In vitro Inhibition of \textit{Helicobacter pylori} by Some Spices and Medicinal Plants Used in Iran

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Abstract: \textit{Helicobacter pylori} is the most common cause of gastric infections worldwide. The antibacterial activity of methanol extracts of \textit{Satureja hortensis} L. (Lamiaceae), \textit{Artemisia dracunculus} L. (Asteraceae), \textit{Carthamus tinctorius} L. (Asteraceae), \textit{Apium petroselinum} L. (Apiaceae), \textit{Citrus sinensis} L. (Rutaceae) and \textit{Punica granatum} L. (Punicaceae) were evaluated against \textit{H. pylori}. Inhibition tests were carried out using the disc diffusion method against 27 clinical isolates of \textit{H. pylori} for initial screening and then agar dilution method to determine minimum inhibitory concentration (MIC). Among the crude extracts, \textit{P. granatum} showed the highest inhibition (27.96±0.97mm) and the MICs of its butanol and aqueous fractions were 156 and 195.12 µg/mL, respectively. While its chloroform fraction had no activity against tested \textit{H. pylori} isolates. All extracts except safflower preserved their anti-\textit{Helicobacter pylori} activity at 121°C. All the extracts were capable of inhibiting the \textit{in vitro} growth of \textit{H. pylori} and the butanol fraction of \textit{P. granatum}, showed excellent anti-\textit{H. pylori} activity.

Key words: \textit{H. pylori} • Antibacterial activity • Extract • Medicinal plants

INTRODUCTION

After of \textit{Helicobacter pylori} in 1982 by Warren and Marshall, it was shown that most duodenal and peptic ulcers are caused by this organism. \textit{H. pylori} is an important etiological agent of gastritis, peptic ulcer and gastric cancer [1, 2] Current therapy is based on a combination of antibiotics, bismuth sub citrate and proton pump inhibitors [3]. Although the triple therapy is considered highly efficient, only 80% of patients show absence of pathogen and the infection sometimes returns after treatment completion [4, 5]. The main reason for eradication failure can be \textit{H. pylori}’s resistance to one of the antibiotics used for treatment [6]. Also chemical drugs have side effects such as diarrhea and colitis. Therefore it is necessary to introduce alternative remedial regimens. One of these resources is medicinal plants that some of their therapeutic properties have been recognized in folk medicine. Most people have positive attitude toward natural products due to their natural origin and lesser toxicity [7]. Study of anti-\textit{Helicobacter pylori} effects of these plants, especially those that are traditionally used for gastric problems are important.

The present study was carried out to evaluate \textit{in vitro} anti-\textit{Helicobacter pylori} activity of some spices of native medicinal plants in Iran [\textit{Satureja hortensis} L. (Lamiaceae), \textit{Artemisia dracunculus} L. (Asteraceae), \textit{Carthamus tinctorius} L. (Asteraceae), \textit{Apium petroselinum} L. (Apiaceae), \textit{Citrus sinensis} L. (Rutaceae) and \textit{Punica granatum} L. (Punicaceae)] that have been traditionally used in folk medicine.

MATERIALS AND METHODS

Plant Materials: The names of plants, collection sites and parts used in this study are illustrated in Table 1. All plants were identified by Herbarium of Department of Pharmacognosy, Mashhad Faculty of Pharmacy (Mashhad University, Iran). A voucher specimen was maintained for reference in the Herbarium.

Preparation of Extracts: Plants were dried at room temperature in the shade and ground separately before extraction. Each material was extracted using maceration with methanol. The powder of materials was macerated in methanol for 3 days and the mixture was subsequently filtered and concentrated under reduced pressure at 40°C. Dried extracts were stored at 4°C.
Dried methanol extract of pomegranate rind was further fractionated by successive solvent extraction with n-butanol saturated with distilled water (three times) and then with chloroform (three times). The solvents were omitted under reduced pressure at 45°C. Butanol (BuOH), chloroform (CHCl₃) and aqueous (R-H₂O) fractions were used for activity testing.

**Microbiological Studies:** In this study 27 clinical isolates of *H. pylori* were obtained from the Laboratory of 17-Shahrivar Hospital (Mashhad-Iran). These isolates were identified by colony morphology, Gram stain, microscopic morphology, colalase’, oxidase’, rapid urease’, nalidixic acid resistance and H₂S [8]. Growth inhibition was performed on Mueller-Hinton agar (Pronadisa-Madrid) containing 7% egg yolk emulsion and 4 mg triphenyl tetrazolium chloride (Merck) by the filter paper disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [9, 10]. Standard discs containing 2 mg of herbal extract were placed on egg yolk emulsion agar plate, previously inoculated with 0.1 mL bacterial suspension in sterile normal saline. The turbidity of the bacterial suspension was equivalent to Mc-Farland’s tube No. 4 (10⁶ CFU/mL) [11]. Inhibition growth assay was performed using standard commercial discs of amoxicillin (25 µg/disk) and, metronidazole (5 µg/disk) as positive control and discs containing evaporated methanol and distilled water as negative control. All plates were incubated for 2-3 days at 37°C under microaerophilic condition (10% CO₂), 90-100% humidity. Minimum inhibitory concentration (MIC) values were determined by the agar dilution method for four isolates of *H. pylori* with the highest sensitivity. MICs were reported as the lowest concentration of the plant extract that inhibited visible bacterial growth [12]. The minimum bactericidal concentrations (MBC) were established by the lack of growth upon re-inoculation from extract- treated plates to Mueller- Hinton agar plates [12]. All experiments were carried out three times. After primary screening of anti-*Helicobacter pylori* activity at concentration of 2 mg/disc (Table 2). Among the crude extracts, pomegranate rind showed the highest inhibition (27.96±0.97 mm) and orange peel (13.56±0.51 mm) and Sweet fennel (13.70±0.45 mm) showed the least activity against *H. pylori* clinical isolates. The effect of pomegranate rind and sawflower seed showed bactericidal effect against *H. pylori* isolates.

**Statistical Analysis:** Data were the means of inhibition zones of antibacterial activity of plant extracts experiments which have been repeated three times. The results were analyzed initially using analysis of variance (ANOVA) and then Duncan test. A value of p < 0.05 was considered statistically significant.

**RESULTS**

All herbal extracts showed anti-*Helicobacter pylori* activity at concentration of 2 mg/disc (Table 2). Among the crude extracts, pomegranate rind showed the highest inhibition (27.96±0.97 mm) and orange peel (13.56±0.51 mm) and Sweet fennel (13.70±0.45 mm) showed the least activity against *H. pylori* clinical isolates. The effect of pomegranate rind and sawflower seed showed bactericidal effect against *H. pylori* isolates.

As the extract of pomegranate rind was the most efficient, it was decided to test its fractions against *H. pylori* isolates. The mean of inhibition zone of butanol fraction (25.3±0.86 mm) was more than aqueous

**Table 1: Characteristics of the plants used in this study**

<table>
<thead>
<tr>
<th>Plant</th>
<th>Scientific name</th>
<th>Family</th>
<th>Collection sites</th>
<th>Part</th>
</tr>
</thead>
<tbody>
<tr>
<td>Safflower</td>
<td><em>Carthamus tinctorius</em> L.</td>
<td>Asteraceae</td>
<td>Khorasan</td>
<td>Flower</td>
</tr>
<tr>
<td>Sweet fennel</td>
<td><em>Satureja hortensis</em> L.</td>
<td>Lamiaceae</td>
<td>Mashhad-Khorasan</td>
<td>Leaves</td>
</tr>
<tr>
<td>Tarragon</td>
<td><em>Artemisia dracunculus</em> L.</td>
<td>Asteraceae</td>
<td>Mashhad-Khorasan</td>
<td>Leaves</td>
</tr>
<tr>
<td>Orange</td>
<td><em>Citrus sinensis</em></td>
<td>Rutaceae</td>
<td>North of Iran</td>
<td>Peel of fruit</td>
</tr>
<tr>
<td>Pomegranate</td>
<td><em>Punica granatum</em></td>
<td>Puniceae</td>
<td>Saveh-Markazi</td>
<td>Peel of fruit</td>
</tr>
<tr>
<td>Parsley</td>
<td><em>Apium petroselinum</em> L.</td>
<td>Apicioae</td>
<td>Neishabur-Khorasan</td>
<td>Seeds</td>
</tr>
</tbody>
</table>

**Determination of Temperature and pH Stability of Extracts and Constituents:** Methanol extracts dissolved in sterile distilled water and either were incubated at 80°C for 30 min or autoclaved at 121°C for 20 min. In order to determine pH stability, methanol extracts were mixed with 0.1M sterile phosphate buffer solution of various pH values (5, 6, 7 and 8) in separate tubes and were incubated at room temperature for 3 [13]. Then, discs containing extracts were prepared after exposure to the different thermal and pH conditions and tested as above for anti-*Helicobacter pylori* assay.
mg/mL by aqueous extract of thyme whereas in solid medium complete inhibition was attained at 4-5 mg/mL [17]. The MIC of black myrabalan (Terminalia chebula Retz) aqueous extract against *H. pylori* has been reported to be 125 mg/L [12]. The methanol and chloroform extracts of *Byrsonima crassa* inhibited, *in vitro*, the growth of *H. pylori* with MIC value of 1024 µg/mL [20]. The MICs of crude urushiol extract from the sap of the Korean lacquer tree (*Rhus vernicifera* Stokes) against 3 strains of *H. pylori* ranged from 0.064 mg/mL to 0.256 mg/mL [21]. The MIC$_{50}$ and MIC$_{90}$ of oil extract of *Chamomilla recutita* flowers against *H. pylori* were 125 and 62.5 mg/mL, respectively [22].

Aqueous and butanol fractions of pomegranate peel showed good activity on *H. pylori* clinical isolates with MICs of 156 and 195.12 µg/mL, respectively, but chloroform fraction had no effect on *H. pylori*. Methanol extract and hexane fractions of rhizome and leaves of *Aristolochia paucinervis* exhibited an inhibitory activity at a concentration of ≥ 128 µg/mL against *H. pylori*. Hexane fraction of rhizome demonstrated a higher inhibitory activity (MIC: 4 µg/mL) than hexane fraction of leaves (MIC: 16 µg/mL) [23]. The dichloromethanic fraction of *Calophyllum brasiliense*, a medicinal tree that grows particularly in the hilly and forested regions of Brazil, appeared to have the most active and potent fraction *in vitro* against *H. pylori* growth with an MIC of 31 µg/mL [24].

Many of chemical materials that are used today for preservation, coloring or flavoring of food products are hazardous for human health.

Pomegranate peels are used as a medicinal plant as well as for making dye. Anti-bacterial, anti-parasitic, antiviral effects [25-27], anti-oxidant and anti-mutagenic activities [27, 28] have been reported for pomegranate peel, up to now. The use of this extract is recommended for making food products instead of chemical coloring material due to its useful effects. The presence of phenolic compounds (proanthocyanin) and tannins (Ellagic tannins) in pomegranate peel have been reported and anti-microbial effects of these compounds have been recognized [29, 30]. It is possible that some of anti-*Helicobacter pylori* activity of pomegranate peel is related to tannin and phenolic compounds.

Although the activity of orange peel on *H. pylori* was less than other extracts, it can still be used for flavoring cakes and sweets instead of chemical agents such as vanilla that in high doses is poisonous [31]. A traditional Iranian cake is made with a grated unpeeled orange. There is also a rice dish and a jam prepared with orange peels [32].

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**Table 2: Inhibition zone (mean±SE) of herbal methanol extracts at concentration 2 mg/disc against 27 *H. pylori* clinical isolates**

<table>
<thead>
<tr>
<th>Herbal extract</th>
<th>MIC (µg/mL)</th>
<th>MBC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carthamus tinctorius</td>
<td>18.77±0.56</td>
<td>16.44±0.57</td>
</tr>
<tr>
<td>Satureja hortensis</td>
<td>13.70±0.45</td>
<td>16.44±0.57</td>
</tr>
<tr>
<td>Artemisia dracunculus</td>
<td>15.07±0.48</td>
<td>24.41±3.78</td>
</tr>
<tr>
<td>Citrus sinensis</td>
<td>13.56±0.51</td>
<td>27.96±0.97</td>
</tr>
<tr>
<td>Punica granatum</td>
<td>27.96±0.97</td>
<td>37.14±2.22</td>
</tr>
<tr>
<td>Apium petroselimum</td>
<td>37.14±2.22</td>
<td>41.15±2.31</td>
</tr>
<tr>
<td><em>significant at p &lt; 0.05</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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**Table 3: MICs and MBCs of the herbal extracts against *H. pylori* clinical isolates**

<table>
<thead>
<tr>
<th>Herbal extract</th>
<th>MIC (µg/mL)</th>
<th>MBC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carthamus tinctorius</td>
<td>691.25</td>
<td>691.25</td>
</tr>
<tr>
<td>Artemisia dracunculus</td>
<td>691.25</td>
<td>827.5</td>
</tr>
<tr>
<td>Citrus sinensis</td>
<td>729</td>
<td>1041.5</td>
</tr>
<tr>
<td>Punica granatum</td>
<td>321.25</td>
<td>338.5</td>
</tr>
<tr>
<td>Apium petroselimum</td>
<td>729</td>
<td>729</td>
</tr>
</tbody>
</table>

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**DISCUSSION**

In the present study, among the plant crude extracts tested, that of pomegranate peel which showed the highest activity on *H. pylori* growth. The MIC of pomegranate peel was 312.5 µg/mL and it had bacteriostatic effect. Several authors have previously reported that some extracts of plants exhibit an inhibitory activity against *H. pylori* [12, 16-19]. It was reported that MICs for the aqueous garlic extract against nineteen strains of *H. pylori* range from 2 to 5 mg/mL [19]. *H. pylori* growth in liquid medium was completely inhibited at 3-5
Considered to be stable at 121°C, all extracts except safflower are therefore suitable for process of food preparation without any effect on anti-Helicobacter pylori of these herbal extracts. Whereas black and green tea lose their effectiveness at 85°C for 5 min as was reported by Diker and Hascelik [33].

The results of this study showed these herbs might have a considerable potency at preventing or aiding the treatment of H. pylori infections.

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