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LABORATORY RESEARCH

Bacteriostatic effect of dill, fennel, caraway and cinnamon extracts against *Helicobacter pylori*

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Abstract
Background. Helicobacter pylori is one of the most prevalent gastric pathogens, causing gastric dysfunction, ulceration and, eventually, cancer. Antibiotic resistance, a rapidly growing problem, may interfere with the success of eradication therapy. The aim of this study was to evaluate the anti-*H. pylori* effect of crude extracts derived from dill, fennel, caraway and cinnamon, all of which are common dietary additives in Iran.
Design. In vitro bactericidal measures.
Methods. The sensitivity of *H. pylori* isolates from gastric fluids to herbal extracts was evaluated using two standard *ex vivo* techniques.
Results. The results showed that dill extract had the greatest antibacterial activity. Flow cytometric analysis of bacterial viability, however, demonstrated bacteriostatic properties of all test extracts.
Conclusion. The possible synergistic effects of different dietary combinations of these extracts may be a factor in the possible protection afforded by the traditional Iranian diet against *H. pylori* infection. We concluded that these extracts might be useful as dietary supplements, at least, to complement and expedite current treatments.

Keywords: Helicobacter pylori, *herbal extract, gel diffusion, flow cytometry*

Introduction

Helicobacter pylori, a common cause of human bacterial infection, is the most prevalent gastric microbial pathogen. Although chronic *H. pylori* infection may be asymptomatic in many cases, it may eventually lead to gastritis, peptic ulceration and malignancies [1]. Recently, chronic infection with *H. pylori* has been associated with the development of such disorders as iron deficiency and iron-deficiency anaemia [2], ischaemic heart disease [3,4], anterior uveitis [5], and autoimmune pancreatitis [6]. Although the infection is seemingly acquired early in life

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[7], the mode of transmission of *H. pylori* is still poorly understood. Transmission from person to person and especially from mother to child has been proposed [8].

The eradication of *H. pylori* involves combination therapy, including a proton pump inhibitor and two to three antibiotics taken twice daily for 7–14 days [9,10]. However, antibiotic resistance is a growing and serious problem that interferes with the success of eradication therapy. With the increasing resistance of microbes to conventional therapies, alternative treatments are being re-explored [11,12]. This study was undertaken to assess the anti-*H. pylori* effects of crude extracts derived from dill, fennel, caraway and cinnamon, which are usually used as natural dietary additives in the Iranian diet. The herbal extracts, prepared using the percolation method, were assessed for their antibacterial activities using both a bacterial susceptibility test and flow cytometric analysis, in which viable bacteria absorbed a fluorescent dye, rhodamine 123.

Materials and methods

Subjects

Samples of gastric secretions were taken from the gastric antrum under gastroscopy of 30 patients with gastrointestinal discomfort who attended Ekbatan Hospital, Hamedan, in western Iran. Although the subjects underwent endoscopy for routine diagnostic purposes, written consents were taken from them prior to the procedure. The research design was approved scientifically and ethically by the Higher Education Committee of Hamedan University of Medical Sciences.

Bacteriological tests

Part of the sample was used for the urease rapid test and the remainder was sent to the microbiology laboratory in thiogylcollate medium at 4°C for direct smear, Gram staining and culture. Samples were cultured in *Campylobacter* selective agar base (Merck) supplemented with *Campylobacter* selective supplement (Merck) (containing 2 mg vancomycin, 50 µg polymyxin, and 1 mg trimethoprim) and 2 mg l^{-1} amphotricin B, 5–10% sheep red blood cells and 7% horse serum. Microaerophilic conditions, using an anaerobic jar with gas pack C (8–10% CO_2 and 5–7% O_2) in a 37°C incubator, were followed. Urease and oxidase tests were also carried out for further confirmation of the bacteriological diagnosis. Those isolates with positive test results for direct smear, urease and culture were used for further experiments.

Herbal extraction

Herbal extracts were prepared using the percolation method. Briefly, herbs were incubated in 70% ethanol at 37°C overnight. This procedure was repeated three times for complete extraction. The solvent was then evaporated using the evaporator system. The material left was further dried by incubating at 50°C overnight, and then weighed and used to make different concentrations.

Disc preparation from herbal extracts

Different concentrations of each herbal extract (10, 20, 40, 80, 100, 250 and 500 mg ml^{-1}) were made in dimethyl sulphoxide (DMSO), from which 30 µl was added to blank discs (Padtan-Teb). To evaporate the solvent the discs were incubated at 37°C for 1 hour.

Bacterial susceptibility test

The test was performed as originally described by Kirby-Bauer [13]. Briefly, a microbial suspension with turbidity equal to 0.5 McFarland standard unit in brain–heart infusion broth (BHIb) was prepared and inoculated into blood Mueller–Hinton agar. Herbal extract discs (cinnamon, fennel, dill and caraway) together with negative and positive control discs (DMSO and tetracycline, respectively) and amoxycillin (10 μg), tetracycline (30 μg) and ciprofloxacin (5 μg) discs were also transferred into culture plates, which were then incubated at 37°C under microaerophilic conditions (anaerobic jar with gas pack C).

Flow cytometry

One millilitre of *H. pylori* suspension in BHIb with turbidity equal to 1 McFarland unit was mixed with an equal volume of each herbal extract in different concentrations (10, 20, 80, 100, 250, 500 mg ml^{-1}) and 1 ml of antibiotic solutions, i.e. ciprofloxacin (1.5, 3.0, 6.5, 12.5, 25, 50 and 100 μg ml^{-1}) and tetracycline (100, 150, 300, 1000 and 2000 μg ml^{-1}) in separate flow cytometric tubes, which were then incubated for 30 min under culture conditions, as described above. Microbial suspensions without any special treatment (antibiotic or herbal extract) or with 5% sodium hypochlorite were also used as positive and negative controls, respectively. After the incubation period, the tubes were centrifuged at 1000***g*** for 10 min. The sediment was washed once with phosphate-buffered saline (PBS) and then 2 ml rhodamine 123 was added to each tube and incubated in the dark at room temperature for 30 min. The cells were washed again with PBS and after centrifugation 0.5 ml of 10% formaldehyde was added to the sediment to fix the stain on the cells. A cell suspension containing 10^8 bacteria was then analysed by flow cytometer (FACSCalibur, Becton Dickinson).

Statistical analyses

Data were analysed using McNemar and Cochran tests. The predetermined upper limit of probability for significance throughout this study was $p<0.05$. All statistical analyses were performed using Windows 2000/SPSS 10 package.

Results

Disc diffusion

In bacterial susceptibility testing with three antibiotics, 100% of the isolates were sensitive to both ciprofloxacin and tetracycline and 100% were resistant to amoxycillin. Herbal extracts, although not effective against *H. pylori* at concentrations of 10–100 mg ml^{-1}, inhibited bacterial growth at 250–500 mg ml^{-1} (Table I). At a concentration of 500 mg ml^{-1}, the Cochran test showed that the inhibitory effects of various herbal extracts were significantly different ($p=0.007$) (Table II). The inhibitory effects of dill (Table III), caraway (Table IV) and fennel (Table V) extracts were all more than that of cinnamon (Table VI), although the only significant difference was between dill and cinnamon (McNemar test, $p=0.008$). At a concentration of 250 mg ml^{-1}, the inhibitory effects of the herbal extracts were also significantly different (Cochran test, $p<0.0001$; Table VII). In an evaluation of the inhibitory effects of the extracts using the McNemar test, dill was found to be significantly stronger than caraway ($p=0.004$), fennel ($p=0.031$) and cinnamon ($p=0.008$).

Table I. Comparison of antibacterial effects of different herbal extracts at two concentrations against *Helicobacter pylori*.

	Diameter of growth inhibition (mm)	
Herbal extract	250 mg ml^{-1}	500 mg ml^{-1}
Dill	8–10	9–12
Fennel	8–9	9–12
Caraway	–	9–12
Cinnamon	9	9–12

Table II. Comparison of *Helicobacter pylori* sensitivity to different herbal extracts at 500 mg ml^{-1}. The antibacterial effects of the test extracts were significantly different (Cochran test, $p=0.007$).

	Sensitive		Resistant		Total	
Herbal extract	*n*	%	*n*	%	*n*	%
Dill	14	100	0	0	14	100
Fennel	9	64.3	5	37.5	14	100
Caraway	11	78.6	3	21.4	14	100
Cinnamon	6	42.9	8	57.1	14	100

Table III. Comparison of *Helicobacter pylori* sensitivity to two concentrations of dill extract. Although the antibacterial effect of the extract at 500 mg ml^{-1} was stronger, the difference was not statistically significant (McNemar test, $p=0.063$).

	Sensitive		Resistant		Total	
Concentration	*n*	%	*n*	%	*n*	%
250 mg ml^{-1}	9	64.3	5	35.7	14	100
500 mg ml^{-1}	14	100	0	0	14	100

Table IV. Comparison of *Helicobacter pylori* sensitivity to caraway extracts. A significant difference was observed between the two concentrations (McNemar test, $p=0.001$).

	Sensitive		Resistant		Total	
Concentration	*n*	%	*n*	%	*n*	%
250 mg ml^{-1}	0	0	14	100	14	100
500 mg ml^{-1}	11	78.6	3	21.3	14	100

Table V. Comparison of *Helicobacter pylori* sensitivity to fennel extracts. A significant difference was observed between the two concentrations (McNemar test, $p=0.031$).

	Sensitive		Resistant		Total	
Concentration	*n*	%	*n*	%	*n*	%
250 mg ml^{-1}	3	21.4	11	78.6	14	100
500 mg ml^{-1}	9	64.3	5	35.7	14	100

Table VI. Comparison of *Helicobacter pylori* sensitivity to cinnamon extracts. No significant difference was observed between the two concentrations (McNemar test, $p=0.063$).

	Sensitive		Resistant		Total	
Concentration	*n*	%	*n*	%	*n*	%
250 mg ml^{-1}	1	7.1	13	92.9	14	100
500 mg ml^{-1}	6	42.9	8	57.1	14	100

Table VII. Comparison of *Helicobacter pylori* sensitivity to different herbal extracts at 250 mg ml^{-1}. The antibacterial effects of the test extracts were significantly different (Cochran test, $p<0.0001$).

	Sensitive		Resistant		Total	
Herbal extract	*n*	%	*n*	%	*n*	%
Dill	9	64.3	5	35.7	14	100
Fennel	3	21.4	11	78.6	14	100
Caraway	0	0	14	100	14	100
Cinnamon	1	7.1	13	92.9	14	100

Flow cytometry

Flow cytometric analysis revealed the percentage of killed bacteria, as well as viable bacteria, as only viable bacteria were stainable with rhodamine. The evaluation of the viability of 14 *H. pylori* isolates using flow cytometry showed that 0.18% of sodium hypochlorite-treated bacteria (negative control) and 99.9% of pure bacterial suspension (positive control) were alive (Figure 1). On the other hand, 99.9% of tetracycline-treated bacteria were also viable, but only 0.28% of ciprofloxacin-treated bacteria remained viable (Figure 2). Interestingly, 99.1% of dill extract-treated bacteria, 99.58% of fennel-treated bacteria, 98.9% of caraway-treated bacteria and 97.7% of cinnamon-treated bacteria absorbed rhodamine, indicating that they were still alive (Figure 3).

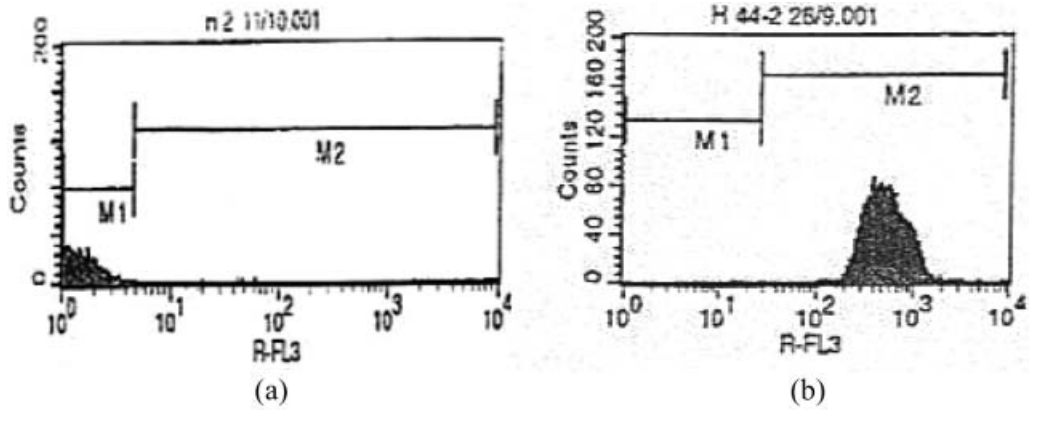


Figure 1. Flow cytometric analysis of (a) 5% sodium hypochlorite-treated *Helicobacter pylori* (negative control) and (b) pure *H. pylori* suspension (positive control). M1, killed bacteria [99.8% (a) vs. 0.01% (b)]; M2, viable bacteria [0.2% (a) vs. 99.9% (b)].

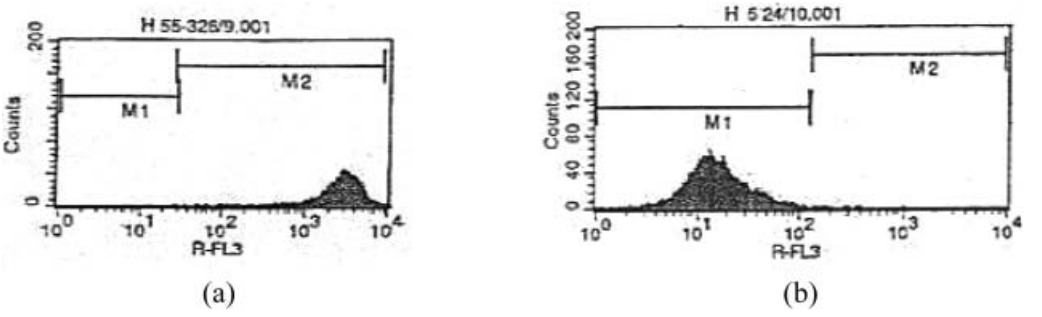
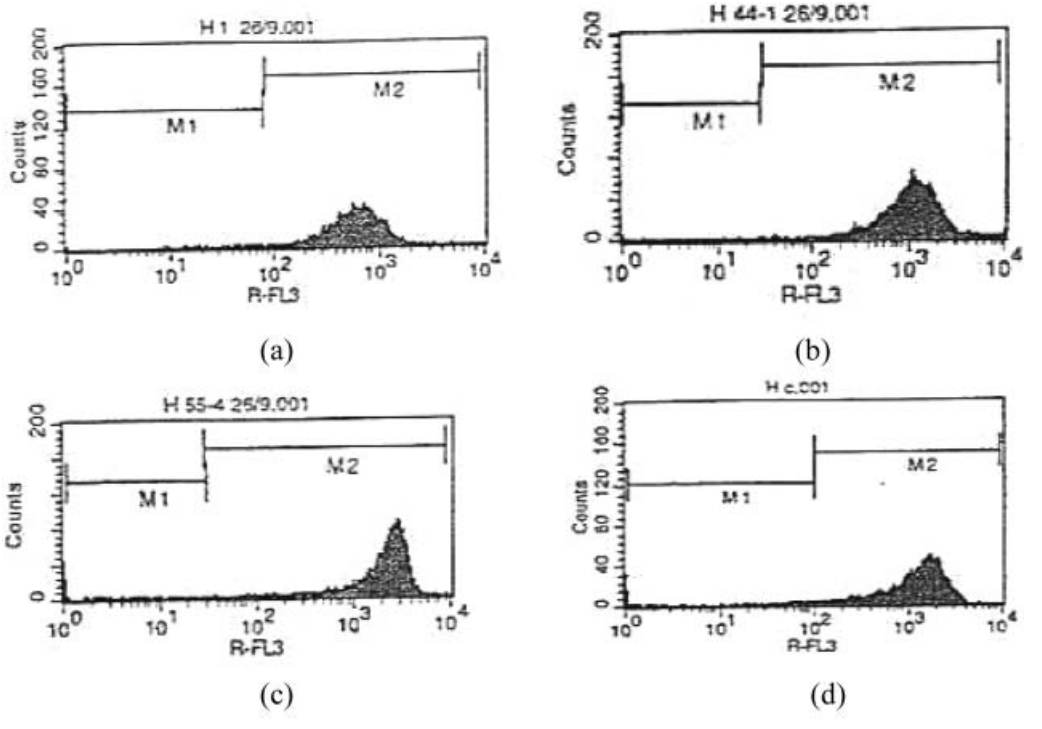


Figure 2. Flow cytometric analysis of the effect of (a) tetracycline and (b) ciprofloxacin on *Helicobacter pylori*. Tetracycline had no effect on bacteria, as almost all of the bacteria absorbed rhodamine. However, almost all of the ciprofloxacin-treated bacteria were killed. M1, killed bacteria [0.1% (a) vs. 99.7% (b)]; M2, viable bacteria [99.9% (a) vs. 0.3% (b)].



	a	b	c	d
M1	0.9%	0.4%	1.06%	2.3%
M2	99.1%	99.6%	98.94%	97.7%

Figure 3. Flow cytometric analysis of the anti-*Helicobacter pylori* effect of the extracts derived from (a) dill, (b) fennel, (c) caraway and (d) cinnamon. M1, killed bacteria; M2, viable bacteria.

Discussion

Although the antibacterial properties of some herbal extracts *in vitro* have been reported by many investigators [1–6], in none of them has flow cytometric analysis of bacterial viability been used. Flow cytometry has been proved to be a useful tool to evaluate the viability of bacteria [14–16] and of their spores [17,18]. This method allowed the determination of bactericidal or bacteriostatic effects of different agents *in vitro*. Based on gel diffusion and flow cytometric data, it was concluded that all the herbal extracts examined were bacteriostatic. Despite *in vitro* antibacterial effects of herbal extracts, the efficacy of none of them has been proven in clinical settings [19]. It must be noted that, in this study, crude whole extracts were evaluated. If the effective constituent(s) were used, antibacterial effects would be expected to be stronger.

In a study of the antibacterial activities of the essential oils derived from the leaves of two clones of cinnamon (A and B) and their chemical constituents on nine bacterial species (other than *H. pylori*), the inhibitory effect of indigenous cinnamon B leaf essential oils with minimum inhibitory concentrations between 250 and 500 $\mu g\,ml^{-1}$ was observed [20]. Similar antibacterial effects of cinnamon essential oil, among some other herbal extracts, at the dilution of 1:100 against food spoilage bacteria, with a direct relationship between the inhibitory effect of essential oils and the presence of eugenol and cinnamaldehyde, have been reported [21]. In our study, cinnamon crude extract showed almost the least antibacterial effect at both concentrations of 250 and 500 $mg\,ml^{-1}$, which were an order of magnitude higher than the concentrations tested as essential oils in other studies (20). It is likely that there are trace amounts of effective antibacterial constituents in cinnamon than in the other herbs studied.

Fennel essential oils have antibacterial [22] and antioxidant [23] properties. The antibacterial effect of herbal extracts such as fennel oil have been shown to be potentiated when in combination with benzoic acid derivatives such as methyl paraben (methyl 4-hydroxybenzoic acid), as judged by studies on *Listeria* and *Salmonella* species [24]. Based on our findings, the antibacterial effect of fennel against *H. pylori*, which has been studied less, must be taken more into consideration.

Essential oils extracted from dill seeds have been found to have antibacterial effects [22,25]. Interestingly, the strength and spectrum of inhibition for the fractions of the essential oils may exceed those of crude oils and mixing fractions may lead to additive, synergistic or antagonistic effects against individual test micro-organisms [25]. The metabolites of 30-day-old dill have been established as having a reliable bacteriostatic effect [26]. Caraway oil in combination with peppermint oil has been used to treat dyspepsia [27]. It is unknown whether this curative effect of caraway is due to its inhibitory effect against *H. pylori*. Although *H. pylori* has been reported as one of the causes of dyspepsia [28,29], some clinical trials failed to show a beneficial effect of the bacterial eradication on gastric acidity and gastroesophageal reflux [30]. Although herbal essential oils alone may not be efficient enough against *H. pylori in vivo*, they may be used as dietary supplements to complement current therapies [31].

The antibacterial effects of herbs may be relevant to their antioxidant properties as it was shown that a combination of antioxidants could protect guinea pigs against *H. pylori* infection. In animal studies, antioxidant intake should be low to optimize the development of *H. pylori*-associated disease [32]. Speculation on the association between the antioxidant and antibacterial properties of herbs needs further investigation.

Conclusion

Crude extracts derived from dill, fennel, cinnamon and caraway all showed bacteriostatic effects against *H. pylori in vitro*, as judged by gel diffusion and flow cytometric analysis. Dill

had the highest antibacterial effect at both 250 and 500 mg ml^{-1} concentrations. The possible synergistic effect between different combinations of these extracts must be taken into account. These extracts may be used as dietary supplements to complement and expedite current treatments. This speculation needs further study.

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