I Rasooli
S Shayegh
SDA Astaneh

The effect of *Mentha spicata* and *Eucalyptus camaldulensis* essential oils on dental biofilm

Authors' affiliations:
Iraj Rasooli, *Shakiba Darvish Alipoor Astaneh*, Department of Biology, Shahed University, Tehran, Iran
Shojaedin Shayegh, College of Dentistry, Shahed University, Tehran, Iran

Correspondence to:
Iraj Rasooli
Department of Biology
Shahed University
Opposite Imam Khomeini's shrine
Tehran-Qom High way
Tehran
Iran
Tel.: +98(21)55277647
Fax: +98(21)55277639
E-mail: rasooli@shahed.ac.ir

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Abstract: *Objectives:* To assess the antimicrobial effects of *Mentha spicata* and *Eucalyptus camaldulensis* essential oils and chlorhexidine against *Streptococcus mutans* and *Streptococcus pyogenes*, with a particular focus on *in vitro* and *in vivo* biofilm formation. *Methods:* The essential oils were analysed by gas chromatography (GC) and GC-mass spectrometry. *In vitro* and *in vivo* antimicrobial and biofilm preventing activities of the oils were studied. *Results:* Fifteen and 21 compounds were identified in the essential oils of *M. spicata* and *E. camaldulensis* respectively. Minimal bactericidal concentrations (MBC) of the *M. spicata* and *E. camaldulensis* oils were found to be 4 and 2 mg ml^{-1}, and those of chlorhexidine (2%) were 8 and 1 mg ml^{-1} for both *S. mutans* and *S. pyogenes* respectively. Decimal reduction time of *S. mutans* by *M. spicata* and *E. camaldulensis* oils at their MBC levels was 2.8 min, while that of cholrhexidine was 12.8 min. *D*-value of *S. pyogenes* exposed to the MBC levels of *M. spicata* and *E. camaldulensis* oils and of chlorhexidine were 4.3, 3.6 and 2.8 min respectively. Antibacterial and *in vivo* biofilm preventive efficacies of all the concentrations of eucalyptus oil were significantly ($P < 0.001$) higher than that of *M. spicata* oil and chlorhexidine. In conclusion, essential oils are capable of affecting biofilm formation. *Conclusion:* The essential oils from *E. camaldulensis* and *M. spicata* significantly retard biofilm formation that can contribute to the development of novel anticaries treatments.

Key words: biofilm; chlorhexidine; essential oils; *Eucalyptus camaldulensis; Mentha spicata; Streptococcus mutans*

Introduction

Controlling the dental biofilm is one of the major approaches to reducing dental caries and periodontal diseases (1, 2). Possible alternative means of antibacterial therapy for controlling infectious diseases have recently focused on affecting biofilm formation and bacterial accumulation (3). Bacterial cells are known to exhibit very different membrane compositions when grown in a biofilm situation compared with their planktonic counterparts (4). The ability of *Streptococcus mutans* and other cariogenic species to produce excess amounts of lactic acid in the presence of fermentable carbon sources is considered a major cause for tooth decay (5–7). Antimicrobial resistance in bacteria raises serious concern for the continued efficacy of antimicrobial agents in medicine, agriculture and industry (8). With the increase in the prevalence of microbial resistance to conventional antiseptics and antibiotics, attention is now turning to the use of natural antimicrobial compounds (9). Chlorhexidine is regarded as the 'gold standard' antiplaque treatment and is particularly effective against gingivitis and widely used as an adjunct treatment for periodontitis (10). Antiseptic mouthwashes have the potential to meet most of these criteria relative to gingivitis. Essential oils have been formulated into several over-the counter oral hygiene products, and the efficacy of the essential oil-containing mouthrinse, listerine, has been reported since the 1890s (11). Peridex is a 0.12% solution of chlorhexidine, a bisbiguanide antiseptic (12), while the active ingredients in Listerine are four essential oils: thymol 0.064%, eucalyptol 0.092%, methyl salicylate 0.060% and menthol 0.042% (13). Chlorhexidine has a strong affinity for tooth and tissue surfaces, and these serve as reservoirs even after rinsing or irrigation with the agent is completed (14). The escalating demand for new antimicrobials has prompted several investigations into the antimicrobial effects of phytochemicals extracted from a range of botanic origins, most of which have been used traditionally for many years. The antimicrobial properties of essential oils have been known for many years and have been used against a wide variety of bacteria and fungi, including oral pathogens (15–17). Essential oils are odorous, volatile products of plant secondary metabolism, and are found on many leaves and stems. The use of essential oils as antimicrobial agents has been described qualitatively for many years (18) and, in the specific case of oral microorganisms, essential oil mouthwashes have been demonstrated to be beneficial, safe components of daily oral health routines (19). But little is known about the spectrum of action of essential oils against oral microorganisms. Ideal antibacterial substances must be effective against more microorganisms, act rapidly, maintain activity at low concentrations, have no side-effects and be usable without causing discomfort. In this study, we compare the antibacterial activities of chlorhexidine with those of phytochemical essential oils from *Mentha spicata* and *Eucalyptus camaldulensis* against two oral bacteria.

Materials and Methods

Strains and culture conditions

The primary causative agent of dental caries, i.e. *S. mutans* PTCC 1601, was our main target in this study. A clinical isolate of *Streptococcus pyogenes* was taken as a model oral pathogen to find out possible simultaneous antimicrobial effects of anti-*S. mutans* agents. All strains were cultured on blood agar.

Oil extraction and analysis

The plant materials were steam distilled for 90 min in full glass apparatus. The oils were isolated using a Clevenger-type apparatus. The extraction was carried out after a 4-h maceration in 500 ml of water. The oils were stored in dark glass bottles in a freezer until they were used. Gas chromatography (GC) analyses were performed using a Shimadzu-9A gas chromatograph (Kyoto, Japan) equipped with a flame ionization detector, and quantitative analysis was carried out on Euro Chrom 2000 from Knauer (Berlin, Germany) by the area normalization method neglecting response factors. The analysis was carried out using a DB-5 fused-silica column (30 m × 0.25 mm, film thickness 0.25 μm; J & W Scientific Inc., Rancho Cordova, CA, USA). The operating conditions were as follows: injector and detector temperature, 250 and 265°C respectively; carrier gas, helium. Oven temperature programme was 40–250°C at the rate of 4°C min^{-1}.

Gas chromatography-mass spectrometry

The GC-mass spectrometry (GC-MS) unit consisted of a Varian Model 3400 gas chromatograph (Varian Inc., Palo Alto, CA, USA) coupled to a Saturn II ion trap detector. The column was the same as GC, and the GC conditions were as mentioned earlier. Mass spectrometer conditions were: ionization potential 70 eV; electron multiplier energy 2000 V. The identities of the oil components were established from their GC retention indices, relative to C7–C25 n-alkanes, by comparison of their MS spectra with those reported in the literature (20, 21), and by computer matching with the Wiley 5

mass spectra library, whenever possible, by co-injection with standards available in the laboratories.

Oil dilution solvent

1/2, 1/4 and 1/8 dilutions of the oils were made with dimethylsulphoxide (DMSO). These dilutions were used in antibacterial analysis. Undiluted oil was taken as dilution 1. Bacterial strains were streaked on Mueller Hinton agar plates using sterile cotton swabs. Sterile blank filter paper discs of 6 mm diameter loaded with 5 µl of DMSO were placed on the agar plates and incubated at 37°C for 24 h. No antibacterial activity was observed on the plates and hence DMSO was selected as a safe diluting agent for the oil. Five microlitre from each oil dilution followed by sterilization using a 0.45-µm membrane filter was added to sterile blank discs. The solvent also served as control.

Preparation of essential oil blended toothpastes

A concentration of 10, 20, 30 and 40 µl of each essential oil was mix blended with 50 ml of non-fluoride toothpastes aseptically to obtain 0.2, 0.4, 0.6 and 0.8 mg ml^{-1} of oil in each toothpaste. The pastes were then reintroduced to the tubes and capped tightly.

Disc diffusion method

The agar disc diffusion method was employed for the determination of antimicrobial activities of the essential oils in question. Briefly, 0.1 ml from 10^8 CFU ml^{-1} bacterial suspension was spread on the Mueller Hinton agar plates. Filter paper discs (6 mm in diameter) were impregnated with 5 µl of the oil and were placed on the inoculated plates. These plates, after remaining at 4°C for 2 h, were incubated at 37°C for 24 h. The diameters of the inhibition zones were measured in millimetres. All tests were performed in triplicate.

Determination of minimum inhibitory and bactericidal concentrations

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were assessed according to our modified procedure (22). MIC was determined by a broth dilution method in test tubes as follows: 40 µl from each of various dilutions of the oils was added to 5 ml of brain heart infusion (BHI) tubes containing 10^7 CFU ml^{-1} of live bacterial cells. The tubes were then incubated on an incubator shaker to evenly disperse the oil throughout the broth in tubes. The highest dilution (lowest concentration) showing no visible growth was regarded as MIC. A 0.1 ml of the cell suspensions from the tubes showing no growth was subcultured on BHI agar plates in triplicate to determine if the inhibition was reversible or permanent. MBC was determined as the highest dilution (lowest concentration) at which no growth occurred on the plates.

Bactericidal kinetics of the oils

Forty microlitres of each oil at the dilution determined by MBC was added to each 5 ml of BHI broth tubes containing bacterial suspension of 10^7 CFU ml^{-1}, and the tubes were then incubated at 37°C in an incubator shaker. A 0.1 ml samples were taken after 5, 10, 15, 20, 25, 30, 45, 90, 120, 150, 180, 210, 240, 270 and 300 min. The samples were immediately washed with sterile phosphate buffer pH 7.0, centrifuged at 9500 g per 1 min, re-suspended in the buffer, spread cultured on BHI agar and incubated for 24 h at 37°C. Phosphate buffer was used as a diluent when needed. Bactericidal experiments were performed three times. Microbial colonies were counted from triplicates after the incubation period, and the mean total number of viable cells per ml was calculated.

Determination of decimal reduction time (*D*-value)

The mean total number of viable bacteria from bactericidal kinetics experiments at each time interval was converted to $\log_{10}$ viable cells using routine mathematical formulae. The time required to decrease the microbial population in a sample by 90% on the basis of logarithmic value of one was regarded as *D*-value.

Specific biofilm formation assay

As a result of its biofilm forming properties, *S. mutans* alone was employed in this part of the study (23). Bacteria were grown in 14-ml polystyrene culture tubes containing 2 ml of Lauria-Bertani medium and various concentrations of essential oils or individual chemicals. The total volume of the test compounds added never exceeded 1.5% of the culture volume. Nine identically prepared tubes were used for each concentration. Three of the nine tubes were used to measure growth in suspended culture (*G* tubes), three tubes were used to measure biofilm growth (*B* tubes) and three tubes served as controls for abiotic factors (NC tubes). Inocula were grown to the late log phase. Subsequently, the *B* and *G* tubes received 20 µl of inoculum, and all tubes (*B*, *G* and NC tubes) were incubated in

an orbital cabinet shaker for 17 ± 1 h. Following incubation, cells in the *G* tubes were mixed well by gentle vortexing, and the optical densities of the cultures at 600 nm (OD_{600}) were measured. The *B* and NC tubes each received 125 µl of a 0.3% solution of crystal violet (CV). After 15 min, the suspended culture was poured out, and the tubes were rinsed well with distilled deionized water (six rinses, approximately 4 ml per rinse). Any remaining CV was dissolved in 2 ml of an ethanolacetone (80:20) solution, and the absorbance at 570 nm of each resultant solution was measured spectrophotometrically. Biofilm accumulation was normalized with respect to growth, which yielded the specific biofilm formation (SBF). SBF was determined by using the following formula: SBF = (*B* − NC)/*G*, where *B* is the CV retained in the *B* tubes, NC is the amount of CV that adhered to the polystyrene tubes because of abiotic factors and *G* is the optical density of cells grown in suspended culture. At least two replicate experiments were performed for each concentration of chemical that was tested.

In vivo studies

Equal number of male and female dentistry students of Shahed university, Tehran, volunteered to participate in this study. Their age range was 20–30 years. All students showed an overall healthy mouth status. A total of 100 volunteers took part in this study. Three major groups were designed in this study. Each group or subgroup consisted of 10 individuals. Group 1 brushed using a standard toothpaste. This group served as control. Group 2 and its eight subgroups brushed using the same type of toothpastes as in the control group that was blended with different concentrations of essential oils. Group 3 brushed using toothpastes blended with 0.2% chlorhexidine. After the baseline examinations, we randomly assigned subjects to one of the three groups. Each subject then received a complete dental prophylaxis to remove plaque, stain and calculus, which was confirmed by the use of disclosing solution. The groups were instructed to brush twice daily with an ADA-accepted toothbrush (Oral-B 35). We instructed all subjects to brush thoroughly twice daily and gave them toothbrushes and dentifrice as needed. We allowed the subjects to follow their usual dietary habits, but instructed them to refrain from using any oral care products other than what we provided them for the study. We permitted limited interdental cleaning in all groups in instances of considerable food entrapment. All the subject groups were examined once a week for 4 weeks. On each examination, after using a disclosing solution on the teeth, we scored the plaque area using the Turesky modification of the Quigley-Hein Plaque Index (24) on six surfaces of all scorable teeth as follows: no plaque (0); separate flecks or discontinuous band of plaque at the gingival (cervical) margin (1); thin (up to 1 mm), continuous band of plaque at the gingival margin (2); band of plaque wider than 1 mm but less than one-third of surface (3); plaque covering one-third or more, but less than two-thirds, of surface (4) and plaque covering two-thirds or more of surface (5).

Statistical analysis

The values were calculated as the mean percentage of bacterial adherence, compared with control groups. Differences between two means were evaluated by the Student's paired *t*-test and were used to compare each test with the control. The level of significance was determined at $P < 0.05$. A one-way ANOVA was performed for comparison of multiple means.

Results

Gas chromatography and GC-MS analysis of the essential oils of *M. spicata* L. and *E. camaldulensis* var. *obtuse* led to identification of 15 and 21 compounds respectively (Tables 1 and 2). *Mentha spicata* L. oil had limonene (48%), piperitone (20.27%) and caryophyllene (7.9%) as the major compounds. 1,8-Cineole (64%), γ-terpinene (7%), myrcenol (7.4%) and caryophyllene oxide (3.7%) were the major compounds of *E. camaldulensis* oil. The results of the agar diffusion tests are summarized in Table 3. The essential oils exerted variable antimicrobial effects on different microorganisms taken under study with greater antibacterial effect than chlorhexidine (Table 3). Minimal inhibitory concentrations (MIC) of the *M. spicata* and *E. camaldulensis* oils tested in 5 ml tubes were found to be 2

Table 1. **Chemical composition of essential oil from *Mentha spicata* L**

No.	Compound	RI	%
1	α-Pinene	928	2.35
2	Sabinene	963	2.27
3	β-Pinene	968	3.37
4	Myrcene	979	2.9
5	Limonene	1022	48
6	Menthol	1172	4.7
7	Piperitone	1228	20.27
8	Isopiperitinone	1239	0.7
9	Geranial	1249	1.3
10	Piperitinone	1311	0.9
11	Piperitinone oxide	1343	0.3
12	β-Cubebene	1385	2
13	Caryophyllene	1420	7.9
14	α-Humulene	1445	0.8
15	Germacrene-D	1475	0.96

Table 2. **Chemical composition of essential oil from *Eucalyptus camaldulensis* var. *obtuse***

No.	Compound	Kovats	%
1	α-Pinene	935	9.6
2	Sabinene	975	0.4
3	β-Myrcene	991	0.4
4	α-Phellandrene	1004	1.6
5	1,8-Cineole	1027	64
6	γ-Terpinene	1055	7
7	Terpinolene	1084	0.7
8	Myrcenol	1111	7.4
9	Nopinone	1136	1.4
10	Pinocarvone	1161	0.4
11	Terpin-4-ol	1175	1.5
12	α-Terpineole	1185	0.3
13	Dihydro carveol	1193	0.4
14	Neo-iso-Dihydro carveol	1227	0.3
15	Aromadendrene	1436	1.9
16	α-Humulene	1457	0.7
17	Bicyclogermacrene	1492	0.5
18	Elemol	1556	0.6
19	Caryophyllene oxide	1578	3.7
20	Humulene epoxide II	1601	0.5
21	α-Acorenol	1623	0.6

Table 3. **Disc diffusion assay of antibacterial properties of essential oils and chlorhexidine**

	Mean zone of bacterial growth inhibition (mm)	
Antibacterial agent	*Streptococcus mutans*	*Streptococcus pyogenes*
Mentha spicata	60 ± 0	46 ± 2
Eucalyptus camaldulensis	47 ± 2.65	48.33 ± 2.89
Chlorhexidine (0.2%)	14 ± 1	22 ± 1

and 1 mg ml^{-1} for both *S. mutans* and *S. pyogenes* respectively. MBC of the *M. spicata* and *E. camaldulensis* oils tested in 5 ml tubes were found to be 4 and 2 mg ml^{-1} for both *S. mutans* and *S. pyogenes* respectively. MIC/MBC values of chlorhexidine (2%) were 6/8 and 1/1 mg ml^{-1} with respect to *S. mutans* and *S. pyogenes* respectively. Using these values, kinetics of bacterial death was studied to determine the decimal reduction time (*D*-value) (Figs 1 and 2). Decimal reduction time of *S. mutans* brought about by each of the essential oils from *M. spicata* and *E. camaldulensis* at their MBC levels was 2.8 min, while that of chlorhexidine was 12.8 min. Lower amount of eucalyptus oil could completely kill *S. mutans* within 20 min of exposure, which is comparable with the same killing time with double amount of *M. spicata* oil (Fig. 1). *D*-value of *S. pyogenes* on exposure to the MBC levels of *M. spicata* and *E. camaldulensis* oils and of chlorhexidine were 4.3, 3.6 and 2.8 min, indicating higher efficacy of chlorhexidine ($P < 0.05$). *Streptococcus pyogenes* was affected by both oils and

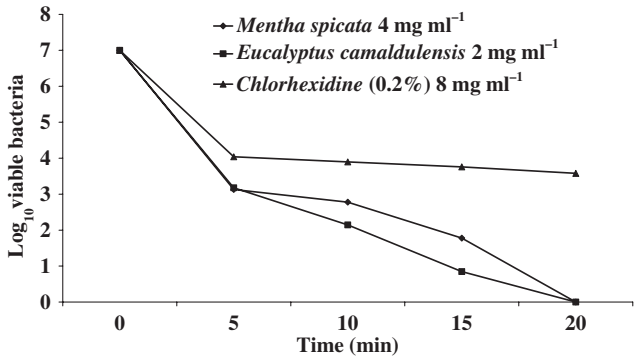

Fig. 1. Decimal reduction time (*D*-value) of *Streptococcus mutans* exposed to the MBC levels of essential oils and chlorhexidine.

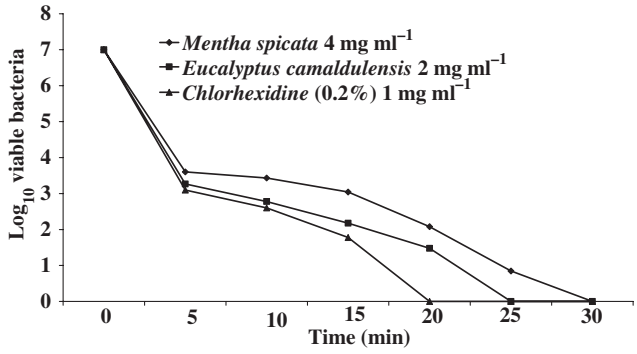

Fig. 2. Decimal reduction time (*D*-value) of *Streptococcus pyogenes* exposed to the MBC levels of essential oils and chlorhexidine.

by lower amount of chlorhexidine (Fig. 2). *In vitro* inhibition of biofilm formation by *S. mutans* was studied at three concentrations of 1, 2 and 4 mg ml^{-1} of essential oils and chlorhexidine (Fig. 3). It was found that chlorhexidine was more effective at 1 mg ml^{-1} concentration, while *E. camaldulensis* oil showed stronger activity at 2 and 4 mg ml^{-1} concentrations. Lowest inhibitory activity on *in vitro* biofilm formation was noted with *M. spicata* oil (Fig. 3). *In vivo* experiments confirmed the results obtained from the *in vitro* experiments. The inhibitory activity of all the concentrations of eucalyptus oil were significantly ($P < 0.001$) higher than *M. spicata* oil and chlorhexidine. Chlorhexidine (2 mg ml^{-1}) inhibited maximum of 13.9% dental biofilm formation at the end of the fourth week. At the end of the same period, 2, 4, 6 and 8 mg ml^{-1} concentrations of *M. spicata* and *E. camaldulensis* oils had 2.43%, 7.20%, 7.44%, 9.66% and 14.46%, 20.25%, 22.93%, 39.24% inhibition on dental biofilm formations respectively (Fig. 4).

Discussion

The major components play the primary role in the antibacterial activities of the essential oils. *Streptococcus mutans* showed

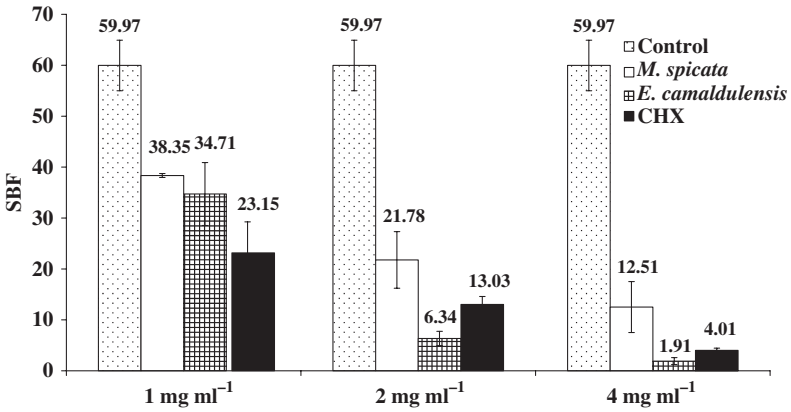

Fig. 3. In vitro biofilm formation by *Streptococcus mutans* exposed to various levels of different essential oils and chlorhexidine.

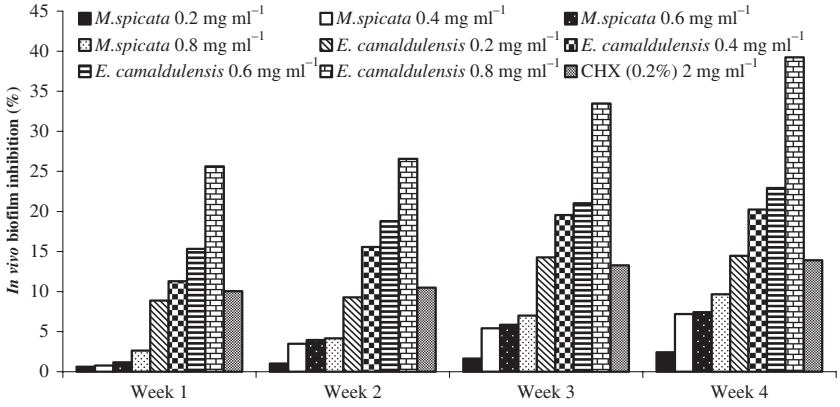

Fig. 4. Per cent *in vivo* inhibition of biofilm formation compared with the control groups using Quigley-Hein Plaque Index.

significantly ($P < 0.05$) higher sensitivity to *M. spicata* oil than *S. pyogenes*, while this difference was not significant when the organisms were exposed to *E. camaldulensis* oil (Table 3), indicating equal antistreptococcal effect of *Eucalyptus* oil. Chlorhexidine (0.2%) could inhibit both bacterial growth significantly ($P < 0.05$) (Table 3). Because of the variation of diffusion and solubility properties of the different oils, the results obtained by the disc diffusion method are not directly comparable with those obtained with the microdilution broth assay (25). However, the oils that exhibited large inhibition zones for a given bacteria were confirmed as those with the lower MIC values. The essential oil of *E. camaldulensis*, characterized by the presence of high concentrations of 1,8-cineole with well-documented antimicrobial activity (26), inhibited the growth of both streptococci under study. In this study, we sought to evaluate the effect of the essential oils with chlorhexidine with a view to reducing the formation of biofilm *in vitro* (Fig. 3) and *in vivo* (Fig. 4). The results show higher efficacy of chlorhexidine at 1 mg ml^{-1}, while eucalyptus oil was the most effective agent inhibiting biofilm formation at higher concentrations (Fig. 3). Cinnamon essential oil has been reported to exhibit the greatest antimicrobial potency at 1.25–2.5 mg ml^{-1} concentration against planktonic and biofilm cultures of *S. mutans* (27). Our results show effective antimicrobial potency at doses of 2 and 4 mg ml^{-1} for *E. camaldulensis* and *M. spicata* oils in planktonic cultures (Fig. 3). *In vivo* experiments (Fig. 4) indicated that lower concentrations of *M. spicata* oil were significantly ($P < 0.001$) ineffective during the first 2 weeks as compared with that of eucalyptus oil and chlorhexidine. The efficacy of *M. spicata* was comparable with, but lower than, that of chlorhexidine during the 3rd and 4th week (Fig. 4). Chlorhexidine has been studied in a number of controlled trials for periods of 6 months or longer. In these studies, plaque reduction has ranged from 16% to 61% (28–30) as against our results of 10.05% to 13.90%. These differences could be attributed to the mode of application of chlorhexidine, such as mouthrinse and toothpaste formulations, and to the duration of study. Minor changes in plaque scores were recorded in a 1-year study on 10 patients who had been treated non-surgically for periodontal disease in which the subjects were instructed to use chlorhexidine (0.2%) twice daily as a rinse, but were not given any other formal oral hygiene instructions (31). Similarly designed studies for Listerine showed plaque reductions ranging from 22% to 36% (32). These clinical studies also clearly demonstrate that essential oil mouthwashes have excellent safety and tolerability profiles. They showed no evidence of extrinsic tooth stain compared with

controls, and intra-oral soft-tissue examinations showed no aberration of any kind (32–36). In addition, the users reported no changes in taste perception and showed no increase in calculus formation (32, 33). However, there are side-effects to chlorhexidine treatment, such as an objectionable taste, tooth discoloration and desquamation and soreness of the oral mucosa (10). Its activity is pH dependent and is greatly reduced in the presence of organic matter (37). Recent reports have suggested that chlorhexidine was ineffective against dental caries in clinical trials (38), and it has been implicated as the potential cause of the selection and persistence of bacteria with low level antibiotic resistance (37, 39). This study shows that essential oils are capable of affecting biofilm formation. They significantly decreased bacterial adhesion and affected bacterial viability in biofilms. Thus, essential oils have the ability to act as novel agents in affecting biofilm formation and represent a step in preventing biofilm-associated diseases.

Conclusion

This study demonstrates that the use of essential oil-containing toothpastes provides a meaningful and clinically significant incremental benefit to a recommended regimen of brushing twice daily. Dental professionals should consider recommending a brush regimen using essential oil blended toothpastes to their patients to maintain dental and gingival health.

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