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Antibacterial Efficacy of Eucalyptus camaldulensis and Mentha spicata Essential Oils against Eikenella corrodens-Induced Halitosis

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ABSTRACT

Eikenella corrodens (*E. corrodens*) is one of the main bacteria responsible for halitosis due to its metabolism of amino acids, which produces volatile sulfur compounds (VSCs). High demand for new antibiotic formulations, due to increased resistance to the available types, has triggered attempts to find plants with antibiotic properties. This study aimed to assess the antibacterial effects of *Eucalyptus camaldulensis* (EC) and *Mentha spicata* (MS) essential oils on *E. corrodens*. The antibacterial efficacy of EC and MS against *E. corrodens* was evaluated using the well diffusion technique. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of EC and MS essential oils against *E. corrodens* were determined using the broth microdilution method. Data were analyzed using Kruskal-Wallis, Mann-Whitney and Student's t tests. The mean diameter of the growth inhibition zone was 8.8 ± 0.837 mm for MS and 16 ± 4.637 mm for EC. The MIC values were 0.55 and 0.45 mg/mL for EC and MS, respectively. The MBC values were 1.8 and 2.4 mg/mL for EC and MS, respectively. Based on our findings, MS and EC essential oils possess bacteriostatic properties against *E. corrodens*. Mouthwashes containing EC and MS essential oils may effectively eliminate bad breath.

Introduction

Usually, human breath has a neutral odor known as the human odor. The gas-emitting bacteria on the tongue and in the gingival sulci are mainly responsible for rotten breath, also known as halitosis, bad breath, malodor, *fetor oris* and mouth odor. [6] Yaegaki and Coil in 2000 were among the first to discuss the biochemical mechanism and pathogenesis of halitosis. [22] It has been reported that approximately half the world's population suffers from halitosis, [3] which can be considered as a social dilemma rather than a medical concern. [19] Non-pathological oral malodors include morning breath and bad breath due to fatigue, starvation, puberty, pregnancy, menstruation, menopause and nutrition.[1-3,5,7-12,14-18,21] Pathological halitosis has an intra-oral origin in 90% of cases.[2] Extra-oral causes, comprising the remaining 10% of cases, include a wide range of conditions from

The intraoral causes mainly include the metabolic and proteolytic activities of anaerobic Gram-negative bacteria on tooth surfaces and the dorsal surface of the tongue as well as in periodontal pockets. [14] It has been shown that bacteria can metabolize amino acids (e.g., cysteine and methionine) and produce sulfur-containing gases known as volatile sulfur compounds (VSCs) (e.g., hydrogen sulfide and methyl mercaptan), which are responsible for halitosis. [1] Studies have shown that methyl mercaptan is the main cause of malodor of the dorsal tongue surface in periodontal patients.[8,16] However, in patients with healthy periodontium, hydrogen sulfide is mainly responsible for bad breath. These two gases comprise 90% of all VSCs. [8,15,16] In addition, sulfur-containing protein compounds are present in the saliva and gingival crevicular fluid, which are comprised of oral epithelial cells, leukocytes, other blood cells, food residues and bacteria.[8] VSCs have a destructive effect on periodontal tissues, and in turn, destruction of tissue increases the level of these gases. In the oral environment, bacteria accumulate on the dorsal tongue surface and get trapped in extraction sockets, necrotic tissue, extensive cavities, open contacts (sites of food impaction), crowns, defective restorations, inflammatory lesions and wounds in gingivitis and periodontitis patients. [6,8,15] Studies have shown that *Prevotella intermedia*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Micromonas micros*, *Campylobacter rectus*, *Eikenella corrodens* and *Treponema denticola* play major roles in the production of VSCs. [3,8,15,17] *E. corrodens* is among the normal oral flora and is present in 60% of dental plaque samples. It is naturally resistant to clindamycin and aminoglycosides but its isolates are sensitive to penicillin, cephalosporin, amoxicillin, chloramphenicol, carbenicillin and imipenem *in vitro*. [7]

The increased prevalence of antibiotic resistence has urged researchers to focus on the antimicrobial efficacy of herbal extracts and essential oils. For this purpose, since 1890, herbal extracts and essential oils have been used in oral hygiene products and formulations of mouthwashes. [18] The essential oils that constitute the focus of the present study, *Eucalyptus camaldulensis* (EC) and *Mentha spicata* (MS), are used in the formulation of mouthwashes and oral care products such as Listerine. [18]

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EC and MS are among the Lamiaceae, a large family of flowering plants with about 200 genera and 4000 species. [11] They are aromatic and include herbs such as basil, mint, rosemary, sage, savory, marjoram, oregano, hyssop, thyme, lavender and perilla. MS, commonly known as spearmint, is famous for its carminative oil (spearmint oil). Its distinctive smell is attributed to its main constituent, carvone. It also contains limonene, dihydrocarvone and 1,8-cineol. [11]It is often used as a flavor for toothpastes. The River Red gum (*E. camaldulensis*) is a tree of the genus *Eucalyptus*. It grows in many parts of the world, but is native to Australia. Eucalyptus oil is also often added to the formulations of fragrances, soaps, foods and drinks.[9]

A recent study showed that the effect of MR and EC on the bacteria present in dental biofilm was even greater than that of chlorhexidine (CHX), which is the gold standard anti-plaque mouthwash for treatment of gingivitis and periodontitis. [18] Therefore, the current study aimed to assess the antibacterial efficacy of MS and EC essential oils against *E. corrodens* to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) values of these two herbal essential oils.

Materials and Methods

This *in vitro* experimental study was conducted on the standard bacterial strain *E. corrodens* (PTCC 1391). The study design was approved by the ethics committee of Shiraz University of Medical Sciences. All phases of bacterial culture and tests were performed in the Bacteriology Department of Shiraz University of Medical Sciences.

Preparation of essential oils

The essential oils of MS and EC were purchased from Kesht va sanat Kazerooncompany (Kazeroon, Iran). To assess the composition of the oils and their antimicrobial constituents, data sheets showing the results of Gas Chromatography Mass Spectrometry (GC-MS) analysis of the extracts were obtained from the manufacturer. GC-MS is among the most advanced analytical devices available and combines gas chromatography and mass spectrometry for the detection and isolation of essential oil constituents. [21]

Bacteria

All tests were performed on standard strain *E. corrodens* (PTCC 1391), which was obtained from the Scientific and Industrial Research Organization of Iran. *E. corrodens* is a Gram-negative, slow-growing, anaerobic rod and is part of the human normal oral flora. *E. corrodens* grows in different culture media such as Mueller-Hinton agar with 5% sheep's blood, chocolate agar, Trypticase soy agar with blood and Brucella agar with blood under anaerobic, aerobic or 5% CO_2 (microaerophilic) conditions. [1]

Dilution of essential oils

Dimethyl sulfoxide (DMSO) solvent was used for dilution of the essential oils. This solvent is widely used in bacterial analyses. 128 mg/mL concentration of the essential oils was prepared using DMSO. The baseline concentration of the essential oils was assumed to be 100% (pure). To prepare the desired concentration, 100 µL of each essential oil was weighed using a digital scale. This mass was 92 mg for EC and 95 mg for MS. DMSO was added to reach a volume of 1000 µL and a concentration of 128 mg/mL of each essential oil. To ensure that DMSO would not adversely affect the bacteria being studied, a sample of bacteria was cultured in Mueller Hinton Agar culture medium and then 6 mm diameter sterile discs were loaded with 5µL of DMSO and placed on the culture medium. The culture medium was then incubated at 37°C for 24 hours.

Preparation of microbial suspension

A 0.5 McFarland standard solution was prepared by adding 99.5 mL of 1% sulfuric acid to 0.5 mL of 1.175% barium chloride. The prepared solution had an optical density (absorbance) of 0.13-0.80 at 625 nm wavelength. The prepared 0.5 McFarland standard solution caused a turbidity equal to a bacterial suspension containing 1-1.5x10^8 colony forming units per milliliter (CFU/mL).[5] To prepare the microbial suspension, we required bacteria cultured for 24 hours. Thus, 24 hours prior to the experiment, bacterial vials stored at -70°C were freshly cultured on blood agar. Colonies that formed were dissolved in 1 ml of Mueller Hinton Agar culture medium to obtain a 0.5 McFarland standard concentration. To ensure the concentration of suspension, some of it was poured into a sterile screw-top tube and its turbidity was measured with a spectrophotometer at 635 nm wavelength. By doing so, the turbidity of the bacterial suspension could be compared with that of 0.5 McFarland standard solution. [5]

Evaluation of growth inhibition zones

The inhibitory effects of EC and MS essential oils on *E. corrodens* were evaluated using the well diffusion technique. This method is commonly used for the assessment of the antimicrobial activity of plant extracts and essential oils. For this method, Mueller Hinton Agar culture medium enriched with 5% sheep blood was used. The culture medium was inoculated with 0.5 McFarland standard bacterial suspension. The streaking was done using a swab. Next, wells measuring 6mm in diameter and 4mm in depth were created at an appropriate distance from one another using a Pasteur pipette to create 5 wells at the periphery and 1 at the center of plate; 100µL of each essential oil at 16, 32, 64 and 128 mg/mL concentrations were transferred to the wells. 100µL of 0.2% CHX was transferred to a well as a positive control. Plates were stored in a microaerophilic incubator at 37°C for 24 hours. The diameter of the growth inhibition zone around the wells was measured by a ruler in millimeters (5 wells were allocated to each essential oil at each concentration) .[18]

Evaluation of minimum inhibitory concentration (MIC)

The MIC of each essential oil was determined using the broth microdilution method. For this purpose, 100 µL of the brain heart infusion broth was transferred to a horizontal row of 12 wells on a standard 96-well microplate. Next, 100µL of the highest concentration of each essential oil (128 mg/mL) was added to the first well; 100 µL of the first well was transferred to the second well and so on to the tenth well; 100µL from the tenth well was discarded to maintain all volumes constant. Thus, a dilution range of 64 to 0.125 mg/mL was obtained in the first 10 wells. The eleventh well was the negative control, containing only culture medium, and the twelfth well was the positive control. 5µL of the 0.5 McFarland standard bacterial suspension was added to all wells except for the eleventh well (negative control). The plate was stored in a microaerophilic incubator at 35°C for 24 hours. In each row, the first well with turbidity was marked and the concentration of essential oil in the previous well (the last clear well) was considered to be the MIC for the respective essential oil against *E. corrodens* .[18]This experiment was repeated 10 times.

Evaluation of minimum bactericidal concentration (MBC)

To evaluate the MBC of the essential oils, 10 µL of the solution in the MIC wells (before showing turbidity due to bacterial growth) was cultured on blood agar enriched with 5% sheep's blood and stored in a microaerophilic incubator at 37°C for 24 hours. The lowest concentration with no sign of

bacterial growth was considered to be the MBC .[18] This experiment was repeated 10 times.

Statistical analysis

All data were obtained from 10 repetitions of the well diffusion, MIC and MBC tests for the two essential oils against *E. corrodens*. The inhibition zone diameters were compared between groups using Kruskal-Wallis and Mann Whitney tests. Student's t-test was used to compare the mean MIC and MBC values between the two essential oils. SPSS version 17.0 was used for data analysis.

Results

Results of GC-MS confirmed that the main constituents of EC included 1,8-cineole (66%), α- pinene (15.57%), γ-terpinene (7%) and globulol (3.46%). The main constituents of MS were carvone (65%) and limone (20%), with 1,8-cineole, beta myrcene, menthol, cis-dihydrocarvone and β-Bourbonene each comprising 1% of the extract.

The mean diameter of the growth inhibition zone in the presence of 128, 64 and 32 mg/mL concentrations of essential oils was analyzed and measured. The mean diameters of the growth inhibition zones at each concentration are tabulated in Tables 1 and 2. The mean diameter of the growth inhibition zone was the highest (16 mm) in the presence of 128 mg/mL concentration of EC. The growth inhibition zones were 12.2 and 9 mm in the presence of 64 and 32 mg/mL concentrations of EC, respectively. The mean diameter of the growth inhibition zones were 8.8 and 6.4 mm at 128 and 64 mg/mL concentrations of MS, respectively. Thus, the values for MS were higher than the corresponding values for EC. No growth inhibition zone was seen in the presence of 32 mg/mL MS. Also, no growth inhibition zone was seen around the blank disc containing DMSO at each time of culture for different concentrations of the two essential oils. Statistical analysis showed that the 128 mg/mL concentrations of EC and MS exhibited significantly larger zones of inhibition ($P<0.05$) than lower concentrations. Moreover, EC (128 mg/mL) exhibited a significantly larger zone of inhibition ($P<0.05$) than MS (128 mg/mL). The same trend was observed for other concentrations of MS, with EC showing more powerful growth inhibition.

Table 1.Comparison of E. corrodens growth inhibition zone in presence of different concentrations of the essential oils.

Group	min	max	median(mean±SD)	P^*
1:*Mentha spicata* 128 $\frac{mg}{ml}$	8	10	9.00 (8.8±0.837) A	<0.001
2: *Mentha spicata* 64 $\frac{mg}{ml}$	6	8	6.00 (6.4±0.548) B	
3: *Eucalyptus cama.* 128 $\frac{mg}{ml}$	12	23	15.00 (16±4.637) C	
4: *Eucalyptus cama.* 64 $\frac{mg}{ml}$	10	15	12.00 (12.2±2.28) C	
5: *Eucalyptus cama.* 32 $\frac{mg}{ml}$	8	10	9.00 (9±1) A	

T : Kruskal-Wallis H test

Median (mean±SD) values with at least a same letters indicate that there is no significant difference between groups (Mann-whitney U test)

The results of MIC and MBC measurements are presented in Table 2. The mean MIC was found to be 0.55 mg/mL for EC and 0.45 mg/mL for MS against *E. corrodens*. Values were compared using Student's t-test, which revealed no significant difference between the MIC values of the two essential oils (P=0.372). Hence, both essential oils inhibited the growth of *E. corrodens* at similar concentrations (Table 2). Table 3 shows the results of MBC for MS and EC against *E. corrodens*. The mean MBC was 1.8 mg/mL for EC and 2.4 mg/mL for MS. MBC values were compared using Student's t-test, which revealed no significant difference in MBC values of the two extracts (P=0.156) showing that both extracts showed comparable bactericidal effects against *E. corrodens*.

Table 2. Pairwise comparison of E. corrodens growth inhibition zone in presence of different concentrations of the two essential oils (all statistically significant).

Comparative analysis			p-value
Mentha spicata 128 $\frac{mg}{ml}$	v.s	*Mentha spicata* 64 $\frac{mg}{ml}$	.008
Mentha spicata 128 $\frac{mg}{ml}$	v.s	*Eucalyptus cama.* 128 $\frac{mg}{ml}$	.008
Mentha spicata 128 $\frac{mg}{ml}$	v.s	*Eucalyptus cama.* 64 $\frac{mg}{ml}$	.016
Mentha spicata 64 $\frac{mg}{ml}$	v.s	*Eucalyptus cama.* 128 $\frac{mg}{ml}$	.008
Mentha spicata 64 $\frac{mg}{ml}$	v.s	*Eucalyptus cama.* 64 $\frac{mg}{ml}$	.008
Mentha spicata 64 $\frac{mg}{ml}$	v.s	*Eucalyptus cama.* 32 $\frac{mg}{ml}$	.008
Eucalyptus cama. 128 $\frac{mg}{ml}$	v.s	*Eucalyptus cama.* 32 $\frac{mg}{ml}$	.008
Eucalyptus cama. 64 $\frac{mg}{ml}$	v.s	*Eucalyptus cama.* 32 $\frac{mg}{ml}$	.032

Table 3. The MIC and MBC values of the two essential oils against E. corrodens.

Group	MBC	P	MIC	P
Eucalyptus camaldulensis	1.80±0.422	0.156	0.55±0.260	0.372
Mentha spicata	2.40±1.174		0.45±0.230	

Discussion

The main constituents of an essential oil determines their antibacterial properties. In EC, 1,8 cineole is the main constituent, whereas in MS, carvone is. The antimicrobial properties of 1,8 cineole have already been documented in the literature. [10] In our study, we found that *E. corrodens* was more sensitive to EC than to MS at similar concentrations. EC at 128 and 64 mg/ml concentrations caused similar growth inhibition zones on agar medium. The growth inhibition zone formed in the presence of 32 mg/ml EC was similar to the zone formed in the presence of 128 mg/mL MS. Considering the fact that the well diffusion method is based on the distribution of the antimicrobial agent in agar medium while the micro-dilution method is based on the dilution of extract in wells, and also a different number of bacteria were used in the two techniques, the results of these two methods cannot be compared. [18] Also, this would result in obtaining different concentrations in well diffusion compared to serial dilution method.

Several studies have evaluated the antimicrobial properties of herbal essential oils such as EC and MS; however, to the best of our knowledge, no previous study has evaluated the effects of these essential oils against *E. corrodens*. Rasooli et al. evaluated the antimicrobial effects of EC and MS against *Streptococcus pyogenes* and *Streptococcus mutans* using the disc diffusion and broth microdilution techniques. [18] Similar to our study, they reported that these essential oils have antimicrobial activity. Moreover, Akin et al. evaluated the antimicrobial activity of EC and *Myrtus*

communis essential oils against 7 Gram-positive and Gram-negative bacteria using the disc diffusion and agar dilution techniques. [1] and reported that the antimicrobial efficacy of EC was lower than we found in this study. This difference in results might be due to differences in the composition of the EC essential oils, related to different geographical locations, and also to the differences in the bacteria being studied as well as the methods used.

Additionally, Hussain et al. evaluated the antibacterial effect of MS extract and its active component, corvone, on 10 bacterial strains, including *E. coli* and *S. aureus*, using the disc diffusion and microdilution techniques. The reported antibacterial efficacy for MS in their study was comparable to our data. [12] Others have concluded that MS extract is bactericidal against 8 strains of Gram-positive and Gram-negative bacteria using the disc diffusion and microdilution techniques at dilutions lower than 1/100. In dilutions of over 1/1000, the bacteriostatic properties depended on the dosage of the effective component. [20] Imai et al. analyzed the antibacterial properteis of 3 main constituents of MS and 4 main constituents of peppermint essential oils on *Helicobacter pylori*, *Salmonella enteritidis*, *Escherichia coli* 0157:H7, methicillin resistant *Staphylococcus aureus* and methicillin sensitive *Staphylococcus aureus* using the broth microdilution method. They concluded that MS is an effective agent against growth and proliferation of these pathogens. [13] In another study, Hussain et al. assessed the effect of 4 genera of Mentha harvested in the summer and winter on 10 bacterial strains using the broth microdilution and disc diffusion methods. The MS essential oil with the active component of carvone was bacteriostatic against all 10 strains, irrespective of when the oil was harvested. [11]

It has been reported that several factors including the geographical location, plant growth, genera and age of plant, method of essential oil extraction, type of extract, type of solvent, concentration, bacterial culture medium and type of bacteria all play a role in the antibacterial efficacy of plant extracts and essential oils. [4] In the current study, the constituents of the two essential oils were determined using GC-MS. It would also be valuable to extract and purify the main components of these essential oils as well as those of other eucalyptus, mint and herbal extracts to test their antibacterial efficacy against *E. corrodens* and other bacteria responsible for oral malodor. Future *in vivo* studies are required on patients suffering from halitosis with particular emphasis on this bacterium as well as other anaerobes in the oral environment. Further investigations of this topic may lead us towards an efficient formulation for a mouthwash of herbal essential oils with no or minimal side effects.

Conclusion

We reported that MS and EC essential oils possess bacteriostatic properties against *E. corrodens*. Considering the role of *E. corrodens* in halitosis, it appears that decreasing its microbial activity in the oral environment can greatly help eliminate bad breath. Thus, mouthwashes containing EC and MS essential oils may be effective for resolution of halitosis.

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