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The impact of a-tomatine on shear bonding strength in different dentin types and on cariogenic microorganisms: an in vitro and in silico study

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Abstract

Introduction The objective of this study is to investigate the shear bonding strength of a glycoalkaloid, also a novel matrix metalloproteinase enzyme known as α-tomatine, on two different surfaces of dentin (sound & caries-affected) and its efficacy against cariogenic microorganisms using in vitro and in silico methods.

Methods The effect of a-tomatine at different concentrations (0.75 / 1 / 1.5 µM) on shear bonding strength in caries-affected and sound dentin was also investigated ($n = 10$; each per subgroup). The analysis of shear bonding and failure tests was conducted after a 24-hour storage period. Fracture surfaces were examined under a scanning electron microscope. A stock solution 3 mM of a-tomatine was prepared for antimicrobial evaluation. Antimicrobial activities of the agents against *Streptococcus mutans* ATCC 25175, *Lactobacillus casei* ATCC 4646, and *Candida albicans* ATCC 10231 standard strains were investigated by microdilution method. In addition, through the method of molecular docking and dynamic analysis, the affinity of a-tomatine for certain enzymes of these microorganisms was examined.

Results The pretreatment agent and dentin type significantly influenced shear bonding strength values ($p < 0.05$). As the molarity of a-tomatine increased, the bonding value decreased in sound dentin, while the opposite was true in caries-affected dentin. According to molecular docking and dynamic analysis, the highest affinity was observed in *L. casei*'s signaling protein. Microdilution assays revealed a-tomatine to exhibit fungicidal activity against *C. albicans* and bacteriostatic effects against *S. mutans*. No antimicrobial effect was observed on *L. casei*.

Conclusion a-tomatine demonstrates a positive impact by serving as both a pretreatment agent for bonding strength and an inhibitor against certain cariogenic microorganisms.

Keywords a-tomatine, Candida albicans, Caries-affected dentin, Cariogenic microorganisms, Dental caries, Lactobacillus casei, Streptococcus mutans

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Introduction

Dental caries is an infectious multifactorial disease that induces damage to the hard tissue of the tooth [1]. The onset of the demineralization process in the tooth occurs as a result of various factors converging, a phenomenon often referred to as the Keyes diagram, with the most significant factors being the formation of dental plaque on the tooth surface due to its adhesion capability and the subsequent presence of microorganisms leading to caries [2]. Many bacteria mentions in the formation of dental caries, especially *mutans streptoccocci* and *lactobacilli* stand out as the primary bacteria and called cariogenic microorganisms. Among these microorganisms, mutans streptococci are particularly notable for their ability to synthesize extracellular polysaccharides, sustain persistent colonization on the hard surfaces of teeth, contribute to the development of the biofilm matrix, metabolize carbohydrates (acidogenicity), and survive in low pH environments (aciduricity), thereby initiating the first stage of caries development [3]. As stated in the extended ecological plaque hypothesis, the balance of acidogenic and aciduric phases within the biofilm is maintained through the activities of other microorganisms, thus perpetuating the progression of carious lesions [4, 5]. Furthermore, it has been determined that high counts of these microorganisms in oral environment also show a positive correlation with DMFT (Decayed - Missing - Filled Teeth) scores [6, 7]. In recent years, studies have also been put forward that adopt *candida* to play a pathogenic role on dental caries [8].

With ongoing research advancements in adhesive technology, coupled with the introduction of new restorative materials to the market, there has been notable progress in adhering resin-based materials to dental tissues, particularly caries-affected dentine, following the adoption of a minimal invasive principle [9–11]. The latest generation of universal adhesives combines primer with adhesive resin, offering a structure characterized by low technique sensitivity and enabling rapid application in clinical settings. Moreover, they can be applied in different modes such as self-etch and etch-and-rinse [10, 12].

In recent years, various innovative methods have been explored to enhance the bonding strength of adhesive systems. Nanotechnology has been employed to incorporate different materials into adhesives, aiming to augment their adhesive properties [13, 14]. Additionally, the use of dentin deproteinization agents [15], the application of adhesives in different methods (such as extended primer) [16], and the utilization of specific materials such as silver diamine fluoride [17] have been investigated. Moreover, diverse matrix metalloproteinase (MMP) inhibitors, primarily chlorhexidine, have been explored as innovative approaches [14, 18–20] to enhance bonding durability.

Many agents which are mentioned above are applied to dentin surfaces with the aim of enhancing bond strength [19, 21], and research is underway to discover new agents for this purpose [18, 22, 23]. One of these agents is a-tomatine, a typical glycoalkaloid found in the roots, which has an important role against potential pathogens and can be detected in all tomato genotypes [24] (Fig. 1). Tomatine consists of a tomatidine (the aglycone part), two glucose molecules, one xylose, and one galactose contained within a ß-lycotetraose (the tetrasaccharide part) [25, 26]. It has been found that a-tomatine can inhibit certain matrix metalloproteinase (MMP-2 & MMP-9) enzymes, thereby preventing metastasis in various cancer studies [27, 28]. Furthermore, It is stated that a-tomatine has antimicrobial effects on various parasites, bacteria and fungi [29]. In the context of superior bond strength related to a-tomatine via inhibition of MMP-2
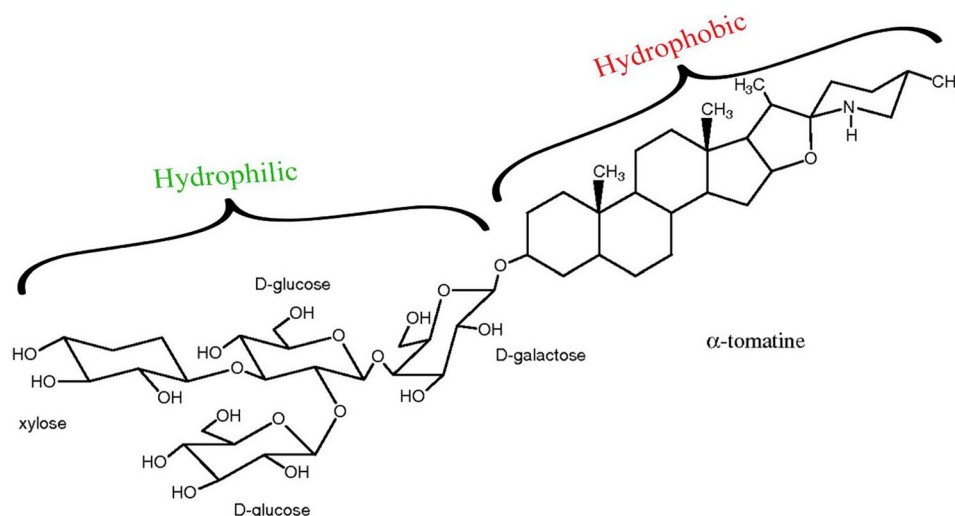

Fig. 1 The chemical structure of a-tomatine

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& 9, limited research is available in the literature, which has been found that a-tomatine provides better short- and long-term outcomes in terms of bonding strength compared to chlorhexidine [18]. Nevertheless, no studies have been conducted to investigate the efficiency of a-tomatine on cariogenic microorganisms in the oral microbiota.

In light of this information, this study aims to explore the antimicrobial activity of a-tomatine against a range of cariogenic microorganisms, to evaluate this antimicrobial efficacy using in silico methods, specifically through molecular docking techniques, and to assess the comparative effectiveness of a-tomatine on shear bonding strength in different types of dentin (caries-affected and sound). The first hypothesis asserts that a-tomatine exhibits antibacterial/antifungal efficacy against cariogenic microorganisms, a claim further validated by in silico methodologies. The second hypothesis suggests that increasing the molarity of a-tomatine will result in higher bond strength values regardless of dentin types.

Materials and methods

Sample calculation and ethical committee approval

Prior to commencing the study, approval was obtained from the Altinbas University Clinical Research Ethics Committee (File no: 2023/198). Power analysis was conducted using the G*Power (3.1.9) software to determine the sample size. The study's power, denoted as 1-β (β = Type II error probability), was calculated with a "d" value of 2.60, obtained using data from the study by [23]. It was determined that, to achieve a 95% power level at an α:0.05 significance level, at least 5 teeth should be used in each subgroup. Accounting for potential losses in each group, the study was planned to be conducted with 10 teeth.

The formation of the caries-affected and sound dentin and determination of experimental groups

In the study, All molar teeth extracted due to carious or periodontal reasons were employed. Before the study,

the teeth were soaked in a chloramine T solution for one week and then stored in a refrigerator at 4 °C in distilled water until the experimental phase. The teeth were prepared for measurement by fixing them onto sleeve-like silicone molds using cold acrylic (Takilon, Istanbul, Türkiye), ensuring only the crown portions were visible. Then, were embedded up to the cervical level and their occlusal surfaces were dried using the air/water spray. Subsequently, a caries-detecting device called DIAGNOdent (KaVo Dental, Biberach, Germany) was employed to distinguish between sound and caries-affected teeth. According to the manufacturer's instructions, the device was used with the A tip designed for fissure and proximal surfaces to evaluate teeth in terms of caries. Based on measurement values, teeth with scores ranging from 0 to 5 were categorized as sound, while those with scores between 26 and 35 were identified as caries-affected teeth. Afterward, a low-speed microtome (Isomet High Speed Pro, Buehler Precision Saw, Düsseldorf, Germany) with a diamond blade under water cooling was employed to cut the enamel-dentin border from 2 to 3 mm below. For teeth with caries-affected dentin, potential enamel prisms were removed by sanding with 400-grit followed by 600 grit silicon carbide paper for 60 s. In sound dentin, only the 600-grit sanding process was performed.

The preparation for a-tomatine and dimethyl sulfoxide

The powdered a-tomatine (phyproof© PhytoLab, Germany) material was dissolved in dimethyl sulfoxide liquid (DMSO) with the help of a precision balance [28]. Firstly, 9.31 mg of a-tomatine was weighed and dissolved in DMSO (Merck, Sigma Aldrich, Darmstadt, Germany-M116743.1000) to obtain a stock concentration of 3 mM. According to the literature, the percentages of a-tomatine materials were determined as 0.75, 1, and 1.5 μM [18]. Also, DMSO was used in a purity form. All consumable materials used in the study are presented in Table 1.

Table 1 The content information of the materials used in the study

Material	Composition	Manufacturer
Dimethyl Sulfoxide Extra Pure	Dimethyl Sulfoxide C_2H_6OS (Purity ≥ 99.0%)	Merck, Sigma Aldrich, Germany
Etching Dental Gel	37.5% phosphoric acid gel	Kerr, Gel Etchant, Orange, USA
Clearfil Tri-S Bond Universal	10-MDP, Bis-GMA, HEMA, colloidal silica, ethanol, silane, sodium fluoride, camphoquinone, ethanol, water	Kuraray Noritake Dental Inc., Okayama, Japan
FiltekTM Z250	Organic matrix: BIS-GMA, UDMA, BIS-EMA Inorganic matrix: zirconia/silica as a filler, the loading of the inorganic filler (without treatment with silane) is 60% by volume with particle size in the range of 0.01–3.5 μm.	3 M ESPE, St Paul, MN, USA
a-tomatine	a-tomatine (powdered)	PhyProof, PhytoLab GmbH Dutendorfer, Germany

The stage of restoration and the calculation of shear bond strength

An untreated group served as the control group, resulting in a total of 10 subgroups. A total of 100 molar teeth were used in the study, with 10 teeth used for each group. Prior to restoration, DMSO and a-tomatine materials with different molarities were applied as pretreatment agents to the obtained flat dentin surfaces using an applicator for 20 s. The excess agent was removed with absorbent paper [18]. Subsequently, to ensure standardization, a uniform composite restorative material (Filtek Z250, 3 M ESPE, USA) and a universal type adhesive agent (Clearfil™ Tri-S Bond, Kuraray Noritake Dental Inc:, Tokyo, Japan) were applied in an self & etch mode by a single operator (M.K.Ü) and polymerized (Valo, Ultradent Inc., Utah, USA). During the restoration phase, restorations with a diameter of 2 mm and a thickness of 2 mm were built up using a silicone model. Before the shear bond test, all specimens were stored at 37 °C for 24 h. Subsequently, shear bond strengths were measured using a universal testing machine by applying a force ranging from 0 to 400 N to each specimen with a knife-edge type blade at a crosshead speed of 1 mm/min (Universal test device, Mod Dental, Turkey). The values at the moment of fracture were recorded and converted from N to mPa to calculate the bonding strength. All data were averaged to obtain a single bond strength value for each tooth. After the shear bond strength test, fractured surfaces were examined under a stereomicroscope at ×40 magnification. Failure modes were classified as cohesive, adhesive, or mixed failure. SEM imaging was performed with one sample from each group at x10000 magnification.

Bacteria repertoire and evaluating the antimicrobial activity of a-tomatine

In the present study, the antibacterial and antifungal activities of a-tomatine against *S. mutans*, *L. casei* and *C. albicans* were investigated. 3mM stock solution of a-tomatine was used for experimental solution while 2% chlorhexidine gluconate (CHX) solution was chosen as the positive control and DMSO was used as the negative control group. *S. mutans* ATCC 25175, *L. casei* ATCC 4646 and *C. albicans* ATCC 10231 strains was used in this study. Each experiment was pre-cultured by placing a single colony into 10 ml of brain-heart infusion medium (BHI, Merck KGaA 64271 Darmstadt, Germany) for bacteria and Sabouraud dextrose agar (SDA, Merck KgaA) for the yeast. It was then incubated at 37 °C for an overnight under anaerobic conditions. The preculture was diluted 1:20 in BHI and then the bacterial suspensions were incubated for an extra 2.5 h. The turbidity of the suspension was adjusted to the McFarland 0.5 turbidity standard (final concentration of $\sim 1.5 \times 10^8$ cfu/ml).

The Minimum inhibitory concentration (MIC) of the experimental and positive control groups against the strains were tested by broth microdilution method approved by CLSI and performed in 96 flat-bottomed microtiter plates (TPP, Switzerland). Each well of the test solutions was added to the first test well and mixed. Serial two-fold dilutions was then prepared BHI or SDA broth with a final volume of 100 µL, and 100 µl of the bacterial suspensions was then added to each well. The microplates were incubated at 37^0C for 24 h. MIC was defined as the lowest concentration of the test compound to inhibit growth.

The minimum bactericidal/fungicidal concentration (MBC/MFC) was evaluated by subculturing 10 µl of microbial suspension from wells showing no visible growth in the MIC tests on agar plates. MBC/MFC was determined as the lowest concentration without microbial growth after 24 h of incubation at 37 °C.

The microbicidal effect was obtained by calculating the MBC or MFC/MIC ratio. Accordingly, products with a ratio of ≥4 were considered to be microbiostatic (bacteriostatic/fungustatic) effective, and products with a ratio of <4 were considered to be microbicide (bactericide/fungusic) effective. Experiments were conducted in triplicate, and the median of the three independent experiments was taken as the MIC and MMC values.

Molecular docking analysis
Computational method
The Maestro [30] interface of Schrödinger software company and its embedded modules were used in all molecular modeling studies.

Preparation of ligands
Three-dimensional structures of a-tomatine molecule used in molecular docking and dynamics calculations were downloaded from PubChem. Preparation of the ligands for molecular docking was done with the Lig-Prep [31] module in Maestro. EPIK [32] embedded software was used to estimate the ionization states of the two ligands around the oral pH of 6.4 and the pH state was set to 6.9, giving a standard deviation of 0.5 [33]. Tautomerization was considered and minimization was performed using the OPLS3e [34] force field.

Preparation of proteins
The Protein Preparation Wizard [35] in Maestro was used for all protein construct preparation. The protein crystal structure of the dextranase enzyme isolated from *S. mutans* with PDB ID 3VMP [36] was used to model a-tomatine's inhibition of plaque formation induced by *S. mutans*. The purpose of choosing this enzyme is that it has a high potential for action against the enzymes produced by the bacteria. The intracellular

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signal transducing protein crystal structure, PDB ID 5MTU [37], isolated from *L. casei*, was chosen because inhibition of this enzyme by a-tomatine has the potential to reduce the survival rate of *L. casei* in the life cycle. Aspartic Protease enzyme isolated from *C. albicans* with PDB ID 1ZAP code was preferred in molecular modeling studies because it would reduce the survival rate of the yeast by acting as a protease inhibitor of a-tomatine. All of the identified target proteins were subjected to separate protein preparation. The protein preparation process included the methods we used in our previous studies [38–40]. Accordingly, the target protein was imported with Maestro's Protein Preparation Wizard. The missing amino acids of the imported proteins, if any, were completed and the status of ionizable amino acid residues was estimated by preprocessing using PROPKA [41] with a pH value of 6.9 and a standard deviation of 0.5. Since molecular dynamics calculations will be performed in the following stages of the study, the crystal waters were left unerased in order to obtain more realistic results. The hydrogen bonds were then optimized for pH 6.9. Finally, the protein structure was prepared by minimization using the OPLS3 [42] force field.

SiteMap and receptor grid generation
Since the structures of cogtene ligands in protein structures have quite different molecular shapes than bulky ligands such as a-tomatine, the cognate ligands were deleted and the binding sites, including the regions where the cognate ligands were found, were redetermined using Maestro's SiteMap tool [43]. These regions were recorded for use in molecular docking with the Receptor Grid Generation tool.

Ligand docking
The affinities of a-tomatine molecule prepared for molecular docking to protein structures were determined using Maestro Glide [44] module. The Ligand Docking tool in Maestro was used for this purpose. Considering the state of the protein structure, Scalling Factor was set to 0.75 and Partial Charge Cutoff to 0.17 in Ligand Docking and molecular docking was initiated by keeping the other settings at default values.

System setup
In preparation for molecular dynamics simulations, protein-ligand complexes obtained by molecular docking were immersed in a solvent box of 10 Å x 10 Å x 10 Å water molecules. The water molecules in the solvent box were requested to be at the SPC [45, 46] setting to obtain more realistic results. In the SPC setting, the bond lengths in water molecules are fixed to 1 Å and the angle between hydrogen atoms is kept at 109.5 degrees. Finally, in order to model the physiological environment

accurately, NaCl at a concentration of 0.15 M was added to the solvent box using the Monte Carlo method and the System Setup process was carried out using the OPLS3 force field.

Molecular dynamics simulation
The final step of our computational approach involved performing molecular dynamics simulations. Molecular dynamics simulation was carried out for each protein structure separately, with the same settings, using the Desmond [47] module in Maestro, and specific protocols were applied. When molecular dynamics simulations each involved examining the motion of protein-ligand complexes in the solvent environment within a small time step, precise control of the parameters of temperature, pressure, and particle number was required. For this purpose, the pressure was kept constant at 1.01325 bar using the [48] barostat, the temperature was kept constant at 310 K using the Nosé–Hoover thermostat [49], and Ensemble Class NPT was selected. The simulation was created in two stages. One of these included a relaxation phase of 2 ps and a simulation production phase of 100 ns. Molecular dynamics simulations were initiated for the a-tomatine & aspartic protease, a-tomatine & signaling protein, and a-tomatine & dextranase complex with these settings.

Scanning electron microscobe (SEM) analysis
The SEM images of samples from each group ($n=1$; each per group) were captured using the FEI Versa 3D Dual Beam SEM equipped with a secondary electron (SE) detector at an acceleration voltage of 2.0 kV, to investigate the impact of different types of dentin and pretreatment agents on shear bonding strength. The morphology of the samples obtained using SE detectors is presented in Figs. 2 and 3. The images were captured at a magnification of x10000 for a clearer view of the fracture surfaces.

Statistical analysis
All data were subjected to statistical analysis using a software program (GraphPad Prism 10, 225 Franklin Street, Boston, USA). Descriptive statistical methods (mean, standard deviation, median, minimum, maximum) were employed for data analysis.

The appropriateness of the normal distribution for quantitative data was evaluated utilizing a Kolmogorov–Smirnov test, considering the sample size $n>50$, as well as examining skewness–kurtosis values and conducting graphical evaluations through Q-Q Plots. Subsequently, a homoscedasticity test was conducted to determine whether the variances were distributed homogeneously. Additionally, two-way ANOVA followed by post-hoc Tukey (HSD) test was conducted to analyze the data. The significance level was considered at least $p<0.05$.
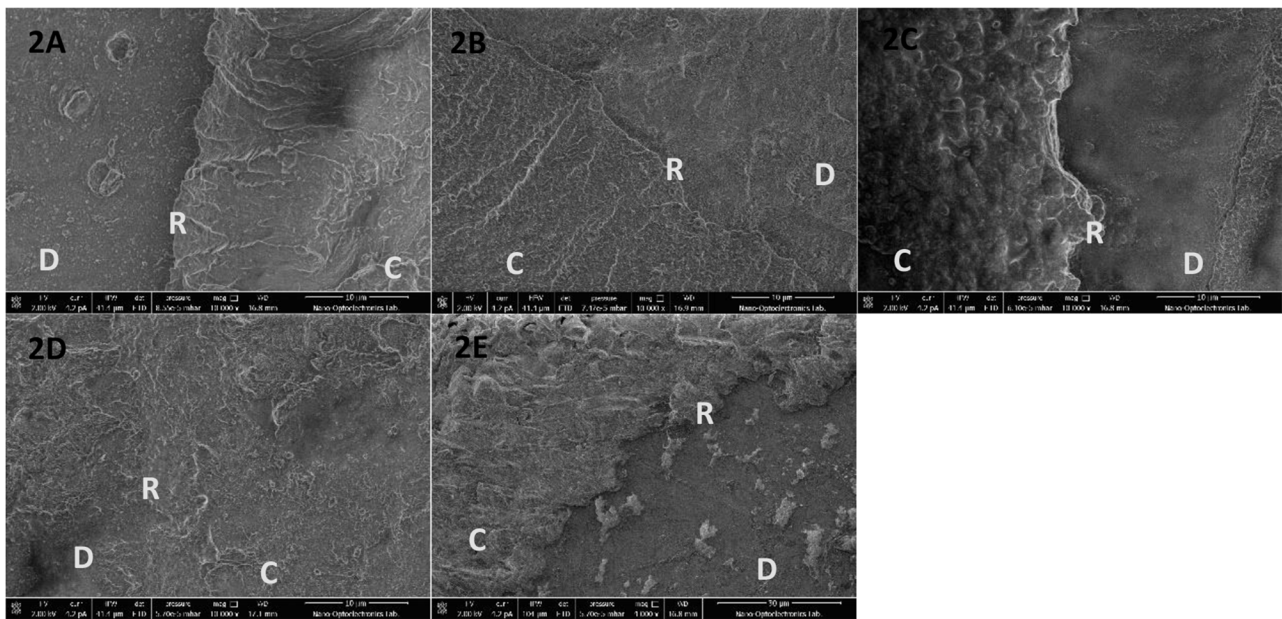
Fig. 2 SEM images of each subgroup in sound dentin (**A**: control, **B**: DMSO, **C**: a-tomatine with 0,75 µM, **D**: a-tomatine with 1 µM, **E**: a-tomatine with 1.5 µM). *Abbreviations*: Composite (C), Dentin (D), Resin Tag (R)

Fig. 3 SEM images of each subgroup in caries-affected dentin (**A**: control, **B**: DMSO, **C**: a-tomatine with 0,75 µM, **D**: a-tomatine with 1 µM, **E**: a-tomatine with 1.5 µM). Abbreviations: Composite (C), Dentin (D), Resin Tag (R)
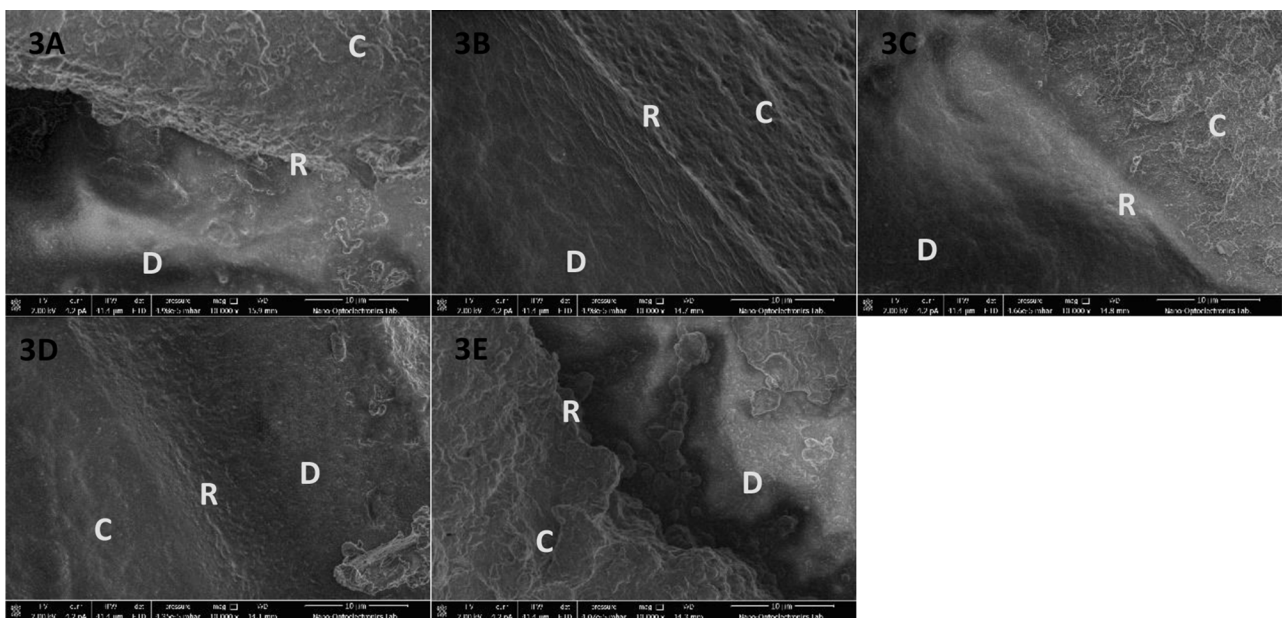
Table 2 The two-way ANOVA findings for dentin type and pretreatment agents (tests of between-subjects effects)

Source	Sum of squares	F	p
Pretreatment agent	155.2	5.811	**0.0003***
Dentin type	71.45	10.70	**0.0015***
Pretreatment agent * Dentin type	369.9	13.85	**<0.0001***

*$p < 0.05$ $p < 0.01$**

Results

The findings of shear bond strength

The bond strength values exhibit significant variations contingent upon both the dentin type and the specific pretreatment agent employed ($p < 0.05$). When both variables are concurrently considered, a notably robust and statistically significant distinction emerges ($p < 0.0001$) (Table 2). In sound dentin, the DMSO group manifested the lowest recorded bond strength value, whereas in

Table 3 The results of shear bond strength values according to different dentin types and pretreatment agents

	Control	DMSO	a-tomatine (0.75 µM)	a-tomatine (1 µM)	a-tomatine (1.5 µM)
Sound	15.28 ± 2.46 **B, Ca**	12.10 ± 2.83 **Ca**	19.44 ± 1.56 **Aa**	17.01 ± 4.35 **A, Ba**	16.14 ± 0.75 **A, Ba**
Caries-affected	10.63 ± 1.06 **Bb**	17.25 ± 2.25 **Ab**	13.57 ± 2.73 **A, Bb**	14.81 ± 2.58 **Aa**	15.26 ± 3.21 **Aa**

Two-way ANOVA with Tukey's post-hoc analysis ($p < 0.05$)

Different uppercase letters in the same row indicate a statistically significant difference between the columns

Different lowercase letters in the same column indicate a statistically significant difference between the rows

Table 4 The number and percentage representation of failure mode

Dentin type	Pretreatment agent	Adhesive	Cohesive	Mixed
Control	Control	4 (%40)	4 (%40)	2 (%20)
	DMSO	4 (%40)	2 (%20)	4 (%40)
	a-tomatine (0.75 µM)	3 (%30)	4 (%40)	3 (%30)
	a-tomatine (1 µM)	7 (%70)	1 (%10)	2 (%20)
	a-tomatine (1.5 µM)	8 (%80)	1 (%10)	1 (%10)
Caries-affected	Control	6 (%60)	3 (%30)	1 (%10)
	DMSO	2 (%20)	6 (%60)	2 (%20)
	a-tomatine (0.75 µM)	6 (%60)	2 (%20)	2 (%20)
	a-tomatine (1 µM)	5 (%50)	1 (%10)	4 (%40)
	a-tomatine (1.5 µM)	4 (%40)	3 (%30)	4 (%40)

caries-affected dentin, the DMSO group yielded the highest values. Particularly in sound dentin, it was observed that the group treated with 0.75 µM a-tomatine exhibited the highest bond strength value, while an increase in the molarity of a-tomatine resulted in a decrease in bond strength values. Conversely, in caries-affected dentin, a linear relationship was observed, with bond strength values increasing as the molarity of a-tomatine increased. In addition, the control group within the caries-affected group displayed the lowest bond strength value (Table 3). The distribution of fracture types resulting from the shear bond strength test is presented in Table 4.

The detection of antibacterial efficacy and the verify of the findings via molecular docking analysis

The results of the antimicrobial activities of the study and control groups against the tested bacterial and fungal strains are presented in Table 5. a-tomatine was found to be fungicidal against *C. albicans*, while it was found to be bacteriostatic against *S. mutans*. No antimicrobial effect was found on *L. casei*. Chlorhexidine was found to be bacteriostatic on *S. mutans* and bactericidal and fungicidal on *L. casei* and *C. albicans*, respectively. DMSO had no antimicrobial effect on the tested organisms (Table 5).

Molecular docking

The molecular docking method was successfully applied to determine the affinities of a-tomatine compound to the dextranase enzyme isolated from *S. mutans*, the signal transducing protein isolated from *L. casei*, and the aspartic protease enzyme isolated from *C. albicans*. Validation of the dockings to PDB ID:3VMP coded dextranase enzyme, PDB ID:5MTU coded signal transduction protein and PDB ID:1ZAP aspartic protease enzyme were performed by removing the cognate ligands from their active sites and reinserting them and are shown in Fig. 4. It has been previously proven that Maestro and its modules provide reliable results in the molecular docking method [50, 51]. Docking scores obtained by molecular docking method are given in Table 6.

a-tomatine exhibited significant affinity for the enzyme dextranase isolated from the bacterium *S. mutans*. The hydrophilic region consisting of D-glucose, D-galactose and xylose structures of a-tomatine compound is associated with dextranase. The Asp258, Glu496, Tyr307, Asp385 residues in the Dextranase enzyme and the hydroxyl ends attached to the sugar structures in the hydrophilic region in the a-tomatine compound support this interaction by hydrogen bonding. In addition, the pockets formed by the hydrophilic regions in Dextranase are in complete agreement with a-tomatine and it is seen in Fig. 5 that the a-tomatine compound covers these

Table 5 MIC, MBC and MBC/MIC ratio for the formulations against the selected microorganisms

No	Name	S.mutans ATCC 25175			L.casei ATCC 4646			C.albicans ATCC 10231		
		MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MFC	MFC/MIC
1	a-tomatine (3mM)	0.25 *(0.75)*	-	-	0,5	-	-	0.125 *(0.375)*	0.125 *(0.375)*	1*
2	Chlorhexidine (%2)	0.0004 *(8×10^{-6})*	0.0016 *(32×10^{-6})*	4	0.0009 *(18×10^{-6})*	0.0018 *(36×10^{-6})*	2*	0.0625 *(125×10^{-5})*	0.0625 *(125×10^{-5})*	1*
3	DMSO	-	-	-	-	-	-	-	-	-

*Bactericidal/fungicidal MBC & MFC / MIC < 4

(The values in the table indicate dilution ratios. Accordingly, the initial concentrations for the dilutions can be calculated by multiplying the values in the table with the respective MIC or MBC. For example, the MIC value of a-tomatine for S. mutans is 3 mM x 0.25 = 0.75 mM, whereas the MIC and MFC values for C. albicans are equal, calculated as 3 mM x 0.125 = 0.375 mM. The MIC value of chlorhexidine for S. mutans is $0.02 \times 0.0004 = 8 \times 10^{-6}$, while the MBC value is $0.02 \times 0.0016 = 32 \times 10^{-6}$. The corresponding concentrations are presented in italics in the table)
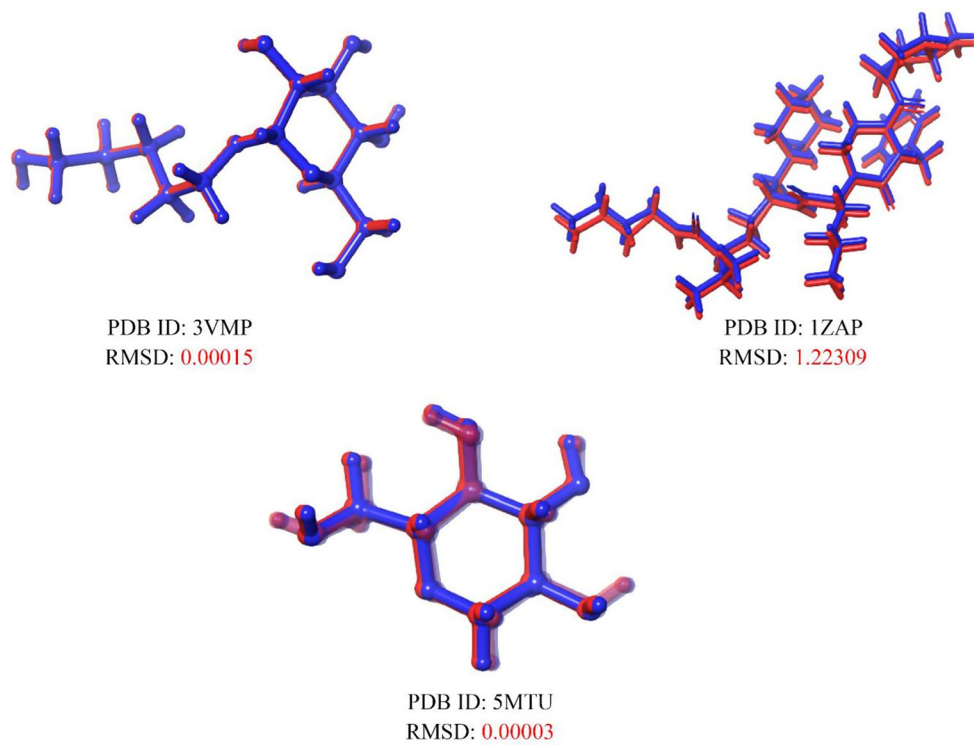
Fig. 4 Validation of all protein structures used in molecular docking calculations. The blue colour represents the cognate ligand and the red colour redocking the reattached ligands

Table 6 Docking scores(kcal/mol) of a-tomatine compound

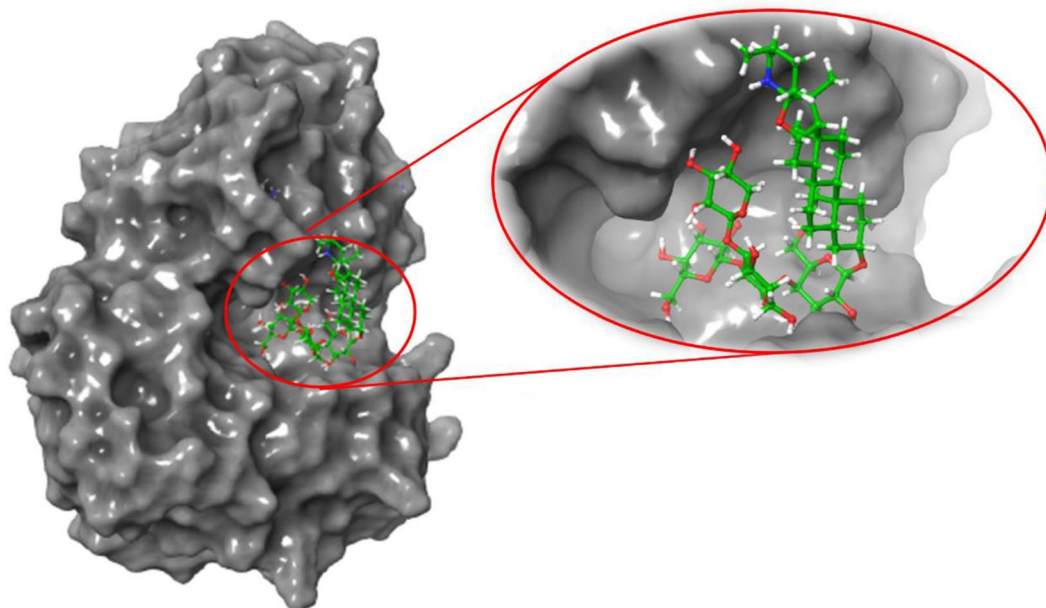|	Streptococcus mutans Dextranase PDB ID: 3VMP	Lactobacillus casei Signal Protein PDB ID: 5MTU	Candida albicans Aspartic Protease PDB ID; 1ZAP
a-tomatine	-14.15	-18.54	-11.55

Fig. 5 How a-tomatine binds to the enzyme *Streptococcus mutans*-Dextranase (PDB ID: 3VMP)

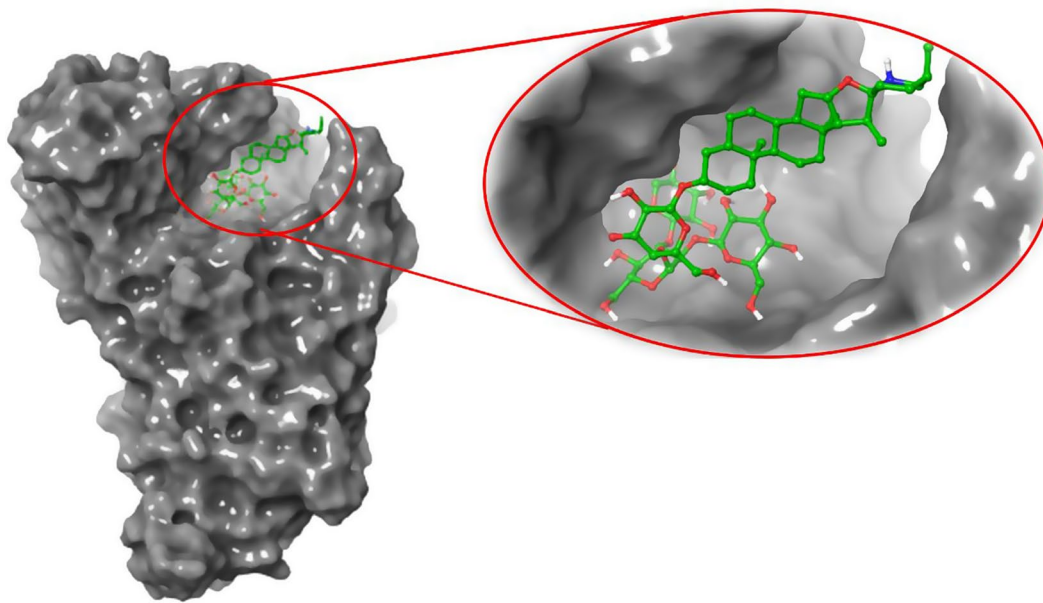Ucuncu *et al. BMC Oral Health*   (2024) 24:1220

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Fig. 6 How a-tomatine binds to the *Lactobacillus casei*-Signal Transduction Protein (PDB ID: 5MTU)
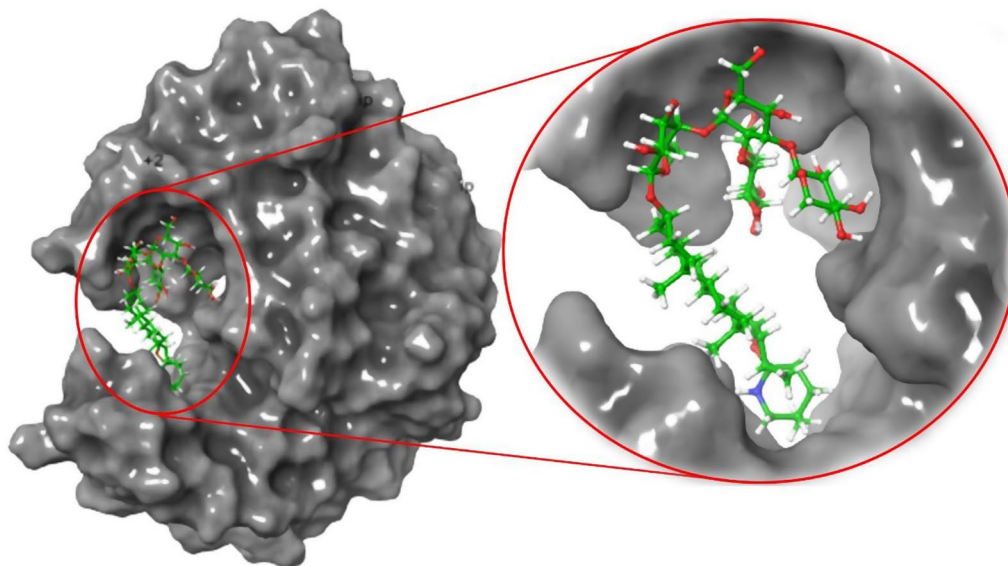

Fig. 7 How a-tomatine binds to the enzyme *Candida albicans*-Proteaz (PDB ID: 1ZAP)

pockets well. While the hydrophilic region in the a-tomatine compound is embedded in the protein structure, the hydrophobic region in the compound protrudes outside the protein structure without any interaction.

Figure 6 shows that both the hydrophilic region and the hydrophobic region of a-tomatine are embedded in the *L. casei*-signal protein structure. This docking on the signal transduction protein takes place especially around the Asn216 residue. Asn216 residue is inserted between D-galactose and D-glucose structures. In addition, potential strong interactions with residues Ans235, Asn55 and

Asn162 were identified as a result of molecular modeling studies.

Figure 7 shows the protein structure of the aspartic protease enzyme isolated from *C. albicans* resembling a "U" shape and the docked structure of the a-tomatine compound. Both the hydrophilic part and the hydrophobic part of the a-tomatine compound interact with the aspartic protease enzyme. In particular, the hydrophilic part does not appear to be located in the ligand binding pocket in the protein structure and the hydrophobic part of the compound interacts with the ends of the "U" shape. These data suggest that a-tomatine is a potential inhibitor

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of the aspartic protease enzyme in *C. albicans.* The xylose and D-glucose moieties in the a-tomatine compound exhibit very close interactions with the Asp120 residue in the protein structure and form hydrogen bonds. Glu10 and Asp53 are other residues that form hydrogen bonds with a-tomatine.

Molecular dynamics

100 ns molecular dynamics simulations were successfully applied to evaluate the time-dependent stability and affinity of protein-ligand complexes formed by molecular docking.

Trajectory analysis

The interactions between the Dextranase enzyme isolated from *S. mutans* and a-tomatine remained effective throughout the molecular dynamics simulations, and the ligand remained bound to the protein structure throughout the molecular dynamics simulation. The hydrophilic region of the compound was embedded into the protein structure and the molecular dynamics simulation was started. The hydrophobic part was outside the protein structure, and small conformational changes occurred in these hydrophobic parts at the beginning of the simulation. After 32 ns of the simulation, although the D-galactose part of the a-tomatine compound showed small fluctuations, the other parts forming the hydrophilic part of the compound maintained their high stability. Halfway through the simulation time, an equilibrium and plateau level occurred and the molecular dynamics simulation ended with these small movements.

A 100 ns molecular dynamics simulation of the interactions between signal transducing protein isolated from *L. casei* and a-tomatine was examined in detail. While the hydrophilic parts in the a-tomatine compound begin the molecular dynamics simulation by being embedded deep within the signal-transmitting protein structure, the hydrophobic part is positioned parallel to the protein structure and is positioned quite compatible with the recesses and protrusions in the protein structure. However, this situation of the hydrophobic part changed at 7 ns, right at the beginning of the simulation. While the hydrophilic part maintained its position within the protein structure, the hydrophobic part moved towards the solvent environment. As a result of this movement, this part of the a-tomatine compound began to fluctuate in the solvent environment. When the simulation reached 36 ns, small fluctuations and conformational changes were also observed in the hydrophilic part. At 41 ns, the hydrophilic part performed a rotational movement covering the entire region within the binding pocket, provided that it remained within the protein structure. No other significant conformational changes were observed during the remaining time of the simulation, but the hydrophobic part that emerged from the binding pocket at the beginning of the simulation always continued to be released in the solvent environment. The simulation became stable with these movements and formed a plateau level.

The interactions of the a-tomatine compound with the aspartic protease enzyme isolated from *C. albicans* began with the interactions described in the molecular docking study. While the hydrophobic end of the a-tomatine compound interacted with the end parts of the "U" shape-like structure of the protein structure, after 5 ns, it was released as a result of a conformational change made by this part of the protein structure and began to be released into the solvent environment. While these were happening in the hydrophobic part, the hydrophilic part always maintained its affinity for the binding pocket located in the center of the protein structure and its conformational changes remained very limited. At 56 ns of the simulation, the hydrophilic end showed almost no conformational change, while the hydrophobic end curled over the oxygen atom that provided its connection to the hydrophilic end and took a parallel position to the hydrophilic end. The existence of intramolecular interactions was effective in the formation of this position. Molecular dynamics simulation ended with these conformational changes in the a-tomatine compound, creating a stable structure and plateau level.

Root mean square deviation (RMSD) of ligands and proteins

Root-Mean-Square Deviation data was used to gain insights into the changing average positions of the protein and ligand as time progresses in the molecular dynamics simulation. RMSD plots are generated relative to other frames in the simulation that are aligned to the first frame, giving insight into the average deviation. The varying RMSD data of protein and ligand complexes are shown collectively in Fig. 8. When the RMSD data of the dextranase & a-tomatine complex are analyzed, it is seen that the average position of the a-tomatine compound deviates by an average of 2.5 Å from its initial binding. If this deviation value is below 3 Å, it is considered that the average position does not change much. When the ligand fit on protein data is examined, it is seen that a plateau level occurs up to 80 ns of the simulation, but after this time it makes a sudden and sharp increase. The ligand fit on protein plot increased to around 7.5 Å. The change of the average position of the protein and the ligand in the solvent environment of the interactions between a-tomatine and the signal transducing protein is largely in agreement at each time step in the simulation. The average position of the a-tomatine compound during the molecular dynamics simulation is again below 3 Å. a-tomatine remained at its initial binding site and moved in harmony with the protein structure. The ligand fit on protein plot
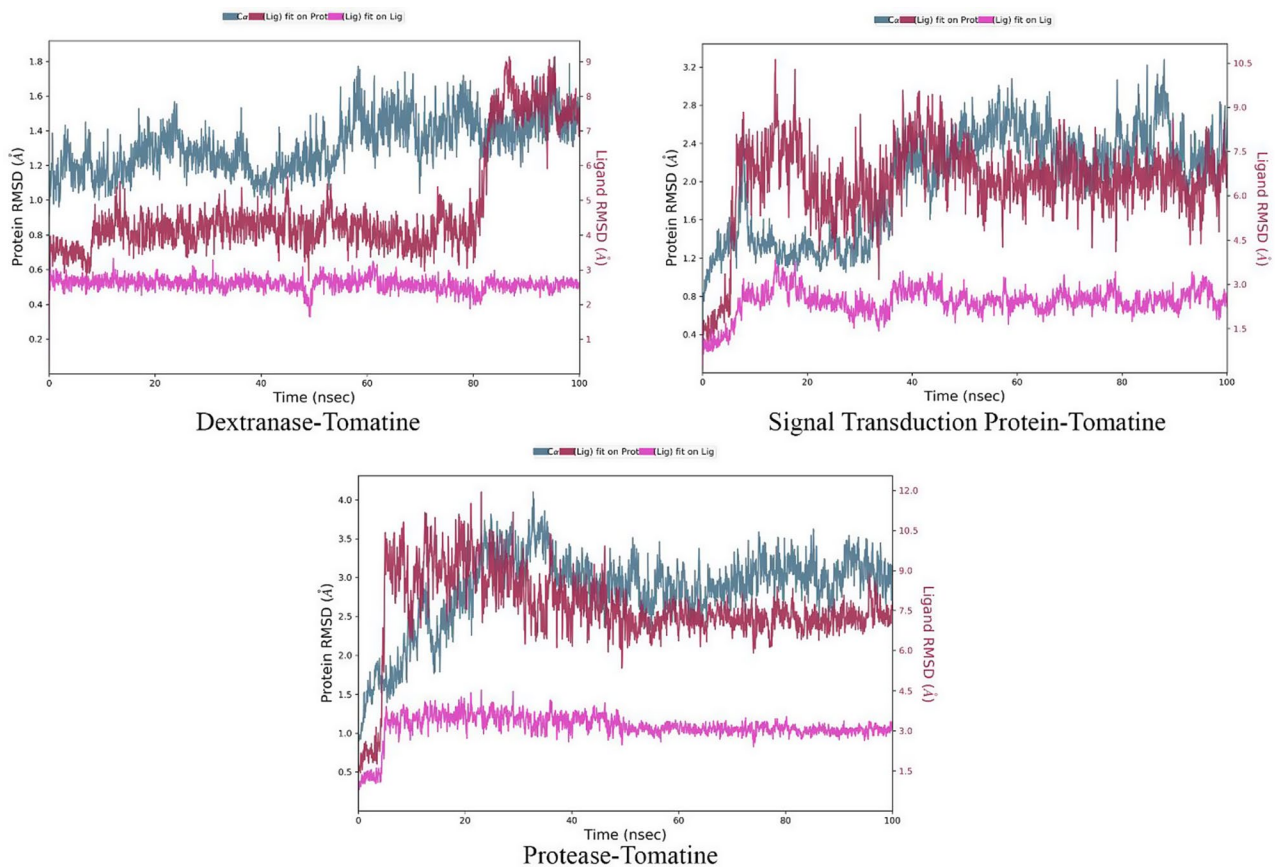
Fig. 8 RMSD data for protein ligand complexes. Pink color; when the ligand is alone, red; for ligand fit on protein, blue; RMSD data for protein alpha-carbon

increased to around 7.5 Å. The average position of the a-tomatine compound in the time-dependent variation of a-tomatine & aspartic protease enzyme interactions is around 3 Å. The ligand fit on protein plot is in good agreement with the RMSD plot of the ligand. The value of the ligand fit on protein plot increased up to around 9 Å during the simulation period.

Root mean square fluctuation (RMSF) of ligands and proteins
RMSF plots were used to analyze local fluctuations in protein structures and to see the effect of ligand contacts. Fluctuations in amino acid residues in RMSF graphs are shown in Fig. 9. High values indicate large fluctuations in that region. Globular proteins are known to fluctuate less by nature. The protein structure of the enzyme dextranase is also close to globular and its local fluctuations were limited. The regions of ligand contact are in the regions where fluctuations peak and it is seen that they limit the fluctuations in these regions of the protein structure. In the signal transducing protein & a-tomatine complex, local fluctuations are limited by the rigid structure of the protein, but the regions of ligand contact are the extremes of the peaks and limit the fluctuation motion. The aspartic protease & a-tomatine complex is different from the other two proteins because its shape is

quite different from theirs. The highly fluctuating contact points around 300 and 250 correspond to the extremes of the protein structure and mark the hydrophobic points in the a-tomatine compound. Other contact regions show fluctuations in the interior of the protein structure and contact is present at the peaks of the peaks. This means that the fluctuations are limited by ligand contact.

SEM analysis and evaluations
Since a universal type adhesive was applied to the dentin surfaces in self-etch mode, the surface exhibited less mineral removal and predominantly closed dentinal tubules compared to phosphoric acid etching. Short resin tags, not extending in different directions, were observed. It can be stated that all subgroups displayed partially similar micromorphological appearances. The images obtained from carious dentin showed significantly less pronounced dentinal tubules compared to sound dentin, and irregular lines were observed between the adhesive-dentin interfaces (Figs. 2 and 3).

Discussion
In light of the findings obtained, the first hypothesis was partially accepted. a-tomatine exhibited fungicidal efficacy against *C. albicans*, while it provided bacteriostatic
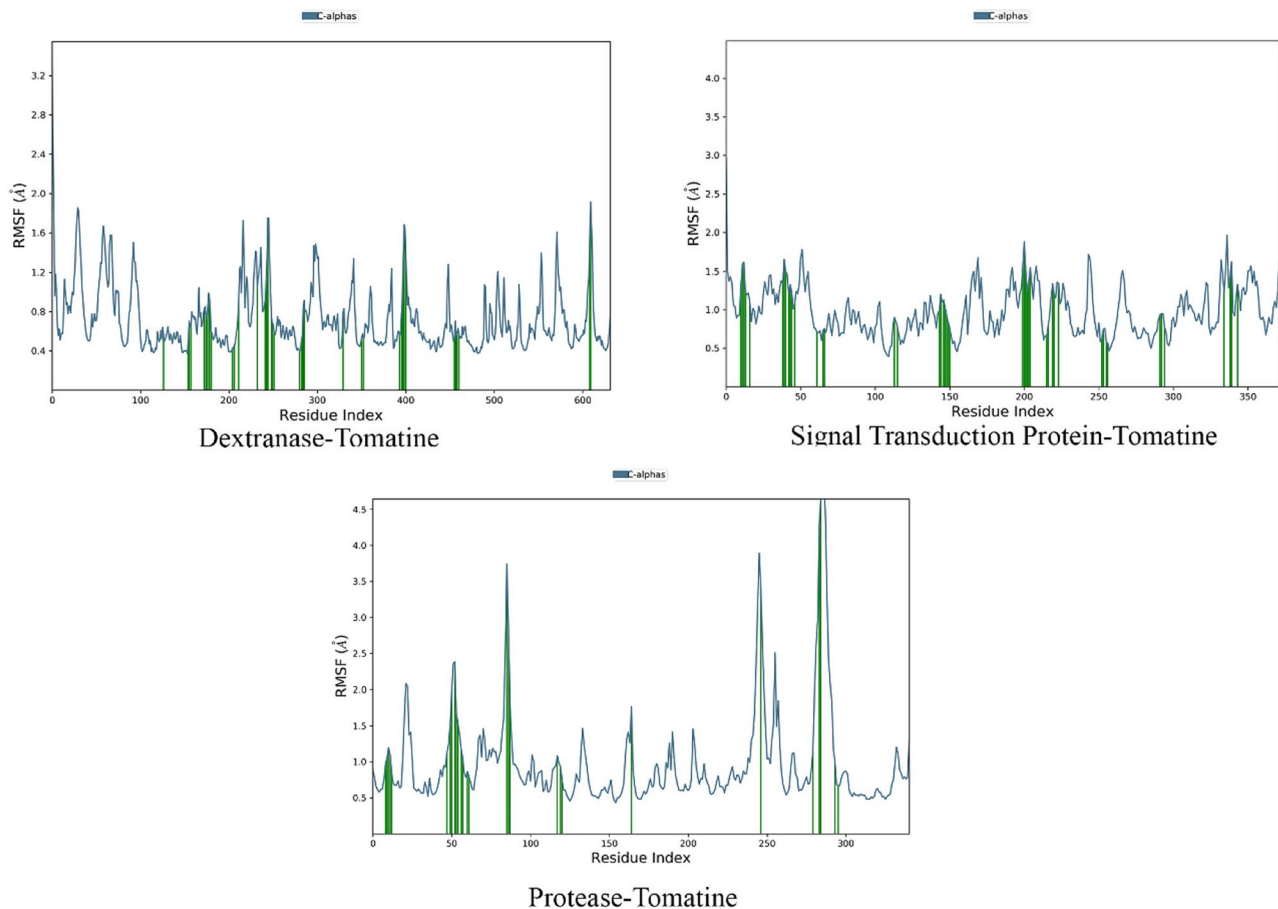
Fig. 9 RMSF data for protein (alpha-carbon)-ligand complexes. The green vertical lines indicate amino acid residues with ligand contact

activity against *S. mutans*. However, it was not effective against *L. casei*. The second hypothesis was also partially accepted. An increase in molarity positively influenced bond strength values in caries-affected dentin, whereas the opposite was observed in sound dentin.

Various restorative materials applied on caries-affected dentin exhibit lower bond strength values compared to sound dentin. It has been observed that the values obtained in our study are consistent with the literature [9, 52]. Chemical and structural changes, such as partial demineralization and a more irregular structure in caries-affected dentin, could contribute to the lower bond strength [53, 54]. In caries-affected dentin, the presence of morphological changes in the collagen structure, deeper demineralization zones, and lower mineral content may lead to lower bond strength values compared to sound dentin. In summary, regardless of the adhesive type, bonding to caries-affected dentin is more challenging than to sound dentin [9]. Either DMSO or the application of a-tomatine has resulted in better bond strength values in caries-affected dentin compared to the control group. This occurs through the synergistic effect of a-tomatine dissolved in DMSO, which acts as an

inhibitor on MMPs [28], facilitating deeper penetration into the dentin tissue, dissolution of cross-linked collagens into sparse fibrils, and ensuring the integrity of the hybrid layer [55, 56].

Of course, caries-affected dentin is inherently softer and more porous than sound dentin, owing to its partial demineralization. Moreover, it tends to have narrow and obstructed dentinal tubules due to the deposition of intratubular dentin, leading to the formation of a thicker layer [57]. Therefore, it is expected that the bond strength values obtained in caries-affected dentin would be lower compared to sound dentin. In this study, naturally obtained caries-affected dentin was utilized, and the categorization and differentiation of these teeth relied on a caries diagnostic tool, DIAGNOdent. Caries-affected dentin can be created using methods such as pH cycling, detection with a caries detector [52, 58]. Nevertheless, it has been reported that the difference between the two types is not significantly influential, especially in bond strength studies [52, 59]. However, the use of DIAGNOdent device is recommended for detecting caries-affected dentin [60]. According to the signals obtained from the tooth, a value ranging from 0 to 99 is observed on the

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device's indicator panel, with an increase in this numerical value indicating an increased likelihood of caries [61]. In light of this information, considering the use of naturally occurring carious dentin in this study, caries-affected and sound teeth were categorized according to DIAGNOdent with the manufacturer's instructions.

Universal adhesives can be applied in both etch & rinse or self-etch modes [10]. It has been observed that the inclusion of an etching step before the application of a universal adhesive, regardless of aging, enhances dentin penetration but does not affect the bond strength values [62]. In this context, the use of an MMP inhibitor agent before the application of universal adhesives, especially when applied in the self-etch mode, can be considered an important step in enhancing bond strength values [63]. Not only MMP inhibitors but also additional methods such as extra hydrophobic coating, extended application time, and additional adhesive layer application contribute to increasing the bond strength of universal adhesives [63]. However, it should be noted that the pH level of self-etch adhesives, whether mild or strong, also affects bond strength values. It has been noted that mild adhesives exhibit greater resistance to degradation compared to ultra-mild adhesives and achieve better bond strength when used in conjunction with various MMP inhibitor agents [64]. In this study, a universal adhesive (Tri-S Bond Universal) was applied in the self-etch mode. Universal adhesives applied in the self-etch mode do not exhibit changes in dentin collagen structure similar to the destabilization pattern caused by treatment with phosphoric acid [65]. Therefore, the use of an MMP inhibitor was aimed at enhancing bond strength, and this is why the universal adhesive was applied in the self-etch mode. In a study comparing Clearfil SE Bond and Clearfil Tri-S Bond, it was found that Clearfil Tri-S Bond has a less acidic nature compared to SE Bond (pH: 2.7), resulting in lower bond strength values. This might be due to the effective removal of the smear layer and the creation of sufficient porosities in dentin [66]. This is precisely why the groups treated with a-tomatine achieved higher bond strength values compared to the control groups without pretreatment agents. Furthermore, in sound dentin, as the molarity of a-tomatine increased, bond strength values decreased, which can be explained by the possibility that a-tomatine may have increased calcium release from the dentin tissue in certain ratios [67]. Consequently, this raises concerns about the activation of metallic ion-activated proteolytic enzymes in dentin, leading to degradation [65]. However, in line with caries-affected dentin being more denatured and the disruption of the helical structure compared to sound dentin [65]. a-tomatine has contributed to the stabilization of this structure. Therefore, the improved values with increasing a-tomatine concentration can be attributed to this

effect. Additionally, the fact that a-tomatine is dissolved in DMSO, an organic solvent, suggests the possibility of enhanced interpeptide bonding, especially in caries-affected dentin [68].

The use of DMSO as a pretreatment agent is a subject of debate. Some publications suggest that this organic solvent material has a positive effect [20, 69] on bond strength, while others report a neutral impact [70]. DMSO, as a polar aprotic solvent with both hydrophilic and hydrophobic properties [71], can enhance penetration into dentin tissue and achieve this by dissolving cross-linked collagens in the dentin matrix into sparse fibrils [55, 56]. It not only facilitates the adhesive to penetrate deeper but also plays a role in inhibiting the endogenous MMP enzymes in dentin, thus ensuring the integrity of the hybrid layer [56]. Although it is reported that DMSO may have a long-term effect on bond strength and that bond strength values may be better than those of control groups [20, 56], in the short term, bond strength values are influenced by the brand of adhesive used and the method of adhesive application, leading to different results [69]. In the present study, DMSO appeared to be less effective in sound dentin compared to the control group. However, in caries-affected dentin, it exhibited improved bond strength values compared to the control. Similarly, in fluorotic dentin, the use of DMSO as a pretreatment agent was observed to enhance bond strength values [23]. This effect was reported to be due to the sparser network structure of collagen fibrils and the prevention of the degradation of type I collagen. In summary, it can be said that the effectiveness of DMSO is influenced by the type of dentin. In this context, the debate continues regarding whether the focus should be on achieving higher bond strength values or establishing a more long-term stable bond [22], and further long-term studies are still needed.

Oral mycobiome is a crucial component of the human oral microbiome that consists of fungi. Every healthy person contains between 9 and 23 fungal genera, with Candida species being the most commonly found oral fungi [72]. *Candida app.* gain pathogenicity thanks to their ability to form a dental biofilm on the teeth structure and to produce extracellular polysaccharides [8, 73]. According to literature, it is demonstrated that the presence of *C. albicans* modifies the oral bacteriome, specifically by increasing the numbers of *S. mutans* in the dental plaque of early childhood caries [74]. Previous in vivo studies indicate that *C. albicans* interacts synergistically with *S. mutans*, which is prevalent in severe early childhood caries [75]. *C. albicans* may enhance mixed biofilm accumulation with *S. mutans*, hence boosting microbial transport in vivo and resulting in a severe caries lesion [76]. This study is the first to examine the efficacy of a-tomatine against cariogenic microorganisms in the oral

microbiota. A-tomatine showed minor activity against *S.mutans* however, it was detected that a-tomatine, such as CHX, had bactericidal/fungicidal effectiveness against *C.albicans.*

The increase in acidity between the biofilm and the tooth interface is a milestone for the onset of dental caries. Loss of calcium and phosphate from the tooth surface disrupts the balance between the enamel and the surface [77]. *C. albicans* which contributes that defect is dangerous not just in terms of dental caries [74]. Because of the bacterial interaction, it can also induce periodontitis [78], endodontics [79], and denture stomatitis [80]. Simultaneously with the discovery of a-tomatine, research was conducted that revealed the molecule exhibited antimicrobial capabilities against several fungus and human infections [67]. According to a recent study, it has been reported that a-tomatine inhibits *C. albicans* inducing vaginal infection and has no activity against beneficial *lactobacilli spp.* In addition, powders prepared from various parts of tomatoes have been proven to have anti-bacterial and anti-fungal properties [81]. a-tomatine may affect by binding to the sterols of the structure, thereby eliminating the polarization of cell membranes and inducing leakage of cell contents. This may be an indication of its effectiveness on bacteria [82, 83]. Furthermore, *Candida spp.* may be susceptible to growth suppression in multiple ways, and a-tomatine may have a mode of action similar to the antifungal drug fluconazole [81]. According to Tam et al., although a-tomatine has no inhibitory activity against beneficial *lactobacilli spp.* [81], it has been stated that it can inhibit various *lactobacilli spp.* by a-tomatine & lactic acid conjugation [84]. The substance tested in present investigation is known as a-tomatine. It is noted that a-tomatine is a toxic chemical common ingredient in green and unripe tomato fruit however, there is evidence that regular eating of tomato types has no negative consequences [85]. According to EFSA (European Food Safety Authority) Panel on Contaminants in the Food Chain, a-tomatine does not pose a risk. Both the FDA (Food and Drug Administration) and EFSA have not established a limit for this ingredient in food [86].

Few studies have reported the antimicrobial and antifungal activity of CHX against *S. mutans* and *L. acidophilus* and *C. albicans* [87–90]. Chlorhexidine can be used in dentistry for both therapeutic and prophylactic purposes. CHX is a versatile agent that can show antimicrobial and antifungal properties by affecting aerobic and anaerobic bacteria even at very low concentrations and can also be incorporated into adhesives [91–93]. Thanks to the positive charge of chlorhexidine, it is attracted by the negative charge on the bacterial cell wall. Phosphate on the surface of the bacterial cell provides the adsorption of the bacteria with chlorhexidine. After adsorption, the cell wall is overcome and penetration takes place, this is called bacteriostatic activity. Disruption of cell integrity, outflow of cytoplasm, cytoplasmic coagulation, and phosphorylation of nucleic acids reveal the bactericidal effect [94]. In the present study. CHX, which has a minimum inhibitory concentration against *S.mutans* not exceeding 1 mg/mL [94], showed bacteriostatic activity against *S.mutans*. Bactericidal/fungicidal activity was detected against *L. casei* and *C. albicans*.

Molecular docking studies have demonstrated that the a-tomatine compound exhibits affinity for all three protein structures. In particular, the data demonstrate affinity for protein structures isolated from Candida albicans and indicate strong interactions with the Asp120, Glu10 and Asp53 residues. Furthermore, our studies concentrated on the dextranase enzyme derived from *S. mutans*. By employing the molecular docking method and molecular dynamics simulations, along with modeling the interactions within a limited time frame to reflect conditions in the oral environment, we observed that the a-tomatine compound possesses a remarkable capacity to influence these protein structures. In particular, the hydrophobic component of a-tomatine appears to be highly efficacious. In all MD simulations of protein structures in which the a-tomatine compound was present, the compound remained in the initial binding pocket. The data on RMSD and RMSF indicate that the compound has the potential to inhibit the protein, although there are significant conformational limitations. The stability and plateauing of the complexes provide compelling evidence that their stability will be consistently maintained in the oral environment. The conformational alterations of the complexes throughout the MD simulations are readily discernible in Figs. 7 and 8. A robust interaction exists between the signal transduction protein of *L. casei* and a-tomatine. A-tomatine exerts its inhibitory effect on the function of this protein structure through both hydrophobic and hydrophilic moieties. While the hydrophilic moieties are more prominent in terms of inhibition intensity, the movement of release in the solvent medium appears to direct the hydrophilic portion of the compound deep into the protein structure. However, the opposite was observed in the in vitro data. This is because the hydrophobic part of the a-tomatine compound largely prevents the entire compound from entering the cell. The information obtained at this stage of the study provides important evidence that the inhibition of this enzyme can be achieved by modifying the a-tomatine compound. This information will inform the design of new compounds. This is not the case for the *C. albicans* Aspartic Protease & a-tomatine complex. Aspartic protease, an extracellular enzyme, is closely related to bacterial survival and the a-tomatine compound strongly inhibits aspartic protease [95]. The

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a-tomatine compound strongly interacts with Asp120, which is located in the catalytic site of aspartic protease. These interactions, which were determined as a result of analysis by molecular docking method, continue during molecular dynamics simulation. This is quite parallel with in vitro data. a-tomatine compound appears to be effective against *C. albicans* in in vitro experiments. In silico studies also concluded that there is a strong inhibition interaction between the a-tomatine compound and the catalytic site of the Aspartic Protease enzyme. Even though the selected material exhibits higher affinity for a specific enzyme, this circumstance should always be scrutinized through in vitro or alternative study methodologies, and the outcomes of both study types need to be compared. Additionally, the efficacy of a-tomatine at different concentrations should be explored concerning shear bond strength testing. Furthermore, the contribution of a-tomatine to remineralization in carious dentin or demineralized areas created through artificial methods remains open to discussion and investigation. It was concluded that the reason for this was the difficulty in penetration into the cell due to the hydrophilic and hydrophobic properties of a-tomatine.

Docking studies offer theoretical insights but may not consistently reflect the precise biological activity. It can be advantageous to validate these results with experimental assays to authenticate the real inhibitory impacts of a-tomatine on the studied enzymes. One limitation of the study is that the effectiveness of a-tomatine on MMP inhibitors has not been directly or indirectly examined on bonding strength. Gelatin zymography (SDS Page) [19], in situ zymography [96], total MMP assay measurements [97] can be used to evaluate this effectiveness, and these are among the goals of our future studies. This study only includes bond strength values for a 24-hour period. Assessment of the efficacy of a-tomatine not only on dentin but also on enamel bonding, as well as testing restorations for restoration consistency, marginal integrity, or leakage, may be the focus of future studies. Bond strength values should be compared with longer-term aging (at least 6 months) for comparative analysis. Additionally, a single operator (M.K.Ü) was responsible for performing the restorative procedures and subsequent mechanical testing. While this scenario could potentially increase the likelihood of subjectivity and inconsistency in the results depending on the experience and skill level of the clinician involved, the operator (M.K.Ü) in this study is adequately experienced and has previously conducted similar studies. Therefore, to ensure consistency and standardization, the procedures were conducted by a single operator.

Conclusion

In caries-affected dentin, bonding strength values are enhanced with a-tomatine or DMSO. Additionally, a-tomatine exhibits fungicidal activity against *C.albicans*, and bacteriostatic activity against *S.mutans.* Despite the identified high affinity for the signal transduction protein in *L. casei*, no inhibitory effect on *L. casei* proliferation was observed.

Declarations

Ethics approval and consent to participate
All techniques conducted on human subjects in studies conformed to the ethical norms of the institutional and/or national research committee as well as the 1964 Helsinki Declaration and its subsequent revisions or other similar ethical standards. The Altinbas University Faculty of Dentistry Clinical Research Ethics Committee approved the study's design (no: 2023/198). Informed consent has been obtained from all participants prior to their inclusion in the study. In this study, experiments involving extracted teeth samples collected from participating individuals are confirmed to have been conducted in accordance with relevant guidelines and regulations by all authors.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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