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Original article

Antibacterial Activity of Twenty Iranian Plant Extracts Against Clinical Isolates of *Helicobacter pylori*

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Abstract

Objective(s)
Due to increasing emergence of drug-resistance in *Helicobacter pylori* isolates, traditional plants are potentially valuable sources of novel anti-*H. pylori* agents. In this research, anti-*H. pylori* activity of the organic extracts of twenty native Iranian plants was determined against ten clinical isolates of *H. pylori*.
Materials and Methods
Disc diffusion was used to determine the biological activity of 20 plant extracts as well as 8 antibiotics commonly used to treat *H. pylori* infections. Minimum inhibitory concentrations were also measured by tube and agar dilution methods for the biologically active plant extracts.
Results
Of the twenty plant extracts analyzed, sixteen exhibited good anti-*H. pylori* activity, using disc diffusion. The ten most active extracts were *Carum bulbocastanum, Carum carvi, Mentha longifolia, Saliva limbata, Saliva sclarea, Ziziphora clinopodioides, Thymus caramanicus, Glycyrrhiza glabra, Xanthium brasilicum* and *Trachyspermum copticum.* Minimum inhibitory concentrations measured for the 10 biologically active plant extracts were within the range of 31.25 to 500 μg/ml.
Conclusion
Among the ten plant extracts effective against *H. pylori* clinical isolates, *Carum carvi, Xanthium brasilicum* and *Trachyspermum copticum* showed the highest activity.

Keywords: Anti-*Helicobacter pylori,* Iranian plants, Organic extracts

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Introduction

Helicobacter pylori is well recognized as a major etiologic factor in gastrodudenal diseases such as chronic gastritis, peptic ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (MALToma) (1-4). The current treatment regimes for *H. pylori* infections are based on the combination of a proton pump inhibitor and two antibiotics (triple therapy) (5, 6). However, eradication is not always successful and the use of these antibiotics occasionally causes emergence of resistant clones and various harmful adverse effects (5, 7-9). Thus, development of new effective therapeutic agents could represent a significant advance in treatment of these infections. Traditional uses of plants for medicinal purposes provide a basis for the use of specific plants for specific medical conditions. In fact, a number of drugs and natural substances such as various essential oils, extracts of the lichen *Certaria islandica*, Chinese green tea and several native Iranian plants have been shown to have *in vitro* antibacterial activity against *H. pylori* (10-14). In the present study, we investigated the anti-*H. pylori* activity of twenty native Iranian plant extracts against clinical isolates of *H. pylori* from adults by disc diffusion and measured the minimum inhibitory concentrations of the effective extracts.

Materials and Methods

H. pylori strains and culture conditions

Thirty two gastric biopsies were received from patients with gastrointestinal disease (age range 40 to 90 years old) at the Atrac Endoscopy Clinic (Gonabad, Iran). Biopsies were placed in a modified Campy–Thio medium, composed of thioglycolate base medium (Difco, Detroit, Michigan, USA), 10% sheep defibrinated blood, 8 mg/l polymyxin B, 2 mg/l amphotericin B and 6 mg/l vancomycin (Fluka, Sigma- Aldrich, Taufkirchen, Germany). The tubes were incubated at 37 °C under microaerophilic atmosphere for 2 to 5 days at which time, 20 µl of each specimen was streaked onto Brucella agar plates (Difco, Detroit, Michigan, USA) containing 10% defibrinated sheep blood and the three antibiotics. Identification was carried out by Gram stain morphology, catalase, oxidase and urea hydrolysis activities. Isolates were maintained in skim milk containing 15% glycerol and 10% fetal calf serum at –80 °C. *H. pylori* ATCC strain 43504 was also used as control in all experiments. Human studies were approved by the local Ethics Committee.

Plant collection and preparation of extracts

Plant names and the areas of collection are shown in Table 1. Plants were kindly identified by M. Yousefzadi (Department of

Table 1. Native Iranian plants chosen for the study, collection areas and plant parts collected.

Plant name	**Collection area / Province**	**Parts collected**	**Local name** (15)
Carum bulbocastanum	Yazd	Fruit	Zireh Irani
Carum carvi	Yazd	Fruit	Zireh siah
Mentha longifolia	Tar lake/Firozkooh	Aerial	Pouneh
Saliva limbata	Shahre kord/Chaharmahale Bakhteyari	Aerial	Maryam goli
Saliva sclarea	Tehran	Aerial	Maryam goli
Ziziphora clinopodioides	Arak	Aerial	Kakouti
Urtica dioica	Tehran	Aerial	Gazaneh
Gundelia tournefortii	Kermanshah	Leaf	Kangar
Codoncephalum stenocalathium	Ilam	Aerial	Sarzangi
Cannabis sativa	Baloochestan	Aerial	Shahdaneh
Lactuca viminea	Lorestan	Aerial	Kahoo
Rumex ephedroides	Khoozestan	Aerial	Torshak
Caccinia actinobole	Tehran	Aerial	Gavzaban asa
Glycyrrhiza glabra	Northern border / Golestan	Root	Shirin baian
Thymus caramanicus	Damavand/Tehran	Aerial	Avishan
Apocynum venetum	Baladeh/Tehran	Aerial	Shahdaneh Canadaii
Avicennia marina	Gheshm Island	Root	Harra
Hypericum olivieri	Velenjak/Tehran	Aerial	Gol Raii
Xanthium brasilicum	Chitgar Park/Tehran	Aerial	Zardineh Brezili
Trachyspermum copticum	Lar / Phars	Fruit	Badian

Ecology and Systematic, Research Institute of Applied Science, ACECR, Tehran, Iran) and Dr. Mozaffarian (Department of Botany, Research Institute of Forests and Rangelands, Tehran, Iran) and voucher numbers are kept at the Shahid Beheshti University Herbarium. To prepare organic extracts, dried plant parts were soaked in an equal mixture of methanol, diethyl ether and petroleum benzene (Merck, Darmstadt, Germany) at a ratio of 1:10 (w/v) at room temperature for 24 hr before filtration. The filtrates were concentrated, using a Rotavapor (Eyela, Tokyo, Japan) to about 5 ml and placed at –15 ^{o}C to remove heavy hydrocarbons and lipids. The extracts were then diluted with cold methanol, filtered, and the filtrates were evaporated to leave a solid pellet which was then reconstituted in methanol (10%, w/w) (14).

Anti- Helicobacter pylori activity measured by disc diffusion

Suspensions of fresh cultures were made in saline and turbidity was adjusted to 9 x 10^8 or 1.5×10^8 bacteria/ml (corresponding to McFarland standards 3 and 0.5 respectively). It has been reported that a McFarland standard of 3 or 4 yields actual counts of 5×10^6 cells /ml for *H. pylori* (16). Hence, both inocula were used to test anti-Helicobacter activity of the plant extracts. In each case, 200 µl of the appropriate microbial suspension was placed on a large (50 ml) Mueller Hinton agar plate (Merck, Darmstadt, Germany) containing 10% fetal calf serum (Gibco/BRL, Paisley, UK) and spread evenly in all directions.

Antibiotic discs including metronidazole (5 µg), amoxicillin (30 µg), ampicillin (10 µg), tetracycline (30 µg), furazolidone (50 µg), azithromycin (15 µg), erythromycin (15 µg) (HiMedia Laboratories, Pvt. Limited, Mumbai- 400 086 India) and clarithromycin (15 µg) (Becton Dickinson, Abbot Laboratories, Chicago, IL., USA) were placed on the bacterial lawns and the plates were incubated at 37 ^{o}C under microaerophilic conditions for 2-5 days. For the plant extracts, sterile blank discs (6 mm) were inoculated with 25 µl (2.5 mg) of each extract or solvent controls and were dried at 30-35 ^{o}C for 12-24 hr before being placed on the surface of the bacterial lawns.

Measurement of minimum inhibitory concentrations

Minimum inhibitory concentrations (MIC) were measured within the range of 0.015 to 1000 µg/ml, using tube and agar dilution methods. Fresh bacterial suspensions were prepared in saline and adjusted to McFarland turbidity standard 3. For the tube assay, serial 2-fold dilutions were made from a stock of crude plant extract in dimethyl sulfoxide (2 mg/ml) (Merck, Darmstadt, Germany) in 0.5 ml of Mueller Hinton broth containing 10% fetal calf serum. Finally, 0.5 ml of a 1:200 dilution of bacteria adjusted to MacFarland standard 3 in Mueller Hinton broth was added to the tubes. For the agar dilution assay, the extracts were added in 2-fold decreasing concentrations to Mueller Hinton agar containing 10% serum and 2 µl of the undiluted bacterial suspensions were spot inoculated on each plate. Controls of bacteria without extract or extracts without bacteria were also included. The tubes and plates were incubated for 2-5 days, before recording minimum inhibitory concentrations.

Results

Ten isolates of *H. pylori* were recovered from the 32 gastric biopsies and their susceptibility was determined to eight antibiotics and the crude organic plant extracts by disc diffusion and employing MacFarland standard 3. Table 2 shows that the highest degree of antibiotic resistance was observed for metronidazole (9/10 isolates) followed by amoxicillin, tetracycline, erythromycin (4/10 isolates) and finally, ampicillin, furazolidone, azithromycin, clarithromycin (3/10 isolates). For plant extracts, an inhibition zone of 15 mm was arbitrarily chosen as the cut-off point for bacterial susceptibility. All *H. pylori* isolates (10/10 isolates) were sensitive to the extracts of *C. bulbocastanum, C. carvi, M. longifolia, S. limbata, S. sclarea, Z. clinopodioides, T. caramanicus, G. glabra, T. copticum* and *X.*

brasilicum. Eight were sensitive to *Urtica dioica* extract, seven to the extracts of *Avicennia marina, Hypericum olivieri, Gundelia tournefortii* and *Codoncephalum stenocalathium* and six were susceptible to *Cannabis sativa* extract. The extracts of *Lactuca viminea, Rumex ephedroides, Caccinia actinobole* and *Apocynum venetum* showed no antibacterial activity. In addition, using the 10 most effective plant extracts, the disc test was carried out against two bacterial inocula (1.5×10^8 and 3×10^9 cells/ml). As shown in Table 3, all extracts showed high anti-*H. pylori* activity, using both inocula.

Table 4 shows the results of minimum inhibitory concentrations determined for the 10 effective plant extracts against *H. pylori* isolates, using broth and agar dilution methods. The results were identical for both assays and were within the range of 125-500 µg/ml for organic extracts of *S. limbata* and *S. sclarea*, 62.5-250 µg/ml for organic extracts of *M. longifolia, T. caramanicus, G. glabra* and *X. brasilicum* and 31.25-125 µg/ml for organic extracts of *Z. clinopodioides, C. bulbocastanum, C. carvi* and *T. copticum*. Such values are significant given that the crude extracts were used.

Table 2. Anti- *Helicobacter pylori* activity of 8 antibiotics and organic extracts of 16 native Iranian plants against 10 clinical isolates of *H. pylori* shown by disc diffusion.

Isolate number	*T. copticum*	*X. brasilicum*	*H. olivieri*	*A. marina*	*T. caramanicus*	*G. glabra*	*C. sativa*	*C. stenocalathium*	*G. tournefortii*	*U. dioica*	*Z. clinopodioides*	*S. sclarea*	*S. limbata*	*M. longifolia*	*C. carvi*	*C. bulbocastanum*	Azithromycin	Clarithromycin	Erythromycin
1	28	29	R	R	29	22	R	R	R	R	25	19	20	23	25	27	R	R	R
2	25	28	R	R	24	25	R	R	17	R	25	20	22	25	38	40	22	R	R
3	26	25	20	19	26	22	18	22	22	18	28	19	20	31	26	38	26	25	25
4	30	26	21	20	24	20	20	20	R	24	20	15	18	18	25	26	25	29	26
5	29	28	22	19	22	30	17	R	R	25	20	18	18	28	25	28	R	R	R
6	31	28	20	19	26	25	R	30	19	21	25	12	20	25	16	25	28	26	24
7	32	30	R	R	25	28	R	20	20	25	28	19	22	31	22	24	R	24	R
8	30	35	21	21	27	26	17	18	18	25	30	22	23	40	20	25	29	26	25
9	39	39	22	22	32	31	21	26	24	24	35	28	28	38	29	30	31	28	28
10	40	35	23	21	31	30	21	27	25	25	34	31	30	40	32	32	30	31	30
Control	40	35	30	18	42	42	20	20	25	21	37	30	28	35	30	35	nt	30	nt

Numbers shown in the table represent zones of inhibition measured in mm. For plant extracts, zones of > 15 mm show sensitivity and R, indicates resistance. *H. pylori* ATCC 43504 was used as the susceptible control strain, nt, not tested.

Table 3. Comparison of anti- *Helicobacter pylori* activities of the organic extracts from ten Iranian plants using MacFarland standards 0.5 and 3, measured by disc diffusion.

Isolate number	*C. bulbocastanum*		*C. carvi*		*M. longifolia*		*S. limbata*		*S. sclarea*		*Z. clinopodioides*		*G. glabra*		*T. caramanicus*		*X. brasilicum*		*T. copticum*	
	MF#0.5	MF#3	MF#0.5	MF#3	MF#0.5	MF#3	MF#0.5	MF#3	MF#0.5	MF#3	MF#0.5	MF#3	MF#0.5	MF#3	MF#0.5	MF#3	MF#0.5	MF#3	MF#0.5	MF#3
1	29	27	27	25	25	23	22	20	20	19	27	25	24	22	31	29	32	29	31	28
2	42	40	39	38	28	25	23	22	23	20	28	25	27	25	27	24	32	28	28	25
3	40	38	28	26	33	31	25	20	22	19	30	28	25	22	29	26	28	25	29	26
4	30	26	28	25	21	18	22	18	18	15	28	20	24	20	29	24	28	26	33	30
5	31	28	27	25	31	28	21	18	22	18	24	20	32	30	26	22	32	28	33	29
6	27	25	18	16	27	25	23	20	24	22	30	25	27	25	29	26	32	28	34	31
7	27	24	24	22	34	31	23	22	22	19	32	28	30	28	29	25	34	30	36	32
8	27	25	24	20	42	40	24	23	24	22	33	30	29	26	31	27	37	35	36	30
9	35	30	31	29	40	38	30	28	30	28	37	35	33	31	34	32	42	39	42	39
10	34	32	34	32	42	40	32	30	33	31	36	34	36	30	34	31	39	35	43	40
Control	36	35	31	30	36	35	29	28	31	30	40	37	44	42	45	42	36	35	42	40

Numbers shown in the table represent zones of inhibition measured in mm. For plant extracts, zones of > 15 mm were chosen as sensitivity cut off point. *H. pylori* ATCC 43504 was used as the susceptible control strain.

Table 4. Minimum inhibitory concentrations (MIC) measured for 10 organic extracts from native Iranian plants against clinical isolates of *H. pylori*.

	Isolate number										
Plant Extract	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Control**
C. bulbocastanum	125	31.25	31.25	62.5	62.5	62.5	125	125	62.5	62.5	62.5
C. carvi	62.5	31.25	62.5	62.5	62.5	125	125	125	62.5	31.25	125
M. longifolia	125	125	62.5	250	250	125	62.5	62.5	62.5	62.5	62.5
S. limbata	250	250	500	500	500	250	250	125	125	125	250
S. sclarea	250	500	500	500	250	500	250	125	125	125	250
Z. clinopodioides	125	125	125	125	125	62.5	62.5	31.25	31.25	31.25	31.25
G. glabra	250	125	125	125	62.5	125	62.5	62.5	62.5	62.5	15.6
T. caramanicus	125	250	125	125	250	125	62.5	62.5	62.5	62.5	15.6
X. brasilicum	125	250	250	125	62.5	62.5	62.5	62.5	62.5	62.5	125
T. copticum	125	125	125	62.5	62.5	62.5	31.25	62.5	31.25	31.25	62.5

MIC was determined by tube and agar dilution methods, using MacFarland standard # 3 and values are shown in µg/ml. *H. pylori* ATCC 43504 was used as control.

Discussion

Due to increasing antibiotic resistance in clinical isolates of *H. pylori*, the search for new and safe anti-*H. pylori* compounds are of utmost importance and native medicinal plants seem to be a logical source for seeking new agents.

We determined the biological activity of twenty native Iranian plants against 10 clinical isolates of *H. pylori* and found that sixteen had good anti- *H. pylori* activity. As a matter of fact, inhibition zones for the plant extracts obtained by disc diffusion were equal or larger than those of the eight antibiotics commonly used to eradicate *H. pylori* infections. These findings suggest the potential of natural agents for treatment of *H. pylori* infections. Minimum inhibitory concentration values were also encouraging since only a fraction of the crude extract is expected to have antimicrobial activity. We have previously shown the antibacterial activity of *Xanthium brasilicum* from Iran and identified *Xanthatin (8-Epi-xanthatin)* as its active antibacterial compound (14, 17 and unpublished data). Antibacterial and anti-ulcerogenic activities of *Xanthanolides* have also been reported by other researchers (17-20). There are some reports on anti-*H. pylori* activity of a number of plant extracts including some species within the genera used in this study (21-29). However, *in vitro* susceptibility does not necessarily mean success *in vivo*. Graham *et al*. showed that neither garlic nor red pepper containing capsaicin had any *in vivo* effect on *H. pylori* despite their anti- *H. pylori* activity *in vitro* (30). Therefore, a wide range of plants with anti- *H. pylori* activity must be screened before exploring the potential use of effective agents for *in vivo* use. More extensive experiments are also needed to determine the *in vivo* activities of these edible medicinal plants and furthermore, identifying the active components of each plant as well as their therapeutic values.

Conclusion

Majority of the plant extracts used in this study had considerable *in vitro* activity against clinical isolates of *H. pylori*. Considering that these plants are edible and are traditionally used for treatment of a number of ailments, their anti-*H. pylori* activity is quite significant and could present alternative treatments for *H. pylori* infections.

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