Original Article

The antibacterial activity of "*Satureja hortensis*" extract and essential oil against oral bacteria

Leila Golpasand Hagh[1], Atefe Arefian[1], Ahmad Farajzade[2], Sana Dibazar[3], Neda Samiea[1]

Departments of [1]Periodontology and [3]Operative and Esthetic Dentistry Dental Faculty, Ahvaz Jundishapur University of Medical Sciences, [2]Department of Microbiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

ABSTRACT

Background: Recently, there has been an increasing growth in research on medical plant's effect on dental plaque bacteria. The aim of this study was to determine the antibacterial effects of *Satureja hortensis* extract and its essential oil (EO) on *Streptococcus salivarius¸ Streptococcus sanguis,* and *Streptococcus mutans* as important bacteria in early supragingival dental plaque formation.

Materials and Methods: In this *in vitro* study, different concentrations of *S. hortensis* extract and its EO were prepared using double dilution method. The disc diffusion method was used to determine antibacterial activity. Based on these measurements, the minimal inhibitory concentration value was reported for each bacterium. Antibiotics used as positive controls in this study were erythromycin (15 μg) and tetracycline (30 μg). *t*-test and ANOVA were used for statistical analysis ($P < 0.05$).

Results: Aqueous and methanolic extract did not show significant antibacterial activity, but the EO significantly inhibited the growth of the test bacteria compared to positive control ($P < 0.05$). High concentrations of EO processed greater antimicrobial effects against three oral bacteria than other low concentrations ($P < 0.0001$). For *S. mutans*, the inhibition effect of tetracycline 30 μg was similar with 50% ($P = 0.789$) and 25% ($P = 0.158$) dosages of the EO. For *S. salivarius,* the effect of tetracycline 30 μg was similar to 50% dosages of the EO ($P = 0.122$). For *S. sanguis,* the effect of erythromycin 15 μg was lower than 50% ($P = 0.0006$) and 25% ($P = 0.003$) dosages of the EO. The inhibition effects of all concentrations of EO were higher for *S. sanguis*. *S. salivarius* and *S. sanguis* are more sensitive than *S. mutans* to *S. hortensis* EO.

Conclusion: Due to the strong antibacterial effect of *S. hortensis* EO on the oral bacteria growth, it can be served as herbal mouth rinse, while to confirm this antibacterial effect, further clinical studies are necessary.

Key Words: *Satureja, Streptococcus mutans, Streptococcus salivarius, Streptococcus sanguis*

INTRODUCTION

Dental caries and periodontal diseases are two of the most important infectious diseases in the community at present.[1,2] Accumulation of microbial plaque on tooth surfaces is one of the first stages of caries process and periodontal diseases.[3] The microbial plaque consists of a wide spectrum of bacteria with complex

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interactions. The dominant microbial composition of the dental plaque, which is often affected by the oral environment, can determine the potential for damage.[4] The basic stage in plaque formation is the ability of adherence for microorganism to dental and tissue surfaces. In early dental plaque formation, primarily Gram-positive *cocci* can attach to dental surfaces.[5] Recently, molecular methods such as proteomics and 16S rRNA sequencing demonstrate the predominant species of *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus sanguis,* and *Streptococcus mitis* in the supragingival plaque.[6] Over time, Gram-negative bacteria are added to the plaque and complicate the plaque environment and so increase the potential for damage.[7]

Plaque control and good oral hygiene might be helpful in prevention and achievement of good treatment outcomes in this respect.[8] Different mechanical and chemical plaque control techniques have been introduced. However, many individuals do not properly use mechanical plaque control techniques.[9,10] It has been demonstrated that even if adequate time is spent, only half of the plaque can be removed.[11]

In 2002, emphasis was put on the use of mouth rinses by the International Association for Dental Research as an adjunct to control plaque.[12] In this context, various mouth rinses were introduced to help control plaque. Of all these, chlorhexidine (CHX) is used as the most important antiplaque mouth rinse.[13] A systematic study has shown that CHX at concentrations of 0.06% to 0.2% is effective in decreasing bacterial plaque. In this respect, its overall dose is important for its efficacy.[14] Studies have shown that the optimal dose of CHX is 20 mg twice daily.[15] There is a direct relationship between the concentration of CHX and the incidence of complications at doses >0.1%.[16-18] Due to their side effects, including a change in taste and formation of stains on the teeth, and also the presence of chemical agents such as alcohol, preservatives, and synthetic pigments, many patients are not interested in the long-term use of chemical mouth rinses.[19-21] Therefore, in recent years, the idea of the use of herbal agents with antibacterial effects in the formulation of mouth rinses has drawn some attention to minimize complications.

Satureja L. (savory, saturei) is a plant with 30 different species belonging to the Lamiaceae family. This herbal medicine is indigenous to the Mediterranean area.[22] The aerial parts of the plant are white to pale pink-violet in color and its odor is a stimulant and has invigorating effects.[22,23] Previous studies have reported a wide range of biologic properties for this plant, including antioxidative, anti-inflammatory, and analgesic effects.[24,25]

A study by Adiguzel *et al.* revealed the antifungal and antibacterial effects of this plant against the fungi and bacteria in foodstuff.[26] In addition, a study by Sabzghabaee *et al.* showed the positive effect of its extract on the treatment of denture stomatitis due to *Candida albicans*.[27]

The aim of the present *in vitro* study was to evaluate the effect of *Satureja hortensis L.* extract on three important bacteria in early dental plaque formation, with *S. mutans* being the most important etiologic agent for dental caries pathogenicity and *S. sanguis* and *S. salivarius* as predominant microorganisms in pit and fissures of new erupted teeth and tongue covering plaque, respectively, through determination of minimal inhibitory concentration (MIC).[28-31]

MATERIALS AND METHODS

In this *in vitro* study, to evaluate the antibacterial activity of *S. hortensis* extract and essential oil (EO) on test bacteria, different concentrations of aqueous and methanol extracts and EO were classified under six groups as follows:
- Group 1 = 1.5625%
- Group 2 = 3.125%
- Group 3 = 6.25%
- Group 4 = 12.5%
- Group 5 = 25%
- Group 6 = 50%.

Plant material
The plant (*S. hortensis L.)* used in this work was collected from Hamidiyeh (Ahvaz, Khuzestan, Iran) in February 2015. After identifying the species by the herbarium section staff, the plant leaves were separated from shadow dried materials and then were powdered in a grinder.

Preparation of the aqueous and methanol extracts
The dried and powdered plant leaves (100 g) were extracted successively with 500 cc of methanol and 500 cc of water using Soxhlet extractor for 48 h at a temperature not exceeding the boiling point of the solvent. The aqueous and methanol extracts were filtered through Whatman filter paper and then concentrated in vacuo at 40°C by means of a rotary

evaporator. The residues obtained were stored in a freezer until future tests.[32-34]

Isolation of the essential oil

One hundred gram of the fresh aerial parts of the plants collected was submitted to 500 cc of water distillation for 5 h using a Clevenger-type apparatus. Then, the EO was stored until future tests.

Microbial strains

The extracts and the EO were tested against three oral bacteria (*S. mutans* PTCC1683, *S. salivarius* PTCC1448, and *S. sanguis* PTCC1449). These bacteria were provided by the Iranian Type Culture Collection and then the lyophilized strains were inoculated on blood agar and then incubated for 18 h at 37°C. The precultures of bacteria were prepared for the susceptibility tests. For this purpose, the bacteria strains were taken by sterile inoculating loop touching to 4–5 colonies raised from pure microorganism culture, and these strains were inoculated in physiologic serum at the concentration of 1×10^8 CFU/ml (in order to achieve the McFarland no: 0.5 density) and then incubated at 37°C.

Disc diffusion assay

Extracts and EO were diluted in dimethyl sulfoxide (DMSO) to the different test concentrations (for extract test, concentrations were 5, 2.5, 1.25, 0.625, 0.3125, and 0.15625 mg/ml, and for EO test, concentrations were 50%, 25%, 12.5%, 6.25%, 3.125%, and 1.5625%). Antimicrobial tests were carried out by the disc diffusion method. The discs (6 mm diameter) were impregnated with 30 µl of the extracts and oil dilution and placed on the inoculated blood agar. Negative controls were prepared using the same solvents to dissolve the extracts and EO (DMSO). Tetracycline (30 µg) and erythromycin (15 µg) were used as positive reference standards to determine the sensitivity of a strain of each tested microbial species. The inoculated plates were incubated at 37°C for 18 h. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test microorganisms. The least concentration of each extract and EO showing a clear zone of inhibition were taken as the MIC. The assays were performed three times for each bacterium.

Statistical analysis

Analysis of the results was performed by *t*-test and ANOVA using SPSS statistics software (version 16; SPSS Inc, Chicago, IL, USA) $P > 0.05$ was considered to be significant.

RESULTS

According to the results of this study, aqueous extract and methanol extract did not show significant antibacterial activity against the test bacteria compared to positive control as there was no inhibition zone even in high concentrations. However, the EO significantly inhibited the growth of the test bacteria.

Average and standard deviation of the inhibition zones of the bacteria by EO, negative control (DMSO), and positive control (tetracycline 30 µg and erythromycin 15 µg) are summarized in Table 1.

The results demonstrated that there was a significant difference in inhibition zones of different concentrations. High concentrations of EO processed greater antimicrobial effects against three oral bacteria than other low concentrations ($P < 0.0001$).

For all bacteria, negative control DMSO did not show any inhibition zone.

The inhibition zone of different concentrations, except 1.5625% and 3.125% of concentrations for *S. mutans* and 1.5625% for *S. salivarius* and *S. sanguis,* was significant compared to negative control ($P < 0.05$).

For *S. mutans*, the inhibition effect of tetracycline 30 µg was similar to 50% ($P = 0.789$) and 25% ($P = 0.158$) dosages of the EO and was higher compared to lower concentrations.

For *S. salivarius,* the effect of tetracycline 30 µg was similar to 50% dosages of the EO ($P = 0.206$) and was higher compared to lower concentrations.

Table 1: Average and standard deviation and *P* values of inhibition zone of bacteria

Microorganism		Groups						Negative control (−)	Positive control (+)
		1	2	3	4	5	6		
Average of inhibition zone (mm)/SD/(*P*)	*S. mutans*	-	-	13.3±2.08	15.3±1.52 (0.011)	18±1.73 (0.158)	20.3±1.52 (0.789)	-	20±1
	S. salivarius	-	11.5±0.86	12.16±0.76	16.3±0.57	19±1.32 (0.004)	23.6±0.57 (0.206)	-	24.6±1
	S. sanguis	-	11.3±1.15	17±1	18.6±0.57 (0.073)	22±1 (0.003)	28.83±1.89 (0.0006)	-	17±1

Negative control: Dimethyl sulfoxide, Positive control: Tetracycline 30 kg/disc for *S. mutans* and *S. salivarius*, erythromycin 15 kg/disc for *S. sanguis*. *S. sanguis*: *Streptococcus sanguis*, *S. salivarius*: *Streptococcus salivarius*, *S. mutans*: *Streptococcus mutans*, SD: Standard deviation

For *S. sanguis,* the effect of erythromycin 15 μg was lower than 50% (P = 0.0006) and 25% (P = 0.003) dosages of the EO and was similar to other concentrations.

The inhibition effects of 6.25% and 50% of concentrations of the EO were higher for *S. sanguis* and similar for *S. salivarius* and *S. mutans*.

The inhibition effects of 12.5% and 25% of concentrations of the EO for *S. sanguis* were higher amounts than *S. mutans,* and the differences were not significant for *S. salivarius*.

The MIC values of EO on target bacteria were as follows: *S. mutans* 3.125%, *S. salivarius* 1.5625%, and *S. sanguis* 1.5625%. Hence, *S. salivarius* and *S. sanguis* are more sensitive than *S. mutans* to *S. hortensis* EO [Table 2].

DISCUSSION

The results of the present study showed that the aqueous extract tested had no antibacterial effects on the three microorganisms evaluated; however, EO had strong antibacterial effect on *S. mutans, S. salivarius,* and *S. sanguis*. In the present *in vitro* study, to evaluate the effect of the extract and EO of *S. hortensis* on the three dominant microorganisms in the oral cavity, the MIC technique was used. In the majority of cases, MIC, minimum bactericidal concentration, minimum fungicidal concentration, MIC50, and lethal dose 50 (minimum lethal concentration) are used to evaluate the efficacy and effect of herbal components and for comparison of the required concentration to exhibit the growth of microorganisms.[35] The results of MIC test in the present study showed the antibacterial effects of 3.125% concentration on *S. mutans* and 1.56% concentration on *S. sanguis* and *S. salivarius*.

EOs are herbal components that have been prepared using hydrodistillation, steam distillation, or solvent extraction techniques and usually have a molecular weight <500 d.[36]

Table 2: Minimal inhibitory concentration values of *Satureja hortensis* essential oil against oral bacteria

Microorganism	PTCC	MIC (%)
S. mutans	1683	3.125
S. salivarius	1448	1.5625
S. sanguis	1449	1.5625

MIC: Minimal inhibitory concentration, *S. sanguis*: *Streptococcus sanguis*, *S. salivarius*: *Streptococcus salivarius*, *S. mutans*: *Streptococcus mutans*

Herbal medicines have been used in different fields of traditional medicine. Currently, their efficacy has been shown with the use of new techniques for the analysis and identification of their chemical structure with scientific evidence.[37] EOs are usually the product of a combination of terpenoids and phenylpropanoids.[38] Several studies on different aspects of EOs have demonstrated their different properties, including antineoplastic (with necrosis and apoptosis of cancerous cells),[35,39] antimutagenic (through inhibition of synthesis of P450),[40] antifungal and antioxidative (due to the presence of thymol and carvacrol),[41,42] antiviral (through inhibition of viral proliferation), and anti-inflammatory (by inhibition of the release of free radicals) properties.[37] These products are lipophilic and therefore have the capacity to penetrate through the cell wall and cellular membranes. These compounds increase the permeability after they affect polysaccharides, phospholipids, and fatty acids of the cell membrane. In addition, they can affect the proton-pump mechanism and deactivate cellular enzymes after denaturing the plasma proteins to cause cellular death.[43-45]

The chemical structure and EO content of different species of *Satureja L.* are different. Evaluation of EO in *S. hortensis L.* has shown a high content of phenolic components, including carvacrol and γ-terpinene.[46] The concentrations of these compounds in descending order are as follows: carvacrol, cymene, α-pinene, terpineol, thymol, β-pinene, linalool, and borneol.[47] It should be pointed out, apart from the genus of the plant, the conditions affecting its growth, climate and geographical location, seasonal and temperature changes, and even conditions after its harvest affect the chemical composition of EOs.[48]

In a study by Zeidán-Chuliá *et al.* on the antibacterial properties of *S. hortensis L.* EO and *M. Salvia fruticosa* on *Fusobacterium nucleatum*, it was shown that both herbal EOs exhibited effective antibacterial properties against bacterial species above. However, the effect of *S. hortensis L.* was stronger due to a higher concentration of terpenes.[49]

In addition, Sharifi-Rad *et al.* evaluated the antineoplastic and antibacterial effects of EO of *Satureja intermedia C.A.* on *S. salivarius* and *S. mutans* and reported their effect on these bacterial species. The difference in MIC between the present study and the study above might be attributed to

differences in concentrations of the ingredients of the herbal extracts; in this context, monoterpene hydrocarbons, γ-terpinene, thymol, and p-cymene exhibited the highest concentrations, respectively, and carvacrol comprised only a small percentage of the extract.[22]

Carvacrol is the chief ingredient of EO of this plant and an increase in the concentration of carvacrol and thymol results in an increase in its antibacterial activity; however, it should be pointed out that the antibacterial activity does not increase with only an increase in concentrations of these two compounds and the presence of other ingredients at proper concentrations will improve the performance of other ingredients with their synergistic effects.[50,51] The most important mechanism of action of the EO of the plant under study is its ability to penetrate into the bacterial cell and its effect on the vital organelles of the microorganism after disrupting the permeability of the cell wall and cell membrane through its effect on the lipids of the cell membrane.[52] In addition, the ability of EOs to penetrate into microbial biofilms that are resistant to the penetration and effect of antibiotics[53] is an advantage apart from their antimicrobial properties, which might induce persistent and more effective effects in mouth rinses.

It should be emphasized that *in vitro* nature of the present study and other similar studies is some of the limitations here. In this context, the clinical use of EO in mouth rinses is associated with some complex issues. Based on the results of a study by Mohtashami *et al.*, the concentrations of ingredients of EOs do not remain unchanged when they are stored under different conditions, with an increase in the concentration of carvacrol and a decrease in the concentration of ingredients with a low boiling point.[46]

Given the reciprocal effects of EO ingredients to induce proper antimicrobial effects, further studies are necessary to evaluate the antimicrobial effects of this extract under different storage conditions. In addition, the ingredients in the extract that are used for chemotherapy purposes in addition to their antimicrobial properties possibly exert toxic effects on the oral cavity mucosa, possibly inducing allergic reactions.[54] On the other hand, the oral cavity is a dynamic environment with different enzymes; therefore, it is possible that the ingredients of EO might undergo changes under the influence of oxidation and enzymatic reactions.[55,56] Therefore, further and more comprehensive clinical studies are necessary before their clinical applications.

CONCLUSION

Aqueous extract and methanol extract did not show significant antibacterial activity against the test bacteria, but the EO significantly inhibited the growth of the test bacteria. *S. salivarius* and *S. sanguis* are more sensitive than *S. mutans* to *S. hortensis* EO.

So due to the strong antibacterial effect of *S. hortensis* EO on the oral bacteria growth, it can be served as a herbal mouth rinse, but further clinical studies are necessary.

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Conflicts of interest
The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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