***In vitro* antimicrobial activity of *Salvadora persica* extract on *Helicobacter pylori* strains isolated from duodenal ulcer biopsies**

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Abstract

The aim of this study was to evaluate the *in vitro* antimicrobial activity of a methanolic extract of *Salvadora persica* solution on *Helicobacter pylori* isolated from duodenal ulcer. Over 22 strains of *H. pylori* were isolated from duodenal ulcer from August 2010 to June 2011. The *S. persica* stem was purchased from a local herb market and finely powdered. Extraction was performed with 60% methanol using a soxhlet extractor for 48 h until the solvent turned colorless while being incubated in an oven at 40°C for 48 h till dried. Dry powder was used to determine antimicrobial activity by the agar ditch method. Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the extract were determined by the agar dilution method. At concentrations of 10, 100, 200, 500 µg/mL, no zone of inhibition around the ditches was observed while a clear zone of inhibition (12 mm) was detected at 1000 µg/mL concentration for all the isolates. The best antimicrobial activity was observed at MIC 1000 µg/mL ($P \leq 0.05$). Furthermore, 10 out of 22 isolates were inhibited at 750 µg/mL of the extract. The MBC results showed that at a concentration of 1000 µg/mL all cells were dead while at a concentration of 750 µg/mL of *S. persica* a few *H. pylori* cells were still able to form colonies on Brucella agar supplemented with sheep red blood cells and antibiotics. From the above results it can be concluded that high concentration of *S. persica* could inhibit the growth of *H. pylori* and MIC and MBC were similar at that concentration.

Introduction

Salvadora persica is a small tree or shrub with a crooked trunk, seldom more than one foot in diameter. Its bark is scabrous and cracked, whitish with pendulous extremities. The root bark of the tree is similar to sand and the inner surfaces are an even lighter shade of brown. It has a pleasant fragrance and a warm and pungent taste. Young branches are green in color with a slightly rough bark, grayish-brown on the main stem, paler elsewhere. Leaves are oblong-elliptic to almost circular measuring 3×7 cm, light to dark green in color. They are rather fleshy, sometimes with wart-like glandular dots and dense, rather loose hairs. It sheds its leaves from late December to January. The leaves break with a fine crisp crackle when they are trodden on.¹

S. persica is a popular chewing stick (Miswak) throughout the Indian subcontinent, as well as the wider Muslim world.^{2,3} A chemical and phytochemical analysis of *S. persica* has identified carbohydrates and trimethylamine, an alkaloid which may effectively be salvadorine, chlorides, sulfur, terpenes, vitamin C glycosides, large amounts of fluoride and silica, small amounts of tannins, saponins, flavonoids and sterols.⁴

There has been little research into the antimicrobial activity of *S. persica* on different microorganisms.^{3,5,6} The antimicrobial activity of *S. persica* solution (Miswak) as a root canal cleaner was studied by Al-Sabawi *et al.*⁷ The results of the *in vitro* antimicrobial effect of alcoholic extract of *S. persica* (1%, 5%, 10%, 15% and 20%) against aerobic and anaerobic bacteria revealed that the best antimicrobial effect for *S. persica* extract was observed at 15% concentration according to a broth microdilution method. Antimicrobial activity of Miswak extract (*S. persica*) in commercially available non-alcoholic mouth rinses was compared and it was found that *S. persica* has good activity against oral microflora.⁸ In one study in Pakistan, the antimicrobial effect of aqueous extract of seven different types of chewing sticks was compared.⁹ It was found that *S. persica* extract can inhibit the growth of *Enterococcus faecalis* at a concentration of 50%.

Another study showed that *Streptococcus mutans* was more susceptible to Miswaks antimicrobial activity than *Lactobacilli*.^{10,11}

The effect of mouth wash extracted from *S. persica* (Miswak) on dental plaque formation was investigated¹² and it was found that crude *S. persica* extract solution was well tolerated by experimental animals. A disc diffusion test showed a marked antibacterial effect *in vitro* and this effect was concentration dependent. The extract had an *in vivo* effect but this was not considered absolute.¹³ Al-Otaibi *et al.*¹⁴

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studied the effect of chewing sticks and tooth brushing on plaque removal and gingival health. Similarly, Geetha *et al.*¹⁵ investigated the control of urinary risk factors of stone formation by *S. persica* in experimental hyperoxaluria.

H. pylori is a gram negative motile microaerophilic short rod bacterium that is responsible for most peptic ulcers and many cases of stomach inflammation (chronic gastritis).¹⁶

Those living in developing countries or in crowded, insanitary conditions are most likely to contract the bacterium which is passed from person to person. *H. pylori* only grows in the stomach and is usually the cause of more than half the peptic ulcers worldwide.¹⁷ The bacterium causes peptic ulcer by damaging the mucous coating that protects the stomach and duodenum. Damage to the mucous coating allows powerful stomach acid to get through to the sensitive lining beneath. Together, the stomach acid and *H. pylori* irritate the lining of the stomach or duodenum and cause an ulcer contracted during childhood.¹⁸

Each year a considerable amount of time and money is wasted on treating infection caused by *H. pylori*. The drugs on the market are generally tetracycline, claritromycin, metronidazole, a proton pump inhibitor, and bismuth subsalicylate. Drug resistant strains are to be found growing in different parts of the

world and this makes therapy for infection caused by this bacterium more complicated. Furthermore, *H. pylori* treatment may cause side effects in some patients. Therefore, investigations into anti-*H. pylori* plant extracts with negligible side effects are very important.

There is a little information on the antimicrobial effect of *S. persica* on *H. pylori*. *in vitro* susceptibility of *H. pylori* to licorice extract showed that it inhibited *H. pylori* strains with a minimum inhibitory concentration (MIC) range of 50-400 mg/mL.¹⁹

Teymournejad *et al.*²⁰ studied the effect of different concentrations of the *S. persica* extract on the *H. pylori* in the mouth (dental plaque). It was observed that at high concentrations of the plant extract the growth of bacteria was inhibited.

In this investigation, we evaluated the antimicrobial activity of *S. persica* extract on *H. pylori* strains isolated from duodenal ulcer biopsies of patients presenting with duodenal ulcer and vomiting at the Afzalipoor gastroenterology unit in Kerman, Iran.

Materials and Methods

Specimen collection

More than 22 *H. pylori* positive specimens were collected from patients of both sexes with abdominal discomfort, dyspepsia and poor appetite referred to the endoscopic section of the Gastroenterology Division of the Afzalipoor Hospital in Kerman, Iran, from August 2010 to June 2011. Some of them had bloody or black stools and vomiting. Three biopsies were taken from curvature of the antrum and duodenum (2-3 cm from pilor valve) of patients in the morning session between 8 to 12 am. All endoscopic examinations were performed with only local anesthesia.

One specimen was used for rapid urease test and two others were transferred into two tubes containing 500 μ L of sterile brain heart infusion (BHI) broth and brought to the Department of Microbiology Laboratory for further analysis. There, 100 μ L of the specimens were inoculated into a sterile Brucella agar (Merck-Germany) plate containing 10% sheep blood cells and antibiotics such as vancomycin, terimethoprine, and amphotericin-B added as supplements. The plates were incubated under microaerophilic conditions for three days at 37°C as described previously.²¹

Bacterial identification

The identification of *H. pylori* from specimens was carried out by biochemical tests, as well as morphology and gram staining. Colony characteristics on sheep blood agar,

urease, oxidase, and catalase testes were also studied.²²

Plant material

Dried stems of *S. persica* were purchased from a local medicinal herb market in Kerman, in the south east of Iran and identified by our pharmacognosist at the Herbal and Traditional Medicines Research Center, Kerman University of Medical Sciences.

Extraction method

Plant materials (stems) were cut by using a sharp knife and ground to a powder with a commercially available food blender. One hundred grams of the powdered stems (mesh 40) were extracted with 60% methanol (Merck-Germany) using a soxhlet extractor for 48 h until the solvent turned colorless. The solvent was then removed from the extractor using a rotary vacuum evaporator at 40°C for four hours until a concentrated extract was obtained. The extract was incubated in an oven at 40°C until it yielded 8 g dry powder which was then stored in sterile screw capped vials in the refrigerator at 4°C until use.¹³

Antimicrobial activity

The ditch plate method was used to study the antimicrobial activity of *S. persica* extract. Three to four colonies of the isolates were suspended in 3 mL of sterile distilled water, gently shaken and a lawn culture was spread on the sterile Brucella agar plate containing 10% sheep blood and antibiotic supplements. Then 0.1 mL of different aliquots of the plant extract (10, 100, 200, 750, 1000 μ g/mL) were pipetted into the ditches made at the center of petri dishes. The plates were then incubated at 37°C for three days as described previously and examined for inhibition zones of the growth of the bacteria around the ditches. The average size of these zones was recorded in millimeters.

Determination of minimum inhibitory concentration of extract by the agar dilution method

In this method, 6 flasks containing 25 mL of Muller-Hinton agar were prepared and autoclaved at 120°C (15 LB) for 15 min. We then added 10 mL sheep blood (Darvash Ltd., Iran) and different concentrations of *S. persica* plant extract (10 μ g/mL, 100 μ g/mL, 200 μ g/mL, 500 μ g/mL, 750 μ g/mL, 1000 μ g/mL) at 45°C and the preparation was allowed to solidify at room temperature. The bacterial suspensions were spot inoculated at a concentration of 1×10^7 CFU/mL onto each plate.

MIC was determined after three days of incubation at 37°C under microaerophilic conditions. MIC was defined as the lowest concen-

trations of the extract that inhibited visible bacterial growth on the plates. At the same time, a negative control (inability to grow) containing Muller-Hinton agar plate and a positive control (able to grow) containing Brucella agar supplemented with 10% sheep blood were prepared and inoculated with the *H. pylori* isolates and incubated under microaerophilic conditions in the incubator at 37°C.

Determination of minimal bactericidal concentration of the extract

Minimal bactericidal concentration (MBC) of the *S. persica* extract against *H. pylori* clinical isolates was measured by inoculating sterile swab from the MIC spot onto Brucella agar supplemented with 10% sheep red blood cells and antibiotics. After three days of incubation under microaerophilic conditions the growth (visible colonies) were checked.

Statistical analysis

All analyses were performed using SPSS, version 16.0 (SPSS Inc., Chicago, IL, USA). All P values were two-tailed; $P \leq 0.05$ was considered statistically significant. Means and standard deviations (SD) were calculated as required for numerical variables.

Results and Discussion

From August 2010 to June 2011, more than 22 *H. pylori* were collected from patients undergoing endoscopic follow up for the early detection of gastric and duodenal ulcer at the Gastroenterology Division of the Afzalipoor Hospital in Kerman, Iran. Both male and female patients were included; average age 63 ± 0.8 years. Figure 1 shows the colony characteristics of *H. pylori* isolates on Brucella agar medium supplemented with 10% sheep blood and vancomycin, terimethoprine, and amphotericin-B antibiotics.

The inhibitory concentrations of *S. persica* methanolic extract against *H. pylori* isolates by the agar ditch method are presented in Table 1. As shown, at concentrations of 10, 100, 200, 500 μ g/mL there was no zone of inhibition of the growth around the ditches while a clear zone of inhibition (12 mm) was observed at a concentration of 1000 μ g/mL of the extract for all the isolates. Furthermore, 10 *H. pylori* isolates (1, 2, 4, 6, 7, 8, 13, 15, 20 and 21) showed a zone of inhibition (8 mm) at a concentration of 750 μ g/mL concentration of the *S. persica* extract ($P \leq 0.05$). These results were further confirmed by MIC determination as shown in Table 2.

Our results clearly showed that *S. persica* extract exerted antimicrobial activity against *H. pylori* clinical isolates only at a high concen-

Figure 1. Colony characteristics of *H. pylori* on Brucella agar medium supplemented by 10% sheep blood and antibiotics.

Figure 2. Minimum bactericidal concentration of *S. persica* extract on *H. pylori* isolated from stomach ulcer.

Table 1. Antimicrobial activity of methanolic extract of *S. persica* against *H. pylori* isolates by agar ditch method.

Antimicrobia activity	Zone diameter (mm)	Extract concentration (µg/mL)	N. isolates show zone of inhibition (%)
-	0	≥500	0
+	7±0.8	750	45.4
+	12	1000	100

$P \leq 0.05$ was considered as significant of association. The above results are mean of 2 independent experiments. Means and standard deviations (SD) were calculated as required for numerical variables. At the same time a negative control consists of *H. pylori* positive selective medium without extract and a positive control with the extract was run alongside the tests.

Table 2. Minimum inhibitory concentration of *S. persica* extract against *H. pylori* strains isolated from the Gastroenterology Division of the Afzalipoor Hospital in Kerman, Iran.

N. of isolates (µg/mL)	Extract concentration (µg/mL)							MIC
12	10	100	200	500	750	1000		1000
	+	+	+	+	+	-		
10	+	+	+	+	-	-		750

+ growth of the isolates on that concentration; - absence of growth of the isolates on that concentration. MIC, minimum inhibitory concentration. The above results are average of 2 independent experiments.

tration (1000 µg/mL) and was ineffective at low concentrations.

The best antimicrobial effect for *S. persica* extract was observed at a concentration of 1000 µg/mL. The difference between this and the other concentrations was highly significant ($P \leq 0.05$). Furthermore, 10 of 22 *H. pylori* isolates were inhibited at a concentration of 750 µg/mL of extract. MBC results showed no bacterium was alive at a concentration of 1000 µg/mL and all cells were dead, while at a concentration of 750 µg/mL *S. persica* there were still a few *H. pylori* cells able to form colonies to the same MIC value on Brucella agar supple-

mented with sheep red blood cells and antibiotics (vancomycin, trimethoprim, and amphotericin-B, (Figure 2).

The potential antimicrobial activity of *S. persica* was studied by Al-Sabawi et al.⁷

Results revealed that 15% alcoholic extract of *S. persica* had significant antimicrobial effect. There was no significant difference in these results compared to sodium hypochlorite and chlorhexidine, while there was a significant difference compared to normal saline.

Similarly, the disc-diffusion method was used to determine the susceptibility of 3 *H. pylori* isolates to methanol extracts of 23

Iranian plants.²² All tests were performed in triplicate. Among them, the extracts of *Punica granatum* and *Juglans regia* had remarkable anti-*H. pylori* activity with a mean inhibition zone diameter of 39 and 16 mm at 100 µg/disc-1, respectively. In view of the results obtained with *P. granatum* (pomegranate), the peel extracts of nine cultivars of pomegranate were further assayed against the clinical isolates of *H. pylori*. The results revealed that all Iranian pomegranate cultivars, except for Alak-e-Shirin, showed significant *in vitro* activity against the clinical isolates of *H. pylori*. Similarly, *H. pylori* were isolated from 10 clinical and environmental samples from water and biopsies in Tehran, Iran.²⁰ At a 1/16 dilution of stock there was complete inhibition of *H. pylori* growth.

One study²³ reported the inhibitory effects of hot water extract of cacao mass (cocoa extract) on the adhesion of *H. pylori* to gastric epithelial cells. The inhibition rate of cocoa extract was significantly higher than that obtained with the extract of oolong tea, black tea, green tea and coffee. It was also shown that cocoa extract had bactericidal effect on *H. pylori* at a final concentration of 3.5-10%.

Antifungal activities of *S. persica* and *Juglans regia* on different *Candida* species were studied by Nouri et al.²⁴ Methanol, ethylacetate and diluted acetone extracts of *S. persica* and *J. regia* were screened for *in vitro* activity against *Candida* and it was found that *S. persica* exerted more antifungal activity than *J. regia*.

Treatment of peptic ulcer caused by *H. pylori* with current antibiotics is cumbersome, time consuming and some of them, such as tetracycline or clarithromycin²⁵ have considerable side effects. Therefore, a plant extract like *S. persica* can replace antibiotics in the treatment of *H. pylori* peptic ulcer.

Conclusions

Overall, our study revealed that *S. persica* at a high concentration can exert antimicrobial activity against *H. pylori* isolated from duodenal ulcer. The results of preliminary tests by the ditch method were further confirmed by determination of MIC of the plant extract. Furthermore, the MBC results showed that at high concentration of *S. persica* no *H. pylori* isolates were capable of forming colonies on Brucella agar containing sheep red blood cells.

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