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Anti-*Helicobacter pylori* activity of Swertianolin, isolated from swertia herb

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Abstract

Helicobacter pylori is a Gram-negative, spiral-shaped, motile bacterium present in human stomachs that causes gastric ulcers. A preliminary screening revealed that a methanolic extract of swertia herb demonstrated anti-*H. pylori* activity. Swertia herb (*Swertia japonica* Makino, Gentianaceae) is a well-known Japanese traditional medicine to treat gastrointestinal diseases. In this study, we explored the active compounds in methanolic extract of swertia herb. The dried extract was dissolved in water and partitioned with *n*-hexane, ethyl acetate, and *n*-butanol, successively. The part soluble in ethyl acetate showed effective anti-*H. pylori* activity, and two compounds, swertianolin (**1**) and isoorientin (**2**), were isolated. The IC_{50} values of **1**, **2**, and amoxicillin (AMPC) which is used as positive control were 6.1, 177.0, and 0.044 μM, respectively. The minimum bactericidal concentration (MBC) values of **1** and AMPC were 91.7 and 0.21 μM, respectively. The MBC of **2** could not be determined (>892.9 μM). Furthermore, synergy was observed when compound **1** was used in combination with AMCP. Therefore, **1** could be considered as one of the active compounds of swertia herb. To our knowledge, the anti-*H. pylori* activities of methanolic extract of swertia herb and its isolated compound have never been reported.

Keywords: *Swertia japonica*, *Helicobacter pylori*, Swertianolin

Introduction

Helicobacter pylori is a micro-aerophilic Gram-negative, spiral-shaped, motile bacterium that infects the human gastric mucosa, and is estimated to inhabit approximately 45% of the global human population [1]. The Japanese people show a high incidence of *H. pylori* [2]. This bacterium is associated with gastric disorders such as chronic gastritis, duodenal ulcer, and gastric cancer [3]. In general, triple therapy consisting of a proton pump inhibitor or a potassium-competitive acid blocker combined with amoxicillin and clarithromycin or metronidazole, is employed for the eradication treatment of *H. pylori* [4]. Many medicinal herbs have been traditionally used to treat stomach problems since ancient times in Japan. We screened the anti-*H. pylori* activities of traditional natural medicines using *in vitro* assays. In the present study, swertia herb (*Swertia japonica*, Gentianaceae), which is listed in the Japanese Pharmacopoeia [5] as SWERTIAE HERBA, was selected as a candidate against *H. pylori*. This herb is a well-known Japanese folk medicine to treat gastrointestinal diseases and contains secoiridoid glycosides, such as swertiamarin and gentiopicroside, which are bitter-tasting substances [6]. The hepatoprotective effect against D-galactosamine/lipopolysaccharide-induced liver injury in mice [7] and stimulation of gastric emptying and gastrointestinal motility by inhibiting the dopamine D2 receptor [8] were also reported in previous studies of swertia herb. We

used the whole herb of *Swertia japonica* Makino (Gentianaceae), collected during the blooming season. In this study, we evaluated anti-*H. pylori* activities of *n*-hexane, ethyl acetate, *n*-butanol, and aqueous soluble fractions prepared from the methanolic extract of swertia herb.

Material and methods

Plant materials

Swertia herb (H862903) (whole plant of *Swertia japonica*) was purchased from UCHIDAWAKANYAKU Ltd. (Arakawa-ku, Tokyo, Japan).

Preparation of extracts

The whole parts of *S. japonica* (2.0 kg) were extracted with methanol by reflux for an hour, and the cycle was repeated three times. The methanolic extracts were filtered through a filter paper and evaporated to yield dried residues. The residues were suspended in water and partitioned with *n*-hexane. The water soluble portion was partitioned with ethyl acetate and *n*-butanol to afford corresponding soluble parts (*n*-hexane part, 11.7 g; ethyl acetate part, 1.7 g; *n*-butanol part, 47.1 g; and water part, 5.5 g). The ethyl acetate part was subjected to chromatography on a silica gel open column (Silica gel 60, ϕ 40 × 560 mm; KANTO CHEMICAL CO., INC., Chuo-ku, Tokyo, Japan) with chloroform–methanol (8:2, v/v) as a solvent to yield 21 fractions. Compound **1** (7.1 mg) was isolated

as pure compound from Fraction 19. Fractions 16 and 17 (134 mg) were chromatographed on an ODS column (ODS-SM 50C, ϕ26 × 300 mm; Yamazen Corporation, Osaka, Osaka, Japan) with water–methanol (2:1 → 3:2 → 1:1) to isolate compound **2** (24.4 mg).

Identification

Compounds **1** and **2** were identified as swertianolin and isooreintin, respectively. Their structures were confirmed by comparing their NMR and MS spectroscopic data [9, 10].

Measurement of anti-*H. pylori* activity

A strain of *H. pylori* (ATCC43504) was purchased from American Type Culture Collection (Biofluid, Inc. Rockville, MD, USA). *H. pylori*-selective agar culture plate was purchased from Nissui Pharmaceutical Co., LTD. (Taito-ku, Tokyo, Japan). The micro-dilution broth method was used to determine the IC_{50}. Brain Heart Infusion (BHI) broth, containing 10% fetal bovine serum (FBS) (Biofluid, Inc. Rockville, MD, U.S.A.) and 0.1% glucose, was used as the medium and the strain of *H. pylori* (ATCC43504) was cultured in a jar conditioned with AnaeroPack (Campylo) (Mitsubishi Gas Chemical Co., Inc. Chiyoda-ku, Tokyo, Japan). Briefly, *H. pylori* was inoculated on the selective agar culture plate and cultured at 37 °C for 5 days. The collected bacterial colonies were dispersed and diluted to 10^7 colony forming unit (CFU)/mL with the medium. The fractions were dissolved in dimethyl sulfoxide, and then diluted with the medium. To the

solution of the fractions, a suspension of bacteria was added to make 10^6 CFU/100 μL/well. The mixture was incubated at 37°C for 3 days. The IC_{50} values of the fractions were determined by observation of the turbidity with microplate reader [11]. The MBC was determined by inoculating the *H. Pylori* selective agar culture plate from the IC test well plate.

Evaluation of the combination of isolated compounds and AMCP

The combination of **1** and AMPC, and **2** and AMPC against a *H. pylori*, was determined by Checkerboard test [12-14]. Isolated compounds and AMPC were serially diluted in DMSO and then dissolved in micro-dilution broth medium. These were mixed in microplates. FIC was calculated as follows:

$$\text{FIC} = \frac{\text{MIC of drug A in combination}}{\text{MIC of drug A alone}} + \frac{\text{MIC of drug B in combination}}{\text{MIC of drug B alone}}$$

The FIC index is interpreted as follows: ≤0.5, synergy; 0.5–1, additive; 1–4.0, indifference; >4.0, antagonism.

Results and Discussion

Our previous study of screening natural medicines with anti-*H. pylori* activity revealed

the efficacy of the methanolic extract of swertia herb. Therefore, in this study, we aimed to elucidate the active compounds of swertia herb. The methanolic extract was dissolved in water and *n*-hexane, ethyl acetate, and *n*-butanol soluble parts were obtained by partitioning the water fraction. The *n*-hexane and ethyl acetate-soluble parts demonstrated anti-*H. Pylori* activity, which is shown in Table 1. Although the *n*-hexane fraction showed the highest potency (0.45±0.15 mg/mL), the ethyl acetate fraction (1.85±0.65 mg/mL) was chosen for isolation of active compounds considering chromatographic efficiency based on TLC analysis (Supplementary Material, Fig. S1). Incidentally, the extraction of *n*-hexane was conducted for defatting. As the result, good separations of EtOAc soluble part were obtained compared to *n*-hexane soluble part on TLC analysis. To identify the active compounds responsible for anti-*H. pylori* activity of swertia herb, we fractioned the ethyl acetate soluble part by chromatography. Compounds **1** and **2** were isolated and identified as swertianolin and isooreintin, respectively (Fig. 1). Their structures were confirmed by spectroscopic analysis including MS and NMR (Supplementary Material, Fig. S2−5). The isolated compounds **1** and **2,** including swertiamarin, which is the representative compound of swertia herb, were tested for anti-*H. pylori* activity *in vitro*. The degrees of inhibitory activity were expressed as MBC and IC_{50} values. As shown in Table 2, **1** showed the strongest anti-*H. pylori* activity (MBC; 91.7±28.1 μM and IC_{50};

6.1±1.5 μM). The effect of **1** was not as effective as AMPC, which was used as a positive control. The activity of **2** was weaker than that of **1** (MBC; >892.9 μM, IC_{50};177.0±27.6 μM). These results suggested that **1** and **2** had bactericidal and bacteriostatic effects, respectively. Although the methanolic extract of swertia herb exhibited anti-*H. pylori* activity, the representative compound of swertia herb, swertiamarin did not show any inhibitory activity. Furthermore, the combination of **1** and AMPC, and **2** and AMPC were examined by the checkerboard method and showed synergistic and additive effects, respectively (Table 3). Thus, the isolated compound **1** could potentially be used as an adjunctive agent to *H. pylori* infection prophylaxis and eradication therapy. To the best of our knowledge, the anti-*H. pylori* activities of methanolic extract of swertia herb and its isolated compound have never been reported. Multi-drug resistance in *H. pylori* is of significant clinical concern [15]. In the future, we aim to screen an appropriate antibiotic combination with **1,** using the Break-point Checkerboard Plate [16]. We also plan to evaluate the mechanism of action of **1** by screening its urease activity. At least, it was demonstrated that swertianolin has anti-*H. pylori* activity using a metronidazole-resistant *H. pylori* strain (ATCC 43504) [17]. It has been reported that the 1,3,5,6-oxygenated function comprising substitutions at C-4 and at 3-hydroxyl positions in xanthone nucleus played important roles for strong inhibitory activity against the *H. pylori* [18]. In fact,

swertianoline possesses a methoxyl group at C-3 position on xanthone skeleton. Furthermore, bellidifolin which is the aglycone of **1**, is also reported to show antibacterial activities [19]. It is important to clarify which is active component aglycon or glycoside for medical application. The type of substitution group and position are considerable for the activity of xanthone derivatives against *H. pylori* [20]. On the other hand, K-J Huang et al. reported that xanthone-related compounds were discovered as antibiotics through virtual screening for enzyme I of bacterial phosphoenolpyruvate-dependent phosphotransferase system (PTS) inhibitors. The PTS is ubiquitous in eubacteria and enzyme I inhibitor could be a drug target to develop antimicrobial agents [21]. Although further investigation is required, xanthones might show anti-*H. pylori* activity by the inhibition of enzyme I.

Conclusions

In this study, we demonstrated anti-*H. pylori* activity of methanolic extract of swertia herb, a well-known herb that is used as a gastroenteric drug in Japanese traditional medicine. Swertianolin (**1**), isolated from the methanolic extract, was found to be effective against *H. pylori*. It is interesting to note that a new gastroenteric activity of swertia herb was elucidated in this experiment.

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Table 1 Anti-*Helicobacter pylori* activity of methanolic extract of swertia herb and soluble parts prepared from the extract.

Sample	MBC (mg/mL)[a]
MeOH extract	1.05 ± 0.26
n-Hexane soluble part	0.45 ± 0.15
EtOAc soluble part	1.85 ± 0.65
n-BuOH soluble part	2.5 ± 0.00
H_2O soluble part	NA

Results are expressed as the mean value ± SD

Four experiments were performed to determine these values

NA not applied due to low activity

[a]The minimum bactericidal concentration against *Helicobacter pylori*.

Table 2 Anti-*Helicobacter pylori* activity of isolated compounds and swertiamarin.

Compound	MBC (μM)[a]	IC_{50} (μM)[b]
1	91.7 ± 28.1	6.1 ± 1.5
2	>892.9	177.0 ± 27.6
Swertiamarin	>1000	>1000
Amoxicilin	0.21±0.08	0.044±0.003

Results are expressed as the mean value ± SD

Four experiments were performed to determine these values

[a]The minimum bactericidal concentration against *Helicobacter pylori*.

[b]The half-maximal inhibitory concentration against *H. pylori.*

Amoxicilin was used as positive control.

Table 3 Fractional inhibitory concentration values of isolated compounds and Amoxicillin.

Compound	FIC[a]	Outcome
1 + Amoxicillin	0.45	synergy effect
2 + Amoxicillin	1.00	additive effect

Figure Legend

Fig. 1 The structures of swertianolin (**1**), isooreintin (**2**), and swertiamarin