Iranian Journal of Basic Medical Sciences
Vol. 11, No. 2, Summer 2008, 91-96
Received: May 10, 2008; Accepted: Aug 11, 2008

Inhibition of *Helicobacter pylori* Growth *in vitro* by Saffron (*Crocus sativus* L.)

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Abstract

Objective(s)
Anti-*Helicobacter pylori* effects of saffron *(Crocus sativus* L., Iridacea) and its major constituents, crocin and safranal, were evaluated.

Materials and Methods
Macerated aqueous and methanol extracts tested against 45 clinical isolates of *Helicobacter pylori*, using paper disc diffusion method (DDM) on modified egg yolk emulsion agar (EYE agar). Four antibiotics also tested against all isolates as positive control.

Results
Although there were small differences in sensitivity among the isolates tested, but all isolates were susceptible to methanol and aqueous extracts. The minimum inhibitory concentrations (MIC) of methanol extract, crocin and safranal measured as 677, 26.5 and 16.6 μg/ml, respectively, using agar dilution method. The results showed that high temperature did not have any effect on the activity of extracts, crocin and safranal. The effect of pH on the activity of methanol extract indicated no significant difference at pH 5 to 8, in comparison with the control.

Conclusion
The results indicated that saffron has a moderate anti-*Helicobacter* activity.

Keywords: Anti-*Helicobacter pylori*, Crocin, *Crocus sativus,* Saffron, Safranal

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Introduction

Helicobacter pylori cause upper gastrointestinal tract disorders such as chronic gastritis, peptic ulcer disease and gastric carcinoma (1). Although there are several drug treatment regimens for these significant infections including colloidal bismuth subcitrate (CBS) together with antibiotics such as amoxycillin and metronidazole (2) but sometimes eradication failure is seen. Increased resistance of *H. pylori* strains may develop leading to relapse (3). Incomplete cure, side effects of antibiotics and resistant strains cause search for new sources of drugs. Medicinal plants have been used for centuries to cure stomach disorders and they could be a useful source of novel antibacterial compounds or lead compounds for development of new antibacterial agents.

Crocus sativus L. (Iridacea), commonly known as saffron and used as a spice and food colorant has been used in folk medicine for various purposes such as flatulent colic, increase of appetite, relieve of abdominal pain (4), aphrodisiac, antispasmodic and expectorant (5). Several pharmacological activities have been demonstrated for the saffron extracts or its chemical constituents, including antitumor (6-8), radical scavenger, hyperlipaemic (9), anticonvulsant (10), improve activity on learning and memory (9, 11), cytotoxic and antigenotoxic activity (12). Several constituents have been isolated from stigma, leaves, petals and pollen of *Crocus sativus* (13), including carotenoids (crocin and crocetin), monoterpenoids (saffranol and picrotoxin), flavanoids (quercetin) and anthocyanins (kaempferol). Among the constituents of saffron extract, crocin and saffranal are mainly responsible for the above biological activities. A recent study showed that saffron extract has anti-ulcerogenic activity (14). The Present study was carried out to evaluate the anti-*Helicobacter pylori* activity of extracts of saffron and its major constituents, safranal and crocin, also, to find a rational for its use in traditional medicine.

Materials and Methods

Chemicals

Crocin and safranal purchased from Fluka (Buchas, Switzerland). All reagents and solvents were commercially available and of analytical grades.

Plant materials

Plants collected from Qaen (located south of Khorasan province, Iran) in October 2004. The stigma separated and dried in dark at room temperature. The *Crocus sativus* L. identified at Ferdowsi University, Mashhad, Iran (by Mr. Joharchi) and voucher samples, preserved for reference in the Herbarium of School of Pharmacy, Mashhad, Iran (143-0319-1).

Preparation of Extract

The powder of stigma extracted using maceration with either water or methanol. The powdered stigma macerated in water or methanol for 3 days, and the mixture subsequently filtered and concentrated under reduced pressure at 40 ^{o}C. The yield was 50% and 58% (w/w), respectively.

Bacterial isolates

Clinical isolates of *H. pylori* obtained from the 17 Shahrivar Hospital (Mashhad, Iran). A total of 45 clinical isolates of biopsy specimens were used. Primary isolation was performed on selective Brucella agar (Merck, Germany), supplemented with horse blood 5-7% (v/v), starch (0.1% w/v, Merck, Germany), vancomycin (5 mg/l), trimethoprim (5 mg/l), polymyxine B (2500 u/l), and amphotricine B (10 mg/l). Bacteria incubated for 5-7 days under microaerophillic condition (10% CO_2 and 90-100% humidity) at 37 °C. Following primary isolation, *H. pylori* bacterial cells identified according to colony morphology, Gram staining, rapid urease^{+}, catalase^{+}, oxidase^{+}, Indole^{-}, H_2S^{-} and nalidixic acid resistant (15,16).

Anti-Helicobacter pylori assay

Growth inhibition was performed by the filter paper disc diffusion method (DDM) on modified egg yolk emulsion agar (EYE agar) at 37 °C under microaerophilic conditions.

Briefly, each 100 ml of EYE agar contained 3.8 g Mueller-Hinton agar (Pronadisa-Madrid), 7-10% egg yolk emulsion and 4 mg triphenyl tetrazolium chloride (Merck) (17).

Standard discs (d=6 mm) containing 2 mg of plant extracts were placed on egg yolk emulsion agar plate, previously inoculated with 0.1 ml bacterial suspension in sterile normal saline. The turbidity of bacterial suspension was equivalent to McFarland tube No. 4 (10^8 CFU/ml). Inhibition growth assay of antibiotics versus saffron extracts, crocin and safranal performed using standard commercial discs of amoxycillin (25 μg/disc), metronidazole (5 μg/disc), ampicillin (10 μg/disc) and clarithromycine (15 μg/disc). The plates incubated for 3-4 days, at 37 °C under microaerophillic conditions (18). The zones of inhibition measured and reported in millimeters (average diameter by two repetitions).

The MIC of the methanol extract was determined using an agar dilution method (19), by adding various amounts (0-1250 μg/ml) of saffron methanol extract, crocin or safranal to solid or liquid media (brain heart infusion broth + 7% FCS, v/v) for 4 clinical isolates of *H. pylori* with highest sensitivity.

Various concentrations of the extracts were poured in the melted (45 °C) Mueller–Hinton agar medium, followed by inoculation of the plates with 0.1 ml of bacterial suspension in sterile normal saline (10^8 CFU/ml). All plates incubated for 4-5 days at 37 °C under microaerophillic conditions. The MIC expressed as the lowest concentration of the extract that inhibited visible growth. Minimum bactericidal concentrations (MBC) were established by the lack of growth upon re-inoculation from extract-treated plates to Mueller-Hinton Agar plates.

Temperature and pH stability of saffron extract and its stability during storage

In order to determine pH stability, 50 mg methanol extract of saffron mixed with 0.1 M sterile phosphate buffer solution (PBS) of various pH values (5, 6, 7 and 8) in separate tubes and incubated at room temperature for 3 hrs (20). Then, as above for temperature, after exposure of the extract to different pH conditions the discs containing 2 mg saffron extract were prepared and tested.

Results

Mean diameters of *H. pylori* growth inhibition by extract of saffron are presented in Table 1. All isolates exhibited sensitivity to both methanol and ethanol extracts. Amoxicillin, ampicillin, metronidazole and clarithromycine were active against all isolates whereas 11% of isolates (5 isolates) resisted metronidazole. Both extracts at concentration of 2 mg/disc showed less anti-*H. pilori* activity compared to amoxicillin, ampicillin, mertonidazole and clarithromycine. Considering the preliminary results presented in Table 1, safranal and crocin as major components of saffron extract tested against seven isolates that were more sensitive to either extracts or standard antibiotics. Results indicated that safranal was more active against all seven isolates tested than crocin. While, the MIC was 677, 26.5, 16.6 μg/ml, for methanol extract, crocin and safranal respectively.

Fifty mg of methanol extract of saffron dissolved in 5 ml of sterile distilled water and either was incubated at 80°C for 30 mins or autoclaved at 121 °C for 20 mins. For the determination of stability during storage similar extract also, stored at 4 °C and room temperature for 4 weeks. After exposure of the extract to different thermal conditions, the discs containing 2 mg saffron extract were prepared and tested.

The effect of thermal stability on anti-*H. pylori* activity was also, studied on 3 isolates sensitive to methanol extract. As shown in Table 2, the methanol extract of saffron, preserved its antibacterial activity against three isolates, after either heating or autoclaving.

In the present study, the effect of pH on bactericidal activity of saffron methanol extract investigated. The extract was stable at pH 5, 6, 7 and 8 with no loss of anti *H. pylori* activity (Table 3). The effect of pH on the activity of methanol extract, indicated no significant difference at pH 5 to 8, in comparison to the control (Table 3).

Table 1. Inhibitory effects of methanol extracts of *Crocus sativus* L. and some antibiotics on *H. pylori* in 45 clinical isolates.

Isolates number	Inhibition zone (mm)*					
	Metronidazole (5 µg/disc)	Amoxycillin (25 µg/disc)	Ampicillin (10 µg/disc)	Clarithromycin (15 µg/disc)	Aqueous extract (2 mg/disc)	Methanol extract (2 mg/disc)
1	12	25	10	46	15	16
2	9	28.5	12	45	18.5	18
3	9	24	35	44	19	20
4	-¶	28.5	20	-	15	16
5	28	25	21	-	18	23
6	-	40	22.5	-	21	19
7	10	33	13.5	54	13	15.5
8	-	24	19.5	-	13.5	15
9	6	20	16	47.5	14	14.5
10	41.5	13	8	37.5	15	18
11	-	25	17.5	-	17.5	20
12	11	36	15	35	13.5	14
13	50	20	42	47	14.5	13.5
14	51	32	37.5	46	13	17.5
15	6	50	51	56	10	15
16	16.5	38	28	23	13	10
17	6	35	35	30.5	12.5	19
18	6	30	24	36	11	13.5
19	9.5	54	44	60	18	22
20	55	40	53.5	51.5	17.5	22
21	9.5	35	50	48	16.5	23.5
22	10	30	40.5	50.5	18	22
23	29.5	40	32	33	12	15
24	50.5	50	37	59	10.5	17
25	-	34.5	41	-	11	13
26	46	42	44	41	11	12
27	52	36	31.5	51	14	18.5
28	10.5	45	39.5	49	16	19
29	6	44.5	60.5	58	17.5	18
30	28	50	47	50.5	16	22.5
31	19	38	30	39	12.5	15
32	47.5	60.5	51.5	52	12	12.5
33	10	20	16	38	12	14
34	9.5	37	32.5	-	15	18
35	10	46	40	49.5	18	16.5
36	-	48.5	31	-	12.5	17
37	9	44	39.5	41	10.5	16
38	50.5	49	35.5	59	14	17
39	13	22	12.5	45.5	15	15
40	11	28	22.5	-	15	16
41	51	35	30.5	50.5	14	18.5
42	48	40.5	38	45	12.5	17.5
43	9	36.5	41	35	15.5	21
44	10.5	28	24	34	13.5	14.5
45	46	37	36	38	12	18

* Diameter of each disc was 6 mm and values are mean of duplicate discs

¶ -, Not determined

Table 2. Effect of temperature treatment on the anti-*H. pylori* activity of saffron extract.

Isolate number	Inhibition zone (mm)		
	Control	80 ºC	121 ºC
Saffron (2 mg/disc)			
Strain 10	17	17.5	18.5
Strain 42	12.5	12	12
Strain 43	18	17.5	18

Table 3. Effect of pH on the anti-*H. pylori* activity of methanol extract of saffron.

Isolate number	Inhibition zone (mm)			
	pH = 5	pH = 6	pH = 7	pH = 8
Strain 10	17	17.5	18	15
Strain 42	17.5	17	17.5	13
Strain 43	15	14	14.5	11

Discussion

Considering the increased development of resistance of *H. pylori* to antibiotics, searching for new antibiotics with natural origin is valuable especially in the third world countries where most people get their medication from local market based on traditional medicine. It has been reported that saffron is used in stomach disorders in traditional medicine and the rational for using saffron in gastro-intestinal disorders could be due to its anti-*H. pylori* activity. Analysis of the chemical composition of saffron revealed more than 150 volatile and several non-volatile compounds of which 40-50 constituents have been already identified (21). Although several studies on the anti-*Helicobacter* activity of plant extracts have been reported (17, 22-25) but surprisingly, no antimicrobial activity has been demonstrated for either saffron extract or its constituents.

In this research, it was shown that the extract of saffron and its major constituents, crocin and safranal, had anti-*H. pylori* activity. Results indicated that both safranal and crocin were more potent than the methanol extract indicating that the anti-*H. pylori* activity of saffron could mainly attributed to these constituents. The MIC values reported for other plant extracts in the literature showed that saffron extract, safranal and crocin exhibited lower MIC values, ranging from 16.6 to 677 µg/ml, indicating that all have high anti-*H. pylori* activity. Different degrees of anti-*H. pylori* activities have been reported for plant extracts in the literature. MICs for the aqueous garlic extract against nineteen strains of *H. pylori* ranged from 2 to 5 mg/ml (26). The extracts of *Pteleopsis suberosa* have shown anti-*H. pylori* activity against the standard and clinical strains, with MIC ranging from 0.0625 to 0.5 mg/ml (27).

Screening of Turkish anti-ulcerogenic folk remedies for anti-*H. pylori* activity showed active plant extracts with MIC in the range of 1.95-250 µg/ml (28). Seven Greek herbal medicine extracts were active against the standard and clinical strains of *H. pylori* with MIC ranging from 0.625 to 5 mg/ml (29). Extract of *Curcuma longa* and curcumin inhibited the growth of *H. pylori* strains with MIC range of 6.25-50 µg/ml (24). Fourteen Chinese herbal medicines proved to be active against *H. pylori* with MIC of 40-60 µg/ml (23). Five Taiwanese folk medicinal plants showed strong anti-*H. pylori* activity with MIC ranging from 0.64 to 10.24 mg/ml (22).

The results of thermal stability indicated that the active components of saffron were thermally stable and it appeared that the volatile components had no responsibility for the observed activity.

It has been shown that the efficacy of antibiotics is pH dependent. For example, amoxicillin is the most effective at neutral pH and tetracycline presents greater activity at low pH (30). The results obtained in present research indicated that the active constituents of saffron were not affected by the pH of environment.

Conclusion

Saffron and its major constituents had a strong activity against most of the *H. pylori* strains tested. Further microbiological and clinical trials are needed to validate the anti-*H. pylori* activity of saffron *in vivo*.

Acknowledgment

We would like to thank the Vice Chancellor for Research of Mashhad University of Medical Sciences for providing financial support to perform this research.

References

1. Tytgat GN, Rauws EA. Campylobacter pylori and its role in peptic ulcer disease. Gastroenterol Clin North Am 1990; 19:183-96.
2. Edwards DI. Nitroimidazole drugs action and resistance mechanisms. Two mechanisms of resistance. J Antimicrob Chemother 1993; 31:9-20.
3. Goddard AF, Logan RP.H. Antimicrobial resistance and *Helicobacter pylori*. J Antimicrob Chemother 1996;37: 639-643.

4. Said HM, Saeed A, D'Silva LA, Zubairy HN, Bano Z. *Medicinal Herbal:* a testbook for medicinal students and doctors. Hamdard Foundation Pakistan 1996. Vol. 1 p. 1-282.
5. Zargari A. Medicinal Plant. Tehran University Press; 1990. Vol. 4. p. 574-578.
6. Nair SC, Pannikar B, Panikkar KR. Antitumour activity of saffron *(Crocus sativus*). Cancer Lett 1991; 57:109-114.
7. Nair SC, Kurumboor SK, Hasegawa JH. Saffron chemoprevention in biology and medicine: a review. Cancer Biother 1995; 10:257-264.
8. Salomi MJ, Nair SC, Panikar KR. Inhibitory effects of *Nigella sativa* and saffron (*Crocus sativus*) on chemical carcinogenesis in mice. Nutr Cancer 1991; 16:67-72.
9. Abe K, Saito H. Effects of saffron extract and its constituent, crocin, on learning behavior and long-term potentiation. Phytother Res 2000; 14:149-152.
10. Hosseinzadeh H, Khosravan V. Anticonvulsant effect of *Crocus sativus* stigma aqueous and ethanolic extracts in mice. Arch Iran Med 2001;5:44-47.
11. Zhang Y, Shoyama Y, Sugiura M, Saito H. Effects of *Crocus sativus* on the ethanol-induced impairment of passive avoidance performances in mice. Biol Pharm Bull 1994 ;17:217-221.
12. Abdullaev FI, Riveron-Negrete L,Caballero-Ortega H, Hernandez JM, Perez-Lopez I, Pereda-Miranda R, *et al*. Use of *in vitro* assays to assess thr potential antigenotoxic and cytotoxic effects of saffron (*Crocus sativus* L.). Toxicol *In Vitro* 2003;17:731-736.
13. Rios JL, Recio MC, Giner RM, Mane S. An update review of saffron and its active compounds. Phytother Res 1996; 10:189-193.
14. Al-Mofleh IA, Alhaidar AA, Mossa JS, Al-Sohaibani MO, Qureshi A, Rafatullah S. Antigasteric ulcer studies on saffron (*Crocus sativus* L.) in rats. Pak J Biol Sci 2006; 9:1009-1013.
15. Goodwin CS, Armstrong JA. Microbiological aspects of *Helicobacter pylori*. Eur J Clin. Microbiol Infect Dis 1990; 9:1-13.
16. Boyanova L,Koumanova R, Lazarova E, Jelev C. 2003. *Helicobacter pylori* and *Helicobacter heilmannii* in children. A Bulgarian study. Diagn Microbiol Infect Dis 2003 ;46:249-252.
17. Tabak M, Armon R, Neeman I. Cinnamon extracts' inhibitory effect on *Helicobacter pylori*. J Ethnopharmacol 1999; 67:269-277.
18. McNulty C, Owen R, Tompkins D, Hawtin P, McColl K, Price A. 2002. *Helicobacter pylori* susceptibility testing by disc diffusion. J Antimicrob Chemother 2002; 49:601-609.
19. Shahamat M, Mai UEH, Paszko-Kolva C, Yamamoto H, Colwell RR. 1991. Evaluation of liquid media for growth of *Helicobacter pylori*. J Clin Microbiol 1991; 29:2835-2837.
20. Ten Brink B, Minekus M, Vander Vossen J, Leer RJ, Huis J HJ. 1994. Antimicrobial activity of *Lactobacilli:* preliminary characterization and optimization of production of acidocin B, a novel bacteriocin produced by *L. acidophilus* M46. J Appl Bacteriol 1994; 77:140-148.
21. Das I, Chakrabarty RN, Das S. Saffron can prevent chemically induced skin carcinogenesis in Swiss albino mice. Asian Pas J Cancer Prev 2004; 5:70-76.
22. WangY, Huang T. Screening of anti-*Helicobacter pylori* herbs driving from Taiwanese folk medicinal plants. FEMS Immunol Med Microbiol 2005; 43:295-300.
23. Li Y, Zhang Q, Liu JL, Ren X T. *In vitro* anti-*Helicobacter pylori* action of 30 Chinese herbal medicines used to treat ulcer diseases. J Ethnopharmacol 2005; 98:329-333.
24. Mahady GB, Pendland S L,Lu Z Z. Turmeric (*Curcuma longa*) and curcumin inhibit the growth of *Helicobacter pylori*, a group 1 carcinogene. Anticancer Res 2002; 22:4179-4181.
25. Boyanova L, Derejian S, Koumanova R, Katsarov N, Gergova G, Mitov I, *et al* 2003. Inhibition of *Helicobacter pylori* growth *in vitro* by Bulgarian propolis: preliminary report. J Med Microbiol 2003;52:417-419.
26. Cellini L,Campli ED, Masulli M, Bartolomeo SD, Allocati N. Inhibition of *Helicobacter pylori* by garlic extract (*Allium sativum*). FEMS Immunol Med Microbiol 1996; 13:277-279.
27. Germano MP, Sanogo R, Guglielmo M, Pasquale RD, Crisafi G. Effects of *Pteleopsis suberosa* extracts on experimental gastric ulcers and *Helicobacter pylori* growth. J Ethnopharmacol 1998; 59:167-172.
28. Yesilada E, Gurbuz I, Shibata H. Screening of Turkish anti-ulcerogenic folk remedies for anti-*Helicobacter pylori* activity. J Ethnopharmacol 1999; 66:289-293.
29. Stamatis G, Kyriazopoulos P, Golegou S, Basayiannis A, Skaltsas S, Skatsas H. *In vitro* anti-*Helicobacter pylori* activity of Greek herbal medicines. J Ethnopharmacol 2003; 88:175-79.
30. Worrel JA, Stoner SC. Eradication of *Helicobacter pylori*. Med Updates Psychiatr 1998; 3:99-104.