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Anti-*Helicobacter pylori* Compounds from *Polygonum cuspidatum*

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Abstract – Anti-*Helicobacter pylori* activity guided fractionation led to the isolation of five anthraquinones, two stilbenes and one naphthoquinone from the EtOAc fraction of *Polygonum cuspidatum,* using silica gel column chromatography, Sephadex-LH20, MPLC and recrystallization. The chemical structures were identified to be physcion (**1**), emodin (**2**), anthraglycoside B (**3**), *trans*-resveratrol (**4**), anthraglycoside A (**5**), polydatin (**6**), 2-methoxy-6-acetyl-7-methyljuglone (**7**) and citreorosein (**8**) by UV, ^{1}H-NMR, ^{13}C-NMR and mass spectrometry. Anti-*Helicobacter pylori* activity including MIC values of each compound was evaluated. All of the isolates exhibited anti-*H. pylori* activity of which MIC values were lower than that of a positive control, quercetin. Compounds **2** and **7** showed potent growth inhibitory activity. Especially, a naphthoquinone, compound **7** displayed most potent antibacterial activity with MIC_{50} value of 0.30 μM and MIC_{90} value of 0.39 μM. Although *anti-H. pylori* activity of this plant was previously reported, this is the first report on that of compounds isolated from this species. From these findings, *P. cuspidatum* roots or its isolates may be useful for *H. pylori* infection and further study is needed to elucidate mechanism of action.

Keywords – *Polygonum cuspidatum*, Anti-*Helicobacter pylori* activity, Emodin, 2-Methoxy-6-acetyl-7-methyljuglone

Introduction

H. pylori is a gram-negative, spiral-shaped, microaerophilic, flagellated human pathogen bacterium that successfully colonizes gastric mucosa.[1,2] *H. pylori* produce urease which breaks down urea of stomach into ammonia and CO_2, and the ammonia neutralizes the acidic environment of stomach. This bacterium also generates vacuolating toxin (VacA) and the product of the cytotoxin-associated gene (CagA), which have been reported to be responsible in the virulence.[3] Since *Helicobactor pylori* was identified in the pyloric region of chronic gastritis patients in 1983, by Marshall and Warren,[4] *H. pylori* has been known to be involved in gastrointestinal disorders such as gastritis, duodenal ulcer and stomach cancer.[2,5] In addition, recent studies have revealed relationships between this bacterium and other diseases such as Parkinson's disease and chronic hepatitis C.[6,7]

Although the first-line triple therapy, which prescribes one proton pump inhibitor, amoxicillin and clarithromycin or metronidazole, sequential therapy and bismuth-based quadruple therapies combined with triple therapy or sequential therapy have been tried for treatment of *H. pylori* infection, clarithromycin resistance, relapse and other mild side effects such as vomiting and diarrhea are still emerging.[8,9] Therefore, interest on natural products which can be used as adjuvant therapy with less adverse effect has been increasing.[10]

Polygonum cuspidatum Siebold & Zucc. (*Reynoutria japonica* Houtt.) belongs to the family Polygonaceae, and is a large, herbaceous perennial plant. This plant has hollow and erect stems with distinct raised nodes, with red or purple spots.[11] This plant is widely distributed in Asia and North America.[12] The root of this plant is known to have major secondary metabolites including emodin, polydatin, resveratrol, physcion and anthraglycoside B.[13] Recently, over sixty seven compounds have been isolated and identified from this plant. They are quinones, stilbenes, flavonoids, coumarins, lignans and others. Over one hundred prescriptions containing this crude drug has been used to treat diseases such as inflammation, jaundice and skin burn.[14] Modern investigations have revealed that *P. cuspidatum* have many pharmacological effects including anti-shock, anti-inflammatory, antioxidant, anticancer, hepatoprotective, antibacterial, lipid regulating, antiviral, and antifungal activities.[15-19] The present study was undertaken to isolate and identify bioactive constituents

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from 70% ethanol extract of *P. cuspidatum* roots, which showed significant anti-*H. pylori* activity, and to evaluate the antibacterial activity of isolated compounds.

Experimental

General – The nuclear magnetic resonance (NMR) spectrometer used here was a Bruker DRX-300 and a Bruker DRX-500 spectrometer (Germany), and chemical shifts were recorded as δ values. FAB/MS and EI/MS were obtained on a JEOL JMS-700 (Akishima, Japan). Medium pressure liquid chromatography (MPLC) was performed on YMC GEL ODS-A (12 nm, S-150 μM) (YMC Co. Ltd., Kyoto, Japan) and 25 g with Biotage Isolera One system (Charlotte, NC). TLC was done on Silica gel 60 F_{254} (Merck, Germany). Column chromatography was performed on silica gel 60 (0.063 - 0.43 mm; Merck KGaA, Damstadt, Germany) and Sephadex LH-20 (GE Healthcare, Uppsala, Sweden). A CO_2 incubator, ASTEC SCA-80DS (Japan) was used for bacterial culture. All chemicals used in bioassay were of biochemical reagent grade. Quercetin was purchased from Sigma (St. Louis, MO) and used as a positive control. The other chemicals were extra grade.

Plant Material – The roots of *Polygonum cuspidatum* was purchased from Kyung-Dong oriental market (Jegi-dog, Seoul) in October 2013. The plant was identified by professor Mi-Jeong Ahn, College of Pharmacy, Gyeongsang National University. The voucher specimen (No. GSC-104) was deposited in the Herbarium of the College of Pharmacy, Gyeongsang National University.

Extraction and Isolation – The dried roots of *Polygonum cuspidatum* (4 kg) were ground and extracted with 70% ethanol at room temperature. The ethanolic extract was concentrated through rotary evaporator to give a crude extract (1.12 kg). This extract was then suspended in H_2O and partitioned successively with *n*-hexane, ethyl acetate and *n*-butanol, respectively, to give *n*-hexane Fr. (14.6 g), EtOAc Fr. (407 g), *n*-BuOH Fr. (174 g) and water Fr. (468 g) fractions, respectively. The ethyl acetate fraction was subjected to silica column chromatography (CC) with a gradient elution of Hexane, CH_2Cl_2 and MeOH mixture (1:1:0 → 0:1:0 → MeOH) to give seven fractions (fr.1-fr.7). The fr.2 and fr.3 were applied on silica gel CC using a mixture of hexane and EtOAc (100:0 → 0:100) as an eluting solvent to give subfractions, fr.2a-fr.2c and fr.3a-fr.3b, respectively. Compounds **1** (894 mg) and **2** (18 g) were isolated from fr.2b and fr.3b, respectively, by recrystallization. Compound **7** (80 mg) was obtained from fr.3a through repeated silica gel CC using a mixture of hexane and CH_2Cl_2 mixture. Compound **8** (7 mg) was isolated from the same subfraction by silica gel CC using a mixture of hexane and CH_2Cl_2 mixture followed by Sephadex LH-20 CC using methanol as developing solvent. Other two fractions (fr.4 and fr.5) were divided into subfractions, fr.4a-fr.4e and fr.5a-fr.5c, respectively, by silica gel CC using a CH_2Cl_2 and MeOH mixture (100:0 → 0:100) as an eluting solvent. Compounds **3** (345 mg), **4** (42 mg) and **5** (120 mg) were given by recrystallization from fr.4c, fr.5a and fr.5b, respectively. A subfraction fr.5c gave compound **6** (11 g) by MPLC using water and methanol mixture (100:0 → 0:100) as eluting solvent.

Physcion (1) – Yellowish powder, $C_{16}H_{12}O_5$; EI-MS (*m/z*): 284 $[M]^+$; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 12.34 (1H, s, 1-OH), 12.14 (1H, s, 8-OH), 7.64 (1H, d, J = 1.2 Hz, H-4), 7.38 (1H, d, J = 2.6 Hz, H-5), 7.10 (1H, d, J = 1.2 Hz, H-2), 6.70 (1H, d, J = 2.6 Hz, H-7), 3.96 (3H, s, 6-OCH_3), 2.47 (3H, s, 3-CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz): see Table 2.

Emodin (2) – Orange needles, $C_{15}H_{10}O_5$; EI-MS (*m/z*): 270 $[M]^+$; ^{1}H-NMR (DMSO-d_6, 300 MHz): δ 12.07 (1H, s, 1-OH), 12.01 (1H, s, 8-OH), 7.46 (1H, br d, H-4), 7.15 (1H, br d, H-2), 7.09 (1H, d, J = 2.4 Hz, H-5), 6.57 (1H, d, J = 2.4 Hz, H-7), 2.47 (3H, s, 3-CH_3); ^{13}C-NMR (DMSO-d_6, 125 MHz): see Table 2.

Anthraglycoside B (3) – Yellowish powder, $C_{21}H_{20}O_{10}$; FAB-MS (*m/z*): 433.2 $[M+H]^+$; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 13.23 (1H, br s, OH-1), 7.46 (1H, br s, H-4), 7.27 (1H, d, J = 2.4 Hz, H-5), 7.16 (1H, br s, H-2), 6.98 (1H, d, J = 2.4 Hz, H-7), 5.05 (1H, d, J = 7.6 Hz, H-1'), 3.72~3.24 (glc-H 2'~6'), 2.41 (3H, s, 3-CH_3); ^{13}C-NMR (DMSO-d_6, 125 MHz): see Table 2.

***trans*-Resveratrol (4)** – Pale white powder, $C_{14}H_{12}O_3$; EI-MS (*m/z*): 228 $[M]^+$; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 9.54 (4'-OH), 9.18 (3, 5-OH) 7.39 (2H, d, J = 8.6 Hz, H-2', 6'), 6.93 (1H, d, J = 16.5 Hz, H-b), 6.81 (1H, d, J = 16.5 Hz, H-a), 6.75 (2H, d, J = 8.6 Hz, H-3', 5'), 6.38 (2H, d, J = 2.0 Hz, H-2, 6), 6.12 (1H, t, J = 2.0 Hz, H-4); ^{13}C-NMR (DMSO-d_6, 125 MHz): see Table 2.

Anthraglycoside A (5) – Yellowish powder, $C_{22}H_{22}O_{10}$; ESI-MS (*m/z*): 445.1 $[M-H]^-$; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 13.10 (1-OH), 7.50 (1H, br s, H-4), 7.37 (1H, br d, H-5), 7.19 (2H, br d, H-2, 7), 5.18 (1H, d, J = 7.7 Hz, H-1'), 3.51~3.19 (glc-H 2'~6'), 3.97 (3H, s, 6-OCH_3), 2.41 (3H, s, 3-CH_3); ^{13}C-NMR (DMSO-d_6, 125 MHz): see Table 2.

Polydatin (6) – White powder, $C_{20}H_{22}O_8$; FAB-MS (*m/z*): 390.1 $[M]^+$; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 7.40 (2H, d, J = 8.6 Hz, H-2', 6'), 7.03 (1H, d, J = 16.3 Hz, H-

b), 6.87 (1H, d, J = 16.3 Hz, H-a), 6.76 (2H, d, J = 8.6 Hz, H-3', 5'), 6.74 (1H, br t, H-2), 6.57 (1H, br t, H-6), 6.34 (1H, br t, H-4), 4.81 (1H, d, J = 7.6 Hz, glc H-1"), 3.18~3.49 (glc H-2"~6"); ^{13}C-NMR (DMSO-d_6, 125 MHz): see Table 2.

2-Methoxy-6-acetyl-7-methyljuglone (7) – Red needles, $C_{14}H_{12}O_5$; EI-MS (*m/z*): 260 $[M]^+$; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 12.53 (1H, s, 5-OH), 6.13 (1H,s, H-3), 7.54 (1H, s, H-8), 3.95 (3H, s, 2-OCH_3), 2.61 (3H, s, 6-$COCH_3$), 2.37 (3H, s, 7-CH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz): see Table 2.

Citreorosein (8) – Yellowish powder, $C_{15}H_{10}O_6$; EI-MS (*m/z*): 286 $[M]^+$; ^{1}H-NMR (DMSO-d_6, 300 MHz): δ 12.10 (2H, s, 1, 8-OH), 7.65 (1H, br s, H-4), 7.26 (1H, br s, H-2), 7.13 (1H, d, J = 2.4 Hz, H-5), 6.59 (1H, d, J = 2.4 Hz, H-7), 4.61 (2H, s, 3-CH_2); ^{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 – Anti-*H. pylori* activity of total extract and the fractions was evaluated with impregnated paper disc according to our previously reported method.[20] Each 20 μL of sample solution in DMSO was applied to paper discs (Advantec, 8 mm diameter and 0.7 mm thickness, Toyo Roshi, Japan). 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 – Minimal inhibitory concentrations (MICs) were determined with broth dilution method as shown in our previously report.[20,21] After incubation for 24 - 48 hr, MIC value was assessed as the lowest concentration to inhibit the bacterial growth. Growth was evaluated by reading optical density at 600 nm. MIC_{50} and MIC_{90} were defined as the lowest concentration of inhibiting growth by 50 and 90%, respectively, and the values were calculated from GraphPad Version 5.01 (GraphPad Software, Inc., San Diego, CA). All of the values were obtained from triplicate determinations and two independent experiments.

Results and Discussion

The anti-*H. pylori* activity of total extract and the fractions from *P. cuspidatum* was evaluated with paper disc diffusion method. As a result, total extract, hexane and EtOAc fractions showed much larger clear inhibition zone than quercetin, the positive control, while BuOH Fr. and water Fr. exhibited similar inhibitory activity to quercetin (Table 1). Since EtOAc Fr. exhibited the largest clear inhibition zone, bioactivity-guided isolation was carried out for this fraction. Five anthraquinones, two stilbene and one naphthoquinone compounds were isolated from this fraction using silica gel column chromatography, Sephadex-LH20, MPLC and recrystallization. Based on the spectroscopic data including UV, ^{1}H-NMR, ^{13}C-NMR and MS data, the chemical structures were identified to be physcion (**1**), emodin (**2**), anthraglycoside B (emodin-8-*O*-*β*-D-glucoside) (**3**), *trans*-resveratrol (**4**), anthraglycoside A (physcion-8-*O*-*β*-D-glucoside) (**5**), polydatin (*trans*-resveratrol-3-*O*-*β*-D-glucoside) (**6**), 2-methoxy-6-acetyl-7-methyljuglone (**7**) and citreorosein (**8**) (Fig. 1) (Table 2).[21-26]

Anti-*Helicobacter pylori* activity including MIC values of each compound was evaluated with a broth dilution

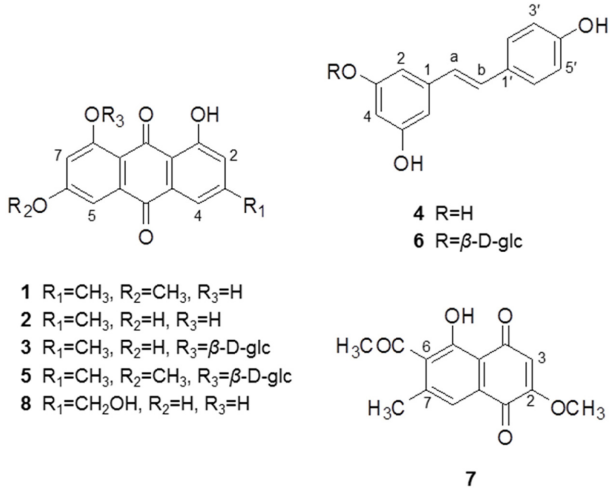

Fig. 1. Chemical structures of compounds **1** - **8** isolated from the roots of *Polygonum cuspidatum*.

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

Sample	DMSO	Quercetin	Total Ex.	Hexane Fr.	EtOAc Fr.	BuOH Fr.	Water Fr.
Clear zone (mm)	–	11	16	19	21	12	10

Table 2. ^{13}C-NMR chemical shifts of compounds **1** - **8**

C	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1	165.2	164.9	162.2	139.7	161.2	139.8	179.1	161.9
2	121.3	120.9	124.6	104.7	124.7	103.2	161.0	121.2
3	148.5	148.7	147.3	159.0	147.6	159.4	109.6	153.2
4	124.5	124.6	119.7	102.2	119.8	105.2	190.3	117.5
5	108.2	109.4	109.1	159.0	107.0	158.8	158.1	109.3
6	162.5	161.9	165.5	104.7	165.2	107.7	130.5	165.0
7	106.8	108.4	108.9		108.7		143.5	108.4
8	166.6	166.3	161.7		162.2		121.6	166.3
9	190.8	190.6	186.7		187.0		136.7	190.0
10	182.1	181.8	182.7		182.4		112.4	181.9
4a	133.2	133.3	132.6		132.6			133.4
8a	110.3	109.3	113.4		115.0			109.5
9a	113.7	113.8	115.0		114.9			114.6
10a	135.3	135.5	136.9		136.8			135.6
a				126.1		125.7		
b				128.3		128.5		
1'				128.5		129.0		
2'				128.3		128.4		
3'				116.0		116.0		
4'				157.7		157.8		
5'				116.0		116.0		
6'				128.3		128.4		
1"			101.3		101.1	101.2		
2"			73.8		73.7	73.8		
3"			76.9		77.1	77.2		
4"			69.9		70.3	70.2		
5"			77.8		77.9	77.6		
6"			61.0		61.2	61.2		
6-OCH_3	56.1				56.6			
3-CH_3	22.2	22.0	21.9		21.9			
3-CH_2OH								62.5
2-OCH_3							56.8	
6-$\underline{C}OCH_3$							202.9	
6-$CO\underline{C}H_3$							31.9	
7-CH_3							20.0	

method. All of the isolates exhibited anti-*H. pylori* activity of which MIC values were lower than those of a positive control, quercetin[27] (Table 3). Compounds **2** and **7** showed potent growth inhibitory activity. Especially, a 1,4-naphthoquinone, compound **7** displayed most potent antibacterial activity with MIC_{50} of 0.30 μM and MIC_{90} of 0.39 μM. Compounds **4** and **8** exhibited moderate activity with MIC_{50} of 59.3 and 37.8 μM, respectively.

The difference in anti-*H. pylori* activity of emodin (**2**) and anthraglycoside B (**3**) suggests that glycosylation at α-position of anthraquinone nucleus reduce the anti-bacterial activity. Glycosylation at C-3 position of stilbenes also reduced the activity as shown in *trans*-resveratrol (**4**) and polydatin (**6**). The weak activity of physcion (**1**) might be ascribed to the poor solubility in the aquatic assay media, which comes from the substitution of a hydroxy group at C-6 position in emodin (**2**) with a methoxy group. From the comparison of emodin (**2**) and citreorosein (**8**), it can be deduced that a hydroxymethyl group on C-3 position of α-hydroxy anthraquinones lowers the anti-*H. pylori* activity. A methoxy group at C-2 position and a hydroxyl group at C-5 position have been

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

Samples	Quercetin	1	2	3	4	5	6	7	8
MIC (μM)	50	12.5	3.13	12.5	6.25	12.5	12.5	0.25	6.25
MIC_{50} (μM)	> 100	> 100	27.0	> 100	59.3	> 100	> 100	0.30	37.8
MIC_{90} (μM)	–	–	48.8	–	> 100	–	–	0.39	100

reported to increase anti-*H. pylori* activity of 1,4-naphthoquinones.[28,29]

Although anti-*H. pylori* activity of this plant, compounds **2** and **4** were previously reported,[30] this is the first report on that of compounds isolated from this species and the other isolated compounds. From these findings, *P. cuspidatum* roots or its isolates may be useful for *H. pylori* infection and further study is needed to elucidate mechanism of action.

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