Study of antibacterial activity of selected Iranian plant extracts on Helicobacter pylori

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Abstract: Helicobacter pylori is a major contributor to chronic gastritis, peptic, duodenal ulcers, and is associated with distal adenocarcinoma. Increasing resistance of H. pylori to common antibiotics is becoming an issue and finding new treatments are crucial. Aqueous and ethanolic extracts of Glycyrrhiza glabra, Filipendula ulmaria, Argentina anserine, Crataegus douglasii, Rubus fruticosus were tested, by agar diffusion and agar dilution method, against H. pylori clinical isolates. Among the aqueous extracts studied, G. glabra extract had the highest antibacterial effect on H. pylori (mean diameter of inhibition zone 14.7 mm) followed by Filipendula ulmaria, Rubus fruticosus, Argentina anserine, and Crataegus douglasii. Among the ethanolic extracts, Glycyrrhiza glabra extract was the most effective one (mean diameter of inhibition zone 9.9 mm), followed by Filipendula ulmaria, Argentina anserine, Rubus fruticosus, and Crataegus douglasii. Given the high levels of antibacterial activities of aqueous and ethanolic extracts of Glycyrrhiza glabra and Filipendula ulmaria, the next step is to identify the antimicrobial constituents of these plants.

Keywords: Helicobacter pylori, Glycyrrhiza glabra, Filipendula ulmaria

1. Introduction

Italian researcher, Bizzozero, in 1983, found helical microbes in stomach and in the following years, 1987; Goodwin and Marshall discovered a bacterium which was named Campylobacter. Lastly in 1998, a new genus called Helicobacter was proposed by Goodwin and his colleagues. The discovery of this microorganism opened a new era in the pathology of digestive system [1-3]. Helicobacter pylori, found in biopsy samples of digestive system, has drawn more attention because there are evidences of this bacterium’s pathogenic activities. H. pylori is found in stomach mucus, sometimes in duodenal mucus, can form colonies in Barrett’s esophagus and is known as a probable cause of esophagitis [3,4]. More importantly, there are evidences of gastric adenocarcinoma development caused by H. pylori infections[5,6].

H. pylori is an active, gram-negative, microaerophilic, spirally shaped bacterium with lophotrichous flagellation. It grows in the form of curved rods in agar medium and cocccoid form in old desiccated cultures. H. pylori is oxidas, urease, catalase, and alkaline phosphatase positive.

H. pylori eradication therapy is carried out through therapeutic treatments as single or multidrug regimens[7,8]. The antibiotics amoxicillin, clarithromycin, and metronidazole inhibit gastric acid secretion. In addition, H2 blockers and bismuth salts are used in H. pylori treatment. Unsuccessful treatments in full removal of infections, recurrent infections, and increasing resistance of H. pylori to therapeutic treatments (5 - 20% of treatments have been reported to fail due to H. pylori resistance to treatments) all have led to continuous research on finding new ways of treatments[9,10].

Many studies now focus on anti H. pylori effects of medicinal plants, which are of limited side effects on tissues. For centuries, medicinal plants have been used in traditional medicine to treat a wide range of diseases including digestive disorders such as ulcers. Aqueous extract of thyme and ethanolic extract of cinnamome have anti H. pylori effects[11-13].

It has been suggested that thyme is more effective than cinnamon since it inhibits the urease activity of H. pylori[14]. In addition, wild thyme is among the herbs which have been studied for anti H. pylori effects, particularly because of its easily produced aqueous extract, and its effects have been reported to be potentially more significant than those of cinnamon[15,16].

Antimicrobial activities of cinnamon and turmeric, which are used globally as food additive, are particularly known in the eastern hemisphere. Antibacterial effects of turmeric oil on several bacteria, including Escherichia coli, have been studied widely [17,18]. Ginger’s inhibiting effects on Micrococcus luteus have also been examined [19,20]. Since some food additives have antibacterial effects on H. pylori, they can be used in H. pylori infection treatment, and in control of its transmission[21]. Therefore, studies should be conducted to examine antibacterial activities of herbal extracts and identifying their potent constituents.
II. Materials and methods

2.1 Strains and growth conditions

Gastric biopsy samples taken from patients, provided by the endoscopy department of Shafa medical care center, diagnosed with peptic ulcer, duodenal ulcer, gastritis and stomach cancer were used in this study. The samples were transported to the laboratory in transport kits and, were finely chopped in tissue grinder; 1 ml of sterile saline solution was added and, then were vortexed at high speed. Microscopic examination of smears was conducted, as a standard bacteriology test, to preselect the positive specimens. Phase-contrast microscopy can be utilized for examination of biopsies allowing us for a quick diagnosis of typical spirals morphology of Helicobacter.

In the next step, serial dilutions of the positive homogenates were prepared (up to $10^{-5}$ of original concentration). 100μl of the last two dilutions were cultured, direct plating, on serum added brain heart agar medium for primary isolation of bacteria. For selective isolation campylobacter blood agar containing 5% defibrinated sheep blood, vancomycin (10 mg/l), polymyxin B (0.25 mg/l), trimethoprim (5 mg/l), and amphotericin B (2 mg/l) were used. The plates were incubated in 100% humidity at 37°C for 5-7 days in microaerophilic Jar (CO$_2$, 10%, O$_2$, 5%). The agar plates were checked for growth from day 3 through day 7. An isolate was identified as $H$. pylori on the basis of positive catalase, oxidase, and urease reactions, typical colony morphology (small, round colonies), and the presence of characteristic curved gram-negative bacilli on Gram-stained smears. Suspect colonies were skipped routinely [7,22-24].

2.2 Plant extract preparation

$G$. glabra root, $F$. ulmaria flower, $A$. anserine root, $C$. douglasii berry, and $R$. fruticosus leaf, plant parts, were washed under running tap-water, dried in shade, powdered by electric grinder and then extracts were prepared aqueous and ethanolic. Ten grams of each plant powder was put in water and ten grams in ethanol (maceration with an herb-to-solvent ratio of 1:10) then the solutions were incubated for 72 hours at 32 °C to improve and speed up the maceration process. Supernatant was filtered (Whatman no. 1 filter paper disc), and concentrated using vacuum distillation. The concentrated extracts were then placed on watch glass and heated, 40 °C, to speed the evaporation of solvents. Finally, dried extracts of the plants were obtained [25-28].

2.3 Evaluation of $H$. pylori sensitivity to aqueous and ethanolic extracts of $G$. glabra, $F$. ulmaria, $A$. anserine, $C$. douglasii, $R$. fruticosus using agar dilution

Ten $H$. pylori clinical isolates (ulcer samples; 4, gastritis samples; 4, and stomach cancer samples; 2) were used to investigate the antibacterial effects of these plants. Aqueous extracts of $G$. glabra, $F$. ulmaria, $A$. anserine, $C$. douglasii, $R$. fruticosus were added to Brucella Blood Agar (BRU) with 5% blood (at concentration series of 50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 μg/ml). Suspensions of bacteria were prepared with $3 \times 10^7$ bacteria per ml.

5μl of each suspension was dripped onto the BRU containing the extracts, and the plates were then placed into the anaerobic jar. After addition of distilled water to the gas-pack, we then tightened the jar lid and incubated the jar at 37 °C in an incubator with 5% CO$_2$. After five days, minimum inhibitory concentrations (MIC) of the extracts were measured [29].

2.4 Evaluation of $H$. pylori sensitivity to ethanolic extracts of $G$. glabra, $F$. ulmaria, $A$. anserine, $C$. douglasii, $R$. fruticosus using agar diffusion

Isolated bacteria grown on plates, containing campylobacter blood agar with 5% defibrinated sheep blood, were used to prepare the bacterial suspension, $3 \times 10^7$ bacteria per ml. Then, 50 μl of the suspension was added to Müller-Hinton agar with 5% blood. Sterile blank discs were put on the plates and 10 μl of ethanolic extracts of $G$. glabra, $F$. ulmaria, $A$. anserine, $C$. douglasii, $R$. fruticosus were dropped to the blank discs at 400 μg/ml concentration. After two days of incubation, the plates were examined for inhibition zone [30-32].

III. Results

In a parallel study, 108 gastric mucosal biopsy specimens were obtained from 36 consecutive dyspeptic patients (25 males and 11 females) undergoing endoscopy. Utilizing phase-contrast microscopic examination of smears and the selective cultivation, $H$. pylori was detected in 10 patients (6 males and 4 females). All samples were oxidase, catalase, and urease-positive. In addition, they were sensitive to cephalothin and resistant to nalidixic acid.

Minimum inhibitory concentrations were of 150-300 μg/ml for $G$. glabra, 250-350 μg/ml for $F$. ulmaria, 300-400 μg/ml for $A$. anserine, 350-450 μg/ml for $C$. douglasii, 400-450 μg/ml for $R$. fruticosus for different clinical isolates of $H$. pylori (Table III).

Aqueous extracts of $G$. glabra (inhibition zone 12-17 mm) was of stronger antibacterial effects compared to its ethanolic extracts. Among aqueous and ethanolic extracts investigated, the greatest effect was recorded for
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*G. glabra*, notably significant, followed by aqueous extracts of *F. ulmaria*, *A. anserine*, *R. fruticosus*, and *C. douglasii* (Tables I and II).

The results suggest greater inhibition effects of aqueous extracts for *F. ulmaria*, *A. anserine*, *C. douglasii*, and *R. fruticosus* compared to their ethanolic extracts. The difference however was not statistically significant.

### Table I: Antibacterial effects of aqueous extracts on 10 clinical isolates of *H. pylori*

<table>
<thead>
<tr>
<th>Medicinal plants</th>
<th>Aqueous Extract : Inhibition zone (mm) in fresh clinical isolates of <em>H. pylori</em></th>
<th>Average result</th>
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### Table II: Antibacterial effects of ethanolic extracts on 10 clinical isolates of *H. pylori*

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<th>Medicinal Plants</th>
<th>Ethanolic Extract : Inhibition zone (mm) in fresh clinical isolates of <em>H. pylori</em></th>
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### Table III: MIC values of Aqueous and Ethanolic extracts on selected isolates of *H. pylori* (µg/ml)

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**IV. Discussion**

*H. pylori* is a major contributor to chronic gastritis, and peptic and duoental ulcers, and is also strongly associated with distal adenocarcinoma. Scientists worldwide are extensively searching for new sources of bioactive compounds, especially antimicrobial agents of natural origins considering the prevalence of antibiotic resistance among the pathogens. Antimicrobial agents could be of plant origin, medicinal plant derived in the form of essential oil and ethanolic or aqueous extracts. Also antimicrobial antibiotics could be of bacterium source, marine or terrestrial e.g. antifungal and antibacterial metabolites produced by *Streptomyces* [33].

Given the prevalence of infections in different communities and its increasing resistance to antibiotics, it is extremely important to find alternative treatments and medications for *H. pylori* infections.

In a series of studies, Hill et al. examined anti *H. pylori* effects of garlic powder and oil [34]. Sato et al. identified the gallic acid in *Terminalia chebula* and recorded its ethanolic extract’s antibacterial effects on methicillin-resistant *staphylococci* [35].

In a previous study, we found that *Allium ascalonicum* (MIC$_{50}$ 128 µg/ml) has antimicrobial effect on vancomycin resistant *Staphylococcus epidermidis* another significant pathogenic bacterium, one of major causes of nosocomial infection[36].
Another study in China examined the inhibitory effects of *allium* in patients diagnosed with stomach cancer. The results suggested that; increased consumption dose of *allium* vegetables (e.g. garlic, onion, shallot, etc) would reduce the risk of stomach cancer by 40%[37]. The results had shown that vegetables of *allium* family can inhibit the tumor growth, *H. pylori* infections, and vancomycin resistant *S. epidermidis*.

A study by Li et al. on 30 species of herbs commonly used in Chinese traditional medicine to treat gastric ulcer, anti *H. pylori* activities of the following families were examined against the standard *H. pylori* strain ATCC43504 as well as against *H. pylori* clinical isolates: Apiaceae, Aristolochiaceae, Asteraceae, Elaeagnaceae, Fabaceae, Lauraceae, Lamiaceae, Magnoliaceae, Meliaceae, Menispermaceae, Myrtaceae, Orchidaceae, Papaveraceae, Piperaceae, Polyporaceae, Primulaceae, Rutaceae, and Zingiberaceae. The findings indicated that a significant number of species examined in the study (both aqueous and ethanolic extracts) have inhibitory effects on *H. pylori*[38].

A study, in Japan, by Funatogawa et al. examined inhibitory effects of hydrolysable tannins extracted from plants on treating infections caused by *H. pylori*. The results showed that these compounds (particularly monomeric tannin) have significant anti *H. pylori* activities due to their impact on lipid bilayer membranes[39]. Ehasnifar examined anti *H. pylori* effects of several plants, including pennroyal and flowering oregano, and found that pennroyal had no significant effect of growth inhibition while oregano significantly inhibited *H. pylori* growth[40].

Our findings indicated the highest level of anti *H. pylori* activity in aqueous extract of *G. glabra* followed by extracts of *F. ulmaria*, *R. fruticosus*, *A. anserine* and *C. douglasii*.

*G. glabra* (Leguminosae family) is a plant that has long been used in treatment of gastritis. Analysis of *G. glabra* has revealed that its constituents are of flavonoid, isoflavonoid, chalcone and glycosides groups and those may contribute to its anti *H. pylori* effects. Given the results of this study, it is of great importance to examine the clinical applications of the extract and to develop new food products that can contain *G. glabra* as a food additive.

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**References**

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