showed significantly lower dentin loss than those treated with the negative control solutions (all $P < 0.05$). No significant differences were noted between the deionized water and ethanol groups (all $P > 0.05$). Additionally, the dentin loss of Q150 and Q300 was significantly less than that of CHX (all $P < 0.05$).

3.2 Raman spectra

Figures 1 and 2 show Raman spectra of quercetin, dentin in the native state and dentin after quercetin treatment recorded in a wavelength range of 500-1800 cm^{-1}. When dentin is treated with quercetin, one can observe a shift of the 1666 cm^{-1} amide I band towards 1664 cm^{-1} and simultaneously a shift of the 1242 and 1270 cm^{-1} amide III bands towards lower frequencies 1241 and 1268 cm^{-1}, respectively (Fig. 1). Additionally, the ratio between CN-stretch/NH-deformations was decreased after treatment with Q300 (1.057 for native state dentin vs. 0.946 for quercetin-treated dentin).

3.3 The penetration depth of quercetin into dentin

The C_2=C_3 stretching modes ($\nu_{str}C_2$=C_3) at 1609 cm^{-1} arising from quercetin did not overlap with the major vibrational peaks observed for dentin (Fig. 1). Thus, this spectral peak was used as a Raman probe to track the spatial distribution of quercetin in dentin. Additionally, as shown in Fig. 1, $\nu_{str}C_2$=C_3 was clearly visible in the spectra of

quercetin-treated dentin, representing clear evidence of the presence of quercetin in the dentin.

Given that Raman peak intensity is directly related to the quercetin concentration, $\nu_{str}C_2{=}C_3$ intensities were utilized to track the amount of quercetin in dentin at different depths. Fig. 2 shows the 2D imaging (XY = 400 × 400 μm^2, Z = 0 μm) of the coloured plane obtained by detecting the $\nu_{str}C_2{=}C_3$ signal on a uniform area by means of a confocal Raman spectrophotometer. Quercetin was distributed evenly throughout the dentin surface. Fig. 3a shows the 3D reconstruction (XYZ = 40 × 40 × 40 μm^3) of the coloured volume. Nine selected XY slices from the 3D Raman map showed the distribution of quercetin with different dentin depths, as shown in Figure 3b. The amount of quercetin decreased rapidly with increasing dentin depth, and the maximum penetration depth of quercetin reached approximately 25-30 μm. However, minimal or even no penetration of quercetin in dentin was noted at 30-40 μm. Furthermore, three 3D reconstructed videos showing the precise position of quercetin in the dentin are provided in Supplementary Material Video S1.

3.4 Effects of quercetin on the inhibition of dentin-derived MMPs

For the negative controls (deionized water and ethanol), intense green fluorescence indicating gelatinolytic activity was observed in demineralized dentin (Fig. 4). Treatment with quercetin and CHX resulted in a significant decrease in the fluorescence intensity, indicating an inhibition of dentin-derived MMP activity (Fig. 4). The inhibitory percentages of Q75 (32.24 ± 5.22%) and Q150 (41.37 ± 1.67%) were

significantly lower than that of CHX (59.68 ± 6.00%) (all $P < 0.05$), but no significant difference was found between Q300 (60.26 ± 5.35%) and CHX ($P = 0.58$).

3.5 Degrees of crosslinking

The results showed that crosslinking degrees exhibited concentration-dependent increases (all $P < 0.05$) (28.51 ± 2.64%, 43.54 ± 1.46%, and 54.41 ± 3.30% for Q75, Q150, and Q300, respectively) (Figure 5).

3.6 Effects of quercetin on the ultimate microtensile strength of collagen

The beams treated with different concentrations of quercetin exhibited significantly higher μUTS values (23.47 ± 8.62, 24.29 ± 7.33, and 29.56 ± 8.55 MPa for Q75, Q150, and Q300, respectively) than control samples (9.90 ± 3.62 MPa for ethanol and 10.18 ± 3.37 MPa for deionized water) (all $P < 0.05$). The μUTS value of Q300 was significantly higher than those of Q75 and Q150 (all $P < 0.05$).

4. Discussion

Based on the present findings, the null hypotheses were all rejected. The present results showed that quercetin was able to reduce dentin loss and that Q150 and Q300 were more effective than the well-established product CHX, indicating the great potential of quercetin in preventing dentin erosion and abrasion. In addition, our results demonstrated that quercetin could penetrate approximately 25-30 μm into the dentin and further prevent dentin from erosion and abrasion by inhibiting dentin-derived MMP activity as well as enhancing the mechanical properties of the DOM.

Dental erosion is widely regarded as a multifactorial condition caused by chemical processes that are usually accompanied by mechanical factors [2]. The softened surface layer produced by erosive challenge, especially in eroded dentin, is more susceptible to mechanical forces, such as abrasion [26]. Thus, the erosive/abrasive model was adopted, and toothbrushing was selected because it is a suitable method for simulating intraoral frictional forces [29]. Because biological factors, such as saliva, dental pellicle or dental plaque, cannot be simulated adequately *in vitro*, both erosion and abrasion of the specimens were performed intraorally in the present study. Behaviours, such as brushing twice daily with fluoride toothpaste and brushing the upper front teeth for 30 s are sufficient for individuals with good oral hygiene [59]. In addition, fluoridated toothpastes account for greater than 90% of the market internationally [60]. Therefore, in the present study, the specimens were subjected to 4 erosive challenges/day and 2 abrasive challenges/day. Although the efficacy of delayed brushing in increasing the

abrasion resistance of eroded enamel and dentin is still questioned, delayed brushing may be more relevant to the clinical situation than immediate brushing [26]. Therefore, according to previous studies [13,29,61], a 30-min interval between erosive and abrasive challenge was adopted in this study.

Raman spectroscopy is an analytical technique for determining the chemical composition of materials and obtaining structure composition based on molecular vibration of the molecules in the sample. This technique has evolved as a convenient approach to characterize the spatial distributions of organic compounds and inorganic compounds with a spatial resolution of approximately 1 μm [45–47]. In this and our previous studies [12], quercetin was preapplied only once for 2 min, and the preventive effect of quercetin on dentin erosion and abrasion was quite remarkable. This finding could be attributed to the low molecular weight of quercetin, which allows it to attain greater and faster penetration in the dentin collagen matrix [62]. The spatial distribution of quercetin in dentin was analysed using Raman spectroscopy for the first time, and the hypothesis was confirmed. The results showed a rapid decrease in amount with depth, and the penetration depth of quercetin was at most 25-30 μm. After 7 d of erosive/abrasive challenges, the dentin loss in all groups was < 2 μm and considerably lower than the maximum penetration depth of quercetin to sound dentin. It can be hypothesised that quercetin penetrates deeper into demineralized dentin, which possesses greater porosity than healthy dentin [63]. Theoretically, quercetin could be

used in sound and demineralized dentin, indicating promising application potential in the prevention and treatment of dentin erosion and abrasion.

Based on previous studies [18,56,61,64], the impact of quercetin on dentin erosion and abrasion may be related to the inhibition of dentin-derived MMPs and the improvement in the mechanical properties of dentin collagen fibre. In the present study, *in situ* zymography was performed using EnzChek gelatinase/collagenase assay kits. CHX, a common protease inhibitor, was selected as a positive control at its most commonly used concentration [16,65]. The present results showed that quercetin strongly affected the activation of dentin-derived MMPs, especially at a concentration of 300 μg/mL which exhibited the same inhibitory activities as CHX. Given that MMPs are a family of Zn^{2+}/Ca^{2+}-dependent enzymes, the main inhibitory mechanism of quercetin on MMPs can be attributed to the two vicinal hydroxyl groups of quercetin leading to powerful metal-chelate characteristics [66,67]. Moreover, molecular docking (supplementary material S2) and previous studies have determined that quercetin can protrude into the S1′ pocket of MMPs, which is the main site for substrate recognition by hydrophobic interactions and electrostatic interactions [54,55].

Collagen, as a structural framework for the dentin organic matrix and other extracellular matrix components, functions mainly to provide tensile strength [69]. The Raman spectra of collagen can be correlated directly to their backbone conformation. As previously revealed, Raman spectra of amide I and amide III are sensitive to the secondary structure of the polypeptide chain and can be used to analyse the protein

conformational change after different treatments [39,70]. For native dentin, the main contribution to the amide I band is the stretching vibrations of peptide C=O groups at 1666 cm^{-1}, whereas the amide III band originates from the N–H bending vibrations at 1270 cm^{-1} coupled to the C–N stretching mode at 1242 cm^{-1}. As shown by Raman analysis, characteristic shift in both amide-I and -III bands after treatment with quercetin, which is consistent with previous studies, suggests crosslinking [71,72] probably through the formation of a hydrogen bond between phenolic hydroxyl groups and the amino side chain [53,73]. It has been showed that quercetin has a unique polyphenol structure, particularly the phenolic hydroxyl groups, which can form stable hydrogen bonds with the side chains of hydroxyl, carboxyl-amine or amide groups of collagen molecules [74,75].

The ninhydrin assay was used to detect the crosslinking degree. According to the present results, the crosslinking degrees exhibited a concentration-dependent increase. Q300 showed the highest degree of crosslinking. Cross-linking could alter the mechanical and physicochemical properties of dentin collagen. It has been shown that the degree of collagen crosslinking is significantly associated with tensile strength. A higher tensile strength mainly indicates a higher level of crosslinking in biological matrices [76]. In the current work, therefore, the feasibility of applying quercetin to enhance the mechanical properties of DOM was explored via tensile strength. According to the present results, treatment with quercetin resulted in increased μUTS of the DOM, and the enhancement effect was concentration dependent. The

crosslinking ability of quercetin, at least to some extent, explains why quercetin has a relatively lower or similar dentin-derived MMP inhibition ability than CHX; however, quercetin led to a significantly lower dentin loss after erosive/abrasive challenge than CHX.

This study has several limitations. First, the penetration depth of quercetin was evaluated preliminarily in a semiquantitative manner, and quantitative information between Raman peak intensity and quercetin concentration requires evaluation in future studies. In addition, influencing factors such as treatment time and concentration of quercetin should be considered in future studies. Second, the clinical potential of quercetin is limited by its poor solubility in water, but this feature also indicates that saliva does not efficiently wash away quercetin in a complex oral environment [77]. Further research is also needed to develop appropriate vehicles for quercetin.

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Table 1 Means and standard deviations of the dentin loss (μm) for each group

Group	Dentin loss
Deionized water	1.83±0.42 [a]
Ethanol	1.84±0.53 [a]
CHX	1.24±0.36 [b]
Q75	0.94±0.38 [bc]
Q150	0.85±0.39 [c]
Q300	0.73±0.37 [c]

* Values marked with the same superscript letter were not significantly different ($P > 0.05$).

Figure 1. Average Raman spectra (5000-1800 cm^{-1}) of quercetin (red) and dentin before (*green*) and after (blue) Q300 treatment obtained with 785-nm excitation and baseline correction.

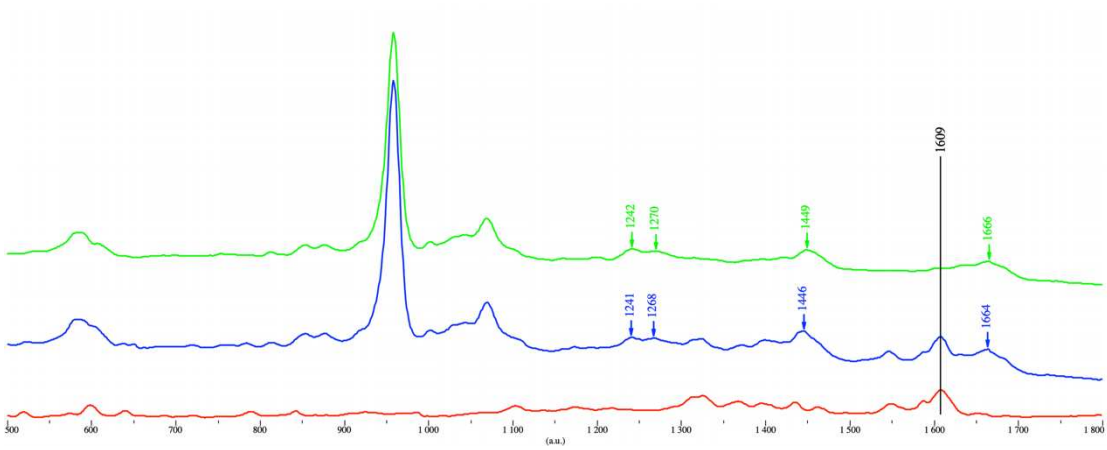
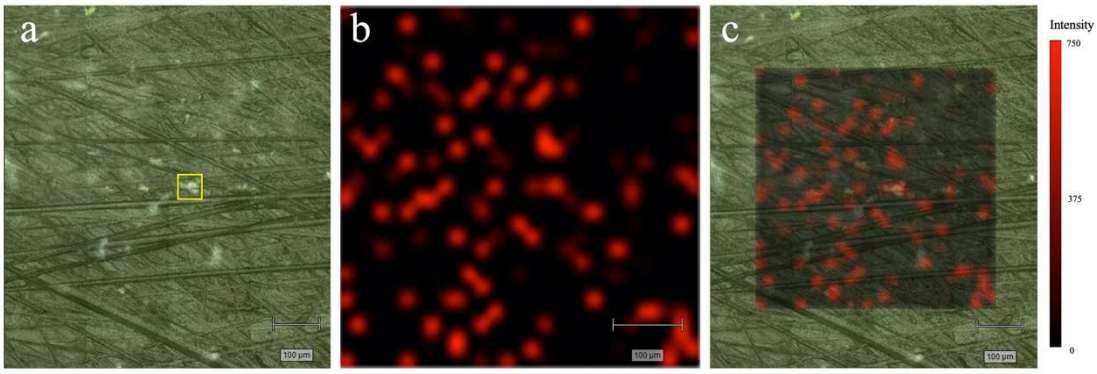


Figure 2. 2D images of quercetin-treated dentin were obtained with a step size of 2 μm in the x–y plane (400 μm × 400 μm, containing 40,000 points). The intensity of *vstr*C2=C3 was recorded at each image point (pixel). The different colours correspond to the *vstr*C_2=C_3 peak intensities (the bar on the right). (a) White light image; (b) 2D Raman image; (c) merged image. Yellow box: the area selected for 3D Raman volume imaging.



Yellow box: the area selected for 3D Raman volume imaging.

Figure 3.3D Raman maps of quercetin-treated dentin were obtained with a step size of 2 μm using the x–y–z stage (40 μm × 40 μm × 40 μm, containing 64,000 points). The intensity of *vstr*C2=C3 was recorded at each image point (pixel). The different colours correspond to the vstrC2=C3 peak intensities (the bar in the lower right corner). (a) XY slices from the different depths; (b) 3D reconstruction (XYZ = 40 × 40 × 40 μm^3) of the coloured volume.

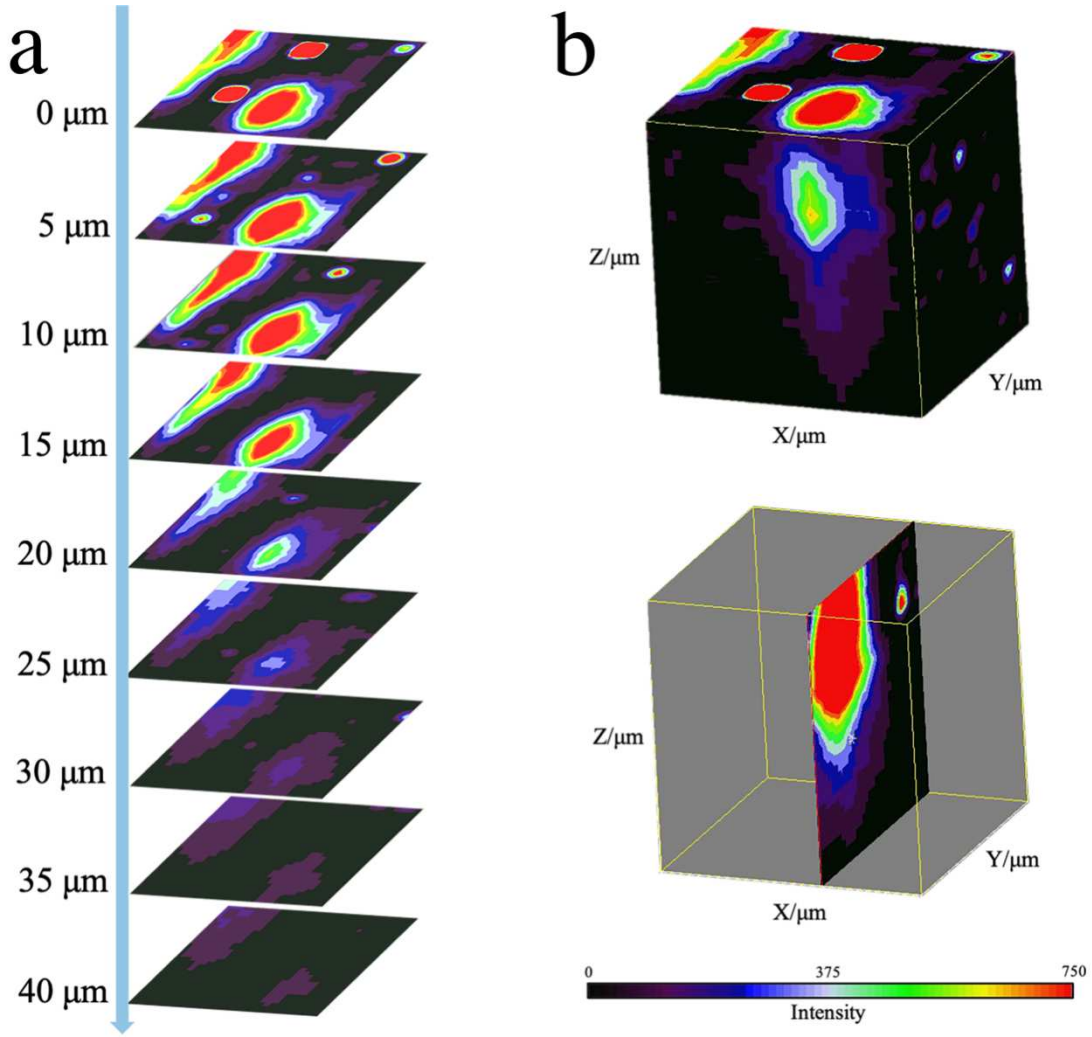


Figure 4. CLSM of the *in situ* zymography of the group with different pretreatment solutions after incubation for 24 h. Green fluorescence represents collagenolytic activity originating from quenched FITC-conjugated collagen breakdown by MMP. The figures of deionized water and ethanol represent the control groups and show the intense activity of MMP (green staining). Pretreatment with CHX, Q75, Q150 and Q300 resulted in lower fluorescence signals, indicating reduced endogenous enzymatic activity, especially in the CHX and Q300 groups.

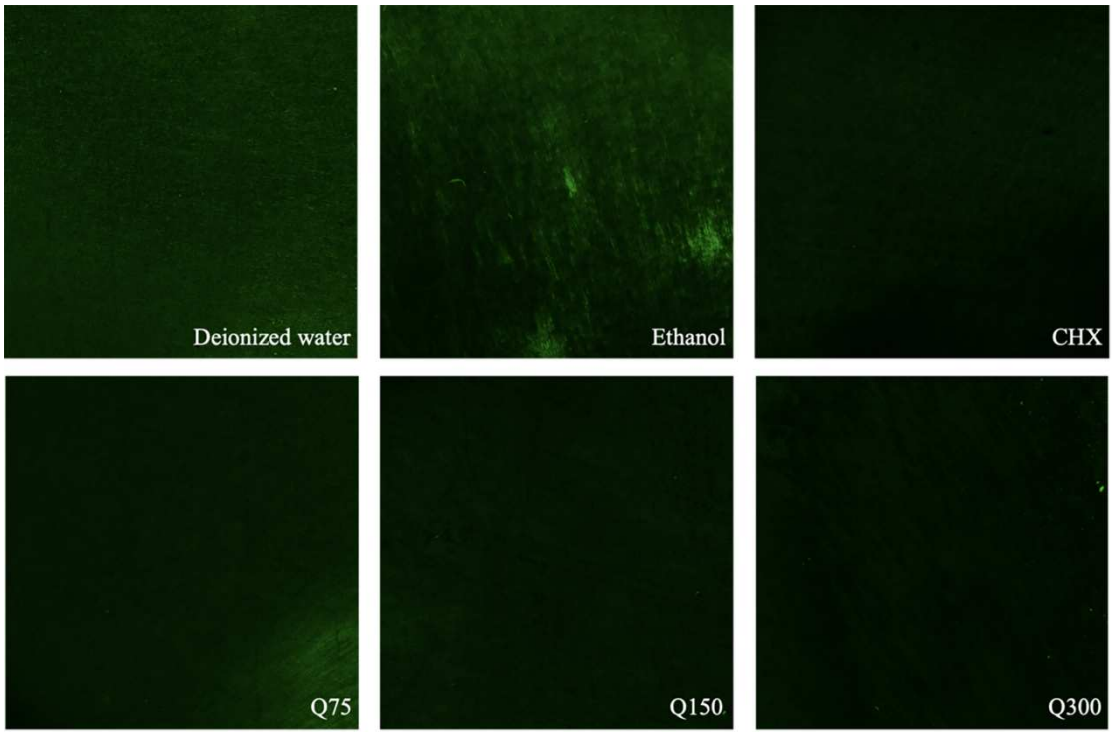
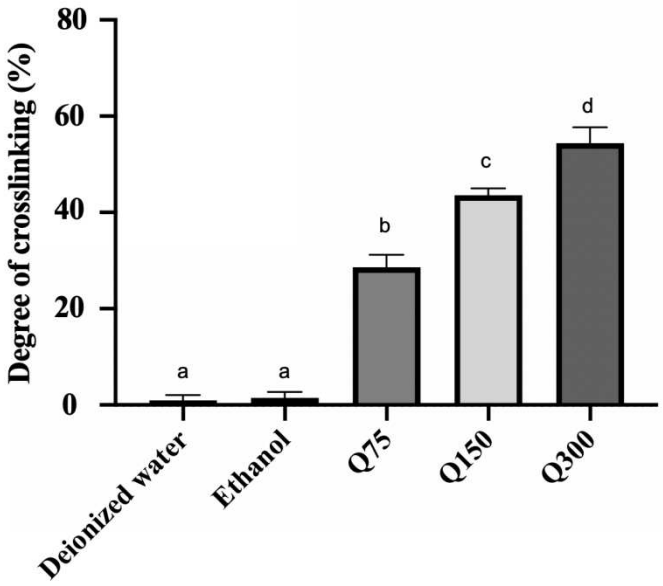


Figure 5. Means and standard deviations of the degree of crosslinking (%) for each group



* Values marked with the same superscript letter were not significantly different ($P > 0.05$).