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Anti-inflammatory Compounds from the Leaves of *Ailanthus altissima*

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Abstract − In our ongoing search for biological components from the Korea endemic plants, the MeOH extract of *Ailanthus altissima* leaves (Simaroubaceae) showed cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) dual inhibitory activity by assessing their effects on the production of prostaglandin D_2 (PGD_2) and leukotriene C_4 (LTC_4) in mouse bone marrow-derived mast cells (BMMCs). In further study, eight compounds, squalene (1), β-sitosterol (2), scopoletin (3), quercetin (4), luteolin (5), astragalin (6), scopolin (7), and daucosterol (8) were isolated, the chemical structures were elucidated on the basis of physicochemical and spectroscopic data and by comparison with those of published literatures. Among the compounds, 2, 4, and 5 strongly inhibited both the COX-2-dependent PGD_2 generation with IC_{50} values of 2.6, 7.3 and 2.5 μM, respectively and the generation of LTC_4 in the 5-LOX dependent phase with IC_{50} values of 2.0, 5.1 and 1.8 μM, respectively, which suggest that the anti-inflammatory activity of *A. altissima* might occur in part *via* the inhibition of both PGD_2 and LTC_4 generation by 2, 4 and 5.

Keywords: *Ailanthus altissima*, Simaroubaceae, Chemical constituents, Cyclooxygenase-2, 5-Lipoxygenase, Anti-inflammatory activity

INTRODUCTION

Ailanthus altissima (*A. altissima*), commonly known as the 'tree of heaven', is used in traditional medicine in many parts of Asia including China to treat cold and gastric diseases. Extract of *A. altissima*, has demonstrated anti-proliferative and central nerve depressant activities (O'Neill *et al.,* 1986; Crespi Perellino *et al.,* 1988; De Feo *et al.,* 2005). Among the bioactive compounds isolated from *A. altissima*, quassinoids have various biological activities such as anti-tumor, anti-viral, anti-tuberculosis and anti-plasmodial activities (Kubota *et al.,* 1997; Ohno *et al.,* 1997; Rahman *et al.,* 1997; Tamura *et al.,* 2002; Okunade *et al.,* 2003; Tamura *et al.,* 2003).

Eicosanoids are inflammatory mediators that are biosynthesized in many cell types by cyclooxygenases (COX) and lipoxygenases (LOX). They are strongly associated with inflammatory disorders, acute as well as chronic inflammation. The inhibition of eicosanoid production is one of the important therapeutic strategies in various inflammatory diseases. Among the eicosanoid generating enzymes, COX-2 was found to be essential for the production of PGs in inflammatory sites (Seibert *et al.,* 1994). LTs produced by 5-LOX also participate in the pathogenesis of bronchial asthma (Arm, 2004). Therefore, the dual inhibitors of COX-2/5-LOX are believed to be the ideal treatment for allergic diseases and asthma as nonsteroidal anti-inflammatory drugs (NSAIDs) (Fiorucci *et al.,* 2001).

Recently, we reported that anti-inflammatory activity of ethanol extracts of *A. altissima* through the COX-2 and 5-LOX assays (Jin *et al.,* 2006). However, the bioactive constituents of the leaves of *A. altissima* have not yet been characterized. This paper describes the isolation compounds from *A. altissima* and evaluation of COX-2 and 5-LOX inhibitory activities of the isolates.

MATERIALS AND METHODS

Plant material

The leaves of *A. altissima* were collected in August 2005 at Herbal Garden of Chungnam National University, Daejeon and identified by one of the authors (K. Bae). A voucher specimen (CNU 1539) was deposited at the her-

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barium in the College of Pharmacy, Chungnam National University.

Instruments and reagents

The melting points were measured using an Electrothermal melting point apparatus and are uncorrected. The UV and IR spectra were recorded with a Beckman Du-650 UV-VIS recording spectrophotometer and a Jasco Report-100 infrared spectrometer, respectively. The NMR spectra were recorded on a JEOL 300 FT-NMR spectrometer (^{1}H, 300 MHz, ^{13}C, 75 MHz) with tetramethylsilane (TMS) as the internal standard. The EIMS data were obtained using a Hewlett-Packard 5989B spectrometer. Column chromatography was carried out with silica gel (Kieselgel 60, 70-230 mesh and 230-400 mesh, Merck). Thin layer chromatography (TLC) was performed on pre-coated silica gel 60 F_{254} (0.25 mm, Merck), visualized with a UV lamp, and also by spraying with a 10% H_2SO_4 solution and heating.

Extraction and isolation

The leaves of *A. altissima* (2.6 kg) were extracted with MeOH twice under reflux for 3 h, then filtered and concentrated to yield the MeOH extract (220 g). The MeOH extract was suspended in H_2O and extracted successively with n-hexane, EtOAc and then n-BuOH to give n-hexane fraction (62 g), EtOAc fraction (48 g), n-BuOH fraction (50 g) and water residue, respectively. The n-hexane fraction was subjected to column chromatography with silica gel and eluted using a stepwise gradient of n-hexane and EtOAc (70:1→1:1) to afford 3 subfractions (H1-H3). Fractions H2 was chromatographed on a silica gel column using n-hexane-EtOAc (30:1) to give compounds 1 (15 mg), and 2 (30 mg).

The EtOAc fraction was applied to silica gel column and eluted with increasing polarity of n-hexane-acetone mixtures (10:1→1:1), to yield 5 subfrations (E1-E5). Fractions E2 was further purified over a silica gel column, with n-hexane-EtOAc (10:1) as the solvent system, resulting in compound 3 (65 mg). Fraction E3 was further purified by silica gel column chromatography with hexane-acetone (6:1) to afford compound 4 (15 mg), 5 (12 mg) and 6 (9 mg). Compound 7 (20 mg) were obtained from subfraction E4 by MPLC system [ODS column (11 mm×300 mm, Yamazen), MeOH-H_2O (2:1), flow rate: 2 ml/min, UV 254 nm]. The n-BuOH fraction was subjected to LH20 column chromatography using MeOH-H_2O gradient (1:100→100:1) to give 5 subfractions. Compound 8 (40 mg) was obtained from subfraction 4 by using MPLC system [ODS column (11 mm×300 mm, Yamazen), MeOH-H_2O (2:1), flow rate: 2 ml/min, UV 254 nm].

Squalene (1)

Colorless oil; UV λ_{max} (MeOH) nm (log ε): 220 (3.7); FABMS m/z: 433 $[M+Na]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 5.16 (6H, m, H-4, 9, 14), 2.10 (20H, m, CH_2), 1.70 (6H, s, H-2), 1.62 (18H, s, H-1, 8, 13). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 135.4 (C-12 and C-7), 131.6 (C-1), 124.8 (C-4), 124.7 (C-14 and C-9), 40.1 (C-6 and C-11), 28.6 (C-15), 27.1 (C-5 and C-10), 26.0 (C-1), 18.0 (C-3), 16.4 (C-8 and C-13).

β-sitosterol (2)

Colorless needle; mp 140-141°C; IR ν_{max} (KBr) cm^{-1}: 3320 (OH), 1642 (C=C), 1050 (C-O); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 5.36 (1H, d, J=5.4 Hz, H-6), 3.52 (1H, m, H-3), 1.02 (3H, s, H-19), 0.94 (3H, d, J=6.6 Hz, H-21), 0.87 (3H, d, J=6.9 Hz, H-26), 0.84 (3H, t, J=6.4 Hz, H-29), 0.79 (3H, d, J=6.8 Hz, H-27), 0.70 (3H, s, H-18). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 141.2 (C-5), 122.1 (C-6), 72.1 (C-3), 57.2 (C-14), 56.5 (C-17), 50.6 (C-9), 46.3 (C-24), 42.7 (C-13 and C-4), 40.2 (C-12), 37.7 (C-1), 36.9 (C-10), 36.3 (C-20), 34.4 (C-22), 32.3 (C-8) 32.0 (C-2 and C-7), 29.6 (C-27), 28.6 (C-16), 26.5 (C-23), 24.7 (C-15), 23.5 (C-25), 21.5 (C-11), 20.2 (C-19), 19.8 (C-29), 19.5 (C-28), 19.1 (C-21), 12.4 (C-26), 12.3 (C-18).

Scopoletin (3)

Yellow crystal; mp 202-204°C; UV λ_{max} (MeOH) nm (log ε): 210 (4.3), 300 (3.0), 338 (3.5), 350 (3.1), 352 (3.2), 358 (3.7), 366 (3.4); IR ν_{max} (KBr) cm^{-1}: 3340 (OH), 3106, 3031, 2990, 1710 (pyrone ring), 1600 (C=C); ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 10.25 (1H, s, OH), 7.90 (1H, d, J=9.3 Hz, H-4), 7.21 (1H, s, H-5), 6.78 (1H, s, H-8), 6.21 (1H, d, J=9.3 Hz, H-3), 3.82 (3H, s, OCH_3). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 161.5 (C-2), 152.0 (C-9), 150.3 (C-7), 146.1 (C-6), 145.3 (C-4), 112.5 (C-3), 111.4 (C-10), 110.5 (C-5), 103.6 (C-8), 56.9 (OCH_3).

Quercetin (4)

Yellow powder; negative ES-MS m/z: 301 [M-H]-; ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 12.54 (1H, s, OH-5), 9.39 (3H, br, OH-3), 7.68 (1H, dd, J=2.2, 8.5 Hz, H-2'), 7.54 (1H, dd, J=2.2 Hz, H-6'), 6.88 (1H, d, J=8.5 Hz, H-3'), 6.40 (1H, d, J=2.0 Hz, H-8), 6.19 (1H, d, J=2.0 Hz, H-6). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 160.7 (C-2), 135.7 (C-3), 175.8 (C-4), 163.9 (C-5), 98.2 (C-6), 167.7 (C-7), 93.3 (C-8), 147.5 (C-9), 102.7 (C-10), 120.1 (C-1'), 115.0 (C-2'), 125.0 (C-3'), 146.8 (C-4'), 142.0 (C-5'), 121.5 (C-6').

Luteolin (5)

Yellow powder; positive FAB-MS m/z: 287.1 $[M+H]^+$;

^{1}H-NMR (300 MHz, DMSO-d_6) δ: 6.64 (1H, s, H-3), 6.42 (1H, dd, J=2.0 Hz, H-8), 6.16 (1H, d, J=2.0 Hz, H-6), 7.37 (1H, d, J=2.0, 7.8 Hz, H-2'), 6.87 (1H, d, J=8.0 Hz, H-3'), 7.40 (1H, d, J=2.0 Hz, H-6'). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 164.5 (C-2), 103.4 (C-3), 172.2 (C-4), 162.1 (C-5), 99.5 (C-6), 165.0 (C-7), 94.5 (C-8), 158.0 (C-9), 104.2 (C-10), 121.9 (C-1'), 113.9 (C-2'), 146.4 (C-3'), 150.6 (C-4'), 138.6 (C-5'), 119.6 (C-6').

Astragalin (6)

Yellow powder; positive FAB-MS m/z: 453.1 $[M+H]^+$; ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 3.08-3.66 (m, sugar-H), 5.45 (d, J=7.2 Hz, H-1"), 6.19 (d, J=1.9 Hz, H-6), 6.42 (d, J =1.8 Hz, H-8), 6.88 (d, J=6.8 Hz, H-3', 5'), 8.04 (d, J=6.9 Hz, H-2', 6'), 12.60 (s, OH-5). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 156.1 (C-2), 133.1 (C-3), 177.3 (C-4), 161.1 (C-5), 98.7 (C-6), 164.5 (C-7), 93.6 (C-8), 156.4 (C-9), 103.8 (C-10), 120.8 (C-1'), 130.8 (C-2', 6'), 115.0 (C-3', 5'), 159.9 (C-4'), 100.8 (C-1"), 74.1 (C-2"), 77.4 (C-3"), 69.8 (C-4"), 76.3 (C-5"), 60.8 (C-6").

Scopolin (7)

White powder; mp 217-219°C; UV λ_{max} (MeOH) nm (log ε): 205 (4.9), 220 (4.2), 310 (4.3), 350 (4.9); IR ν_{max} (KBr) cm^{-1}: 3440 (OH), 1700 (-pyrone ring), 1610 (C=C), 1280, 1080 (C-O); FAB-MS m/z: 377 $[M+Na]^+$ and 355 $[M+H]^+$; ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 7.97 (1H, d, J=9.6 Hz, H-4), 7.30 (1H, s, H-5), 7.16 (1H, s, H-8), 6.33 (1H, d, J=9.6 Hz, H-3), 5.09 (1H, d, J=7.5 Hz, H-1'), 3.82 (3H, s, OCH_3), 3.69 (1H, dd, J=9.6, 5.4 Hz, H-6'), 3.16-3.45 (4H, m, H-2',3',4',5'). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 161.4 (C-2), 150.7 (C-9), 149.8 (C-7), 146.8 (C-6), 145.1 (C-4), 114.2 (C-3), 113.1 (C-10), 110.5 (C-5), 103.8 (C-8), 100.4 (C-1'), 77.9 (C-3'), 77.5 (C-5'), 73.9 (C-2'), 70.4 (C-4'), 61.5 (C-6'), 56.9 (OCH_3).

Daucosterol (8)

Colorless crystal; mp 280-282°C; IR ν_{max} (KBr) cm^{-1}: 3391 (OH), 2361, 1635 (C=C), 1107 (C-O). ^{1}H-NMR (300 MHz, CD_3OD) δ: 5.30 (1H, d, J=5.4 Hz, H-6), 4.83 (1H, d, J=7.5 Hz, H-1'), 4.38 (1H, m, H-3), 0.96 (3H, s, H-19), 0.90 (3H, d, J=6.3 Hz, H-21), 0.80 (3H, d, J=6.0 Hz, H-26), 0.79 (3H, t, J=6.6 Hz, H-29), 0.66 (3H, s, H-18). ^{13}C-NMR (75 MHz, CD_3OD) δ: 141.3 (C-5), 122.0 (C-6), 101.7 (C-1'), 77.8 (C-3'), 77.6 (C-3 and C-5'), 74.4 (C-2'), 71.0 (C-4'), 61.5 (C-6'), 57.0 (C-14), 56.5 (C-17), 50.5 (C-9), 46.0 (C-24), 42.7 (C-13 and C-4), 40.2 (C-12), 37.7 (C-1), 36.9 (C-10), 36.3 (C-20), 34.4 (C-22), 32.3 (C-8), 32.0 (C-2 and C-7), 29.6 (C-27), 28.6 (C-16), 26.5 (C-23), 24.7 (C-15), 23.5 (C-25), 21.4 (C-11), 20.2 (C-19), 20.0 (C-29), 19.8 (C-28), 19.5 (C-21), 12.7 (C-26), 12.7 (C-18).

Preparation and activation of bone marrow-derived mast cells (BMMCs)

Bone marrow cells from male Balb/cJ mice were cultured for up to 10 weeks in 50% enriched medium (RPMI 1640 containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, antibiotics and 10% fetal calf serum) and 50% WEHI-3 cell conditioned medium as a source of IL-3. After 3 weeks more than 98% of the cells were found to be BMMCs when checked by the previously described procedure (Muarakami *et al.,* 1994).

Determination of prostaglandin D_2 (PGD_2)

In order to measure the inhibitory activity on COX-2 by samples, the cells were suspended in enriched medium at a cell density of 5×10^5 cells/ml and preincubated with aspirin (10 μg/ml) for 2 h to irreversibly inactivate any preexisting COX-1. After washing, the BMMC were activated with c-kit ligand (KL, 100 ng/ml), IL-10 (100 U/ml) and LPS (100 ng/ml) at 37°C for 8 h in the presence or absence of the samples previously dissolved in dimethylsulfoxide (DMSO). All reactions were quenched by centrifugation at 120 g at 4°C for 5 min. The supernatant and cell pellets were frozen immediately in liquid N_2 and stored at −80°C until needed for further analysis. Concentrations of PGD_2 in the supernatant were measured using PGD_2 assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to manufacturer's instruction. Under these conditions, the COX-2-dependent phases of PGD_2 generation reached 1.6 ng /10^6 cells. The data is reported as the arithmetic mean of triplicate determinations.

Determination of leukotriene C_4 (LTC_4)

The BMMCs suspended in enriched medium at a density of 1×10^6 cells/ml were pretreated with the samples for 15 min at 37°C and stimulated with KL (KL; 100 ng/ml). After 20 min stimulation, the supernatants were isolated and analyzed by EIA. The LTC_4 level was determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacture's instructions. Under these conditions, the LTC_4 reached up to 500 pg/10^6 cells. The data is reported as the arithmetic mean of triplicate determinations.

RESULTS AND DISCUSSION

During our search for biologically active compounds derived from the endemic species in Korea, the n-hexane and EtOAc fractions of the MeOH extract of the leaves of

A. altissima were shown to possess COX-2 and 5-LOX dual inhibitory activities by assessing their effects on the production of the PGD_2 and LTC_4 in mouse bone marrow-derived mast cells (BMMCs) (Table I). Repeated chromatography resulted in the isolation of eight compounds (Fig. 1). Based on comparison of ^1H and ^{13}C-NMR spectral data of these compounds with published data, the isolated compounds were identified as 1-8 (Pistelli *et al.,* 1996; Thuong *et al.,* 2005).

Murakami *et al.* reported that BMMCs exhibit biphasic PGD_2 biosynthetic responses over time, as well as COX-1-dependent immediate and COX-2-dependent delayed responses (Murakami *et al.,* 1994). The immediate generation of PGD_2 that occurs within 2 h of culturing is associated with the coupling of COX-1, while the delayed generation of PGD_2, which occurs after several hours of culturing (2-10 h), is associated with the de novo induction and function of COX-2 after the stimulation with particular cytokines and LPS combinations (Murakami *et al.,* 1994). This cell model also appears to be suitable for assessing the effect of 5-LOX inhibitors, since the immediate generation of LTC_4 elicited by the IgE-dependent or cytokine-initiated stimulus occurs in BMMCs through 5-LOX (Murakami *et al.,* 1995). Therefore, the BMMC system is useful for screening selective COX-1/COX-2 or 5-LOX and COX-2/5-LOX dual inhibitors from various sources (Moon *et al.,* 1999; Lee *et al.,* 2004; Son *et al.,* 2005; Son *et al.,* 2006).

Table I. Inhibitory effect of MeOH extract and solvent fractions against COX-2 and 5-LOX

Extract and fractions	COX-2 inhibition (%) (50 μg/ml)	5-LO inhibition (%) (50 μg/ml)
MeOH	49.0	98.9
Hexane	57.4	99.8
EtOAc	53.3	96.7
BuOH	0	99.0
H_2O	0	0

The generation of PGD_2 in the COX-2-dependent phase and the generation of LTC_4 in the 5-LOX dependent phase were tested after the BMMCs were activated with a combination of KL, IL-10 and LPS in the presence or absence of each compound. As the results, 2, 4, and 5 strongly inhibited both the COX-2-dependent PGD_2 generation with IC_{50} values of 2.6, 7.3 and 2.5 μM, respectively and the generation of LTC_4 in the 5-LOX dependent phase with IC_{50} values of 2.0, 5.1 and 1.8 μM, respectively (Table II). In this experiments, NS398 (COX-2 selective inhibitor) and AA861 (5-LOX inhibitor) were used as positive control (Makino *et al.,* 1986; Ouellet *et al.,* 1995). Under the same conditions, NS398 and AA861 strongly inhibited PGD_2 and LTC_4 generation of bone marrow-derived mast cells (BMMC) in a concentration-dependent manner with an IC_{50} of 1.6×10^{-4} μM and 3.2×10^{-2} μM, respectively.

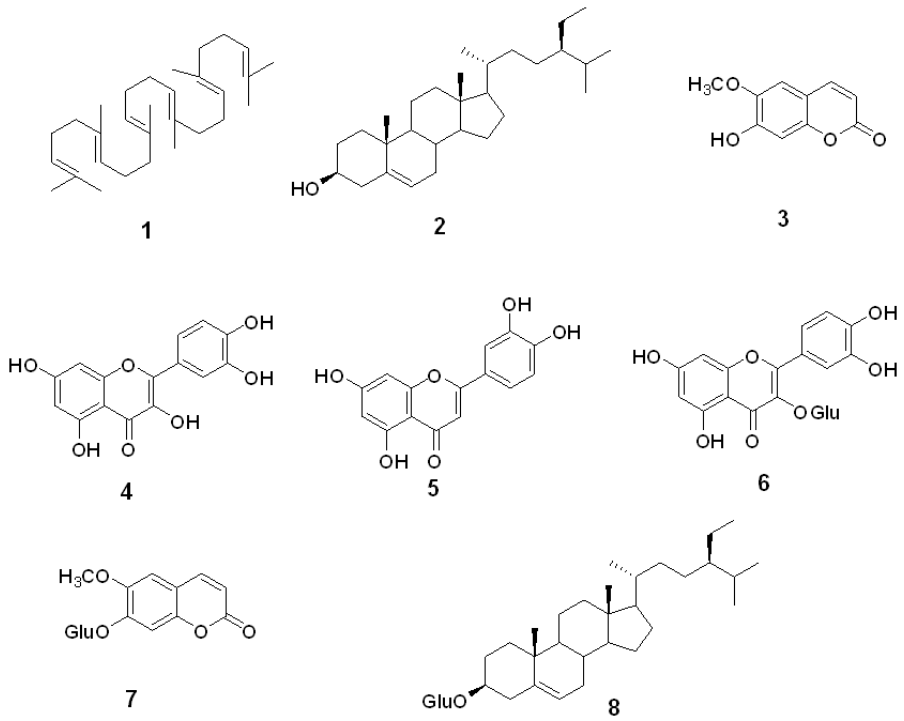

Fig. 1. Chemical structures of isolated compounds (1-8).

Table II. Inhibitory effect of isolated compounds against COX-2 and 5-LOX

Compounds	COX-2 inhibition (IC_{50}, μM)	5-LOX inhibition (IC_{50}, μM)
1	>100	1.8
2	2.6	2.0
3	39.2	59.9
4	7.3	5.1
5	2.5	1.8
6	>100	>100
7	>100	>100
8	>100	>100
NS398[a]	1.6×10^{-4}	—
AA861[a]	—	3.2×10^{-2}

[a]These compounds were used as positive controls.

These results suggest that the anti-inflammatory activity of *A. altissima* might occur *via* the inhibition of both PGD_2 and LTC_4 generation by 2, 4 and 5. It is interesting that glucosylation of 2, 4 and 5 to 8, 7 and 6, respectively, lead to complete loss of inhibitory activities in both PGD_2 and LTC_4 generation. COX-2 and 5-LOX inhibitory activity of luteolin and β-sitosterol isolated from the root of *Dystaenia takeshimana*, but no IC_{50} values of them, has been reported (Kim *et al.,* 2006). COX-2 inhibitory activity, but no 5-LOX inhibitory activity of quercetin, has been reported (Raso *et al.,* 2001). Further study will be needed to clarify the mechanisms for the action of these compounds isolated in this study.

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