In vitro assessment of *Tribulus terrestris* aqueous extract and Benzoaxacin fraction against *Helicobacter pylori* isolates from biopsy samples of Iranian patients

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**Abstract**

**Background & Objectives:** *Helicobacter pylori* (Hp) is related to gastritis, gastric ulcer, duodenal ulcer, and mucosal carcinoma. Emergence of multidrug resistant Hp strains encouraged researchers to find new effective drugs, especially medicinal herbs and plants which usually show fewer side effects. The aim of this study was an in vitro assessment of anti Hp activity of total extract of *Tribulus terrestris* (*T. terrestris* Benzoaxacin), a local Iranian medicinal plant and its fraction Benzoaxacin.

**Method and Materials:** Total aqueous extract of the plant was prepared and liquid extraction with petroleum ether was used to separate its components. LC/MS system proved the existence of Benzoaxine derivative in the water fraction and the third's fraction. Anti (Hp) effects of total extract and its third fraction were examined by cup plate method and using standard MacFarland. 50 biopsy samples of antrum were detected from patients who were endoscopic candidates in Milad and Fayazbaksh hospitals of Tehran during 2011. All samples were isolated, diagnosed based on standard methods and biochemical tests and confirmed by PCR method for ureC gene, too. Different dilutions (250, 500, 750 and 1000 mg/ml) of total extract were prepared. Clarythromycin (Cl\(^\text{r}\) ) E-test strips and an identified Hp OC1096 was used, simultaneously.

**Results:** Of 50 biopsy samples, 12 Hp strains were isolated. Rapid urease test were positive in all except one biopsy sample. Existence of ureC gene in all isolates was confirmed except for one strain by PCR. By cup plate method, resistance to concentrations of 1000 and 750mg/ml wase detected in 50% of Hp isolates and 66.6% of them were resistant to concentrations 250 and 500 mg/ml. Also, 83.3% of Hp strains were resistant to Benzoaxacin fraction. Clarythromycin sensitivity was detected in 83% of Hp isolates, simultaneously.

**Conclusion:** This study was done as a pilot study for in vitro evaluation of antibacterial effect of total extract of *T. terrestris* by cup plate method. Existence of high resistant rate (≥50%) to different concentrations *T. terrestris* aqueous extract renders doing plate test on more Hp strains in future studies highly recommended. In contrast of the similarity of Benzoaxin structure to Ofloxacin, existence of 83.3% resistance among tested isolates showed no anti Hp effectiveness of this fraction.

**Keywords:** *Helicobacter pylori*, *Tribulus terrestris*, Benzoaxacin, antibacterial resistance

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Background & Objectives

*Helicobacter pylori* (Hp) is a gram negative bacteria. Infectivity of peoples in different ages around the world is high (50%) and is (≥80%) among Iranian people. Usually Hp eradication is possible by using triple therapy including two antibiotics (clarithromycin and tetracycline) and bismuth sulfite, and among the antibiotics the role of clarithromycin is critical. Based on different reports, the key role of clarithromycin is related to its effect in treatment failure of resistance strains. It means if the Hp strain is resistant to clarithromycin the chance of Hp eradication is very low and vice versa.

In the other hand, Iran is a good place of growing medicinal herbs or plants and there are a lot of documents about their usage as an alternative treatment. Among them *Tribulus terrestris*, which is a local plant of Kerman province of Iran has many confirmed properties as steroidal glycosides, anti-kidney stones, anti blood pressure, anti diabetic, anti inflammation, increaser of fertility and as a pain killer. Based on some studies, some of the derivates of Benzoxacin which is one of the constituent of Tribulus spp showed anti fungi and anti microbial property specially with anti-chlamydia effect.

Based on one of our study, the total extract of *T. terrestris* was effective against *Bacillus subtilis, Pseudomonas aeruginosa* and *E.coli*. Also, to identify the existence of Benzoxacin in that study, petroleum ether was added to total extract and consequently, three aquatic, etherdipetrol and intermediate phases were separated. All 3 phases were evaluated by LC/MS method for detection of the Benzoxacin as a derivates of Terrosoxazine. In addition, the antibacterial effect of Benzoxacin was evaluated against those bacteria, however no antibacterial effect was detected. Also, Funatogawas et al identified that the hydrolysable monomers of tannin from herbal extracts had anti Hp effect.

So, in this study the anti *Helicobacter pylori* effect of total extract of *T. terrestris* was evaluated by cup plate method. Also, based on the structural similarity between Benzoxacin fraction and Levofloxacin which is an effective antibiotic in Hp eradication, the other aim of this study was evaluation of anti Hp effect of this fraction.

Methods & Materials

**Biopsy collection and Bacterial strains:** This study is a descriptive experimental study done as a pilot study for the first time. Therefore, there were no any basic data for sample size determination. So, as of a pilot study, an expert gastrologist detected 50 biopsy samples from antrum of patients who were candidate for endoscopy and referred to Milad and Fayazbakha hospitals. From each patient two antrum biopsy samples were collected, one for rapid urease test and the other for bacterial culture.

All biopsy samples were transferred in to a special transfer buffer and in a cold container then sent to microbiology research center of Shahid Beheshti University of Medical Sciences (SBMU). At the time of reception at lab, all biopsy samples were homogenized by a sterile homogenizer and cultured on a blood agar medium supplemented by 3 antibiotics (Amphoterin B, sulamethoxazole trimethoprim and vancomycin) and incubated at 37°C and microaerophilic conditions (using Gaspak type C, Merck) for 48h to 72h. Consequently, gram staining, oxidase and catalase tests were used to confirm the *Helicobacter spp* identification. Consequently, DNA extraction was done for all Helicobacter spp strains by boiling method and using Tris-HCl (pH 7.5) and NaOH (50mM). Further PCR for ureC gene was performed to confirm isolates as *Helicobacter pylori*. The used primers and PCR program are mentioned at tables 1 and 2. The identified strains were cultured at BHI and glycerol medium and kept at -70°C immediately for further investigation.

**Plant collection:** The aerial parts of *T. terrestris* were collected from Kerman a province of Iran during 2011. The plant identification was done by pharmaceutical department of Pharmacology School, Shahid Beheshti University of Medical Sciences.

**Total extraction:** For total extraction, 100 g of fruits of *T. terrestris* were weighted, washed, dried and grounded. A liter of water was added, boiled for 5 min and filtered after cooling. Then concentrated by rotary evaporator to volume 50ml and kept at 4°C until antibacterial assay.
**Benzoaxacin fractionalize:** 10 g of total extract was solved in 250 ml of water and purred in a separation decanter. Then ether do petrol organic solvent was added and left to stay for hours. Consequently, three aquatic, ether do petrol and intermediate phases were separated. All 3 phases were added to LC/MS for Benzoaxacin detection.

**Cup plate method:** First of all, a serial dilution from 1000 mg to 250 mg was prepared from total extract by DMSO10% for antibacterial assay. Then cups were made by a sterile pipette pasture in Muller Hinton Agar. The bacterial suspension with turbidity equal to 3 McFarland was prepared and cultured on each punched agar by a sterile swab. The plates were left at Room temperature for a while and after that the wells were purred with the different concentration of the total extract. The clarythromycin was used as a positive control simultaneously. The plates were incubated at 37°C for 48h at microaerophilic conditions.

The diameter of the zone of inhibition was determined based on mm. A strain of Hp OC1096 which was collected from Gasterology research center, Shahid Beheshti University of Medical Sciences was used as control strain, simultaneously. In addition, the resistance or sensitivity of H. pylori strains to clarythromycin strips (Lieflkchem, Denmark) was evaluated, in parallel.

**Test accreditation:** To accredit the results, all concentrations of total extract and DMSO were cultured in wells to identify their presumptive microbial infection. Also, clarythromycin and water were used as positive and negative control, respectively.

<table>
<thead>
<tr>
<th>Table 1: The sequence of ureC primers</th>
<th>The gene</th>
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<tr>
<td>5′ taagccttaagggtgtgagg4′</td>
<td>ureC</td>
</tr>
<tr>
<td>5′ gctactttctacatacgcgc3′</td>
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<th>Table 2: PCR program of ureC</th>
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<tr>
<td>The program</td>
<td>Pre- denaturation 94 °C 4′</td>
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<tr>
<td></td>
<td>Denaturation 94 °C 1′</td>
</tr>
<tr>
<td></td>
<td>Annealing 54 °C 1′</td>
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<tr>
<td></td>
<td>Extension 72°C 1′</td>
</tr>
<tr>
<td>X 35 cycles</td>
<td>72°C 5′</td>
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</table>

**Results**

Of 50 biopsy samples, 12 strains were confirmed as *Helicobacter pylori* based on gram negative staining bacteria, positive catalase and oxidase tests and a 297bp band of ureC was detected in all strains except one sample by PCR (Figure1).

![Figure 1: The 297bp band of ureC gene](image1)

**Discussion**

The main aim of this study was the determination of the anti H.pylori properties of total extract of *T. terrestris* and its fraction Benzoaxacin by cup plate method. In this study 50 biopsy samples were detected from patients of Milad and Fayazbakhsh...
hospitals of Tehran who were candidate for endoscopy. Rapid urease test was done for all biopsy samples and 12 of them were H.pylori positive. The ureC band of 297bp was present in all H.pylori strains except one. It seems that one of the isolates was a member of Helicobacter but not pylori.

The anti H.pylori effect of T. terrestris was detected among 50% of isolates in concentrations of 1000 and 750 mg/ml. Also 66.6% were resistant to concentrations of 500 and 250mg/ml. Benzoxacin resistance was detected in 83.3% of isolates. Existence of resistance to clarithromycin in 17% of isolates was another data of this study.

In other studies, the metanolic extract of T. terrestris had a good antibacterial effect against E.coli, S.aureus, P.aeruginosa, and Enterococcus fecalis 16, 17.

In our other study which was performed as a student thesis, we showed that the total extract of T. terrestris was effective against P.aeruginosa (ATCC9027) and E.coli (ATCC8739) with MIC 125mg/ml and B.subtilis (ATCC6633) 250 mg/ml. In addition a meaning correlation between increased sensitivity with increasing the concentration was detected. Also in that study it was clear that Benzoxacin was not effective against none of tested bacteria 14.

Conclusion

Despite the satisfying results of our other study which showed standard strains of E.coli, P.aeruginosa and B.subtilis to be sensitive to total extract of T. terrestris, in the present study, only 50% of H.pylori strains were sensitive to the concentration of 1000 mg/ml therefore evaluation of more H.pylori strains in the future studies is recommended. In contrast of the similarity of Benzoxacin structure to Ofloxacin, existence of 83.3% of resistance among tested isolates showed no anti Hp effectiveness of this fraction.

References