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# Anti-*Helicobacter pylori* Activity of Compounds Isolated from *Fraxinus mandshurica* Bark

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**Abstract** – *Helicobacter pylori* is a well-known pathogen that is responsible for gastric disorders. Overcoming of the antibiotic-resistance is a main barrier to treat *H. pylori* infection. In our search for anti-*H. pylori* compounds from natural resources, bioactivity-guided isolation on the ethyl acetate fraction of *Fraxinus mandshurica* bark that had shown anti-*H. pylori* activity gave twelve compounds (**1** - **12**) of six coumarins, three phenylethanoids, two secoiridoids, and a lignan using silica gel column chromatography, Sephadex-LH 20, and recrystallization. The chemical structures were identified by spectroscopic data analysis, including 1D, 2D NMR, and mass spectrometry. Among them, compounds **2**, **10**, and **11** showed moderate growth inhibitory activity against three strains of *H. pylori*, compared with positive controls of quercetin and metronidazole. Compounds **5**, **6**, **8**, and **12** exhibited the inhibitory activity against strains 26695 or 43504. This is the first report on the anti-*H. pylori* activity of this plant and the isolated compounds.
**Keywords** – *Fraxinus mandshurica,* Anti-*Helicobacter pylori,* Fraxetin, Calceolarioside A, Calceolarioside B

## Introduction

*Helicobacter pylori* is a gram-negative bacteria that is considered as one of the most predominant human pathogens in the world.[1] Infection by this bacteria plays a vital role, not only in causing the peptic ulcer disease, but also in gastric cancer, which is the third most common cause of cancer-related deaths worldwide.[1,2] Moreover, *H. pylori* is linked to autoimmune conditions such as Sjogren's syndrome, primary biliary cirrhosis, primary sclerosing cholangitis, autoimmune hepatitis or hepatitis C virus-related liver diseases that trigger autoimmune sequelae.[3] Therefore, treatment and elimination of *H. pylori* is an important public health intervention. Treatment of *H. pylori* infection has been approached by the widely used standard triple therapy or quadruple therapy containing a proton-pump inhibitor and the antibiotics of clarithromycin, amoxicillin, and metronidazole with the mild side effects such as vomiting and diarrhea.[4-6] Meanwhile, *H. pylori* has been included in the list of antibiotic-resistant "priority pathogens" by the World Health Organization (WHO), with a catalog of both antimicrobial resistance and common microbiota side effects.[1] To reduce the severity of antimicrobial resistance, it is necessary to search for new natural products that can inhibit the growth of pathogens with minimum adverse effects.

*Fraxinus mandshurica*, known as the Manchurian ash (Oleaceae) is a tree native to China, Korea, Japan, and Russia, and is widely used for making furniture because of its moderate hardness and beautiful texture.[7,8] Some countries like Canada and the United States occasionally cultivated it as an ornamental tree as it makes an excellent landscape in moist areas. On the contrary, the dried bark of *F. mandshurica* has been used as "Qinpi" (秦皮, Cortex Fraxini), a traditional folk medicine in Korea, China, and Japan, to treat diseases such as rheumatic arthritis, inflammatory diseases, urinary retention, fever, and so on.[9-11] Several studies showed that the isolated compounds from *F. mandshurica* such as hydroxy-coumarins, a monolignol, lignans, phenylethanoids, and secoiridoids, possess various biological activities both *in vitro* and *in vivo*.[12] Fraxetin has the potential to treat osteolytic diseases and can prevent osteoblast apoptosis.[13] Oleuropein modulates glucose consumption and increases insulin sensitivity in skeletal muscle cells through multiple mechanisms.[14] Calceolarioside A can act as an immunosuppressant through inhibition of IL-2 and IgE

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production in mouse spleen cells and U266 cells respectively.[9] Although *F. mandshurica* has shown antimicrobial activity against *Staphylococcus aureus*, there is no systemic report about its activity against *H. pylori* with the same species yet.[15]

Therefore, the goal of the present study was to isolate specific bioactive compounds from *F. mandshurica* bark, of which total extract had shown anti-*H. pylori* activity, and to evaluate the growth inhibitory activity of the isolated compounds.

## Experimental

**General** – Extra pure grade solvents (Daejung, Siheung, Korea) were used for sample extraction, fractionation and isolation. The 1D and 2D NMR spectra were recorded using a Bruker DRX-300 and a Bruker DRX-500 spectrometer (Karlsruhe, Germany), respectively. NMR solvents were purchased from Cambridge Isotope laboratories, Inc. (Andover, MA, USA). EI-MS and FAB-MS spectral data were recorded on a JEOL JMS-700 (Akishima, Japan). Optical density was determined on a Optizen Pop UV/Vis spectrophotometer (Mecasys, Daejeon, Korea). Column chromatography was performed on silica gel 60 (0.063 - 0.43 mm, Merck, Darmstadt, Germany) and Sephadex LH-20 (GE Healthcare, Uppsala, Sweden). Medium pressure liquid chromatography (MPLC) was performed on 120 g of YMC GEL ODS-A (12 nm, S-150 lM) (YMC Co. Ltd., Kyoto, Japan) with Biotage Isolera One system (Charlotte, NC, USA). TLC was performed on silica gel 60 $F_{254}$ (Merck, Darmstadt, Germany). A $CO_2$ incubator (Sanyo, Sakata, Japan) was used for *H. pylori* culture. Quercetin and metronidazole used as positive controls were purchased from Sigma (St. Louis, MO, USA).

**Plant material** – The stem bark of *Fraxinus mandshurica* Rupr. was collected from Sicheon-Myeon of Sancheong province in June, 2016. The sample was identified by Professor Mi-Jeong Ahn, College of Pharmacy, Gyeongsang National University, and the voucher specimen (PGSC No. 561) was deposited in the Herbarium of the College of Pharmacy, Gyeongsang National University.

**Extraction and isolation** – The dried bark of *F. mandshurica* (960 g) was ground and extracted with 100% methanol at room temperature. The methanolic extract was concentrated through the rotary evaporator to give a crude extract (187 g). This methanolic extract was suspended in water and partitioned successively with *n*-hexane, dicholomethane, ethyl acetate and *n*-butanol, respectively, to yield *n*-hexane fr. (12 g), $CH_2Cl_2$ fr. (6 g), EtOAc fr. (60 g), *n*-BuOH fr. (88 g) and aqueous fr. (14 g) fractions, respectively. The EtOAc fraction was subjected to open silica column chromatography (CC) with a gradient elution of $CHCl_3$ and MeOH mixture (100:0 → 0:100) give seven subfractions (Fr.1-Fr.7). Compound **1** (32 mg) was isolated from the Fr.1 by the silica gel CC using a mixture of Hexane and EtOAc, followed by Sephadex LH-20 CC using methanol as eluting solvent. Compound **2** (52.4 mg) was isolated from another subfraction, Fr.2 by recrystallization. A subfraction, Fr.3 was applied on Sephadex LH-20 CC with MeOH which gave three subfractions (fr.3.1-fr.3.3). Compound **3** (4.5 mg) was isolated from fr.3.2 by recrystallization. The Fr.4 was applied to open silica CC using a mixture of $CHCl_3$ and MeOH (100:0 → 0:100) as an eluting solvent to give four subfractions (fr.4.1-fr.4.4). A subfraction, fr.4.3 was divided into five subfractions (fr.4.3.1-fr.4.3.5) by silica CC using a mixture of EtOAc and MeOH as an eluting solvent. Compounds **4** (2.8 g) and **5** (800 mg) were isolated from a subfraction, fr.4.3.2 and fr.4.3.3, respectively, by recrystallization. Compound **6** (1.5 g) was isolated from the fr.4.3.4 by reverse phase liquid chromatography (MPLC) with a gradient elution of water : MeOH (100:0 → 0:100). Fr.5 fraction was applied on MPLC using a mixture of $CH_2Cl_2$ and MeOH (100:0 → 0:100) as an eluting solvent to give five subfractions (fr.5.1-5.5). Compound **7** (20 mg) was isolated from a subfraction, fr.5.5 by recrystallization. Compounds **8** (600 mg) and **9** (15 mg) were isolated from fr.5.2.2 and fr.5.3.2 through reverse phase MPLC with a gradient elution of water : MeOH (100:0 → 0:100) and normal MPLC with the mixture of EtOAc and MeOH as an eluting solvent, respectively. The Fr.6 was divided into five subfractions (fr.6.1-fr.6.5) by silica gel CC using a mixture of EtOAc and MeOH as an eluting solvent. Compound **10** (10 mg) was isolated from a subfraction fr.6.2 by reverse phase MPLC with a gradient elution of water and MeOH mixture (100:0 → 0:100), which gave two subfractions (fr.6.2.1-fr.6.2.2). A subfraction, fr.6.3 yielded compound **11** (6.3 mg) by MPLC using a mixture of $CHCl_3$ and MeOH (100:0 → 0:100) as an eluting solvent. Compound **12** (15 mg) was isolated from a subfraction, fr.6.5 through Sephadex LH-20 CC with MeOH.

**Fraxinol (1)** – White crystal, $C_{11}H_{10}O_5$; EI-MS (*m/z*): 222 $[M]^+$; $^1$H-NMR ($CDCl_3$, 500 MHz): δ 7.97 (1H, d, $J$ = 9.7 Hz, H-4), 6.65 (1H, s, H-8), 6.29 (1H, d, $J$ = 9.7 Hz, H-3), 5.51 (1H, s, H-6), 4.05 (3H, s, 7-$OCH_3$), 3.99 (3H, s, 5-$OCH_3$); $^{13}$C-NMR ($CDCl_3$, 125 MHz): δ 161.4 (C-2), 151.1 (C-7), 148.7 (C-9), 141.9 (C-5), 138.8 (C-4), 134.7 (C-6), 113.0 (C-3), 107.5 (C-10), 94.8 (C-8), 61.5

(7-$OCH_3$), 56.6 (5-$OCH_3$).

**Fraxetin (2)** – Pale yellowish amorphous powder, $C_{10}H_8O_5$; FAB-MS (*m/z*): 209.2 $[M+H]^+$; $^1$H-NMR (DMSO-$d_6$, 300 MHz): δ 7.89 (1H, d, *J* = 9.5 Hz, H-4), 6.80 (1H, s, H-5), 6.22 (1H, d, *J* = 9.5 Hz, H-3), 3.82 (3H, s, 6-$OCH_3$); $^{13}$C-NMR (DMSO-$d_6$, 75 MHz): δ 161.0 (C-2), 145.8 (C-7), 140.8 (C-8), 140.0 (C-6), 133.3 (C-9), 112.2 (C-3), 110.7 (C-10), 100.8 (C-5), 56.5 (6-$OCH_3$).

**Tyrosol (3)** – Brownish amorphous powder, $C_8H_{10}O_2$; EI-MS (*m/z*): 138 $[M]^+$; $^1$H-NMR ($CDCl_3$, 300 MHz): δ 7.12 (2H, d, *J* = 8.5 Hz, H-2, 6), 6.81 (2H, d, *J* = 8.5 Hz, H-3, 5), 3.86 (2H, t, *J* = 6.6 Hz, H-8), 2.83 (2H, t, *J* = 6.6 Hz, H-7); $^{13}$C-NMR ($CDCl_3$, 125 MHz): δ 154.3 (C-4), 130.2 (C-2, 6), 115.4 (C-3, 5), 63.9 (C-8), 38.1 (C-7).

**Ligstroside (4)** – Brownish sticky powder, $C_{25}H_{32}O_{12}$; FAB-MS (*m/z*): 547.1 $[M+Na]^+$; $^1$H-NMR ($CD_3OD$, 500 MHz): δ 7.53 (1H, s, H-3), 7.07 (2H, d, *J* = 8.5 Hz, H-4′, 8′), 6.74 (2H, d, *J* = 8.5 Hz, H-5′, 7′), 6.10 (1H, q, *J* = 7.2 Hz, H-8), 5.94 (1H, s, H-1), 4.83 (1H, d, *J* = 7.2 Hz, H-1″), 4.25 (1H, m, H-1′a), 4.13 (1H, m, H-1′b), 3.99 (1H, dd, *J* = 9.2, 4.5 Hz, H-5), 3.89 (1H, dd, *J* = 12.1, 1.8 Hz, H-6″b), 3.73 (3H, s, H-$COOCH_3$), 3.69 (1H, m, H-6″a), 3.43 (1H, d, *J* = 9.0 Hz, H-3″), 3.35 (1H, m, H-4″), 3.34 (1H, m, H-5″), 3.33 (1H, m, H-2″), 2.84 (2H, t, *J* = 6.9 Hz, H-2′), 2.72 (1H, dd, *J* = 14.1, 4.5 Hz, H-6a), 2.45 (1H, dd, *J* = 14.1, 4.5 Hz, H-6b), 1.67 (3H, dd, *J* = 7.2, 1.4 Hz, H-10); $^{13}$C-NMR ($CD_3OD$, 125 MHz): δ 171.8 (C-7), 167.2 (C-11), 155.7 (C-6′), 153.7 (C-3), 130.0 (C-4′, 8′), 129.1 (C-9), 128.6 (C-3′), 123.5 (C-8), 114.9 (C-5′, 7′), 108.0 (C-4), 99.5 (C-1″), 93.7 (C-1), 77.0 (C-5″), 76.5 (C-3″), 73.4 (C-2″), 70.1 (C-4″), 65.5 (C-1′), 61.4 (C-6″), 50.8 (11-$OCH_3$), 39.8 (C-6), 33.8 (C- 2′), 30.4 (C-5), 12.2 (C-10).

**Mandshurin (5)** – White amorphous powder, $C_{17}H_{20}O_{10}$; FAB-MS (*m/z*): 385.2 $[M+H]^+$; $^1$H-NMR (DMSO-$d_6$, 500 MHz): δ 8.00 (1H, d, *J* = 9.7 Hz, H-4), 6.90 (1H, s, H-8), 6.28 (1H, d, *J* = 9.7 Hz, H-3), 4.95 (1H, d, *J* = 5.6 Hz, H-1′), 3.97 (3H, s, 5-$OCH_3$), 3.87 (3H, s, 7-$OCH_3$), 3.58 (1H, m, H-6′a), 3.39 (1H, m, H-6′b), 3.25 (1H, m, H-5′), 3.22 (1H, m, H-2′), 3.13 (1H, m, H-4′), 3.05 (1H, m, H-3′); $^{13}$C-NMR (DMSO-$d_6$, 125 MHz): δ 160.7 (C-2), 157.4 (C-7), 151.5 (C-9), 149.4 (C-5), 139.7 (C-4), 134.6 (C-6), 112.7 (C-3), 107.2 (C-10), 102.9 (C-1′), 96.7 (C-8), 77.8 (C-3′), 77.0 (C-5′), 74.5 (C-2′), 70.4 (C-4′), 61.3 (C-6′), 62.7 (5-$OCH_3$), 57.2 (7-$OCH_3$).

**Pinoresinol-4′-*O*-*β*-D-glucopyranoside (6)** – White sticky powder, $C_{26}H_{32}O_{11}$; FAB-MS (*m/z*): 543.2 $[M+Na]^+$; $^1$H-NMR ($CD_3OD$, 500 MHz): 7.17 (1H, d, *J* = 8.4 Hz, H-5′), 7.03 (1H, d, *J* = 1.8 Hz, H-2′), 6.96 (1H, d, *J* = 1.8 Hz, H-2), 6.92 (1H, dd, *J* = 8.4, 1.8, Hz, H-6′), 6.81 (1H, dd, *J* = 8.1, 1.8 Hz, H-6), 6.78 (1H, d, *J* = 8.1 Hz, H-5), 4.89 (1H, d, *J* = 7.4 Hz, H-1″), 4.75 (1H, d, *J* = 4.5 Hz, H-7′), 4.71 (1H, d, *J* = 4.5 Hz, H-7), 4.24 (2H, m, H-$9_{eq}$, $9'_{eq}$), 3.87 (3H, s, 3′-$OCH_3$), 3.87 (1H, s, H-6″a), 3.86 (3H, s, 3-$OCH_3$), 3.83 (2H, m, H-$9_{ax}$, $9'_{ax}$), 3.71 (1H, m, H-6″b), 3.52 (1H, s, H-2″), 3.50 (1H, s, H-3″), 3.42 (1H, s, H-4″), 3.41 (1H, s, H-5″), 3.12 (2H, m, H-8′, 8); $^{13}$C-NMR ($CD_3OD$, 125 MHz): δ 149.5(C-3′), 147.8 (C-3), 146.1 (C-4′), 145.9 (C-3), 136.1 (C-1′), 132.4 (C-1), 118.7 (C-6), 118.4 (C-6′), 116.6 (C-5′), 114.7 (C-5), 110.2 (C-2′), 109.6 (C-2), 101.4 (C-1″), 86.1 (C-7), 85.7 (C-7′), 76.8 (C-5″), 76.4 (C-3″), 73.5 (C-2″), 71.3 (9, 9′), 69.9 (C-4″), 61.1 (C-6″), 55.4 (3′-$OCH_3$), 55.1 (3-$OCH_3$), 54.1 (C-8′), 53.9 (C-8).

**Fraxin (7)** – Pale yellow amorphous powder, $C_{16}H_{18}O_{10}$; FAB-MS (*m/z*): 371.1 $[M+H]^+$; $^1$H-NMR (DMSO-$d_6$, 500 MHz): δ 7.92 (1H, d, *J* = 9.5 Hz, H-4), 7.07 (1H, s, H-5), 6.26 (1H, d, *J* = 9.5 Hz, H-3), 4.96 (1H, d, *J* = 7.8 Hz, H-1′), 3.83 (3H, s, 6-$OCH_3$), 3.61 (1H, d, *J* = 11.6 MHz, H-6′a), 3.46 (1H, m, H-6′b), 3.36 (1H, s, H-2′), 3.26 (1H, m, H-5′), 3.20 (1H, m, H-4′), 3.17 (1H, m, H-3); $^{13}$C-NMR (DMSO-$d_6$, 125 MHz): δ 160.7 (C-2), 145.9 (C-6), 145.2 (C-4), 144.3 (C-7), 143.2 (C-9), 132.0 (C-8), 112.6 (C-3), 110.5 (C-10), 105.4 (C-5), 104.4 (C-1′), 77.8 (C-3′), 76.7 (C-5′), 74.3 (C-2′), 70.1 (C-4′), 61.2 (C-6′), 56.6 (6-$OCH_3$).

**Oleuropein (8)** – Dark brown sticky powder, $C_{25}H_{32}O_{13}$; FAB-MS (*m/z*): 541.2 $[M+H]^+$; $^1$H-NMR (DMSO-$d_6$, 500 MHz): δ 7.52 (1H, s, H-3), 6.64 (1H, d, *J* = 8.0 Hz, H-7′), 6.60 (1H, d, *J* = 1.9 Hz, H-4′), 6.47 (1H, dd, *J* = 8.0, 1.9 Hz, H-8′), 5.96 (1H, q, *J* = 6.9 Hz, H-8), 5.87 (1H, s, H-1), 4.65 (1H, d, *J* = 7.8 Hz, H-1″), 4.08 (2H, m, H-1′a, 1′b), 3.86 (1H, dd, *J* = 9.2, 4.3 Hz, H-5), 3.69 (1H, d, *J* = 12.1 Hz, H-6″a), 3.65 (3H, s, H-12), 3.46 (1H, m, H-6″b), 3.20 (1H, m, H-5″), 3.17 (1H, m, H-3″), 3.07 (1H, m, H-2″), 3.06 (1H, m, H-4″), 2.68 (2H, t, *J* = 7.2 Hz, H-2′), 2.62 (1H, dd, *J* = 14.5, 4.3 Hz, H-6a), 2.41 (1H, dd, *J* = 14.5, 9.2 Hz, H-6b), 1.65 (3H, d, *J* = 7.1 Hz, H-10); $^{13}$C-NMR (DMSO-$d_6$, 125 MHz): δ 171.1(C-7), 166.6 (C-11), 153.9 (C-3), 145.6 (C-5′), 144.2 (C-6′), 129.6 (C-9), 128.9 (C-3′), 123.5 (C-8), 120.0 (C-8′), 116.6 (C-4′), 116.0 (C-7′), 108.2 (C-4), 99.5 (C-1″), 93.4 (C-1), 77.8 (C-3″), 77.0 (C-5″), 73.7 (C-2″), 70.4 (C-4″), 65.5 (C-1′), 61.6 (C-6″), 51.7 (C-12), 40.6 (C-6), 34.2 (C-2′), 30.6 (C-5), 13.5 (C-10).

**Isofraxidin-7-*O*-*β*-D-glucopyranoside (9)** – White amorphous powder, $C_{17}H_{20}O_{10}$; FAB-MS (*m/z*): 385.1 $[M+H]^+$; $^1$H-NMR (DMSO-$d_6$, 500 MHz): δ 7.97 (1H, d, *J* = 9.5 Hz, H-4), 7.14 (1H, s, H-5), 6.41 (1H, d, *J* = 9.5 Hz, H-3), 5.17 (1H, d, *J* = 7.4 Hz, H- 1′), 3.92 (3H, s, 8-$OCH_3$), 3.82 (3H, s, 6-$OCH_3$), 3.61 (1H, m, H-6′a), 3.40 (1H, m,

H-6′b), 3.27 (1H, m, H-5′), 3.23 (1H, m, H-2′), 3.13 (1H, m, H-3′), 3.05 (1H, m, H-4′); $^{13}$C-NMR (DMSO-$d_6$, 125 MHz): 160.2 (C-2), 149.9 (C-6), 144.8 (C-4), 142.8 (C-9), 142.1 (C-7), 140.7 (C-8), 115.2 (C-3), 115.0 (C-10), 105.9 (C-5), 102.6 (C-1′), 78.0 (C-3′), 77.0 (C-5′), 74.6 (C-2′), 70.3 (C-4′), 61.2 (C-6′), 61.7 (8-$OCH_3$), 57.0 (6-$OCH_3$).

**Calceolarioside A (10)** – Brownish needles; $C_{23}H_{26}O_{11}$; FAB-MS (*m/z*): 479.2 $[M+H]^+$; $^1$H-NMR ($CD_3OD$, 500 MHz): δ 7.61 (1H, d, *J* = 15.9 Hz, H-β), 7.07 (1H, d, *J* = 1.9 Hz, H-2″), 6.98 (1H, dd, *J* = 8.2, 1.9 Hz, H-6″), 6.80 (1H, d, *J* = 8.2 Hz, H-5″), 6.72 (1H, d, *J* = 2.1 Hz, H-2), 6.69 (1H, d, *J* = 8.0 Hz, H-5), 6.59 (1H, dd, *J* = 8.0, 2.1 Hz, H-6), 6.32 (1H, d, *J* = 15.9 Hz, H-α), 4.85 (1H, s, H-4′), 4.38 (1H, d, *J* = 7.8 Hz, H-1′), 4.07 (1H, m, H-8b), 3.74 (1H, m, H-8a), 3.64 (2H, m, H-6′b), 3.56 (1H, m, H-6′a), 3.52 (1H, m, H-5′), 3.31 (1H, m, H-2′), 2.82 (2H, m, H-7); $^{13}$C-NMR ($CD_3OD$, 125 MHz): δ 167.2 (C=O), 148.3 (C-4″), 146.2 (C-β), 145.5 (C-3″), 144.7 (C-3), 143.3 (C-4), 130.1 (C-1), 126.3 (C-1″), 121.7 (C-6″), 119.8 (C-6), 115.7 (C-2), 115.1 (C-5″), 114.9 (C-5), 113.8 (C-2″), 113.3 (C-α), 103.0 (C-1′), 74.7 (C-5′), 74.4 (C-3′), 73.8 (C-2′), 71.1 (C-4′), 70.8 (C-8), 61.1 (C-6′), 35.2 (C-7).

**Calceolarioside B (11)** – Brownish amorphous powder; $C_{23}H_{26}O_{11}$; FAB-MS (*m/z*): 478.2 $[M]^+$; $^1$H-NMR ($CD_3OD$, 500 MHz): δ 7.58 (1H, d, *J* = 15.9 Hz, H-β), 7.05 (1H, d, *J* = 2.1 Hz, H-2″), 6.91 (1H, dd, *J* = 8.2, 2.1 Hz, H-6″), 6.79 (1H, d, *J* = 8.2 Hz, H-5″), 6.69 (1H, d, *J* = 2.0 Hz, H-2), 6.65 (1H, d, *J* = 8.0 Hz, H-5), 6.55 (1H, dd, *J* = 8.0, 2.0 Hz, H-6), 6.31 (1H, d, *J* = 15.9 Hz, H-α), 4.52 (1H, dd, *J* = 9.8, 2.2 Hz, H-6′a), 4.37 (1H, m, H-6′b), 4.34 (1H, d, *J* = 7.8 Hz, H-1′), 3.98 (1H, m, H-8a), 3.73 (1H, m, H-8b), 3.54 (1H, m, H-5′), 3.39 (1H, m, H-4′), 3.37 (1H, s, H-3′), 3.23 (1H, m, H-2′), 2.80 (2H, m, H-7); $^{13}$C-NMR ($CD_3OD$, 125 MHz): δ 167.8 (C=O), 148.2 (C-4″), 145.8 (C-β), 145.4 (C-3″), 144.7 (C-3), 143.3 (C-4), 130.0 (C-1), 126.3 (C-1″), 121.8 (C-6″), 119.9 (C-6), 115.7 (C-2), 115.1 (C-5″), 115.0 (C-5), 113.7 (C-2″), 113.5 (C-α), 103.2 (C-1′), 76.5 (C-3′), 74.1 (C-5′), 73.7 (C-2′), 71.0 (C-8), 70.3 (C-4′), 63.2 (C-6′), 35.3 (C-7).

**Esculin (12)** – White amorphous powder; $C_{15}H_{16}O_9$; FAB-MS (*m/z*): 341.2 $[M+H]^+$; $^1$H-NMR (DMSO-$d_6$, 500 MHz): δ 7.85 (1H, d, *J* = 9.5 Hz, H-4), 7.39 (1H, s, H-5), 6.81 (1H, s, H-8), 6.22 (1H, d, *J* = 9.5 Hz, H-3), 4.77 (1H, d, *J* = 7.3 Hz, H-1′), 3.33 (1H, m, H-3′), 3.30 (1H, m, H-5′), 3.28 (1H, m, H-2′), 3.19 (1H, m, H-4′); $^{13}$C-NMR (DMSO-$d_6$, 125 MHz): 161.3 (C-2), 152.4 (C-7), 151.1 (C-9), 145.0 (C-4), 143.3 (C-6), 115.2 (C-5), 112.2 (C-3), 110.9 (C-10), 103.6 (C-8), 102.8 (C-1′), 77.7 (C-3′), 76.5 (C-5′), 73.7 (C-2′), 70.2 (C-4′), 61.1 (C-6′).

***Helicobacter pylori* culture** – Three *H. pylori* strains (43504, 26695, and 51) were provided by the *Helicobacter pylori* Korean Type Culture Collection, School of Medicine, Gyeongsang National University, Korea. The *H. pylori* strains were grown and maintained under 100% humidity and 10% $CO_2$ for 2 - 3 days at 37 °C. Brucella agar medium (BD Co., Sparks, MD, USA) supplemented with 10% horse serum (Gibco, New York, NY, USA) was used.

**Paper disc diffusion assay** – Anti-*H. pylori* activity of the total extract and the fractions was evaluated with impregnated paper discs according to our previously reported method.[5,6] A total of 30 μL of sample solution in DMSO was applied to the 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 measured after incubation for 2 days. DMSO and quercetin were used as the negative and positive control, respectively.

**MIC determination** – The broth dilution method was used to determine the minimal inhibitory concentration (MIC).[16] A bacterial colony suspension equivalent to 2 - 3 × $10^8$ cfu/mL was prepared. Twenty microliters of the bacterial inoculum were added to Brucella broth media supplemented with 10% horse serum in each well of a 6-well plate containing 20 μL of two-fold serially diluted test samples (6.25 - 100 μM) and controls, so that the final volume was 2 mL. After incubation at 37 °C for 24 h, the MIC value was defined as the lowest concentration of samples at which bacterial growth was inhibited. Growth was assessed by reading the optical density at 600 nm with a spectrophotometer. All of the values were obtained from three independent experiments.

## Results and Discussion

The anti-*H. pylori* activity of the total extract and five fractions from *F. mandshurica* bark was evaluated through a disc diffusion method using three strains of *H. pylori* 43504, 26695, and 51 (Table 1). The inhibitory activity of the total extract was similar to that of the positive control, quercetin. Among the fraction samples, the ethyl acetate fraction (EtOAc fr.) showed the most potent inhibitory activity against the three strains of *H. pylori*. The BuOH fr. and the aqueous fr. failed to show any inhibitory activity. Therefore, bioactivity-guided isolation was carried out for the EtOAc fr., and 12 compounds (**1 - 12**) were isolated from the fraction by successive chromatographic separations including silica gel, YMC gel, and Sephadex LH-20. The chemical structures of the isolated compounds were identified as

**Table 1.** Anti-*H. pylori* activity of total extract and fractions from *F. mandshurica* bark

| | Strains | DMSO[a] | Quercetin[b] | Total extract | Hexane fr. | $CH_2Cl_2$ fr. | EtOAc fr. | BuOH fr. | Aqueous fr. |
|---|---|---|---|---|---|---|---|---|---|
| Inhibition zone (mm) | 43504 | -[c] | 10 | 9 | 14 | 16 | 19 | - | - |
| | 26695 | - | 9 | 10 | 14 | 16 | 19 | - | - |
| | 51 | - | 10 | 10 | 13 | 15 | 18 | - | - |

These values were obtained from three independent experiments and expressed as the mean value (10 mg/mL).
[a]Negative control, [b]Positive control, [c]No inhibition

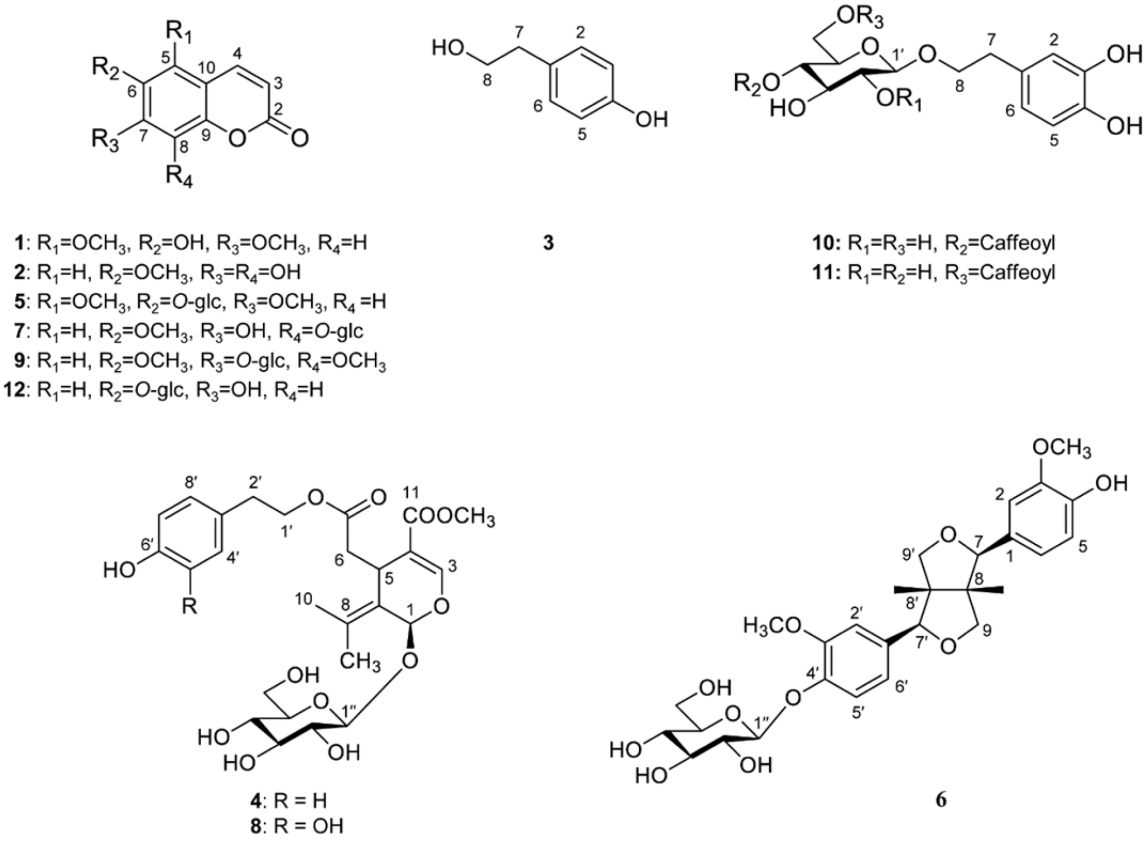


**Fig. 1.** Chemical structures of compounds **1 - 12** isolated from the bark of *Fraxinus mandshurica*.

six coumarins of fraxinol (**1**), fraxetin (**2**), mandshurin (**5**), fraxin (**7**), isofraxidin-7-*O*-*β*-D-glucopyranoside (**9**), and esculin (**12**); three phenylethanoids of tyrosol (**3**), calceolarioside A (**10**) and calceolarioside B (**11**); two secoiridoids of ligstroside (**4**) and oleuropein (**8**); and a lignan, pinoresinol-4′-*O*-*β*-D-glucopyranoside, identified by the extensive analysis and comparing their spectroscopic data (1D and 2D NMR, EI-MS, and FAB-MS) with those previously reported in the literature (Fig. 1).[11,17-25] Among them, isofraxidin-7-*O*-*β*-D-glucopyranoside (**9**) was isolated from the bark of *F. mandshurica* for the first time.

The MIC value of each isolated compound was measured using the broth dilution method. Among the six coumarins, fraxetin (**2**) showed the most potent anti-*H. pylori* activity against all three strains (43504, 26695, and 51) with an MIC value of 12.5 μM. The inhibitory activity of **2** was more potent than that of the positive control, quercetin, and similar to that of metronidazole. The number and position of hydroxy groups on the benzenoid ring of coumarin compounds are known to be related to anti-*H. pylori* activity.[26] Specifically, 7-hydroxy substituted coumarins demonstrated higher inhibitory activity among the test coumarin derivatives.[27] The present study indicated that a catechol moiety at C-7 and C-8 positions enhances the inhibitory activity of **2** against *H. pylori*. In addition, substitution of the hydroxy group at the C-8 position with a glucose moiety reduced the inhibitory effect of fraxin (**7**). Compound **9**, which contains glucose and methoxy moieties at positions 7 and

**Table 2.** MIC values of compounds **1** - **12** against three *H. pylori* strains

| Compounds | Strains | | |
|---|---|---|---|
| | 43504 | 26695 | 51 |
| Fraxinol (**1**) | > 100 | > 100 | > 100 |
| Fraxetin (**2**) | 12.5 | 12.5 | 12.5 |
| Tyrosol (**3**) | > 100 | > 100 | > 100 |
| Ligstroside (**4**) | > 100 | > 100 | > 100 |
| Mandshurin (**5**) | > 100 | 25 | > 100 |
| Pinoresinol-4′-*O*-*β*-D-glucopyranoside (**6**) | > 100 | 25 | > 100 |
| Fraxin (**7**) | > 100 | 50 | > 100 |
| Oleuropein (**8**) | 50 | > 100 | > 100 |
| Isofraxidin-7-*O*-*β*-D-glucopyranoside (**9**) | > 100 | > 100 | > 100 |
| Calceolarioside A (**10**) | 25 | 50 | 50 |
| Calceolarioside B (**11**) | 12.5 | 25 | 12.5 |
| Esculin (**12**) | 25 | > 100 | > 100 |
| Quercetin* | 50 | 50 | 50 |
| Metronidazole* | 12.5 | 6.25 | 12.5 |

These values were obtained from three independent experiments and expressed as the mean value (μM).
*Positive control

8, respectively, did not demonstrate any anti-*H. pylori* activity. Meanwhile, mandshurin (**5**) with a glucose moiety at the position 6 displayed weak inhibitory activity against a strain 26695, whereas fraxinol (**1**) failed to show any activity. These results suggest that a glucose moiety at the position 6 plays a role in the inhibitory activity. While **5** showed growth inhibitory activity against strain 26695 only, esculin (**12**), another coumarin with a glucose moiety at the position 6 (similar to **5**), exhibited inhibitory activity against a strain 43504 only. It can be deduced from this result that the two methoxy groups at positions 5 and 7 of compound **5** confers selective inhibition against the two different strains. These results suggest that not only the number and position of hydroxy groups on the benzenoid ring, but also the presence or specific position of a glucose moiety in the coumarin derivatives can affect the growth inhibitory activity against *H. pylori*. This is the first report on the anti-*H. pylori* activity of coumarin glycosides.

Two phenylethanoid glucosides of calceolarioside A (**10**) and calceolarioside B (**11**), both with one catechol moiety, showed moderate anti-*H. pylori* activity, whereas a simple phenylethanoid, tyrosol (**3**), did not exhibit any activity. Compound **11** with a caffeoyl moiety at position 6′ of the glucose displayed more potent inhibitory activity than compound **10**, which possesses the same moiety at position 4′. The results suggest that a caffeoyl or a glucose moiety could play a role in the inhibitory activity of the compounds. There are previous reports establishing that *N*-(*E*)-caffeoyl-L-glutamic acid showed an inhibitory effect on the adhesion of *H. pylori*; furthermore, 3-*O*-caffeoyl-betulinic acid demonstrated growth inhibitory activity, whereas betulinic acid failed to show any activity.[28,29]

While a secoiridoid, ligstroside (**4**) did not display anti-*H. pylori* activity, another secoiridoid, oleuropein (**8**) with a catechol moiety, showed a weak inhibitory activity with an MIC value of 50 μM against strain 43504. It has been reported that a catechol moiety may affect the inhibitory activity against the urease of *H. pylori*.[30] On the contrary, a lignan, compound **6** showed mild inhibitory activity (MIC value of 25 μM against strain 26695) (Table 2). Although compounds **2**, **3**, **8**, and **11** have been reported to possess anti-microbial properties, this is the first report on anti-*H. pylori* activity of the 12 compounds isolated from *F. mandshurica*.[31-35] In conclusion, *F. mandshurica* bark, or its compounds, would be beneficial against *H. pylori* infection, and further studies are required to investigate the underlying the inhibitory mechanisms.

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