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# Anti-*Helicobacter pylori* flavonoids from licorice extract

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Licorice is the most used crude drug in Kampo medicines (traditional Chinese medicines modified in Japan). The extract of the medicinal plant is also used as the basis of anti-ulcer medicines for treatment of peptic ulcer. Among the chemical constituents of the plant, glabridin and glabrene (components of *Glycyrrhiza glabra*), licochalcone A (*G. inflata*), licoricidin and licoisoflavone B (*G. uralensis*) exhibited inhibitory activity against the growth of *Helicobacter pylori in vitro*. These flavonoids also showed anti-*H. pylori* activity against a clarithromycin (CLAR) and amoxicillin (AMOX)-resistant strain. We also investigated the methanol extract of *G. uralensis*. From the extract, three new isoflavonoids (3-arylcoumarin, pterocarpan, and isoflavan) with a pyran ring, gancaonols A–C, were isolated together with 15 known flavonoids. Among these compounds, vestitol, licoricone, 1-methoxyphaseollidin and gancaonol C exhibited anti-*H. pylori* activity against the CLAR and AMOX-resistant strain as well as four CLAR (AMOX)-sensitive strains. Glycyrin, formononetin, isolicoflavonol, glyasperin D, 6,8-diprenylorobol, gancaonin I, dihydrolicoisoflavone A, and gancaonol B possessed weaker anti-*H. pylori* activity. These compounds may be useful chemopreventive agents for peptic ulcer or gastric cancer in *H. pylori*-infected individuals. D 2002 Elsevier Science Inc. All rights reserved.

: Anti-*Helicobacter pylori*; Flavonoids; Licorice; *Glycyrrhiza*; Gancaonol

## Introduction

*Helicobacter pylori* is a bacterium that lives in the stomach and duodenum. The bacterium is generally recognized as one of the etiological agents of peptic ulcer. Therefore, ulcer patients with *H.*

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*pylori* infection require treatment with antimicrobial agents in addition to anti-secretory drugs, whether on first presentation with the illness or on recurrence [1]. On the other hand, gastric cancer is one of the most frequent cancers and the leading cause of death from cancer in developing countries as well as in Japan and Russia, although it is relatively uncommon in Western nations. Since the discovery of *H. pylori* [2,3], an association between the bacterium and gastric cancer has been suspected. Descriptive epidemiological data indicate that gastric cancer occurs more frequently in some populations that have higher rates of *H. pylori* infection. Furthermore, rates of both *H. pylori* infection and gastric cancer are correlated inversely with socioeconomic status and increase as a function of age and/or intake of dietary salt [4,5]. Recently, Uemura et al. reported the first evidence of the association between *H. pylori* and gastric cancer with a long-term study (range, 1.0 to 10.6 years) [6]. They studied a large group of Japanese patients with duodenal ulcer, gastric hyperplasia, or non-ulcer dyspepsia. During the study, gastric cancer developed in 2.9% of the patients infected with *H. pylori* but in none of the uninfected patients. The association between *H. pylori* infection and gastric cancer ranks with that between smoking and lung cancer [4]. However, most people with chronic *H. pylori* infection have no symptoms of peptic ulcer or gastric cancer, which raises questions regarding preventive agents against these diseases; infected individuals without disease symptoms may be protected by anti-bacterial compounds in the diet and/or medicinal plants used frequently [7 – 21]. Many anti-*H. pylori* agents with a diversity of structures have been isolated from plant sources [22 - 34]. However, their antibacterial activities against the bacterium in stomach are unclear [35] as the bacterium in the narrow interface between the gastric epithelial cell surface and the overlying mucus gel [1,3]. Among these antibacterial compounds, flavonoids could be expected to show anti-*H. pylori* effects *in vivo*, because kaempferol (4V,5,7- trihydroxyflavonol) exhibited antibacterial action in *H. pylori*-infected Mongolian gerbils [25]. As the biological activities of flavonoids are generally weak, the phenolic compounds may act as bacterial suppressors in the stomach.

Licorice (liquorice, kanzoh in Japanese, gancao in Chinese) is the name applied to the roots and stolons of some *Glycyrrhiza* species (*G. glabra, G. uralensis, G. inflata, G. eurycarpa, G. aspera*, and *G. korshinskyi*; Leguminosae) and has been used for medicinal purposes for at least 4000 years. Many isoprenoid-substituted flavonoids have been isolated from these plants [36]. The flavonoid-rich fraction from the extract of *G. uralensis* has been used as an anti-ulcer medicine [37,38]. Anti-*H. pylori* activities of flavonoids from the extract of *G. glabra* were reported previously [39] but there have been no such reports concerning *G. uralensis*. Therefore, we studied anti-*H. pylori* activities of the flavonoids from *G. uralensis*.

## Materials and methods

### *Bacterial strains used and culture conditions*

*H. pylori* ATCC 43504 and ATCC 43526 were obtained from the American Type Culture Collection (Rockville, MD, USA). *H. pylori* ZLM 1007 (KO-1007) and ZLM 1200 were isolated from human stomach biopsy specimens [40]. Clarithromycin (CLAR)-resistant *H. pylori* GP98 was kindly provided by Dr. T. Ito, Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan. All bacteria used in following investigations were spiral form.

### Chemicals

Organic solvents were purchased from Wako Pure Chem. Ind., Ltd. (Osaka, Japan), Godo Solvent Ltd. (Tokyo, Japan), and Isotec Inc. (Miamisburg, OH, USA), and the following chemicals were obtained from the sources indicated: Sephadex LH-20 (Pharmacia Fine Chem. AB, Uppsala, Sweden); Chromatorex octadecylsilyl silica gel (ODS, 100–200 mesh) (Fuji Silysia Chem., Ltd., Kasugai, Japan); Wakogel C-200 and B-5F silica gel ($SiO_2$) (Wako Pure Chem. Ind. Ltd.); Merck RP-18$F_{254S}$ TLC plates (Darmstadt, Germany); amoxicillin (AMOX) and angiotensin VI (Sigma Chem. Ind., St. Louis, MO, USA); α-cyano-4-hydroxycinnamic acid (α-CHCA) (Aldrich, Milwaukee, WI, USA). Glycyrrhizic acid (1), glycyrrhetic acid (2), and licorice-saponin G2 (3) were isolated by the procedure described previously [41]. Licorice flavonoids (4 – 14) used for the assay were produced in our previous study [36].

### Drug samples

Roots of *Glycyrrhiza uralensis* were purchased from Matuura Yakugyo Ltd. (Nagoya, Japan). The plants were purchased in China and prepared for drugs by the company in Japan, Glycyrrhizae Radix lot. 55K3AM5S.

### Apparatus

The UV spectra were measured with a Shimadzu UV-265 spectrophotometer (Shimadzu, Kyoto, Japan). The $^1$H NMR spectra were recorded on Jeol JNM EX-400 or EXP-500 NMR spectrometers (Jeol Ltd., Akishima, Japan). Chemical shifts were reported with respect to acetone-$d_6$ ($\gamma_H$ 2.04 and $\gamma_C$ 206.0) and dimethyl-$d_6$ sulfoxide (DMSO-$d_6$, $\gamma_H$ 2.49). Optical rotations and CD spectra were measured with a Jasco DIP-370 digital polarimeter and J-720W CD spectrometer, respectively (Jasco Co., Hachioji, Japan). Electron ionization (EI) mass spectra (MS) and high-resolution (HR) EI-MS were measured with a Jeol JMS-AMM System II-50 and HX-100 instruments, respectively. HR matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS was measured with a Voyager-DE STR TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA). Matrix used was α-CHCA and calibration was performed with two internal standards, α-CHCA ($[2M + H]^+$) and angiotensin IV ($[M + H]^+$) [42]. HPLC was carried out on a Jasco PU-980 instrument equipped with an UV detector using Pegasil ODS or Silica-120-5 columns (1.0 25.0 cm) (Senshu Science Co. Ltd., Tokyo, Japan).

### Testing for anti-H. pylori activity by disk method

*H. pylori* ATCC 43526 was incubated microaerobically on Brucella agar (Difco Laboratories, Detroit, MI, USA) supplemented with 7% horse serum (Nippon Bio-Test laboratories, Tokyo, Japan) at 37 jC for 3 days in GasPak® jars (BBL Microbiology Systems, Cockeysville, MD, USA) (HSBA) with AnaeroPack® Campylo (Mitsubishi Gas Kagaku, Tokyo, Japan). Colonies were suspended in Brucella broth to give the turbidity equivalent to $OD_{570}$ = 0.25; this resulted in suspensions containing about 2 $10^7$ colony forming units (cfu)/mL. Final concentration of the bacteria used was 4 $10^5$ cfu/mL. All test samples were dissolved in ethyl acetate (EtOAc) at 10 mg/mL. The stock solution was diluted to 1mg/mL with the solvent. Air-dried 8 mm paper disks charged with 50 AL of the sample solution were placed

on agar plates. Diameters of inhibition zones were determined after 3 days of incubation at 37 jC under microaerophilic conditions.

### *Determination of minimum inhibitory concentrations (MICs) by the agar dilution method*

The MIC of the purified substance was determined by the agar dilution method [43]. ***H. pylori*** was incubated as described above. The test compounds were initially dissolved in DMSO and then further diluted in a twofold series with DMSO. The bacterial suspensions were inoculated on HSBA plates containing twofold serial dilutions of the substances with a micro-planter (Sakuma Industry, Tokyo, Japan) capable of delivering 5 AL of suspension. MICs were determined after 3 days of incubation at 37 jC under microaerophilic conditions. MICs were defined as the lowest concentrations of the test compounds inhibiting visible bacterial growth.

### *Extraction of phenolic compounds from Glycyrrhizin uralensis*

The roots of ***G. uralensis*** (300 g) were extracted four times with 2 L of MeOH at room temperature (r.t.) for 2 days each time. The MeOH solutions were combined and then evaporated ***in vacuo*** to yield 69 g of residue (Fr ME). The residue was treated according to the procedure reported previously with slight modifications [44]. A part of the residue (67 g) was extracted with chloroform ($CHCl_3$) at r.t. to give 2.2 g of $CHCl_3$-soluble fraction (Fr CH) and 6.5 g of $CHCl_3$-insoluble fraction (Fr CHI). The Fr CH residue was refluxed with benzene ($C_6H_6$) for 30 min ( 3). The $C_6H_6$-soluble portion of Fr CH (Fr BE) was washed with $H_2O$. The $H_2O$ solution was lyophilized to give Fr A (195 mg). The washed $C_6H_6$ solution (Fr BEW) was extracted successively with 5% $NaHCO_3$ (Fr B) and 5% $Na_2CO_3$ (Fr C) solutions. These basic solutions were neutralized with dil. HCl and then extracted with EtOAc. After the standard treatment, 82 mg of Fr B and 225 mg of Fr C were obtained. The $C_6H_6$-insoluble portion of Fr CH was extracted with 5% $Na_2CO_3$ solution (Fr E) and then treated according to the standad procedure. The acidic fraction (Fr E, 339 mg) and non-acidic fraction (Fr F, 46 mg) were obtained with the above procedure. The antibacterial activities of these fractions are shown in Table 1. Fr C corresponds to the anti-ulcer drug FM100 [37,38,44,45].

Table 1
Anti-***Helicobacter pylori*** activity (paper disk method) of semi-purified fractions of ***G. uralensis***

| | 10 mg/mL | 1 mg/mL |
|---|---|---|
| | (diameter of the inhibition zone) | |
| Fr ME | 19.0 mm | negative |
| Fr CH | 21.5 mm | 14.0 mm |
| Fr CHI | 15.5 mm | negative |
| Fr BE | 20.0 mm | 15.0 mm |
| Fr A | 21.5 mm | negative |
| Fr B | 32.5 mm | 15.0 mm |
| Fr C | 29.0 mm | 16.0 mm |
| Fr D | 16.0 mm | 11.5 mm |
| Fr E | 11.5 mm | negative |
| Fr F | negative | negative |

The inhibition zone of the positive control (AMOX, 5 Ag/mL) was 37.0 mm.

### *Isolation of flavonoids from G. uralensis*

Isolation was guided by TLC analysis compared with the above bioactive fraction (Fr B and Fr C) and the inhibitory activities against growth of methicillin-resistant ***Staphylococcus aureus*** (MRSA), methicillin-sensitive ***S. aureus*** (MSSA) and ***H. pylori*** with the paper disk method (IAHP-PDM). The isolation of anti-MRSA and anti-MSSA flavonoids will be reported elsewhere. The roots of ***G. uralensis*** (5 kg) were treated according to the above procedure to yield 48.6 g of Fr BEW; IAHP-PDM, 23.7 mm (10 mg/mL), 14.5 mm (1 mg/mL). A part of Fr BEW (46 g) was fractionated by $SiO_2$ column chromatography (C.C.) eluted with $C_6H_6$-EtOAc (100:0 - 0:100, column 1). The bioactive fractions (eluted with $C_6H_6$-EtOAc, 9:1 - 3:7, IAHP-PDM, 10 mg/mL: 11.5 - 14.0 mm) were combined (2.6 g) and purified with $SiO_2$ C.C. (*n*-hexane-EtOAc, column 2). From the active fractions of column 2 (*n*-hexane-EtOAc, 80:20 – 76:24, IAHP-PDM, 10 mg/mL: 24.5 mm), 3-*O*-methylglycyrol (**15**, 8 mg) and licoricidin (**11**, 464 mg) were isolated by fractional crystallization. The mother liquor was separated by preparative (p.) TLC ($SiO_2$, *n*-hexane-EtOAc, 3:2, $CHCl_3$-acetone, 20:1) to give 2 mg of gancaonol A (**16**). The other active fractions of the column (*n*-hexane-EtOAc, 74:26, IAHP-PDM, 10 mg/mL: 24.0 - 32.0 mm) were combined (5.7 g) and dissolved in $C_6H_6$. The precipitate (4.3 g) was purified by fractional crystallization from $C_6H_6$-MeOH (1:1), $SiO_2$ C.C. (*n*-hexane-acetone), p. TLC ($SiO_2$, *n*-hexane-acetone, *n*-hexane-EtOAc, $CHCl_3$-EtOAc), and p. HPLC ($SiO_2$, $CHCl_3$-EtOAc, *n*-hexane-EtOAc) to give formononetin (**6**, 22 mg), glycyrol (**12**, 8 mg), isoglycyrol (**13**, 170 mg), vestitol (**17**, 105 mg), licoricone (**18**, 27 mg), glycyrin (**19**, 76 mg), isolicoflavonol (**20**, 7 mg), and gancaonol B (**21**, 2.5 m). The mother liquor (1.4 g, IAHP-PDM, 10 mg/mL: 32.0 mm) was purified with Sephadex LH-20 C.C. (MeOH), ODS C.C. (MOH-$H_2O$), p. TLC (ODS, -$H_2O$, $SiO_2$, *n*-hexane-acetone, *n*-hexane-EtOAc), and p. HPLC ($SiO_2$, n-hexane-EtOAc, $CHCl_3$-EtOAc, ODS, MeOH-$H_2O$, -$H_2O$) to yield **13** (12 mg), **17** (60 mg), **20** (21 mg), lupiwighteone (**22**, 3 mg), 6,8-diprenylorobol (**23**, 3 mg), glyasperin D (**24**, 4 mg), 1-methoxyphaseollidin (**25**, 1 mg), gancaonin I (**26**, 12 mg), gancaonol C (**27**,3 mg), and dihydrolicoisoflavone A (**28**, 1.5 mg; $[a]_D^{24}$ + 30 j, *c* 0.013, EtOH; CD, *c* 2.3 10 $^5$ mol/L, EtOH, u (nm): 0 (236), 2800 (258), 2900 (306), 3900 (344), 0 (395). The original trivial name of the compound was dihydrolicoisoflavone [46], but we use here a tentative name ' 'dihydrolicoisoflavone A' ' for ease of recognition of its structure. The structures of these compounds were identified by comparison of their spectral data with those reported previously or elucidated by spectroscopy [36,46,47]. The known compounds **23** [47] and **28** were first isolated from ***Glycyrrhiza*** species. The anti-***H. pylori*** activities of the isolated compounds are shown in Table 3. Data for compounds **6**, **11** and **13** (Table 2), and compounds unavailable for the test due to their low yield (stock samples of the compounds were used for the test when they were available) are not shown.

### *Gancaonol A (16)*

Amorphous powder. $^1$H NMR (acetone-$d_6$, 400 MHz): y 8.44 (1H, br s, OH-7), 7.99 (1H, br s, H-4), 7.29 (1H, br d, *J* = 9 Hz, H-6V), 6.53, 6.51 (each 1H, d, *J* = 2 Hz, H-6 and H-8), 6.52 (1H, d, *J* = 9 Hz, H-5V), 3.91 (3H, s, OMe-2V), 3.78 (3H, s, OMe-5), 2.84 (2H, t, *J* = 7 Hz, $H_2$-7V), 1.87 (2H, t, *J* = 7 Hz, H-8V), 1.36 (6H, s, $H_3$-10Vand $H_3$-11V). $^{13}$C NMR (acetone-$d_6$, 125 MHz): y 162.1 (C-5), 161.8 (C-2), 159.1 (C-4V), 157.1 (C-7), 156.6 (C-2V), 154.5 (C-8a), 137.9 (C-4), 132.7 (C-6V), 122.3 (C-3), 116.7 (C-4a), 112.9 (C-3V), 108.1 (C-1V), 106.5 (C-5V), 110.5 (C-8), 76.5 (C-9V), 62.5 (OMe-2V), 55.6 (OMe-5), 32.4 (C-8V), 26.9 (C-10Vand C-11V), 17.6 (C-7V). EI-MS *m/z*: 383 $[M + 1]^+$ (17), 382 $[M]^+$ (85), 327 (36), 284