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Platelet Anti-Aggregatory Effects of Coumarins from the Roots of *Angelica genuflexa* and *A. gigas*

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Five coumarins, isoimperatorin (**1**), pabulenol (**2**), isooxypeucedanin (**3**), oxypeucedanin hydrate (**4**) and osthol (**5**) were isolated from the MeOH extract of *Angelica genuflexa* in the course of searching for anti-platelet and anti-coagulant components from plants. Pabulenol (**2**) was isolated from *A. genuflexa* for the first time. The five compounds isolated from *A. genuflexa*, together with decursinol angelate (**6**), decursin (**7**) and nodakenin (**8**) from *A. gigas* were evaluated for their effects on platelet aggregation and blood coagulation. Compounds **2**, **5**, **6** and **7** were observed to be either equally effective or 2~4 times more inhibitory than ASA in both arachidonic acid and U46619 (TXA_2 mimetic) induced platelet aggregations.

Key words: *Angelica genuflexa*, *A. gigas*, Pabulenol, Osthol, Decursinol angelate, Decursin, Platelet anti-aggregation

INTRODUCTION

Platelets play a key role in maintaining physiologic homeostasis in blood (Fritsma *et al.*, 1981). Platelets also play a major role in initiating thrombus formation which occurs with various thrombotic disorders, including hypertension, atherosclerosis and ischemic heart diseases (Becker, 1999). Thrombosis may occur if the hemostatic stimulus is improperly regulated either due to impaired capacity of inhibitory pathway, or more commonly when the capacity of natural anticoagulant mechanism is overwhelmed by the intensity of stimulus (Eisenberg *et al.*, 1999). Platelets are primed by several common risk factors and thrombosis is also an important factor in the pathophysiology of unstable angina and myocardial infarction (Tepper *et al.*, 2000). Therefore, agents with anti-platelet and anti-thrombotic effects may have wide therapeutic potential for circulatory diseases.

Angelica genuflexa Nutt. (Umbelliferae) is a perennial herbaceous plant which has also been variously reported as *A. koreana* Maximowicz and *Ostericum koreanum* Kitakawa (Kitagawa, 1971; Suh *et al.*, 1996). The roots of the plant have been used as traditional herbal medicine in Korea for the treatment of the common cold, headache, neuralgia, arthralgia *etc.* (Woo *et al.*, 1982). The MeOH extract was reported to have strong anti-thrombotic potential in the acute thrombosis model. The extract treated group of mice showed lower mortality and higher recovery rates from the thrombotic challenge than the acetylsalicylic acid (ASA) treated group of mice (Yun-Choi *et al.*, 1995). In our preliminary testing, the MeOH extract and one of the solvent fractions (90% MeOH fr., Fr. II) were observed to have both platelet anti-aggregating and anti-coagulant effects. In the course of searching for active components from this plant, five coumarins, isoimperatorin (**1**), pabulenol (**2**), isooxypeucedanin (**3**), oxypeucedanin hydrate (**4**) and osthol (**5**), were isolated. Pabulenol (**2**) was isolated for the first time from *A. genuflexa*, for the best of our knowledge. The five compounds isolated from *A. genuflexa*, together with decursinol angelate (**6**), decursin (**7**) and nodakenin (**8**) from *A gigas* Nakai, were evaluated for their effects on platelet aggregation and blood coagulation. The three coumarins, which were isolated from *A. gigas* by one of the present authors (Lee, *et al.*, 2002) were also included since *A. gigas* is one of the plant medicine which was previously reported to show anti-platelet effects (Yun-Choi *et al.*, 1985).

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MATERIAL AND METHODS

Instrument and reagents

Melting point was determined on a Büchi B-540 apparatus and uncorrected. IR spectra were recorded on a Jasco FT/IR-5300 spectrometer on KBr. Mass spectra were taken with a Hewlett Packard model 5989 B GC/MS system. ^{1}H- and ^{13}C-NMR spectra were taken at 300 MHz and 75 MHz respectively on a Varian Gemini-2000 spectrometer using TMS as an internal standard. $[\alpha]_D$ was measured on a Jasco P-1020 autopolarimeter. Platelet count was determined on a platelet counter (Excell™ 18 MWI, Inc., DANAM Electronics, U.S.A.). Platelet aggregation was measured on a platelet aggregometer (490-X, Chrono-Log Corp., U.S.A.). The clotting time was determined on a Fibrometer (BBL Fibrosystem, Fisher Scientific, U.S.A). Kiesel gel 60 (70-230 mesh, Art 7734, Merck) was used for column packing. ADP (Adenosine 5'-diphosphate dicyclohexylaminonium salt), Na-AA (Sodium arachidonate) U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostagrandin F2α, TXA_2 mimetic) and TT (thrombin time) agent were purchased from Sigma Chemical Company (U.S.A). Collagen was purchased from Chrono-Log Corp. (U.S.A). CONTACT [aPTT (activated partial thrombin time) reagent] and Thromboplastin-DS [PT (prothrombin time) reagent] were purchased from Fisher Scientific Company (U.S.A). ASA (acetylsalysilic acid) was from Janssen Chemica.

Animals

The rats (Sprague-Dawley, 250±20 g) were bred at the Animal Station of Natural Products Research Institute, Seoul National University. They were fed with a diet of animal chow and tap water and were housed at 20±2°C and 55% humidity in a 12 h light-dark cycle in accordance with the Guide for the Care and Use of Laboratory Animals by Seoul National University.

Plant materials

The roots of *A. genuflexa*, which were cultivated and harvested in Yeongwol, Korea, were purchased from Kyung Dong Herbal Market, Seoul, Korea in May 2002 and identified by Prof. Young Bea Suh, Natural Products Research Institute, Seoul National University. The voucher specimen (#YY200205-1) was deposited at the Herbarium of Natural Product Research Institute, Seoul National University.

Extraction and isolation

The air-dried roots (13 kg) were sliced and percolated in MeOH for one week (the first percolation) and one month (the second percolation) at room temperature. The MeOH extract (3 kg) concentrated *in vacuo* was partitioned with $CHCl_3$ and H_2O and the concentrated $CHCl_3$ layer was partitioned again with *n*-hexane (Fr. I) and 90% MeOH (Fr. II). Fr. II (100 g) was applied to a silica gel (2 kg) column and eluted with $CHCl_3$ and MeOH (10:0~0:10) to give ten fractions (Fr. II-1~Fr. II-10). Fr. II-5 (22 g) was chromatographed on a silica gel (800 g) column, eluting with *n*-hexane and EtOAc (10:0~0:10) afforded nine fractions (Fr. II-5A~Fr. II-5I). On concentration of Fr. II-5E, compound **1** (350 mg) was obtained and the residue of Fr. II-5E (8 g) was subjected to the repeated column chromatography on silica gel (600 g) eluting *n*-hexane and EtOAc (10:0~5:5) to give compounds **2** (475 mg), **3** (497 mg) and **5** (314 mg). Compound **4** (2.7 g) was obtained from Fr. II-7 (8 g) through silica gel (500 g) column eluting with *n*-hexane and EtOAc (6:4~2:8). The structure of each compound was identified on the basis of their physical and spectral data and compared with the literature values.

Pabulenol (2): Pale yellow powder; mp 134oC; [α]D28 -4.55 (c 0.5, EtOH); IR νmax cm–1 (KBr): 3447 (OH), 1705 (C=O, α-pyrone), 1626. 1578, 1456 (aromatic C=C) 1215, 1138 (C-O, benzofuran); EI-MS m/z (rel. int.): 286 [M]+ (14.9), 215 [M-C4H7O]+ (0.8), 202 [M-C5H8O]+ (100), 174 [M-C5H8-CO]+ (42.2); 1H-NMR (300 MHz, CDCl3) : δ 1.83 (3H, s, H-3'-CH3), 2.28 (1H, br s, 2'-OH), 4.39 (1H, dd, J = 9.6, 6.9 Hz, H-1'-a,), 4.47 (1H, dd, J = 9.6, 3.6 Hz, H-1'-b), 4.55 (1H, dd, J = 6.9, 3.6 Hz, H-2'), 5.07 (1H, m(dq), J = 0.6 Hz, H-4'-(E)), 5.20 (1H, m(dq), J = 0.9 Hz, H-4'-(Z)), 6.28(1H, d, J = 9.6 Hz, H-3), 6.97 (1H, dd, J =2.1, 0.9Hz, H-b), 7.15 (1H, br s, H-8), 7.60 (1H, d, J = 2.1 Hz, H-a,), 8.18 (1H, dd, J = 9.6, 0.6 Hz, H-4); 13C-NMR (75 MHz, CDCl3) : δ 18.7 (C-5'), 74.2 (C-2'), 75.6 (C-1'), 94.7 (C-8), 104.7 (C-b), 107.3 (C-4a), 112.9 (C-3), 113.4 (C-4'), 114.1 (C-6), 139.1 (C-4), 143.3 (C-3'), 145.2 (C-a), 148.4 (C-5), 152.5 (C-8a), 158.0 (C-7), 161.1 (C-2).

Preparation of PRP (platelet rich plasma) and PPP (platelet poor plasma)

A male Sprague-Dawley rat was anesthetized with ether and blood was collected from heart after surgery using a syringe containing (9:1, v/v) of 2.2% sodium citrate. The citrated blood was centrifuged at 200×g for 10 min at room temperature to obtain supernatant PRP and PPP was obtained from the residue by centrifugation at 1500×g for 10 more min. PRP was diluted with PPP to adjust the number of platelets to $5\sim6\times10^8$/mL and then was diluted again with physiological saline to make final platelet number of $4.0\sim4.5\times10^8$/mL.

Platelet aggregation

The test sample was dissolved in DMSO to give the final concentration of 1%. The degree of platelet aggregation was monitored by the turbidimetric method according to Born (Born, 1962) using Optical Aggregometer. The re-

duction in turbidity of PRP was measured as the degree of aggregation assuming that PPP represented 100% light transmission and PRP represented 0% transmission. The test sample (or vehicle) was added to the adjusted PRP after 4 min preincubation and an aggregation inducing agent (ADP or collagen) was added at 1 min. Na-AA or U46619 was added 30 sec after the addition of the threshold concentration (0.8~1.2 μg/mL) of collagen, at which only platelet shape change was induced without aggregation. The final concentration of ADP, collagen, AA and U46619 employed were 2~4 μM, 2~5 μg/mL, 30~60 μM and 1~5 μM respectively. The IC_{50} values were determined from the Regression Wizard from the SigmaPlot equation library.

RESULTS AND DISCUSSION

Five compounds (**1~5**) were isolated from Fr. II prepared from the MeOH extract of the roots of *A. genuflexa* which showed strong anti-coagulant and platelet anti-aggregating effects in the preliminary testing (data not shown). Four of them were identified as three furanocoumarins, isoimperatorin (**1**), isooxypeucedanin (**3**) and oxypeucedanin hydrate (**4**), (Fujioka *et al.*, 1999, Harkar *et al.*, 1984), and a dihydrofuranocoumarin, osthol (**5**) (Ito *et al.*, 1990). The chemical structures were determined by the comparison of the spectral data with the reported values.

Compound **2**, which was isolated as pale yellow powder from n-hexane-EtOAc, showed an absorption band due to hydroxy group (3447 cm^{-1}), α,β -unsaturated C=O (1705 cm^{-1}) aromatic C=C bond (1626, 1578, 1356 cm^{-1}) and C-O (1215, 1138 cm^{-1}) in the IR spectrum. The ^{1}H-NMR data showed signals of typical furanocoumarin moiety; two doublets at δ 6.28 (d, *J* = 9.6 Hz) and 8.18 (dd, *J* = 9.6, 0.6 Hz) ascribable to the α-pyrole moiety, another two doublets at δ 6.97 (dd, *J* = 2.1, 0.9 Hz) and 7.60 (d, *J* = 2.1 Hz) from the furan ring and one aromatic singlet at δ 7.15. A proton at δ 4.55 (dd, *J* = 6.9, 3.6 Hz), a proton at δ 4.39 (dd, *J* = 6.9, 9.6 Hz) and an another proton at δ 4.47 (dd, *J* = 3.6, 9.6 Hz) showed the presence of three adjacent protons in ABX system and a hydroxyl proton signal at δ 2.28 (br s) which disappeared with D_2O was observed. The proton singlet at δ 1.83 (3H) is attributed to the vinyl methyl and the signals at δ 5.07 and 5.20 which are doublet-quartet like multiplet showed the two methylene protons. ^{13}C-NMR spectrum with DEPT showed the presence of six quaternary carbons at δ 107.3 (C-4a), 114.1 (C-6), 148.4 (C-5), 152.5 (C-8a), 158.0 (C-7), and 161.1 (C-2) and five protonated carbons at δ 94.7 (C-8), 145.2 (C-a), 112.9 (C-3), 139.1 (C-4) and 104.7 (C-b) which are typical 5-oxygenated furanocoumarin signals (Fujioka, *et al.*, 1999). The two carbon peaks at δ 143.3 (C-3') and 113.4 (C-4') assigned to terminal olefinic carbons and another two oxygenated carbon signals at δ 74.2 (C-2') and 75.6 (C-1') and a methyl carbon signal at δ 18.7 (C-5') were also observed. The spectrum of EI-MS showed a molecular ion at *m/z* 286 and a strong peak at *m/z* 202 $[M-C_5H_8O]^+$. On the basis of above data and with the comparison of the literature values (Adebajo *et al.*, 2000; Basa *et al.*, 1971; Ivie, 1978), compound **2** was identified as pabulenol.

The inhibitory effects of the five compounds, **1**, **2**, **3**, **4** and **5**, isolated from *A. genuflexa* and the three compounds **6**, **7** and **8**, from *A. gigas* were evaluated on platelet aggregation and blood coagulation. Since rat platelets were observed not to aggregate in response to AA or U46619 in the concentration dependent manner, the

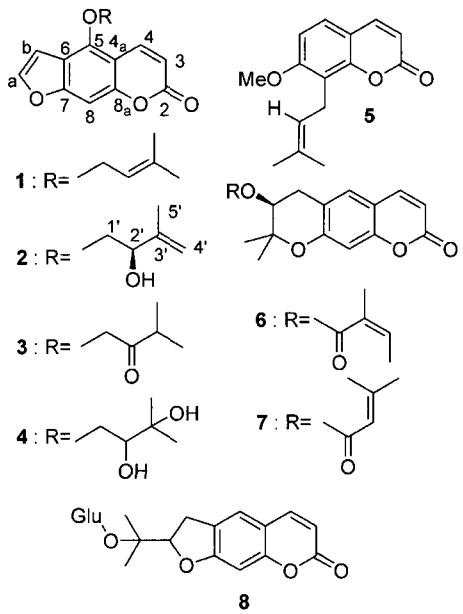

Fig. 1. Coumarins evaluated for anti-platelet activities

Table I. Platelet anti-aggregating effects of the coumarins isolated from *A. genuflexa* and *A. gigas*

Compounds	IC_{50} (μM)		
	Collagen[a]	AA[b,d]	U46619[c,d]
1	307	117	397
2	417	32	194
3	>1000	>1000	>1000
4	198	>300	207
5	>500	29	312
6	222	23	209
7	254	18	113
8	>1000	>1000	>1000
ASA[e]	480	28	464

[a]Collagen (2~5 μg/mL), [b]AA (30~60 μM), [c]U-46619 (1~5 μM), [d]in the presence of the threshold concentration of collagen (0.8~1.2 μg/mL), [e]ASA; acetylsalicylic acid

degree of aggregation was measured in the presence of threshold concentration of collagen (Pyo *et al.*, 2002). U46619, a PGH_2/TXA_2 receptor agonist, induced only shape change but not aggregation in rat platelets (Hanasaki *et al*, 1987). Most of the tested compounds showed dose-dependent inhibitory activities to collagen, AA and U46619 induced platelet aggregation although all of them, including acetyl salicylic acid (ASA) showed only negligible effects on ADP induced aggregation. Compounds **2**, **6** and **7** were either equally active or 2~4 times more inhibitive than ASA, a known anti-platelet compound, to either collagen, AA or U46619 induced aggregations. Compound **1** was equivalent to ASA on collagen and U46619 induced aggregation and compound **5** was comparable to ASA on AA and U46619. Compound **4** was too times more inhibitive than ASA to collagen and U46619 induced platelet aggregation. Compounds **3** and **8** showed only very mild effects to all the stimulators tested. Compounds **1** (370 μM) and **4** (329 μM) were reported having no activities for AA and collagen induced rabbit platelet aggregation (Chen *et al*, 1996). It is assumed that the differences in the assay condition might give the different results.

In summary, compounds **2**, **5**, **6** and **7** were observed to be either equally or 2~4 times more inhibitory than ASA in both AA and U46619 induced platelet aggregation. Disappointingly, all of the tested compounds **1~8** were devoid of anti-coagulant effects (aPTT, PT and TT assay data not shown), although the plant extract and the solvent fraction (fr. II) elongated the coagulation time, suggesting the possibilities of the presence of compounds with anti-coagulant effects.

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