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Original Article

Antibacterial Effects of Eucalyptus camaldulensis and Myrtus communis Methanolic Extracts on Methicillin-Resistant Staphylococcus aureus and Streptococcus mutans

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Abstract

Background: Considering the emergence of resistant microbial species, there is need for safe and effective alternatives to antibiotics. This study evaluated the antibacterial effects of *Eucalyptus camaldulensis* (*E. camaldulensis*) and *Myrtus communis* (*M. communis*) methanolic extracts on methicillin-resistant *Staphylococcus aureus* (MRSA) and *Streptococcus mutans* (*S. mutans*).

Methods: This *in vitro* study evaluated standard strains and clinical isolates of MRSA and *S. mutans*. The *E. camaldulensis* and *M. communis* methanolic extracts were obtained by the maceration technique, and their antibacterial activity against the aforementioned micro-organisms was evaluated by the agar well diffusion technique and measurement of growth inhibition zone diameter. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extracts were also determined against the tested micro-organisms by the broth microdilution technique.

Results: The *M. communis* extract had a MIC of 3.12 *mg/mL* and MBC of 6.25 *mg/mL* against most *S. mutans* isolates, and a MIC=MBC of 6.25 *mg/mL* against most MRSA isolates. The *E. camaldulensis* extract had a MIC=MBC of 12.5 *mg/mL* against most MRSA isolates and a MIC=MBC of 6.25 *mg/mL* against most *S. mutans* isolates. The two extracts had different effects on the two micro-organisms, and the *M. communis* extract caused a significantly larger growth inhibition zone in *S. mutans* culture than MRSA culture (p=0.046); however, the difference in this regard was not significant in use of *E. camaldulensis* (p=0.76).

Conclusion: The *M. communis* extract had significantly superior antibacterial effects on *S. mutans* and MRSA isolates than the *E. camaldulensis* extract.

Keywords: Augar, Anti-bacterial agents, Eucalyptus, Methicillin-Resistant *Staphylococcus aureus*, Microbial sensitivity tests, Myrtus, Plant extracts, *Streptococcus mutans*

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Introduction

Staphylococcus aureus (*S. aureus*) is an important pathogenic micro-organism in humans. Almost all human beings experience *S. aureus* infection at least once in their life time, which may vary in severity from a food poisoning to a serious life-threatening condition. *S. aureus* causes a wide range of diseases, such as bacteremia, staphylococcal scalded skin syndrome, toxic shock syndrome, food poisoning, and extensive abscesses in the organs (1,2).

Staphylococci often show low sensitivity to antibiotics due to possession of several antibiotic resistance mechanisms. Methicillin-resistant *S. aureus* (MRSA) species cause hospital-acquired infections and are currently a medical dilemma worldwide. Hospital-acquired MRSA infections are currently endemic in hospitals of developed and even developing countries. The prevalence of MRSA increased from 14.8% in 1987 to 39.7% in 2005 (3). MRSA species are also problematic in Iran. A previous study reported that 38.6% of the *S. aureus* isolates isolated from hospitalized patients in Shariati Hospital and Children's Medical Center in Tehran were MRSA (4). The majority of *S. aureus* strains (>90%) are penicillin-resistant. MRSA species are resistant to the oxacillin family of antibiotics (nafcillin, methicillin, oxacillin, cloxacillin) and all beta-lactam antibiotics such as penicillin, amoxicillin, and cephalosporines (5). From 2005 to 2011, the rate of MRSA infections decreased by 31%, and the greatest reduction occurred in hospitalized patients (54%).

However, it had an ascending trend in the past decade in communities with poor hygiene practice (6).

Considering the emerging trend of antibiotic resistance, search for effective herbal alternatives is increasing. *Myrtus communis* (*M. communis*) is an aromatic evergreen shrub or small tree. Its leaves have 1.5-2% v/v essence mainly composed of terpinolene, cineol, linalool, and terpineol. It also contains tannins, flavonoids, and vitamin C. It reportedly has strong antibacterial activity against *Porphyromonas gingivalis (P. gingivalis)*, which is a periodontal pathogen (7). Its essential oil has also shown optimal effects on *S. aureus*, *Pseudomonas aeruginosa* (P. *aeruginosa)*, and *Escherichia coli* (*E. coli*) (8-10). It has shown optimal efficacy for resolution of aphthous ulcers (11), and periodontal disease (10).

Eucalyptus camaldulensis (*E. camaldulensis*) is a flowering plant from the family of *Myrtaceae*. Its leaves have medicinal properties, are rich in vitamin C and have anti-oxidant effects (12,13). It has strong disinfecting and antibacterial effects as well (13,14). It can also inhibit dental plaque and biofilm formation (15,16). Obviously, it is crucial to find safe and effective alternatives in cases that MRSA and *Streptococcus mutans* (*S. mutans*) are present. Therefore, this study was conducted to assess the antibacterial effects of *E. camaldulensis* and *M. communis* extracts on MRSA and *S. mutans*.

Materials and Methods

This *in vitro*, experimental study was conducted on standard strains and 10 clinical isolates of *S. mutans* and MRSA and the *E. camaldulensis* and *M. communis* extracts. The protocol of this study was approved by the ethics committee of the Shaid Beheshti University of Medical Sciences (IR.SBMU.DRC.REC.1395.394).

Extraction technique

The *E. camaldulensis* and *M. communis* leaves were dried away from sunlight and powdered in a ball-mill; 10 *g* of the dried leaves was immersed in 100 *mL* of the solvent (Ethyl Acetate) such that the solvent covered the entire surface of the powder. After 48-72 *hrs*, the solution was filtered through a No. 1 Whatman filter paper, and sterilized by using a 0.45 μm membrane filter. After solvent evaporation under vacuum, the dried extract was stored at -20°*C*.

Microbial culture

Standard-strain *S. mutans* (ATCC25175) and MRSA (ATCC25923) were purchased from the Iranian Research Organization for Science and Technology. Clinical isolates were obtained from patients with dental infection, and cultured on blood agar and specific media, and the grown colonies were evaluated in terms of morphology, and also by Gram-staining, catalase test, mannitol salt agar test and, coagulase test for their identification.

MRSA identification

In order to detect MRSA strains, an initial screening was carried out utilizing the cefoxitin disk diffusion

method following the guidelines of the CLSI protocol. Subsequently, molecular confirmation was performed by utilizing the *mecA* gene. The specific primers employed in this process are; forward: TCCAGATTACAACTTCACCAGG and reverse: CCACTTCATATCTTGTAACG (17).

S. mutans identification

S. mutans strains were identified through the use of molecular techniques and PCR for *gtfBC* gene.
The primers are; forward:
ACTACACTTTCGGGTGGCTTGG and reverse: CAGATAAGCGCCAGTTTCATC (18).

Assessment of antibacterial activity of the extracts

Agar well diffusion technique: The microbial suspensions were cultured on blood agar and Mueller Hinton agar plates by a sterile swab. Wells were then created in the plates, and 100 μL of the extracts were added to each well. The plates were then incubated for 24 *hrs*. To ensure accuracy, the tests were repeated in triplicate for each microbial strain. Also, a control plate was used for assessment of bacterial growth in absence of extracts, and another control plate was considered for assessment of diffusion of the extracts in the agar in absence of microorganisms. After 24 *hrs*, the diameter of the growth inhibition zones was measured at the largest area. The plates were also checked to ensure no contamination with other micro-organisms. All procedures were performed near the flame.

Broth microdilution method: According to the CLSI protocol, the extracts were dissolved in 2% dimethyl sulfoxide to prepare 12.5, 6.25, 3.125, 1.56, 0.75, 0.39, and 0.19 *mg/mL* concentrations of the extracts (19). For this purpose, initially 25 *mg* of each extract was dissolved in 1 *ml* of 2% dimethyl sulfoxide. It was heated for a short period of time to enhance the dissolution of the extract. Next, 100 λ of Mueller Hinton broth was added to each well of a 96-well plate; 100 λ of the extract was also added to the first well and sampling was performed. Next, 100 λ of the contents of the first well was collected and transferred to the second well. This process was continued until the 8th well. Next, according to the CLSI protocol, 0.5 McFarland stock solution was diluted 1:20, and 10 λ of it was collected and added to each well. Chlorhexidine (CHX) served as the control group. The concentration of the first well showing no turbidity was recorded as the Minimum Inhibitory Concentration (MIC) of the extract, and the concentration resulting in no bacterial growth was recorded as the Minimum Bactericidal Concentration (MBC).

Statistical analysis

Data were analyzed using SPSS version 16 (SPSS Inc., IL, USA). Considering the presence of two types of micro-organisms and two extracts, data were analyzed by two-way ANOVA followed by Bonferroni correction for subgroup analyses at 0.05 level of significance. Data have been presented in tables.

Results

Agar well diffusion technique

Table 1 presents the mean diameter of the growth inhibition zones in *S. mutans* and MRSA cultures caused by the two extracts. Table 2 presents the diameter of the growth inhibition zones of *S. mutans*

Table 1. Mean diameter of the growth inhibition zones of S. mutans and MRSA caused by the two extracts

	S. mutans			*MRSA*		
	M. communis	*E. camaldulensis*	CHX	*M. communis*	*E. camaldulensis*	CHX
Mean	16.8	12	21.8	15.6	11.8	13.9
Maximum	20	13	27	18	13	17
Minimum	14	11	18	14	11	10
Std.deviation	2.48	0.81	2.57	1.17	0.63	2.23

Eucalyptus camaldulensis (E. camaldulensis); Myrtus communis (M. communis); Streptococcus mutans (S. mutans); Methicillin-Resistant *Staphylococcus aureus* (MRSA); Chlorhexidine (CHX).

Table 2. Diameter of the growth inhibition zones of S. mutans and MRSA clinical isolates caused by the two extracts and CHX (*mm*)

Number	S.mutans			MRSA		
	M. communis	*E. camaldulensis*	CHX	*M. communis*	*E. camaldulensis*	CHX
1	20	12	27	15	13	17
2	14	11	24	16	12	12
3	19	13	22	18	12	10
4	18	11	23	14	12	16
5	14	11	21	14	12	15
6	18	12	22	16	12	13
7	14	13	22	15	12	13
8	14	12	20	16	11	12
9	19	13	19	16	11	16
10	18	12	18	16	11	15

Eucalyptus camaldulensis (E. camaldulensis); Myrtus communis (M. communis); Streptococcus mutans (S. mutans); Methicillin-Resistant *Staphylococcus aureus* (MRSA); Chlorhexidine (CHX).

Table 3. MIC and MBC *(mg/mL)* of the extracts against S. mutans and MRSA

	S. mutans MBC		*S. mutans* MIC		*S. aureus* MBC		*S. aureus* MIC	
	M. communis	*E. camaldulensis*	*M. communis*	*E. camaldulensis*	*M. communis*	*E. camaldulensis*	*M. communis*	*E. camaldulensis*
Maximum	6.25	12.5	6.25	12.5	12.5	25	6.25	12.5
Minimum	3.12	6.25	3.12	3.12	3.12	6.25	3/12	6.25
Mean	5.12	7.95	3.97	6.54	7.95	12.5	5.11	9.35
Std.deviation	1.61	3.019	1.51	2.30	3.94	5.11	1.65	3.29

Minimum Inhibitory Concentration (MIC); Minimum Bactericidal Concentration (MBC). *Eucalyptus camaldulensis (E. camaldulensis); Myrtus communis (M. communis); Streptococcus mutans (S. mutans).*

and MRSA clinical isolates caused by the two extracts and CHX. Two-way ANOVA showed the significant interaction effect of type of micro-organism and type of extract on the diameter of the growth inhibition zones (p=0.05). Subgroup analyses revealed that *M. communis* caused a significantly larger growth inhibition zone in *S. mutans* culture, compared with MRSA culture (p=0.046). However, the difference in the diameter of the growth inhibition zones of *S. mutans* and MRSA cultures was not significant in use of *E. camaldulensis* (p=0.76).

The mean diameter of the growth inhibition zones caused by *M. communis* was significantly larger than that that caused by *E. camaldulensis* (p=0.001).

The largest growth inhibition zone caused by the methanolic extract of *M. communis* was 20 *mm* for MRSA, and 18 *mm* for *S. mutans*. The largest growth inhibition zone caused by the methanolic extract of *E. camaldulensis* was 11 *mm* for both *S. mutans* and MRSA.

MIC and MBC: As shown in table 3, the *M. communis* extract had a MIC of 3.12 *mg/mL* and MBC of 6.25 *mg/mL* against most *S. mutans* isolates, and a MIC=MBC of 6.25 *mg/mL* against most MRSA isolates.

The *E. camaldulensis* extract had a MIC=MBC of 12.5 *mg/mL* against most MRSA isolates and a MIC=MBC of 6.25 *mg/mL* against most *S. mutans* isolates.

In total, the *M. communis* extract had superior antibacterial activity compared with *E. camaldulensis* extract against the tested micro-organisms.

Discussion

This study assessed the antibacterial effects of *E. camaldulensis* and *M. communis* extracts on MRSA and *S. mutans*. The results showed that the *M. communis* extract had a MIC of 3.12 *mg/mL* and MBC of 6.25 *mg/mL* against most *S. mutans* isolates, and a MIC=MBC of 6.25 *mg/mL* against most MRSA isolates. Houshmand *et al* (10) evaluated the effects of *M. communis* extract on *P. aeruginosa* by the disc diffusion and broth microdilution tests. They demonstrated that *P. aeruginosa* had insignificant growth in presence of 2.5% concentration of *M. communis* extract. *P. aeruginosa* is a Gram-negative micro-organism and the cell wall of Gram-positive micro-organisms is more resistant than the membrane of Gram-negative micro-organisms. Hedayati *et al* (7) evaluated the effect of *M. communis* extract on 30 isolates of *P. gingivalis* periopathogenic micro-organism by the broth microdilution technique. They showed strong antimicrobial activity of this extract against *P. gingivalis* isolates. Their results were in line with the present findings despite using a different micro-organism. Consistent with the present results, Teimoory *et al* (20) demonstrated that the alcoholic extract of *M. communis* in 10 *mg/mL* concentration had optimal antibacterial effects on *S. aureus* and *Bacillus cereus*. The results of the current study showed the MIC 5.11 *mg/mL* and this result are lower than study by Teimoory *et al* (20). On the other hand, Taheri *et al* (21) showed significant effect of the hydroalcoholic extract of *M. communis* on *S. aureus* in 0.2 *mg/mL* concentration and the present study result is higher than it.

Hydroalcoholic extract is more effective than methanolic extract and *S. aureus* isolates have different antibiotic resistance mechanisms, which may explain the difference in the reported results. The MIC of hydroalcoholic extract of *M. communis* leaves on *S. mutans* was 5 *mg/mL*, but we can achieve a lower MIC (3.97 *mg/mL*). In both studies hydroalcoholic extract of *M. communis* has been used and the present study results were almost similar. In the present study, the *E. camaldulensis* extract had a MIC and MBC of 9.35 *mg/mL* and 12.5 *mg/mL* against most MRSA, respectively.

The results of a review study in 2019, like the present study, showed impact of the Leaves essential oil of this plant on *S. aureus* strains. But the identified MIC of *E. camaldulensis* on *S. aureus* is lower than this study. However, this review article does not mention whether this report concerns MRSA strains or Methicillin Susceptible Staphylococcus aureus (MSSA) strains. It is worth noting that we examined the MRSA strains. *E. camaldulensis* extract showed MIC and MBC of 6.54 *mg/ml* and 7.95 *mg/ml*, respectively against most *S. mutans* isolate. The 2020 study demonstrated the impact of *E. camaldulensis* on *S. mutans*, which aligns with the findings of the present study.

In total, the *M. communis* extract had superior antibacterial activity compared with *E. camaldulensis* extract against the tested micro-organisms. Rasooli *et al* (15) evaluated the effect of eucalyptus oil along with mint on dental biofilm formation by *S. mutans* and *Streptococcus pyogenes*. They reported an MBC of 2 *mg/mL* for eucalyptus oil against *S. mutans* and showed that dental plaque formation was significantly decelerated in presence of eucalyptus oil. Difference in the reported MBC values in their study and the present study (6.25 *mg/mL*) can be due to using a different solvent and differences in selective bacterial genotypes. A review study also confirmed the considerable antimicrobial properties of *E. camaldulensis* (22). Asiaei *et al* (23) assessed the antimicrobial activity of *E. camaldulensis* essential oil against drug-resistant bacterial growth. They reported its significant activities against some Gram-positive and Gram-negative bacteria including *Klebsiella pneumoniae*, *Salmonella infantis* and *Salmonella enteritidis*. Sattari *et al* (24) indicated optimal antibacterial effects of the aqueous and alcoholic extracts of eucalyptus on *P. aeruginosa* and reported a MIC of 3.2 *mg/mL* for its alcoholic and 17.5 *mg/mL* for its aqueous extract. Differences in the reported values with the present findings are due to evaluation of different micro-organisms and different

types of extracts.

In vitro design and no conduction of chromatography for identification of effective substances were among the limitations of this study, which should be addressed in future investigations. Also, the synergistic effects of extracts with antibiotics should be investigated in further studies.

Conclusion

Within the limitation of the current study, it can be concluded that the *M. communis* extract may have significantly superior antibacterial effects on *S. mutans* and MRSA isolates than the *E. camaldulensis* extract.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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