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Anti-platelet Effect of the Phenolic Constituents Isolated from the Leaves of *Magnolia obovata*

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Abstract – In the course of our work on anti-platelet constituents from plants, eight phenolic compounds, (±)-syringaresinol (**1**), 4-hydroxybenzaldehyde (**2**), 4-hydroxybenzoic acid (**3**), vanillic acid (**4**), 4-hydroxy cinnamic acid (**5**), quercetin 3-O-rhamnoside (**6**), rutin (**7**), and quercetin 3-(2^G-rhamnosylrutinoside) (**8**) were isolated from the methanol extract of the leaves of *Magnolia obovata*. The compounds were identified based on the spectroscopic data. Compound **2**, **3**, **5**, **6**, **7** and **8** were isolated for the first time from genus *Magnolia*. **1** and **6** showed same order of inhibitory potencies as acetylsalicylic acid (ASA) to rat platelet aggregation induced by all the stimulators tested. The remaining six compounds showed only mild effects.

Keywords – *Magnolia obovata*, syringaresinol, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, 4-hydroxy cinnamic acid, quercetin 3-O-rhamnoside, rutin, quercetin 3-(2^G-rhamnosylrutinoside), anti-platelet

Introduction

Over eighty species and varieties of Magnolias are distributed throughout eastern North America and Southeastern Asia (Callaway, 1994). The dried bark of stem, branch, and root of *M. obovata* Thunberg (Magnoliaceae), which is also known as *M. hypoleuca,* has long been used for asthma, stomach problem, fever, headache, anxiety, diarrhea, and stroke as a traditional medicine in Korea and Japan. In our study of anti-platelet compounds, eight phenolic constituents were isolated from the methanolic extracts of dried leaves of *M. obovata*. These eight phenolic compounds were identified as syringaresinol (**1**), 4-hydroxybenzaldehyde (**2**), 4-hydroxybenzoic acid (**3**), vanillic acid (**4**), 4-hydroxy cinnamic acid (**5**), and quercetin 3-O-rhamnoside (**6**), rutin (**7**) and quercetin 3-(2^G-rhamnosylrutinoside) (**8**) on comparison of the spectral data with those of the literature values (Verme, *et al.* 1991,Yaguchi, *et al.*, 1981, Scott, 1972, Bourne, *et al.*, 1963, Harborne & Mabry, 1982, Singh, *et al.*, 2002, Champavier, *et al.*, 2000). Compounds **2**, **3**, **5**, **6**, **7** and **8** were isolated for the first time from genus *Magnolia*. The inhibitory effects of the compounds were evaluated on rat platelet aggregation induced by ADP, collagen, epinephrine, arachidonic acid (AA) and a thromboxane A_2 mimetic agent, U46619.

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Materials and Methods

Materials – Melting point was determined on a Mitamura-Riken melting point apparatus and uncorrected. IR spectrum was recorded on a Jasco FT/IR-5300 spectrometer. ^{1}H- and ^{13}C- NMR spectra were taken at 300 MHz and 75.5 MHz, respectively on a Varian Gemini-2000 spectrometer with tetramethylsilane as the internal standard. Mass spectra were taken with a Hewlett Packard model 5989 B GC/MS system. Platelet count was determined on a PLT-4 (HEMA-1, Texas International Laboratories, Inc., Houston, Texas, U.S.A.). Platelet aggregation was measured on a platelet aggregometer (500VS, Chrono-Log Corp., U.S.A.). Collagen and ADP (adenosine 5-diphosphate dicyclohexylammonium salts) were purchased from Chrono-Log Corp. (U.S.A.). Epinephrine, sodium arachidonate (AA) and U46619 (9,11-dideoxy-11,9-epoxymethanoprostaglandin F_2) were obtained from Sigma Chem. Co. (U.S.A.). The rats (Sprague-Dawley) were bred at the Animal Station of Natural Products Research Institute, Seoul National University. They were maintained and cared in accordance with the Guide for the Care and Use of Laboratory Animals by Seoul National University.

Plant materials – The barks and fruits of *M. obovata* were collected from early July, 1997 at Yungun campus of Seoul National University, Seoul, Korea when two trees were cut down and identified by Prof. Hyung Joon Chi, Natural Products Research Institute, Seoul National University.

Extraction and isolation – The dried leaves of *M. obovata* (5 kg) were grinded and refluxed with methanol two times for six hours each. The MeOH extract concentrated *in vacuo* was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer (184 g), after concentration, was further partitioned between *n*-hexane and 90% MeOH to obtain hexane fraction (fr.) (123 g) and MeOH fr. (61 g). The aqueous layer (317 g) was partitioned with EtOAc and then with BuOH to obtain EtOAc (22 g) or BuOH (45g) fr.

The MeOH fr. (30 g) was applied on a silica gel (1.2 kg) column eluting with $CHCl_3$ containing increasing portion of MeOH to afford compound **1** (40 mg). The EtOAc fr. (22 g) was also subjected to a silica gel (1.0 kg) column eluting with $CHCl_3$MeOH (90:1) and/or n-hexane-EtOAc (1:1) to afford compound **2** (11 mg), compound **3** (13 mg), compound **4** (15 mg), compound **5** (10 mg) and compound **6** (800 mg). The BuOH fr. (45 g) was chromatographed on a silica gel (1.1 kg) column eluting with $CHCl_3$-MeOH-H_2O (90:9:1) and/or EtOAc:MeOH:H_2O (4:5:1) yielding compound **7** (1.2 g) and compound **8** (25 mg).

Compounds **1**, **2**, **3**, **4**, **5**, **6**, **7** and **8** were identified as (±)-syringaresinol (**1**) (Verme, *et al.* 1991), 4-hydroxybenzaldehyde (**2**) (Yaguchi, *et al.*, 1981), 4-hydroxybenzoic acid (**3**), vanillic acid (**4**) (Scott, 1982), 4-hydroxy cinnamic acid (**5**) (Bourne, *et al.*, 1963), quercetin 3-O-rhamnoside (**6**) (Singh, *et al.*, 2002, Harborne & Mabry, 1982), rutin (**7**) (Harborne & Mabry, 1982), and quercetin 3-(2^G-rhamnosylrutinoside) (**8**) (Champavier, *et al.*, 2000) respectively by comparing their spectral data with the literature values and/or by the direct comparison with the authentic samples.

4-Hydroxybenzaldehyde (**2**) – Pale brown needles from *n*-hexane-ether; mp: 116-117°C; IR $\nu_{max}cm^{-1}$(KBr): 3167, 1667, 1597, 1518, 1452, 1286; UV λ_{max} (MeOH) nm: 283; ^{1}H-NMR ($CDCl_3$): δ9.87 (1H, s, CHO), 7.82, 6.98 (2H each, dd, *J*=8.7, 2.1 Hz); ^{13}C-NMR ($CDCl_3$): δ191.3 (C=O), 161.6 (C-4), 132.5 (C-2,6), 129.8 (C-1), 116.0 (C-3,5)

4-Hdroxybenzoic acid (**3**) – White amorphous solid from *n*-hexane-ether; mp: 214-215°C; IR $\nu_{max}cm^{-1}$(KBr): 3387, 1676, 1608, 1595, 1510, 1242; UV λ_{max} (MeOH) nm: 252; ^{1}H-NMR (DMSO-d_6): δ12.39 (1H, br s, COOH), 10.19 (1H, s, OH), 7.77, 6.81 (2H each, ddd, *J*=8.7, 2.7, 1.8 Hz) 6.1 (1H, s, OH); ^{13}C-NMR (DMSO-d_6,): δ167.3 (C=O), 161.8 (C-4), 131.7 (C-2,6), 121.5 (C-1), 115.3 (C-3,5)

4-Hydroxy cinnamic acid (**5**) – Colorless prisms from n-hexane-ether, mp: 213-214°C; IR $\nu_{max}cm^{-1}$(KBr): 3369, 1672, 1628, 1601, 1512, 1244; UV λ_{max} (MeOH) nm: 227, 311; ^{1}H-NMR (DMSO-d_6): δ12.11 (1H, br s, COOH), 9.94 (1H, br s, OH-4), 7.50 (1H, d, *J*=15.9 Hz, H-7), 7.48, 6.77 (2H each, d, *J*=8.4 Hz,), 6.27 (1H, d, *J*=15.9 Hz, H-8); ^{13}C-NMR (DMSO-d_6): δ168.1 (C=O), 159.8 (C-4), 144.4 (C-7), 130.3 (C-2,6), 125.5 (C-1), 115.9 (C-3,5), 115.5 (C-8)

Quercetin 3-O-rhamnoside (**6**) – Yellow amorphous solid from MeOH-$CHCl_3$, mp: 170-172°C; IR $\nu_{max}cm^{-1}$(KBr): 3347, 1659, 1605, 1566, 1505, 1296; UV λ_{max} (MeOH) nm (log ε): 257 9 (4.23), 264 (sh, 4.20), 351 (4.19); (NaOMe) 271 (4.41), 329 (sh, 3.80), 395 (4.32); (NaOAc) 272 (4.39), 324 (sh, 3.79), 392 (4.27); (NaOAc-H_3BO_3) 261 (4.37), 367 (4.28); ($AlCl_3$) 275 (4.40), 433 (4.38); ($AlCl_3$-HCl) 272 (4.30), 300 (sh, 3.28), 3.58 (sh, 4.00) 402 (4.15); ^{1}H-NMR (DMSO-d_6): δ12.65 (1H, s, 5-OH), 10.87, 9.71, 9.35 (1H each, br s, 7, 3', 4'-OH), 7.29 (1H, d, *J*=2.1 Hz, H-2'), 7.24 (1H, dd, *J*=8.4, 2.1 Hz, H-6'), 6.85 (1H, d, *J*=8.4 Hz, H-5'), 6.38 (1H, d, *J*=2.1 Hz, H-8), 6.19 (1H, d, *J*=2.1 Hz, H-6), 5.23 (1H, d, *J*=1.5 Hz, H-1"), 3.94-3.99 (1H, m, H-2") 3.52-3.46 (1H, m, H-3"), 3.3-3.08 (2H, m, H-4", H-5"), 0.80 (3H, d, *J*=5.7 Hz, 5"-CH_3); ^{13}C-NMR (DMSO-d_6,): δ178.0 (C-4), 164.4 (C-7), 161.5 (C-5), 157.5 (C-2), 156.4 (C-9), 148.6 (C-4'), 145.4 (C-3'), 134.4 (C-3), 121.3 (C-1'), 120.9 (C-6'), 115.9 (C-5'), 115.7 (C-2'), 104.3 (C-10), 102.2 (C-1"), 98.9 (C-6), 93.8 (C-8), 71.4 (C-4"), 70.8 (C-2"), 70.5 (C-3"), 70.3 (C-5"), 17.7 (C-6")

Rutin (**7**) – Yellow amorphous solid from MeOH-H_2O, mp: 188-192°C; IR $\nu_{max}cm^{-1}$(KBr): 3424, 1655, 1603, 1508, 1458, 1296, 1065; UV λ_{max} (MeOH) nm (log ε) : 257 (4.37), 267 (sh, 4.31), 357 (4.26); (NaOMe) 273 (4.45), 326 (sh, 4.01), 400 (4.32), 428 (4.19); (NaOAc) 273 (4.43), 327 (sh, 4.04), 398 (4.30); (NaOAc-H_3BO_3) 262 (4.46), 380 (4.34); ($AlCl_3$) 274 (4.44), 434 (4.42); ($AlCl_3$-HCl) 270 (4.38), 300 (sh, 3.92), 403 (4.25); ^{1}H-NMR (DMSO-d_6): δ12.59 (1H, s, 5-OH), 10.81, 9.64, 9.16 (1H each, br s, 7, 3', 4'-OH), 7.53 (1H, dd, *J*=9.0, 2.1 Hz, H-6'), 7.52 (1H, d, *J*= 2.1 Hz, H-2'), 6.83 (1H, d, *J*=9.0 Hz, H-5'), 6.37 (1H, d, *J*=2.1 Hz, H-8), 6.18 (1H, d, *J*=2.1 Hz, H-6), 5.19 (1H, d, *J*=7.5 Hz, H-1"), 4.40 (1H, d, *J*=3.3 Hz, H-1'"), 3.7~3.0 (m, gly and rha H), 0.98 (3H, d, *J*=6.3 Hz, 5'"-CH_3); ^{13}C-NMR (DMSO-d_6): δ177.6 (C-4), 164.2 (C-7), 161.4 (C-5), 156.8 (C-2), 156.6 (C-9), 148.6 (C-4'), 144.9 (C-3'), 133.5 (C-3), 121.8 (C-1'), 121.4 (C-6'), 116.5 (C-5'), 115.4 (C-2'), 104.2 (C-10), 101.4 (C-1"), 100.9 (C-1'"), 98.9 (C-6), 93.8 (C-8), 76.6 (C-3"), 76.1 (C-5"), 74.3 (C-2"), 72.0 (C-4"), 70.8 (C-4'"), 70.6 (C-2'"), 70.2 (C-3'"), 68.4 (C-5'"), 67.2 (C-6"), 17.9 (C-6'")

Quercetin 3-O-(2^G-rhamnosylrutinoside) (**8**) – Bright yellow needles from MeOH-EtOAc, mp: 168-170°C; IR $\nu_{max}cm^{-1}$ (KBr): 3387, 1662, 1610, 1501, 1451, 1304, 1065; UV λ_{max} (MeOH) nm (log ε) : 256 (4.23), 266 (sh, 4.17), 354 (4.17); (NaOMe) 272 (4.31), 328 (sh, 3.88), 397 (4.24); (NaOAc) 273 (4.31), 324 (sh, 3.88), 383 (4.24); (NaOAc-H_3BO_3) 261 (4.32), 374 (4.19), 450 (3.44); ($AlCl_3$) 275 (4.33),

339 (sh, 3.46), 433 (4.29); ($AlCl_3$-HCl) 271 (4.25), 301 (sh, 3.75), 3.59 (3.98) 404 (4.11); ^{1}H-NMR (300 MHz, DMSO-d_6): δ12.65 (1H, s, 5-OH), 7.52 (1H, dd, *J*=8.4, 2.1 Hz, H-6'), 7.47 (1H, d, *J*= 2.1 Hz, H-2'), 6.82 (1H, d, *J*=8.4 Hz, H-5'), 6.36 (1H, d, *J*=2.1 Hz, H-8), 6.16 (1H, d, *J*=2.1 Hz, H-6), 5.51 (1H, d, *J*=7.8 Hz, H-1"), 5.05 (1H, d, J=1.8 Hz, H-1""), 4.32 (1H, d, J=1.8 Hz, H-1'"), 3.8~3.0 (gly and rha H), 0.96, 0.78 (3H each, d, *J*=6.0 Hz, 5'"- and 5""-CH_3); ^{13}C-NMR (75 MHz, CD_3OD): δ179.3 (C-4), 165.7 (C-7), 163.2 (C-5), 158.9 (C-2), 158.5 (C-9), 149.5 (C-4'), 145.9 (C-3'), 134.4 (C-3), 123.5 (C-1'), 123.4 (C-6'), 117.4 (C-2'), 116.0 (C-5'), 105.9 (C-10), 102.6 (C-1""), 102.3 (C-1'"), 100.5 (C-1"), 99.8 (C-6), 94.7 (C-8), 80.0 (C-2"), 78.9 (C-3"), 77.1 (C-5"), 74.1 (C-4""), 73.9 (C-4"), 72.4 (C-2""), 72.3 (C-3", -3""), 72.1 (C-2'"), 71.9 (C-4"), 70.0 (C-5""), 69.7 (C-5'"), 68.3 (C-6"), 17.8 (C-6'"), 17.5 (C-6"")

Platelet aggregation – Blood collected from rat heart using a syringe containing 0.1 volume of 2.2% sodium citrate, was centrifuged at 200 g for 10 min to obtain platelet rich plasma. The supernatant PRP was diluted with saline to adjust the number of platelets (400-450×10^6 platelets/ml) with the aid of platelet counter (PLT-4, Texas International Lab., U.S.A.). The degree of platelet aggregation was measured with platelet aggregometer (Model 500VS, Chrono-Log Corp., U.S.A.). After 3 min pre-incubation of the adjusted PRP, sample or vehicle was added and an aggregation inducing agent [ADP (2-5 μM) or collagen (2-5 μg/ml)] was added at 1 min after the sample addition. Epinephrine-induced rat platelet aggregations were measured by the previously described method (Yun-Choi, 2000) in the presence of threshold concentration of collagen. Briefly, sample was added to PRP 30 sec before the addition of the threshold concentration of collagen (0.8-1.0 μg/ml). Epinephrine (1-4 μM) was added 30 sec after the addition of collagen and the reduction in turbidity of PRP was observed as the degree of aggregation. AA (10-40 μM) and U46619 (1-5 μM) induced platelet aggregation were also measured in the presence of the threshold concentration of collagen. The minimum inducer concentration that elicited maximal aggregation was employed as the control for each PRP. The concentrations of these compounds causing 50% inhibitory effects (IC_{50}) were determined from the Regression Wizard from the SigmaPlot equation library.

Results and Discussion

Eight phenolic compounds were isolated as anti-platelet constituents from leaves of *M. obovata*. These compounds **1**, **2**, **3**, **4**, **5**, **6**, **7**, and **8**, were identified as syringaresinol, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, vanillic acid,

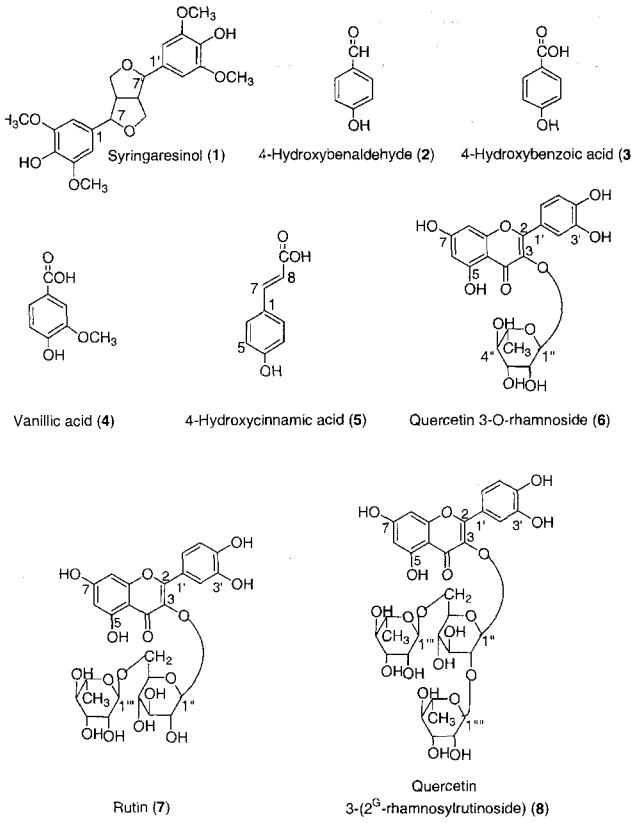

Fig. 1. Compounds evaluated for anti-platelet activities.

4-hydroxy cinnamic acid, quercetin 3-O-rhamnoside, rutin, quercetin 3-(2^G-ramnosylrutinoside), respectively. These pheolic compounds are distributed in various plant sources (Miyazawa, et al, 1992, Pan, *et al.*, 1987, Chae, *et al.*, 1998, Chen, *et al.*, 1976, Yaguchi, *et al.*, 1981, Bourne, *et al.*, 1963, Abegaz, *et al.*, 2002, Chaves, *et al.*, 2001, Kayano, *et al.*, 2002, Hasan, *et al.*, 1995, Champavier, *et al.*, 2000). However, this is the first reported occurrence of compounds **2**, **3**, **5**, **6**, **7**, and **8** from genus Magnolia. The identifications of the compounds were based on various spectral data (^{1}H-, ^{13}C-NMR, IR, UV) in comparison with those of the literature values (Verme, *et al.* 1991, Yaguchi, *et al.*, 1981, Scott, 1972, Bourne, *et al.*, 1963, Singh, *et al.*, 2002, Harborne, & Mabry, 1982, Champavier, *et al.*, 2000) and/or by the direct comparison with the authentic samples.

The inhibitory effects of **1**-**8** on rat platelet aggregation were examined and compared with those of acetylsalicylic acid (ASA) (Table 1). All of the tested compounds showed dose-dependent inhibitory activities to collagen, epinephrine, AA and U46619 induced platelet aggregation. AA is believed to metabolize to prostaglandin endoperoxides, which are subsequently converted to TXA_2 and induce platelet aggregation (Sinakos, 1967). U46619, a PGH_2/TXA_2 receptor agonist, induced only shape change but not aggregation in

Table 1. Platelet anti-aggregating activities of the compounds isolated from the leaves of *M. obovata*

Compounds	IC_{50} (uM)			
	Collagen[a]	Epinephrine[b,e]	AA[c,e]	U46619[d,e]
ASA[f]	420	53	66	340
1	280	35	36	510
2	>1000	520	510	490
3	610	360	390	520
4	480	490	630	360
5	310	360	490	470
6	640	46	38	270
7	>1000	>1000	>1000	380
8	>1000	290	200	560

[a]collagen 2-5 ug/ml.
[b]epinephrine 1- 4 uM.
[c]sodium arachidonate 10-40 uM.
[d]U46619 1-5 uM.
[e]in the presence of threshold concentration of collagen (0.8-1.0 ug/ml).
[f]ASA; acetylsalicylic acid.

rat platelet (Hanasaki, 1981). Since rat platelets were observed not to aggregate in response to epinephrine, AA, or U46619 in the concentration dependent manner, the aggregations were observed in the presence of threshold concentration of collagen. All of the five compounds were not inhibitory to ADP induced aggregation. Compounds **1** and **6** were more inhibitory to epinephrine (IC_{50}; 35 μM and 46 μM, respectively) or AA (IC_{50}; 36 μM and 38 μM, respectively) induced aggregation than to collagen (IC_{50}; 280 μM and 640 μM respectively) or U46619 (IC_{50}; 510 μM and 270 μM, respectively) induced aggregation. In addition, **1** and **6** showed same order of inhibitory potencies to all the aggregation inducing agents as ASA. With the results, it was suggested that both **1** and **6** might show platelet anti-aggregating effects by inhibiting the AA cascade as ASA does. The anti-platelet activities of remaining compounds, simple phenolic compounds and flavonoid di- or tri-glycosides, showed only mild effects to all the stimulators tested.

In summary, eight phenolic compounds were isolated as platelet anti-aggregating components of *M. obovata*. Syringaresinol (**1**) and quercetin 3-O-rhamnoside (**6**) were as effective as ASA and the other six compounds showed only mild effects.

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