

1-1-2008

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AL-BAYATI, FIRAS A. and SULAIMAN, KHUDIR D. (2008) "In Vitro Antimicrobial Activity of *Salvadora persica* L. Extracts Against Some Isolated Oral Pathogens in Iraq," *Turkish Journal of Biology*. Vol. 32: No. 1, Article 9. Available at: <https://journals.tubitak.gov.tr/biology/vol32/iss1/9>

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In Vitro Antimicrobial Activity of *Salvadora persica* L. Extracts Against Some Isolated Oral Pathogens in Iraq

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Received: 05.09.2007

Abstract: Aqueous and methanol extracts of *Salvadora persica* L., a plant used in Iraq for oral hygiene, was investigated for its antimicrobial activities against 7 isolated oral pathogens: *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus faecalis*, *Streptococcus pyogenis*, *Lactobacillus acidophilus*, *Pseudomonas aeruginosa*, and *Candida albicans* using disc diffusion and micro-well dilution assays. According to both antimicrobial assays the aqueous extract inhibited all isolated microorganisms, especially the *Streptococcus* species, and was more efficient than the methanol extract, which was resisted by *Lacto. acidophilus* and *Ps. aeruginosa*. The strongest antibacterial activity was observed using the aqueous extract against *Strep. faecalis* (zone of inhibition: 22.3 mm; MIC: 0.781 mg/ml). Both extracts had equal antifungal activity against *C. albicans* based on the turbidity test (MIC: 6.25 mg/ml).

Key Words: Antimicrobial activity, medicinal plants, *Salvadora persica*, oral hygiene tool

Salvadora Persica L. Bitkisinin Ekstrelerinin Irak'ta İzole Edilen Bazı Ağız Patojenleri Üzerine Etkisi

Özet: Irak'ta ağız hijyeninde kullanılan *Salvadora persica* L. bitkisinin sıvı ve metanol özütü yedi ağız patojenine olan *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus faecalis*, *Streptococcus pyogenis*, *Lactobacillus acidophilus*, *Pseudomonas aeruginosa* ve *Candida albicans* bakteri üzerine antimikrobial aktivitesi disk diffüzyon ve mikro-well seyreltme yöntemi ile çalışılmıştır. Sıvı özüt izole edilen ekstrakt bütün mikroorganizmaların özellikle *Streptococcus* türlerinin büyümesini engellemiş ve metanol ekstraktan daha etkili olmuştur. *L. acidophilus* ve *P. aeruginosa* metanol özüte dirençli bulunmuştur. En güçlü antimikrobiyal aktivite, sıvı özütte *Strep. faecalis*'te (inhibisyon zonu: 22.3 mm; MIC: 0.781 mg/ml) gözlenmiştir. *C. albicans* türüne karşı her iki ekstrakt aynı antifungal etki göstermiştir bulanıklık testi (MIC: 6.25 mg/ml).

Anahtar Sözcükler: Antimikrobiyal aktivite, tıbbi bitki, *Salvadora persica*, oral hijyen

Introduction

In recent years, human pathogenic microorganisms have developed resistance in response to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. This situation, the undesirable side effect of certain antibiotics, and the emergence of previously uncommon infections, has forced scientists to look for new antimicrobial substances from various sources, such as medicinal plants (1,2). The screening of plant extracts and plant products for antimicrobial activity has shown that plants represent a potential source of new anti-infective agents (3,4).

The toothbrush tree, *Salvadora persica*, L., locally called miswak, is a member of the Salvadoraceae family has been used by many Islamic communities as toothbrushes and has been scientifically proven to be very useful in the prevention of tooth decay, even when used without any other tooth cleaning methods (5). Chewing sticks that are made from the roots, twigs, or stems of *S. persica* are commonly used in the Middle East as a means of maintaining oral hygiene. Studies indicate that *S. persica* extract is somewhat comparable to other oral disinfectants and anti-plaque agents, such as triclosan and chlorhexidine gluconate, if used at a very high concentration (6,7).

It has been reported that extracts of miswak possess various biological properties, including significant antibacterial (8), antifungal (9), and anti-plasmodial effects (10). *S. persica* and other related plants are reported to be effective against bacteria that are important for the development of dental plaque.

Despite the wide use of miswak, the plant in Iraq has not received much attention and has not been fully studied. Therefore, the aim of the present study was to evaluate the antimicrobial activity of Iraqi miswak using disc diffusion and micro dilution assays.

Materials and Methods

Plant material

Dried stems of *S. persica* were purchased from a local market in Mosul city, Nineveh province, Republic of Iraq, and identified by an agriculturist and the vendor according to their color and scent.

Extract preparation

Aqueous extracts (H₂O)

The fine-powdered stems of *S. persica* (100 gm) were infused in distilled water until complete exhaustion. The extract was then filtered using Whatman No. 1 filter paper, and the filtrate was then evaporated in vacuo and dried using either a rotary evaporator at 60 °C or a freeze drier (11). The final dried material was stored in labeled sterile bottles and kept in a freezer at -20 °C.

Methanol extracts (MeOH)

The powdered stems (100 gm) were extracted with methanol, using a soxhlet extractor for 10 h or until the solvent turned pure and colorless (12). The solvent was then removed using a rotary vacuum evaporator at 40 °C to give the concentrated extract, which was frozen and freeze-dried until use.

Microbial cultures

The following microorganisms were used to test the activity of the extracts: *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus faecalis*, *Streptococcus pyogenes*, *Lactobacillus acidophilus*, *Pseudomonas aeruginosa*, and *Candida albicans*. All microorganisms were isolated from patients of the general dental clinic of Mosul University, and were very carefully identified using standard microbiological methods (13).

Inoculum preparation

Nutrient broth and Sabouraud dextrose agar (SDA) were used for growing and diluting the microorganism suspensions. Bacterial strains were grown to exponential phase in nutrient broth at 37 °C for 18 h and adjusted to a final density of 10⁸ cfu/ml by diluting fresh cultures and comparison to McFarland density. *C. albicans* was aseptically inoculated on petri dishes containing autoclaved, cooled, and settled SDA medium. The petri dishes were incubated at 31 °C for 48 h to give white round colonies against a yellowish background. These were aseptically subcultured on SDA slants. The yeast colonies from SDA slants were suspended in sterilized 0.9% sodium chloride solution (normal saline), which was compared with McFarland solution. According to the manufacturer's directions, 1 ml of yeast suspension in normal saline was added to 74 ml of sterile medium and kept at 45 °C to give a concentration of 2 × 10⁷ cells/ml.

Antimicrobial screening

Disc diffusion assay

A modified agar diffusion method (14) was used to determine antimicrobial activity. Nutrient agar was inoculated with a microbial cell suspension (200 µl in 20 ml of medium) and poured into sterile petri dishes. Sterile filter paper discs 6 mm in diameter were impregnated with 20 µl of each extract concentration (200, 100, 50, 25, and 12.5 mg/ml), which were prepared using the same solvents employed to dissolve the plant extracts, and then sterilized via pasteurization and membrane filtration (regarding the aqueous extract), and placed on the inoculated agar surface. Standard 6-mm discs containing streptomycin (25 µg/disc) and amphotericin B (10 µg/disc) were used as positive controls. Negative controls were made using paper discs loaded with 20 µl of the solvents. After pre-incubation for 2 h in a refrigerator the plates were incubated overnight at 37 °C for 18-24 h. In contrast, *C. albicans* was incubated at 31 °C for 48 h. At the end of the incubation period antimicrobial activity was evaluated by measuring the zones of inhibition.

Microdilution assay

The minimal inhibitory concentration (MIC) of the *S. persica* extracts were determined based on a microdilution method in 96 multi-well microtiter plates, as previously described (15), with slight modifications. The dissolved extracts were first diluted to the highest

concentration to be tested (12.5 mg/ml), 50 µl of nutrient broth was distributed from the 2nd to the 12th well, a volume of 100 µl from each of the aqueous and methanol extracts initially prepared was pipetted into the 1st test wells of each microtiter line, and then 50 µl of scalar dilution was transferred from the 2nd to the 12th well. To each well was added 10 µl of resazurin indicator solution (prepared by dissolving a 270-mg tablet in 40 ml of sterile distilled water). Finally, 10 µl of bacterial suspension was added to each well. The final concentration of the extracts adopted to evaluate antibacterial activity was included from 12.5 mg/ml to 0.003 mg/ml. Three columns in each plate were used as controls: 1 column with a broad-spectrum antibiotic as a positive control (streptomycin in a serial dilution of 12.5-0.003 mg/ml) and 2 columns containing the solvents, water, and methanol as negative controls. Plates were wrapped loosely with cling film to ensure that bacteria did not become dehydrated and prepared in triplicate, and then they were placed in an incubator at 37 °C for 18-24 h. Color change was then assessed visually. Any color change from purple to pink or colorless was recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value. The average of 3 values was calculated and that was the MIC for the test material.

As for *C. albicans*, a simple turbidity test was used to determine the MIC values of *S. persica* extracts by adding 0.1 ml of each extract concentration (12.5-0.003 mg/ml)

into tubes containing 9.8 ml of sterile nutrient broth, and then the tubes were inoculated with 0.1 ml of yeast suspension and incubated at 31 °C for 48 h. Amphotericin B (12.5-0.003 mg/ml) was used as a positive control. The optical density was determined using a Spectro SC spectrophotometer (LaboMed, Inc.) at 630 nm. The MIC value was the lowest concentration of extract that showed no growth after 48 h of incubation in comparison with the control tube, which included 9.8 ml of nutrient broth and 0.1 ml of yeast suspension in addition to 0.1 ml of each extract concentration (unincubated).

Results

The present study was conducted to investigate the antimicrobial activity of *S. persica* extracts against some isolated oral microorganisms. Among the 40 microbial samples collected from adult patients, 10.63% were identified as *Staph. aureus*, 4.32% as *Strep. mutans*, 6.38% *Strep. faecalis*, 2.12% *Strep. pyogenis*, 5.25% *Lacto. acidophilus*, 2.1% *Ps. aeruginosa*, and 4.3% as *C. albicans*. Table 1 provides the antimicrobial results obtained using the disc diffusion method. The aqueous extract of *S. persica* was active against all oral pathogens and Streptococcus species were the most sensitive; the highest inhibitory activity was seen against *Strep. faecalis* (zone of inhibition: 22.3 mm) using the extract concentration of 200 mg/ml, while the weakest activity was demonstrated against *Ps. aeruginosa*.

Table 1. Antimicrobial activity of aqueous and methanol extracts of *S. persica* stems.

Microorganisms	Inhibition zone (mm)											
	H ₂ O (mg/ml)					MeOH (mg/ml)					Control	
	200	100	50	25	12.5	200	100	50	25	12.5	S	A
<i>Staph. aureus</i>	18.4	17.2	15.9	14.3	13.2	13.6	12.5	11.0	10.3	9.2	20.9	N.T
<i>Strep. mutans</i>	19.3	19.0	17.8	16.5	15.3	16.3	15.0	14.8	13.4	12.0	19.5	N.T
<i>Strep. faecalis</i>	22.3	20.9	18.6	17.1	15.3	17.7	16.8	15.6	14.3	13.4	24.3	N.T
<i>Strep. pyogenis</i>	18.2	17.1	16.3	15.7	13.8	14.5	13.8	12.0	11.2	9.8	19.2	N.T
<i>Lacto acidophilus</i>	14.4	13.7	13.0	12.6	11.1	—	—	—	—	—	18.1	N.T
<i>Ps. aeruginosa</i>	10.8	10.0	9.3	8.2	7.8	—	—	—	—	—	13.2	N.T
<i>C. albicans</i>	12.4	11.3	10.5	9.7	9.1	11.0	10.3	9.8	9.2	8.6	N.T	10.2

—: No activity; S: streptomycin (25 µg); A: amphotericin B (10 µg); N.T: not tested.

On the other hand, the methanol extract of *S. persica* stems showed less inhibitory activity against the tested bacteria than did the aqueous extract. *Lacto. acidophilus* and *Ps. aeruginosa* resisted all methanol extract concentrations, while *Strep. faecalis* was the most susceptible bacteria (zone of inhibition: 17.7 mm) to the highest extract concentration. The aqueous extract exhibited better antifungal results than the methanol extract and the strongest activity was observed using the aqueous extract concentration of 200 mg/ml.

Table 2 summarizes the MIC of the aqueous and methanol extracts of *S. persica* stems. The strongest activity was seen against *Strep. faecalis* (MIC: 0.781 mg/ml) using the aqueous extract, followed by *Strep. mutans* (MIC: 1.56 mg/ml), *Staph. aureus* and *Strep. pyogenis* (MIC: 3.12 mg/ml), and finally *Lacto. acidophilus* and *Ps. aeruginosa* (MIC: 6.25 mg/ml). Furthermore, the methanol extract demonstrated weaker activity against all tested bacteria than did the aqueous extract; the weakest activity was observed against *Staph. aureus* (MIC: 6.25 mg/ml). Both extracts revealed equal antifungal activity against *C. albicans* (MIC: 6.25 mg/ml) using the turbidity test.

The standard drug streptomycin was active against all reference bacteria (zone of inhibition range: 13.2-24.3 mm; MIC range: 0.012-0.781 mg/ml). In addition, amphotericin B demonstrated strong antifungal activity against *C. albicans* (MIC: 0.195 mg/ml).

Discussion

The activity of plant extracts against bacteria have been studied for years, but in a more intensified way during the last 3 decades. During this period, numerous antimicrobial screening evaluations have been published based on the traditional use of Chinese, African, and Asian plant-based drugs (16). Miswak is a common name for *S. persica*, which is commonly used in Saudi Arabia and the Arab world. Miswak wicks clean between the teeth and do not break, regardless of the amount of pressure applied, as they are flexible and strong. The small wicks bend to the appropriate shape to clear plaque and left over food in between teeth and do not damage the gums. The WHO recommended the use of miswak in 1986 and in 2000 an international consensus report on oral hygiene concluded that further research was needed to document the effect of miswak.

In the present study aqueous and methanol extracts of *S. persica* inhibited most bacterial growth, but their effectiveness varied. The zones of inhibition ranged from 7.8-22.3 mm (aqueous extract) to 9.2-17.7 mm (methanol extract). The aqueous extract had promising MIC values against all oral bacteria, especially *Strep. faecalis* and *Strep. mutans*. Previous studies have reported that *S. persica* extracts were effective against *Strep. mutans* (5) and *Strep. faecalis*, even using low extract concentrations (17).

Table 2. MIC values of *S. persica* aqueous and methanol extracts against isolated microorganisms.

Microorganisms	MIC values (mg/ml)			
	Extracts		Control	
	H ₂ O	MeOH	streptomycin	amphotericin B
<i>Staph. aureus</i>	3.12	6.25	0.195	NT
<i>Strep. mutans</i>	1.56	3.12	0.097	NT
<i>Strep. faecalis</i>	0.781	1.56	0.012	NT
<i>Strep. pyogenis</i>	3.12	3.12	0.048	NT
<i>Lacto acidophilus</i>	6.25	N.T	0.390	NT
<i>Ps. aeruginosa</i>	6.25	N.T	0.781	NT
<i>C. albicans</i>	6.25	6.25	N.T	0.195

NT: Not tested.

Both extracts revealed antifungal activity against *C. albicans* (MIC: 6.25 mg/ml), although slight differences were seen using the disc diffusion method. The present study disagrees with a previous report (18) *S. persica* had no antifungal activity against *C. albicans*, which may be due to different types of yeast strains, isolation area, and different assay methods used.

Both antimicrobial assays indicated that the aqueous extract of *S. persica* was more efficient than the methanol extract, which was resisted by *Lacto. acidophilus* and *Ps. aeruginosa*. The resistance of bacteria towards different drugs can be due to modification of the target site, bypass of pathways, decreased uptake (reduced intracellular concentration of the antimicrobial agent, either reducing membrane permeability or by active efflux pump), enzymatic inactivation or modification of the drug, or overproduction of the target (19).

The antimicrobial and cleaning effects of miswak may be attributed to various chemicals contained in its extracts, such as sodium chloride and potassium chloride, as well as salvadorea and salvadorine, saponins, tannins,

vitamin C, silica, and resin (20), in addition to cyanogenic or lignan glycosides (21), alkaloids, terpenoids, and oleic, linoleic, and stearic acids (22).

It could be concluded that miswak and powdered miswak are excellent oral hygiene agents, and their use should be promoted based on scientific knowledge of their benefits and proper use. Because it is widely available in this part of the world and is inexpensive, miswak chewing sticks can be a great help in developing countries with financial constraints and limited oral health care facilities.

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