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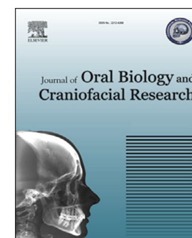

Original Article

Inhibitory activity of Salvadora persica extracts against oral bacterial strains associated with periodontitis: An *in-vitro* study

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ABSTRACT

Aims: The use of natural plant extracts in pharmacology, medicine and dental hygiene has found a growing interest in modern scientific research. *Salvadora persica* is a natural tree whose fibrous branches have been approved by the World Health Organization for oral hygiene. Periodontitis is a highly prevalent adult gingival disease that leads to bone destruction and connective tissue attachment loss. The aim of this research was assessment the antimicrobial activities of methanolic extract of *Salvadora persica* (miswak) on isolated strains from the oral fluid.

Methods: In practical section, 50 female university students (21.4 ± 1 year) participated in the study. Based on examination by a periodontist, they were grouped into (Group I, $n = 21$) and (Group II, $n = 29$) i.e. with and without periodontitis respectively. Their un-stimulated saliva samples were obtained in sterile tubes. While three bacterial genera, *Staphylococcus*, *Streptococcus* and *Lactobacillus* were identified in all subjects, *Enterococcus* and *Escherichia* were only detected in Group I.

Results: A statistically significant difference in colonization levels between the two groups was observed. The effect of methanolic extract of *S. persica* against oral bacterial strains isolated from saliva was investigated using agar disc diffusion and microdilution methods. Although methanolic extract of *S. persica* was effective on growth inhibition of all strains, it was significantly more effective on Gram positive bacteria than Gram negative ones.

Conclusions: Effective substances present in *S. persica* extracts, exhibit a broad range of antibacterial activity and affect almost all bacterial species regardless of the Gram-staining nature.

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1. Introduction

The use of natural plant extracts as mouth wash and oral hygiene has attracted high attention during the last two decades. *Salvadora persica*, commonly referred to as Miswak, *Arak*, *Meswak* or toothbrush tree is a popular chewing stick throughout the Muslim world. Fibrous branches of *S. persica* have been approved by the World Health Organization for use as oral hygiene due to its protective effect on some oral pathogens. Despite its traditional use, up to the present time few to no reports have been found about the antibacterial effects of the various parts of the plant.

Periodontitis is a highly prevalent adult gingival disease that leads to bone destruction and connective tissue attachment loss. The severity of the disease ranges from gingivitis to various classes of periodontitis. Periodontitis differs from many other types of infections since it is not caused by a single bacterium but by a group of bacteria. More than 500 different types of bacteria have been isolated from the oral cavity most of which are innocuous.[1] Periodontitis is associated with members of the indigenous oral microbiota including *Aggregatibacter actinomycetemcomitans*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Enterococcus faecalis* and *Treponema denticola*.[2] The relationship between periodontal microbiota and chronic periodontitis has recently been studied using real time polymerase chain reaction.[3] It has been revealed that mean count of *P. gingivalis* was significantly higher in patients with chronic periodontitis than in periodontally healthy individuals. A considerable association between *P. gingivalis* and chronic periodontitis has been reported.[3] A range of Gram positive and Gram negative bacterial species may be present in oral fluid of subjects suffering from chronic periodontitis.[4] However, bacterial biofilms on teeth, dental restorations, prostheses, and implants consist of more than 700 different bacterial species.[5]

We have previously examined the effect of *S. persica* extract on bacterial quorum sensing *in vitro*.[6] In the present study, the ability of methanol extracts from fibrous braches of *S. persica* was screened on salivary bacterial strains. The strains were isolated form oral fluid of female university students with and without periodontal problems.

2. Materials and methods

2.1. Subjects and clinical tests

The subjects were 50 female university students who volunteered to enter the study. In order to reduce the variables, age and sex were selected in similar ranges. Subjects on special diets such as weight control and vegetarian were excluded. Smoking and antibiotic usage was also among exception criteria. Periodontal tests including probing depth (PD) clinical attachment loss (CAT), gingival index (GI) and plaque index (PI) were performed by one single periodontist. Based on the clinical results they were then grouped into with (Group I, $n = 21$) and without (Group II, $n = 29$) periodontal problems.

2.2. Saliva sampling and measurement of flow rate

The purpose of research was explained to all subjects and they were asked to sign an informed consent. After a rinse of their oral cavity with 10 mL of sterile distilled water, the subjects were told to keep their oral fluid for exactly 3 min. The time was taken by help of technician using a stop watch. This was followed by collecting the un-stimulated saliva samples into sterile tubes. The salivary flow rate was calculated from the volume collected in this period and stated as (ml/min). The saliva samples were first centrifuged and the tubes were sealed completely, kept at 4 °C and transferred to laboratory and incubated at 37 °C for 2 h.

2.3. Saliva culture and bacterial growth

Prior to the culture, the salivary pH was measured using a standard calibrated pH meter. Saliva samples were cultured on a series of non-selective and selective media (Blood agar, Nutritive agar medium and Sabouraud Dextrose Agar SDA), incubated at 37 °C for 24, 48 and 72 h with addition of 5% CO_2 in the atmosphere for lactobacilli and streptococci. The isolated strains were identified using appropriate commercials kits (API Staph, API20E and API50CH, Biomérieux, Marcy l'Etoile, France). The mean of Colony Forming Unit (CFU) count was determined as the mean value of three measurements. The number of microbial colonies was presented as mean ± SD in a logarithmic scale.[7]

2.4. Preparation of S. persica methanol extracts

S. persica plant was collected from Sistan Baluchestan Province, Southeast of Iran in May 2010 (Fig. 1). Its identity was approved by a plant systematic expert, its fibrous barks washed thoroughly, cut into small pieces and left at room temperature for 2 weeks. Thirty grams of dried pulverized barks of plant were mixed with 500 mL of 20% methanol resulting to 60 g dry wt/L each. The dried powder suspension was mixed using a magnetic stirrer for 12 h at room temperature, filtered on Whatman ash-less paper (Cat. No 1442). The mixture was then centrifuged at 9000 rpm for 30 min. The resulting supernatant was separated and left at room

Fig. 1 – Partly dried barks, chewing sticks and leaves of *Salvadora persica*.

temperature for 30 min for evaporation of methanol. The residual methanol was removed using a rotary evaporator and the extract kept at −4 °C until subsequent procedures. Prior to testing, the extract was freshly reconstituted in methanol to final concentration of 400 mg/ml (stock solution). The stock solution was then used to prepare serial dilutions (400-50 mg/ml).[8]

2.5. *Antibacterial activities*

Bacterial strains were grown to exponential phase in nutrient broth and adjusted to a final density of 2 × 108 CFU/ml (OD_{650} = 0.7).[9]

2.5.1. *Agar diffusion method*

100 μl of bacterial suspension was spread smoothly on the Mueller Hinton using a glass spreader. Wells were then bored into the agar using a sterile 6 mm diameter cork borer and the wells filled with various concentrations of sterile methanolic extract made from stock *S. persica* solution (400, 200, 100 and 50 mg/ml). This was followed by 2 h pre-incubation (at room temperature, 25 °C) for proper diffusion of the plant extract into the media. The plates were then incubated at 37° C for 24 h.[10] The mean diameter of complete growth inhibition zone (in mm) was measured and considered as the inhibition zones. Methanol and 20 μg streptomycin were used as negative and positive controls respectively.

2.5.2. *Measurement of minimum inhibitory concentrations (MICs)*

The MIC is defined as the lowest concentration of the extracts inhibiting the growth of microbial strains. A microdilution method in 96 well microtiter plates was used to determine MICs for strains isolated from salivary fluid of both student groups.[11] Serial microdilutions were made from the stock solution of the methanolic extract (400 μg/ml) as follows.

50 μl of nutrient broth was first distributed from the 2nd to the 12th well, followed by addition of 50 μl of sterile methanolic extract of *S. persica* was added into the 1st test well (negative control), then 50 μl of scalar dilution was transferred from 2nd to the 12th well. 50 μl of calibrated microbial suspension was then added to each well. In order to control any dehydration, the plates were wrapped with clinging film prior to incubation at 37 °C for 24–48 h together with 20 μg streptomycin as the positive control. Using a Microplate Absorbance Reader, the growth of bacteria was estimated by measuring well optical density at 620 nm comparing to control wells.

2.6. *Statistical analysis*

Each assay was repeated in duplicate and the results were presented as mean ± SD values. Statistical difference between results was compared by un-paired *t*-test, *p* values less than 0.05 were considered as significant. Significant differences between means were determined by Duncan's multiple range tests. In addition, for antimicrobial activity, Log CFU ≤ Log 1 was considered as significant.[12]

Table 1 – Salivary pH and flow rate of female student with (I) and without (II) signs of periodontitis. Values are expressed as mean ± SD.

Salivary factor	Group I (n = 21)	Group II (n = 29)	Range
pH	6.76 ± 0.12	6.88 ± 0.14	7.15–6.21
Flow rate (ml/min)	0.45 ± 0.13	0.49 ± 0.07	0.33–0.76

3. Results and discussions

3.1. *Salivary flow rate and pH*

The rate of salivary flow and its measured pH values are presented in Table 1. These results are in accordance to the results reported in literature for pH[13] and rate[14] of saliva at various physical and biological conditions. It was observed that the salivary flow rate was not significantly different in the two groups. Table 1 indicates that the pH of saliva was slightly shifted to acidic in Group II (those who suffered from a type of periodontal problem). However, this shift to lower pH was not significant perhaps due to the buffer character of saliva. The change of salivary pH is of prime importance as many bacterial species prefer a certain pH for their growth.[13] The decreased flow rate in Group I compared to healthy Group II is not significant. It is known that the salivary flow rate is influenced by various biological and environmental factors including age[15] and various medications.[16] It has been stated that alternation in flow rate could affect various biochemical concentrations including catecholamine metabolites in the oral cavity.[14] According to the results of this study, a natural plant extract such as methanolic extract of *S. persica* which effectively controls periodontitis, may possibly influence the flow rate of saliva.

3.2. *Clinical examination for periodontitis*

All subjects were examined for signs of periodontitis by measuring the most common indicators probing depth (PD), clinical attachment loss (CAL), gingival index (GI) and plaque index (PI). Table 2 represents the mean value obtained for periodontal indices. According to the data of this table, the subjects were grouped I and II.

PDs and CATs less than 2 and GI and PI less than 0.5 were considered as negative periodontal signs. According to these

Table 2 – Clinical finding for female students with (Group I) and without (II) periodontitis signs. The data are given as mean ± SD.

Periodontal parameter	Group I (n = 21)	Group II (n = 29)
PD (mm)	5.2 ± 0.3	1.6 ± 0.09
CAT (mm)	5.4 ± 0.2	1.8 ± 0.08
GI	1.95 ± 0.05	0.38 ± 0.02
PI	1.56 ± 0.04	0.46 ± 0.03

PD: probing depth, CAL: clinical attachment loss, GI: gingival index and PI: plaque index.

Table 3 – Microbial diversity and aboundance observed in saliva of volunteer female students.

Bacterial strains detected in saliva	*n* (total 50)	%
None (except normal flora)	5	10
Streptococcus	36	72
Lactobacillus	30	60
Staphylococcus	31	62
Escherichia	15	30
Enterococcus	18	36

findings, about 40% of subjects had clinical signs of periodontitis, a result similar to literature reports.[17,18]

3.3. *Bacterial strains identified in saliva samples*

According to the results, an important microbial diversity was found in saliva of volunteer female students (Table 3). Among total of 50 student volunteers, 5 subjects showed no significant sign indicator of bacterial growth, but the normal flora was grown (10%). In 16 cases (32%) one of the 5 bacterial genera appeared as single, pair or triple bacterial strains. Three bacterial genera including *Staphylococcus*, *Streptococcus*, and *Lactobacillus* were most commonly identified genera occurring in 90% of subjects.

On the other hand, *Escherichia* and *Enterococcus* were additionally observed in Group I. This result is supported by our previous study in which we identified *E. faecalis* in saliva of periodontal patients.[8] Supportive to this finding, the presence of *Escherichia coli* has been reported in oral samples from children with caries.[7]

3.4. *Identification of a bacterial genus*

Stock culture of *Enterococcus* was subcultured and initially tested for purity and identity by Gram staining and catalase test prior to disk-diffusion assay. The presence of *E. faecalis* (*E. faecalis*) in oral fluid of Group I was confirmed, while it was observed in the oral fluid obtained from Group II.

3.5. *Effect of methanolic extracts of* S. persica

Using vancomycin and ampicillin as positive tests, it was observed that the growth of all identified bacterial genera was significantly ($p < 0.05$) inhibited in the presence of methanol extracts from *S. persica*. The methanolic extract was most effective when its concentration was 400 mg/ml (Table 4). However, even the highest concentration of extracts is not to be compared with antibiotics as their doses are in μg not mg.

An important drawback with natural plant extracts is that high concentration should be used to achieve a desired result. This is due to the presence of many other compounds in the extract other than the effective compound alone. This problem is only solved if the effective compound is identified and completely purified.

The result of our study showed that the methanolic extract of *S. persica* exhibited a stronger anti-bacterial activity against Gram negative (6.5–12 mm) than Gram positive (1–8 mm) bacteria. It has been suggested that the existence of different lipo-polysaccharids in their membrane could be the reason.[19,20] Diversity of anti-bacterial effect could also be related to different compounds in the methanolic extract such as saponins, tannins, alkaloids and terpenoids.[9] On the other hand, the slight acidic pH of extract may also contribute to growth inhibition of enterobacteria, *Staphylococcus* and enterococci grow.[21] However this slightly low pH could not possibly be the only factor for growth inhibition as a stronger acidic pH may cause complete inhibition.[22,23] In support of the present study, it has been shown that *S. persica* has significant antimicrobial activity against aerobic and anaerobic bacteria obtained from teeth with inflamed gums.[24]

4. Discussions

The rapid increase of antibiotic resistance together with various side effects they cause, has opened a wide range of research area investigating the possible use of natural plant extracts. Among many known synthetic antibiotics vancomycin was used to be most effective on *E. faecalis*. However, the occurrence of vancomycin-resistant *E. faecalis* during the last few years has necessitated the use of natural products.[19] It is known that *E. faecalis* is the primary etiologic agent of chronic periodontitis, indicating that natural medications are mostly important in the case of periodontal problems. In recent approaches, scientists are trying to develop natural products which do not introduce bacterial resistance and cause much lower side effects.[25]

S. persica is a native plant in Iran and the Middle East countries traditionally used for tooth cleaning. In Iran, it is found in south and southeast, mostly in a state called Sistan. According to a literature investigation, only a few reports were found on identification of antimicrobial compounds in *S. persica*. On the other hand, we have previously found interesting results regarding potential anti quorum sensing (AQS) ability of its methanolic extract.[6] In this study, it was revealed that growth of oral bacteria is considerably inhibited by extracts of

Table 4 – The diameter of inhibition zone (mm) in the presence of ampicillin 10 μg (Amp), vancomycin, 20 μg (Van) and *S. persica* extract.

Bacterial strains	Concentration of S. *persica* methanolic extract (mg/ml)						
	400	300	200	100	50	Amp	Van
Escherichia	11.0 ± 0.08	9.0 ± 0.03	8.3 ± 0.03	7.1 ± 0.06	5.4 ± 0.02	4.3 ± 0.04	–
Enterococcus	8.9 ± 0.01	8.0 ± 0.04	7.5 ± 0.04	6.4 ± 0.03	4.5 ± 0.01	5.1 ± 0.04	2.1 ± 0.05
Streptococcus	7.2 ± 0.06	6.5 ± 0.03	5.8 ± 0.02	5.0 ± 0.03	3.6 ± 0.03	4.0 ± 0.04	2.0 ± 0.03
Lactobacillus	6.2 ± 0.05	5.3 ± 0.01	4.7 ± 0.05	3.9 ± 0.04	2.0 ± 0.03	3.2 ± 0.06	1.6 ± 0.04
Staphylococcus	7.5 ± 0.05	5.0 ± 0.03	4.4 ± 0.03	3.0 ± 0.05	1.6 ± 0.05	2.2 ± 0.01	1.4 ± 0.01

the *S. persica*. Considering the less polarity of methanol than water, it is suggested that most of antibacterial agents in *S. persica* have low polarities.

5. Conclusions

Based on the clinical results from this study, it could be concluded that periodontal problems may exist in healthy individuals even at high educational level with almost no apparent sign. On the other hand, effective antibacterial molecules present in *S. persica* could act against growth of most bacterial strains found in saliva. The effective substances in persica extracts, exhibit a broad range of activities in order to affect almost all bacterial species regardless of the Gram-staining nature. It can be concluded that in future work, the antibacterial activity of *S. persica* extracts should be examined against other opportunistic oral pathogens including *Streptococcus mutans*. It is also suggested that further studies performed in order to identify effective chemicals from *S. persica*.

Conflicts of interest

All authors have none to declare.

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