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Inhibitors of Nitric Oxide Production from *Artemisia princeps*

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Abstract – The chromatographic separation of a methanol extract of *Artemisia princes* led to the isolation of two sesquiterpene lactones, artecanin (**1**) and canin (**2**), together with a flavonoid, eupatilin (**3**). Their structures were determined by 1D, 2D-NMR and MS data analysis. All of the isolates were evaluated for their potential to inhibit the LPS-induced production of nitric oxide in murine macrophage RAW 264.7 cells. Compounds **1** - **3** inhibited nitric oxide production with IC_{50} values of 19.5, 20.4 and 25.1 μM, respectively.
Keywords – *Artemisia princeps*, Compositae, Sesquiterpene lactone, Nitric oxide production inhibitor

Introduction

The genus *Artemisia*, one of the largest genera of Compositae, is widespread in the northern hemisphere. *Artemisia princeps* Pampanini is a herbaceous plant that is widely distributed in Korea, China, and Japan. The aerial parts of this plant have been used in Korean traditional medicine for the treatment of inflammation, gastric ulcer, colic pain, vomiting and diarrhea, and irregular bleeding from uterus (Jung and Shin, 1990; Tan *et al.*, 1998). Previous studies of phytochemical components of *Artemisia* have led to the isolation of many compounds, such as monoterpenes, sesquiterpenes, triterpenes, phenylpropanoids, lignans, and flavones (Tan *et al.*, 1998).

It has been reported that *A. princes* has anti-diabetic (Jung *et al.*, 2007; Nakasugi *et al.*, 2000) and anti-atherosclerosis effects (Han *et al.*, 2009). Yomogin, an eudesmane sesquiterpene isolated from *A. princeps*, possesses an inhibitory effect of the production of nitric oxide (NO) in LPS-activated RAW 264.7 cells by suppressing iNOS enzyme expression (Rhu *et al.*, 1998; Ryu *et al.*, 2000). Flavone derivatives such as eupatilin and jaceosidin isolated from *Artemisia* species inhibit the expression of TNF-α, IL-1β, iNOS, and COX-2 via regulation of NF-κB activation (Moscatelli *et al.*, 2006; Kim *et al.*, 2008; Lee *et al*, 2008; Min *et al.*, 2009). Sesquiterpenes such as artemisolide and arteminolide B also inhibit NF-κB activation and the LPS-induced production of NO and PGE_2 via down-regulation of iNOS and COX-2 expression (Jin *et al.*, 2004; Reddy *et al.*, 2006).

As part of our research program for the discovery of plant-derived inhibitors of NO production, we found that the methanolic extract of the aerial parts of *A. princeps* inhibited the production of NO in RAW 264.7 cells.

In this study, we identified two known guaianolide sesquiterpene lactones, artecanin (**1**) and canin (**2**), together with a flavone, eupatilin (**3**). All isolates were examined for their inhibitory effects on the production of NO in murine macrophage RAW 264.7 cells.

Materials and Methods

General experimental procedures – Melting points were measured on an Electroghermal 9100 instrument without correction. Optical rotations were determined using a JASCO DIP-100 polarimeter. UV and IR spectra were obtained on a JASCO UV-550 and JASCO Report-100 spectrometer, respectively. NMR spectra were acquired with a Bruker AMX 500 instrument at room temperature. ESI-MS and HRFAB-MS were measured on a Finnigan LCQ Fleet and a JEOL JMS-HX/HX110A tandem mass spectrometer, respectively. Silica gel (70 - 230 mesh, Merck, Germany), Lichroprep RP-18 (40 - 63 μM, Merck, Germany), and Sephadex LH-20 (25-100 μM, Amersham Biosciences, Sweden) were used for open column chromatography. Thin layer chromatography (TLC) was per-

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formed on a pre-coated silica gel 60 F_{254} (0.25 mm, Merck, Germany). All other chemicals and reagents were analytical grade.

Plant material – The aerial parts of *A. princeps* were collected at Cheongju, Chungbuk, Korea, in June 2008 and identified by Emeritus Professor Kyong Soon Lee, College of Pharmacy, Chungbuk National University. A voucher specimen (CBNU 0813) has been deposited at the Herbarium of College of Pharmacy, Chungbuk National University, Korea.

Extraction and isolation – The dried aerial parts of *A. princeps* (0.7 kg) were extracted three times with MeOH (5 L × 3) at room temperature. The combined extracts were concentrated under vacuum.. The MeOH extract (50 g) was suspended in H_2O and partitioned with n-hexane (1.5 L × 3) and CH_2Cl_2 (1.5 L × 3). The CH_2Cl_2 extract (10.7 g) was subjected to silica gel column (4.5 × 40 cm), eluting with a step gradient of CH_2Cl_2-MeOH (100 : 0, 50 : 1, 20 : 1, 10 : 1, 5 : 1, 1 : 1, each 1.5 L) to give six fractions (AP-1 – AP-6). Fraction AP-3 (1.8 g) was further chromatographed over silica gel column, eluting with mixtures of hexane-EtOAc (20 : 1, 10 : 1, 5 : 1, 1 : 1, 0 : 1, 0.6 L) to afford four combined fractions (AP-3A – AP-3D). Fraction AP-3B was further chromatogrphed over a Sephadex LH-20, eluting with CH_2Cl_2-MeOH (1 : 1) to yield four sub-fractions (AP-3B1 – AP-3B4). Sub-fraction AP-3B2 was subjected to flash column chromatography on RP-18, eluting with MeOH : H_2O (20, 40, 60 and 80% MeOH) to afford compounds **1** (4.0 mg) and **2** (3.5 mg). Sub-fraction AP-3B3 was recrystallized to yield a compound **3** (10 mg).

Artecanin (1) – Colorless oil; $[\alpha]^{20}_D$ +15.2; IR (KBr) ν_{max} 3510, 1750, 1655 cm^{-1}; HRFAB-MS: *m/z* 279.1235 $[M+H]^+$, calcd for $C_{15}H_{19}O_5$, 279.1232; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 1.

Canin (2) – Colorless needle crystal; m.p. 242 - 244 °C; $[\alpha]^{20}_D$ −14.7; IR (KBr) ν_{max} 3500, 1755, 1655 cm^{-1}; HRFAB-MS: *m/z* 279.1236 $[M+H]^+$, calcd for $C_{15}H_{19}O_5$, 279.1232; ^1H NMR (Pyridine-d_5, 500 MHz) and ^{13}C NMR (Pyridine-d_5, 125 MHz), see Table 1.

Eupatilin (3) – Yellow amorphous powder; m.p. 228 - 230 °C; UV (MeOH) λ_{max} nm : 274, 340; IR (KBr) ν_{max} 3390, 3265, 1655, 1620, 1584, 1460, and 1425 cm^{-1}; ESI-MS: *m/z* 344 $[M]^+$; ^1H NMR (DMSO-d_6, 500 MHz): δ 3.75 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 3.88 (3H, s, OCH_3), 6.63 (1H, s, H-3), 6.97 (1H, s, H-8), 7.11 (1H, d, *J* = 8.5 Hz, H-5'), 7.56 (1H, br s, H-2'), 7.67 (1H, br d, *J* = 8.5 Hz, H-6'), 10.8 (1H, br s, OH-4'), 13.0 (1H, br s, OH-5); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 163.4 (C-2), 104.2 (C-3), 182.2 (C-4), 152.8 (C-5), 131.3 (C-6), 157.3 (C-7), 94.4 (C-8), 152.4 (C-9), 103.4 (C-10), 122.9 (C-1'), 109.4 (C-2'), 149.0 (C-3'), 152.1 (C-4'), 111.6 (C-5'), 120.0 (C-6'), 60.0 (OCH_3), 55.9 (OCH_3), 55.7 (OCH_3).

Assay for inhibitory activity of nitric oxide production – The nitrite concentration was determined by measuring the amount of nitrite in the cell culture supernatant as previously described (Hong *et al.*, 2008). Briefly, RAW 264.7 cells (2 × 10^5 cells/mL) were stimulated

Table 1. ^1H (500 MHz) and ^{13}C NMR (125 MHz) data of compounds 1 and 2.[a]

Carbon No	1		2	
	δ_C	δ_H	^d_C	δ_H
1	79.1	–	78.5	–
2	58.6	3.66 br s	58.9	3.48 br s
3	58.5	3.54 br s	58.5	3.29 br s
4	73.3	–	73.9	–
5	43.5	2.82 d (11.5)	50.5	2.55 d (11.8)
6	80.8	4.47 dd (10.3, 11.5)	80.0	4.23 dd (10.2, 11.8)
7	50.3	3.99 m	45.6	3.40 m
8	24.4	2.48 m, 1.45 m	23.9	2.17 m, 1.64 m
9	33.9	2.02 m, 1.85 m	34.5	2.00 m, 1.82 m
10	72.3	–	72.5	–
11	141.7	–	139.7	–
12	169.7	–	169.6	–
13	118.8	6.19 d (3.3), 5.38 d (3.0)	120.3	6.21 d (3.4), 5.50 d (3.2)
14	27.5	1.24 s	27.4	1.15 s
15	19.8	1.64 s	19.5	1.56 s

[a] Chemical shift are shown in the δ scale with *J* values (Hz) in parentheses.

with or without 1 μg/mL of LPS in the presence or absence of compounds. After incubation at 37 °C for 24 h, 100 μL of cell-free supernatant was mixed with 100 μL of Griess reagent containing equal volumes of 2% (w/v) sulfanilamide in 5% (w/v) phosphoric acid and 0.2% (w/v) of N-(1-naphthyl)ethylenediamine solution to determine nitrite production. The viability of the cells remaining after the Griess assay was determined using a CCK-8 assay (Cell counting kit-8, Dojindo, Tokyo, Japan).

Results and Discussion

Repeated column chromatographic separation of the CH_2Cl_2 extract of the aerial parts of *A. princeps* resulted in the isolation of two known guaianolide sesquiterpene lactones, artecanin (**1**) and canin (**2**), together with a flavone, eupatilin (**3**). Compound **3** was identified by comparing ^1H, ^{13}C NMR and MS data with those reported in the literatures (Liu and Mabry, 1981a; Nakasugi *et al.*, 2000; Kang *et al.*, 2008).

Compound **1** was obtained as colorless oil, and the molecular formula was determined as $C_{15}H_{18}O_5$ by HRFAB-MS at *m/z* 279.1235 $[M+H]^+$ (calcd *m/z* 279.1232), indicating seven double-bond equivalents in the molecule. The IR absorption bands at 3510 and 1750 cm^{-1} suggested the presence of hydroxyl and γ-lactone functionalities. The ^1H NMR spectrum of **1** showed an *exo*-methylene signals [δ_H 6.19 (1H, d, J = 3.3 Hz, H-13a), and 5.38 (1H, d, J = 3.0 Hz, H-13b)], three oxygenated proton signals [δ_H 4.47 (1H, dd, J = 10.3 and 11.5 Hz, H-6), 3.66 (1H, br s, H-2), and 3.54 (1H, br s, H-3)], and two methyl groups [δ_H 1.24 (3H, s, CH_3-14) and 1.64 (3H, s, CH_3-15)]. The ^{13}C-NMR spectrum of **1** revealed the presence of 15 carbon signals, including two methyls, two methylenes, an *exo*-methylene, five methines, and five quaternary carbons. These observations suggested that compound **1** was a 1,2;3,4-diepoxyguaianolide sesquiterpene lactone with two tertiary methyls and a hydroxyl group (Ohno *et al.*, 1980; Hewlett *et al.*, 1996). The two methyl groups were located at C-4 and C-10 on the basis of HMBC correlations from CH_3-15 (δ_H 1.64) to C-3 (δ_C 58.5), C-4 (δ_C 73.3), and C-5 (δ_C 43.5) and from CH_3-14 (δ_H 1.24) to C-1 (δ_C 79.1), C-9 (δ_C 33.9), and C-10 (δ_C 72.3), respectively. The five-membered ring stereochemistry of compound **1** was determined by interpretation of chemical shift of H-5 and C-5. In the ^1H and ^{13}C NMR spectrum, it is clearly observed that H-5 (vicinal to the epoxide) of 1β,2β;3β,4β-diepoxyguaianolide compound **1** is deshielded (δ_H 2.82), where as C-5 of **1** is shielded (δ_C 43.5) in comparison with corresponding data of its

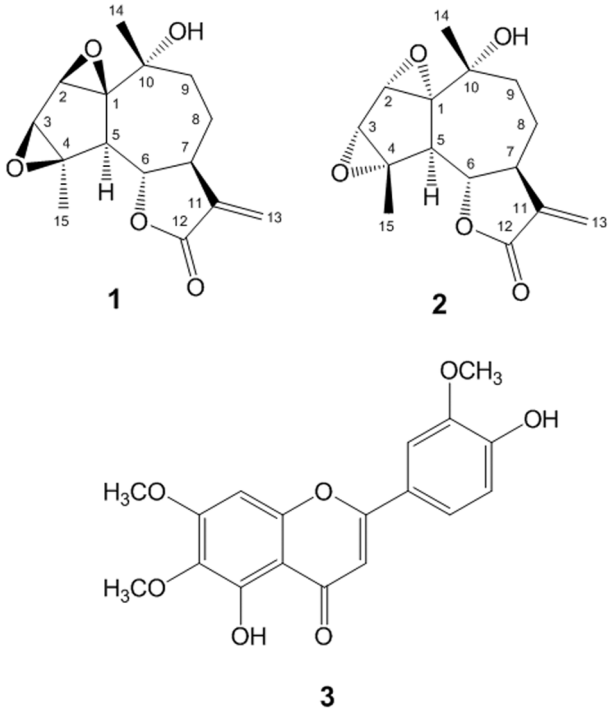

Fig. 1. Structures of compounds **1** - **3**.

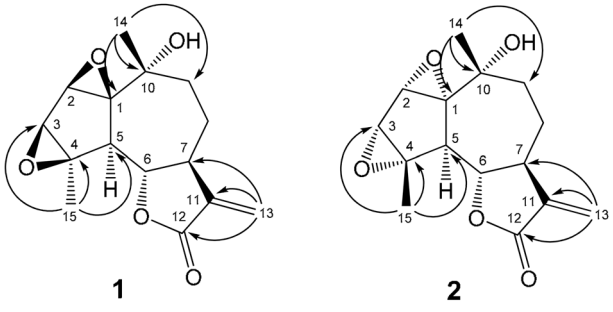

Fig. 2. Selected HMBC correlations of compounds **1** and **2**.

1α,2α;3α,4α-diepoxyguaianolide compound **2** (δ_H 2.55 and δ_C 50.5) (Hewlett *et al.*, 1996; Trifunovic *et al.*, 2008). The NOESY experiment further confirmed the relative configuration of compound **1**, wherein the correlations were observed from H-5 to CH_3-15 and from H-2 to CH_3-14. These results indicated that 1,2;3,4-diepoxide, C-14, and C-15 methyl groups had β, β, and α-orientations, respectively. Therefore, compound **1** was identified as artecanin (chrysartemin B), 1β,2β;3β,4β-diepoxyguaianolide lactone, isolated from *Artemisia*, *Achillea*, and *Tanacetum* species (Ohno *et al.*, 1980; Liu and Mabry, 1981b; Hewlett *et al.*, 1996; Trifunovic *et al.*, 2006).

Compound **2** was obtained as colorless needle crystals. The positive HRFAB-MS at *m/z* 279.1236 $[M+H]^+$ (calcd

m/z 279.1232) revealed the same molecular formula ($C_{15}H_{18}O_5$) as compound **1**. The overall appearance of the ^1H and ^{13}C NMR spectra of compound **2**, very similar to those of **1**, as well as HMBC correlations, indicated the same gross structure and a diastereomeric relationship between **1** and **2**. The relative stereochemistry of compound **2** was determined by NOESY NMR experiment and the comparison of H-5 and C-5 chemical shift data of compound **1**. This indicated that **2** had the opposite (1α,2α;3α,4α) diepoxy configuration (Trifunovic *et al.*, 2008). The relative stereochemistry of two methyl groups at C-14 and C-15 was further determined by NOE correlations H-6/CH_3-15 and H-6/CH_3-14, indicating β, β-orientations, respectively. Therefore, compound **2** was identified as canin (chrysartemin A), 1α,2α;3α,4α-diepoxy diasteroisomer of compound **1** (Liu and Mabry, 1981b; Begley *et al.*, 1989; Hewlett *et al.*, 1996).

Nitric oxide, synthesized via oxidation of L-arginine by a family of nitric oxide synthase (NOS), plays an important role in physiological and pathological processes. However, excessive production of NO by iNOS in macrophages is involved in various inflammatory diseases (Alderton *et al.*, 2001). Therefore, inhibitors of NO production in macrophages are an important target for the discovery of anti-inflammatory agents.

Compounds **1** - **3** were examined for their inhibitory effects on the production of NO in LPS-stimulated RAW 264.7 cells. A well-known iNOS inhibitor, aminoguanidine, as a positive control, inhibited the production of NO with IC_{50} value of 19.8 μM. 1,2;3,4-Diepoxyguaianolide sesquiterpene lactone derivatives, artecanin (**1**) and canin (**2**), showed strong inhibitory effects with IC_{50} values of 19.5 and 20.4 μM, respectively, confirming the importance of the α-methylene-γ-lactone moiety as an active center. A flavone derivative, eupatilin (3) showed moderate inhibitory effect with IC_{50} value of 25.1 μM. However, further studies for the mechanism on the inhibition of NO are needed to fully characterize the effects of the compounds.

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