Natural Product Sciences
21(1) : 49-53 (2015)

Anti-*Helicobacter pylori* Compounds from *Maackia amurensis*

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Abstract – Eight isoflavonoid compounds were isolated from the EtOAc fraction of *Maackia amurensis* which had shown the highest anti-*Helicobacter pylori* activity among the fractions, using medium pressure liquid chromatography and recrystallization. Based on the spectroscopic data including ^{1}H-NMR, ^{13}C-NMR, HMBC and MS data, the chemical structures of the isolates were determined to be (−)-medicarpin (**1**), afromosin (**2**), formononetin (**3**), tectorigenin (**4**), prunetin (**5**), wistin (**6**), tectoridin (**7**) and ononin (**8**). Anti-*H. pylori* activity of each compound was evaluated with broth dilution assay. As a result, (−)-medicarpin (**1**), tectorigenin (**4**) and wistin (**6**) showed anti-*H. pylori* activity. (−)-Medicarpin (**1**) exhibited the most potent growth inhibitory activity against *H. pylori* with the minimal inhibitory concentration $(MIC)_{90}$ of 25 μM, and tectorigenin (**4**) with MIC_{90} of 100 μM ranked the second. This is the first study to show the anti-*H. pylori* activity of *M. amurensis*, and it is suggested that the stem bark of *M. amurensis* or the EtOAc fraction or the isolated compounds can be a new natural source for the treatment of *H. pylori* infection.
Keywords – *Maackia amurensis*, Anti-*Helicobacter pylori* activity, (−)-Medicarpin, Tectorigenin

Introduction

A gram-negative bacillus, *Helicobacter pylori* was firstly isolated from the pyloric region of chronic gastritis patients in 1983, and successfully cultured by Marshall and Warren.[1] As incubation time becomes longer, the curved shape changes into circular. This spiral-shaped bacterium grows well at 30 - 37 ºC, pH 7.0 - 7.4, and microaerophilic condition.[2,3] *H. pylori* produces urease which hydrolyzes urea of gastric mucosa to ammonia and CO_2, and so neutralize the strong acidity of stomach to survive. Vacuolating toxin (VacA) and the product of the cytotoxin-associated gene (CagA) generated by *H. pylori* has been reported to be involved in the virulence.[4] In addition, this bacterium has high motility in viscous environments owing to flagella.[5] *H. pylori* is known to be one of the main causes of gastrointestinal disorders such as chronic gastritis, duodenal ulcer and stomach cancer.[6,7]

Although non-bismuth or bismuth-based quadruple therapies prescribing one proton pump inhibitor and two or three antibiotics (eg., amoxicillin, clarithromycin or tetracycline) have been preferred for treatment of *H. pylori* infection, resistance to the antibiotics, relapse and other mild side effects such as vomiting and diarrhea are still emerging.[8] Therefore, interest on natural products which can be used as adjuvant therapy with less adverse reaction has been increasing. It has been reported that total extracts from the natural sources including *Paeonia lactiflora*, *Portulaca oleracea* and *Scutellaria baicalensis* showed anti-*H. pylori* activity. 1,2,3,4,6-Penta-*O*-galloyl-*β*-D-glucopyranose, quercetin, baicalein, and so on have been isolated to be the active compounds.[9-13]

Maackia amurensis (Fabaceae) is distributed in Korea, China, Japan and Russia. The stem bark and branches of *M. amurensis* have been used as the folk medicine for inflammatory diseases like arthritis, cholecystitis and hepatitis.[14] Flavonoids, isoflavonoids and alkaloids have been reported as the main constituents of this plant, and these compounds are known to have the anti-inflammatory, insecticidal and hepatoprotective activities.[15-17] Previous studies on *M. amurensis* have been mainly focused on

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their function as lectins, carbohydrate-binding proteins in the immune system.[18] There have been no prior studies on the anti-bacterial activity against *H. pylori*. The present study was undertaken to isolate and identify the bioactive constituents from the 70% ethanol extract of *Maackia amurensis* stem bark which showed significant anti-*H. pylori* activity, and to evaluate the antibacterial activity of the isolated compounds.

Experimental

General – ^{1}H and ^{13}C NMR spectra were taken on a Bruker DRX-300 and a Bruker DRX-500 spectrometer (Germany), and chemical shifts were recorded as d values. FAB/MS and EI/MS were obtained on a JEOL JMS-700 (Akishima, Japan). Medium pressure liquid chromatography (MPLC) was performed on SNAP cartridge KP-Sil 100 g and 25 g with Biotage Isolera One system (USA). TLC was carried out on silica gel Silica gel 60 F_{254} (Merck, Germany). A CO_2 incubator, ASTEC SCA-80DS (Japan) was used for bacterial culture. All chemicals used in bioassay were of biochemical reagent grade. Quercetin used as a positive control was purchased from Sigma (St. Louis, MO). The other chemicals were extra grade.

Plant Material – The stem of *Maackia amurensis* Rupr. et Max. was purchased from Kyung-Dong oriental market (Jegi-dog, Seoul) in September 2012. The plant was identified by professor Jong Hee Park, College of Pharmacy, Pusan National University. In this study, the stem bark of *M. amurensis* was used after peeling the cork layer. The voucher specimen (No. PKC-2012-49) was deposited in the Herbarium of the College of Pharmacy, Gyeongsang National University.

Extraction and Isolation – Dried stem bark (150 g) of *M. amurensis* was extracted with 70% ethanol. The total extract (25 g) was suspended in water, and then partitioned successively with *n*-hexane, methylene chloride, ethyl acetate, and *n*-butanol, respectively, to give hexane Fr. (1.8 g), CH_2Cl_2 Fr. (3.5 g), EtOAc Fr. (2.9 g), BuOH Fr. (4.9 g) and water Fr. (11.5 g) fractions, respectively. The ethyl acetate fraction was subjected to silica gel column packed in methylene chloride. The column was then eluted in gradient ratios with methylene chloride-methanol (100 : 0 → 0 : 100) and this fraction was divided by MPLC into six subfractions (fr.1-fr.6). Compound **1** (6 mg) was obtained from fr.1 by MPLC using hexane-ethyl acetate as eluting solvent. Another subfraction, fr.2 was separated into five subfractions (fr.2a-2e) by MPLC using methylene chloride-methanol as eluting solvent. Compounds **2** (8 mg) and **5** (7 mg) were given by recrystallization from fr.2a and fr.2d, respectively. Two sunfractions, fr.2c and fr.2d were concentrated under reduced pressure to give compounds **3** (30 mg) and **4** (12 mg), respectively. A subfraction, fr.5 was divided into four subfractions (fr.5a-5d) by MPLC using methylene chloride-methanol as developing solvent. Compounds **6** (20 mg), **7** (8 mg) and **8** (70 mg) were obtained from fr.5a, fr.5b and fr.5c, respectively.

(–)-Medicarpin (1) – White powder. $C_{16}H_{14}O_4$; EI/MS (*m/z*): 270 $[M]^+$; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 7.32 (1H, d, *J* = 8.4 Hz, H-1), 7.06 (1H, d, *J* = 8.8 Hz, H-7), 6.48 (1H, dd, *J* = 8.4, 2.5 Hz, H-2), 6.39 (1H, d, *J* = 2.2 Hz, H-8), 6.38 (1H, d, *J* = 2.4 Hz, H-4), 6.35 (1H, d, *J* = 2.4 Hz, H-10), 5.42 (1H, d, *J* = 6.8 Hz, H-11a), 4.16 (1H, dd, *J* = 10.9, 5.0 Hz, H-6eq), 3.55 (1H, t, *J* = 10.9 Hz, H-6ax), 3.46 (1H, m, H-6a), 3.70 (3H, s, 9-OCH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz,): δ 161.6 (C-9), 161.1 (C-10a), 157.5 (C-3), 157.1 (C-4a), 132.6 (C-1), 125.1 (C-7), 119.5 (C-6b), 112.67 (C-11b), 110.1 (C-2), 106.9 (C-10), 104.1 (C-4), 97.3 (C-8), 78.9 (C-11a), 67.0 (C-6), 39.9 (C-6a), 55.9 (9-OCH_3).

Afromosin (2) – White powder. $C_{17}H_{14}O_5$; EI/MS (*m/z*): 298 $[M]^+$; ^{1}H-NMR (DMSO-d_6, 300 MHz): δ 8.32 (1H, s, H-2), 7.50 (2H, d, *J* = 8.8 Hz, H-2', 6'), 7.42 (1H, s, H-5), 6.99 (2H, d, *J* = 8.8 Hz, H-3', 5'), 6.94 (1H, s, H-8), 3.87 (3H, s, 6-OCH_3), 3.78 (3H, s, 4'-OCH_3); ^{13}C-NMR (DMSO-d_6, 75 MHz): see Table 2.

Formononetin (3) – White needle crystal. $C_{16}H_{12}O_4$; FAB/MS (*m/z*): 269.1 $[M + H]^+$; ^{1}H-NMR (DMSO-d_6 + D_2O, 500 MHz): δ 8.31 (1H, s, H-2), 7.96 (1H, d, *J* = 8.8 Hz, H-5), 7.49 (2H, d, *J* = 8.8 Hz, H-2', 6'), 6.98 (2H, d, *J* = 8.8 Hz, H-3', 5'), 6.94 (1H, dd, *J* = 8.8, 2.2 Hz, H-6), 6.86 (1H, d, *J* = 2.2 Hz, H-8), 3.77 (3H, s, 4'-OCH_3); ^{13}C-NMR (DMSO-d_6 + D_2O, 125 MHz): see Table 2.

Tectorigenin (4) – White powder. $C_{16}H_{12}O_6$; EI-MS (*m/z*): 300 $[M]^+$; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 8.30 (1H, s, H-2), 7.37 (2H, d, *J* = 8.5 Hz, H-2', 6'), 6.82 (2H, d, *J* = 8.5 Hz, H-3', 5'), 6.47 (1H, s, H-8), 3.75 (3H, s, 6-OCH_3); ^{13}C-NMR (DMSO-d_6, 75 MHz): see Table 2.

Prunetin (5) – White needle crystal. $C_{16}H_{12}O_5$; EI/MS (*m/z*): 284 $[M]^+$; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 8.28 (1H, s, H-2), 7.44 (1H, s, H-8), 7.40 (2H, d, *J* = 8.5 Hz, H-2', 6'), 6.95 (1H, s, H-6), 6.82 (2H, d, *J* = 8.5 Hz, H-3', 5'), 3.89 (3H, s, 7-OCH_3); ^{13}C-NMR (DMSO-d_6, 125 MHz): see Table 2.

Wistin (6) – White powder. $C_{23}H_{24}O_{10}$; FAB/MS (*m/z*): 461.2 $[M + H]^+$; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 8.44 (1H, s, H-2), 7.55 (2H, d, *J* = 8.7 Hz, H-2', 6'), 7.50 (1H, s, H-5), 7.34 (1H, s, H-8), 7.01 (2H, d, *J* = 8.7 Hz, H-3', 5'), 5.19 (1H, d, *J* = 7.1 Hz, H-1"), 3.72 (2H, m, H-6"a),

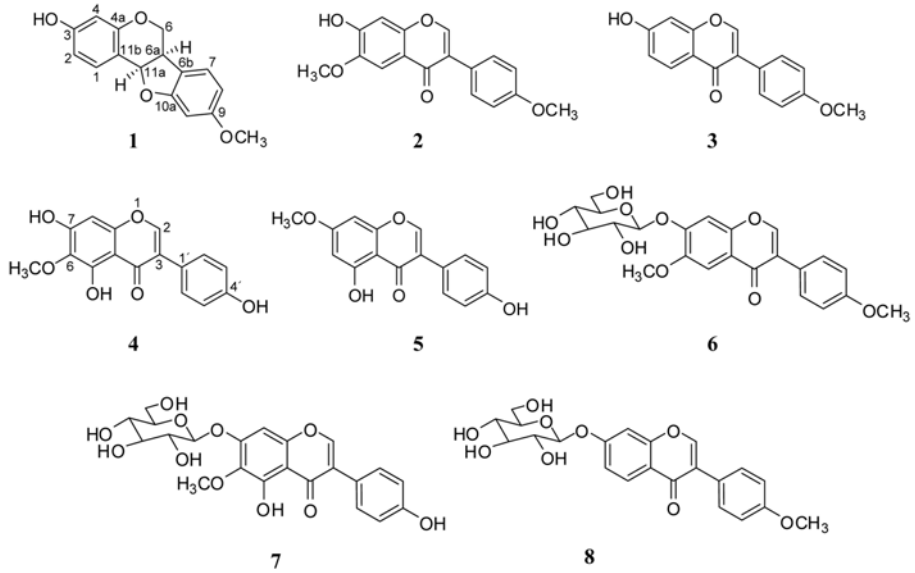

Fig. 1. Chemical structures of compounds **1 - 8** from the stem bark of *M. amurensis*.

Table 1. Anti-*Helicobacter pylori* activity of total extract and the fractions from *M. amurense*

Sample	DMSO	Quercetin	Total Ex.	Hexane Fr.	CH_2Cl_2 Fr.	EtOAc Fr.	BuOH Fr.	Water Fr.
Clear zone (mm)	–	12	21	17	19	23	13	–

3.49 (1H, m, H-6"b), 3.46 (1H, m, H-3"), 3.33 (2H, m, H-2", 5"), 3.18 (1H, m, H-4"), 3.90 (3H, s, 6-OCH_3), 3.80 (3H, s, 4'-OCH_3); ^{13}C-NMR (DMSO-d_6, 125 MHz): see Table 2.

Tectoridin (7) – White crystalline powder. $C_{22}H_{22}O_{11}$; FAB/MS (*m/z*): 463.2 $[M + H]^+$; ^{1}H-NMR (DMSO-d_6 + D_2O, 500 MHz): δ 8.41 (1H, s, H-2), 7.39 (2H, d, J = 8.6 Hz, H-2', 6'), 6.88 (1H, s, H-8), 6.84 (2H, d, J = 8.6 Hz, H-3', 5'), 5.08 (1H, d, J = 7.2 Hz, H-1"), 3.68 (1H, m, H-6"a), 3.47 (1H, m, H-6"b), 3.45 (1H, m, H-5"), 3.34 (1H, m, H-2"), 3.33 (1H, m, H-3"), 3.19 (1H, m, H-4"), 3.77 (3H, s, 6-OCH_3); ^{13}C-NMR (DMSO-d_6 + D_2O, 125 MHz): see Table 2.

Ononin (8) – Pale yellow powder. $C_{22}H_{22}O_{11}$; FAB/MS (*m/z*): 431.3 $[M + H]^+$; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 8.44 (1H, s, H-2), 8.07 (1H, d, J = 8.9 Hz, H-5), 7.54 (2H, d, J = 8.8 Hz, H-2', 6'), 7.25 (1H, d, J = 2.3 Hz, H-8), 7.16 (1H, dd, J = 8.9, 2.3 Hz, H-6), 7.01 (2H, d, J = 8.8 Hz, H-3', 5'), 5.11 (1H, d, J = 7.3 Hz, H-1"), 3.72 (1H, m, H-6"a), 3.49 (1H, m, H-6"b), 3.47 (1H, m, H-3"), 3.33 (1H, m, H-5"), 3.32 (1H, m, H-2"), 3.18 (1H, m, H-4"), 3.77 (3H, s, 4'-OCH_3); ^{13}C-NMR (DMSO-d_6, 125 MHz): see Table 2.

***Helicobacter pylori* culture** – *H. pylori* 43504 strain used in this study was provided by the *Helicobacter pylori* Korean Type Culture Collection, School of Medicine, Gyeongsang National University, Korea. *H. pylori* was grown and maintained on Brucella agar medium (BD Co., Sparks, MD, USA) supplemented with 10% horse serum (Gibco, New York, USA). Incubation was done for 2 - 3 days at 37 ºC, 100% humidity and 10% CO_2 conditions.

Paper disc diffusion assay – Impregnated paper disc bioassay method was applied to the anti-*H. pylori* activity evaluation of total extract and the fractions. The cultured bacterial broth which showed an optical density of 0.02 at 600 nm was diluted with Brucella agar medium to give the final concentration of 2%. Each 30 μL of sample solution in DMSO was applied to paper discs (Advantec, 8 mm diameter and 0.7 mm thickness, Toyo Roshi, Japan). After drying, the discs were placed on the agar surface that was inoculated with *H. pylori*. The sample concentration was 10 mg/mL, and diameters of the inhibition zones were recorded after incubation for 2 days. The negative and positive control discs received DMSO and quercetin, respectively.

MICs determination – Broth dilution method was used to determine the minimal inhibitory concentration (MIC).[19] The bacterial colony suspension equivalent to 2 - 3×10^8 cfu/mL was prepared. Twenty microliter of the bacterial inoculum was added to Brucella broth media supplemented with 10% horse serum in each six well

Table 2. ^{13}C-NMR chemical shifts of compounds **2 - 8**

C	**2**	**3**	**4**	**5**	**6**	**7**	**8**
2	153.7	156.2	154.3	152.8	156.6	154.9	154.0
3	124.9	127.3	122.1	123.4	123.2	122.5	123.8
4	174.7	177.8	180.8	174.7	174.5	181.1	175.1
5	105.0	130.4	153.6	153.4	105.2	152.9	124.4
6	147.5	118.3	132.0	103.2	147.9	132.7	116.0
7	153.3	165.6	153.2	147.4	152.0	156.8	161.8
8	103.2	105.2	94.4	105.2	103.9	94.5	103.8
9	152.3	160.6	158.6	152.1	151.6	152.9	157.4
10	116.5	119.7	104.9	116.6	118.2	106.8	118.8
1'	123.1	126.3	121.7	123.2	124.7	121.5	127.3
2', 6'	130.5	133.2	130.5	130.4	130.5	130.5	130.5
3', 5'	114.0	116.7	115.4	115.3	118.2	115.4	114.0
4'	159.3	162.1	157.8	157.5	159.3	157.6	159.4
1"					100.0	100.5	100.4
2"					73.4	73.3	73.5
3"					77.2	76.5	77.6
4"					70.0	69.9	70.0
5"					77.6	77.1	76.8
6"					61.1	60.9	61.0
-OCH_3	56.2	58.2	60.2	56.2	56.3	60.7	55.5
4'-OCH_3	55.6				55.5		

Table 3. Anti-*Helicobacter pylori* activity of compounds **1 - 8**

Sample	Quercetin	1	2	3	4	5	6	7	8
MIC_{50} (μM)	50	6.25	> 100	> 100	25	> 100	100	> 100	> 100
MIC_{90} (μM)	100	25	–	–	100	–	–	–	–
MBC (μM)	> 100	50	–	–	> 100	–	–	–	–

plate containing 20 μL of two-fold serially diluted test sample (6.25 - 100 μM) or controls, so that the final volume was 2 mL. After incubation for 24 - 48 hr, MIC_{50} and MIC_{90} were defined as the lowest concentration of inhibiting growth by 50 and 90%, respectively. Growth was evaluated by reading optical density at 600 nm. After cultures were incubated for 24 hr, 20 μL of aliquots were seeded on blood agar plates and incubated by another 48 hr in order to determine the minimal bactericidal concentration (MBC). MBC was defined as the minimal sample concentration required to kill 99.9% of the organisms in the medium. Triplicate experiments were performed.

Results and Discussion

The anti-*H. pylori* activity of total extract and the fractions from *M. amurensis* was evaluated with paper disc diffusion method (Table 1). Hexane, CH_2Cl_2 and EtOAc fractions including total extract exhibited larger clear inhibition zone than quercetin, the positive control, while BuOH Fr. showed similar inhibitory activity to quercetin. Since EtOAc Fr. showed the greatest antibacterial activity against *H. pylori*, bioactivity-guided isolation was executed using MPLC and recrystallization. As results, eight isoflavonoid compounds were isolated from this fraction. Based on the spectroscopic data including ^{1}H-NMR, ^{13}C-NMR, HMBC and MS data, the chemical structures of the isolates were determined to be (–)-medicarpin (**1**), afromosin (**2**), formononetin (**3**), tectorigenin (**4**), prunetin (**5**), wistin (**6**), tectoridin (**7**) and ononin (**8**) (Table 2).[20-24]

Anti-*H. pylori* activity of each compound was evaluated with a broth dilution method. Among the isolated isoflavonoid compounds, (–)-medicarpin (**1**), tectorigenin (**4**) and wistin (**6**) showed anti-*H. pylori* activity. (–)-Medicarpin (**1**) exhibited the most potent growth inhibitory

activity against *H. pylori* with MIC_{50} of 6.25 μM and MIC_{90} of 25 μM, and tectorigenin (**4**) with MIC_{50} of 25 μM and MIC_{90} of 100 μM ranked the second. These two compounds showed more potent growth inhibitory activity against *H. pylori* than quercetin. Wistin (**6**) showed weak anti-*H. pylori* activity. The other five isolates did not show the antibacterial activity. Although formononetin has been reported to inhibit *H. pylori* growth,[25] this compound (**3**) failed to show the antibacterial activity in this study. The difference in anti-*H. pylori* activity of tectorigenin (**4**) and tectoridin (**7**) suggests that glycosylation at 7-OH lower the antibacterial activity. From the comparison of wistin (**6**) and tectoridin (**7**), it can be deduced that methoxy group at C-4' elevates the growth inhibitory activity than hydroxy group. However, other structural characteristics may be involved in their activity since afromosin (**2**) and formononetin (**3**) with 4'-OCH_3 and 7-OH did not show anti-*H. pylori* activity. For example, free rotation between B and C ring in isoflavonoid structure could give negative effect on the antibacterial activity of these compounds.

To the best of our knowledge, this is the first study that demonstrates the anti-*H. pylori* activity of *M. amurensis* extract and its components including (−)-medicarpin and wistin. The results of this study suggest that stem bark of *M. amurensis* or the EtOAc fraction or the isolated compounds can be a new natural source for the treatment of *H. pylori* infection.

Acknowledgments

This research was financially supported by the Ministry of Trade, Industry & Energy (MOTIE), Korea Institute for Advancement of Technology (KIAT) through the Inter-ER Cooperation Projects (Grant # A004500005), and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2014R1A1A3050570).

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Received October 21, 2014
Revised December 8, 2014
Accepted December 9, 2014