Anti-*Helicobacter pylori* Activity of the Methanolic Extract of *Geum iranicum* and its Main Compounds

Somayeh Shahani[a], Hamid R. Monsef-Esfahani[a], Soodabeh Saeidnia[b], Parastoo Saniee[c], Farideh Siavoshi[c], Alireza Foroumadi[d], Nasrin Samadi[e], and Ahmad R. Gohari[b,*]

[a] Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
[b] Medicinal Plants Research Center, Tehran University of Medical Sciences, Tehran, P. O. Box 14155-6451, Iran. Fax: +98-21-64122330. E-mail: goharii@tums.ac.ir
[c] Microbiology Department, Faculty of Sciences, University of Tehran, Tehran, Iran
[d] Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran, Iran
[e] Department of Drug and Food Control, Faculty of Pharmacy and Biotechnology Research Center, Tehran University of Medical Sciences, Tehran, Iran

* Author for correspondence and reprint requests

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Geum iranicum Khatamsaz, belonging to the Rosaceae family, is an endemic plant of Iran. The methanol extract of the roots of this plant showed significant activity against one of the clinical isolates of *Helicobacter pylori* which was resistant to metronidazole. The aim of this study was the isolation and evaluation of the major compounds of *G. iranicum* effective against *H. pylori.* The compounds were isolated using various chromatographic methods and identified by spectroscopic data (^{1}H and ^{13}C NMR, HMQC, HMBC, EI-MS). An antimicrobial susceptibility test was performed employing the disk diffusion method against clinical isolates of *H. pylori* and a micro dilution method against several Gram-positive and Gram-negative bacteria; additionally the inhibition zone diameters (IZD) and minimum inhibitory concentrations (MIC) values were recorded. Nine compounds were isolated: two triterpenoids, uvaol and niga-ichigoside F1, three sterols, *β*-sitosterol, *β*-sitosteryl acetate, and *β*-sitosteryl linoleate, one phenyl propanoid, eugenol, one phenolic glycoside, gein, one flavanol, (+)-catechin, and sucrose. The aqueous fraction, obtained by partitioning the MeOH extract with water and chloroform, was the most effective fraction of the extract against all clinical isolates of *H. pylori*. Further investigation of the isolated compounds showed that eugenol was effective against *H. pylori* but gein, diglycosidic eugenol, did not exhibit any activity against *H. pylori*. The subfraction D_4 was the effective fraction which contained tannins. It appeared that tannins were probably the active compounds responsible for the anti-*H. pylori* activity of *G. iranicum*. The aqueous fraction showed a moderate inhibitory activity against both Gram-positive and Gram-negative bacteria. The MIC values indicated that Gram-positive bacteria including *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Bacillus subtilis* are more susceptible than Gram-neagative bacteria including *Escherichia coli* and *Pseudomonas aeruginosa*.

Key words: Geum iranicum, *Helicobacter pylori*, Eugenol

Introduction

Helicobacter pylori colonizes the stomachs of about 50% of the world's human population. This organism is the main risk factor for peptic ulceration as well as gastric mucosal-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma. The prevention of *H. pylori* colonization could potentially provide primary prevention of the mentioned diseases (Fauci *et al.*, 2008). The common treatments of *H. pylori* infection, including antibiotics and a proton pump inhibitor, may fail for several reasons. The main reason was found to be *H. pylori* resistance to antibiotics like clarithromycin and metronidazole (Mégraud, 2004). Therefore, the research for new anti-*H. pylori* drugs from plant sources is ongoing (Nariman *et al.*, 2004).

The genus *Geum*, belonging to the Rosaceae family, is a perennial rhizomatous herb with five species in Iran of which *G. iranicum* Khatamsaz is an endemic one (Khatamsaz, 1992; Mozaffarian,

1996). Some *Geum* species are used as medicinal plants in folk medicine (Vollmann and Schultze, 1995; Gruenwald, 2004). The roots of *G. urbanum* and *G. rivale* are employed against diarrhea (Gruenwald, 2004), and *G. japonicum* has been used as diuretic and astringent in traditional Chinese medicine and Japanese folk medicine (Ming *et al.*, 2000). In an Iranian folk remedy, the infusion of the root of *G. iranicum* is employed to treat gastrointestinal disorders like diarrhea, and a decoction of the whole plant is combined with wheat flour and used as a poultice for frostbite (Abutorabi, 2001). Previous studies on extracts of *Geum* species have shown that they can be effective in the treatment of some diseases. A new triterpene acid, 2α,19α-dihydroxy-3-oxo-urs-12-en-28-oic acid, from the extract of *G. japonicum* showed potent inhibitory activity against HIV-1 protease (Xu *et al.*, 2000). In addition, the extract of *G. japonicum* was active against HSV-1 and HSV-2 (herpes simplex virus) (Kurokawa *et al.*, 1995) and CMV (cytomegalo virus) (Yukawa *et al.*, 1996). Some tannins isolated from the extract of this plant showed potent anticoagulant activity (Zeng *et al.*, 1998). Other *Geum* species showed antioxidant (Russo *et al.*, 2005), antimicrobial (Panizzi *et al.*, 2000), and anti-inflammatory (Tunón *et al.*, 1995) effects, respectively. In another study, antimicrobial activity was reported for the extract and essential oil of *Geum kokanicum* that was collected in Iran (Faramarzi *et al.*, 2008). Furthermore, the polar extract of this species showed a potent inhibitory effect on matrix metalloproteinase activity at minimal cytotoxic doses (Khorramizdeh *et al.*, 2006).

A literature review revealed that the anti-*H. pylori* activity of *Geum* species has not been investigated. In the present study we aimed therefore to evaluate the activity of various extracts, sub-fractions, and main components of *G. iranicum* against clinical isolates of *H. pylori* (resistant to metronidazole). Furthermore, the evaluation of the antibacterial activity (against Gram-positive and Gram-negative bacteria) of fractions, together with the isolation and identification of the separated compounds, is reported.

Material and Methods

General experimental procedures

Silica gel 60 F_{254} pre-coated plates (Merck, Darmstadt, Germany) and anisaldehyde/H_2SO_4 reagent followed by heating were used for detecting the compounds. NMR experiments were performed on a Bruker Avance 500 DRX (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer (Rheinstetten, Germany). EI-MS spectra were measured on an Agilent Technology (Palo Alto CA, USA) instrument with a 5973 Network mass selective detector (MS model). A CO_2 incubator (Heraeus, Hamburg, Germany) was used for the antibacterial susceptibility tests.

Plant collection

Roots of *Geum iranicum* were collected from the Gloul Sarani protected area, 75 km north of Shirvan, province of Khorasan-e-Shomali, Iran, at 2460 m above sea level, during the flowering stage in June 2009. A voucher specimen (6714 THE) was deposited at the herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

Antimicrobial susceptibility test

Three clinical isolates of *H. pylori*, Is.1, Is.2, and Is.3, were obtained from patients with chronic gastritis who had been referred to the Endoscopy Unit at Shariati Hospital, Tehran, Iran. Antral biopsies with positive rapid urease tests were cultured in the microbiology laboratory as described in our previous study (Siavoshi *et al.*, 2010). Antimicrobial susceptibility was tested by the disk diffusion method according to the guidelines of the NCCLS (2006). Serial dilutions of test samples were made in dimethyl sulfoxide (DMSO) (100, 50, 25, 12.5 μg/ml). Bacterial suspensions were prepared in normal saline with the turbidity of McFarland standard No. 2 (equivalent to $6 \cdot 10^8$ cell/ml). The surface of blood agar plates was inoculated with 100 μl of each bacterial suspension. Then, plates were dried at room temperature for about 10 min. The sterile blank disks (6 mm in diameter) were placed on the surface of inoculated plates and impregnated with 10 μl of each sample dilution. Control plates included blank disks impregnated with 10 μl of DMSO. Plates were incubated at 37 °C under microaerobic conditions and examined after 3–5 d. The inhibition zone diameters (IZD) were recorded.

Antibacterial activity of the aqueous fraction was also tested against several Gram-positive and Gram-negative bacteria including *Staphylococcus aureus* ATCC 6538, *Staphylococcus epider-*

midis ATCC 12228, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 8739, and *Pseudomonas aeruginosa* ATCC 9027 by a micro dilution method using 96 U-shaped wells plates (NCCLS, 2006). A 200-μl aliquot of stock solutions of the fraction (128 mg/ml) and ciprofloxacin (100 μg/ml) as a standard antibiotic compound in Mueller-Hinton broth (MHB) were transferred into the first well in each row and serially diluted by mixing with 100 μl of MHB in subsequent wells. Then 100 μl of bacterial suspension ($1 \cdot 10^6$ CFU/ml) were added to each well to reach the final inoculum size of about $5 \cdot 10^5$ CFU/ml. After 24 h of incubation at 35 °C, the microdilution trays were tested for the absence or presence of visible growth. The endpoint minimum inhibitory concentration (MIC) is the lowest concentration of the fraction at which the test strain does not demonstrate visible growth.

Extraction and isolation procedure

Dried roots of *G. iranicum* (1 kg) were cut into small pieces and extracted with EtOAc and MeOH (3 × 4 l for each solvent), successively, by percolation at room temperature to obtain EtOAc (6 g) and MeOH (75 g) extracts. The EtOAc extract was subjected to silica gel column chromatography (CC) (4 × 20 cm) using *n*-hexane/$CHCl_3$ (7:3, 3:7 v/v), EtOAc, and MeOH as eluents to give 7 fractions (A–G). Fraction A was further separated by silica gel CC (3 × 20 cm) with *n*-hexane, *n*-hexane/$CHCl_3$ (7:3, 3:7 v/v) and $CHCl_3$ to give 5 fractions (A_1–A_5) of which fraction A_3 contained compound **1** (15 mg). Silica gel CC (2 × 20 cm) was used for separation of fraction B to yield 7 fractions (B_1–B_7) with *n*-hexane/$CHCl_3$ (7:3, 6:4, 4:6 v/v) and $CHCl_3$. Fraction B_2 was **2** (22 mg). Fraction C was fractionated with *n*-hexane/$CHCl_3$ (9:1, 5:5 v/v) by silica gel CC (2 × 20 cm) to give fractions C_1–C_5; compound **3** (141 mg) was isolated from fraction C_4. Compound **4** (5 mg) was obtained from fraction D using silica gel CC (2.5 × 20 cm) with *n*-hexane/EtOAc (8:2 v/v).

The MeOH extract (75 g) was suspended in water and extracted with $CHCl_3$ to obtain $CHCl_3$ and aqueous fractions. The $CHCl_3$ fraction was further chromatographed with *n*-hexane/$CHCl_3$ (7:3, 3:7 v/v) and $CHCl_3$ to give 6 fractions of which fraction 5 was compound **5** (17 mg). The aqueous portion of the MeOH extract (70 g) was subjected to silica gel CC (10 × 20 cm) with EtOAc, EtOAc/MeOH (9:1, 7:3, 4:6 v/v) and MeOH to yield 6 fractions (A–F). Fraction A was separated by silica gel CC (2.5 × 20 cm) with $CHCl_3$/MeOH (19:1, 9:1, 8:2 v/v) to yield 5 fractions (A_1–A_5). Fraction A_4 was compound **6** (129 mg). Fraction C was subjected to silica gel CC (2.5 × 20 cm) with $CHCl_3$/MeOH (8:2, 7:3 v/v) to give 4 fractions (C_1–C_4). Compound **7** (13 mg) was obtained from C_2 using silica gel CC (1.5 × 40 cm) with $CHCl_3$/MeOH (75:25 v/v). Fraction D was chromatographed on a silica gel column (4 × 20 cm) with $CHCl_3$/MeOH (8:2, 6:4 v/v) and MeOH to result in factions D_1–D_4 of which D_2 was compound **8** (70 mg). Compound **9** (960 mg) was obtained from fraction E using silica gel CC (2.5 × 20 cm) with EtOAc/MeOH (6:4 v/v).

β-Sitosteryl acetate (**1**): R_f = 0.14 in *n*-hexane/$CHCl_3$ (7:3 v/v). – ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H = 0.69 (3H, s, H-18), 0.82 (3H, d, J = 6.8 Hz, H-27), 0.84 (3H, d, J = 6.8 Hz, H-26), 0.86 (3H, t, J = 7.3 Hz, H-29), 0.93 (3H, d, J = 6.5 Hz, H-21), 1.03 (3H, s, H-19), 2.05, (3H, s, OCH_3), 4.61 (1H, m, H-3), 5.38 (1H, brd, J = 5 Hz, H-6). – ^{13}C NMR ($CDCl_3$, 500 MHz): δ_C = 11.8 (C-18), 11.9 (C-29), 18.8 (C-21), 19.0 (C-27), 19.3 (C-19), 19.8 (C-26), 21.0 (C-11), 21.4 ($COOCH_3$), 23.1 (C-28), 24.3 (C-15), 26.1 (C-23), 27.7 (C-2), 28.2 (C-16), 29.1 (C-25), 31.8 (C-8), 31.9 (C-7), 33.9 (C-22), 36.1 (C-20), 36.6 (C-10), 36.9 (C-1), 38.1 (C-4), 39.7 (C-12), 42.3 (C-13), 45.8 (C-24), 50.0 (C-9), 56.0 (C-17), 56.7 (C-14), 73.9 (C-3), 122.6 (C-6), 139.6 (C-5), 170.5 (C=O). – EI-MS: *m/z* (%) = 396 [M–HOAc]$^+$ (100), 381 (15), 288 (9), 275 (6), 255 (18), 213 (21).

β-Sitosteryl linoleate (**2**): R_f = 0.37 in *n*-hexane/$CHCl_3$ (7:3 v/v). – ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H = 0.68 (3H, s, H-18), 0.81 (3H, d, J = 7 Hz, H-27), 0.83 (3H, d, J = 7 Hz, H-26), 0.84 (3H, m, H-29), 0.88 (3H, t, J = 6.5 Hz, H-18'), 0.92 (3H, d, J = 7 Hz, H-21), 1.03 (3H, s, H-19), 1.25–1.38 (14H, m, H-4', 5', 6', 7', 15', 16', 17'), 1.59–1.65 (2H, m, H-3'), 2.03–2.08 (4H, m, H-8', 14'), 2.29 (2H, t, J = 7.5 Hz, H-2'), 2.76 (2H, t, J = 6.5 Hz, H-11'), 4.6 (1H, m, H-3), 5.30–5.40 (5H, m, olefinic protons). – ^{13}C NMR ($CDCl_3$, 500 MHz): δ_C = 11.8 (C-18), 11.9 (C-29), 14.1 (C-18'), 18.8 (C-21), 19.0 (C-27), 19.3 (C-19), 19.8 (C-26), 21.0 (C-11), 22.7 (C-17'), 23.1 (C-28), 24.3 (C-15), 25.1 (C-3'), 25.6 (C-11'), 26.0 (C-23), 27.2 (C-8',14'), 27.8 (C-2), 28.3 (C-16), 29.1 (C-25), 29.2 (C-4'), 29.3 (C-5'), 29.4 (C-15'), 29.6 (C-6'), 29.7 (C-7'), 31.6 (C-16'), 31.8

(C-7), 31.9 (C-8), 33.9 (C-22), 34.4 (C-2'), 36.2 (C-20), 36.5 (C-10), 37.0 (C-1), 38.2 (C-4), 39.7 (C-12), 42.3 (C-13), 45.8 (C-24), 50.0 (C-9), 56.0 (C-17), 56.7 (C-14), 73.7 (C-3), 122.6 (C-6), 127.9 (C-10'), 128.0 (C-12'), 130.0 (C-9'), 130.2 (C-13'), 139.7 (5), 173.3 (C=O). – EI-MS: *m/z* (%) = 676 $[M]^+$ (1), 396 (100), 381 (15), 288 (12), 275 (14), 255 (16), 213 (12).

β-Sitosterol (**3**): R_f = 0.13 in *n*-hexane/EtOAc (9:1 v/v). – ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H = 0.68 (3H, s, H-18), 0.79 (3H, d, *J* = 6.5 Hz, H-27), 0.82 (3H, d, *J* = 6.5 Hz, H-26), 0.83 (3H, t, *J* = 7.5 Hz, H-29), 0.91 (3H, d, *J* = 6.5 Hz, H-21), 0.99 (3H, s, H-19), 3.5 (1H, m, H-3), 5.3 (1H, brd, *J* = 5 Hz, H-6). – ^{13}C NMR ($CDCl_3$, 500 MHz): δ_C = 11.8 (C-18), 11.9 (C-29), 18.8 (C-21), 19.0 (C-27), 19.4 (C-19), 19.8 (C-26), 21.1 (C-11), 23.0 (C-28), 24.3 (C-15), 26.0 (C-23), 28.3 (C-16), 29.1 (C-25), 31.6 (C-2), 31.8 (C-7), 31.9 (C-8), 33.9 (C-22), 36.1 (C-20), 36.5 (C-10), 37.2 (C-1), 39.7 (C-12), 42.3 (C-13), 45.8 (C-24), 50.1 (C-9), 56.0 (C-17), 56.8 (C-14), 71.8 (C-3), 121.7 (C-6), 140.7 (C-5). – EI-MS: *m/z* (%) = 414 $[M]^+$ (100), 399 (38), 396 (32), 381 (40), 303 (75), 273 (37), 231 (41), 213 (67).

Uvaol (12-ursen-3*β*,28-diol) (**4**): R_f = 0.23 in *n*-hexane/EtOAc (8:2 v/v). – ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H = 0.80 (3H, s, H-25), 0.81 (3H, d, *J* = 5.8 Hz, H-30), 0.94 (3H, d, H-29), 0.95 (3H, s, H-24), 0.99 (3H, s, H-26), 1.00 (3H, s, H-27), 1.11 (3H, s, H-23), 3.19 (1H, d, *J* = 11 Hz, H-28a), 3.23 (1H, dd, *J* = 4.9, 11.3 Hz, H-3), 3.53 (1H, d, *J* = 11 Hz, H-28b), 5.14 (1H, t, *J* = 3.5, H-12). – ^{13}C NMR ($CDCl_3$, 500 MHz): δ_C = 15.6 (C-24), 15.7 (C-25), 16.8 (C-29), 17.3 (C-26), 18.3 (C-6), 21.3 (C-30), 23.3 (C-27), 23.4 (C-11), 26.0 (C-16), 27.2 (C-2), 28.1 (C-23), 29.7 (C-15), 30.6 (C-21), 32.8 (C-7), 35.2 (C-22), 36.8 (C-17), 38.0 (C-10), 38.8 (C-1), 38.8 (C-4), 39.3 (C-19), 39.4 (C-20), 40.0 (C-8), 42.8 (C-14), 47.6 (C-9), 54.0 (C-18), 55.1 (C-5), 69.9 (C-28), 79.0 (C-3), 125.0 (C-12), 138.7 (C-13). – EI-MS: *m/z* (%) = 442 $[M]^+$ (5), 411 (35), 234 (42), 203 (100), 189 (42), 133 (29).

Eugenol (**5**): R_f = 0.26 in *n*-hexane/$CHCl_3$ (3:7 v/v). – ^{1}H NMR ($CDCl_3$, 500 MHz): δ_H = 3.32 (2H, d, *J* = 6.65 Hz, H-7), 3.87 (3H, s, OCH_3), 5.05 (2H, m, H-9), 5.48 (1H, brs, OH), 5.95 (1H, m, H-8), 6.69 (2H, m, H-3 and H-5), 6.84 (1H, d, *J* = 8.5 Hz, H-6). – ^{13}C NMR ($CDCl_3$, 500 MHz): δ_C = 39.9 (C-7), 55.8 (OCH_3), 111.1 (C-3), 114.2 (C-6), 115.5 (C-9), 121.2 (C-5), 131.9 (C-4), 137.8 (C-8), 143.9 (C-1), 146.4 (C-2).

Catechin (**6**): R_f = 0.37 in $CHCl_3$/MeOH (8:2 v/v). – ^{1}H NMR (CD_3OD, 500 MHz): δ_H = 2.51 (1H, dd, *J* = 16.1, 8.2 Hz, H-4a), 2.86 (1H, dd, *J* = 16.1, 5.4 Hz, H-4b), 3.97 (1H, m, H-3), 4.57 (1H, d, *J* = 7.5 Hz, H-2), 5.86 (1H, d, *J* = 2.2 Hz, H-8), 5.93 (1H, d, *J* = 2.2 Hz, H-6), 6.72 (1H, dd, *J* = 8.1, 2 Hz, H-6'), 6.76 (1H, d, *J* = 8.1, H-5'), 6.84 (1H, d, *J* = 2 Hz, H-2'). – ^{13}C NMR (CD_3OD, 500 MHz): δ_C = 28.4 (C-4), 68.6 (C-3), 82.7 (C-2), 95.4 (C-8), 96.2 (C-6), 100.7 (C-10), 115.1 (C-2'), 116.0 (C-5'), 119.9 (C-6'), 132.0 (C-1'), 146.1 (C-3'), 146.1 (C-4'), 156.8 (C-9), 157.4 (C-5), 157.6 (C-7).

Niga-ichigoside F1 (2*α*,3*β*,19*α*,23-tetrahydroxy-urs-12-en-28-oic acid *β*-D-glucopyranosyl ester) (**7**): R_f = 0.47 in $CHCl_3$/MeOH (75:25 v/v). – ^{1}H NMR (CD_3OD, 500 MHz): δ_H = 0.7 (3H, s, H-24), 0.78 (3H, s, H-26), 0.93 (3H, d, *J* = 6.5 Hz, H-30), 1.04 (3H, s, H-25), 1.21 (3H, s, H-29), 1.34 (3H, s, H-27), 2.52 (1H, s, H-18), 3.25 (1H, d, *J* = 11 Hz, H-23a), 3.49 (1H, d, *J* = 11 Hz, H-23b), 3.67 (1H, m, H-2), 3.35–3.82 (5H, H_{Glc}-2–6), 5.31 (2H, d, *J* = 8.1 Hz, H_{Glc}-1, H-12). – ^{13}C NMR (CD_3OD, 500 MHz): δ_C = 13.9 (C-24), 16.6 (C-30), 17.6 (C-26), 17.7 (C-25), 19.2 (C-6), 24.7 (C-27), 24.8 (C-11), 26.5 (C-16), 27.1 (C-29), 27.2 (C-21), 29.6 (C-15), 33.5 (C-7), 38.3 (C-22), 38.9 (C-10), 40.8 (C-8), 42.8 (C-14), 42.9 (C-20), 44.1 (C-4), 48.0 (C-1), 48.2 (C-9), 48.4 (C-5), 54.9 (C-18), 66.4 (C-23), 69.7 (C-2), 73.6 (C-19), 78.3 (C-3), 129.7 (C-12), 139.5 (C-13), 178.5 (C=O); glucose: 62.4 (C-6'), 71.1 (C-4'), 73.8 (C-2') 78.3 (C-5'), 78.5 (C-3'), 95.8 (C-1'). – EI-MS: *m/z* (%) = 504 $[M–C_6H_{10}O_5]$ (4), 426 (23), 344 (100), 264 (15), 246 (18), 239 (22), 219 (17), 201 (34), 164 (72).

Gein (eugenyl vicianoside) (**8**): R_f = 0.2 in $CHCl_3$/MeOH (8:2 v/v). – ^{1}H NMR, ^{13}C NMR, and HMBC (CD_3OD, 500 MHz): see Table I.

Sucrose (**9**): R_f = 0.28 in EtOAc/MeOH (6:4 v/v). – ^{1}H NMR (D_2O, 500 MHz): δ_H = 3.31 (1H, t, *J* = 9.5 Hz, H_{Glc}-4), 3.4 (1H, dd, *J* = 10, 3.8 Hz, H_{Glc}-2), 3.51 (2H, s, H_{Fru}-1), 3.59 (1H, t, *J* = 9.5 Hz, H_{Glc}-3), 3.66 (4H, m, H_{Glc}-6 and H_{Fru}-6), 3.69 (1H, m, H_{Glc}-5), 3.72 (1H, m, H_{Fru}-5), 3.89 (1H, t, *J* = 8.5 Hz, H_{Fru}-4), 4.04 (1H, d, *J* = 8.7 Hz, H_{Fru}-3), 5.25 (1H, d, *J* = 3.8 Hz, H_{Glc}-1). – ^{13}C NMR (D_2O, 500 MHz): δ_C = 60.1 (C_{Glc}-6), 61.3 (C_{Fru}-1), 62.3 (C_{Fru}-6), 69.2 (C_{Glc}-4), 71.0 (C_{Glc}-2), 72.4 (C_{Glc}-5), 72.5 (C_{Glc}-3), 73.9 (C_{Fru}-4), 76.3 (C_{Fru}-3), 81.3 (C_{Fru}-5), 92.1 (C_{Glc}-1), 103.6 (C_{Fru}-2).

Table I. NMR data of compound **8** in CD_3OD.

Carbon No.	^{13}C NMR (ppm)	^{1}H NMR (ppm)	HMBC
1	146.1		H-3, H-5, H-6, H-1'
2	150.7		OCH_3, H-3, H-6
3	114.0	6.82 (d, J = 2 Hz, 1H)	H-5, H-7
4	136.4		H-3, H-5, H-6, H-7, H-8
5	122.2	6.76 (dd, J = 8.2, 2 Hz, 1H)	H-3, H-7
6	118.3	7.11 (d, J = 8.1 Hz, 1H)	
7	40.7	3.33 (d, J = 6.6 Hz, 2H)	H-3, H-5, H-8, H-9
8	139.0	5.95 (m, 1H)	H-7
9	115.8	5.04 (m, 2H)	H-7
OMe	56.6	3.84 (s, 3H)	
Glc			
1'	102.7	4.86 (d, J = 7.1 Hz, 1H)	
2'	74.9	3.48 (m, 1H)	
3'	77.6	3.46 (m, 1H)	H-4'
4'	71.4	3.40 (m, 1H)	
5'	77.3	3.58 (m, 1H)	H-4', H-6'
6'	69.1	3.75 (m, 1H)	H-4', H-1"
		4.08 (dd, J = 11.5, 2.1 Hz, 1H)	
Ara			
1"	104.7	4.28 (d, J = 6.8 Hz, 1H)	H-6'a, H- 6'b, H-2", H-5"a, H-5"b
2"	72.4	3.56 (m, 1H)	
3"	74.1	3.48 (m, 1H)	H-1", H-2", H-4"
4"	69.4	3.76 (m, 1H)	H-5"a, H-5"b
5"	66.6	3.44 (m, 1H)	H-1"
		3.81 (dd, J = 12.3, 3.3 Hz, 1H)	

Results

The inhibition zone diameters (IZD) of test samples and antibiotics against *H. pylori* are summarized in Tables II and III. No inhibition zone was observed for DMSO in control plates. The MIC values of the aqueous fraction and ciprofloxacin (as a positive control) against several Gram-positive and Gram-negative bacteria are shown in Table IV.

From the EtOAc and MeOH extracts of the roots, nine compounds including two triterpenoids, uvaol (**4**) (Mahato and Kundu, 1994) and niga-ichigoside F1 (**7**) (Bowen-Forbes *et al.*, 2009), three sterols, *β*-sitosterol (**3**), *β*-sitosteryl acetate (**1**) (Goad and Akihisa, 1997), and *β*-sitosteryl linoleate (**2**) (Huh *et al.*, 2010; Dyas *et al.*, 1991), one phenyl propanoid, eugenol (**5**), one phenolic glycoside, gein (**8**) (Takeda *et al.*, 1998; Shimoda *et al.*, 2007; Shigenaga *et al.*, 1985), one flavanol, (+)-catechin (**6**) (Banavides *et al.*, 2006), and sucrose (**9**) (Agrawal, 1992) were isolated by column chromatography and identified by comparison of their spectroscopic data with those in the literature (Fig. 1). Spectroscopic data such as ^{1}H and ^{13}C NMR, HMQC, HMBC, ^{1}H-^{1}H COSY and EI-MS were employed for identification of the isolated compounds. In the previous study, the NMR data of gein were reported in $CDCl_3$ and pyridine-d_5 (Shigenaga *et al.*, 1985). To the best of our knowledge, there is no report on the 2D-NMR correlations of this compound, so that this is the first report on the NMR data of gein in CD_3OD and HSQC and HMBC correlations (Table I and Fig. 2).

Discussion

The MeOH extract of *G. iranicum* was considered an effective extract against one of the clinical isolates of *H. pylori* which was resistant to metronidazole. As shown in Table II, the chloroform fraction, obtained from the MeOH extract, was not effective against *H. pylori*, but the aqueous fraction showed a potent activity (Table II). Subfractions of the aqueous part were evaluated for their antibacterial activity against all isolates. Fraction D was the only one that displayed antibacterial activity (MIC = 25 μg/ml). Therefore, the fractions obtained from D fractionation were

Table II. Inhibition zone diameters (mm) of some extracts of *G. iranicum.*

Sample	Mean inhibition zone diameter [mm][a]											
	100 μg/ml			50 μg/ml			25 μg/ml			12.5 μg/ml		
	Is.1[b]	Is.2	Is.3	Is.1	Is.2	Is.3	Is.1	Is.2	Is.3	Is.1	Is.2	Is.3
Ethyl acetate extract	-	-	-	-	-	-	-	-	-	-	-	-
Methanol extract	15±0.5	-	-	-	-	-	-	-	-	-	-	-
Chloroform fraction	-	-	-	-	-	-	-	-	-	-	-	-
Aqueous fraction	35±0.5	30±0.5	24±0.5	28±0	26±0.5	18±0.25	24±0.57	24±0.5	16±0	20±0.5	19±0.5	14±0
Fraction D	22±0.5	21±0.25	12±0.5	15±0.5	12±0.5	10±0.5	12±0	10±0.5	-	-	-	-
Fraction D_4	17±0.25	16±0.5	20±0	12±0.57	12±0.5	10±0.5	10±0.5	10±0.28	-	10±0.25	9±0	-
Eugenol	20±0.5	22±0.28	10±0.5	-	-	-	-	-	-	-	-	-
Catechin	-	-	-	-	-	-	-	-	-	-	-	-
Niga-ichigoside F1	-	-	-	-	-	-	-	-	-	-	-	-
Gein	-	-	-	-	-	-	-	-	-	-	-	-

[a] The results are shown as mean ± SD (n = 3).
[b] Is, clinical isolate of *H. pylori*.

Table III. Inhibition zone diameters (mm) of some antibiotics against clinical isolates of *H. pylori.*

Antibiotic [μg/ml]	Inhibition zone diameter [mm]		
	Is.1[a]	Is.2	Is.3
Furazolidone (MIC 0.5 μg/ml)			
2	34	19	25
1	22	15	21
0.5	11	10	18
0.25	-	-	13
Tetracycline (MIC 2 μg/ml)			
4	20	-	-
2	13	-	-
1	-	-	-
0.5	-	-	-
Amoxicillin (MIC 0.5 μg/ml)			
2	40	30	36
1	30	24	25
0.5	20	12	15
0.25	11	-	10
Clarithromycin (MIC 2 μg/ml)			
4	-	35	33
2	-	28	27
1	-	24	24
0.5	-	21	19
Metronidazole (MIC 8 μg/ml)			
32	-	-	-
16	-	-	-
8	-	-	-
4	-	-	-

[a] Is, clinical isolate of *H. pylori*.

Table IV. Minimum inhibitory concentrations of the aqueous fraction of *G. iranicum* against several Gram-positive and Gram-negative bacteria.

Bacterial strain[a]	Minimum inhibitory concentration	
	Aqueous fraction [mg/ml]	Ciprofloxacin [μg/ml]
S. aureus	16	0.19
S. epidermidis	16	0.39
B. subtilis	16	0.19
E. coli	32	0.013
P. aeruginosa	32	0.39

[a] The bacterial strains are *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027.

evaluated again and resulted in one active fraction D_4 (Table II). Finally, the isolated compounds from the chloroform and aqueous fractions (eugenol, gein, catechin, and niga-ichigoside F1) were examined for anti-*H. pylori* activity. Among the tested compounds, eugenol (**5**), isolated from the chloroform fraction, showed an antibacterial effect at a concentration of 100 μg/ml with inhibition zones of 20, 22, and 10 mm against the isolates 1, 2, and 3 respectively. Eugenol is a major compound in the root oil of *G. iranicum* (Shahani *et al.*, 2011) and is employed as a flavour-

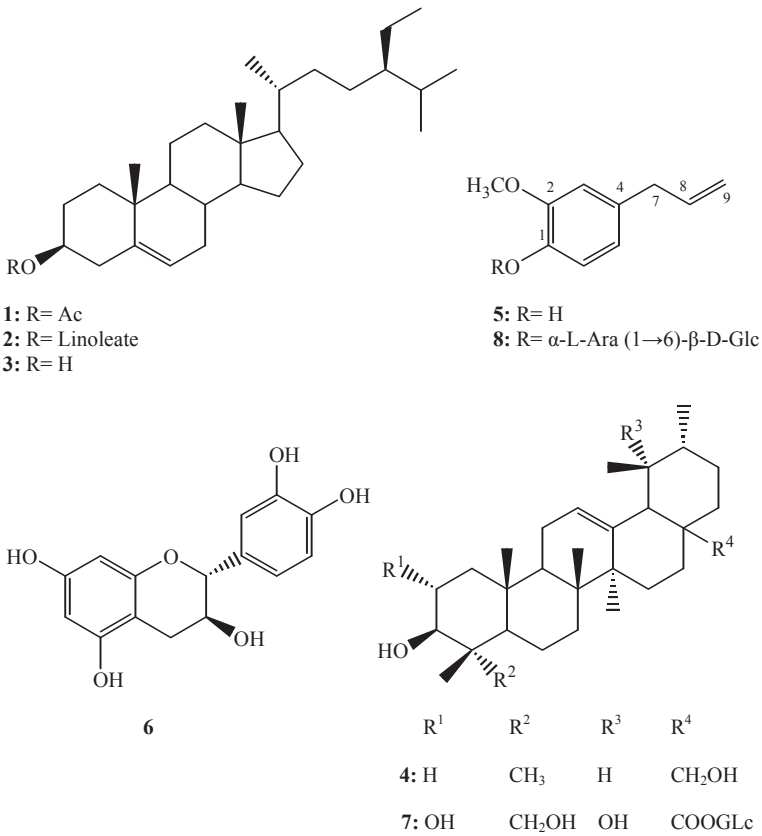

Fig. 1. Chemical structures of the isolated compounds.

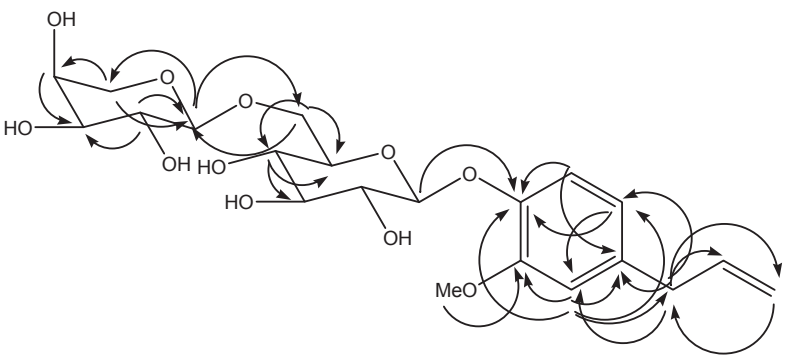

Fig. 2. HMBC correlations of compound **8** (H→C).

ing agent in cosmetics and food products, as well as a cement material in dentistry (Atsumi *et al.*, 2005). Furthermore, eugenol has been found to exhibit a broad range of biological activities including antibacterial (Devi *et al.*, 2010), antifungal (Campaniello *et al.*, 2010), antiviral (Benencia and Courreges, 2000), antioxidant (Atsumi *et al.*, 2005), anti-inflammatory (Yogalakshmi *et al.*, 2010), anti nociceptive (Daniel *et al.*, 2009), and antidepressant activity (Tao *et al.*, 2005). A literature review shows that the minimal bactericidal concentration (MBC) of eugenol (obtained from essential oils) was 100 μg/ml against one strain of *H. pylori*, which had been isolated from a pa-

tient with non-ulcer dyspepsia (Bergonzelli *et al.*, 2003). Elsewhere it was reported that eugenol (commercial preparation) inhibited the growth of 30 strains of *H. pylori* at a concentration of 2 μg/ml (Ali *et al.*, 2005). Our antibacterial data for eugenol agree with the results reported by Bergonzelli *et al.* (2003).

Diglycosylated eugenol, named gein (**8**), produced no inhibition zone. Gein has been isolated from other *Geum* species (Shigenaga *et al.*, 1985), and no biological activity has been reported for this compound. It seems that glycosylation of eugenol causes a decrease in anti-*H. pylori* activity. Also other purified compounds like catechin (**6**) and niga-ichigoside F1 (**7**) did not display any antibacterial activity at all concentrations.

Fraction D_4 exhibited antibacterial activity as shown in Table II. Further investigation of fraction D_4 indicated that this fraction can precipitate a solution of gelatin (1%), containing sodium chloride (10%), and give a blue-black precipitate with ferric chloride. Therefore, this fraction should include polar compounds, especially hydrolysable tannins (Evans, 2009). There is a report in the literature on the antibacterial activity of hydrolysable tannins derived from medicinal plants against *H. pylori* (Funatogawa *et al.*, 2004). For this reason, it seems that tannins from *G. iranicum* might be one of the important groups of compounds active against *H. pylori.*

The aqueous fraction exhibited a moderate inhibitory activity against both Gram-positive and Gram-negative bacteria. The Gram-positive bacteria were inhibited at 16 mg/ml, while Gram-negative bacteria were inhibited at about 32 mg/ml.

In conclusion, considering the increasing resistance of *H. pylori* to antibiotics like metronidazole and clarithromycin, there is great interest in finding new drugs from natural sources. The results of this study show that *G. iranicum* has a significant antibacterial activity against resistant clinical isolates of *H. pylori*. Eugenol is one of the effective compounds, whereas other purified compounds (like gein, catechin, and niga-ichigoside F1) were not effective. Finally, the tannin-containing fraction of *G. iranicum* showed considerable anti-*H. pylori* activity. Further investigation is required to purify the active tannins from this plant.

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