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ORIGINAL ARTICLE

Efficacy of theaflavin-based dental varnishes against erosive demineralizatıon and matrix metalloproteinase expression

G. Ozan[1] · H. Sar Sancakli[1] · S. Batu[2] · A. Gurbuz Yurtsever[3] · M. S. Erdal[3] · A. Sengelen[4] · E. Onay Ucar[4] · S. Ozel Yildiz[5] · B. Bicak[6] · S. Kecel Gunduz[6]

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Abstract

The objective of the present study was to evaluate the efficacy of experimental varnishes containing theaflavin (TF-2a/2b) against erosive demineralization and matrix metalloproteinase enzymes (MMPs) and investigate the bonding properties of TFs to MMPs. Enamel and dentin samples, measuring 3 × 5 × 2 mm, were extracted and immersed in citric acid solution for 60 s to induce artificial erosive lesions. The samples were then randomly allocated into six experimental groups and one control (fluoride) group ($n = 6$). Experimental groups were treated with different varnish formulations of resin-, polymer-, or resin + polymer-based varnishes containing either TF-2a or TF-2b. Following immersion in artificial saliva, all samples were subjected to a 5-min pH cycle for 5 days. Mineral density (MD) values were assessed using MicroCT, and expression levels of MMPs were evaluated by Western Blot. In silico analyses of both TFs were conducted against MMP-2 and MMP-9. Mineral density values of all groups increased significantly following the pH cycle [TF-2b resin + polymer-based ($p = 0.023$); all other groups ($p = 0.026$)]. Varnishes based on TF-2b demonstrated numerically superior results in MD scores, but resin-polymer-based TF-2a showed better MMP expressions than other groups. The binding energies of both tested TFs with MMP-2 and MMP-9 were determined to be similar. Profile analysis of both TFs are also indicated to be effective against the activity of MMPs. Therefore, resin-based TF-2a and TF-2b experimental varnishes may serve as viable natural alternatives to fluorides in managing dental erosion.

Keywords Dental erosion · Theaflavin · Dental varnish · MMP expression · Microtomography · In silico analyses

Introduction

In the past decade, the dental profession has increasingly recognized dental erosion as a significant issue, largely due to patients' growing acidic oral habits that lead to erosive tooth wear (ETW) [1]. ETW commonly affects multiple teeth, resulting in worn dentition and associated symptoms, such as dentinal sensitivity, pain, reduced vertical dimension, discomfort, and aesthetic concerns [2]. In addition, tissue loss due to ETW is irreversible, with limited restorative treatment options available in the early stages. Thus, it is essential to implement preventive strategies to avoid further damage. According to the latest consensus from the European Federation of Conservative Dentistry (EFCD), while restorative treatments can alleviate clinical symptoms, they should be combined with preventive measures [3]. Numerous studies have explored various components, including gels, solutions, pastes, and varnishes [4, 5]. Recent findings indicate that fluoride formulations are particularly effective

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✉ G. Ozan
gunce.saygi@istanbul.edu.tr

1 Faculty of Dentistry, Department of Restorative Dentistry, Istanbul University, Istanbul, Turkey

2 Faculty of Dentistry, Department of Biochemistry, Istanbul University, Istanbul, Turkey

3 Faculty of Pharmacy, Department of Pharmaceutical Technology, Istanbul University, Istanbul, Turkey

4 Faculty of Science, Department of Molecular Biology and Genetics, Istanbul University, Istanbul, Turkey

5 Faculty of Medicine, Department of Biostatistics, Istanbul University, Istanbul, Turkey

6 Faculty of Science, Department of Physics, Atomic And Molecular Physics Division, Istanbul, Turkey

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in controlling dental erosion [6]. Fluoride's strong remineralization effect and its ability to adhere to enamel offer significant protection against ETW. However, the scenario becomes more complicated when tissue loss extends beyond enamel, affecting the structural properties of dentin.

Recent research into dental erosion has focused on endogenous enzymes, specifically matrix metalloproteinases (MMPs) and cysteine cathepsins (CCs), which are present in saliva and dental hard tissues, particularly in higher concentrations in dentin [4, 7]. These enzymes can degrade the organic collagen matrix in dentin, impacting the ionic diffusion of demineralized tooth surfaces and thereby mitigating further erosive damage [8, 9]. This has prompted investigations into various compounds, such as polymers, nanohydroxyapatite, sodium trimetaphosphate, and protease inhibitors, for their potential to counteract endogenous damage [8]. Among these, epigallocatechin-3-gallate (EGCG) has been extensively studied due to its polyphenolic properties. However, recent studies suggest that theaflavins (TFs), another class of polyphenolic compounds, also exhibit significant protective properties. Besides, theaflavins can adsorb onto the pellicle, enhancing its thickness with salivary proteins and thereby forming a more robust protective barrier against acid attacks [10]. Given their structural similarities to EGCG—featuring aromatic rings with phenol hydroxyl and galloyl groups—the aflavins are hypothesized to interact similarly by chelating ions on the tooth surface [11, 12]. Preliminary investigations into theaflavins have yielded encouraging results [12, 13].

The inhibitory effects of black tea theaflavins have been extensively studied in medicine due to their antioxidant, anti-inflammatory, and anti-carcinogenic properties [14, 15]. Research has shown that both theaflavin gallate and theaflavin digallate can inhibit the activity of type IV collagenase MMPs, including MMP-2 and MMP-9, across various human tissues [14]. However, only one recent study has explored the inhibitory effects of theaflavins specifically on dentinal MMPs, finding that TFs can inhibit these enzymes in a concentration-dependent manner [12]. Furthermore, comparative studies of epigallocatechin-3-gallate (EGCG) and theaflavin derivatives have not shown significant differences in their effectiveness against ETW [11, 13]. In addition, polyphenols like theaflavins are reported to act as "processing cofactors" in biomaterials such as dental varnishes, enhancing their mechanical and functional properties [16]. Thus, incorporating theaflavin derivatives into preventive strategies may be advantageous for avoiding further ETW.

In the present study, two types of theaflavins (2a and 2b) were incorporated into dental varnish formulations aimed at preventing the early stages of ETW. Given that varnishes provide a more durable application to dental tissues compared to solutions or rinses [4], the current study investigates the effectiveness of experimental dental varnishes containing TF-3-gallate (2a) and TF-3'-gallate (2b) against erosive demineralization and MMP enzymes (MMP-2 and MMP-9). In addition, it was also managed to prove the mechanism of binding of TFs to MMPs and their intermolecular interactions by conducting in silico analyses. Thoroughly, the tested varnish formulations based on TFs would be linked not only at the clinical level but also with the molecular level. The null hypotheses of this study are that (i) there will be no significant difference in erosive demineralization and (ii) anti-MMP activity among the tested TF varnishes.

Methods

The present study followed a randomized and blinded design utilizing an in vitro and in silico model. The research is conducted in accordance with the 1964 Helsinki Declaration and approved by the ethical committee of Istanbul University Faculty of Dentistry (No. 2021/17).

Sample preparation

Freshly extracted, non-erupted third molars ($n = 180$) were utilized to prepare enamel and dentin slabs measuring $3 \times 5 \times 2$ mm. The occlusal enamel surfaces were exposed using a high-speed automatic polisher (Buehler MetaSeru 250 Grinder-Polisher, Düsseldorf, Germany). Subsequently, dentin slabs were obtained from the middle third of the horizontal axis of the teeth, which were then positioned in the holding area of a microtome machine (Isomet 1000 Precision Buehler, Esslingen, Germany). The slabs were preserved in a 0.1% thymol solution (pH 7.1) at 4 °C for 1 week, followed by storage in a 0.9% isotonic solution for an additional week to ensure the removal of thymol residues. Each sample was then embedded in epoxy resin, with a 2 mm overlay on the surface. After the resin had been set, all surfaces were polished to prevent surface inclination using the aforementioned automatic polisher, with water coolant applied during the process. The samples were maintained in artificial saliva until the commencement of the experimental period.

Experimental period

All samples were subjected to demineralization using citric acid (CA-0.3%, pH 3.2) for 60 s to simulate initial erosive lesions. Following demineralization, the samples were rinsed under running water for 30 s and subsequently stored in distilled water in a refrigerator at + 4 °C. To avoid misidentification, the bottom surfaces of the epoxy resin blocks were marked with a pencil. The samples were then subjected to measurements. An illustration depicting the entire experimental procedure is presented in Fig. 1.

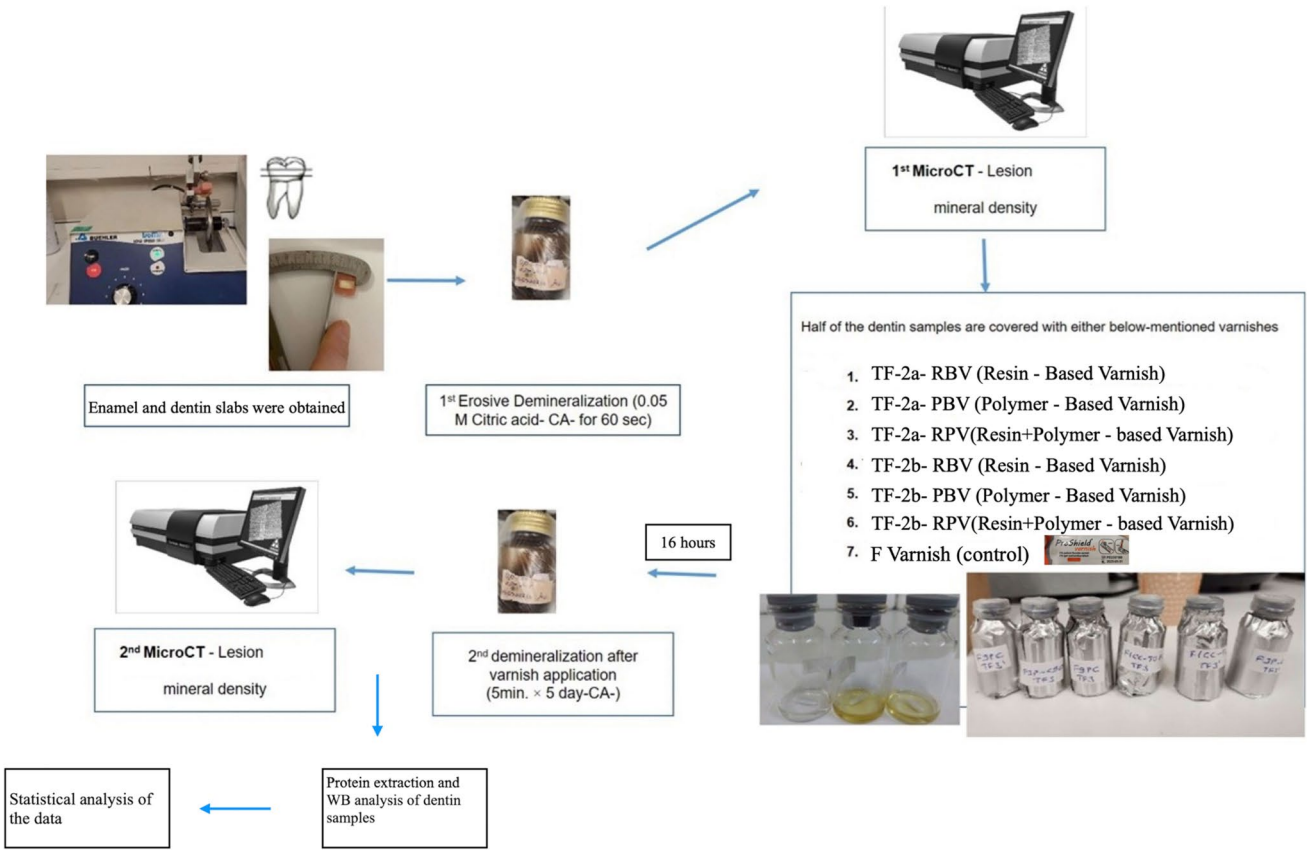


Fig. 1 Experimental period of the evaluation of erosive demineralization

Baseline MicroCT measurements

Following the induction of initial erosive lesions, mineral density levels were assessed at designated points using a computerized tomography system (SkyScan 1172, Bruker, Kontich, Belgium) at the Sabancı University Nanotechnology Research and Application Center, Istanbul. Prior to examination, the surfaces of the samples were gently dried with a tissue to prevent desiccation. Three distinct points on the enamel surfaces were selected to define the volume of interest (VOI) for the lesions and were marked with a pencil to ensure consistent measurement locations at the end of the experimental period. The turntable of the tomography system was calibrated to ensure that the X-ray beams were directed perpendicularly to the resin cylinder. The X-ray tube was operated at 80 kV and 128 mA, equipped with aluminum filters. The samples underwent scanning with a 180° rotation and a rotation step of 0.4°. Two-dimensional images were then reconstructed using NRecon software (SkyScan, Bruker, Kontich, Belgium). Following the imaging process, the samples were stored in distilled water at + 4 °C for subsequent experimental procedures.

Application of the varnishes and erosive cycling

Following the initial MicroCT measurements, samples were allocated to six experimental groups and a control according to the material combinations ($n = 6$): (1) TF-2a–RBV (TF-2a resin-based varnish); (2) TF-2a-PBV (TF-2a polymer-based varnish); (3) TF-2a-RPV (TF-2a resin + polymer-based varnish); (4) TF-2b -RBV (TF-2b resin-based varnish); (5) TF-2b-PBV (TF-2b polymer-based varnish); (6) TF-2b-RPV (TF-2b resin + polymer-based varnish); and (7) commercial fluoride varnish (ProShield Varnish, President Dental, Germany) as control. The experimental varnishes were formulated differently due to the main compounds, which are either an artificial resin (Colophony), a coating polymer (Kollicoat Mae-100P) or a combination of both. Ethanol was used as the main solvent, and deionized water was also included. TF-2a and -2b are also soluble in ethanol; thus, all solid ingredients were slowly added to ethanol in vigorous stirring until the homogenous varnishes were obtained. Varnishes were constituted at Istanbul University Faculty of Pharmacy, Department of Pharmaceutical Technology, and kept in glass containers away from sunlight exposure.

Varnishes were applied to the surfaces using a micro brush and allowed to dry completely for 10 min. Subsequently, the samples were immersed in artificial saliva (composed of 0.1 mol/L Tris-buffer, 1.5 mmol/L Ca^{2+}, 0.9 mmol/L PO_4, 150 mmol/L KCl, pH 7.0) for 16 h. Then, the samples were dried using a water/air spray and subjected to pH cycling, which involved exposure to citric acid (CA, 0.3%, pH 3.2) for 5 min per cycle, repeated daily for 5 days. The citric acid solution was refreshed each day, and after each exposure, the samples were rinsed under running tap water. Between erosive cycles, the samples were again stored in artificial saliva.

Final MicroCT measurements

The mineral density was measured with micro-computed tomography from the same point of the lesions that were specified as VOI at the baseline measurements. In addition, the same laboratory operator performed the final measurements as the same as at the baseline under the supervision of one of the authors (G.O.) as well.

Sample preparation for MMP expression analysis

To assess the effectiveness of varnishes on the expression levels of MMP-2 and MMP-9, dentin samples were prepared again as previously described ($n = 6$). An additional control group consisting of "sound dentin" samples, which did not receive varnish application, was included, resulting in a total of 8 groups. The samples were immersed in citric acid (CA) solution with the same concentration and pH (0.3%, pH 3.2) for 60 s to induce primary erosive demineralization. Following this, the samples were rinsed under running tap water for 30 s and stored in a refrigerator at + 4 °C until varnish application. No varnish was applied to the "sound dentin" group. Varnishes were applied to the surfaces of the other groups using a micro-brush and left to dry until the commencement of erosive cycling. During the experimental procedure, the samples were kept in artificial saliva, as previously described, and subjected to erosive cycling as outlined above. After the erosive cycling, all samples were washed with distilled water and air-dried for 3 s. Then, all samples were kept in artificial saliva for 16 h before the protein assessments.

Protein extraction and Western Blot (WB) analysis

Tooth samples (dentin fragments) were frozen in liquid nitrogen and pulverized with a mortar/pestle. Then, dentin powders were homogenized in ice-cold lysis buffer (50 mM tris–HCl/pH.8.4, 5 mM $CaCl_2$, 100 mM NaCl, 0.1% Triton X-100, 0.1% NP-40, 0.02% NaN_3) with protease inhibitor cocktail (Roche, Darmstadt/Germany) [17]. The total protein concentration was quantified using a bicinchoninic acid (BCA) kit (iNtRON Biotechnology, Seongnam/Korea). As described previously [18], equal amounts (30 µg/well) of extracted proteins were separated under reducing conditions by SDS–PAGE and electrotransferred to the PVDF membrane. The transfer was checked by staining the membrane with Ponceau S, which was used for total protein normalization [19]. After removing Ponceau S with 0.1 N NaOH, the membrane was blocked using 5% non-fat dry milk (Sigma, St. Louis/USA) in tris-buffered saline/Tween-20; TBST) for 2 h at RT and incubated with the following primary antibodies (overnight at 4°C): anti-MMP2 (#MA5-13,590, 1:500 dilution, host: mouse) and anti-MMP9 (#MA5-32,705, 1:500 dilution, host: rabbit) were obtained from Invitrogen/Thermo (Carlsbad, CA, USA). After washing five times with TBST (5 min each), the incubation with specific anti-mouse or anti-rabbit HRP-linked secondary antibodies (Cat. no. BA1050, BA1054, respectively, [1:5000 dilution] Boster Biological Technology, Valley Ave, Pleasanton, CA, USA) was performed for 2 h at RT. Following the washing away of excess antibodies, blots were visualized using enhanced chemiluminescence (ECL) plus substrate kit (Thermo, Kwartsweg/Holland) and ChemiDoc-XRS-system/ImageLab 6.0.1software (BioRad, Hercules/USA).

In silico analysis (molecular docking and ADMET)

In silico molecular docking of the present study, the molecular structures of two types of theaflavins (2a and -2b) were obtained from PubChem and Chemicalbook [20, 21]. Theaflavin-3-gallate (TF2a) (PubChem ID: 169,167) and Theaflavin-3'-gallate (TF2b) (CBNumber: CB2463115) were downloaded to establish the starting molecular structures for docking analysis. Both molecules were optimized with the Gaussian 09 [22] program using the DFT theory level, RB3LYP calculation method, 6-31G(d,p) basis set. The optimized geometric structures were then used as ligands for docking analysis. In molecular docking analysis calculations, the 'ligand preparation' and 'receptor preparation wizards' in the Schrödinger Glide module [23, 24] were used. The optimized ligands, TF2a and TF2b, were further refined using the OPLS force field in the LigPrep tool. This process produced different conformers of the ligands, which were then used for docking analysis. Due to the inhibitory effects of theaflavins on the activity of dentinal matrix metalloproteinases (MMPs), particularly type IV collagenase MMPs, including MMP-2 and MMP-9, these enzymes were selected as potential receptors and obtained from the Protein Data Bank [25].

Crystal structure of human MMP-2 (PDB: 7XGJ) [26] with 2.80 Å resolution and 168 sequence length and MMP-9 (PDB:5CUH) [27] with 1.83 Å resolution and 164 sequence length were downloaded. Preliminary

preparations were made by leaving only the A chain and Zn^{+2} and Ca^{+2} ions in the receptors.

The water in the structures of MMP enzymes, specifically MMP-2 and MMP-9, was removed, and polar hydrogen atoms were added to these receptors. The charges were adjusted to a neutral pH using PROPKA [28], and the heavy atoms were refined by selecting an RMSD value of 0.3 Å. Each receptor underwent a pre-preparation process, an optimization process, and finally a minimization process using the OPLS3 force field [29]. To identify the active binding sites of the receptors, a grid process was conducted, allowing for the selection of residues that could move freely within the binding site. Following this, molecular docking analysis was performed. The docking process revealed the interactions between each ligand and the residues in the receptors' binding sites, and it also assessed and documented their binding affinities.

Statistical analysis

Statistical analysis was performed with SPSS (v.25; IBM, New York, USA) and GraphPad Prism statistical software v10.2.3 (La Jolla/USA). The Shapiro–Wilk test was used to evaluate the homogeneity of the variables. Since all of the groups did not meet the assumptions of normality, Kruskal–Wallis tests were performed. To evaluate the mineral density levels and the significance among different formulations of the varnishes, post-hoc pairwise analyses were analyzed with the Wilcoxon test with Bonferroni corrections. Besides, paired samples and independent samples tests were used to differentiate the effect of varnishes of TF-2a or -2b. A one-way analysis of variance (ANOVA) followed by Tukey post-hoc test was performed for multiple comparisons. The criterion for statistical significance was $p < 0.05$.

Results

MicroCT evaluations (MD scores)

The mineral density (MD) scores of all groups are presented in Table 1.

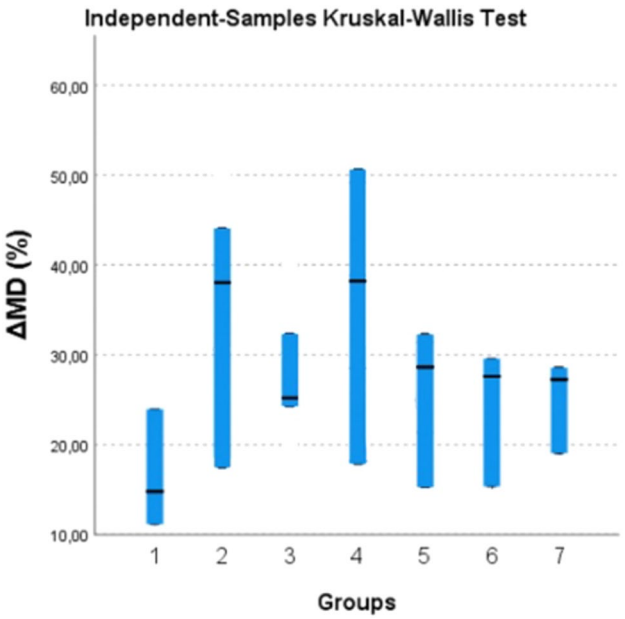
The results indicate that there were no significant differences between baseline and final mineral density (MD) scores within any of the groups. In addition, there were no significant differences in final MD scores among the tested groups ($p > 0.05$). To quantify changes in mineral density, percentage mineral change (ΔMD) values were calculated using the MD scores from baseline and final measurements, as described by the formula provided in Table 2.

Table 1 Median, SD scores and statistical significance of MD (mineral density) values of baseline and final MicroCT measurements

Groups	Baseline Median ± SD	Final Median ± SD	*p*
(1) TF-2a–RBV	1.92 ± 0.04	2.19 ± 0.19	0.026
(2) TF-2a–PBV	1.76 ± 0.14	2.22 ± 0.13	0.026
(3) TF-2a–RPV	1.72 ± 0.25	2.15 ± 0.35	0.026
(4) TF-2b–RBV	1.78 ± 0.16	2.24 ± 0.13	0.026
(5) TF-2b–PBV	1.91 ± 0.09	2.24 ± 0.14	0.026
(6) TF-2b–RPV	1.81 ± 0.06	2.19 ± 0.09	0.023
(7) F–V (control)	1.76 ± 0.10	2.25 ± 0.03	0.026

$$\frac{MD_{FINAL} - MD_{BASELINE}}{MD_{BASELINE}} \times 100$$

It is observed that (Graphic 1) there were no significant differences among ΔMD values of all tested groups ($p = 0.058$).



Graphic 1. Distribution of the ΔMD scores of tested groups

Pairwise analyses were conducted to determine any superiority between TF-2a and TF-2b varnishes. Given that not all groups were normally distributed, the Kruskal–Wallis test along with Bonferroni corrections revealed significant differences in baseline and final mineral density (MD) scores among varnishes containing TF-2a or TF-2b ($p < 0.001$). However, no significant differences were observed in the percentage mineral change (ΔMD) values among these varnishes (Table 3). To further evaluate the effects of similar varnish components (Resin, Polymers, Resin + Polymer),

Table 2 Significance of difference of MD (ΔMD- %) values of tested groups

Groups		Mean	*p*
1	TF-2a–RBV	14.81	
2	TF-2a–PBV	38.07	
3	TF-2a–RPV	25	
4	TF-2b–RBV	30.60	
5	TF-2b–PBV	27.27	
6	TF-2b–RPV	27.62	
7	F–V (Control)	27.27	
			0.058

pairwise analyses were repeated. This analysis also showed no significant differences in baseline and final MD scores ($p = 0.028$ and $p = 0.027$, respectively) or ΔMD values ($p = 0.831$) among varnishes with identical formulas (Table 4). In addition, Fig. 2 presents examples of the VOIs from random samples of Groups 1 and 4, which represent TF-2a and TF-2b varnishes, respectively.

Evaluation of MMP expression

According to the immunoblotting analysis in Fig. 3, the positive control (PC, Fluoride varnish) group showed a statistically significant decrease in MMP-2 and MMP-9 levels compared to negative control (NC-Sound dentin without any varnishes). Remarkably, among the six tested samples, the TF-2a-RPV (Group 3) showed the closest result to PC; a highly significant decrease of > 75% in MMP-2 and MMP-9 levels was determined. The lowest effect in TF-2a group samples was obtained in the RBV sample, with 46% and 43% reduction in MMP-2 and MMP-9 levels, respectively, and still had a marked impact compared to NC. There was no statistically significant difference between the TF-2b groups on the MMP-9 levels. Notably, there was no statistically significant difference between the reducing effect of TF-2a-RPV and TF-2b-RPV samples on MMP-2 and MMP-9 levels. Overall, it was determined that varnishes of RPV had higher potential protective properties against dentin erosion, with the highest effect compared to the untreated NC group in dentin samples and the closest effect to the fluoride, which was the PC group.

Table 3 Statistical significance of baseline and final MD (mineral density) scores and ΔMD (%) values of experimental varnishes regarding their content of TF-2a and TF-2b

	Baseline	Final	ΔMD (%)
Groups	Mean ± SD	Mean ± SD	
TF-2a-based varnishes (Groups 1–2–3)	1.73 ± 0.22	2.17 ± 0.25	25.64
TF-2b-based varnishes (Groups 4–5–6)	1.78 ± 0.13	2.25 ± 0.13	27.12
p	< 0.001	< 0.001	0.778

Molecular docking and ADMET results

The activity of experimental dental varnishes containing TF-2a and TF-2b against MMP enzymes (MMP-2 and MMP-9) was investigated in terms of docking energy scores and binding site interactions by in silico molecular docking analysis.

The minimum docking energy scores from 32 docking results between MMP-2 and TF-2a and TF-2b are −7.40, and −6.80 kcal/mol, respectively. The visualization of the docking analysis with 32 different docking scores and hydrogen bonds formed between the key amino acid residues in MMP-2 that provide binding to TF-2a and other interactions are given in Fig. 4. The results show that TF-2a formed hydrogen bonds of 2.33 Å, 2.05 Å, 2.26 Å, 1.75 Å, and 1.72 Å with the key amino acid residues Ala86, Pro141, Leu83, and Glu130 in the MMP-2 binding site, respectively, and had metal coordination interactions of 2.30 and 2.40 with Zn201.

Then, the TF-2b–MMP-2 complex was visualized in Fig. 5. TF-2b formed hydrogen bonds with Ala84, Pro141, Glu130, and Arg7 residues of 2.37 Å, 2.16 Å, 1.95 Å, 1.47 Å, and 1.91 Å, respectively, and made a metal coordination interaction of 2.08 Å length with the same Zn. As a result of these interactions, it exhibited a binding energy of −6.80 kcal/mol to MMP-2.

The minimum binding energy between TF-2a and TF-2b with MMP-9 was calculated as -8.52 and -7.75 kcal/mol, respectively. TF-2a was attached to the central cavity of MMP-9 and formed hydrogen bonds with Glu227(2.09 Å),

Table 4 Statistical significance of baseline and final MD (mineral density) scores and ΔMD (%) values of tested experimental varnishes regarding their formula

	Baseline	Final	ΔMD (%)
Groups	Mean ± SD	Mean ± SD	
Resin-based varnishes (Groups 1 and 4)	1.86 ± 0.17	2.21 ± 0.11	24.90
Polymer-based varnishes (Groups 2 and 5)	1.79 ± 0.14	2.23 ± 0.14	28.62
Resin + polymer-based varnishes (Groups 3 and 6)	1.73 ± 0.21	2.17 ± 0.28	25.62
p	0.028	0.027	0.831

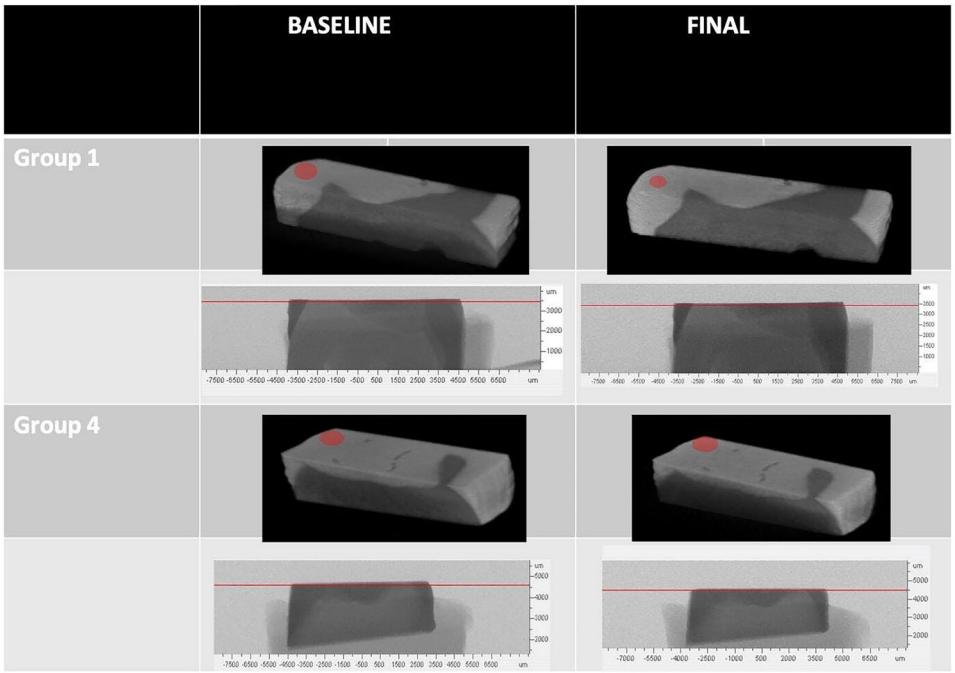


Fig. 2 Volume of Interests (VOI) of random samples of Groups 1 and 4.* *The data were collected from the areas indicated by red dots

Ala191(1.86 Å), Asp235(2.14 Å) and Tyr218(1.76 Å) as seen in Fig. 6. The lowest binding energy docked complex of TF-2b with MMP-9 was also shown in Fig. 7. TF-2b formed four hydrogen bonds with Asp235, Pro246, Glu227, and Leu188 residues with 2.44 Å, 2.30 Å, 1.84 Å, and 2.74 Å, respectively. Moreover, Zn301 interacted with metal coordination interaction with TF-2b.

SwissADME profiles and pkCSM models widely comprehend the physicochemical properties, drug-likeness scores, and toxicity of TF-2a and TF-2b compounds (Supplementary file). According to these predictions, it was evaluated that TF-2a and TF-2b have the potential to be CYP2C9 inhibitors. CYP2C9 inhibitors are important compounds that suppress these enzymes and can slow down drug metabolism and increase their levels in the blood. Besides, it was also observed that the tested TFs exceeded some drug-likeness rules according to the Lipinski, Ghose, and Veber rules. Furthermore, it was determined that the predicted water solubility values of TF-2a and TF-2b compounds were calculated as −2.898 and −2.892 log(mol/L) units, respectively. Besides, the absorption of all compounds in the gastrointestinal system was low. According to the pkCSM model, all compounds were identified as P-gp substrates, and TF-2a and TF-2b were also evaluated as P-glycoprotein I inhibitors, while they were evaluated as P-glycoprotein II inhibitors. Moreover, it was observed that the intestinal absorption of the compounds is moderate. According to the pkCSM model, all compounds had values of −2.735 (log Kp), so they had low skin permeability properties. It was also estimated that the compounds did not have the potential to pass through the blood–brain barrier (BBB permeability). Toxicity assessment of all compounds according to the pkCSM prediction tool was performed, and AMES toxicity, skin sensitization, and hepatotoxicity were predicted as negative. The maximum recommended tolerated dose (MRTD) calculated in the present study for TF-2a and TF-2b values were 0.453 and 0.438 (log mg/kg/day), respectively.

Discussion

The methodology of this study focused on evaluating the potential effects of theaflavins on enamel mineralization and the preservation of the dentin organic matrix (DOM), specifically in relation to preventing erosive tooth wear (ETW). To achieve these objectives, the study investigated erosive lesion volumes using MicroCT and assessed the levels of MMP enzymes following the application of varnishes. These enzymes contribute to the degradation of the DOM and were analyzed through Western blot (WB) techniques. Erosive lesions were treated with varnishes containing either TF-2a or TF-2b, both at approximately 1% concentration, in solutions with cosolvents and polymers designed to optimize viscosity and solubility. The hypothesis of the study proposed that the mineral density of erosive lesions would increase due to the anti-MMP and anti-erosive properties of theaflavins. However, despite some promising results, the first hypothesis was rejected, as there were no significant differences observed in mineral density alterations (ΔMD) among the different TF varnishes. Conversely, the second hypothesis was accepted, as significant differences in MMP levels were observed following the application of the various varnishes.

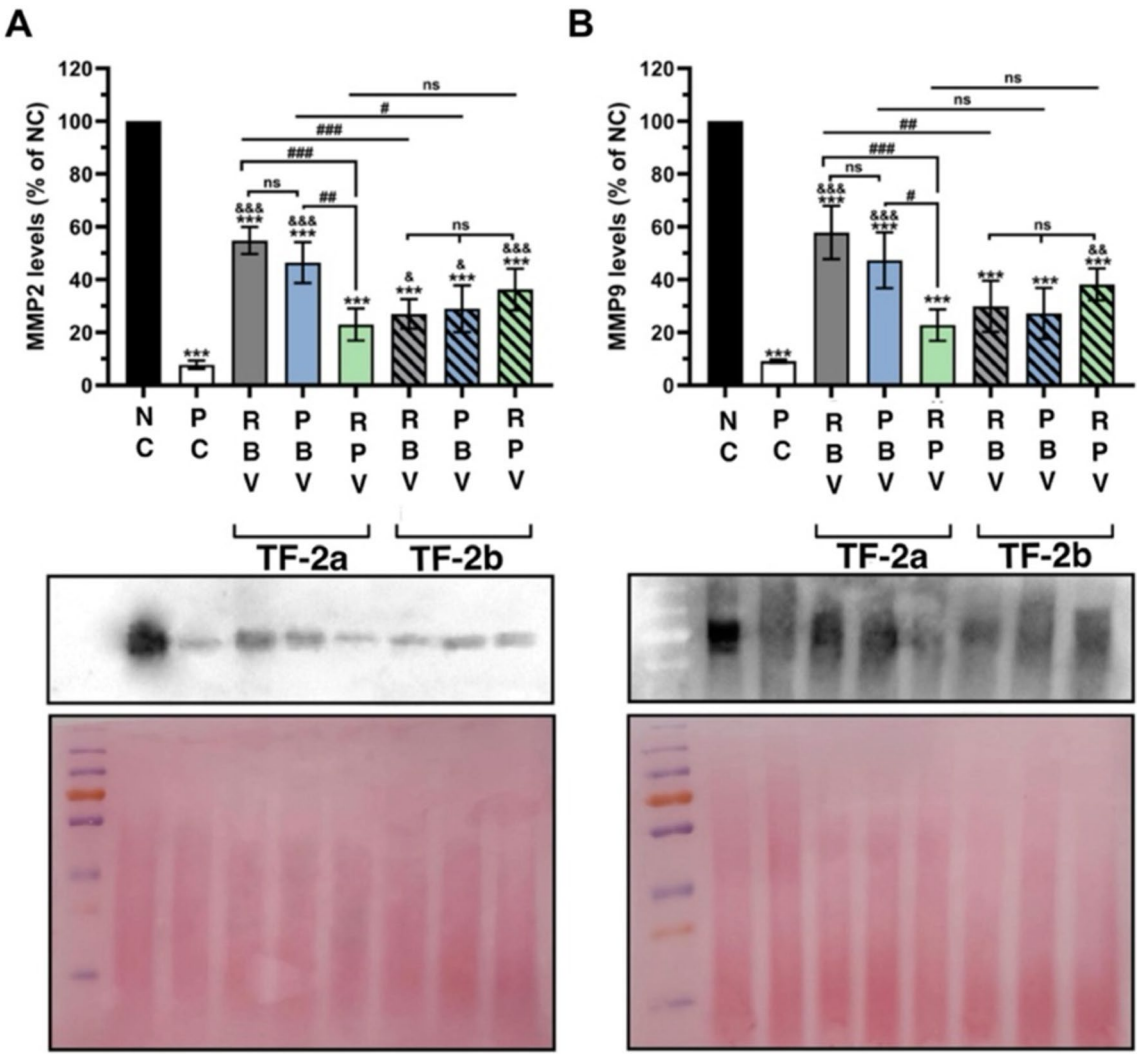


Fig. 3 Matrix metalloproteinase (MMP-2 and -9) levels of dentin samples following the application of varnishes*. *Immunoblot analysis of **A** MMP-2 and **B** MMP-9. Ponceau S staining was used for total protein normalization. Data are presented as mean ± SD. ***$P < 0.001$ versus negative control (NC). $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, and $^{\&\&\&}P < 0.001$ versus positive control (PC). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ indicate multiple comparisons between groups. *ns*—not significant (indicates $P > 0.05$). Statistical differences were analyzed by one-way ANOVA followed by Tukey post-hoc test

In the current study, erosive demineralization was assessed by measuring mineral density using MicroCT. Although microradiographic methods are commonly used for evaluating dental caries [30–32], MicroCT has also been reported as effective for measuring erosive lesions [31]. Only two previous studies [33, 34] have utilized MicroCT to investigate erosive depth, both demonstrating its capability to reveal the extent of lesions and areas of significant demineralization. Given that dental erosion involves both surface softening and sub-surface demineralization, it is generally recommended to employ multiple monitoring techniques [33]. However, in this study, only MicroCT was used to evaluate erosive lesions, consistent with the approach used for assessing MMP levels via Western blot (WB). This focus allowed for a comparative analysis of the experimental varnishes and their efficacy against erosive demineralization. On the other hand, in silico analyses assisted MMP expression scores by revealing the binding sites and docking energy scores, also showing many intermolecular interactions (Figs. 4–7). Both at the molecular level and clinical application (varnish), TF-2a and -2b were investigated in detail.

In studies of ETW, the mineral content of lesions is critical for evaluating the effects of erosion. Previous research has associated reduced mineral content with surface softening [31], highlighting the importance of monitoring lesion depth, mineral loss, and density. In the current study, immersion in citric acid (CA) solution with a pH of 3.2 for

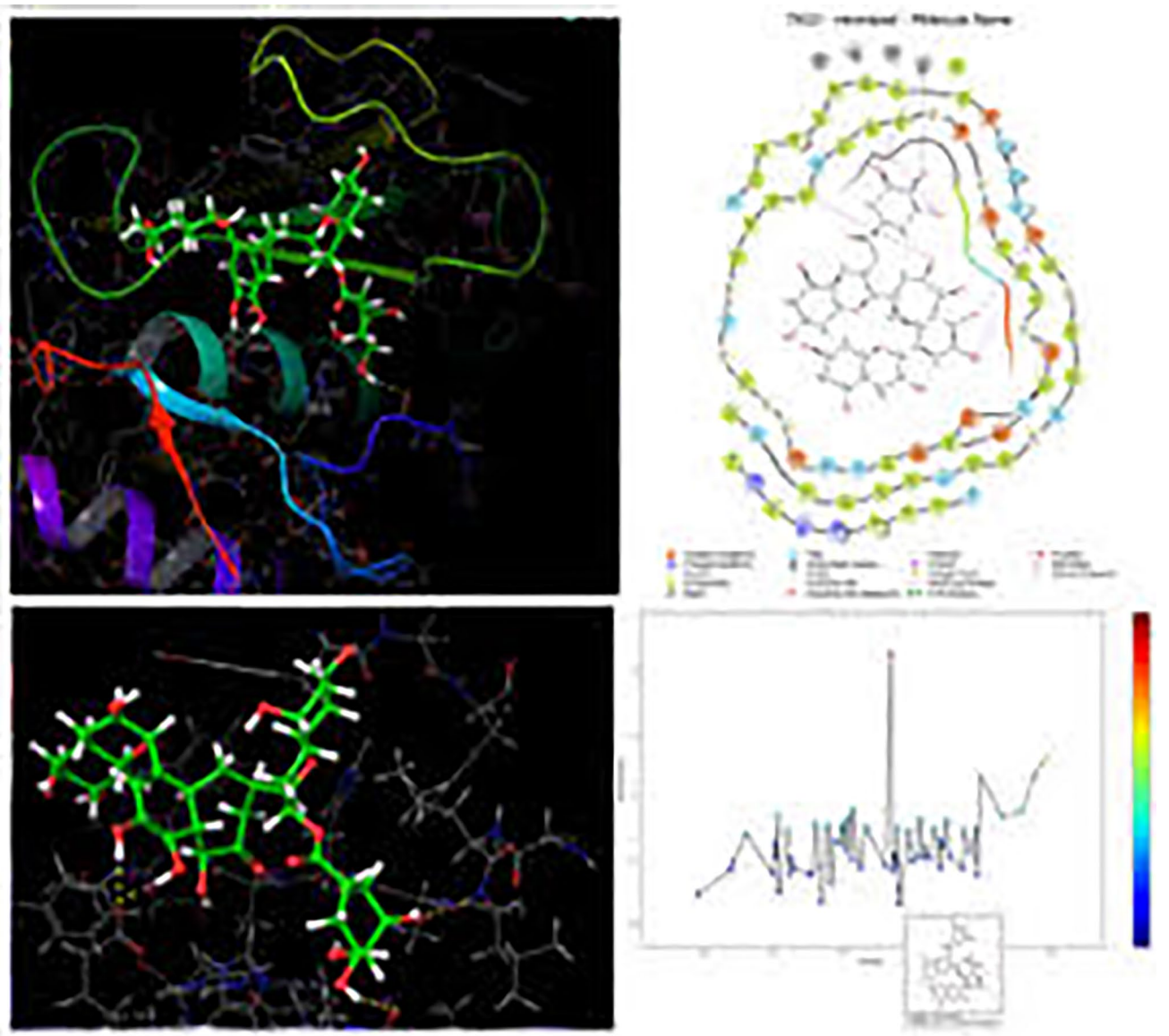


Fig. 4 Docked structure of TF-2a with MMP2 enzyme, 32 docked results, 2D illustration of binding interactions of TF-2a and interacting residues, 3D hydrogen bonds between TF-2a and MMP2

30 min was reported to produce a lesion depth of approximately 1 µm [35]. In this context, a brief 60-s immersion was expected to induce surface softening, simulating early lesions effectively. Subsequently, samples were subjected to an additional 25 min of CA exposure during erosive cycling to evaluate the effects of the tested TF varnishes on lesions with depths greater than 1 µm. Mineral density measurements using MicroCT were particularly effective for monitoring softening within early lesions.

Dental erosion progresses differently in sound teeth compared to those already exhibiting ETW [36]. In the current study, varnishes were applied to surfaces with pre-existing erosive demineralization. Notably, the calcium release was found to be numerically higher in teeth with ETW compared to sound teeth [36], underscoring the importance of protective measures, such as anti-erosive varnishes, for early stage ETW lesions. The tested varnishes improved mineral density values in the samples by 14.81–38.07%. These results, with mineral density values ranging from 1.72 to 2.25, are consistent with those reported by Akkuc et al. [34], who evaluated the effects of kinds of toothpaste on erosive lesions.

It is important to note that the experimental varnishes were applied as a single layer. Applying a thicker layer of varnish might enhance ion chelation, potentially leading to higher mineral density values and results comparable to those of the fluoride group. In addition, excessive layers

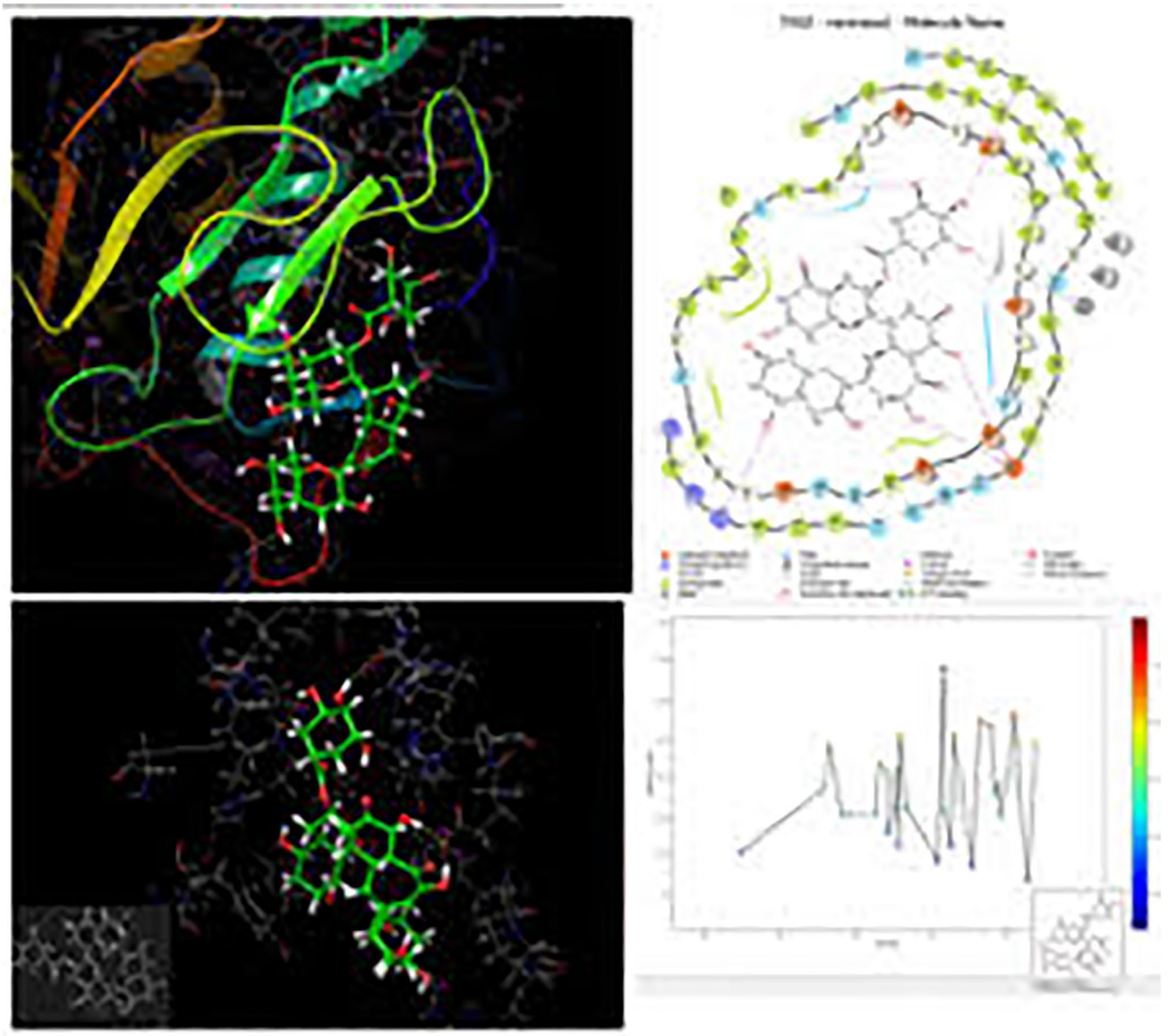


Fig. 5 Docked structure of TF-2b with MMP-2 enzyme, 32 docked results, 2D illustration of binding interactions of TF-2b and interacting residues, 3D hydrogen bonds between TF-2b and MMP-2

could affect the MMP enzymes more, and thicker TF layers may result in increased adsorption of salivary proteins. Therefore, clinical studies are expected to yield higher mineral density scores. Given the limited number of similar studies, further comparisons regarding the efficacy of these varnishes are currently not feasible.

Treatments for ETW commonly include fluorides in various forms, which are beneficial, particularly for early erosive lesions, but do not provide complete prevention [3]. This underscores the relevance of exploring novel anti-erosive agents. Theaflavins, recently identified as cross-linkers [12] and shown to prevent dentin loss [13] and exhibit anti-caries effects [37], present a promising alternative. However, their mechanism of action has not been fully explained. Evaluating the structures of both MMPs and TFs would signify a major advancement in the development of new dental products. Therefore, in silico analyses and different servers were utilized to evaluate the compounds, such as SwissADME [20] and pkCSM server systems (showed in Supplementary file) [21]. The study of ADMET properties of a compound, namely, absorption, distribution, metabolism, excretion, and toxicity, provides an important insight into drug development processes [20, 21]. The results of these properties presented various data on the possible dental products such as current experimental TF varnishes. One of the parameters tested was polar

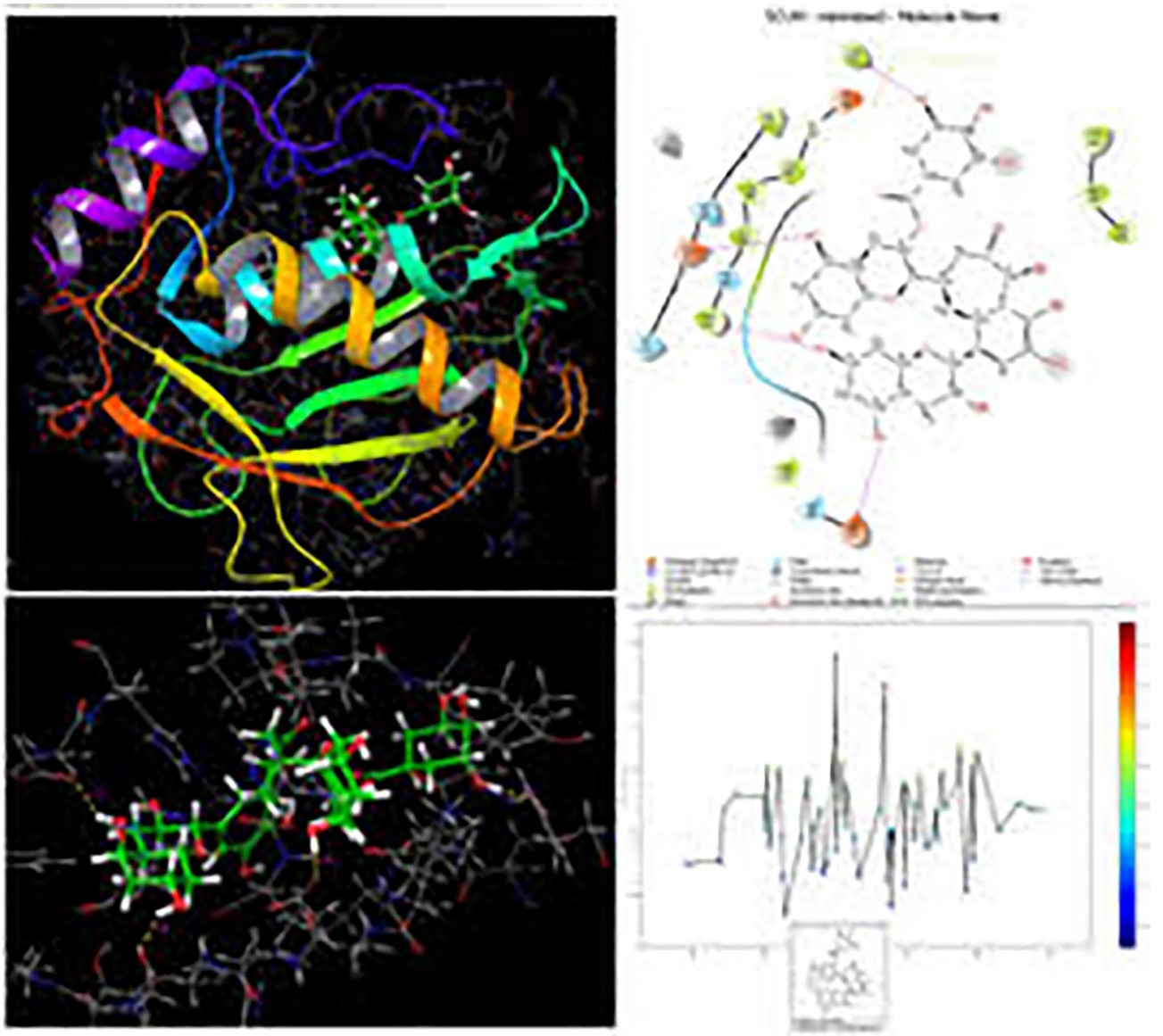


Fig. 6 Docked structure of TF-2a with MMP-9 enzyme, 32 docked results, 2D illustration of binding interactions of TF-2a and interacting residues, 3D hydrogen bonds between TF-2a and MMP-9

surface area (PSA) which is related to the transport and absorption of drug molecules [23], and the determination of topological PSA (TPSA) can provide an estimate of whether the drug is a carrier for cell permeability (< 140 $Å^2$) and/or a carrier at the blood–brain barrier (< 90 $Å^2$) [24–26]. By looking at the TPSA values of each molecule, it could be assumed that they do not play a transporter role in cell permeability or the blood–brain barrier.

The pkCSM model (showed in Supplementary file) was also performed just to predict the physicochemical properties and possible toxicity of the compounds. Intestinal absorption values relate to absorption from the human intestine and if absorption of a compound is less than 30%, it is considered poorly absorbed. Accordingly, the absorption of the compounds was found as "moderate". On the other hand, if the skin permeability constant log Kp (cm/h) has a value greater than -2.5, the skin permeability is considered as low, in which the results provided that tested TFs were showing low skin permeability properties.

To add more, the ability of compounds to appear as oral products could be tested by their toxicity or tolerance level so, this ability was measured by a few parameters in the current study. The maximum recommended tolerated dose (MRTD), which provides an estimate of the toxic dose threshold for chemicals in humans, is considered low if it is less than or equal to 0.477 and high if it is greater than

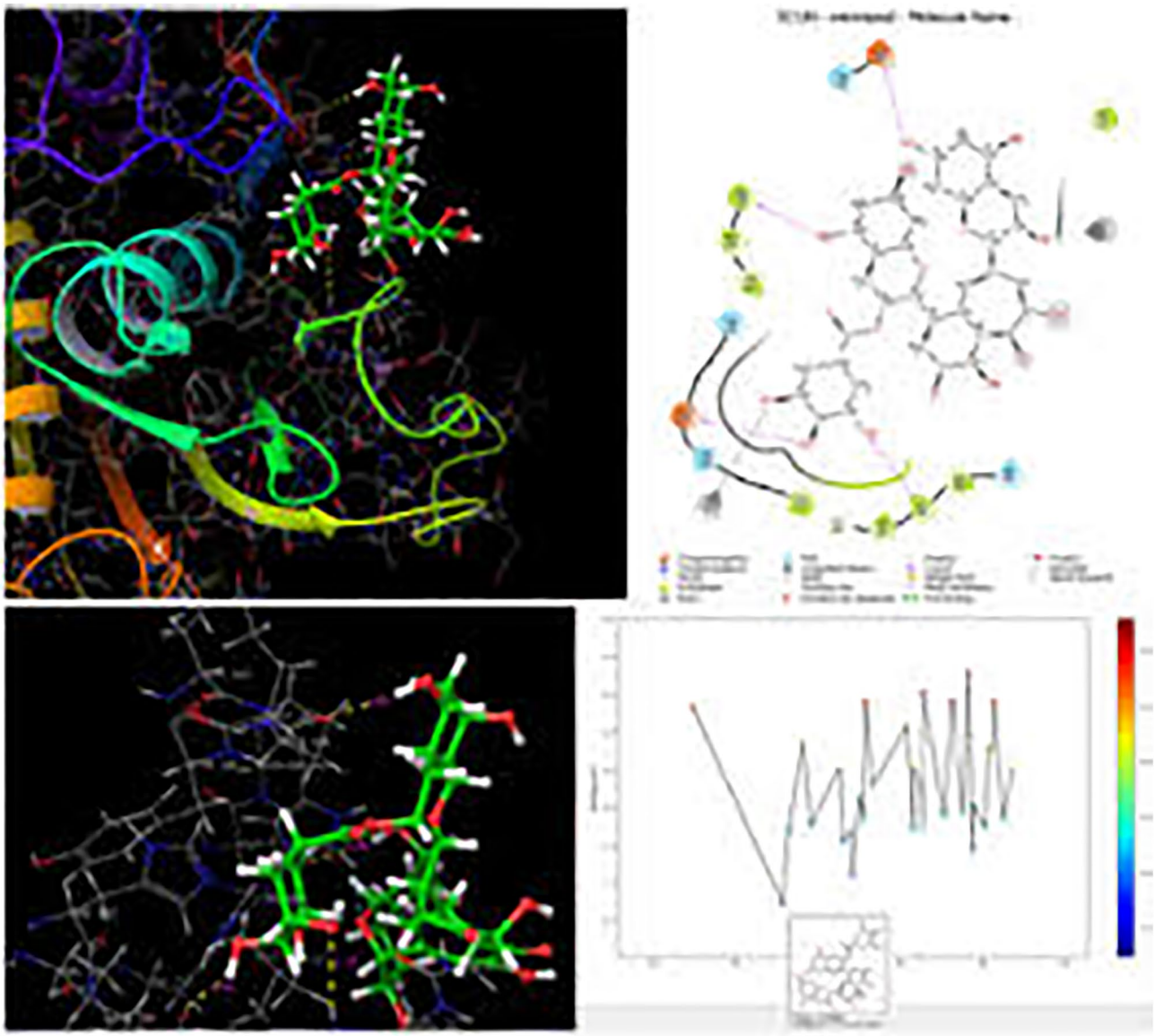


Fig. 7 Docked structure of TF-2b with MMP-9 enzyme, 32 docked results, 2D illustration of binding interactions of TF-2b and interacting residues, 3D hydrogen bonds between TF-2b and MMP-9

0.477. In the current study, TF-2a and TF-2b values were calculated as 0.453 and 0.438 (log mg/kg/day), respectively. Thus, the scores are found low at toxicity. Apart from that, P-glycoprotein (P-gp) is also involved in removing foreign substances from cells. According to the pkCSM model, tested TFs were identified as P-gp substrates. The modulation of P-gp-mediated transport can have a substantial impact on the pharmacokinetics of P-gp substrates, potentially influencing their absorption, distribution, metabolism, and excretion. Considering pkCSM results, TF-2a and TF-2b were evaluated as both P-glycoprotein I and II inhibitors. Evaluating the excretion properties, OCT2 is an important transport protein in the renal clearance of drugs and endogenous compounds. Besides, OCT2 substrates may have the potential to cause adverse interactions when used with OCT2 inhibitors. Examining whether a candidate molecule can be transported by OCT2 provides useful information not only about clearance but also about possible contraindications. In this study, it was predicted that not all compounds have the potential to be OCT2 substrates, which is also in favor of forming TF-based oral products.

Among the polyphenolic compounds in black tea (Camellia sinensis), TF-2a and TF-2b are frequently studied [12, 37], which justifies the evaluation of varnishes containing these compounds. In addition to the active ingredients (TF-2a and TF-2b), the composition of dental varnishes should

include resin to ensure film formation on tooth surfaces, mechanical stability, and controlled release [38]. Analysis of the results revealed that varnishes containing only polymers (PBV groups) achieved superior mineral density (MD) results compared to those with only resin (RBV groups) or combined formulations (RPV groups) (Table 4). While resins may enhance adhesion to tooth surfaces, the polymers used in this study appear to improve the release of TFs, thereby reducing mineral loss. In addition, black tea's ability to adsorb onto the salivary pellicle and increase its resistance to surfactants [10] may contribute to the improved anti-erosive potential observed. In the current experiment, TF-2b varnishes yielded better MD results than TF-2a formulations, although the differences were not statistically significant (Table 3). This finding is consistent with the study by Guo et al. [12]. Future research should focus on optimizing TF-2b formulations to enhance their anti-erosive effects.

The current study also steps into the possible effects of the experimental TF varnishes on the MMP enzymes and compares it to a commercial Fluoride varnish. Specifically, MMP-2 and -9 contribute to the progression of ETW by initiating their proteolytic action if they are activated in an acidic environment [39]. The dentin surfaces were initially immersed in CA solution to stimulate the endogenous MMPs in dentin, and erosive cycling was performed following the application of both control (Fluoride) and experimental varnishes. Fluorides are, especially NaF, known for their anti-MMP activities by avoiding Zn^{+2} and Ca^{+2} ions, which are compulsory to activate the catalytic activities [40]. Regarding the results, it is plausible to obtain Fluoride varnish (5% NaF) as the "Control group (positive)" providing significantly the lowest MMP-2 and -9 levels. Significantly superior results of the control group may be attributed to the higher NaF concentration of 50,000 ppm (according to the manufacturer). It was reported that there is an inhibitory effect of fluorides that is irreversible at higher concentrations, such as 5,000 ppm, on MMP enzymes [41]. On the other hand, there is only one study that evaluated the MMP levels of theaflavins in dental tissues. Even though higher concentrations, such as 4% and 8%, were reported to prevent dentin erosion, only 1% of TF solution had significantly reduced the MMP-2 and -9 levels [12]. The TF concentrations added to the experimental varnishes are approximately 1%, but still, a decrease of > 75% in MMP-2 and MMP-9 levels was determined in the current study. Even though the high binding energy scores of both TFs were proved, it is also plausible that the tested TF-based varnishes have the possibility to inhibit the actions of MMP-2 and -9. TF-2b varnishes based on either resin (RBV) or polymer (PBV) determined significantly lower levels of MMP than all the groups based on TF-2a, except for the RPV group. It is noticeable that the tested fluoride varnish is also resin-based. Thus, it may be beneficial to utilize the advantages of tested TF-2b varnishes specifically with the resin-based ones on the early ETW cases to avoid further DOM deterioration caused by MMPs. TF-2a–RPV provided superior MMP expressions without any significantly different ΔMD results than other groups. In the current study, the docking results provided information about the binding mechanism, including binding mode, key amino acids involved in binding sites, and intermolecular interactions. According to the results, TF-2a showed numerically higher binding energy scores than TF-2b. The binding energy of TF-2a with MMP-2 and MMP-9 was found to be −7.40 to −8.52 kcal/mol, and the binding energy of TF-2b with MMP-2 and MMP-9 was found to be −6.80 to −7.75 kcal/mol, indicating that TF-2a and TF-2b could be effective against the activity of MMPs. The negative values of the binding energies of all complexes indicated that these interactions occurred thermodynamically spontaneously and were also found to be better than the previously found binding energies of −5.17 and −5.93 kcal/mol for the same molecules [12].

The numerous polyphenols and OH^- groups that form the structure of TF-2a ensured binding by making five hydrogen bonds in the MMP-2 binding site. Besides, according to ADME profiles, the molecular weights of TF-2a and TF-2b are obviously high (718,61 and 716,60 g/mol, respectively). The OH^- distribution and the high molecular weight of the structure are supposed to increase binding to the receptors, and that exact structure results in an intermolecular reaction with MMPs; thus, assuming an inhibitory effect. It was also determined that TF-2b interacted with similar residues in the binding site of MMPs. As a result of ADMET analyses (showed in Supplementary file), it was estimated that TF-2a and TF-2b comply with many of the drug-likeness rules, and gastrointestinal absorption is not high. It was determined by various prediction tools that there are no blood–brain barrier, CNS permeability, or hepatotoxic effects; thus, they could be reliably involved in oral formulations.

Within the limitations of the present study, it may be assumed that theaflavin-based varnishes showed promising results in terms of higher ΔMD values of Theaflavin 2b-based varnish (Group 4) on eroded dentin. Besides, the expression level of the resin–polymer-based varnish (Group 3) and binding energy of Theaflavin 2a to MMP enzymes were remarkable. However, artificial saliva was added to simulate the oral cycle completely so, it may have accelerated the MD values numerically. Thus, it may be appropriate to use distilled water in further methodologies.

Conclusion

The current study determined to find the superior TF (2a or 2b) and form a suitable, non-sticky varnish that can be spread by a microbrush so, the same concentrations of TFs (approx. 1%) were used. Higher TF concentrations may be evaluated in different acidic media to simulate both intrinsic and extrinsic etiologies of dental erosion. Last but not least, further studies may also focus on the investigations to analyze the clinical effects, such as release rate by time or remineralization.

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Data availability Data sharing could not be applicable to this article since there is no repository for the data.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

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