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 dishes 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=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.

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

There has been little research into the antimicrobial activity of S. persica on different microorganisms. The antimicrobial activity of S. persica solution (Miswak) as a root canal cleaner was studied by Al-Sabawi et al. 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. In one study in Pakistan, the antimicrobial effect of aqueous extract of seven different types of chewing sticks was compared. It was found that S. persica extract can inhibit the growth of Entrococcus fecalis at a concentration of 50%.

Another study showed that Streptococcus mutans was more susceptible to Miswaks antimicrobial activity than Lactobacilli. The effect of mouth wash extracted from S. persica (Miswak) on dental plaque formation was investigated 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. Al-Otaibi et al. studied the effect of chewing sticks and tooth brushing on plaque removal and gingival health. Similarly, Geetha et al. 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).

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. 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.

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, claritomycine, metronidazole, a proton pump inhibitor, and bismuth subsilate. Drug resistant strains are to be found growing in different parts of the
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 pilar 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 µL of sterile brain hearth infusion (BHI) broth and brought to the Department of Microbiology Laboratory for further analysis. There, 100 µL of the specimens were inoculated into a sterile Brucella agar (Merck-Germany) plate containing 10% sheep blood and antibiotics supplements. Then 0.1 mL of different aliquots of the plant extract (1, 10, 100, 200, 750, 1000 µg/mL) were pipetted onto the plates 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 dishes. The average size of these zones was recorded in millimeters.

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 (1, 10, 100, 200, 750, 1000 µg/mL) were pipetted into the ditches made at the center of petri dishes. The plates were then incubated at 37°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.

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 µg/mL, 100 µg/mL, 200 µg/mL, 500 µg/mL, 750 µg/mL, 1000 µg/mL) at 45°C and the preparation was allowed to solidify at room temperature. The bacterial suspensions were then inoculated at a concentration of 1x10^8 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 concentration 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.

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±8.0 years. Figure 1 shows the colony characteristics of H. pylori isolates on Brucella agar medium supplemented with 10% sheep blood and vancomycin, terimethoprime, and amphotericin-B antibiotics.

The inhibitory concentrations of S. persica methanolic extract against H. pylori isolates by the agar method are presented in Table 1. As shown, at concentrations of 10, 100, 200, 500 µg/mL there was no zone of inhibition of the growth around the dishes while a clear zone of inhibition (12 mm) was observed at a concentration of 1000 µg/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 µg/mL concentration of the S. persica extract (P<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 concent-

[Microbiology Research 2012; 3:e9]
Table 1. Antimicrobial activity of methanolic extract of \textit{S. persica} against \textit{H. pylori} isolates by agar ditch method.

<table>
<thead>
<tr>
<th>Antimicrobial activity</th>
<th>Zone diameter (mm)</th>
<th>Extract concentration (µg/mL)</th>
<th>N. isolates show zone of inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>0</td>
<td>≥500</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>7±0.8</td>
<td>750</td>
<td>45.4</td>
</tr>
<tr>
<td>+</td>
<td>12</td>
<td>1000</td>
<td>100</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>N. of isolates (µg/mL)</th>
<th>Extract concentration (µg/mL)</th>
<th>MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>10 100 200 500 750 1000</td>
<td>1000</td>
</tr>
<tr>
<td>10</td>
<td>+ + + + + + - - - - - - - - -</td>
<td>750</td>
</tr>
</tbody>
</table>

+ 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 1 independent experiments.

The best antimicrobial effect for \textit{S. persica} extract was observed at a concentration of 1000 µg/mL. The difference between this and the other concentrations was highly significant (P≤0.05). Furthermore, 10 of 22 \textit{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 \textit{S. persica} there were still a few \textit{H. pylori} cells able to form colonies to the same MIC value on Brucella agar supplemented with sheep red blood cells and antibiotics (vancomycin, trimethoprim, and amphotericin-B, (Figure 2).

The potential antimicrobial activity of \textit{S. persica} was studied by Al-Sabawi et al.\textsuperscript{7} Results revealed that 15% alcoholic extract of \textit{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 \textit{H. pylori} isolates to methanol extracts of 23 Iranian plants.\textsuperscript{22} All tests were performed in triplicate. Among them, the extracts of Punica granatum and Juglans regia had remarkable anti-\textit{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 \textit{P. granatum} (pomegranate), the peel extracts of nine cultivars of pomegranate were further assayed against the clinical isolates of \textit{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 \textit{H. pylori}. Similarly, \textit{H. pylori} were isolated from 10 clinical and environmental samples from water and biopsies in Tehran, Iran.\textsuperscript{20} At a 1/16 dilution of stock there was complete inhibition of \textit{H. pylori} growth.

One study\textsuperscript{23} reported the inhibitory effects of hot water extract of cacao mass (cocoa extract) on the adhesion of \textit{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 \textit{H. pylori} at a final concentration of 3.5-10%.

Antifungal activities of \textit{S. persica} and Juglans riga on different Candida species were studied by Noumi et al.\textsuperscript{24} Methanol, ethylacetate and diluted acetone extracts of \textit{S. persica} and \textit{J. riga} were screened for in vitro activity against Candida and it was found that \textit{S. persica} exerted more antifungal activity than \textit{J. riga}.

Treatment of peptic ulcer caused by \textit{H. pylori} with current antibiotics is cumbersome, time consuming and some of them, such as tetracycline or clarithromycin\textsuperscript{25} have considerable side effects. Therefore, a plant extract like \textit{S. persica} can replace antibiotics in the treatment of \textit{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 gar containing sheep red blood cells.

References