Original Article

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

Mojdeh Hakemi Vala[1,2]*, Hossein Goudarzi [1], Siavashe Naseri Moghadam[3], Mohammad Kamalinejad[4], Sahar Jahangiri[1], Mehrdad Gholami [1]

[1] Infectious diseases and tropical medicine research center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2] Department of Microbiology, Medical school, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[3] Digestive Disease Research Center (DDRC), Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran
[4] Department of Pharmaceutics, Pharmacology School, Shahid Beheshti University of Medical Sciences, Tehran, Iran

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* Benzoxacin), a local Iranian medicinal plant and its fraction *Benzoxacin.*

Method and Materials: Total aqueous extract of aerial parts of the plant was prepared and liquid extraction with petroleum ether was used to separate its components. LC/MS system proved the existence of Benzoxazine 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 Fayazbakhsh 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^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 Benzoxacin 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 test on more Hp strains in future studies highly recommended. In contrast of the similarity of Benzoxazin structure to Ofloxacin, existence of 83.3% resistance among tested isolates showed no anti Hp effectiveness of this fraction.

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

***Corresponding Author:** Mojdeh Hakemi Vala, Kodakyar ST, Daneshjo Blvd, Velenjak Tehran-Iran, Post box: 19835-151
Tel: +982123872556. Email: mojdeh_hakemi@yahoo.com

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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 [1, 2]. Usually Hp eradication is possible by using triple therapy including two antibiotics (clarythromycin and tetracycline) and bismuth sulfite, and among the antibiotics the role of clarythromycin is critical[3]. Based on different reports, the key role of clarythromycin is related to its effect in treatment failure of resistance strains. It means if the Hp strain is resistant to clarythromycin the chance of Hp eradication is very low and vice versa [4].

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 [5-12]. 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[13].

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, etherdopetrol 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 [14].

Also, Funatogawas et al identified that the hydrolysable monomers of tannin from herbal extracts had anti Hp effect [15].

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 anthrum of patients who were candidate for endoscopy and referred to Milad and Fayazbakhsh hospitals. From each patient two anthrum 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 (Amphotrisin B, sulfamethoxazole 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.

Benzoxacin 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 Benzoxacin 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 [16]. 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 (Lioflichem, 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 1: The sequence of ureC primers

The primers	The gene
5’ taagcttttaggggtggtagggg3’ 5’gcttactttctaacactaacgcgc3’	ureC

Table 2: PCR program of ureC

The program		
	Pre- denaturation 94 ºC	4’
	Denaturation 94 ºC	1’
	Annealing 54 ºC	1’
	Extension 72ºC	1’
X 35 cycles	72ºC	5’

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).

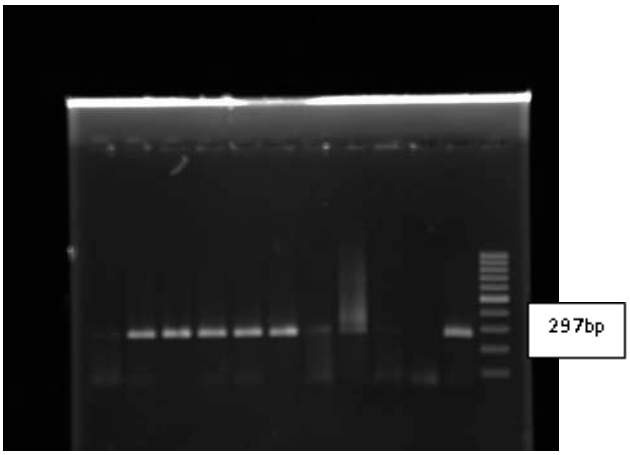

Figure 1: The 297bp band of ureC gene

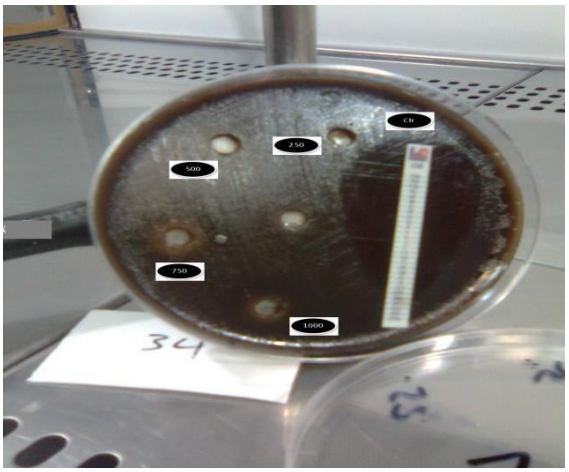

Figure 2: A cup plate assay for T. terrestris against H.pylori

A resistant Hp strain (No.34) to all concentrations of T. terrestris and clarythromycin

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 Benzoxacin 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 clarythromycin 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 Benzoxazin structure to Ofloxacin, existence of 83.3% of resistance among tested isolates showed no anti Hp effectiveness of this fraction.

References

1. Movahedi M, Afsharfard A, Moradi A, Nasermoaddeli A, Khoshnevis J, Fattahi F, et al. Survival rate of gastric cancer in Iran. J Res Med Sci. 2009;14(6):367–73.
2. Glocker E, Berning M, Gerrits MM, Kusters JG, Kist M. Real-time PCR screening for 16S rRNA mutations associated with resistance to tetracycline in Helicobacter pylori. Antimicrob Agents Chemother. 2005 Aug;49(8):3166-70.
3. Oleastro M, Menard A, Santos A, Lamouliatte H, Monteiro L, Barthelemy P, et al. Real-time PCR assay for rapid and accurate detection of point mutations conferring resistance to clarithromycin in Helicobacter pylori. J Clin Microbiol.. 2003 Jan;41(1):397-402.
4. Broutet N, Tchamgoue S, Pereira E, Lamouliatte H, Salamon R, Megraud F. Risk factors for failure of Helicobacter pylori therapy--results of an individual data analysis of 2751 patients. Aliment Pharmacol Ther. 2003 Jan;17(1):99-109.
5. Hamed AI, Oleszek W, Stochmal A, Pizza C, Piacente S. Steroidal saponins from the aerial parts of Tribulus pentandrus Forssk. Phytochemistry. 2004 Nov;65(21):2935-45.
6. Abdel-Hameed el SS, El-Nahas HA, El-Wakil EA, Ahmed WS. Cytotoxic cholestane and pregnane glycosides from Tribulus macropterus. Z Naturforsch C.. 2007 May-Jun;62(5-6):319-25.
7. Sharifi AM, Darabi R, Akbarloo N. Study of antihypertensive mechanism of Tribulus terrestris in 2K1C hypertensive rats: role of tissue ACE activity. Life Sci. 2003 Oct 24;73(23):2963-71.
8. Phillips OA, Mathew KT, Oriowo MA. Antihypertensive and vasodilator effects of methanolic and aqueous extracts of Tribulus terrestris in rats. J Ethnopharmacol. 2006 Apr 6;104(3):351-5.
9. Gauthaman K, Adaikan PG. Effect of Tribulus terrestris on nicotinamide adenine dinucleotide phosphate-diaphorase activity and androgen receptors in rat brain. J Ethnopharmacol. 2005 Jan 4;96(1-2):127-32.
10. Li M, Qu W, Wang Y, Wan H, Tian C. [Hypoglycemic effect of saponin from Tribulus terrestris] Zhong Yao Cai. 2002 Jun;25(6):420-2.
11. Su L, Chen G, Feng SG, Wang W, Li ZF, Chen H, et al.
Steroidal saponins from Tribulus terrestris. Steroids. 2009 Apr-May;74(4-5):399-403.
12. Deepak M, Dipankar G, Prashanth D, Asha MK, Amit A, Venkataraman BV. Tribulosin and beta-sitosterol-D-glucoside, the anthelmintic principles of Tribulus terrestris. Phytomedicine. 2002 Dec;9(8):753-6.
13. Rothstein DM, van Duzer J, Sternlicht A, Gilman SC. Rifalazil and other benzoxazinorifamycins in the treatment of chlamydia-based persistent infections. Arch Pharm. 2007 Oct;340(10):517-29.
14. Makhmor M. Evaluation the anti microbial effect of total extract of Tribulus terrestris and its fraction Benzoxazine. 2010. Thesis of Pharmaceutical Sciences Branch , Islamic Azad University, Tehran-Iran.
15. Funatogawa K, Hayashi S, Shimomura H, Yoshida T, Hatano T, Ito H, et al. Antibacterial activity of hydrolyzable tannins derived from medicinal plants against Helicobacter pylori. Microbiol Iimmunol. 2004;48(4):251-61.
16. Mehrgan H MF, Pakdaman S, Poursaeed M. Antibacterial Activity of Thymus pubescens Methanolic Extract. Iran J Pharma Res. 2008;7(4):291-5.
17. Kiyanbakht S JF. Evaluation of antibacterial activity of Tribulus terrestris L. Growing in Iran. Iran J Pharma Y Ther. 2003;2(1).