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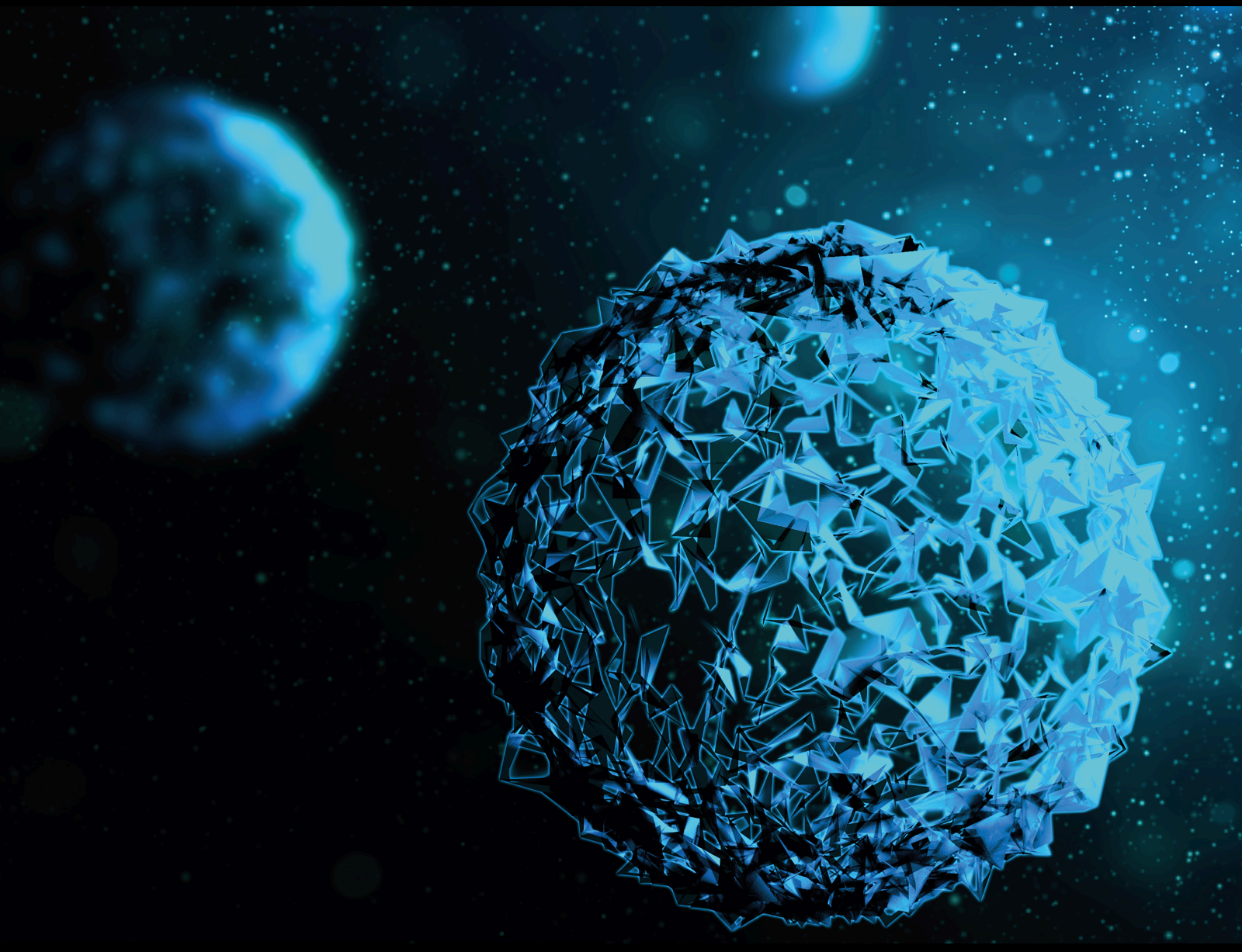

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Research Article

Chemical Characterization and Cytotoxic/Antibacterial Effects of Nine Iranian Propolis Extracts on Human Fibroblast Cells and Oral Bacteria

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Multimicrobial infections caused by pathobionts are called dysbiotic multimicrobial illnesses. Commercial mouthwashes, such as chlorhexidine, have negative side effects that can prevent tooth decay and infection. The present study aimed to determine the antifungal, antibacterial, and cytotoxicity characteristics of the propolis extracts from different areas (Iran). The ethanolic extract of propolis was prepared. GC/MS carried out the characterization to determine the thymol, carvacrol, and menthol extracts, and also, total phenol and flavonoid were assed for all samples. The antimicrobial and antibiofilm effects were evaluated against *S. mutans*, *S. mitis*, *S. salivarius*, *L. acidophilus*, *E. coli*, *S. aureus*, and *C. albicans*. The cytotoxic effect of extracts was measured on human fibroblast cells by MTT test. The MIC values in mg mL^{-1} were ranged as follows: *S. salivarius* (0.003 to 0.048), *S. mutans* (0.003 to 0.029), *S. mitis* (0.007 to 0.058), *L. acidophilus* (0.007 to 0.117), *C. albicans* (0.014 to 0.234), *E. coli* (0.007 to 0.058), and *S. aureus* (0.007 to 0.058), while MBC were, respectively, *S. mutans* (0.007 to 0.058), *S. salivarius* (0.007 to 0.117), *S. mitis* (0.007 to 0.117), *L. acidophilus* (0.014 to 0.234), *C. albicans* (0.029 to 0.468), *E. coli* (0.014 to 0.234), and *S. aureus* (0.007 to 0.117). Cariogenic bacteria and *Candida albicans* were demonstrated to be resistant to propolis extracts. Therefore, propolis extracts may make good mouthwashes.

1. Introduction

A variety of factors contribute to dental caries. Biological fermentation produces lactic acid, which contributes to dental caries. The presence of dental biofilm promotes the progression of periodontal disease and caries [1]. Public health issues such as dental caries affect millions [2]. It is believed that bacteria, primarily *Streptococcus mutans*, contribute to the initiation of caries. However, caries does not always require the presence of bacteria for its development [1, 3]. Tooth decay is dependent upon *Streptococcus mutans'* ability to produce extracellular polysaccharides (mainly glucans). The bacteria use glucosyltransferases to turn nutritious carbohydrates (GTF) into glucans [1]. *S. mutans* has been successfully removed from the oral cavity after repeated attempts. Dental cavities can often be reduced with antibiotics such as ampicillin, penicillin, and tetracycline. These compounds are also associated with negative effects, such as increased susceptibility to bacteria, diarrhea, vomiting, and tooth discoloration when ingested in large quantities. This plant has broad spectrum antibacterial activity against oral bacteria, including *Sanguinaria canadensis*. A unique oral product is due to its powerful antibacterial properties. The use of this drug was limited due to its association with oral leukoplakia. In light of these challenges, more research is needed on natural antibacterial materials that are safe and effective against oral microorganisms [4]. Propolis is a brownish waxy product produced by the honeybee from plant leaves, buds, and exudates. Propolis, known from ancient times, possesses anti-inflammatory, antimicrobial, antioxidant, hepatoprotective, immunostimulating, and cytostatic properties [5].

Pollen, flavonoids, phenolic acids, waxes, and aromatic balsam constituents of propolis are what it is primarily made up of. Depending on where and how it is made, propolis varies in composition based on what kind and what kind of plants are used for making it [5]. Flavonoids have an essential role in the biological activity of propolis [5]. The biochemical properties of flavonoids are binding biological polymers and heavy metal ions, scavenging free radicals and catalysis of electron transport [5]. The flavonoids inhibit the integration of uridine, thymidine, and leucine into tumoral cells and inhibit DNA synthesis and cause the antitumoral effect of propolis [5]. Bees use propolis to seal their hives and thus check the entry of microbes. The synergistic effect of its compounds causes antimicrobial properties of propolis [5]. Propolis works against harmful bacteria by affecting the integrity of the membrane and thus inhibiting bacterial enzyme activity and motility. Propolis is effective against antibiotics-resistant bacteria [5]. Propolis has a wide range of applications. It contains urinary tract infection, cancer, treatment of open wounds, influenza, sinus congestion, gastritis, ear disease, periodontal disease, intestinal infections, arthritis, headaches, Parkinson's disease, conjunctivitis, and warts [5]. Propolis is used against invasive fungi, bacteria, and even larvae [6]. Several studies have demonstrated the antimicrobial activities of propolis [6–21]. The effectiveness of propolis against *Streptococcus mutans* was reported by many studies [1]. In this study, it was examined whether propolis alcoholic extract from different parts of Iran has any effect on normal fibroblast cells and how effective it is at controlling oral microbes.

2. Materials and Methods

2.1. Materials. In this study, we were interested in testing how propolis extract affected different bacteria that cause oral infections. This led to the selection of a range of bacteria that cause oral disorders. Iranian University of Medical Sciences provided *S. salivarius*, *S. mutans*, *S. mitis*, *C. albicans*, *L. acidophilus*, *S. aureus*, *E. coli*, and human fibroblast cells. MTT Kit was obtained from Bioidea (Iran). YPD broth, BHI agar and broth, and crystal violet were provided from Merck (Darmstadt, Germany). Trypsin, DMEM, PBS, FBS antistreptomycin, and beta-glycerol were bought from Gibco (New York, USA). DMSO was obtained from Sigma-Aldrich.

2.2. Propolis Sampling and Extraction

2.2.1. Propolis Sampling. Raw propolis was collected in 2020 from Tabriz (East Azerbaijan), Kurdistan, Khalkhal (Ardabil Province), Sarab (East Azerbaijan Province), Neor Lake (Ardabil Province), Fasa (Fars Province), Qaleh Rudkhan (Gilan Province), Fereydun Shahr (Isfahan Province), and Kermanshah (Figure 1).

2.2.2. Propolis Extract Preparation. Samples were frozen (-20°C) and then grounded. Raw propolis samples were extracted (under stirring (by tenfold volume of ethanol (70%)) in firmly closed flasks in the dark environment, at ambient temperature for three days. Then the suspensions were frozen (-20°C, 24 h) and filtered to remove less soluble substances and waxes (Whatman filter paper (No. 1). This process was repeated (three times). What remains at the end is ethanol extract of propolis (EEP). By a rotary evaporator (rotary evaporator), the solutions were evaporated (under reduced pressure at 64°C) to near dryness. Then the solutions were freeze-dried to obtain a powder [3].

2.3. Gas Chromatography/Mass Spectrometry (GC/MS). The GC/MS was performed using a GCMS (QP2010S (Shimadzu, Japan)). In 10 mL of 50% ethanol, freeze-dried propolis (1 g) was dissolved. In this experiment, EEP (25 mg) was evaporated under nitrogen conditions, then derivatized (by one percent TMCS, 100 L BSTFA, 50 g pyridine, and one cc hexane after one day), and dissolved in one cc hexane. As a carrier, helium gas (one liter) was used (at a flow rate of 0.05 mL/min) (in a splitting ratio of 1 : 25). Capillary column was connected to a quadrupole mass spectrometer. Specifically, the head pressure was adjusted at 53.1 kPa, the injector temperature was adjusted at 230°C, and the transfer line heater temperature was adjusted at 250°C. With GC/MS Postrum Analysis, the mass spectra were as follows: 1-s scan time, 35–450 m/z scan range, 220°C source temperature, 70 eV electron energy, and 3-min filament delay time [22].

2.4. Total Phenolic Compounds Analysis. This study was conducted using the Folin-Ciocalteu spectrophotometric

FIGURE 1: Nine samples of propolis were gathered from various parts of Iran.

technique with gallic acid as the standard. Extraction was carried out in ethanol (0.1 mg.min^{-1}) with a concentration of 0.01. In the next step, sodium carbonate (7.5 percent) and Folin-Ciocalteu solution (2.5 mL) (10 percent) were added to the solution. A 50-degree bath was used to soak the solution for 5 minutes. Spectrophotometers (765 nm) were used to measure absorbances. In this study, gallic acid standard curves (mg EGA/g) were compared with the raw data. This process was repeated three times [23].

2.5. Flavonoid Content Analysis. A spectrophotometer (415 nm) was used to measure the flavonoid content of EEP. Methanol was mixed 1 : 1 with aluminum chloride 2.0 percent to create the solutions. Standard solutions of quercetin were used to set the curves. A blank sample was evaluated for flavonoid content (mg EQ/g) and represented as quercetin equivalents [23]. Samples were analyzed three times [23].

2.6. MTT Assay. Several doses of EEP (12.5 to 0.006 mg/mL) were used in 96-well plates to culture human gingival fibroblasts. An assay for the determination of cell survival was performed using 3-(4,5-dimethylthiaziazol-2-yl) 2,5-diphenyl tetrazolium bromide. The cells were plated at 2105 cells/mL in each well. In the following step, EEP samples in DMEM (without serum) (100 L/well) were diluted to a variety of concentrations. Cells without extracts served as a control. A humid environment containing 5% CO2 with 37°C and a humid atmosphere was used for 24 hours to incubate the colonies. During the next phase, cell growth was measured using MTT solution (5 mg/mL). A 5% CO_2 atmosphere and 37°C were used to incubate plates with MTT solutions for four hours. Dimethylsulfoxide (DMSO) was added to the well's medium. Crystals were dissolved in DMSO. ELISA reader (EL X 808) was used to examine the plates after 10 minutes at room temperature (lambda wavelength 570 nm, reference wavelength 630 nm). An MTT-based technique was used to determine mitochondrial activity after 24 and 48 hours of training. Cell metabolic and mitochondrial activities were examined using MTT tests [24, 25]. This data is presented as a percentage (control value = 100%). Each test was repeated three times. Calculating the viability percentages involved the following equation:

$$\text{The percentage of cell viability} = \frac{\text{Samples (OD)}}{\text{Control (OD)}} \times 100 \quad (1)$$

2.7. Antimicrobial Activity of the Propolis Extracts

2.7.1. Bacterial Strain and Inoculum Preparation for Evaluation of MIC and MBC. *Streptococcus mitis*, *S. mutans*, *S. salivarius*, *L. acidophilus*, *S. aureus*, *E. coli*, and *C. albicans* were the bacteria and fungus strains employed in this investigation. In BHI medium (37°C, 5% CO_2), bacteria are reactivated after 48 hours. A loop of BHI Broth medium (25 mL) was then added to the bacteria (Merck, Darmstadt, Germany). Incubation for 24 hours at 37°C yielded the concentration of cells. In a spectrophotometer (at 625 nm), 1.0108 CFU/mL was measured (absorbance of 0.18) in a spectrophotometer [26]. A sterile YPD broth was used to prepare the suspension of albicans from the stock culture of albicans. In the MIC test, 1.0×10^5 CFU/mL were used [3].

2.7.2. Determination of MIC, MBC, and MFC Tests. A 96-well microtiter plate was injected with 100 mL of BHI broth or YPD broth to determine the MIC. EEP (100 L) was then injected into the wells' first column. A concentration of 15 mg/ml was used. As well content (100 L) was moved from the highest to the lowest concentration [26], the EEP was gradually diluted (1 : 1 v/v) from 15 to 0.007 mg/mL [26]. After the previous column was discarded, 100 liters were added to the new column. A total of 100 L of bacteria and fungi (1.05 105 CFU/mL) were injected in the last step. In these studies, there were three control groups: growth control (only microbiological content) (no antimicrobials), antimicrobial control (CHX 0.2 percent), and sterility control (only sterile culture medium). A temperature of 37°C with 5% CO_2 was used for incubation of the microplates for 24 hours [3].

2.7.3. Disk Agar Diffusion Test (DAD). Many strains of bacteria were cultured in BHI and YPD agar and then suspended in NaCl solution. Using McFarland 0.5, they were corrected to spectrophotometric measurement using a spectrophotometer. Propolis suspensions (400 mL) were combined with BHI and YPD agar (40 mL, 45°C). On top of the BHI agar, a layer was added. Inoculations were then made using sterile swabs on plates. YPD agar (3 108/mL concentration) was used to streak strains on BHI agar and YPD agar. For each experiment, 0.08 mL of 2× MBC Propolis, 0.2 percent CHX, and 0.2 percent CHX (positive control) were applied to EEP plates. A 48-hour incubation was carried out at 37°C. An analysis of the inhibition zones was performed [13, 27].

2.7.4. Biofilm Formation and Degradation Evaluation. Biofilm formation was studied using crystal violet staining. Agar

plates were cultivated with 1% sucrose and sterilized BHI and YPD agars. Two microplates of each EEP were grown under anaerobic conditions (37°C, 5% CO_2). In order to remove nonadherent bacteria, we rinsed the microplates with PBS three times after the broths were removed. After forty-five minutes, the microplates were dried at 60°C. After the crystal violet solution was added (100 L, 1% v/v), the reaction was completed. A 15-minute incubation period followed. The microplates were then washed with PBS. 125 μL of ethanol (95 percent) was poured into each well to test the production of biofilms. The optical density of wells was measured at 590 nm using a microplate reader for comparison with a control biofilm (without EEP) [28]. EEP percentage inhibition was calculated for the various concentrations of propolis samples using the following formula: We calculated the mean absorbances of the propolis samples, and the EEP percentage inhibition was calculated for each concentration using the following formula:

$$\text{The biofilm formation rate} = \frac{\text{Samples (OD)}}{\text{Control (OD)}} \times 100 \quad (2)$$

$$\text{The biofilm reduction rate} = 100 - \left(\frac{\text{Samples (OD)}}{\text{Control (OD)}}\right) \times 100 \quad (3)$$

where $OD_{treatment}$ with samples and $OD_{control}$ without samples (570 nm).

2.8. Statistical Analysis. An ANOVA of one-way and Tukey post hoc tests were used to compare means between groups. The statistical analysis was carried out using SPSS statistics model 20.

3. Results

3.1. Determination of Flavonoids and Phenolic Compounds. The obtained results of the flavonoid and phenolic analysis are presented in Table 1. The range of phenolic compounds was from 5575 to 35500 mg/kg. Propolis from Fereydunshahr had the highest phenolic compounds, and propolis from Kermanshah had the lowest phenolic compounds. The range of flavonoids compounds was from 2285 to 63309 mg/kg. Propolis from Khalkhal had the highest flavonoids compounds, and also, propolis from Kermanshah had the lowest flavonoids compounds.

3.2. GC/MS Analysis of EEP. Components of different EEPs were recognized including menthol, thymol, carvedilol. The chemical composition of nine extracts was analyzed by GC/MS technique. Figures 2–9 show that the amount of carvacrol was more than thymol and menthol in Kermanshah, Fasa, Tabriz, Sarab, Gilan, Khalkhal, Kurdistan, and Fereydun Shahr EEPs that had the highest amount of carvacrol. In addition, the amount of carvacrol in Kermanshah, Fasa, Sarab, and Fereydun Shahr EEPs was more than Tabriz and Neor EEPs, and also, carvacrol amount in Tabriz and Neor EEPs was more than Gilan, Khalkhal, and Kurdistan EEPs that had the lowest amount of the carvacrol among the samples. Figure 10 shows that the amount of menthol was more than the carvacrol and thymol in Neor EEPs. Figures 5, 6, 8, and 10 show that the amount of menthol in Neor EEPs was more than Sarab, Gilan, and Kurdistan EEPs. In addition, menthol amount of Sarab, Gilan, and Kurdistan EEPs was more than Kermanshah, Fasa, Tabriz, Khalkhal, and Neor EEPs. The Neor EEPs had the highest amount of menthol among samples. The amount of thymol was more in Kermanshah, Fasa, Tabriz, Neor, Sarab, and Fereydun Shahr EEPs compared to Gilan, Khalkhal, and Kurdistan EEPs (Figure 2; GC of Kermanshah EEPs, Figure 3; GC of Fasa EEPs, Figure 4; GC of Tabriz EEPs, Figure 5; GC of Neor EEPs, Figure 6; GC of Sarab EEPs, Figure 7; GC of Gilan EEPs, Figure 8; GC of Khalkhal EEPs, Figure 9; GC of Kurdistan EEPs, Figure 10; GC of Fereydun Shahr EEPs).

Table 1: Flavonoid and phenolic contents of the EEPs.

Propolis	Phenolic compounds (mg/kg)	Flavonoids (mg/kg))
Kermanshah	5575	2285
Fasa	35400	10096
Tabriz	14050	35962
Neor lake	19300	22203
Sarab	19500	22705
Gilan	15250	30471
Khalkhal	12000	63309
Kurdistan	16950	33618
Fereydun Shahr	35500	8192

3.3. Cell Viability Evaluation. Cultured cells were incubated with different extract concentrations (0.97 to 500 mg/mL). Cell viability was determined by the MTT assay. In a dose- and time-dependent manner, extracts significantly reduced the number of viable cells. Following treatment with the samples for incubation durations of 24 and 48 hours, optical density of viable cells was used to calculate the viability percentages for both cell lines and the control group. According to MTT data, the viability of Fasa, Neor Lake, Khalkhal, and Kurdistan propolis was greater than 50% with 500 mg/mL over 24 and 48 hours. Cell viability was also enhanced when all concentrations were reduced. The results are shown in Figures 11 and 12.

3.4. Antimicrobial Analysis

3.4.1. MIC. MIC values were calculated using the broth microdilution technique. There was a range in MIC values ($mg\,mL^{-1}$) (Table 2) for *S. salivarius* (0.003 to 0.048), *S. mutans* (0.003 to 0.029), *S. mitis* (0.007 to 0.058), *L. acidophilus* (0.007 to 0.117), *C. albicans* (0.014 to 0.234), *E. coli* (0.007 to 0.058), and *S. aureus* (0.007 to 0.058) (Table 2). There were statistically difference between groups 2, 4, 5, 6, 7, and 9 with 1, 3, 8 (P-value <0.001). There were statistically difference between groups 2, 4, 5, 6, 7, and 9 (P-value <0.05).

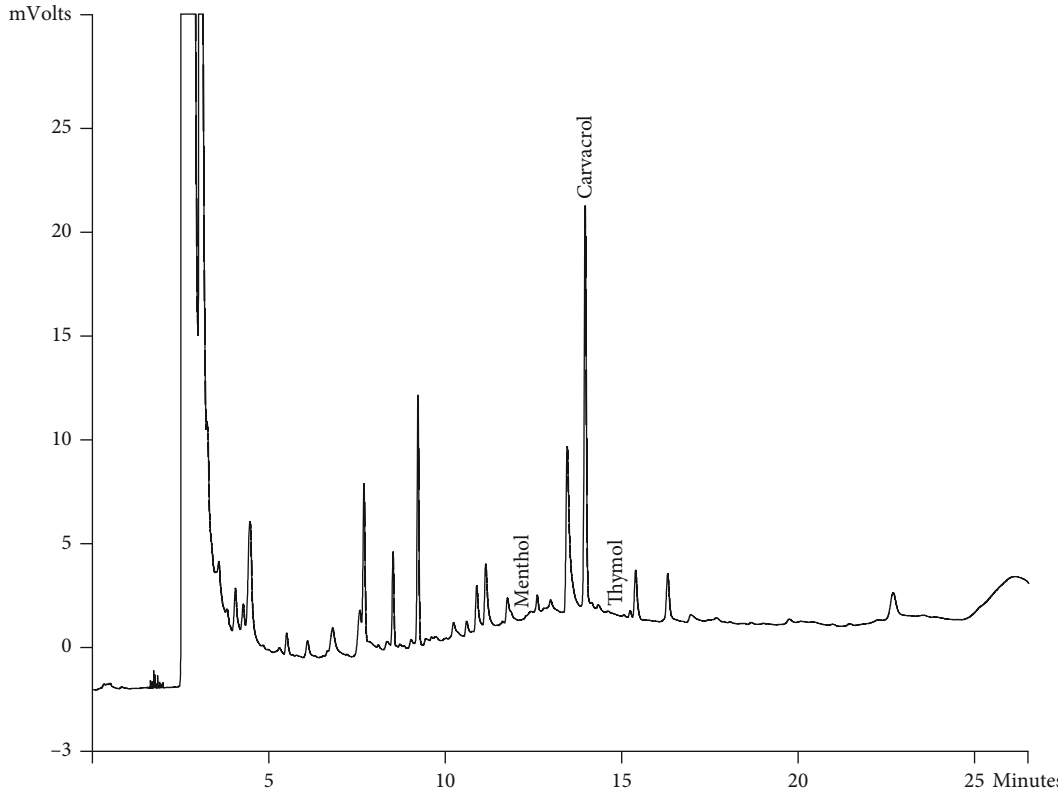

Figure 2: Gas chromatogram of Kermanshah EEPs (GC/MS profile) showing thymol, carvacrol, and menthol as the significant constituents.

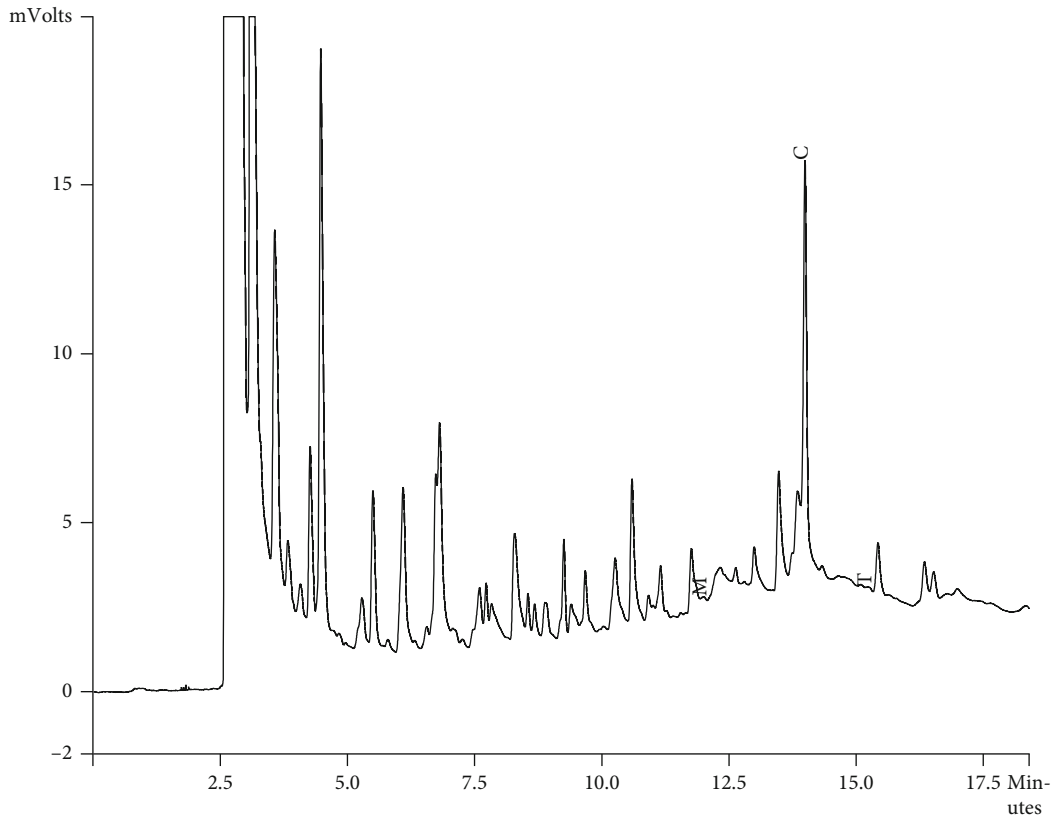

Figure 3: Gas chromatogram of Fasa EEPs (GC/MS profile) showing thymol, carvacrol, and menthol as the significant constituents.

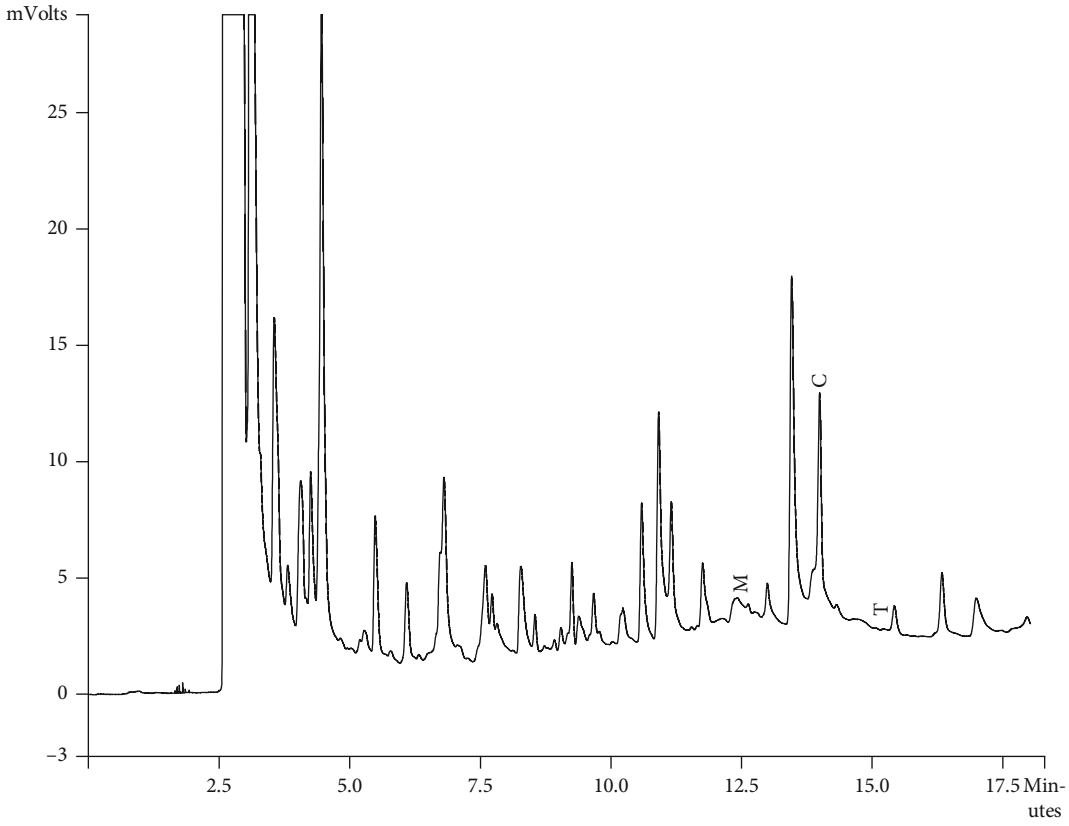

Figure 4: Gas chromatogram of Tabriz EEPs (GC/MS profile) showing thymol, carvacrol, and menthol as the significant constituents.

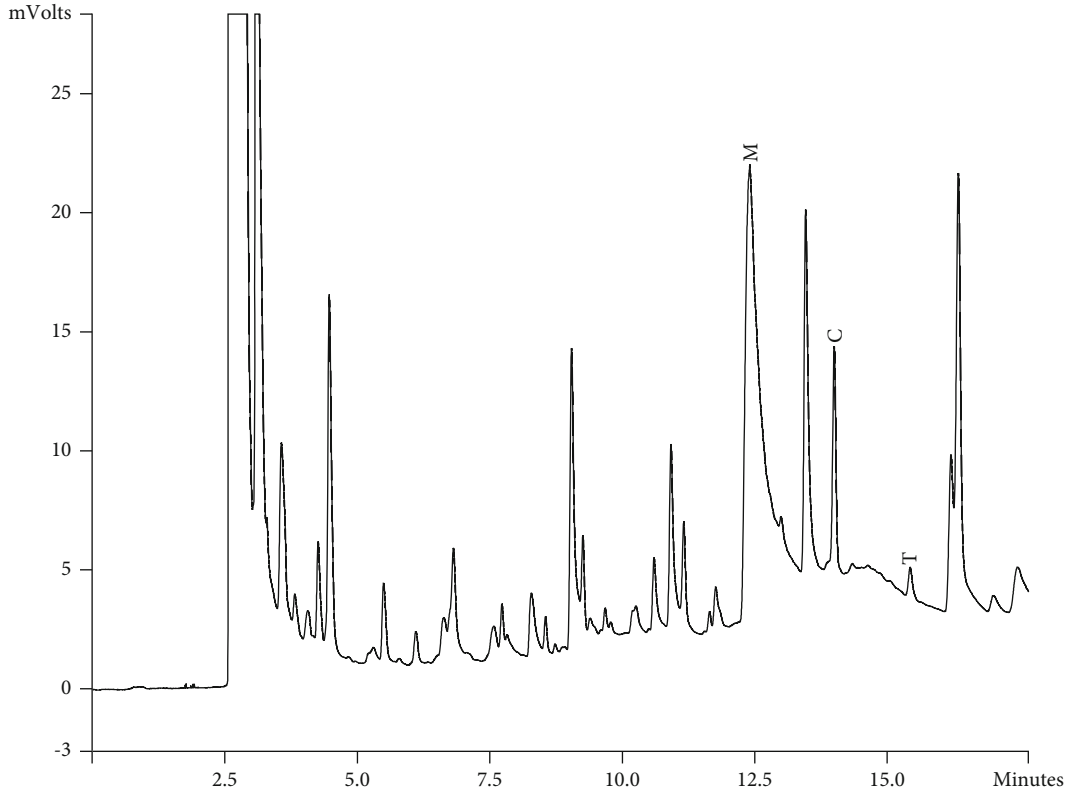

Figure 5: Gas chromatogram of Neor EEPs (GC/MS profile) showing thymol, carvacrol, and menthol as the significant constituents.

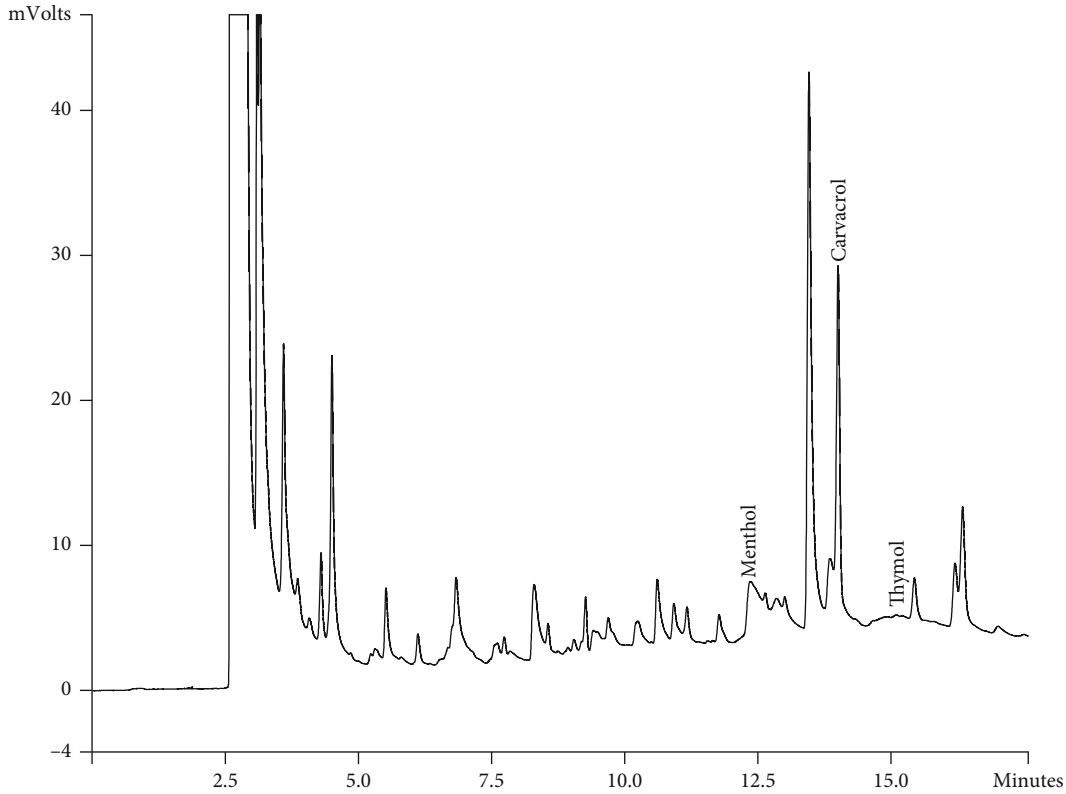

Figure 6: Gas chromatogram of Sarab EEPs (GC/MS profile) showing thymol, carvacrol, and menthol as the significant constituents.

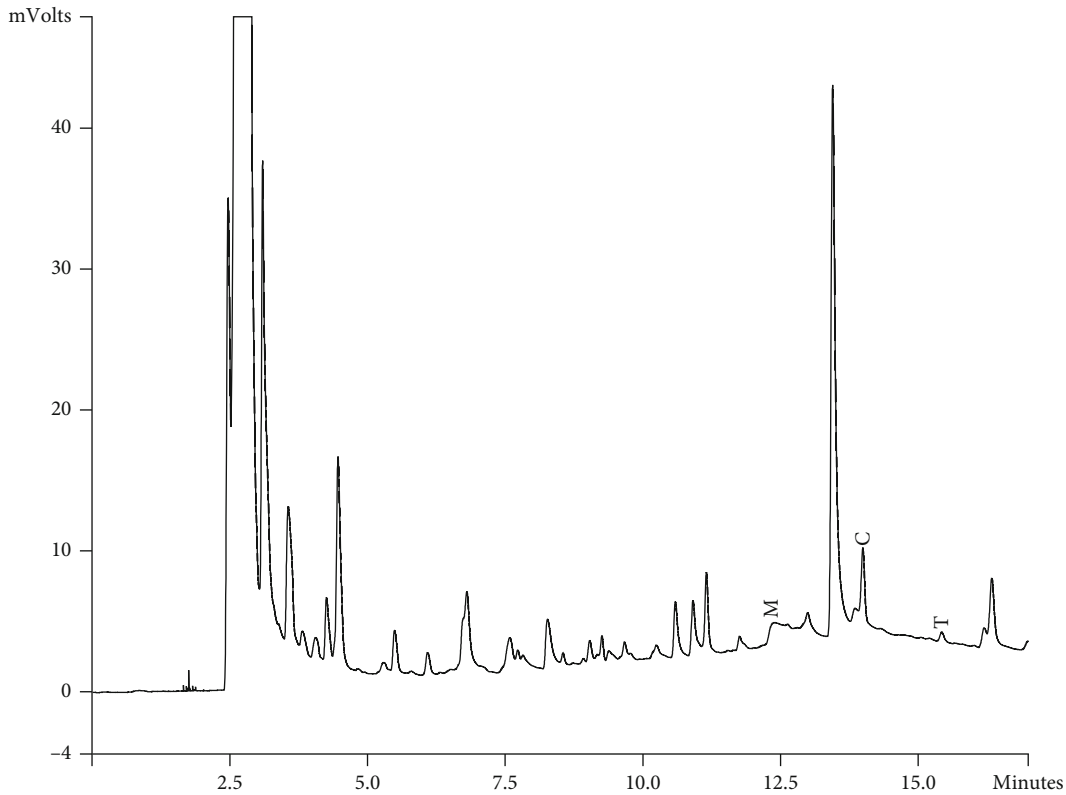

Figure 7: Gas chromatogram of Gilan EEPs (GC/MS profile) showing thymol, carvacrol, and menthol as the significant constituents.

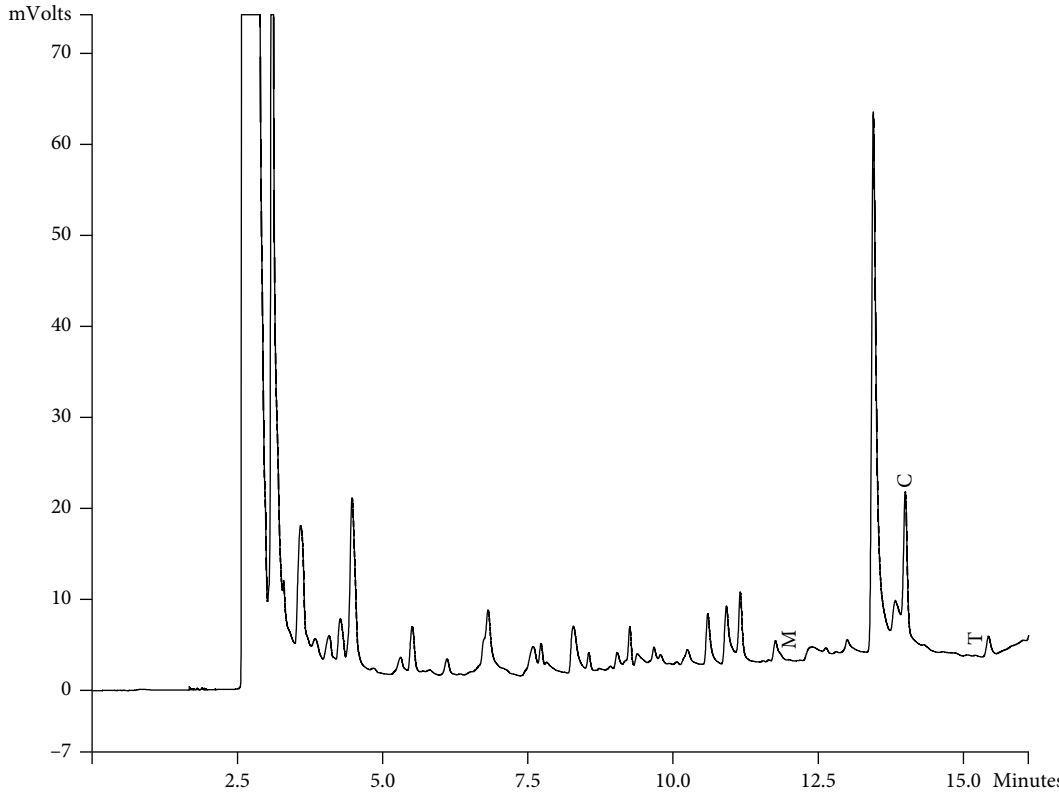

Figure 8: Gas chromatogram of Khalkhal EEPs (GC/MS profile) showing thymol, carvacrol, and menthol as the significant constituents.

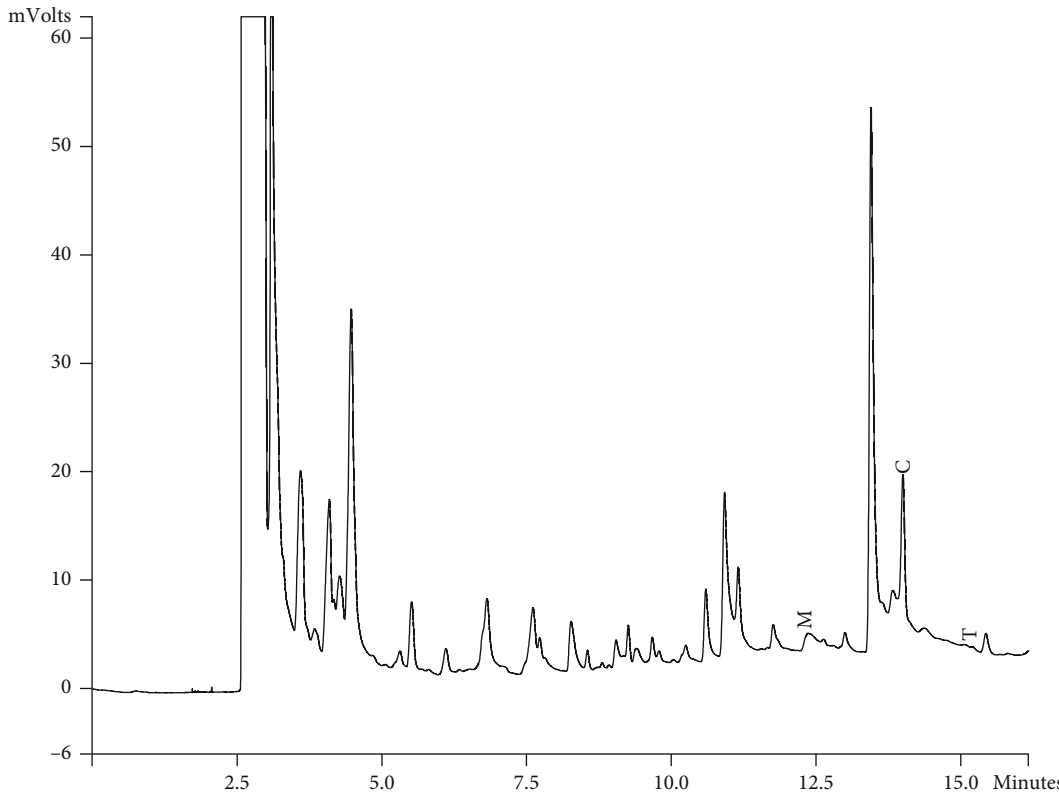

Figure 9: Gas chromatogram of Kurdistan EEPs (GC/MS profile) showing thymol, carvacrol, and menthol as the significant constituents.

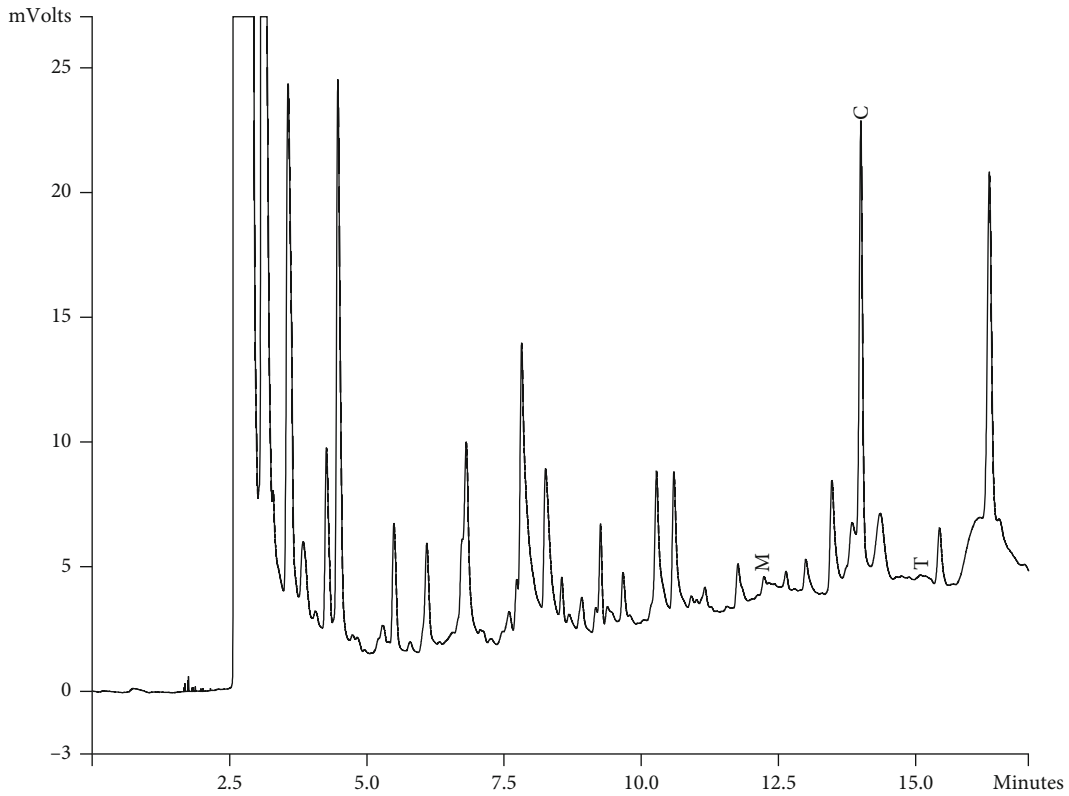

Figure 10: Gas chromatogram of Fereydun Shahr EEPs (GC/MS profile) showing thymol, carvacrol, and menthol as the significant constituents.

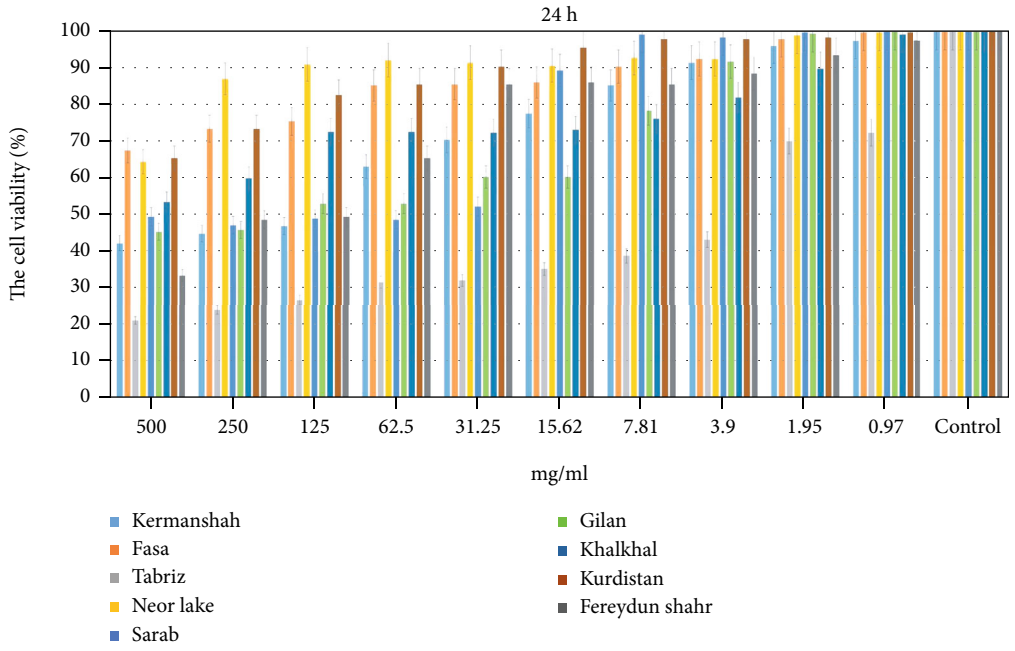

Figure 11: The percentage of cell viability on fibroblast cell lines by MTT assay (24 h). Results are shown as mean ± SD ($n = 3$). 1, Kermanshah; 2, Fasa; 3, Tabriz; 4, Neor Lake; 5, Sarab; 6, Gilan; 7, Khalkhal; 8, Kurdistan; 9, Fereydun Shahr.

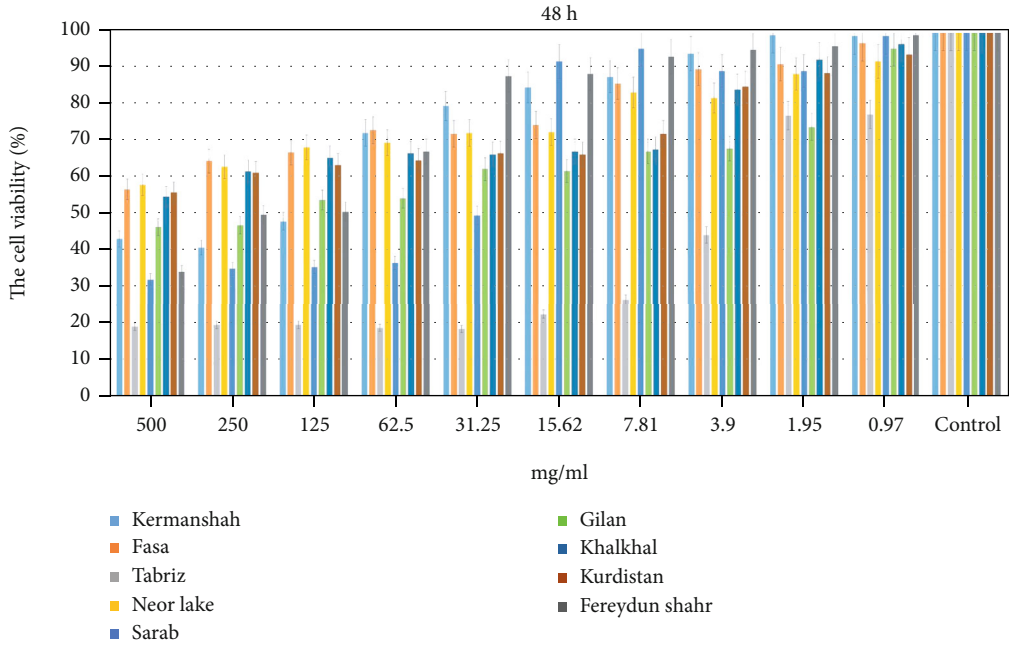

Figure 12: The percentage of cell viability on fibroblast cell lines by MTT assay (48 h). Results are shown as mean ± SD ($n = 3$). 1, Kermanshah; 2, Fasa; 3, Tabriz; 4, Neor Lake; 5, Sarab; 6, Gilan; 7, Khalkhal; 8, Kurdistan; 9, Fereydun Shahr.

Table 2: MIC in mg mL^{-1} of EEP obtained using the broth microdilution method.

Samples	1	2	3	4	5	6	7	8	9	CHX
S. mutans	0.003	0.029	0.007	0.029	0.029	0.029	0.029	0.003	0.007	0.0000305
S. salivarius	0.003	0.029	0.007	0.029	0.029	0.048	0.048	0.003	0.014	0.0000305
S. mitis	0.007	0.029	0.007	0.058	0.058	0.058	0.058	0.014	0.014	0.0000305
L. acidophilus	0.007	0.058	0.014	0.058	0.058	0.117	0.058	0.007	0.029	0.0000152
C. albicans	0.014	0.117	0.014	0.058	0.234	0.234	0.058	0.029	0.058	0.0000152
E. coli	0.007	0.029	0.014	0.058	0.058	0.058	0.058	0.014	0.029	0.0000305
S. aureus	0.007	0.029	0.007	0.058	0.048	0.058	0.058	0.014	0.029	0.0000152

∗Kermanshah (sample 1), Fasa (sample 2), Tabriz (sample 3), Neor Lake (sample 4), Sarab (sample 5), Gilan (sample 6), Khalkhal (sample 7), Kurdistan (sample 8), and Fereydun Shahr (sample 9). There were statistically difference between groups 2, 4, 5, 6, 7, and 9 (P-value < 0.05). There was a statistical difference between groups 1, 3, and 8 (P-value < 0.05). There were statistically difference between groups 2, 4, 5, 6, 7, and 9 with 1, 3, and 8 (P-value<0.001).

Table 3: MBC and MFC in mg mL^{-1} of EEP obtained using the broth microdilution method.

Samples	1	2	3	4	5	6	7	8	9	CHX
S. mutans	0.007	0.058	0.014	0.029	0.058	0.058	0.058	0.007	0.014	0.000244
S. salivarius	0.007	0.058	0.014	0.058	0.058	0.117	0.117	0.007	0.029	0.000244
S. mitis	0.014	0.058	0.007	0.117	0.029	0.117	0.117	0.014	0.029	0.000244
L. acidophilus	0.014	0.117	0.029	0.117	0.117	0.234	0.117	0.014	0.058	0.000122
C. albicans	0.058	0.234	0.029	0.234	0.468	0.468	0.117	0.058	0.117	0.000976
E. coli	0.014	0.117	0.014	0.117	0.117	0.234	0.117	0.014	0.058	0.000122
S. aureus	0.014	0.058	0.007	0.058	0.029	0.117	0.117	0.014	0.029	0.000122

There were statistically difference between groups 1, 3, and 8 (P-value <0.05).

3.4.2. MBC and MFC. The range of MBC and MFC values in mg mL^{-1} for *S. mutans* was (0.007 to 0.058), *S. salivarius* (0.007 to 0.117), *S. mitis* (0.007 to 0.117), *L. acidophilus* (0.014 to 0.234), *C. albicans* (0.029 to 0.468), *E. coli* (0.014 to 0.234), and *S. aureus* (0.007 to 0.117 (Table 3). There were statistically difference between groups 2, 4, 5, 6, 7, and 9 with 1, 3, and 8 (P-value <0.001). There were statistically

Table 4: Mean area of microbial growth inhibition zones in mm ($n = 3$) provided by the EEP samples.

Samples	2 × MBC concentrations of each Propolis									CHX 0.2%
	1	2	3	4	5	6	7	8	9	
S. mutans	9.5	16	14	15	15	15.5	16	10	14.5	20
S. salivarius	11	14	12	14	14	15	16	11	13	20
S. mitis	12.5	15	7.5	16.5	14	15.5	17	12	14.5	19.5
L. acidophilus	10.5	13.5	11	13.5	14	15	14	9	12	21
C. albicans	10.5	12	9.5	12.5	13.5	13	11.5	12	11	19
E. coli	10.5	14	11	13.5	14	15.5	14	9	11.5	20
S. aureus	12	15	8	16	14	15.5	16	12	14	20

∗Kermanshah (sample 1), Fasa (sample 2), Tabriz (sample 3), Neor Lake (sample 4), Sarab (sample 5), Gilan (sample 6), Khalkhal (sample 7), Kurdistan (sample 8), and Fereydun Shahr (sample 9). There were statistically difference between groups 2, 4, 5, 6, 7, and 9 (P-value < 0.05). There was a statistical difference between groups 1, 3, and 8 (P-value < 0.05). There were statistically difference between groups 2, 4, 5, 6, 7, and 9 with 1, 3, and 8 (P-value<0.001).

difference between groups 2, 4, 5, 6, 7, and 9 (P-value <0.05). There were statistically difference between groups 1, 3, and 8 (P-value<0.05).

3.4.3. Disk Agar Diffusion Analysis. The results were affected by the strains and EEP samples. As a result, propolis samples inhibited bacterial growth in various zones for *S. mutans* (9.5 to 16), *S. salivarius* (11 to 16), *S. mitis* (7.5 to 17), *L. acidophilus* (9 to 15), *C. albicans* (11 to 13.5), *E. coli* (9 to 15.5), and *S. aureus* (8 to 16) (Table 4). There were statistically difference between groups 2, 4, 5, 6, 7, and 9 with 1, 3, and 8 (P -value <0.001). There were statistically difference between groups 2, 4, 5, 6, 7, and 9 (P-value<0.05). There was a statistical difference between groups 1, 3, and 8 (P-value <0.05).

3.4.4. The Results of Biofilm Formation. To determine whether samples are effective in preventing biofilm development, microdilution was used. Figure 4 illustrates the percentage of samples that developed biofilm. These percentages are based on comparing the OD of each well with that of the control group (at 570-nm wavelength) in order to assess biofilm formation in the tested microorganisms (Table 5). Propolis sampled from different areas had different antibacterial and antifungal properties. Khalkhal propolis had the highest antibacterial and antifungal properties. On the other hand, Kurdistan, Sarab, and Gilan propolis were ranked after Khalkhal propolis. Tabriz and Neor propolis had fewer antibacterial and antifungal properties than Kurdistan, Sarab, and Gilan Propolis. Kermanshah, Fasa, and Fereydunshahr propolis had the lowest antibacterial and antifungal properties.

3.4.5. The Results of Biofilm Degradation. Biofilms were also investigated by using similar methods. In this case, the biofilm reduction rate was calculated as a percentage (Table 6). Khalkhal propolis had the highest antibacterial and antifungal properties. On the other hand, Kurdistan, Sarab, and Gilan propolis were ranked after Khalkhal propolis. Tabriz, Kermanshah, and Neor propolis had less antibacterial and antifungal properties than Kurdistan, Sarab, and Gilan Propolis. Fasa and Fereydunshahr propolis had the lowest antibacterial and antifungal properties.

4. Discussion

Dental caries can be prevented in part by reducing consumption of fermentable carbohydrates, by using fluoride mouthwash, by keeping teeth clean, and by a number of other methods. Caries control coadjutants can also be derived from natural sources. The herbal extract can replace synthetic antimicrobials. Caries is caused by an abundance of bacteria. *S. mutans* is not the only factor related to the onset of caries. In many cases, antibacterial compounds are tested on the biofilm of *S. mutans* [26]. Critical components of natural materials with antimicrobial activities are phenolic compounds. Phenolic compounds inhibit the enzyme glycosyltransferase [29].

Critical components of natural materials with antimicrobial activities are phenolic compounds. Phenolic compounds inhibit the enzyme glycosyltransferase [29]. *S. mutans* uses the enzyme glycosyltransferase to adhere to the tooth surface. The phenolic component artepillin C in propolis is effective against MRSA infections. The extract of propolis kaempferide is used to treat infections caused by *S. mutans*. Quercetin is a flavonoid component of propolis that binds to the DNA gyrase of *E. coli* to delay bacterial activity. Propolis can affect bacterial proteins and cause fractional bacterial lysis. *S. mutans* uses the glycosyltransferase enzyme to stick to the tooth surface. Artepillin C is one of the numerous phenolic components of propolis that showed antibacterial activity against MRSA. Kaempferide is an extract of propolis and is used to treat *S. aureus* skin infections. Also, Kaempferide was highly effective against *E. faecalis*, *S. saprophyticus*, and *L. monocytogenes* [29]. Quercetin is a flavonoid component of propolis that binds to the DNA gyrase of *E. coli* to delay bacterial activity. Proteins in bacteria are altered by propolis, causing partial bacterial lysis. Antibacterial properties were also found for pinocembrin and apigenin in propolis. A variety of microorganisms are resistant to cinnamic acid, which is found in propolis. In addition to damaging bacterial cell membranes, cinnamic acid interferes with ATPase activity, biofilm formation, and bacteria division [29].

In this study, the range of phenolic compounds was from 5.5 to 35.5 mg/g. Propolis from Fereydunshahr has the highest phenolic compounds, and propolis from Kermanshah

Table 5: The percentage of microbial biofilm formation.

Samples	2 × MBC/MFC concentrations of each Propolis %									CHX 0.2%
	1	2	3	4	5	6	7	8	9	
S. mutans	30	4	23.5	24	37.5	38	41	29	6.5	86
S. salivarius	24.5%	7	24	22.5	44	32	42.5	39	8.5	84.5
S. mitis	46	16	27	31	44	32	51	42.5	9.5	85
L. acidophilus	33	28.5	31	31.5	39	38.5	44	40	28	83
C. albicans	8	2.5	8	1	8.5	11	13	2.5	2.5	82.5
E. coli	30	28	31	31	36	38	44	42	26	84
S. aureus	45	16	27	31	42	33	51	42	9.5	84

Kermanshah (sample 1), Fasa (sample 2), Tabriz (sample 3), Neor Lake (sample 4), Sarab (sample 5), Gilan (sample 6), Khalkhal (sample 7), Kurdistan (sample 8), and Fereydun Shahr (sample 9). There were statistically difference between groups 2, 4, 5, 6, 7, and 9 (P-value < 0.05). There was a statistical difference between groups 1, 3, and 8 (P-value<0.05). There were statistically difference between groups 2, 4, 5, 6, 7, and 9 with 1, 3, and 8 (P-value < 0.001).

Table 6: The percentage of microbial biofilm degradation.

Samples	2 × MBC/MFC concentrations of each Propolis									CHX 0.2
	1	2	3	4	5	6	7	8	9	
S. mutans	27%	5	21	21	23	27.5	30.5	23	3.5	75%
S. salivarius	16.5	4.5	17	16.5	27.5	20	28	26.5	4.5	79
S. mitis	17	5	13.5	13.5	22.5	17	65.5	23	3.5	75.5
L. acidophilus	13.5	7.5	17	13.5	21	18.5	22.5	25	4.5	73
C. albicans	12	2.5	5.5	4.5	9.5	15	15	2.5	4	74
E. coli	13	7.5	16	14	22	16	23	24	4	73
S. aureus	16	5.5	13	14	22	16	60	21	4	74

∗Kermanshah (sample 1), Fasa (sample 2), Tabriz (sample 3), Neor Lake (sample 4), Sarab (sample 5), Gilan (sample 6), Khalkhal (sample 7), Kurdistan (sample 8), and Fereydun Shahr (sample 9). There were statistically difference between groups 2, 4, 5, 6, 7, and 9 (P-value < 0.05). There was a statistical difference between groups 1, 3, and 8 (P-value < 0.05). There were statistically difference between groups 2, 4, 5, 6, 7, and 9 with 1, 3, and 8 (P-value < Kermanshah (sample 1), Fasa (sample 2), Tabriz (sample 3), Neor Lake (sample 4), Sarab (sample 5), Gilan (sample 6), Khalkhal (sample 7), Kurdistan (sample 8), and Fereydun Shahr (sample 9). There were statistically difference between groups 2, 4, 5, 6, 7, and 9 (P-value < 0.05). There was a statistical difference between groups 1, 3, and 8 (P-value < 0.05). There were statistically difference between groups 2, 4, 5, 6, 7, and 9 with 1, 3, and 8 (P-value < 0.001). 0.001).

has the lowest phenolic compounds [26]. The phenolic content of propolis in Osés et al. study was reported from 65.49 to 228.40 (mg GA/g). According to studies, there were different ranges for phenolic contents of propolis extracts depending on solvent and standard used. Using methanol as solvent and gallic acid as standard, total phenolic contents of Portuguese and Brazilian propolis extracts ranged from 29.5 to 137 (mg/g). For propolis from China, Spain, and Poland, by ethanol as solvent and gallic acid as standard, more quantities of phenolic contents were gained 150–340 (mg/g) [30]. The range of flavonoids compounds was from 2.2 to 63.3 mg/g. Propolis from Khalkhal has the highest flavonoids compounds and propolis from Kermanshah has the lowest flavonoids compounds.

Flavonoids compounds were from 18.48 to 83.76 mg (Q/g) in Osés et al.'s study [30]. Our results were similar to other studies from different geographical areas, with results of 13 to 62 (mg Q/g) flavonoids. Similar results were found for Ethiopian propolis extracts from Ethiopia (from 14.76 to 68.88 (mg C/g)), and lower results were found for propolis extracts from Thailand, with an average of 3.40 (mg C/g) [30]. The MIC values were ranged (mg mL^{-1}) as follows: *S. salivarius* and *S. mutans* (0.003 to 0.029 and 0.003 to 0.048), *S. mitis* (0.007 to 0.058), *L. acidophilus* (0.007 to 0.117), *C. albicans* (0.014 to 0.234), *E. coli* (0.007 to 0.058), and *S. aureus* (0.007 to 0.058). The MBC and MFC values in mg mL^{-1} were range, respectively: for *S. mutans* (0.007 to 0.058), *S. salivarius* (0.007 to 0.117), *S. mitis* (0.007 to 0.117), *L. acidophilus* (0.014 to 0.234), *C. albicans* (0.029 to 0.468), *E. coli* (0.014 to 0.234), and *S. aureus* (0.007 to 0.117). The values found in this study are lower than those of previous studies [3, 31, 32]. And they are higher than some other studies [1, 5]. The chemical composition of extracts is variable, based on their harvest place, the season of harvest, and the type which cause its various biological properties, for instance, anti-inflammatory, antimicrobial, and antioxidant effects. Thus, these results explain the more study of propolis [26].

Surak et al. (2020) studied the cytotoxic properties of some propolis samples that were investigated by MTT assay on MCF7 (human breast adenocarcinoma), MDA-MB-231 (triple-negative human breast adenocarcinoma), HepG2 (human hepatocellular carcinoma), HeLa (human cervical adenocarcinoma), McCoy (normal mouse fibroblasts) cells, and HRT-18 (human colorectal adenocarcinoma). Propolis was effective against tumor cell lines. They concluded that propolis is a substance with antineoplastic properties [33]. Mohamed et al. (2020) studied the cytotoxic properties of

some propolis samples that were investigated by MTT assay on MCF7 and MCF 10A. Propolis was effective against tumor cell lines and inhibited the proliferation of the MCF7 cells [34].

This study, MTT analysis, showed that Fasa, Neor Lake, Khalkhal, and Kurdistan propolis had the highest cell viability with 500 mg/mL during 24 and 48 h. In addition, the cell viability was increased by decreasing the concentration of all groups. In this study, the range of zones of microbial growth inhibition by propolis samples for *S. mutans* was 9.5 to 16; *S. salivarius*, 11 to 16; *S. mitis*, 7.5 to 17; *L. acidophilus*, 9 to 15; *C. albicans*, 11 to 13.5; *E. coli*, 9 to 15.5; and *S. aureus*, 8 to 16. In this study, inhibition zones were higher than in previous studies [13, 35, 36]. We studied the effect of extracts on the degradation and formation of microbial biofilm. Propolis extract from Khalkhal had the highest effect on the formation and degradation, and propolis extract from Fasa had the lowest effect on the degradation and formation of biofilm. In our study, propolis from large areas of Iran was used. The selected areas were located at a considerable distance to study different regions of Iran. Almost all areas where bees were kept and had sufficient vegetation to grow bees and produce bee products were selected. We studied essential microorganisms in oral diseases and other important bacteria.

5. Conclusion

Several antimicrobial studies have found that propolis extracts are effective plaque inhibitors and may be used as a mouthwash. By inhibiting plaque development and by reducing biofilm formation, plaques and biofilms were decreased. In order to overcome the disadvantages of the gold chlorhexidine standard, more long-term clinical trials are necessary to incorporate standardization and certification of mouthwash.

Data Availability

All the data generated or analyzed during this study are included in this article, and also, the datasets analyzed to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

There is no conflict of interest in this study.

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Research Article

Effects of Cold-Light Bleaching on Enamel Surface and Adhesion of *Streptococcus mutans*

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Tooth bleaching is becoming increasingly popular among patients with tooth staining, but the safety of bleaching agents on tooth structure has been questioned. Primarily thriving on the biofilm formation on enamel surface, *Streptococcus mutans* has been recognized as a major cariogenic bacterial species. The present study is aimed at investigating how cold-light bleaching would change enamel roughness and adhesion of *Streptococcus mutans*. Human premolars were divided into 72 enamel slices and allocated into 3 groups: (1) control, (2) cold-light bleaching with 35% hydrogen peroxide (Beyond™), and (3) 35% hydrogen peroxide (Beyond™) alone. Biofilms of *Streptococcus mutans* were cultivated on enamel slices in 5% CO_2 (*v*/*v*) at 37°C for 1 day or 3 days. Enamel surfaces and biofilms were observed using scanning electron microscope (SEM). Atomic force microscopy (AFM) was applied to quantify the roughness of enamel surface, and the amounts of biofilms were measured by optical density of scattered biofilm and confocal laser scanning microscopy (CLSM). Cold-light bleaching significantly increased ($p < 0.05$) surface roughness of enamel compared to controls, but significantly inhibited ($p < 0.05$) adhesion of *Streptococcus mutans* on enamel in the bacterial cultures of both 1 day and 3 days. In conclusion, cold-light bleaching could roughen enamel surface but inhibit *Streptococcus mutans* adhesion at the preliminary stage after the bleaching treatment.

1. Introduction

Tooth bleaching has enjoyed great popularity among patients suffering from intrinsic and extrinsic tooth staining. It can efficiently improve the shade of dental fluorosis, tetracycline pigmentation teeth, and pulpless tooth [1]. Hydrogen peroxide (HP) or carbamide peroxide (CP) is the main component of the majority of bleaching products on the market. When applied on teeth, HP releases HO_2^- and OH [2], which diffuse through enamel and dentin, react with pigments or chromophores, and also change the reflection of tooth surface [3]. CP, which is rapidly decomposed of HP and urea after applied on teeth, shares similar bleaching mechanism with HP. Besides, urea from CP can degrade organic matrix in enamel, facilitating the diffusion of bleaching agents through enamel to reach the dentino-enamel junction [4, 5]. Application of lights can effectively assist the bleaching procedure, and cold-light bleaching has been proved effective to whiten teeth [6]. The efficacy is based on the application of high-concentrated bleaching agents combined with the activation of special blue light (wavelength between 480 nm and 520 nm). The cold-light lamp is equipped with filter to exclude the harmful infrared (wavelength $\lambda > 750$ nm) and ultraviolet ($\lambda < 380$ nm) to reduce the risk of thermal pulp

damage and possible side effects on living cells. In the case of cold-light bleaching, light can activate peroxide to promote the chemical redox reactions in the bleaching process [6].

To investigate the negative effect of bleaching treatment and improve bleaching products, numerous *in vivo* and *in vitro* studies have focused on the changes of morphology and microecology in oral cavity after bleaching treatment. Detrimental alterations in enamel have been reported, including the increase of surface roughness and the decrease of microhardness [5–9]. However, other studies found no significant changes in those aspects [10, 11]. Either is there any consensus on the effect of tooth bleaching on bacteria adhesion to enamel. Given to the increasing popularity of cold-light bleaching, the knowledge of the potential side effects is necessary for both applicants and patients. To our knowledge, nevertheless, only a limited number of studies have reported the effect of cold-light bleaching on enamel surface and adhesion of oral bacteria on enamel at the same time.

As a group of highly adherent bacteria, *Streptococcus mutans* (*S. mutans*) are one of the primary pathogens in the development of dental caries. Producing acids and bacteriocin, they are highly tolerant to acid and possess high-affinity systems for the assimilation of various carbohydrate sources. Thus, this study is aimed at investigating the effect of cold-light bleaching on enamel surface and adhesion of *S. mutans* on enamel. The null hypotheses are as follows: (1) cold-light bleaching has no effect on enamel surface and (2) it has no effect on bacteria adhesion.

2. Materials and Methods

2.1. Tooth Selection. Twenty-four maxillary premolars extracted for orthodontic purpose, with no caries lesions, enamel hypoplasia, cracks, or other defects on axle enamel surfaces, were collected. Volunteers all signed informed consent, and the study gained approbation of West China Hospital of Stomatology Institutional Review Board (WCHSIRB-ST-2015-128). The collected teeth were rinsed under high-pressure water for 10 min to remove the materia alba [6]. Then, they were stored in 2% formaldehyde solution at 4°C till following treatment [12].

2.2. Sample Treatment. Previously described protocol was applied with minor modifications [12]. Every tooth was sectioned into mesiobuccal, distobuccal, and lingual specimens after the removal of root, and enamel surfaces were flattened and polished serially with wet 800-, 1500-, 2000-, 4000-, 5000-grit silicon carbide abrasive papers (Struers, Cleveland, USA). Then, a $3 \times 3 \times 2$ mm enamel slice was cut from every flatted enamel sample using water-cooled saw (Isomet, Buehler, Lake Bluff, IL, USA). Slices were washed in running deionized water for 10 min and dried with compressed air for 5 s before the bleaching treatment.

To control interfering factors from individual variations among every tooth, we randomly allocated three specimens from one tooth into the three groups: (1) control, (2) cold-light bleaching, and (3) 35% hydrogen peroxide (Beyond™) alone.

2.3. Bleaching Procedure. The cold-light bleaching was processed according to the manufacturer's instructions. 35% HP gel (BEYONS II, Beyond™, Beijing, China) was applied on the enamel surface in the treatment group, and the thickness of gel was approximately 2 mm. The bleaching process lasted for 8 min with the cold-light lamp (BY-0398, Beyond™, Beijing, China) vertically 1 cm above enamel slices (power density = 500 mW/cm^2, energy density = 240 J/cm^2). The bleaching procedure was repeated twice, and the gel was removed during the intervals. The control group was mock-treated by 0.9% saline (w/v) along with cold-light bleaching for the same time as the bleached group. After bleached, all enamel specimens were washed in running deionized water again for 1 min and then sterilized [6].

2.4. Bacterial Strains and Growth Conditions. Streptococcus mutans UA159 (ATCC 700610) were maintained in Brain Heart Infusion (BHI) broth. Bacteria were incubated in BHI with 1% (w/v) sucrose (BHIS) for biofilm formation. The biofilms were incubated in the condition of 5% CO_2 (v/v) at 37°C without agitation.

2.5. Growth of Biofilm. A protocol of biofilm formation described previously was conducted with some modifications [13]. All enamel specimens were immersed in filter-sterilized saliva (100 ml) from healthy volunteers at 37°C for 2 h to form acquired pellicle [14]. For the biofilm formation, the bacteria were grown in BHI overnight and then diluted by fresh BHI till the optical density equaled 0.2 at 600 nm. 100 μl prepared planktonic bacteria mixed with 900 μl fresh BHI with 1% (w/v) sucrose (BHIS) was added to each well of 48-well tissue culture plate, in which the specimens were placed with the enamel surface uppermost. The plate was placed in 5% CO_2 (v/v) at 37°C, and specimens were transferred into new wells containing fresh BHIS every 24 h. Biofilms on enamel specimens were collected after cultured for 1 day ($n = 3$ for each group) or 3 days ($n = 3$ for each group). Samples were rinsed by sterilized water to eliminate planktonic bacteria, and biofilm on them was removed by cell scrapers and then suspended in 100 μl saline (0.9%, w/v) in tubes. Biofilms were sonicated for 10 min to separate cells by an ultrasonifier (output control at 8 and duty cycle of 70; Branson Sonifier 450, Fisher Scientific, USA). The turbidity was obtained by optical density (OD) at 595 nm using a microplate reader (SpectraMax 190, Molecular Devices Inc., Sunnyvale, CA) to quantify the amounts of biofilms on enamel.

2.6. Scanning Electron Microscope (SEM) Analysis. The sterile specimens were coated by gold and sent to perform SEM ($n = 3$ for each group). For the scanning of biofilm, the specimens adhered by *S. mutans* were fixed with 2.5% glutaraldehyde at 4°C overnight, dehydrated in series concentration of ethanol ranging from 30% to 100%, and sputter-coated with gold [14]. Specimens were scanned at ×20,000 and ×10,000 magnifications.

2.7. Atomic Force Microscopy (AFM) Analysis. The AFM test was performed according to previous descriptions [15, 16] with some modifications ($n = 3$ for each group). For surface

roughness, the images of morphology and values of roughness average (Ra, nm) were observed by SPM-9600 AFM system (Shimadzu, Kyoto, Japan) in the tapping mode with a silicon nitride tip of NSG11 (NT-MDT, Moscow, Russia) under ambient circumstances. Each specimen was scanned at three randomly selected sites covering an area of 10×10 μm at 1 Hz scanning rate. Adhesion forces of the enamel surface were measured in the contact mode with a tipless cantilever. Seventy force-distance curves were attained at seven random regions for each slice at a scanning rate of 0.5 Hz, ramp size of 18 μm, and trigger force of 5 nN. A built-in software within the STM9700 system was applied to calculate adhesion forces from the curves.

2.8. Confocal Laser Scanning Microscopy (CLSM). Enamel specimens were placed in 48-well plates with 100 μl prepared planktonic bacteria (OD = 0.2 at 600 nm), 900 μl BHIS, and Alexa Fluor 647 (10,000 MW; Molecular Probes Inc., USA) to label formed exopolysaccharide (EPS) as previously described [17]. The plates were incubated in 5% CO_2 (v/v) at 37°C in the dark for 1 day ($n = 3$ for each group) or 3 days ($n = 3$ for each group), during which specimens with biofilm were transferred into new wells with fresh BHIS and Alexa Fluor 647 every 24 h. After incubation, the specimens were rinsed by 0.9% saline (w/v) to remove the planktonic cells and then dried with a sterile filter paper. SYTO 9 nucleic acid stain (Molecular Probes Inc., USA) was applied to label *S. mutans* for 15 min. Specimens were washed and dried again. The whole process was completed in the dark, and the stained specimens were glued on glass slides for laser scanning confocal microscopy (Leica TCS SP2, Leica Microsystems, Wetzlar, Germany) which was equipped with a 60x oil immersion objective lens. 485 and 650 nm were used, respectively, as the absorption maxima wavelength for the nucleic acid stain and the EPS dye, and 498 nm was used as the emission maxima wavelength. At least three randomly selected positions of each specimen were captured. Images were taken from the bottom of the biofilm, section by section to the top layer of the biofilm instructed by previous study [18]. 3D images of the biofilm and the ratio of EPS/bacteria were obtained by IMARIS 7.0.0 (Bitplane, Zürich, Switzerland).

2.9. Statistical Analysis. Each experiment was repeated at least three times independently. All data were analyzed by SPSS 16.0. We set the level of significance to be 0.05. The Shapiro-Wilk test was used to test the distribution of data first. The independent t-test was used to compare the difference between groups for turbidity of scattered biofilm solution, while adhesion forces were analyzed by nonparametric analysis (Kruskal-Wallis test).

3. Results

3.1. The Roughness of Enamel Surfaces Was Increased after Bleaching. We combined the analyses of SEM and AFM to investigate morphological alterations of enamel surfaces caused by bleaching procedure. In the cold-light bleaching (CLB) group and 35% hydrogen peroxide (HP) group, enamel surface morphology became rougher with more pittings in images of SEM (Figure 1) and 3D images of AFM (Figures 2(a)–2(c)). Clear spherical enamel crystal structures could be observed, suggesting the demineralization of tooth enamel surface by bleaching treatment (Figure 1). The changes were more evident in the CLB group than the HP group (Figure 1). The quantitative results of Ra (nm) analyzed from AFM scanning presented higher Ra (nm) value in CLB and HP specimens than control specimens (Figure 2(c)). Besides, the adhesion forces of enamel surface were significantly higher ($p < 0.05$) in the CLB and HP groups than in the control group (Figures 3(a) and 3(b)).

3.2. The Adhesion of S. mutans Was Decreased on Bleached Enamel in Turbidity Test. Next, we tested *S. mutans* adhesion on enamel surfaces via turbidity test. The solution of scattered biofilm cultured for 3 days had significantly higher ($p < 0.05$) OD values comparing to that incubated for 1 day (Figure 4). The OD values of scattered biofilm solution collected from the CLB and HP enamels were significantly lower ($p < 0.05$) than those from the control group, which implicated the possible bacteria inhibitory effect of bleaching procedure (Figure 4). There was no significant difference between the CLB group and HP group ($p > 0.05$).

3.3. The Thickness of Biofilms Was Decreased on Bleached Enamel. SEM was applied to observe biofilms of *S. mutans* on enamel after incubation for 1 day and 3 days. From SEM images of biofilms incubated for 1 day, thinner *S. mutans* biofilms were formed on CLB and HP specimens (Figures 5(c)–5(f)) than on control ones (Figures 5(a) and 5(b)), indicating that *S. mutans* were less likely to adhere to bleached enamel. After incubation for 3 days, there was no significant visual difference in the amounts of bacteria between the bleached (Figures 5(i)–5(l)) and unbleached groups (Figures 5(g) and 5(h)).

3.4. S. mutans Were Less Inclined to Adhere on Bleached Enamel from Analysis of CLSM. Compared to biofilms incubated for 1 day, there was an apparent increase in the thickness of *S. mutans* (green) and EPS (red) in biofilms cultured for 3 days (Figure 6). From 3D reconstruction images (Figure 6), biofilms on CLB and HP specimens were less dense than control ones after incubation for 1 day and 3 days, indicating the comprised adhesion ability of *S. mutans* on CLB and HP enamels.

4. Discussion

Up to now, there is no agreement on whether bleaching treatment exerts adverse effects on enamel structure and adhesion of caries-associated microorganisms. One of the proposed mechanisms of tooth bleaching is the agents' effect on changing the reflection of light of the enamel. The increase in surface roughness after tooth whitening may lead to increased reflectance spectra and therefore to improved digital color reading [3, 19, 20]. Moreover, some studies have suggested that demineralization during tooth bleaching contributed to the efficacy of whitening [3, 21, 22], which was supported by a phenomenon of color regression after bleaching procedure associated with increased mineral uptake [23]. Thus, we evaluated the change of enamel surface morphology after

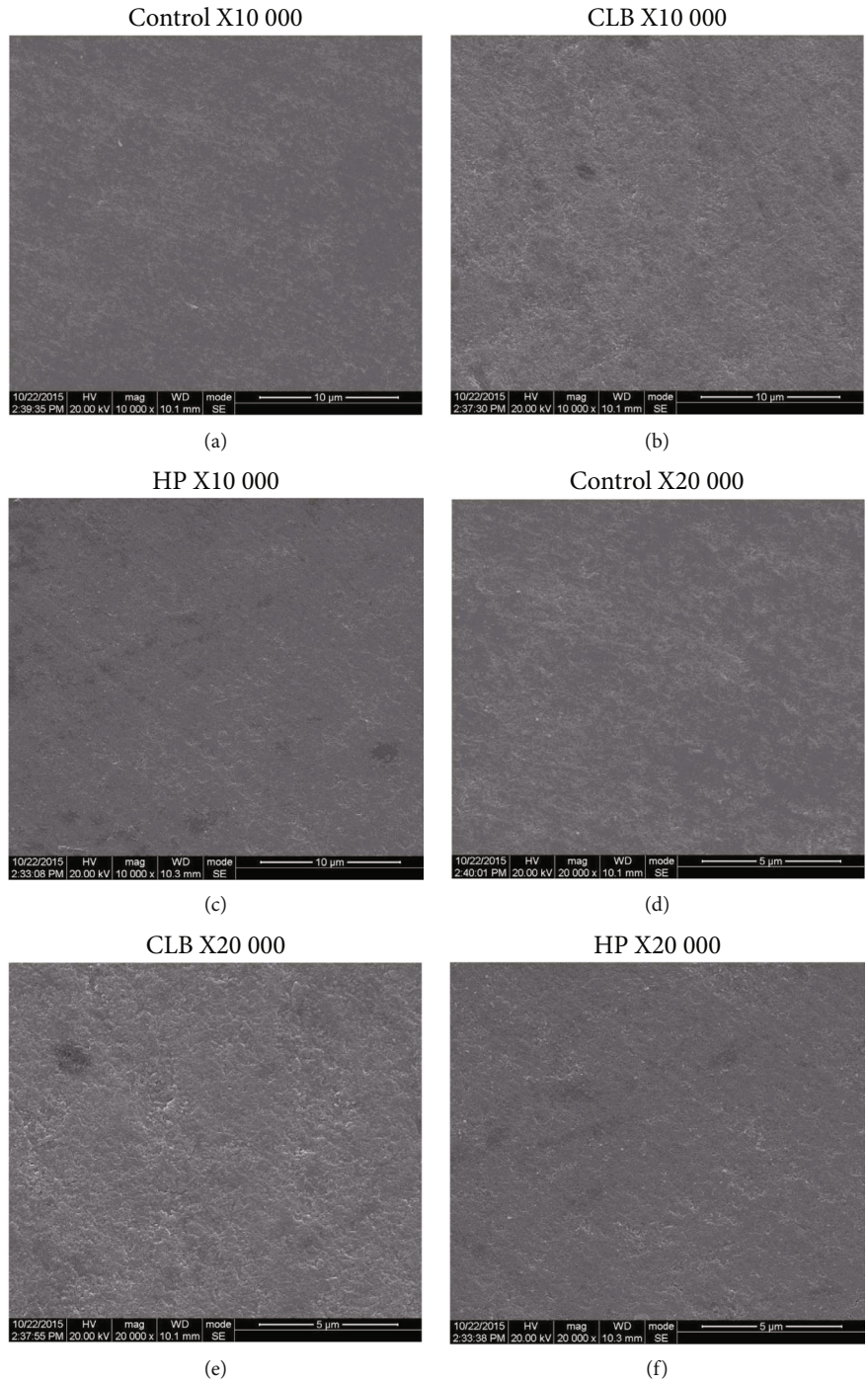
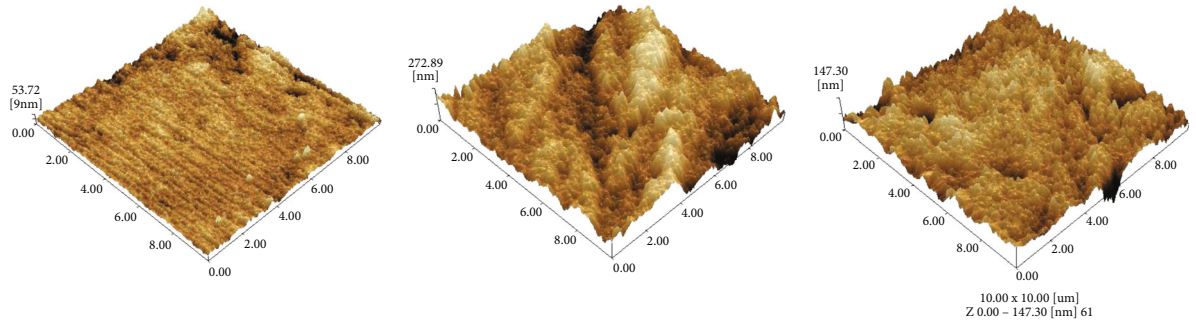


Figure 1: Scanning electron microscope (SEM) images of enamel surface. (a) Control specimen (×10,000 original magnification). (b) Cold-light bleached (CLB) specimen (×10,000 original magnification). (c) Hydrogen peroxide bleached (HP) specimen (×10,000 original magnification). (d) Control specimen (×20,000 original magnification). (e) CLB specimen (×20,000 original magnification). (f) HP specimen (×20,000 original magnification).

cold-light bleaching. Considering the disadvantages of SEM, including the low pressure ambient and acquired gold sputtering which may change natural condition of tested samples [24], we supplemented AFM scanning to provide 3D images of surfaces and more quantitative data to demonstrate the increase of surface roughness after bleaching treatment, especially after cold-light bleaching. The outcome of the morphological alteration of enamel in our study is in accordance with some previous studies. Hosoya et al. concluded that 35% HP could lead to the increase of roughness of enamel according to the test of a noncontact surface roughness shape-measuring apparatus [25]. Besides, rougher enamel surface after cold-light bleaching was reported [6, 26]. It was proposed that demineralization of enamel after bleaching treatment



	Control	CLB	HP
Ra (nm)	9.53±5.50	63.76±26.53*	25.64±12.58*

(d)

Figure 2: Atomic force microscopy (AFM) images of enamel surface. (a) Three-dimensional image of unbleached specimen. (b) 3D image of cold-light bleached specimen. (c) 3D image of hydrogen peroxide bleached specimen. (d) Roughness average (Ra, nm) values of specimens.

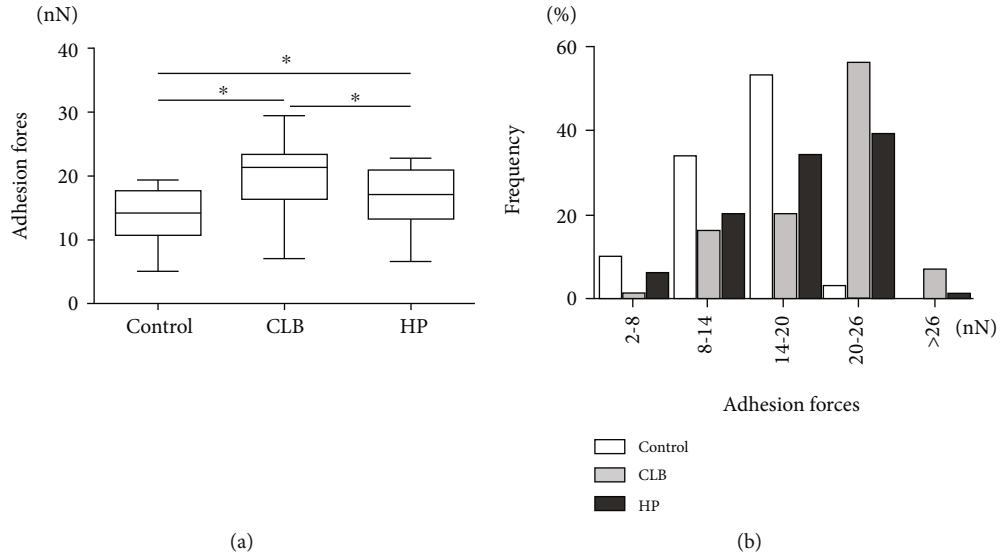


Figure 3: Adhesion forces analyzed from AFM. (a) Adhesion forces presented as mean with 5%-95% percentile ($^*p < 0.05$). (b) Distribution of adhesion forces of specimens.

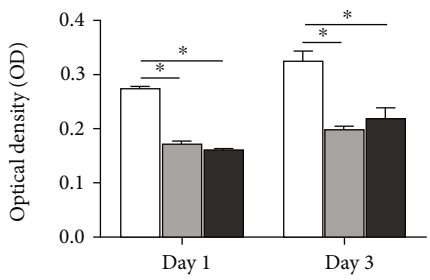


Figure 4: Values of OD of scattered biofilms incubated for 1 day or 3 days on specimens, presented as the mean ± deviation ($^*p < 0.05$).

depended on the pH value of agents rather than peroxide *per se* [27]. We observed rougher enamel surfaces in the CLB group than in the HP alone group, which might be explained by the activation of chemical redox reactions by cold light.

In addition, the inhibited biofilm formation of *S. mutans* on both CLB and HP enamels in our study is also consistent with some previous in vitro and in vivo studies. Yuan et al. [28] found the inhibitory effect of cold-light bleaching on the adhesion of mix bacteria in an artificial oral cavity model for 36 h. In an in vitro study, Zheng et al. [13] reported a decrease of *S. mutans*' adhesion to bleached enamel comparing to unbleached one during the first two weeks after cold-light bleaching. A similar change of bacteria adhesion after cold-light bleaching was also reported in an *in vivo* study [29]. Besides, Gursoy et al. found a declined level of plaque

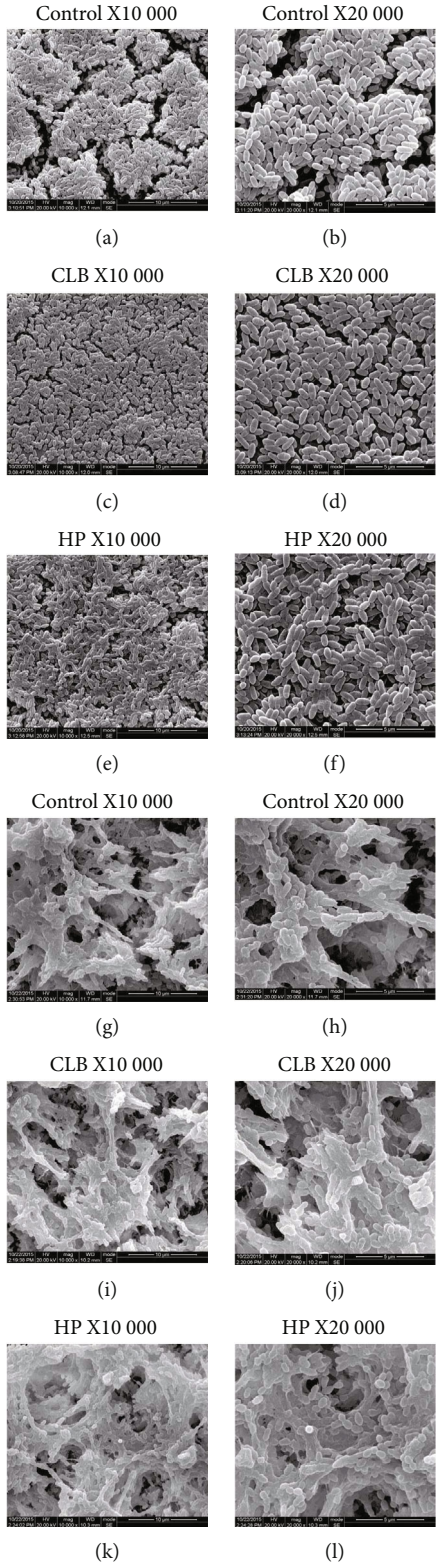


Figure 5: SEM images of biofilms. (a–f) Biofilms incubated for 1 day. (a, c, e) On unbleached, cold-light bleached (CLB), and hydrogen peroxide bleached (HP) enamel (×10,000 original magnification). (b, d, f) On unbleached, CLB, and HP enamel (×20,000 original magnification). (g–l) Biofilms incubated for 3 days. (g, i, k) On unbleached, CLB, and HP enamel (×10,000 original magnification). (h, j, l) On unbleached, CLB, and HP enamel (×20,000 original magnification).

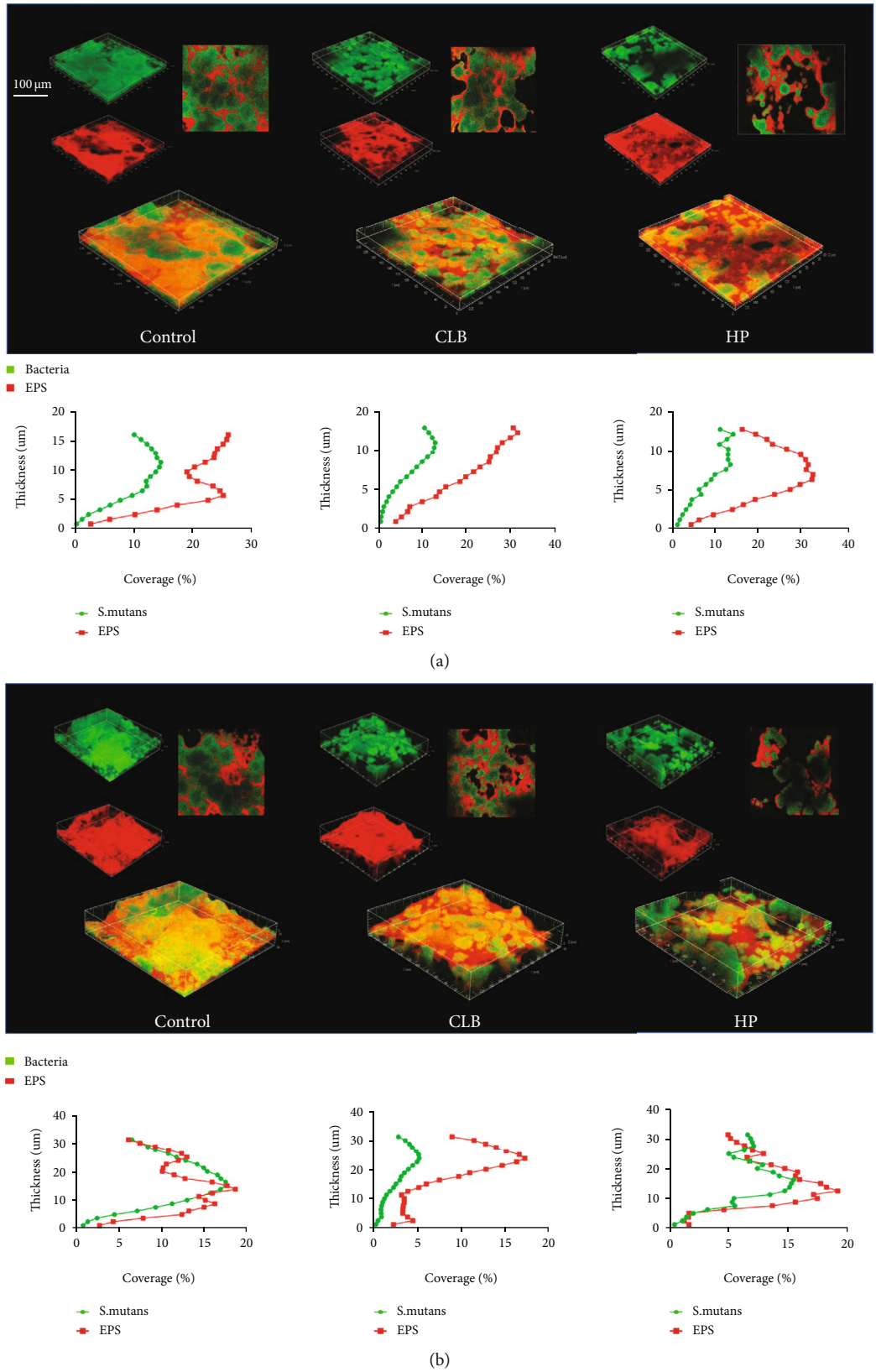


Figure 6: Confocal laser scanning microscopy images of *S. mutans* biofilms. (a) The three-dimensional reconstruction of the *S. mutans* biofilms incubated for 1 day on control and bleached slices and the correspondent distribution of EPS and bacteria of the reconstructed biofilms. (b) The three-dimensional reconstruction of the *S. mutans* biofilms incubated for 3 days on control and bleached slices and the correspondent distribution of EPS and bacteria of the reconstructed biofilms.

index on the third day after treating enamel surface by 35% HP assisted with light [30].

The roughness of enamel surface increased after being bleached, while *S. mutans*' adhesion to bleached enamel decreased. This might be the effect of the residual agents' effect in enamel [31]. The figures of distribution of EPS and bacteria obtained from CLSM also displayed a lower growing speed of bacteria from the bottom of biofilm on bleached specimens, which might indicate the inhibitory effect of residual HP.

However, Ittatirut et al. [14] reported a decrease in enamel surface roughness after being bleached and no significant difference in *S. mutans*' adhesion between bleached and unbleached enamels after incubated for 24 h. Besides, Hosoya et al. [25] reported more *S. mutans* adhering to bleached enamel in the biofilms incubated for 3 days. Indeed, the seemingly contradiction of those studies with ours can probably be explained by the antiseptic effect of bleaching agents itself and its roughening influence on enamel surface. The time it takes to eliminate the residual agents to the minimal inhibitory concentration depends on the types of agents, whitening process, and enamel rinsing procedure after bleaching. Different experimental designs could also have varied impacts on the morphology of enamel, and there is still no agreement on the correlation between the morphology of enamel and adhesion of bacteria.

5. Conclusion

Within the limitation of this in vitro study, we concluded that cold-light bleaching could significantly increase enamel surface roughness but inhibit the formation of biofilms of *S. mutans* till 3 days. In order to investigate the change of biofilm adhesion after bleaching treatment, future researches are needed to investigate the formation of biofilms in a relatively longer period. Also, it is necessary to explore the adverse effects of different sorts of bleaching treatments on enamel and bacteria adhesion to improve products and procedures.

Data Availability

The data that support the findings of the study is available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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