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Anti-Platelet Effect of the Constituents Isolated from the Barks and Fruits of *Magnolia obovata*

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In the course of our work on anti-platelet constituents from plants, five phenolic compounds, magnolol, honokiol, obovatol, methyl caffeate, and syringin, were isolated from the methanol extracts of the barks and fruits of *Magnolia obovata*. The compounds were identified based on the spectroscopic data. Methyl caffeate was isolated for the first time from the genus *Magnolia* and it showed 3 ~ 4-folds higher potency than ASA. The activities of obovatol and honokiol were comparable to ASA. Magnolol and syringin showed only very mild inhibitory effects to all the stimulators.

Key word: *Magnolia obovata*, Methyl caffeate, Magnolol, Honokiol, Obovatol, Anti-platelet

INTRODUCTION

The dried bark of *Magnolia obovata* Thunberg (Magnoliaceae) has long been used as a oriental traditional medicine for the relief of asthma, stroke, fever, headache, anxiety, diarrhea, and abdominal problems In our study of anti-platelet compounds, five phenolic constituents were isolated from the methanolic extracts of dried barks and fresh fruits of *M. obovata*. Four of the phenolic compounds were magnolol, honokiol, obovatol and syringin, whose presence in this plant were already reported (Fujita *et al.*, 1972, 1973; Ito *et al.*, 1982; Niwa, *et al*, 1988). The remaining compound was identified as methyl caffeate on comparison of the spectral data with those of the literature (Bourne *et al.*, 1963; Etzenhouser, *et al*, 2001; Marco, *et al.*, 1988; Xiang, *et al.*, 2001; Nagao, *et al*, 2001). Methyl caffeate was isolated for the first time from the genus *Magnolia*. The inhibitory effects of the compounds were evaluated on rat-platelet aggregation induced by various stimulators.

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 tetramethylsiline 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 dicyclohe-xylammonium salts) were purchased from Chrono-Log Corp. (U.S.A.). Epinephrine, sodium arachidonate (AA) and U46619 (9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$) 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 barks of *M. obovata* (3 kg) were ground and refluxed with methanol three times for six hours each. The

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MeOH extract concentrated *in vacuo* was partitioned between $CHCl_3$ and H_2O, and the $CHCl_3$ layer (400 g), after concentration was further partitioned between *n*-hexane and 90% MeOH to obtain hexane fraction (fr.) (198 g) and MeOH fr. (202 g). H_2O layer was further extracted with EtOAc and BuOH, successively, to give EtOAc soluble fr. (21 g) and BuOH soluble fr. (80g). Fresh fruits of this plant were sliced and percolated in 100% MeOH for two weeks (the first percolation) and 1.5 years (the second percolation) at room temperature. The MeOH extract (680 g) of fruits was fractionated with the same method described above for the extraction and fraction of barks, yielding hexane fr. (19 g), MeOH fr. (61 g), EtOAc fr. (22 g), BuOH fr. (45 g) and H_2O fr. (250 g). The MeOH fr. (30 g) of barks was subjected to silica gel (1.0 kg) chromatography eluting with $CHCl_3$-MeOH (100:1) to afford compound **1** (2.2 g), compound **2** (300 mg), and compound **3** (1.3 g). Compounds **1** (1.8 g), **2** (105 mg), and **3** (1.5 g) were also obtained from MeOH fr. (20 g) of the fruits with the same eluting condition. The EtOAc fr. (13 g) of fruits was applied to a silica gel column (600 g) eluting with H_2O-saturated EtOAc to give compound **4** (23 mg). Compound **5** (45 and 18 mg, respectively) was isolated from BuOH fr. (40 and 15 g, respectively) of the barks and fruits eluting with $CHCl_3$:MeOH:H_2O (100:1:0.1). Compound **1** (colorless prisms from *n*-hexane-ether, $C_{18}H_{18}O_2$, mp 100-102°C), **2** (colorless needles from *n*-hexane-ether, mp 84-86°C), **3** (colorless oils) and **5** (white needles from MeOH-EtOAc, mp 190-192°C) were identified as magnolol (Fujita *et al.* 1973), honokiol (Fujita *et al.*1973, 1973), obovatol (Ito *et al.* 1982), and syringin (Niwa *et al.* 1988), respectively by comparison of spectral data with reported ones.

Methyl caffeate (**4**) - Pale yellow needles (from *n*-hexane-ether); mp: 156-159°C; IR $\nu_{max}cm^{-1}$(KBr): 3478 (OH), 1678 (α, β-unsaturated C=O), 1607, 1535, 1445 (aromatic C=C), 1307 (C-O of COOH), 1281 (aromatic C-O); UV λ_{max} (MeOH) nm: 244, 299, 327; EI-MS (rel. int.): *m/z* 194 (M^+ 93), 163 (100), 152 (37), 134 (47), 117 (34), 89 (52), 77 (29); ^{1}H-NMR (300 MHz, acetone-d_6): δ 7.52 (1H, d, *J*=15.6 Hz, H-7), 7.15 (1H, d, *J*=2.1 Hz, H-2), 7.03 (1H, dd, *J*=8.1, 2.1 Hz, H-6), 6.86 (1H, d, *J*=8.1 Hz, H-5), 6.27 (1H, d, *J*=15.6 Hz, H-8), 3.70 (3H, s, OCH_3); ^{13}C-NMR (75 MHz, acetone-d_6,): 168.2 (C=O), 148.9 (C-4), 146.3 (C-3), 145.9 (C-7), 127.1 (C-1), 122.4 (C-6), 116.2 (C-2), 114.8 (C-5, 8), 51.6 (OCH_3)

Platelet aggregation

Blood collected from rat heart after surgery using 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 uM) or collagen (2-5 ug/ml)] was added at 1 min after the sample addition. Epinephrine-induced rat platelet aggregations were measured by the previously described method (Yun-Choi *et al.*, 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 empolyed 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

Five phenolic compounds were isolated as anti-platelet constituents from barks and fruits of *M. obovata*. Four of them, compounds **1**, **2**, **3** and **5**, were identified as magnolol, honokiol, obovatol, and syringin, respectively, whose presence in this plant have been previously reported (Sugii 1930; Fujita *et al.*, 1972, 1973; Ito *et al.*, *1982*). Compound **4** isolated from EtOAc fr. of fruits was obtained as pale yellow needles (mp 156-159°C) from *n*-hexane-ether. The IR spectrum showed an absorption band due to hydroxy group (3478 cm^{-1}), α, β-unsaturated carbonyl group (1678 cm^{-1}), aromatic C=C (1607, 1535, 1455 cm^{-1}), and carboxylic C-O (1307 cm^{-1}). The mass spectrum of this compound showed the molecular ion peak at *m/z* 194, a base peak $[M-OCH_3]^+$ at *m/z* 163 and a peak $[M-COH]^+$ at *m/z* 134. The ^{1}H-NMR spectrum measured in acetone-d_6 solvent showed two doublets at δ 6.27 and 7.52 (*J*=15.6 Hz) ascribable to the *trans*-olefinic moiety and the signals at δ 6.86 (d, *J*=8.1 Hz), 7.03 (dd, *J*=8.1, 2.1 Hz), and 7.15 (d, *J*=2.1 Hz) due to ABX pattern of 1, 3, 4-trisubstituted aromatic protons and a singlet at δ 3.70 due to OCH_3. The ^{13}C-NMR spectrum with DEPT in acetone-d_6 showed the presence of three aromatic quaternary carbons (δ 127.1, 146.3, and 148.9) and three protonated aromatic carbons (δ 114.8, 116.2, and 122.4). The carbons at δ 145.9 and 114.8 were assigned to olefinic carbons. The carbon of OCH_3 moiety attached to

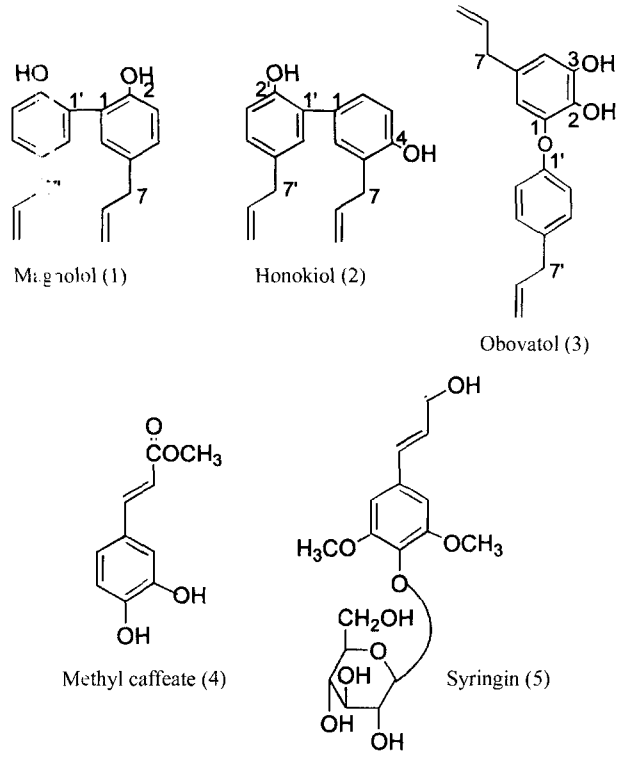

Fig. 1. Compounds evaluated for anti-platelet activities

the aromatic ring generally appears at δ 55-56. The OCH_3 carbon in this compound was shown at δ 51.6 and carbonyl carbon was observed at δ 168.2. Therefore, compound **4** has the ester group. On the basis of above spectral data, compound **4** was identified as methyl caffeate (methyl 3, 4-dihydroxycinnamate). The isolation of compound **4** has been repoted from *Gaillardia pulchella* (Seiichi *et. al.*, 1984), *Clerodendron bunaei* and *C. trichotomum* (Nagao *et al.*, 2001), *Duchesnea chrysantha* (Lee and Yang, 1994), *Perilla frutescens* (Makino *et al.*, 1998), *Artemisia assoana* (Martinez *et al.*,1987), and *Lonicera japonica* (Chang and Hsu, 1992), though it was isolated for the first time from genus *Magnolia.*

The inhibitory effects of **1** - **5** 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 arachidonic acid (AA) and U46619 induced platelet aggregation. AA is believed to be metabolized to prostaglandin endoperoxides, which are subsequently converted to TXA_2 and induce platelet aggregation (Soinakos *et al.*, 1967). U46619, a PGH_2/TXA_2 receptor agonist, induced only shape change but not aggregation in rat platelet (Hanasaki *et al.*, 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 **5**, showed only very mild effects to all the stimulators tested. Compound **2** showed inhibitory effects to epinephrine (IC_{50}; 51 μM) and AA (IC_{50}; 92 μM) comparable to ASA, whereas it exhibited only very mild inhibitory effects to collagen or U46619 induced aggregations. Compounds **3** and **4** were again more inhibitory to epinephrine (IC_{50}; 59 μM and 14 μM) or AA (IC_{50}; 54 μM and 14 μM) induced aggregation than to U46619 (IC_{50}; 790 μM and 330 μM) or collagen (IC_{50}; >1000 μM and 640 μM) induced aggregation. Compounds **1** and **2**, two of the major components of *M. obovata* were reported to show inhibitory effects on either collagen or AA induced rabbit platelet aggregation and the accompanying ATP release without affecting ADP, PAF or thrombin induced aggregation (Teng *et al., 1988*). Compound **4** was reported to possess only very mild inhibitory effects on ADP-induced human platelet aggregation (Chang and Hsu, 1992). Compound **2** (honokiol) was 5~10 times more potent than **1** (magnolol) to AA-induced rat platelet aggregation, The results were in agreement with that those of Teng *et al.* Compound **3** (obovatol), another main components of *M. obovata*, showed similar order of inhibitory potency as ASA to platelet aggregation induced by epinephrine and AA. The inhibitory effects of **4** (methyl caffeate) on collagen or U46619 induced platelet aggregation were equivalent to those of ASA. In addition, **4** showed 3~4-folds higher inhibitory effects than ASA on epinephrine or AA induced rat platelet aggregations. Caffeic acid and methyl caffeate were reported to inhibit 5-lipoxygenase, but not prostagrandin synthesis at all, at least up to 500 μM. On the other hand, platelet aggregation and thromboxane synthesis induced by AA were inhibited by caffeic acid at high (100 μM) concentration (Koshihara *et al.*, 1984). In summary, the three phenolic compounds, honokiol, obovatol, and methyl caffeate were identified as platelet anti-aggregating components of *M. obovata*.

Table. 1. Platelet anti-aggregating activities of the compounds isolated from the barks and fruits 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	>1000	390	460	>1000
2	>1000	51	92	>1000
3	790	59	54	>1000
4	330	14	14	640
5	>1000	>1000	>1000	>1000

[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 the threshold concentration of collagen (collagen 0.8 - 1.0 ug/ml), [f]ASA; acetylsalicylic acid.

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