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Article

Anti-*Helicobacter pylori* Activity of Six Major Compounds Isolated from *Rumex acetosa*

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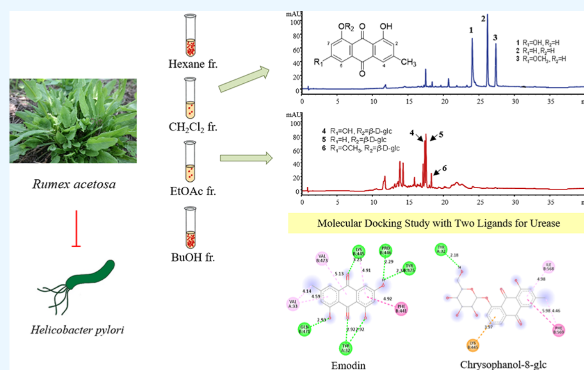
ABSTRACT: Gastric problems are often caused by the well-known *Helicobacter pylori* (*H. pylori*) bacterium. One of the biggest obstacles to the treatment of *H. pylori* infections is increasing the antibiotic resistance. During our search for naturally derived anti-*H. pylori* compounds, six major compounds were isolated from the methylene chloride (CH_2Cl_2) and ethyl acetate (EtOAc) fractions of *Rumex acetosa* that showed anti-*H. pylori* activity. Three anthraquinones and three anthraquinone glucosides were identified as the major chemical constituents of the CH_2Cl_2 and EtOAc fractions, respectively. The chemical structures were identified to be emodin (**1**), chrysophanol (**2**), physcion (**3**), emodin-8-*O*-*β*-D-glucoside (**4**), chrysophanol-8-*O*-*β*-D-glucoside (**5**), and physcion-8-*O*-*β*-D-glucoside (**6**) by UV, ^{1}H NMR, ^{13}C NMR, and mass spectrometry. Anti-*H. pylori* activity, including the minimum inhibitory concentration (MIC) value of each compound, was evaluated against two *H. pylori* strains. All isolates exhibited anti-*H. pylori* activity with different potencies, with an MIC value ranging between 3.13 and 25 *μ*M. However, some variations were found between the two strains. While compound **5** displayed the most potent antibacterial activity with an MIC_{50} value of 8.60 *μ*M and an MIC_{90} value of 15.7 *μ*M against *H. pylori* strain 51, compound **1** exhibited the most potent inhibitory activity against *H. pylori* strain 43504. The two compounds also showed moderate urease inhibitory activity, with compound **1** demonstrating activity higher than that of compound **5**. Furthermore, a molecular docking study revealed the high binding ability of compounds **1** and **5** to the active site of *H. pylori* urease. The present study suggests that the six anthraquinones isolated from *R. acetosa* with the whole parts of this plant may be natural candidates for the treatment of *H. pylori* infection. Further studies are required to determine the exact mechanism of action and to evaluate safety issues in the human body.



1. INTRODUCTION

Rumex acetosa L., a herbaceous perennial plant, belongs to the family Polygonaceae. Its leaf is characteristically arrow-shaped and resembles that of spinach.[1] This plant is widely distributed in Asia, Europe, North America, and South Africa.[2] It is known as sorrel, and the young leaves with a sour taste are consumed as sour soup, sauces, and salad.[3] *R. acetosa* has been used as a folk medicine in Korea for treating mild constipation, arthritis, gastritis, and cutaneous diseases as a substitute for rhubarb.[4] It is also known to have other pharmacological effects, including antitumor,[5,6] antiviral,[7] antibacterial,[8] xanthine oxidase inhibitory,[3] and hypotensive activities.[9] Our previous study has revealed that the administration of *R. acetosa* extract significantly reduced HCl/ethanol-induced gastric ulcer in mice, and the protective activity of ethanol extract is higher than that of water extract.[4] *Helicobacter pylori* is known to be involved in gastrointestinal disorders such as gastritis, duodenal ulcers, and stomach cancer.[10,11] This Gram-negative microaerophilic and flagellated human pathogenic bacterium produces urease, which breaks down the urea in the stomach into ammonia and CO_2, and the ammonia neutralizes the acidic environment of the stomach for its colonization in gastric mucosa.[12,13] Therefore, the present study aimed to isolate the major chemical constituents of the *R. acetosa* extract and evaluate their antibacterial activity against *H. pylori* as potential agents for gastric ulcers.

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Table 1. Anti-*H. pylori* Activity of Total Extract and Fractions from *R. acetosa*

samples	quercetin	total ex.	hexane fr.	CH_2Cl_2 Fr.	EtOAc Fr.	BuOH Fr.	water fr.
clear zone (mm)	15 ± 3	11 ± 1	12 ± 2	15 ± 4	13 ± 3	11 ± 1	

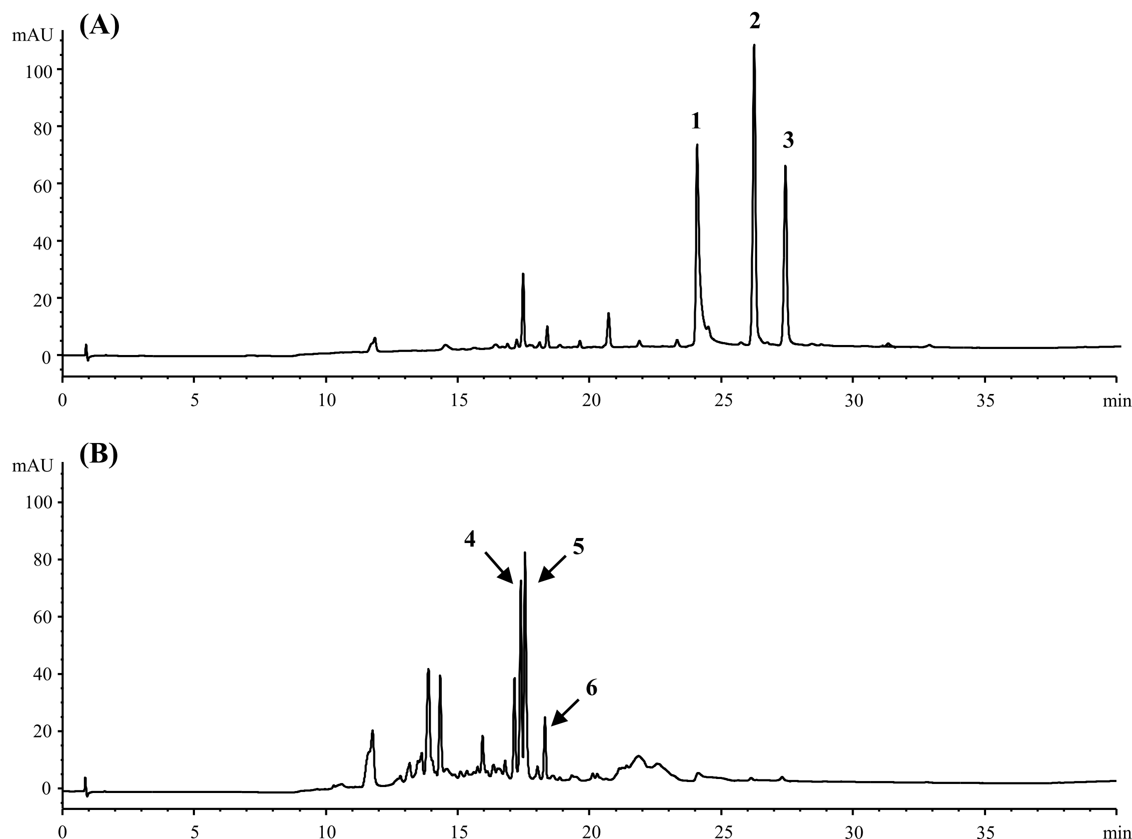


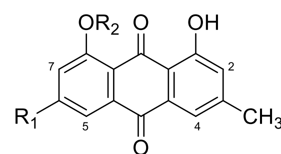
Figure 1. LC chromatograms of methylene chloride (A) and ethyl acetate fractions (B) of *R. acetosa*.

2. RESULTS AND DISCUSSION

The anti-*H. pylori* activity of the extract, fractions, and compounds isolated from *R. acetosa* were evaluated in the current study. The total extract and all fractions, except the aqueous fraction, showed significant anti-*H. pylori* activity (Table 1).

Among them, the methylene chloride (CH_2Cl_2) and ethyl acetate (EtOAc) fractions exhibited a higher activity than others. Three anthraquinones were the major compounds in the CH_2Cl_2 fraction and three anthraquinone glucosides were the major compounds in the EtOAc fraction (Figure 1). The six major compounds were isolated, and their chemical structures were determined by comparing the spectroscopic data including mass spectra and NMR data with previously published data (Figures 2 and S1−S21).[14,15]

H. pylori inhibitory activity, including the minimum inhibitory concentration (MIC) value of the six compounds, was evaluated using a broth dilution method (Table 2). All six compounds exhibited anti-*H. pylori* activity, with an MIC value lower than those of the positive control, quercetin.[16] Among the three anthraquinones, emodin (**1**) showed the most potent anti-*H. pylori* activity with MIC_{50} values of 27.7 and 28.3 μM and MIC_{90} values of 48.3 and 48.5 μM against strains 51 and 43504, respectively. Among the three anthraquinone glucosides, charysophanol-8-*O*-*β*-D-glucoside (**5**) showed the most potent anti-*H. pylori* activity with MIC_{50} values of 8.6 and 36.5 μM and MIC_{90} values of 15.7 and 56.9 μM against strains 51 and 43504,



1 R_1=OH, R_2=H
2 R_1=H, R_2=H
3 R_1=OCH_3, R_2=H
4 R_1=OH, R_2=*β*-D-glc
5 R_1=H, R_2=*β*-D-glc
6 R_1=OCH_3, R_2=*β*-D-glc

Figure 2. Chemical structures of compounds **1**−**6** isolated from *R. acetosa*.

respectively. The MIC_{90} value of compounds **1** and **5** against strain 51 was lower than those of another positive control, metronidazole, used as an antibiotic in clinical field for a gastric ulcer. Their values against a strain 43504 were higher than those of metronidazole.

All six compounds isolated from *R. acetosa* belonged to the anthraquinone family. The three anthraquinones (**1**−**3**) with the same skeleton had different substituents at the C-6 position. Emodin (**1**), with a hydroxyl group at C-6, showed higher inhibitory activity than chrysophanol (**2**) and physcion (**3**), with no substitution or a methoxy group, respectively. The potent anti-*H. pylori* activity of emodin may be attributed to the hydroxyl group attached to the C-6 position.[17] The polar substituents of rhein, emodin, and aloe-emodin are beneficial to

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Table 2. Anti-*H. pylori* Activity of Compounds 1−6 Isolated from *R. acetosa*[a]

strains	MIC (μM)	quercetin[b]	metronida-zole[b]	1	2	3	4	5	6
51	MIC	50	3.13	6.25	6.25	12.5	25	6.25	12.5
	MIC_{50}	>100	22.2	27.7	>100	>100	>100	8.60	>100
	MIC_{90}		59.5	48.3				15.7	
43504	MIC	50	3.13	3.13	6.25	12.5	6.25	6.25	12.5
	MIC_{50}	>100	14.3	28.3	>100	>100	>100	36.5	>100
	MIC_{90}		34.0	48.5				56.9	

[a]These values were obtained from triplicate determinations and two independent experiments, and expressed as the mean value (μM). [b]Positive controls.

Table 3. Urease Inhibitory Activity of Compounds 1 and 5[a,b]

		concentration (μM)				
strains	compounds	6.25	12.5	25	50	100
51	**1**	45.3 ± 5.2[bc]	49.6 ± 2.0[b]	64.0 ± 2.0[a]	68.2 ± 1.3[a]	70.2 ± 0.9[a]
	5	2.9 ± 1.0[e]	36.2 ± 2.1[c]	38.1 ± 1.1[c]	44.0 ± 8.1[bc]	49.3 ± 2.5[bc]
43504	**1**	56.1 ± 0.7[ab]	59.2 ± 0.4[a]	64.9 ± 3.3[a]	65.7 ± 0.4[a]	65.6 ± 4.2[a]
	5	18.5 ± 4.7[d]	44.7 ± 6.3[bc]	47.2 ± 6.6[b]	51.2 ± 0.6[b]	55.8 ± 2.5[b]

[a]Data are expressed as the mean (the average value of inhibitory activity) and SD (the standard deviation value) of three independent experiments. [b]Different superscript letters in the same strain mean significantly different ($p < 0.05$).

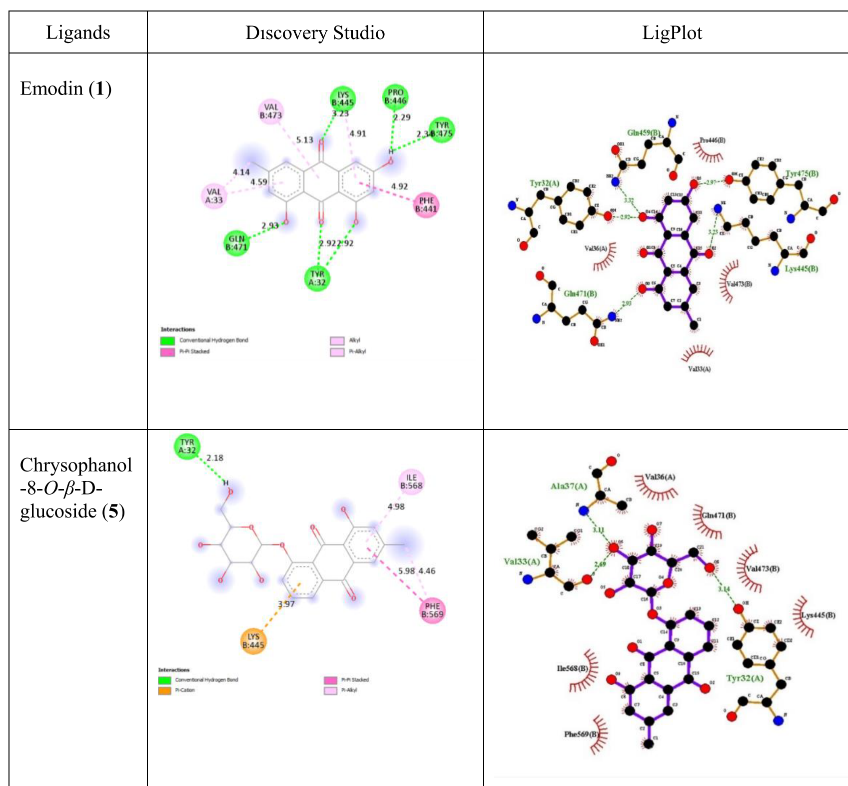

Figure 3. 2D interaction diagrams of two ligands (**1** and **5**) with *H. pylori* urease.

the antibacterial activity of anthraquinone compounds.[18] The difference in the activity of emodin (**1**) and emodin-8-*O*-β-D-glucoside (**4**) suggests that glycosylation at the C-8 position of the anthraquinone nucleus reduces the inhibitory activity.[18] However, these results differ when it comes to chrysophanol (**2**). The addition of a glucose moiety at the C-8 position increased the inhibitory activity against *H. pylori* for compounds **2** and **5**.

The virulence factors of *H. pylori* include motility, exotoxin, mucinase, adhesion, and urease activity.[19] The acid tolerance of *H. pylori* is primarily attributed to its urease activity, an enzyme located in the cytoplasm. The urease produced by *H. pylori* initiates urea hydrolysis, resulting in ammonia production, which creates an alkaline environment in the stomach.[20] This alkaline condition supports the survival of *H. pylori* and triggers inflammation in fibroblasts, uroepithelium, and immune cells, leading to various diseases. Phosphoramidates, hydroxamic acid derivatives, and imidazoles have been used in clinical practice as urease inhibitors.[21] While these inhibitors have shown effectiveness, significant side effects have been reported in various studies. These adverse effects include teratogenicity, psychoneurological disorders, and musculo-integumentary symptoms.[22]

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Table 4. Interacting Residues, Docking Scores, and Binding Energies of Ligand Molecules (1 and 5) against Urease Protein[a]

compounds	binding affinity	hydrogen interaction	hydrophobic interaction	others
emodin (**1**)	−9.2	Gln471(2.93 Å), Tyr32(2.92 Å, 2.92 Å), Lys445(3.23 Å), Pro446(2.29 Å), Pro445(2.34 Å)	Val33(4.14 Å, 4.59 Å), Val 473(5.13 Å), Phe441(4.92 Å)	
chrysophanol-8-*O*-*β*-D-glucoside (**5**)	−7.3	Tyr32(2.18 Å)	Ile568(4.98 Å), Phe569(5.98 Å,4.46 Å)	Lys445(3.97 Å)

[a]For all scoring functions, lower scores indicate more favorable poses. In molecular docking studies, binding energies and docking scores are the primary bases for the selection of active and non-active compounds.

In the urease inhibition assay, concentration-dependent inhibitory activity was observed in compounds **1** and **5**. Urease was extracted from *H. pylori* strains 51 and 43504, respectively. The urease inhibitory activity of emodin (**1**) was higher than that of chrysophanol-8-*O*-*β*-D-glucoside (**5**) (Table 3).

Compound **1** inhibited almost 50% of urea breakdown by urease at a concentration of 6.3 μM, whereas compound **5** showed less than 25% inhibition at the same concentration for both strains. Both compounds showed a higher percentage of inhibition against *H. pylori* strain 43504 than against strain 51. In the previous reports, emodin has shown anti-*H. pylori* effects by inhibiting *β*-hydroxyacyl ACP dehydratase in *H. pylori* strains SS1 and ATCC 43504.[23] Furthermore, the anti-*H. pylori* activity of other anthraquinones similar to that of emodin, such as aloe-emodin and rhein, is known to be related to a decrease in arylamine *N*-acetyltransferase activity in the cytoplasm, which is involved in the nucleic acid synthesis in this bacterium.[24,25] Therefore, it could be inferred that anthraquinones might be responsible for the antibacterial activities with different mechanisms of action.

Molecular docking is an important part of the rational drug design process because it is a practical method that can make accurate predictions on the optimal orientation of a single molecule with respect to its putative target.[26] Among the six isolated compounds, two ligand molecules (compounds **1** and **5**) that have shown significant anti-*H. pylori* and antiurease activities were selected and docked into the urease protein-binding site. The results reveal that the ligand molecules actively bind to the target site. Molecule **1** interacted with the Gln471, Tyr32, Lys445, Pro446, and Pro445 residues, while molecule **5** interacted with the Tyr32 residue (Figure 3). The docking scores and binding energies of the ligands are presented in Table 4. Emodin (**1**) showed a lower docking score and binding energy than chrysophanol-8-*O*-*β*-D-glucoside (**5**). The results from the molecular docking analyses correlate well with the *in vitro* inhibitory properties of compounds **1** and **5** against *H. pylori*. Furthermore, these findings imply that the anti-*H. pylori* activity exhibited by compounds **1** and **5** may be attributed to their capacity to inhibit urease.

In this study, anti-*H. pylori* activity was confirmed in the *R. acetos*a extract, and six anthraquinone compounds were isolated from the CH_2Cl_2 and EtOAc fractions. Among the compounds, potent inhibitory activity was measured from emodin (**1**) and chrysophanol-8-*O*-*β*-D-glucoside (**5**). The antibacterial activities including anti-*H. pylori* activity of emodin have been reported previously,[24,27,28] but the urease inhibitory activity and anti-*H. pylori* activity of the anthraquinone glycoside, chrysophanol-8-*O*-*β*-D-glucoside, were reported for the first time in this study. Although the anti-*H. pylori* activity of *R. acetosa* extract was previously reported,[29] this is the first report on compounds isolated from this plant. These results provide further insights into the design and molecular mechanisms of more potent anthraquinone derivatives for the eradication of *H. pylori*.

3. MATERIALS AND METHODS

3.1. General Experimental Procedures. Nuclear magnetic resonance (NMR) spectra were obtained using Bruker DRX-300 and DRX-500 MHz spectrometers (Germany). A JEOL JMS-700 instrument (Akishima, Tokyo, Japan) was used to obtain EI-MS using JMS-700 M Station software (Tokyo, Japan). ESI-MS was measured by an AB SCIEX X500R and SCIEX OS software (Toronto, Canada). Open column chromatography was performed on silica gel 60 (0.063−0.43 mm; Merck KGaA, Darmstadt, Germany). Medium pressure liquid chromatography (MPLC) was carried out on a 25 g column filled with YMC Gel ODS-A (12 nm, S-150 μM)(YMC Co. Ltd., Kyoto, Japan) using a Biotage Isolera One system (Charlotte, NC, USA). Preparative high-performance liquid chromatography (prep-HPLC) was executed on a YMC Pack ODS-A (250 × 20 mm, 5 μm, YMC Co., Ltd., Kyoto, Japan) column using the Gilson HPLC system consisting of a 321 pump, a UV/vis-155 detector, and a GX-271 liquid handler (Middleton, WI, USA). TLC was done on Silica gel 60 F_{254} (Merck, Darmstadt, Germany). A CO_2 incubator, MCO-18AC (Sanyo, Osaka, Japan), was used to culture the bacteria. The chemicals used in the bioassays were of guaranteed reagent grade, and others were of extra grade. Quercetin and metronidazole used as positive controls were purchased from Sigma (St. Louis, MO, USA).

3.2. Plant Materials. The dried whole parts of *Rumex acetosa* L. were purchased from an oriental market in Sancheong Province, Korea, in April 2014. The plant was identified by Dr. Mi-Jeong Ahn, College of Pharmacy, Gyeongsang National University. The voucher specimen (No. PGSC-172−177) was deposited in the Herbarium of the College of Pharmacy, Gyeongsang National University.

3.3. Extraction and Isolation. The whole part of *R. acetosa* (300 g) was extracted with 70% ethanol at room temperature. The ethanolic extract was concentrated through a rotary evaporator to give a crude extract (82.4 g). This extract was then suspended in water and partitioned successively with *n*-hexane, methylene chloride (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol, respectively, to give *n*-hexane Fr. (4.5 g), CH_2Cl_2 Fr. (3.5 g), EtOAc Fr. (15.4 g), *n*-BuOH Fr. (10.0 g), and water Fr. (48.0 g) fractions, respectively. The CH_2Cl_2 fraction was subjected to open silica column chromatography (CC) with a gradient elution of $CHCl_3$ and MeOH mixture (100:0 → 0:100) to give seven fractions (fr.c1-fr.c7). Compounds **1** (169 mg), **2** (69 mg), and **3** (280 mg) were isolated by recrystallization from fr.c2, fr.c3, and fr.c6, respectively. The EtOAc fraction was subjected to open silica CC with a gradient elution of $CHCl_3$ and MeOH mixture (100:0 → 0:100) to give eight fractions (fr.e1-fr.e8). The fr.6 was divided into six subfractions (fr.e6a−fr.e6f) by MPLC with water and methanol mixture (100:0 → 0:100). Compounds **4** (20 mg, t_R 11.5 min), **5** (30 mg, t_R 12.3 min), and **6** (15 mg, t_R 14.9 min) were given by prep-HPLC using the

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following gradient elution of water (A) and acetonitrile (B) mixture; 40% B for 3 min, 40 to 50% B for 10 min, 50 to 100% B for 1 min, 100% B maintained for 4 min, 100 to 40% for 1 min, and kept the last condition for 6 min. The flow rate was 8 mL/min, and the peaks were detected at 254 nm.

3.3.1. Emodin (1). Orange powder, $C_{15}H_{10}O_5$; EI-MS m/z 270 $[M]^+$; ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.06 (1H, s, 8-OH), 12.01 (1H, s, 1-OH), 7.44 (1H, s, H-4), 7.13 (1H, s, H-2), 7.08 (1H, d, J = 2.4 Hz, H-5), 6.55 (1H, d, J = 2.4 Hz, H-7), 2.39 (3H, s, 3-CH_3); ^{13}C NMR (125 MHz, DMSO-d_6): δ 189.3 (C-9), 182.4 (C-10), 171.2 (C-6), 165.6 (C-8), 162.2 (C-1), 148.6 (C-3), 135.7 (C-10a), 133.6 (C-4a), 124.8 (C-2), 121.1 (C-4), 114.3 (C-9a), 111.1 (C-5), 108.8 (C-7, 8a), 21.6 (3-CH_3).

3.3.2. Chrysophanol (2). Orange powder, $C_{15}H_{10}O_4$; EI-MS m/z 254 $[M]^+$; ^{1}H NMR (300 MHz, $CDCl_3$): δ 12.12 (1H, s, 8-OH), 12.03 (1H, s, 1-OH), 7.83 (1H, dd, J = 8.4, 1.2 Hz, H-5), 7.70 (1H, t, J = 8.4 Hz, H-6), 7.66 (1H, d, J = 1.6 Hz, H-4), 7.30 (1H, dd, J = 8.4, 1.2 Hz, H-7), 7.11 (1H, d, J = 1.6 Hz, H-2), 2.48 (3H, s, 3-CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 192.5 (C-9), 182.0 (C-10), 162.7 (C-1), 162.4 (C-8), 149.4 (C-3), 137.0 (C-6), 133.6 (C-10a), 133.3 (C-4a), 124.6 (C-2), 124.4 (C-7), 121.4 (C-4), 119.9 (C-5), 115.9 (C-8a), 113.7 (C-9a), 22.3 (3-CH_3).

3.3.3. Physcion (3). Orange needle, $C_{16}H_{12}O_5$; EI-MS m/z 284 $[M]^+$; ^{1}H NMR (300 MHz, $CDCl_3$): δ 12.33 (1H, s, 1-OH), 12.14 (1H, s, 8-OH), 7.64 (1H, d, J = 1.6 Hz, H-4), 7.38 (1H, d, J = 2.6 Hz, H-5), 7.10 (1H, d, J = 1.6 Hz, H-2), 6.70 (1H, d, J = 2.6 Hz, H-7), 3.96 (3H, s, 6-OCH_3), 2.48 (3H, s, 3-CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 190.8 (C-9), 182.0 (C-10), 166.6 (C-8), 165.2 (C-1), 162.5 (C-6), 148.5 (C-3), 135.3 (C-10a), 133.2 (C-4a), 124.5 (C-4), 121.3 (C-2), 113.7 (C-9a), 110.3 (C-8a), 108.2 (C-5), 106.8 (C-7), 56.1 (6-OCH_3), 22.2 (3-CH_3).

3.3.4. Emodin-8-O-β-D-glucoside (4). Yellowish powder, $C_{21}H_{20}O_{10}$; ESI-MS m/z 431.0 $[M-H]^-$; ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.2 (1H, s, 1-OH), 7.45 (1H, s, H-4), 7.27 (1H, s, H-5), 7.16 (1H, s, H-2), 6.98 (1H, s, H-7), 5.05 (1H, d, J = 7.5 Hz, H-1′), 3.72 (1H, d, H-6′a), 3.51 (1H, d, H-6′b), 3.44 (1H, d, H-2′), 3.39 (1H, d, H-5′) 3.34 (1H, d, H-3′), 3.25 (1H, d, H-4′), 2.41 (3H, s, 3-CH_3); ^{13}C NMR (125 MHz, DMSO-d_6): δ 186.8 (C-9), 182.7 (C-10), 165.0 (C-6), 162.1 (C-1), 161.6 (C-8), 147.3 (C-3), 137.0 (C-4a), 132.6 (C-9a), 124.7 (C-2), 119.7 (C-4), 115.0 (C-10a), 113.0 (C-8a), 109.0 (C-5), 108.9 (C-7), 101.7 (C-1′), 77.8 (C-5′), 76.9 (C-3′), 73.7 (C-2′), 69.9 (C-4′), 61.0 (C-6′), 21.9 (3-CH_3).

3.3.5. Chrysophanol-8-O-β-D-glucoside (5). Yellowish powder, $C_{21}H_{22}O_9$; ESI-MS m/z 439.0 $[M + Na]^+$; ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.85 (1H, s, 1-OH), 7.88 (1H, d, J = 7.7 Hz, H-5), 7.86 (1H, t, J = 7.7 Hz, H-6), 7.71 (1H, d, J = 7.8 Hz, H-7), 7.54 (1H, s, H-4), 7.21 (1H, s, H-2), 5.17 (1H, d, J = 7.7 Hz, H-1′), 3.72 (1H, s, H-6′a), 3.51 (1H, s, H-6′b), 3.48 (1H, s, H-2′), 3.45 (1H, s, H-5′), 3.33 (1H, s, H-3′), 3.23 (1H, s, H-4′), 2.43 (3H, s, 3-CH_3); ^{13}C NMR (125 MHz, DMSO-d_6): δ 187.9 (C-9), 182.6 (C-10), 162.2 (C-1), 158.7 (C-8), 148.1 (C-3), 136.4 (C-6), 135.3 (C-10a), 132.7 (C-4a), 124.6 (C-2), 122.9 (C-7), 121.0 (C-5, 8a), 119.7 (C-4), 115.3 (C-9a), 101.0 (C-1′), 77.7 (C-5′), 77.0 (C-3′), 73.8 (C-2′), 70.0 (C-4′), 61.1 (C-6′), 22.0 (3-CH_3).

3.3.6. Physcion-8-O-β-D-glucoside (6). Yellowish powder, $C_{22}H_{22}O_{10}$; ESI-MS m/z 469.1 $[M + Na]^+$; ^{1}H NMR (500 MHz, DMSO-d_6): 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′), 3.74 (1H, s, H-6′a), 3.51 (1H, s, H-6′b), 3.48 (1H, s, H-2′), 3.45 (1H, s, H-5′), 3.34 (1H, s, H-3′), 3.25 (1H, s, H-4′), 3.97 (3H, s, 6-OCH_3), 2.41 (3H, s, 3-CH_3); ^{13}C NMR (125 MHz, DMSO-d_6): δ 187.0 (C-9), 182.4 (C-10), 165.2 (C-6), 162.2 (C-8), 161.2 (C-1), 147.6 (C-3), 136.8 (C-10a), 132.6 (C-4a), 124.6 (C-2), 119.8 (C-4), 115.0 (C-8a), 114.9 (C-9a), 108.7 (C-7), 107.0 (C-5), 101.1 (C-1′), 77.9 (C-5′), 77.1 (C-3′), 73.7 (C-2′), 70.3 (C-4′), 61.2 (C-6′), 56.6 (6-OCH_3), 21.9 (3-CH_3).

3.4. *Helicobacter pylori* Culture. *H. pylori* 51 and 43504 strains used in this study were provided by the *H. pylori* Korean Type Culture Collection, School of Medicine, Gyeongsang National University, Korea. Strain 51 had been isolated from the stomach of a Korean patient in 1987, and strain 43504 was isolated from the stomach of an American patient in 2008. Two strains were grown and maintained on Brucella agar medium (BD Co., Sparks, MD, USA) supplemented with 10% horse serum (Gibco, New York, USA). Incubation was done at 37 °C, 100% humidity, and 10% CO_2 conditions. Subculture was accomplished every 2−3 days.

3.5. Paper Disc Diffusion Assay. The anti-*H. pylori* activity of total extract and the fractions was measured with impregnated paper disc according to our previously reported method.[30] Each 30 μL of the 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 the diameters of the inhibition zones were recorded after incubation for 2 days. DMSO was used as the negative control, while quercetin was used as the positive controls.

3.6. MIC Determination. MICs were determined with the broth dilution method as shown in our previous report.[28,30] After incubation for 24 h, growth was evaluated by reading the optical density at 600 nm. MIC_{50} and MIC_{90} were defined as the lowest concentration of inhibiting growth by 50 and 90%, respectively.

3.7. Anti-Urease Activity. The antiurease assay was conducted using phenol red reagent referring to the previously reported paper.[31] The bacterial solution of *H. pylori* was centrifuged at 500 × *g* for 2 min. The resulting precipitate was suspended in lysis buffer followed by three cycles of sonication for 1 min and washed, putting the solution in an ice bath for 10 min. The solution was centrifuged at 100 × *g* for 1 min. Twenty microliters of each compound was reacted with the urease (50 μL) in a 10% CO_2 incubator at 37 °C for 10 min. Subsequently, the mixture was reacted with 0.1 M urea and 1 mM phenol red under 10% CO_2 conditions and 37 °C for 30 min. The absorbance was measured at 570 nm. The activity of the uninhibited enzyme was presumed to be control with an enzyme activity of 100%. The inhibitory activity of compounds **1** and **5** against *H. pylori* urease was obtained based on the following equation: Inhibitory activity (%) = [(absorbance of the control− absorbance of solution with samples)/absorbance of the control] × 100.

3.8. Statistical Analysis. Data were presented as means ± standard deviations of two or triple independent experiments. Statistical significance ($p < 0.05$) was assessed by one-way analysis of variance (ANOVA) using coupled with Dunnett's *t*-tests SPSS Statistics 24.0 software (IBM, Armonk, NY, USA). GraphPad Prism Version 5.01 (GraphPad Software, Inc., San Diego, CA) was used to calculate the MIC_{50} and MIC_{90} values. The values were obtained from triplicate determinations and two independent experiments.

3.9. Molecular Docking. Two selected ligands of emodin (PubChem ID: 3220) and chrysophanol-8-*O*-β-D-glucoside (PubChem ID: 442731) were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). *H. pylori* urease

was obtained from the Protein Databank (https://www.rcsb.org/) (PDB ID: 1E9Y). The AutoDock Vina tool (version 1.5.7) was used to investigate the molecular interactions between the *H. pylori* urease enzyme and the selected ligands. Before the docking analysis, the structure of the enzyme was optimized by removing excess ligands and water molecules using the BIOVIA Discovery Studio 2021 program. Then, all compounds were optimized for energy using the Spartan 14 (Version 1.1.4) program. Polar hydrogen atoms were added to the protein using AutoDock Vina 1.5.7, and the Kollman charges were determined. The partial charges of the compounds were calculated using Computer Gasteiger software. The *x*, *y*, and *z* coordinates were determined to allow the protein to bind to its catalytic site. Finally, the BIOVIA Discovery Studio visualizer and LigPlot were utilized to investigate the molecular interactions and binding types between the selected compounds and the *H. pylori* urease enzyme.[32]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c05282.

MS spectra of isolated compounds; ^{1}H- and ^{13}C NMR spectra; and HMBC spectra of compounds **4−6** (PDF)

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Notes

The authors declare no competing financial interest.

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