Effectiveness of *Salvadora persica* extracts against common oral pathogens

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Abstract *Objective:* The purpose of this study was to evaluate the antibacterial activity of ethanol and hexane extracts of *Salvadora persica* against common oral pathogens.

Materials and methods: Well diffusion, Minimum Inhibition Concentration (MIC), Minimum Bactericidal Concentration (MBC), and Broth microdilution tests were used to determine the optimum antimicrobial concentrations of *S. persica* extracts against *Streptococcus mutans* (*S. mutans*), *Streptococcus sanguis* (*S. sanguis*), and *Streptococcus salivarius* (*S. salivarius*) over 1, 3, 6, 12, and 24 h. Chlorhexidine (CHX) 0.2% was used as a positive control.

Results: The findings showed that the microbial activity of both extracts was concentration-dependent. Ethanol extract of *S. persica* at 25, 50, and 100 mg/ml had more growth inhibitory effect against all isolates compared to hexane extract. In addition, ethanol extract at 8 mg/ml (MBC value) was able to eradicate the growth of all isolates. *S. sanguis* and *S. salivarius* were very sensitive to hexane extract and required 4 mg/ml (MBC value) for their eradication while *S. mutans* was the most resistant (MBC = 8 mg/ml). The statistical findings of CFU counts showed no significant difference ($p = 1.000$) in antibacterial effectiveness between the two extracts against all isolates. A significant decline overtime in CFU counts was noted, except at 12 h and 24 h where no significant difference ($p = 0.793$) was observed and was comparable to CHX.

Conclusion: Ethanol and hexane extracts of *S. persica* were found to exhibit maximum antimicrobial activity against *S. mutans*, *S. sanguis* and *S. salivarius* at high concentrations.

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1. Introduction

Chemical treatment besides mechanical cleaning is needed to maintain gingival health, control plaque and prevent periodontal disease occurrence and progression (Al-Bayaty et al., 2010). However, with the increasing incidence of oral diseases, the global need for alternative prevention and treatment methods that are safe and effective has expanded (Halawany, 2012). Herbal medicine has been used for a long time for dental plaque, microorganism control, and maintenance of oral health (Fine, 1995; Mandel, 1988). The toothbrush tree, *Salvadora persica* (*S. persica*), known locally as "Miswak," is a member of the Salvadoraceae family. It is a small tree with soft, whitish, yellow wood, and has been used in Africa, South America, the Middle East, and Asia as a traditional oral hygiene tool (Noumi et al., 2010; Sofrata et al., 2008). The most common type of Miswak is derived from the Arak tree that grows mainly in Saudi Arabia and in other parts of the Middle East (Batwa et al., 2006).

It has been reported that extracts of *S. persica* possess various biological properties, including significant antimicrobial (Al lafi and Ababneh, 1995; Al-Sohaibani and Murugan, 2012; Masood et al., 2010; Sofrata et al., 2008) and anti-inflammatory (Ibrahim et al., 2011) properties, and lack of toxicity (Balto et al., 2014; Darmani et al., 2006). The antimicrobial and cleaning effects of *S. persica* may be attributed to various chemicals contained in its extracts such as trimethyamin, salvadorine, chloride, fluoride in large amounts, silica, sulfur, mustard, vitamin C, saponins, tannins, cyanogenic glycoside, and benzylisothiocyanate (Akhtar and Ajmal, 1981; Darout et al., 2000a,b). *S. persica* has demonstrated cleansing efficacy, ability to remove the plaque, and decrease ginigival bleeding (Batwa et al., 2006; Darout et al., 2000a,b) when used as a chewing stick. As a mouth wash, *S. persica* has improve periodontal health, reduce microbial plaque accumulation and lower carriage rate of cariogenic bacteria (Al-Otaibi et al., 2004; Khalessi et al., 2004).

Various methods for obtaining *S. persica* extract have been used, mainly aqueous and alcohol extracts (Al-Sabawi et al., 2007; Al-Bayati and Sulaiman, 2008), while others have used *S. persica* pieces without extraction (Sofrata et al., 2008). The antimicrobial effects of *S. persica* against a range of pathogens have been evaluated (Al-Bayati and Sulaiman, 2008; Khalessi et al., 2004; Poureslami et al., 2007). The results of these experiments are variable and sometimes contradictory as to the most effective *S. persica* extract preparation method, its concentrations, and which of the bacterial species are affected by *S. persica* extract.

Balto et al. (2013) have screened the antimicrobial activities of seven *S. persica* extracts against *Enterococcus faecalis* and *Candida albicans*. They have demonstrated that ethanol and hexane extracts exhibit the maximum antimicrobial activity against both microbes. Further study (Balto et al., 2014) has shown that both extracts (ethanol and hexane) were non-cytotoxic on human gingival fibroblast cells. Hexane extract has never been tested against common oral pathogens and in light of the previous promising findings (Balto et al., 2013, 2014), the aim of the current study was to assess the antibacterial activity of ethanol and hexane extracts of *S. persica* against *Streptococcus mutans*, *Streptococcus sanguis*, and *Streptococcus salivarius*.

2. Materials and methods

The study was carried out at the Laboratory of Microbiology, College of Medicine, King Saud University.

2.1. Extracts preparation

The roots of *S. persica* were collected from Al-Makhwah, which is located in the southern region of the Kingdom of Saudi Arabia, in March 2010. The plant was identified by a taxonomist and a voucher specimen (#1745) was deposited at the herbarium, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia for future reference. The stock solution was prepared by extracting the fresh ground roots three times with the following solvents: hexane and 10% ethanol. All extracts were prepared by percolating 100 g of dried powder in each solvent three times every 24 h, with fresh solvent used each time. The extracts were freeze-dried to ensure that the remaining solvent was completely removed. All *S. persica* extracts were suspended in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/ml. The stock solution was sterilized using 10 KG of gamma radiation and kept in a freezer at −20 °C.

2.2. Test organisms

Three Gram-positive strains were used in this study, *Streptococcus mutans* (ATCC25175), *Streptococcus sanguis* (ATCC10556) and *Streptococcus salivarius* (ATCC13419) were taken from frozen stock culture (Dental Caries Research Chair, College of Dentistry, King Saud University), inoculated on a sheep blood agar plate (Oxoid Ltd, Basingstoke) and grown overnight at 37 °C. Cells were collected by centrifugation (900×g for 10 min) and the pellets were re-suspended in brain heart infusion broth (BHIB).

2.3. Tests for antimicrobial activities

2.3.1. Well diffusion method

It is based on the diffusion of the antibacterial substance in the agar. All test isolates were mixed with normal saline to achieve a turbidity equivalent to a 0.5 McFarland standard (approximately 10^8 colony-forming units per milliliter [CFU/ml]). This was further diluted by 1:100 to give a final concentration of 10^6 CFU/ml. Three Muller-Hinton (MH) agar plates with 5% blood (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) were inoculated with microbial suspensions (one plate/ bacteria/ extract). Four small wells were created by indenting the agar with a clean pipette. Each resulting well was approximately 6 mm in diameter and accommodated approximately 90–95 microliters (μl) of extract. Each well was then filled with neat, 1/2, 1/4, and 1/8 dilutions of *S. persica* extracts corresponding to 100 mg/ml, 50 mg/ml, 25 mg/ml, and 12.5 mg/ml, respectively. All experiments were performed in duplicate for each herbal extract. Following incubation at 35 °C for 48 h anaerobically, the zone of herbal diffusion from the well into the agar was measured in millimeters. The shortest distance (mm) from the outer margin of the well to the initial point of microbial growth was considered as the inhibitory zone. Results were

recorded as the average of the two measurements. CHX 0.2% was used as a positive control while sterile saline was used as a negative control (four wells/ plate).

All procedures and microbiological manipulations were carried out in a Class II biological safety cabinet (Baker Scientific Company, Maine, USA).

2.3.2. Broth micro dilution assay for Minimum Inhibitory Concentrations (MIC)

It is the lowest concentration of antimicrobial agent that inhibits the visible growth of a microorganism. MIC of ethanol and hexane extracts of *S. persica* against *Streptococcus mutans* (*S. mutans*), *Streptococcus sanguis* (*S. sanguis*), and *Streptococcus salivarius* (*S. salivarius*) were determined by a triplicate twofold micro-broth dilution method (Mann and Markham, 1998). Briefly, overnight microbial culture at a concentration of 5×10^5 CFU/ml were treated with a concentration range from 16 to 0.125 mg/ml of each extract in sterile 96-well round-bottom microplates (Fisher Scientific). Plates were then incubated at 37 °C for 48 h. After incubation, the MIC of each extract was determined as the lowest concentration at which no growth was observed in the duplicate wells. Twenty microliters of a p-iodonitro-tetrazolium violet solution (0.04%, w/v) (Sigma, USA) was then added to each well. The plates were incubated for a further 25–30 min, and estimated visually for any change in color from yellow to pink indicating reduction of the dye due to bacterial growth. The highest dilution (lowest concentration) that remained yellow corresponded to the MIC. Experiments were performed in duplicate (for each extract and microbe).

2.3.3. Minimal Bactericidal Concentration (MBC) test

It is the lowest concentration of an agent that kills the microorganism. All clear microwells from the MIC test were streaked on Mueller Hinton Agar plate with 5% blood using 1 µl loop. Plates were incubated aerobically at 37 °C for 48 h. The MBC was determined by colony count of more than 99.9% killing.

2.3.4. Colony forming units (CFU)

The stock culture *S. mutans*, *S. sanguis*, and *S. salivarius* was grown in 5 ml of BHIB for 24 h and the inoculums were set at 0.5 McFarland standard. The MBC value of each isolate for ethanol and hexane extract (0.5 ml) was inoculated with final bacteria inoculums of 5×10^5 CFU/ml in sterile Eppendorf tubes. Positive Control tube containing the microbial culture with CHX 0.2% was used. All tests and controls were incubated at 37 °C for 24 h. A single aliquot was assessed periodically at 1, 3, 6, and 12, 24 h by preparing log dilutions in physiological saline. These dilutions were added to BHI agar plates in duplicate and the inoculums were spread with the help

of a sterile glass rod using a plate rotator. Plates were incubated in anaerobic jars (BBL, CA, USA) with gas generating pouches and colonies were counted after 48 h of incubation.

2.4. Statistical analysis

The data of CFU was entered into Microsoft Excel and analyzed using IBM® SPSS® Statistics, Version 20 (IBM Corp., Armonk, NY, USA). One-way analysis of variance was used to compare the mean values of the outcome variable, followed by Tukey's *post hoc* test. The significance level was set at $p < 0.05$.

3. Results

Table 1 shows the antimicrobial potential of *S. persica* extracts at different dilutions. The findings showed that the microbial activity of both extracts was concentration dependent. Ethanol extract of *S. persica* at 25, 50, and 100 mg/ml had more growth inhibitory effect against all isolates compared to hexane extract.

The minimum concentration of the ethanol extract required for inhibition (MIC) or killing (MBC) of *S. mutans* and *S. sanguis* was comparable (8 mg/ml). On the other hand, *S. salivarius* was most sensitive to the extract, and showed a value of 4 mg/ml and 8 mg/ml for MIC and MBC, respectively. The minimum concentration of the hexane extract required for inhibition (MIC) and killing (MBC) of *S. sanguis* and *S. salivarius* was 2 mg/ml and 4 mg/ml, respectively. *S. mutans* showed a value of most resistance to the hexane extract (Table 2).

The statistical findings of CFU counts showed no significant difference ($p = 1.000$) in antibacterial effectiveness between the two extracts

Table 2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of ethanol and hexane extracts of *S. persica*.

S. persica extracts	Microbes	MIC (mg/ml)	MBC (mg/ml)
Ethanol	*Streptococcus mutans*	8	8
	Streptococcus salivarius	4	8
	Streptococcus sanguis	8	8
Hexane	*Streptococcus mutans*	4	8
	Streptococcus salivarius	2	4
	Streptococcus sanguis	2	4
+ ve control	CHX 0.2%	0.2	0.2

Table 1 Zone of microbial inhibition in millimeter provided by ethanol and hexane extracts of *S. persica* at different concentrations.

Microorganisms	Zone of Inhibition (mm)								+ ve control
	Ethanol (mg/ml)				Hexane (mg/ml)				CHX 0.2%
	12.5	25	50	100	12.5	25	50	100	
S. mutans	6	6	8	16	6	6	6	13	23
S. salivarius	6	7	9	18	6	6	7	16	21
S. sanguis	6	9	14	20	6	6	8	14	20

Table 3 The viable count of different strains after exposure to the MBC values of ethanol and hexane extract of *S. persica* during different observation periods.

Time in hours	*S. mutans*		*S. salivarius*		*S. sangius*		Post hoc *p*-value			
	Hexane 8 mg/ml	Ethanol 8 mg/ml	Hexane 4 mg/ml	Ethanol 8 mg/ml	Hexane 4 mg/ml	Ethanol 8 mg/ml	3 h	6 h	12 h	24 h
1 h	4.2×10^5	3×10^5	4.25×10^5	2.8×10^5	4×10^5	2.5×10^5	0.002[*]	0.000[*]	0.000[*]	0.000[*]
3 h	2.7×10^5	1.5×10^5	3.7×10^5	1×10^5	3.1×10^5	1.3×10^5		0.003[*]	0.000[*]	0.000[*]
6 h	1.25×10^5	7×10^4	2.5×10^5	5×10^4	2.8×10^5	6×10^4			0.036[*]	0.001[*]
12 h	NG[**]	NG	NG	NG	1.6×10^5	NG				0.793
24 h	NG	NG	NG	NG	NG	NG				

CHX 0.2% was used as positive control. At all tested intervals no viable count was found for CHX 0.2%.
[*] Statistically significant.
[**] NG = No growth.

of *S. persica* against isolates. A significant decline overtime in CFU counts was noted, except at 12 h and 24 h where no significant difference ($p = 0.793$) was observed (Table 3).

4. Discussion

With the increased continued interest in identifying efficient antibacterial agents with less adverse effects, herbal extracts have been extensively investigated over the last decade. Hence, the purpose of the present study was to assess the antibacterial activity of ethanol and hexane extracts of *S. persica* against common oral pathogens. The antibacterial activity was qualitatively and quantitatively assessed by the presence or absence of inhibition zones, MIC MBC, and CFU values.

S. mutans, *S. sanguis* and *S. salivarius* were Gram-positive strains and were selected in this study as they are most commonly associated with dental caries (Chava et al., 2012; Loesche et al., 1973). Studies have shown that alcoholic solvent has more antimicrobial potency than aqueous when used with *S. persica* extracts (Aassan et al., 2012; Balto et al., 2013), therefore ethanol and hexane have been used as solvents in this study. Although hexane as solvent has been used for preparation of extracts, to the best of our knowledge this is the first time that hexane extract of *S. persica* has been tested for its antibacterial activity against *S. mutans*, *S. sanguis*, and *S. salivarius*.

The well diffusion assay revealed that the microbial activity of both extracts was concentration dependent, and these findings are consistent with Al-Bayaty et al. (2010). The zone of microbial inhibition ranged between 6–20 mm and 6–16 mm for ethanol and hexane extracts, respectively. Such a difference between the two extracts could be attributed to the differential diffusion of the extracts in the surrounding media. Ethanol extract at 100 mg/ml was associated with a wide zone of inhibition (20 mm) against *S. sanguis* and was comparable to the CHX 0.2%, and this result is consistent with other findings in which herbal-based mouthwashes were comparable to triclosan and chlorhexidine gluconate, if used at a very high concentration (Almas, 2002; Almas et al., 2005).

S. salivarius and *S. sanguis* were more sensitive to hexane compared to ethanol while *S. mutans* had similar resistance to both extracts (MIC = 4 mg/ml, MBC = 8 mg/ml). These differences in sensitivity of bacteria have been noted earlier (Al-Delaimy and Ali, 1970). It was found that benzy isothiocyanateis the most effective antibacterial ingredient in *S. persica* extracts prepared from the roots (Al-Bagieh and Weinberg, 1988; Sofrata et al., 2011). These observations suggest that although antibacterial activity of *S. persica* may reside in isothiocyanates, various forms of isothiocyanates with antimicrobial properties might vary in different species. Additionally, it was reported that varying activities of different extracts could be attributed to the presence of several types of compounds belonging to different classes, such as oleoresins in hexane extract (Dapkevicius et al., 1988), sterols and their derivatives, and flavones and flavonoids in semi-polar extract (Guillen and Manzanos, 1988). Therefore, the different reactions of each strain to the various extracts could be due to the fact that each solvent extracted different active ingredients of *S. persica*.

Although there is no significant difference in the antibacterial effect of both extracts against all isolates, ethanol reduced the number of the colony-forming units over time compared to hexane. This observation is consistent with others (AbdElRahman et al., 2002).

Hexane as a solvent has been used for preparation of different extracts. Hexane extract of *Urticadioica* leaves has recently been shown to be an effective antibacterial agent against five clinical isolates of Gram-positive and Gram-negative bacteria (Singh et al., 2012). These findings were further confirmed by Elzaawely et al.(2005) using hexane extract of *Urticadioica* leaves. Hexane extract of the sea urchin, *Temnopleurus alexandri*, performs well as a potent antibacterial agent against a majority of Gram-positive and Gram-negative bacteria, except *Klebsiella pneumonia* (Uma and Parvathavarthini, 2010). Moreover, hexane extract of *Schinus terebinthifolius* (Anacardiaceae) has also been shown to exhibit strong antimicrobial activity against the isolates of *Paracoccidioides brasiliensis*. Collectively, these observations and the findings of the present study indicate that hexane performs well as a solvent in preparation of potent plant extracts (Johannet al., 2010).

5. Conclusion

Ethanol and hexane extracts of *S. persica* were found to exhibit maximum antimicrobial activity against *S. mutans*, *S. sanguis*, and *S. salivarius* at high concentration.

Limitation of the study

This study evaluated the antimicrobial effect of *S. persica* extracts against planktonic bacteria. However, dental plaque existed in the oral cavity as a biofilm. So further studies,

in vitro, are recommended for the evaluation of these extracts as an effective antimicrobial agent against biofilm.

Acknowledgements

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