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Isolation of Constituents with Nitric Oxide Synthase Inhibition Activity from *Phryma leptostachya* var. *asiatica*

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Abstract – Phytochemical studies were performed to identify the active principles of *Phryma leptostachya* var. *asiatica* (Phyrymaceae) for anti-inflammation. The anti-inflammatory activity was assessed by measuring the inhibition rate on nitric oxide (NO) formation in lipopolysaccharide (LPS)-activated macrophage 264.7 cells. Of the five compounds including ursolic acid, phrymarolin I, harpagide, haedoxancoside A, and acteoside isolated from this plant, ursolic acid showed the most prominent inhibition of NO formation. Therefore, ursolic acid may be the anti-inflammatory principle of *Phryma leptostachya* var. *asiatica.*
Keywords – *Phryma leptostachya* var. *asiatica*, Phyrymaceae, nitric oxide, anti-inflammatory, ursolic acid

Introduction

Phrima leptostachya var. *asiatica* (Phrymaceae) is a perennial herb growing in the shading place. This plant is distributed in Korea, Japan, and China as well as Siberia and North-East America. Paripul,the Korean name of this plant, was named because its root has been used to kill flies. In addition, this plant has been also used to treat allergic dermatitis and itching and to prevent cancer disease.[1]

The constituents of the lignans of phrymarolin I and II,[2,3] V and B[4,5] possessing the basic structure of 1,2-dioxygenated-3,7-dioxabicyclo[3.3.0]octane, haedoxancoside A belonging to sesquilignan,[5] and ursolic acid as the triterpene acid[6] were previously reported. Furthermore, the larvicidal activity ofleptostachyol acetate, a lignan of *P. leptostachya* var. *asiatica*, has been also reported.[7,8]

Nitric oxide (NO) is a simple and gaseous mediator produced by nitric oxide synthase (NOS) including inducible nitric oxide synthase (iNOS). NO is involved in pathophysiological conditions such as inflammatory- and autoimmune diseases.[9] The expression of iNOS is induced by pro-inflammatory cytokines and bacterial lipopoly-saccharide (LPS).[10] Therefore, anti-inflammatory effect is assessed by measuring the amount of NO in lipopoly-saccharide (LPS)-induced murine macrophage RAW 264.7 cells.

Jung et al.[1] reported that the root extract of *P. leptostachya* var. *asiatica* has anti-inflammatory effect *via* the mechanism of anti-oxidative and the inhibition of iNOS and cyclooxygenase-2 activities in LPS-activated macrophage cells. However, the active principle of *P. leptostachya* var. *asiatica*for anti-inflammation has not been reported yet. Therefore, in the present study, we aimed to identify which compounds are mainly responsible for anti-inflammatory activity of this plant.

Experimental

Plant material – The whole plant of *Phyryma leptostachya* var. *asiatica* Hara (Phrymaceae), was collected from the mountain area in Wonju city, Gangwon-do, Korea. The plant was washed, dried, and cut for extraction. The plant was identified by Prof. Byung-Min Song, Department of Forest Science, Sangji Univerisity, Korea. A voucher specimen (natchem-#87) was deposited in the Laboratory of Natural Products Chemistry, Sangji University, Korea.

Extraction and fractionation – The plant material (892 g) was extracted with MeOH (each, 5.0 L) three times under reflux. The extracted solution was filtered and concentrated under reduced pressure on a rotatory evaporator. The viscous MeOH extract was further subjected to freeze-drying to give a solid MeOH extract (123.0 g,

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extraction yield 13.3%).

The MeOH extract was suspended in H_2O (2.0 L), and partitioned with hexane (2.0 L) three times. The hexane fraction was further concentrated *in vacuo* to give a $CHCl_3$ fraction (24.0 g). In the same method, the residual MeOH extract was successively fractionated with $CHCl_3$, EtOAc, and BuOH, respectively, to give a $CHCl_3$ fraction (18.5 g), EtOAc fraction (4.55 g), and BuOH fraction (28.0 g).

After that solvent fractionation, the hexane fraction was further fractionated by the column chromatography. The hexane fraction was developed over diaion HP-20 column chromatography with 1.0 L MeOH, and then eluted with 1.0 L MeOH-$CHCl_3$ (1:1). The MeOH-$CHCl_3$ (1:1)-eluted solution was concentrated *in vacuo* to yield a MeOH-$CHCl_3$ fraction.

In addition, the BuOH fraction was further subjected to diaion HP-20 column chromatography to remove sugars and ionic substances. The BuOH fraction was washed with H_2O (2.0 L) over the column, and then eluted with MeOH (2.0 L). The MeOH solution was concentrated to yield 8.29 g MeOH fraction.

Isolation of ursolic acid (1) and phrymarolin I (2) – The MeOH-$CHCl_3$ fraction obtained from the hexane fraction was subjected to silica gel column (40 μm, 165 g, 48 × 170 mm, Hi-Flash column, Yamazen Co., Japan) chromatography using $CHCl_3$-MeOH-H_2O (8:1:1, lower phase) and collected by each 50 mL. After checking TLC, the fractions #25-28 and #36-39 were concentrated to afford PLA-#25-28 and PLA-#36-39, respectively. PLA-#25-28 was washed with MeOH to yield compound **1**. PLA-#36-39 was recrystallized from MeOH to yield compound **2**. Compounds **1** and **2** were identified as ursolic acid (Lee et al., 2002) and phrymarolin I (Taniguchi and Oshima, 1972), respectively, by comparisons of ^{1}H- and ^{13}C-NMR spectroscopic data with literatures.

Compound 1 (ursolic acid) – White powder, UV λ_{max} MeOH (log ε): 220 (2.83) nm; IR ν_{max} (KBr) cm^{-1}: 3400 (broad, OH), 1090(COOH); ^{1}H-NMR (600 MHz, pyridine-d_5) and ^{13}C-NMR (150 MHz, pyridine-d_5) δ: Literature.[6]

Compound 2 (phrymarolin I) – Amorphous powder, IR νmax (KBr) cm^{-1}: 3008 (broad, OH), 1733 (ester), 1634, 1502 (aromatic C=C); ^{1}H-NMR (600 MHz, CD_3OD) and 13C-NMR (150 MHz, CD_3OD) δ: Literature.[2]

Isolation of harpagide (3), haedoxancoside A (4) and acteoside (5) – The MeOH fraction obtained by eluting the BuOH fraction over diaion HP-20 column with MeOH was subjected to silica gel column chromatography (40 μm, 165 g, 48 × 170 mm, Hi-Flash column, Yamazen Co., Japan) with the solvent of $CHCl_3$-MeOH-H_2O (65:35:10, lower phase), and collected by each 50 mL. After checking TLC, the fractions #39-44, #50-58, and #72-88 were concentrated, respectively, and precipitated from MeOH to yield compounds **3**, **4**, and **5**. The three compounds **3**, **4**, and **5** were identified as harpagide,[11] haedoxancoside A,[5] and acteoside[12] by comparisons of spectroscopic data with literatures.

Compound 3 (harpagide) – Amorphous powder frm MeOH, mp 228-229, IR ν_{max} (KBr) cm^{-1}: 3358, 1643, 1250, 1047; 1H-NMR (600 MHz, CD_3OD) and 13C-NMR (150 MHz, CD_3OD) δ: Literature.[11]

Compound 4 (haedoxancoside A) – White powder, mp 181 - 183 ºC, UV λ_{max} (MeOH) nm (log ε) : 293 (4.05), 234 (4.08); IR ν_{max} (KBr) cm^{-1}: 34783006, 2941, 1600, 1500; ^{1}H-NMR (600 MHz, CD3OD) and ^{13}C-NMR (150 MHz, CD3OD) δ: Literature.[5]

Compound 5 (acteoside) – UV λ_{max} (MeOH) nm (log ε) : 328 (4.11); IR ν_{max} (KBr) cm^{-1}: 3403 (broad, O-H), 1698 (C=O), 1631 (olefinic C=C), 1100 – 1000 (glycosidic C-O); 1H-NMR (600 MHz, CD3OD) δ: Literature.[12]

Cell culture – Murine macrophage RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics-antimycotics (PSF; 100 units/ml penicillin G sodium, 100 ng/mL streptomycin, and 250 ng/mL amphotericin B). The cells were incubated at 37 ºC under a humidified atmosphere containing 5% CO_2.

Cell viability assay – After the supernatant was collected for iNOS assay, MTT solution (final concentration of 500 μg/mL) was added to each well and incubated for 4 h at 37 ºC. The culture media was aspirated, and dimethyl sulfoxide (DMSO) was added to dissolve the dye. The absorbance was measured at 570 nm using VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA), and the percent survival was determined by comparison with a control group (LPS+).

iNOS assay - Murine RAW 264.7 cells were seeded in 24-well plates (2×10^5 cells/mL). The next day, culture media were changed to 1% FBS-DMEM with sample treatment. After 1 h, LPS (1 μg/mL) was added, except LPS- control, to stimulate NO production. The amount of NO production in culture media was determined by Griess reaction after 18 h incubation. Briefly, 100 μL of culture media was collected per each well and 180 μL of Griess reagent (0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in H_2O and 1% sulfanilamide in 5% H_3PO_4) was added. The absorbance was measured at 540 nm. The nitrate concentration was determined by

Table 1. Effect of the MeOH extract of *P. leptostachya* var. *asiatica* and its fractions on NO formation in LPS-activated macrophage cells (test concentration of 40 μg/mL)

Sample	Inhibition rate (%)	Cell viability (%)
MeOH extract	41.0	-
Hexane fraction	63.8	-
$CHCl_3$ fraction	48.3	26.4
EtOAc fraction	40.1	-
BuOH fraction	38.2	-

Footnote: -, not cytotoxic

comparison with sodium nitrite standard curve. Percent inhibition was calculated using following formula: [1 – (NO level of test samples/NO levels of vehicle-treated control)] × 100. The IC_{50} value was calculated through non-linear regression analysis using TableCurve 2D v5.01 (Systat Software Inc., San Jose, CA, USA)

Results and Discussion

The iNOS is an enzyme responsible for the production of nitric oxide (NO). Recently, anti-inflammatory activity is frequently assayed by measuring the iNOS inhibition activity.[13] In the present study, iNOS assay was performed to identify the anti-inflammatory compounds since the active compounds from *P. leptostachya* var. *asiatica*have not been reported.

In this study, the iNOS inhibition activity was determined by measuring the amount of nitrate in LPS-activated macrophage 264.7 cells. Cells were pre-treated with 40 μg/ml of each fractions for 1 h and LPS (1 μg/mL) were added to stimulate the cells to produce NO. The iNOS inhibition rate (%) of the MeOH extract and its fractions were shown in Table 1, and cell viability was also tested in the same condition. The MeOH extract significantly reduced the formation of NO by 41.0%. The hexane fraction effectively reduced the NO formation by 63.8% which was more effective than other three fractions. While the MeOH, hexane, and EtOAc fractionsdid not affect the cell viability, the $CHCl_3$ fraction was relatively cytotoxic with 26.4% cell viability at 40 μg/mL.

Therefore, the hexane fraction was chromatographed to isolate the active substance. The two substances of ursolic acid[6] and phrymarolin I[2] were identified by comparison of ^{1}H-NMR and ^{13}C-NMR spectroscopic data with literatures, as shown in Fig. 1. Phrymarolin I possessing the basic skeleton of 1,2-dioxygenated-3,7-dioxabicyclo[3.3.0]octane is known to have a larvicidal activity.[7,8] The three compounds **3**, **4**, and **5** isolated from the BuOH fraction were identified as harpagide,[11] haedoxancoside A,[5] acteoside[12]

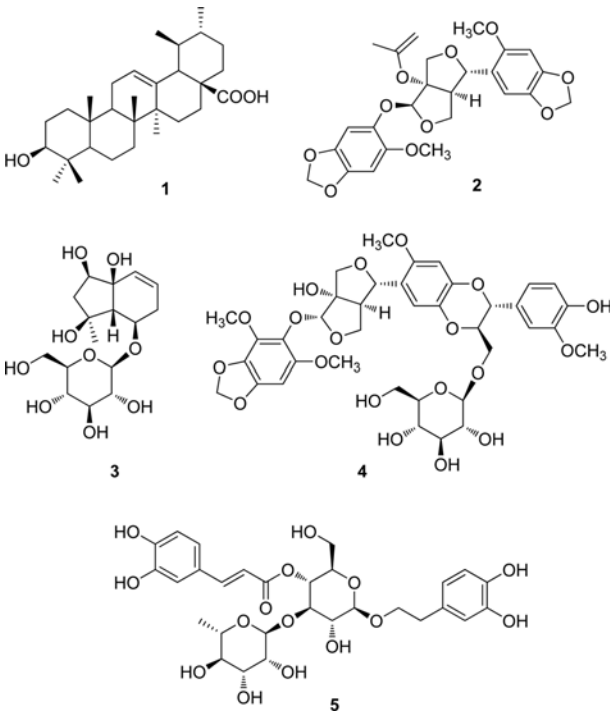

Fig. 1. Structure of compounds **1** – **5** isolated from *P. leptostachya* var. *asiatica.*

Table 2. Effect of compounds **1** – **4** obtained from *P. leptostachya* var. *asiatica* on NO formation in LPS-activated macrophage cells (test concentration of 40 μg/mL)

Compound	Inhibition rate (%)	Cell viability (%)
Ursolic acid (**1**)	80.6	-[a]
Phrymarolin I (**2**)	30.2	-
Haedoxancoside A (**3**)	35.2	-
Acteoside (**4**)	25.6	-
AMT (0.2 μM)[b]	82.4	-

Footnote: -[a], not cytotoxic; [b]AMT: 2-Amino-5,6-dihydro-6-methyl-4H-1,3-thiazine

by ^{1}H-NMR and ^{13}C-NMR spectra. Of the five compounds isolated, harpagide and acteoside has not been reported from *P. leptostachya* var. *asiatica*.

The inhibition rate of the four compounds (ursolic acid, phrymarolin I, haedoxancoside A and acteoside) are shown in Table 2. The AMT (2-Amino-5,6-dihydro-6-methyl-4H-1,3-thiazine), a known iNOS inhibitor, was used as a positive control. Among the four compounds, ursolic acid showed the highest inhibition rate (80.6%), and other compounds showed inhibition rate lower than 50%. Therefore, the inhibition of NO formation and cell viability of ursolic acid were further tested at 10, 20, and 40 μg/mL, respectively (Fig. 2). The IC_{50} value was determined to be 20.8 μg/mL,and the inhibition of NO

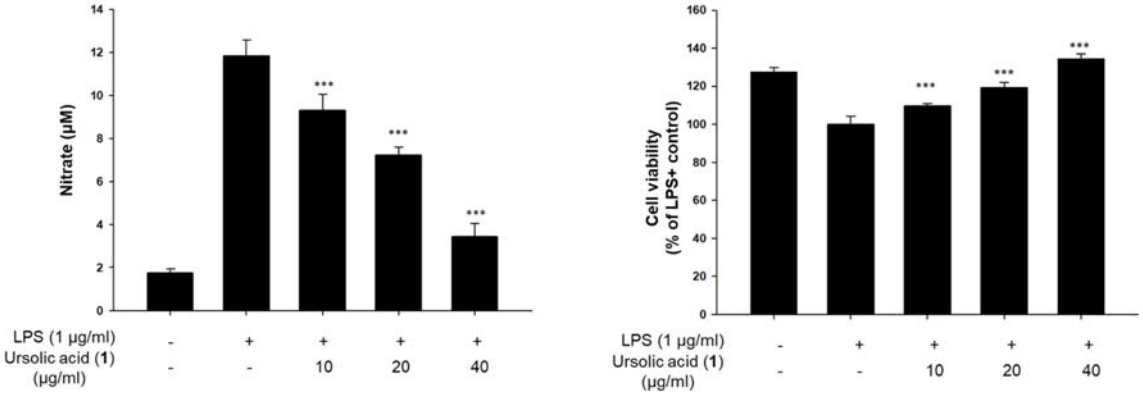

Fig. 2. Effect of compound **1** on NO formation in LPS-activated macrophage 264.7 cells. Cells were pretreated with different concentrations of ursolic acid and stimulated with LPS (1 μg/mL). The amount of nitrate was measured by Griess reaction. The cell viability was measured using MTT. The data are presented as the means ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$ by *t*-test.

production by ursolic acid was not derived from the cell viability.

Although the anti-inflammatory and inhibitory effects of NO formation by ursolic acid have been reported,[14,15] the inhibitory activities of phrymarolin I and haedoxancoside A on NO formation are newly discovered in the present study. In conclusion, ursolic acid exhibited the most potent anti-inflammatory activity without cytotoxicity. In addition, harpagide and acteoside were first isolated from *P. leptostachia* var. *asiatica*

Acknowledgments

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