

Evaluation of Remineralization of White Spot Lesions with Quercetin Nanoparticles

2021 Grants

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Evaluation of Remineralization of White Spot Lesions with Quercetin Nanoparticles

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Biomedical Research Award (BRA)

Period of AAOF Support

July 1, 2021 through June 30, 2023

Institution

Roseman University of Health Sciences

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Dr. Jian-Feng Zhang, Dr. Eduardo Gonçalves Mota

Amount of Funding

\$30,000.00

Abstract

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Respond to the following questions:

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Objectives: The objectives of this study were to synthesize Quercetin nanoparticles (QNPs) and evaluate the remineralization effect of QNPs on artificial white spot lesions (WSLs) when compared to Fluoride solution and Quercetin microparticle solution.

Methods: 76 extracted human molars were divided into 4 pH cycling groups of n=19/group: 1000ppm Fluoride solution as aqueous NaF (group 1), 6.5% w/v Quercetin microparticle solution (group 2), Quercetin Nanoparticle solution at 4000 ppm (group 3) and deionized water (group 4 - control). Teeth samples were prepared and mounted on acrylic blocks and immersed in a demineralizing solution for 4 days at a pH of 4.5, followed by pH cycling 6 times daily for 10 days. For each pH cycle, the specimen were immersed in their respective remineralizing solution (NaF, Quercetin, QNP and DI water) for 10 minutes, followed by immersion in demineralizing solution (pH 4.5). Vicker's microhardness test (VMT), Scanning Electron Microscopy-Energy Dispersive X-ray Spectroscopy (SEM-EDX) and Atomic Force Microscopy (AFM) analysis was conducted to analyze the samples for changes in surface microhardness (SMH), surface roughness average (Ra) and chemical composition [Calcium: Phosphorus (Ca:P) Ratio].

Results: As can be expected, all the groups showed a statistically significant reduction in SMH after demineralization of teeth. SMH measurement values were similar in all experimental groups before ($p=0.858$) as well as after demineralization ($p=0.885$). However, SMH values were statistically significantly different in all remineralization groups ($p<0.001$). Both fluoride treated and QNP treated groups showed significantly higher remineralization values than quercetin and control groups. However, the difference between these two groups (fluoride and QNP) was statistically not significant ($p=0.999$), indicating that these two groups were equally effective. Quercetin treated group showed higher remineralization than control but statistically significantly lower remineralization than other two experimental group ($p<0.001$ as compared all other groups). However, none of the demineralized teeth was able to achieve its baseline SMH level in any group. AFM measurement of surface roughness average (Ra) were similar in all four experimental groups. After remineralization, AFM values were statistically significantly lower in all three experimental groups as compared to control group ($p<0.001$ for all comparisons). In all the four groups, Ca:P values decreased after demineralization and increased after remineralization. However, even after remineralization, the values did not return to the baseline level for any tooth. The changes with demineralization-remineralization process were statistically significantly different only for QNP group, values after demineralization being significantly different from baseline as well as remineralization groups ($p<0.001$ for both comparisons). Ca:P values did not show statistically significant differences between experimental groups at baseline ($p=1.0$), and after demineralization ($p=1.0$). After remineralization values were similar in fluoride and QNP groups ($p=1.0$) suggesting similar improvements. Ca:P values of fluoride and QNP groups were statistically significantly higher than quercetin group ($p=0.007$ and <0.001 respectively) as well as deionized water group (<0.001 for both comparisons).

Conclusions: Within the limitations of this in vitro study, it can be concluded that QNP has higher remineralization potential when compared to Quercetin solution and similar to fluoride solution.

Were the original, specific aims of the proposal realized?*

Yes. The original, specific aims of the proposal were evaluated and within the limitations of this in vitro study, it can be concluded that QNPs have greater remineralization potential than the quercetin solution and are similar to the fluoride solution.

Were the results published?*

No

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To what extent have you used, or how do you intend to use, AAOF funding to further your career?*

AAOF funding has been crucial in undertaking this study and the results of this study will help secure future extramural funding for follow up studies.

Accounting: Were there any leftover funds?

\$7,409.88

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The manuscript has been submitted to Scientific Reports (Nature) journal and is currently under review process.

Comment: *The AAOF commends you on executing and completing this project and looks forward to seeing your contribution in the public domain of published research reports. We encourage you to continue your pursuit of knowledge and AAOF funding, which will advance our specialty.*

Presented

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The results from the study were presented by Dr. Karthikeyan Subramani at the 2024 IADR/AADOCR/CADR General Session [Poster Presentation (Poster #: 2895)], New Orleans LA on March 16, 2024.
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Was AAOF support acknowledged?

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Yes. The AAOF support was acknowledged in the IADR poster presentation and in the submitted manuscript.

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Evaluation of Remineralization of White Spot Lesions with Quercetin Nanoparticles: An *in vitro* Study

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Abstract

This study explored the synthesis of quercetin nanoparticles (QNP) and evaluated the remineralization effect of QNPs on artificial white spot lesions on extracted human teeth. QNPs were successfully synthesized, and their size was measured. Seventy-six extracted human molars were divided into 4 groups of n=19 to undergo a 10-day pH cycling protocol: 1000 ppm fluoride solution as aqueous NaF (group 1), 6.5% w/v quercetin microparticle solution (group 2), 4000 ppm QNP (group 3) and deionized water (group 4). Vickers microhardness tester, scanning electron microscopy-energy dispersive X-ray spectroscopy (SEM-EDX) and atomic force microscopy (AFM) were used to measure the surface microhardness (SMH), Ca:P ratio and surface roughness (Ra), respectively. After remineralization, the SMH values were significantly different among all the experimental groups ($p < 0.001$). The fluoride and QNP groups had significantly greater SMH values than did the quercetin group. The AFM data showed a significant decrease, but the differences were not significant. The Ca:P values were significantly greater than those of the control in all 3 experimental groups, but the QNP and fluoride concentrations were significantly greater than those of quercetin. There were no significant differences between QNPs and fluoride according to any test. It can be concluded from the results of this study that QNPs have similar remineralization potential to fluoride and are more effective than quercetin.

Keywords: White Spot Lesion, Remineralization, Quercetin, Nanoparticles, Orthodontic Treatment

Introduction

Patients undergoing orthodontic treatment tend to exhibit plaque buildup around brackets if oral hygiene is poor. Orthodontic elastomeric ligature ties and chains are more prone to dental plaque buildup. This often results in enamel demineralization and the development of white spot lesions (WSLs) on the enamel surface around brackets¹. WSLs are subsurface enamel porosities from carious demineralization that manifest clinically as a milky white opaque appearance of the enamel². The prevalence of WSLs in patients undergoing orthodontic treatment has been reported to be as high as 46%³. WSLs are caused by an imbalance between the dynamic biological processes of demineralization and remineralization of enamel. These two biological processes depend on various factors, such as calcium (Ca^{2+}), phosphate (PO_4^{3-}) and fluoride (F^-) ions in saliva and plaque and the buffering capacity of saliva and oral hygiene². Natural remineralization of the surface of WSLs from salivary ions [Ca^{2+} , (PO_4^{3-}), F^-] has very little effect on the esthetic appearance and structural properties of deeper WSLs. Current treatment options for WSLs include resin infiltration, microabrasion, vital bleaching, direct/indirect restorations, and the use of remineralizing agents^{4,5}. To aid in the remineralization of deeper WSLs, remineralizing agents such as casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) (MI Paste®)⁶ and fluoride supplements in the form of mouth rinses, gels or topical creams have been used^{7,8}. However, these remineralizing agents have certain limitations. Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) is associated with the milk-derived protein RECALDENT™, which is the active ingredient in MI Paste®. An 11-year-old girl with a milk allergy from California, USA, died due to an adverse allergic reaction to RECALDENT™ after her Dentist prescribed MI

paste for WSL treatment⁹. Fluoride treatment after the formation of WSLs can result in the formation of harder fluorapatite crystals on the surface, leaving milky white discoloration to persist in the deeper layers of enamel. It was also reported that MI paste and fluoride varnish do not appear to be more effective than regular home care (brushing with fluoride toothpaste and flossing) for improving the appearance of WSLs¹⁰. With these limitations of currently available remineralizing agents, newer biocompatible materials need to be explored for the treatment and remineralization of WSLs.

Various biomolecules, including proteins, peptides, nucleic acids, liposomes, carbohydrates and phosphorus-containing biomolecules, have been explored as biotemplates for biomimetic mineralization and for hydroxyapatite crystal formation from calcium and phosphate ions¹¹. The use of phytochemicals (plant chemicals) isolated from dietary plants could balance the oral flora, primarily *Streptococcus mutans*, which metabolizes sucrose to lactic acid that dissolves the hydroxyapatite crystals in enamel, causing demineralization, WSLs and eventual tooth decay¹². The investigation of phytochemicals for the remineralization of WSLs could lead to the development of novel approaches for safer and more natural alternatives to currently available remineralizing agents. Flavonoids are phytochemicals and plant pigments found in almost all fruits, vegetables, and beverages, such as tea and wine^{13,14}. Flavonoids are polyphenolic compounds that are composed of multiple phenol rings with hydroxyl groups (OH) attached to the phenol rings¹⁵. Quercetin is a flavonoid found in red wine, onions, green tea, apples, grapes, citrus fruits and berries; is safer for human use; and has antioxidant, antibacterial, anticancer, anti-inflammatory and cardio- and neuro-protective properties^{13,14,16,17}. Apart from these beneficial bioactive properties, quercetin can also

interact with proteins such as collagen¹⁸ and has been shown to increase bone mass and density¹⁹. The chemical structure of quercetin is shown in Figure 1²⁰. An earlier *in vitro* study showed that quercetin was effective at inhibiting demineralization and enhancing remineralization of artificial root caries lesions²¹. In this study, three flavonoids (6.5% quercetin, 6.5% naringin and 6.5% proanthocyanidin) were tested in solution, and the remineralization effect was compared with that of a 1000 ppm fluoride solution. These three flavonoids had positive effects on artificial root caries remineralization, but the effect was less pronounced than that of 1000 ppm fluoride. As flavonoid microparticles (10^{-6} m) in solution form were used in this study on the dentine surface, the results of such a study need to be re-evaluated to explore whether the nanoparticle (10^{-9} m) form of quercetin has superior remineralization potential. As the nanoparticles are much smaller than the particles at the microscale, there is a possibility of a greater density of deposited nanoparticles in the deeper layers of WSLs.

Quercetin nanoparticles (QNPs) can be prepared from a quercetin solution by anti-solvent precipitation under sonication.²² Due to the limitations of currently available remineralizing agents, evaluating the remineralization potential of naturally occurring bioactive compounds, such as quercetin, in nanoparticulate form is essential. Therefore, this *in vitro* study aimed to evaluate the remineralization potential of QNPs compared to that of fluoride and quercetin microparticle solutions. The null hypothesis tested in this study was that there was no significant difference between the effects of QNPs, quercetin microparticles and NaF solutions on the remineralization of WSLs.

Methods

Specimen preparation and baseline surface microhardness evaluation

All chemical reagents used in this study were purchased from Sigma–Aldrich (St. Louis, MO, USA). The Institutional Review Board (IRB) approved the tooth collection, and the study was approved by the Roseman University IRB and all methods was performed in accordance with relevant guidelines/regulations. Seventy-six extracted permanent human molars which were originally extracted for clinical purposes were collected. Since the patients needed these teeth extracted for clinical purposes, no other patient identifiers were collected/kept along with the teeth. The need for informed consent for collection of teeth was waived by the Roseman University IRB. The collected teeth were stored in 0.1% thymol solution. All the collected teeth were examined under 4.5x magnification (Carson Optical, Ronkonkoma, NY, USA), and the teeth with any hypoplastic lesions, caries, cracks, WSLs, or stains were discarded. All soft and hard tissue debris was removed from the selected teeth and stored in 0.1% thymol solution for further use. Teeth were sectioned 1 mm below the cemento-enamel junction with a slow-speed diamond disc (Kerr Dental, Brea, CA, USA) under running deionized water, and the roots were discarded. 3D-printed cylindrical resin molds were fabricated with a 3D printer (Stratasys, Tucson, AZ, USA), and the teeth were placed in molds with their buccal surface adhered to double-sided adhesive tape on a workbench to ensure that the buccal surfaces remained exposed and parallel to the horizontal plane of the molds. Polymethyl methacrylate resin (Miami Dental Supply, Miami, FL, USA) was poured into the molds,

which were subsequently cured to provide a stable mount. The exposed buccal surfaces were sequentially polished using progressive grit polishing discs (Kerr Dental, Brea, CA, USA) to ensure that the smooth enamel surface was free of microimperfections. A 5 mm × 5 mm window of exposed enamel was created in the middle of the buccal surface by covering the rest of the buccal surface with acid-resistant nail varnish (OPI, New York, NY, USA).

Forty tooth samples were used for surface microhardness measurements, and 36 samples were subjected to atomic force microscopy (AFM) to measure the surface roughness average (Ra) and to scanning electron microscopy (SEM) to analyze the chemical composition (calcium:phosphorus ratio) of the enamel surface. Before artificial WSL formation, the baseline enamel surface microhardness was measured on 40 tooth samples using a Vickers microhardness tester (Metal Testers, New York, NY, USA). Indentations were made at 3 points spaced 500 μm from each other at the center of the exposed window using a 100 g load for 10 seconds. The measurements were averaged, and samples with a Vickers microhardness > 430 or < 340 were excluded from the study.

Synthesis of quercetin nanoparticles and remineralizing solutions

QNPs were synthesized *in vitro* by the antisolvent precipitation method²². Absolute ethyl alcohol against water was used as the solvent and antisolvent at a 1:20 ratio. QNPs were then synthesized by dissolving 100 mg of quercetin in 5 mL of absolute ethyl alcohol. The resulting solution was added to 100 mL of 0.15% (w/v) aqueous solution containing 4:1 w/w hydroxypropyl methylcellulose (HPMC) and sodium lauryl sulfate (SLS). This solution was cooled to 8°C in an ice-water bath and sonicated. The precipitation rate was controlled throughout the process by maintaining the temperature below 8°C using an

ice-water bath. The particle size was reduced with an ultrasonic probe sonicator (Benchmark Scientific, Sayreville, NJ, USA) at an ultrasonic power input of 300 W for 10 min. The suspension was placed in an ROTA evaporator (Heidolph, Wood Dale, IL, USA) at 40°C for 10 min to remove the organic solvent. The QNP suspensions were further homogenized for 30 min to obtain the final preparation. The homogenized suspension was evaluated for particle size distribution with a nanoparticle size analyzer (Zetasizer Nano, Malvern Panalytical, Malvern, UK) and further confirmed using a scanning electron microscope (SEM; JSM-6700F, JEOL Ltd., Tokyo, Japan). A 6.5% (w/v) solution of quercetin in phosphate buffer (0.025 M KH₂PO₄, 0.025 M K₂HPO₄, pH 7.4) was also used in this study to evaluate whether there was a difference between the remineralization potential of the quercetin nanoparticles and the quercetin solution. Sodium fluoride (NaF) solution (1000 ppm) was used as a positive control, and deionized water was used as a negative control. The test groups were designated as follows.

Group 1 – Sodium fluoride (NaF) solution

Group 2 – Quercetin microparticle solution

Group 3 – Quercetin microparticle (QNP) solution

Group 4 - Deionized water

Artificial White Spot Lesion Formation

Artificial WSLs were created by immersing the selected tooth samples in a demineralizing solution (50 mM acetic acid, 10 mM NaH₂PO₄–2H₂O, 2.2 mM CaCl₂–2H₂O, 100 mM NaCl, 1 ppm NaF, 5 mM NaN₃), and the pH was adjusted to 4.5 using 1 M NaOH solution at 37°C for 4 days under continuous, low-speed magnetic stirring (100

rpm) (Benchmark Scientific, Sayreville, NJ, USA).^{23,24} Thereafter, the samples were rinsed with deionized water spray for 15 s followed by ultrasonication in deionized water 3 times (5 min per wash) to terminate demineralization. Following demineralization, surface microhardness measurements were taken on 40 tooth samples to obtain postdemineralization data using the protocol described previously.

***In vitro* remineralization of WSLs**

The tooth samples were then subjected to 10 days of pH cycling for *in vitro* remineralization of artificial WSLs. All the solutions were mixed at 37°C to simulate the oral environment. The plants in each group were pH cycled through the respective treatment solution for 10 minutes, followed by demineralization with acidic buffer for 30 minutes (50 mM acetic acid, 10 mM NaH₂PO₄–2H₂O, 2.2 mM CaCl₂–2H₂O, 100 mM NaCl, 1 ppm NaF, 5 mM NaN₃, pH 4.5) and remineralization with neutral buffer for 10 minutes (20 mM HEPES; 2.25 mM CaCl₂.2H₂O; 1.35 mM KH₂PO₄; 130 mM KCl, pH 7.0).²¹ When the solutions were switched between, the samples were copiously irrigated for 15 seconds using deionized water. The pH of all the solutions was checked daily, and the solutions were confirmed to be stable. Six separate demineralization–remineralization cycles were performed each day for 10 days. All the samples were stored overnight in neutral buffer at 37°C.

Calcium: Phosphorus Ratio Analysis and Surface Roughness Evaluation

Atomic force microscopy (AFM) was used to measure the surface roughness average (Ra), and scanning electron microscopy (SEM)-energy dispersive X-ray

spectroscopy (SEM-EDX) was utilized for chemical composition analysis (calcium:phosphorus ratio) of 36 samples (12 samples before demineralization, 12 samples after demineralization and 12 samples after remineralization). AFM analysis was first performed, followed by SEM-EDX analysis, on the same samples.

For AFM analysis, images were acquired using an XE-70 PSIA AFM (Park Systems, South Korea) in noncontact mode using PPP-NCHR AFM probes (NANOSENSORS, Switzerland) at multiple locations with scan sizes of 5x5, 10x10, 20x20, 40x40, 60x60, 80x80 and 100x100 μm^2 , of which the 60x60 μm^2 images were used for further analysis. Line profiles of pit features and their surrounding areas were extracted and analyzed using Park Systems XEI Imaging and OriginLab Data Analysis Software.

For SEM-EDX analysis, the enamel specimens were cut with low-speed diamond discs (Buehler, U.S.A.) and refined with carbide burs (Komet, U.S.A.) to 4 mm \times 3 mm \times 1 mm pieces. The specimens were then gold-coated with a Cressington Sputter Coater 108 Auto (Cressington Scientific Instruments, United Kingdom) to alleviate charging effects. SEM-EDX was carried out using a JEOL JSM-5600 Scanning Electron Microscope (JEOL, U.S.A.) at 15 kV (aperture 2, spot size 30, working distance 15 mm). Elemental X-ray maps were collected in a 256 \times 256 pixel matrix with 200 scan frames using the INCA Microanalysis Suite (Oxford Instruments, United Kingdom). A 1 mm \times 1 mm area analysis was performed on each specimen, and the calcium-to-phosphorus (Ca:P) ratio was calculated. Due to the gold coating, we could not evaluate each specimen longitudinally. Thus, specimens were taken from multiple teeth to allow for a better determination of the Ca:P ratio.

Evaluation of remineralization and Statistical analysis

The baseline, postdemineralization, and postremineralization data were analyzed with a predetermined significance level of 0.05 with one-way ANOVA and a Tukey HSD post hoc test using SPSS version 29.0 to analyze the changes in surface microhardness (SMH), surface roughness average (Ra) and chemical composition [calcium:phosphorus (Ca:P) ratio].

Results

Quercetin nanoparticle analysis

The average size of the QNPs in suspension was measured with a Zetasizer Nanoparticle Analyzer and was 678.9 nm (Figure 2). Further SEM analysis of the QNPs revealed that the QNPs were rod shaped with lengths ranging from approximately 500 nm to 700 nm and widths of approximately 162 nm (Figs. 3a-d). The quercetin microparticles were approximately 5 µm to 50 µm in length and 5 µm to 10 µm in width (Figs. 3e, f).

Evaluation of the *Surface Microhardness*

Table 1 shows the mean \pm SD for microhardness measured at baseline, after demineralization (WSL formation) and after remineralization. The baseline microhardness [Vickers hardness number (VHN)] of group 1 was 322.39 ± 6.42 , that of group 2 was

321.38 ± 3.54, that of group 3 was 321.04 ± 4.73, and that of group 4 was 322.50 ± 2.74. The microhardness after demineralization was 157.42 ± 6.14 in group 1, 158.11 ± 5.52 in group 2, 157.98 ± 5.89 in group 3 and 159.33 ± 4.2 in group 4. The microhardness after remineralization was 242.85 ± 10.63 in group 1, 216.50 ± 8.20 in group 2, 242.40 ± 7.17 in group 3 and 163.56 ± 4.6 in group 4.

Calcium: Phosphorus Ratio Analysis and Surface Roughness Evaluation

SEM-EDX elemental analysis of the Ca:P ratio (mean ± SD) at baseline, after demineralization and remineralization is shown in Table 2. The baseline Ca:P ratio of group 1 was 2.08 ± 0.12, that of group 2 was 2.08 ± 0.1, that of group 3 was 2.08 ± 0.02, and that of group 4 was 2.08 ± 0.05. The Ca:P ratio after demineralization in group 1 was 1.35 ± 0.86, that in group 2 was 1.36 ± 0.75, that in group 3 was 1.35 ± 0.1, and that in group 4 was 1.35 ± 0.79. The Ca:P ratio after remineralization in group 1 was 1.95 ± 0.01, that in group 2 was 1.56 ± 0.04, that in group 3 was 1.96 ± 0.02, and that in group 4 was 1.38 ± 0.2.

SEM image analysis revealed a smoother enamel surface at baseline (Fig. 4a) and a rougher enamel surface after demineralization with enamel rods exposed (Fig. 4b). SEM images of the particle deposition after 1 round of exposure to fluoride solution (Fig. 4c) and quercetin solution revealed inhomogeneous particle deposition (Fig. 4d). SEM images of the samples analyzed after 1 round of exposure to the QNP solution revealed QNP deposition on the enamel surface (Fig. 4e) and no particle deposition after 1 round of exposure to deionized water (Fig. 4f). SEM images after remineralization with (g) fluoride solution (Fig. 4g) and QNP solution (Fig. 4i) showed uniform remineralized enamel surfaces. SEM images after remineralization with quercetin solution showed an

inhomogeneous remineralized enamel surface (Fig. 4h), and those with deionized water had a rougher enamel surface with a demineralized appearance (Fig. 4j).

The AFM results of the surface roughness average (Ra) in nm (mean \pm SD) measured at baseline, after demineralization and after remineralization are shown in Table 3. The baseline Ra of group 1 was 24.3 ± 4.41 , that of group 2 was 24.63 ± 2.95 , that of group 3 was 24.83 ± 2.77 , and that of group 4 was 24.73 ± 2.13 . The Ra after demineralization in group 1 was 439.87 ± 21.45 , that in group 2 was 439.53 ± 13.96 , and that in group 3 was 439.7 ± 14.92 , and that of group 4 was 439.67 ± 16.95 . The enamel surface was roughest after demineralization (Fig. 5a,b) compared to the baseline surface, which was smoother (Fig. 5c,d). The Ra after remineralization was 269.83 ± 16.54 in group 1, 268.73 ± 11.37 in group 2, 270.53 ± 4.07 in group 3 and 398.6 ± 19.03 in group 4. The AFM images of the enamel surface after remineralization with fluoride (Fig. 5e,f) and QNPs (Fig. 5i,j) revealed a slightly rougher surface, followed by those obtained with quercetin (Fig. 5g,h) and deionized water (Fig. 5k,l).

Discussion

As expected, all the groups exhibited a statistically significant reduction in microhardness after demineralization (Table 1). The microhardness levels were similar in all the experimental groups before ($p=0.858$) and after demineralization ($p=0.885$). However, the microhardness was significantly different among the groups after remineralization ($p<0.001$). The control group (treated with deionized water) exhibited a very small increase in microhardness from demineralized teeth compared to that of all the other

three experimental groups, which was significantly lower than that of all the other groups ($p < 0.001$ from all three experimental groups). The quercetin-treated group exhibited greater remineralization than did the control group but significantly less remineralization than did the other two experimental groups did ($p < 0.001$ compared with all the other groups). The remineralization of the fluoride-treated and QNP-treated groups was significantly greater than that of the quercetin-treated and control groups. However, the difference between these two groups (fluoride and QNP) was not statistically significant ($p = 0.999$), indicating that these two groups were equally effective. However, none of the demineralized teeth were able to achieve their baseline microhardness level in any group.

In all four groups, the SEM-EDX measurements of the Ca:P ratio decreased after demineralization and increased after remineralization. However, even after remineralization, the value did not return to the baseline level for any tooth. The changes associated with the demineralization-remineralization process were significantly different only for the QNP group (Table 2), and the values after demineralization were significantly different from those at baseline and for the remineralization groups ($p < 0.001$ for both comparisons). The change in Ca:P ratios did not significantly differ between the experimental groups at baseline ($p = 1.0$) or after demineralization ($p = 1.0$). After remineralization, the values were similar in the fluoride and QNP groups ($p = 1.0$), suggesting similar improvements. The values in the fluoride and QNP groups were significantly greater than those in the quercetin group ($p = 0.007$ and < 0.001 , respectively) and the deionized water group (< 0.001 for both comparisons).

The AFM measurements of Ra were similar in all four experimental groups at baseline ($p = 0.997$) and after demineralization ($p = 1.0$). After remineralization, the AFM values were

significantly lower in all three experimental groups than in the control group ($p < 0.001$ for all comparisons). An earlier *in vitro* study evaluated the remineralization of artificial root caries on dentin specimens and used three flavonoids in solution form²¹. This study revealed that flavonoids (6.5% quercetin, 6.5% naringin and 6.5% proanthocyanidin) had positive effects on artificial root caries remineralization but had weaker effects than 1000 ppm fluoride. Our study focused on fabricating QNPs and evaluating their remineralization effects compared to those of 6.5% quercetin and 1000 ppm fluoride.

Our results demonstrated that QNPs can be generated from quercetin microparticles, and the SMH and SEM-EDX analyses showed that the QNPs had greater remineralization potential than the quercetin microparticle solution and that the remineralization efficiency of the QNPs was comparable to that of fluoride. One possible explanation for the superior remineralization potential of QNPs compared to microparticles may be their smaller size. Smaller particles can penetrate deeper into the enamel at higher concentrations and act as nucleation sites, providing more effective mineral deposition. Quercetin, a polyphenol, is composed of multiple phenol rings with hydroxyl groups. Hydroxyl groups play a crucial role during remineralization and in the formation of hydroxyapatite.

The results of our *in vitro* study have certain limitations. First, *in vitro* studies lack the complexity and diversity of oral biofilms that exist *in vivo*. Extracted teeth lack blood supply and are more prone to dehydration, which may influence the outcomes of such *in vitro* studies. The present study was limited by the use of VMT, SEM-EDX, and AFM to quantify remineralization. Additional studies could use additional complementary techniques, such as micro-CT, to confirm the findings of this study.

Conclusions

Within the limitations of this *in vitro* study, it can be concluded that QNPs have greater remineralization potential than does the quercetin solution and are similar to the fluoride solution.

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Figure legends

Figure 1. Chemical structure of Quercetin²⁰

Figure 2. Quercetin nanoparticle (QNP) size analysis was performed with a Zetasizer nanoparticle analyzer, which revealed that the average size of the QNPs was 678.9 nm.

Figure 3. Scanning electron microscopy image of QNPs spread on a glass coverslip (a) at 40,000x magnification, (b) at 55,000x magnification, (c) at 100,000x magnification, (d) at 50,000x magnification, (e) at 5000x magnification and (f) at 2,000x magnification.

Figure 4. SEM images of (a) the enamel surface at baseline and (b) after demineralization. SEM images of particle deposition after 1 round of exposure to (c) fluoride solution, (d) quercetin solution, (e) QNP solution, and (f) deionized water; SEM images after remineralization with (g) fluoride solution, (h) quercetin solution, (i) QNP solution and (j) deionized water.

Figure 5. (a, b) 2D and 3D AFM images of the enamel surface at baseline; (c, d) demineralized enamel; 2D and 3D AFM images of the remineralized enamel surface treated with (e, f) fluoride solution, (g, h) quercetin solution, (i, j) QNP solution and (k, l) deionized water.

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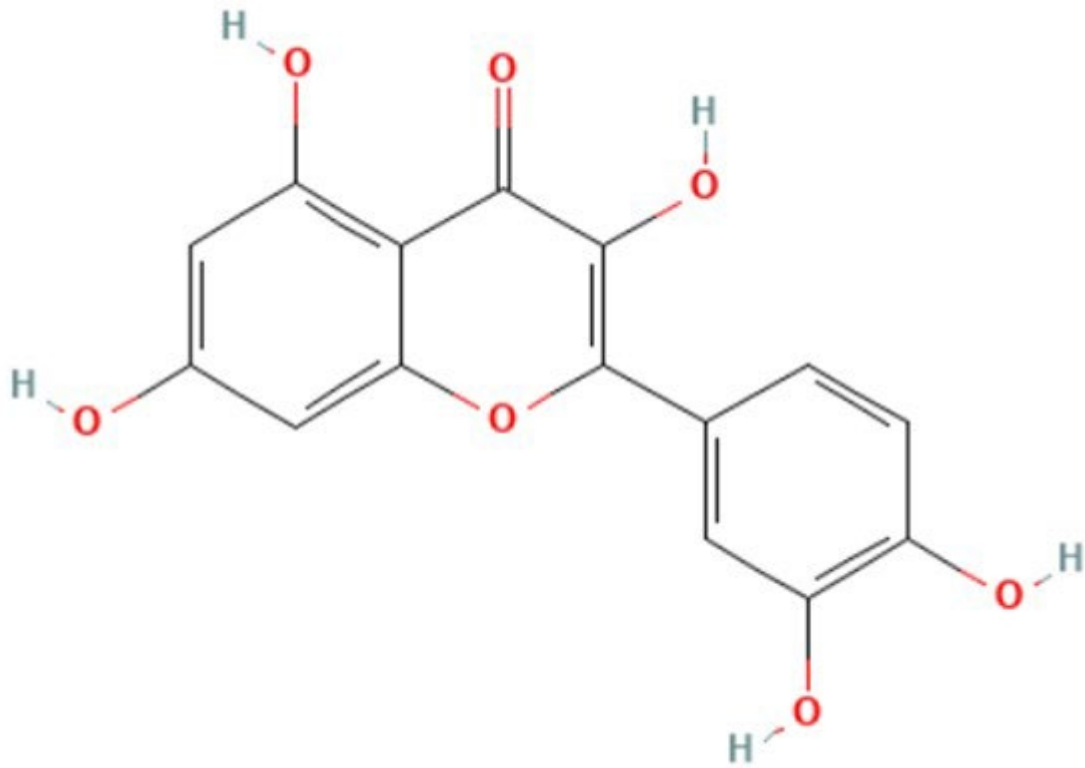


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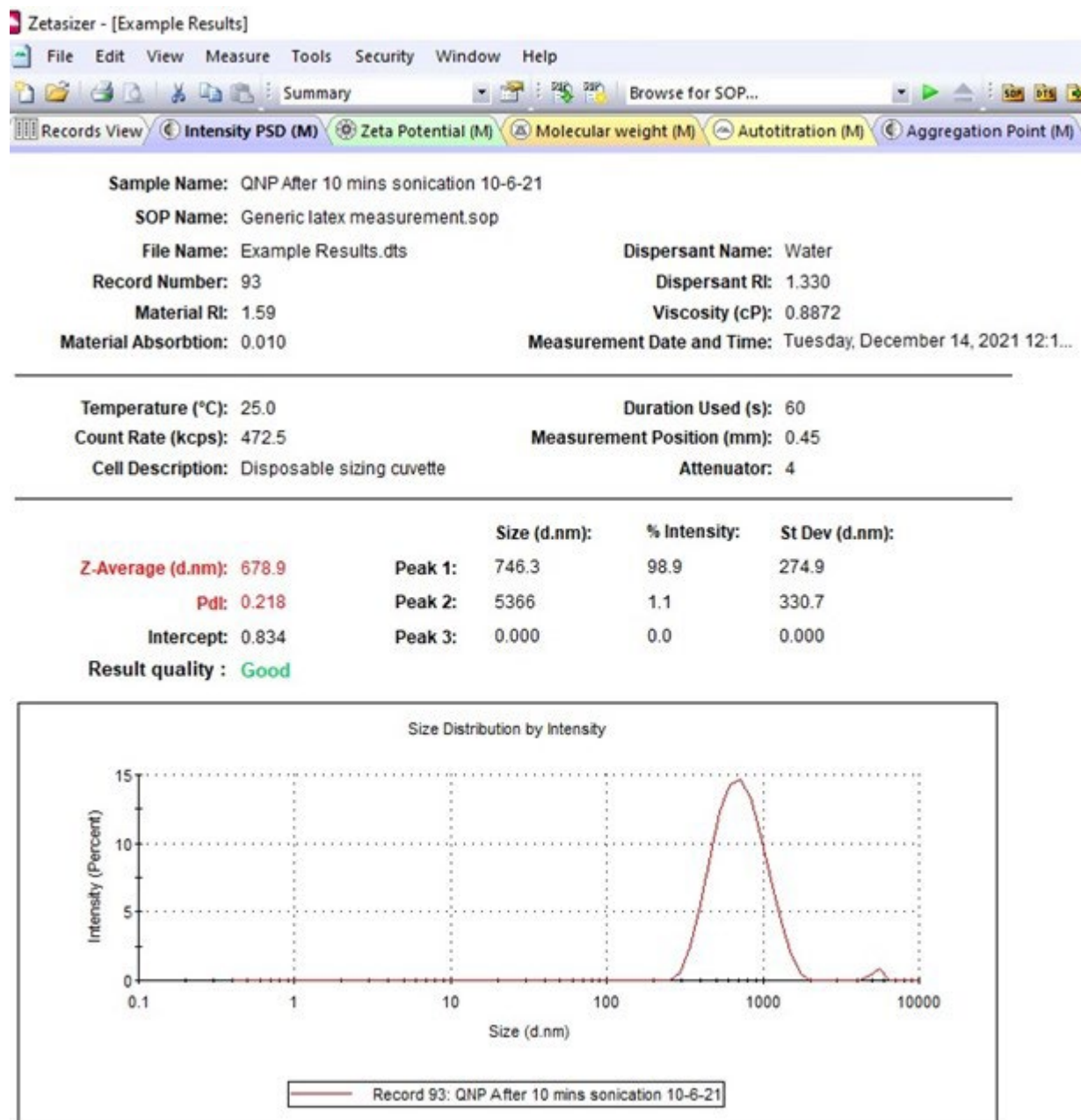


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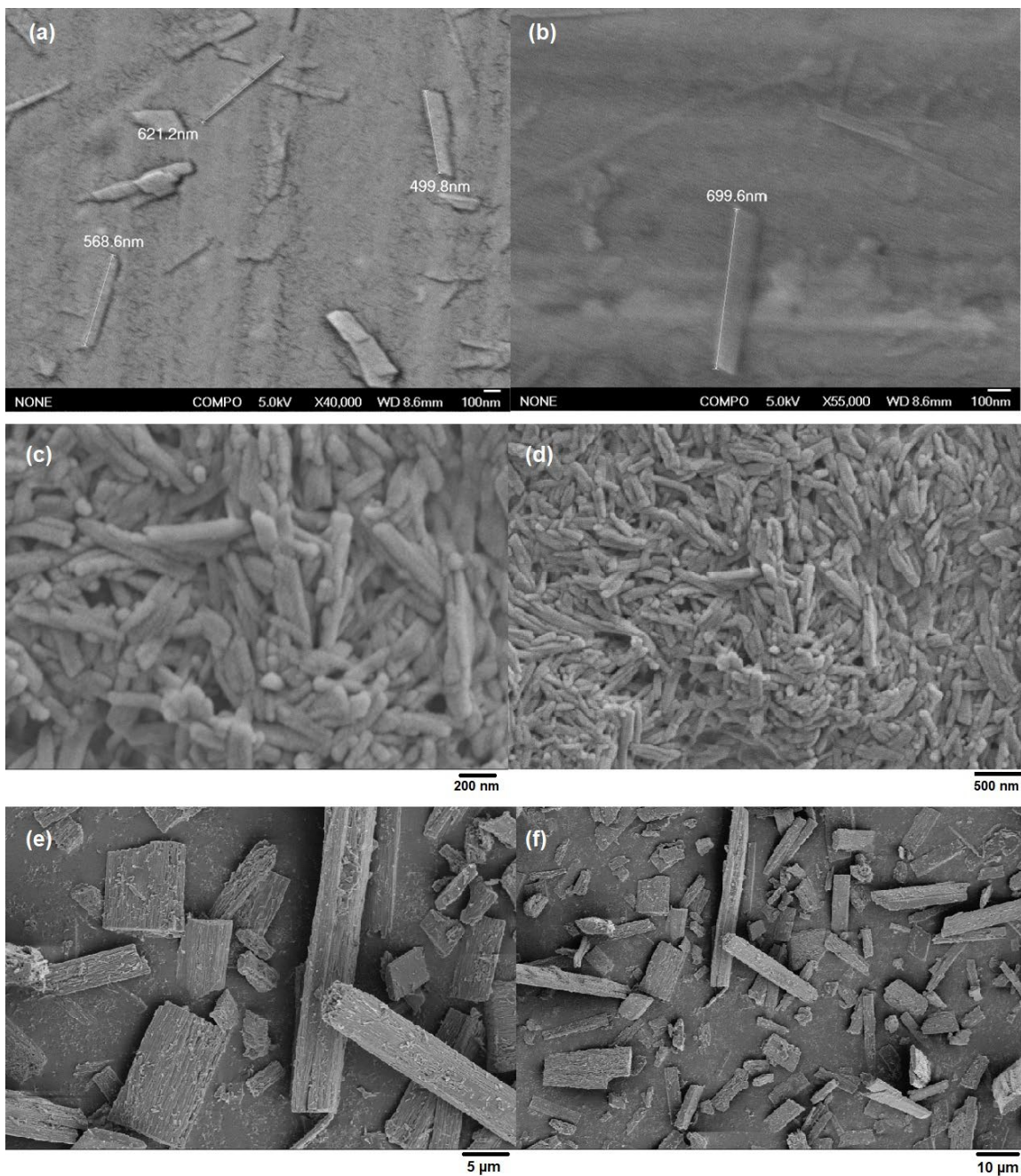


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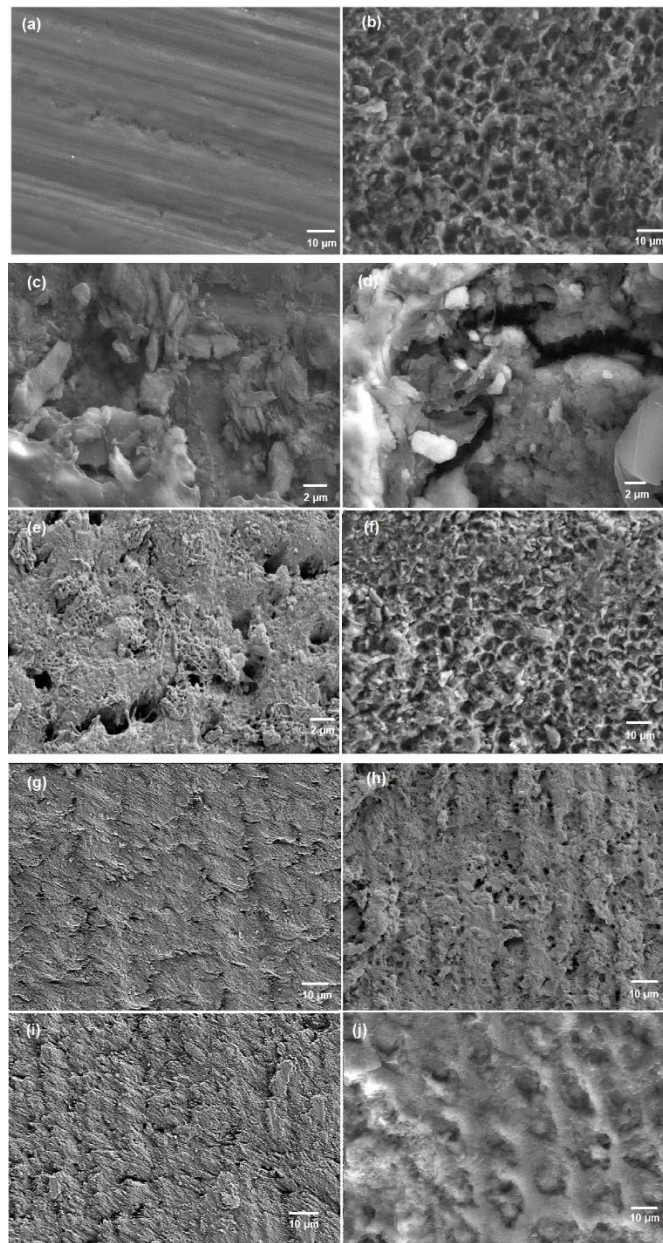


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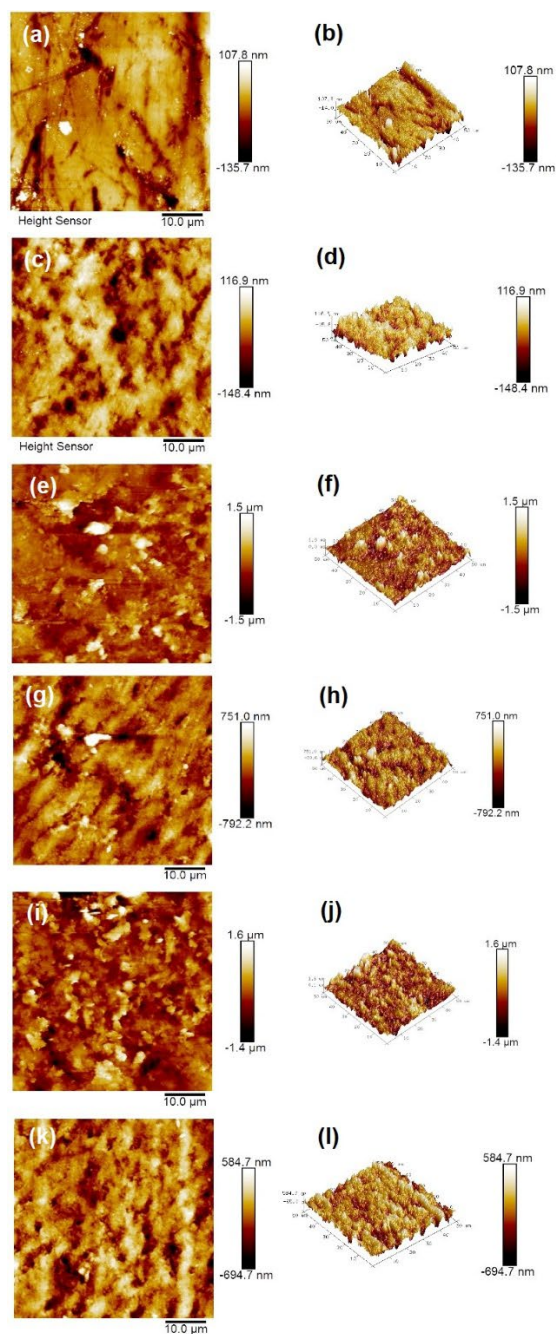


Table 1. Surface microhardness measurements (n=10/group) at baseline, after demineralization (WSL formation) and after remineralization are shown as the Vickers hardness number (VHN) (mean, SD). The measurements are an average of three indentations spaced 500 µm apart from each other in each enamel sample.

Groups	Mean	Std. Deviation	P (t test)		
			B* Vs D*	D* Vs R*	B* Vs R*
Fluoride					
Baseline	322.39	6.42	<0.001		
After Demineralization	157.42	6.14		<0.001	<0.001
After Remineralization	242.85	10.63			
	P (ANOVA) <0.001				
Quercetin					
Baseline	321.38	3.54	<0.001		
After Demineralization	158.11	5.52		<0.001	<0.001
After Remineralization	216.50	8.20			
	P (ANOVA) <0.001				
Quercetin Nanoparticles					
Baseline	321.04	4.73	<0.001		
After Demineralization	157.98	5.89		<0.001	<0.001
After Remineralization	242.40	7.17			
	P (ANOVA) <0.001				
Deionized Water					
Baseline	322.50	2.74	<0.001		
After Demineralization	159.33	4.20		0.058	<0.001
After Remineralization	163.56	4.60			
	P (ANOVA) <0.001				
* B= Baseline; D=Demineralized Teeth; R=Remineralized Teeth.					

Table 2. SEM-EDX measurements of the calcium-to-phosphorus (Ca:P) ratio (mean, SD) of the enamel surface were performed on 36 samples (n=3/group) at baseline, after demineralization (WSL formation) and after remineralization.

Groups	Mean	Std. Deviation	P (t test)		
			B* Vs D*	D* Vs R*	B* Vs R*
Fluoride					
Baseline	2.08	0.12	N/A		N/A
After Demineralization	1.35	0.86		N/A	
After Remineralization	1.95	0.01		N/A	
P (ANOVA) =0.243					
Quercetin					
Baseline	2.08	0.10	N/A		N/A
After Demineralization	1.36	0.75		N/A	
After Remineralization	1.56	0.04		N/A	
P (ANOVA) =0.192					
Quercetin Nanoparticles					
Baseline	2.08	0.02	<0.001		0.096
After Demineralization	1.35	0.10		<0.001	
After Remineralization	1.96	0.02		<0.001	
P (ANOVA) <0.001					
Deionized Water					
Baseline	2.08	0.05	N/A		N/A
After Demineralization	1.35	0.79		N/A	
After Remineralization	1.38	0.20		N/A	
P (ANOVA) =0.175					
* B= Baseline; D=Demineralized Teeth; R=Remineralized Teeth.					

Table 3. AFM measurement of the surface roughness average (Ra) in nm (mean, SD) measured in a 50x50 µm area of the enamel surface on 36 samples (n=3/group)

Groups	Mean	Std. Deviation	P (t test)		
			B* Vs D*	D* Vs R*	B* Vs R*
Fluoride					
Baseline	24.30	4.41	<0.001		
After Demineralization	439.87	21.45		<0.001	<0.001
After Remineralization	269.83	16.54			
P (ANOVA) <0.001					
Quercetin					
Baseline	24.63	2.95	<0.001		
After Demineralization	439.53	13.96		<0.001	<0.001
After Remineralization	268.73	11.37			
P (ANOVA) <0.001					
Quercetin Nanoparticles					
Baseline	24.83	2.77	<0.001		
After Demineralization	439.70	14.92		<0.001	<0.001
After Remineralization	270.53	4.07			
P (ANOVA) <0.001					
Deionized Water					
Baseline	24.73	2.1362	<0.001		
After Demineralization	439.67	16.9542		0.032	<0.001
After Remineralization	398.60	19.0337			
P (ANOVA) <0.001					
* B= Baseline; D=Demineralized Teeth; R=Remineralized Teeth.					