Inhibitory Effect on Nitric Oxide Production of RAW 264.7 Macrophage Cells of an Active Compound from *Alternanthera philoxeroides*

การยับยั้งการสร้างไนตริกออกไซด์ในเซลล์เพาะเลี้ยงแมโครฟาจ RAW 264.7 ของสารสำคัญจากผักเป็ดน้ำ

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ABSTRACT

The present study was undertaken to investigate the effect of a major compound of *Alternanthera philoxeroides*, 3-*O*-β-D-glucopyranosiduronic acid 28-β-D-glucopyranosyl-oleanolate or chikusetsusaponins IVa (compound 1), on the production of nitric oxide (NO) in lipopolydaccharide (LPS)-stimulated RAW 264.7 macrophage cells.The results showed that compound 1 significantly suppressed NO production in a dose-dependent manner. The studies on structure-activity relationship of compound 1 have demonstrated that both acid and alkaline hydrolysis products, compound 1a (oleanolic acid) and compound 1b (oleanolic acid 3-*O*-β-D-glucoronide), also exhibited significant NO inhibitory activity, with the relative potency was compound 1b > compound 1 > compound 1a, respectively.

บทคัดย่อ

การศึกษาผลของสารสำคัญของผักเป็ดน้ำ 3-*O*-β-D-glucopyranosiduronic acid 28-β-D-glucopyranosyl-oleanolate หรือ chikusetsusaponins IVa (compound 1) ต่อการสร้างไนตริกออกไซด์ในเซลล์เพาะเลี้ยงแมโครฟาจ RAW 264.7 ที่มีการกระตุ้นด้วยไลโพโพลีแซคคาไรด์ (LPS) พบว่า สาร compound 1 สามารถลดการสร้างไนตริกออกไซด์ตามความเข้มข้นที่เพิ่มขึ้น และเมื่อศึกษาความสัมพันธ์ของฤทธิ์และ

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โครงสร้างทางเคมีของสาร compound 1 พบว่า ทั้ง compound 1a (oleanolic acid) และ compound 1b (oleanolic acid 3-*O*-β-D-glucoronide) ซึ่งเป็นผลิตผลจากการทำการสลาย compound 1 ด้วยกรดและด่าง สามารถลดการสร้างไนตริกออกไซด์ได้เช่นกัน โดยผลการลดการสร้างไนตริกออกไซด์ของ compound 1b > compound 1 > compound 1a ตามลำดับ

Key Words : *Alternanthera philoxeroides*, chikusetsusaponin IVa, nitric oxide
คำสำคัญ : ผักเป็ดน้ำ ชิคูเซ็นสุซาโปนิน โพร์เอ ไนตริกออกไซด์

Introduction

Inflammation is the first response of the immune system to infection or irritation. Nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) is a key and early inflammatory factor released by monocytes/macrophages and has been implicated as an immune regulator, neurotransmitter, vasodilator in a variety of tissues at physiological concentrations and important mediator under endotoxemia and inflammatory conditions (Ahmad *et al.*, 2002; Kroncke *et al.*, 1997; Wanchu *et al.*, 1999). The high levels of NO have been defined as cytotoxic in inflammation and endotoxemia (Kroncke *et al.*, 1997). Thus, the suppression of NO production by anti-inflammatory drugs is considered to be potential.

Alternanthera philoxeroides (Mart.) Griseb. (Amaranthaceae) is a perennial plant that is widely distributed in Australia, Asia and America. It has been used as a traditional medicine for anti-inflammation and diuretic. In Thai traditional medicine, it is regarded as an antipyretic and dressing for wounds and ulcers. Previous studies reported the inhibitory effect of this plant on the human immunodeficiency virus (Zhang *et al.*, 1988), dengue virus (Jiang *et al.*, 2005) and herpes simplex virus (Rattanathongkom *et al.*, 2009) and also immunodulating activity in mouse splenocytes (Rattanathongkom *et al.*, 2008). The chemical studies of this plant have reported to have phaeophytin a, pheophytin a', oleanoic acid, β-sitosterol, 3β-hydroxystigmast-5-en-7-one, α-spinasterol, 24-methylenecycloartanol, cycloeucalenol, phytol, alternanthin B and N-trans-feruloyl-3,5-dimethoxytyramine (Fang *et al.*, 2007; Fang *et al.*, 2006). However, there are no published report on the isolation and identification of anti-inflammatory bioactive chemical constituents from the plant.

In the present study, the crude extract of *A. philoxeroides* was purified by guiding the inhibitory activity for NO production using lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages cell line to give compound 1 as a major component.

Material and methods

General

^{1}H- and ^{13}C-NMR spectra were recorded on a Varian 400 spectrometer in pyridine-d_5 with tetramethylsilane (TMS) was used as an internal standard. Chromatographic materials were RP-18 (40-60 μm, Merck), silica gel (70-230 mesh), and copolymer of styrene and divinylbenzene (Mitsubishi Chem. Ind. Co. Ltd). Analytical and preparative HPLC was carried out on columns of ODS (150 x 4.6 mm and 150 x 20 i.d., YMC), equipped with a refractive index detector (RID-6A)

with flow rate used 1 and 6 ml/min, respectively. The solvent systems were: (I) Ethyl acetate (EtOAc)-MeOH (9:1), (II) EtOAc-MeOH-H_2O (4:1:0.1), (III) EtOAc-MeOH-H_2O (7:3:0.3), (IV) EtOAc-MeOH-H_2O (6:4:1), (V) 50-80% MeOH.

Chemicals and plant material

RPMI 1640 medium, fetal bovine serum (FBS), and streptomycin/penicillin were purchased from Gibco (Invitrogen, USA). Lipopolysaccharide (LPS), dimethyl sulfoxide (DMSO), sulfanilamide, and N-1 naphthylethylenediamine-dihydrochloride were products from Sigma (St. Louis, MO). All other chemicals were analytical grade.

The whole plant of *A. philoxeroides* was collected from Khon Kaen Province, Thailand. A voucher sample (KKU-A-002) is kept in the Herbarium of the Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand.

Extraction and isolation

The dried whole plant (4.0 kg) was extracted with hot MeOH under reflux at 60°C for 3 times (3h each). Evaporation of the solvent gave a crude MeOH extract (547.1 g). The crude extract was defatted with Et_2O and the aqueous layer was eluted with *n*-BuOH. The *n*-BuOH layer (83.7g) was fractionated to obtain 4 fractions by a column of highly porous copolymer of styrene and divinylbenzene and eluted with water and MeOH. Fraction C was further purified on silica gel column chromatography using solvent systems composed of EtOAc and MeOH to give 7 fractions (Fraction 1-7). Fraction 4 was applied to a reverse-phase (RP18) column eluted MeOH, and received 11 fractions. Fraction 4-8 was further purified by preparative HPLC using 75% MeOH to provide compound 1 (1.57 g) (Figure 1).

Acid hydrolysis

A solution of compound 1 (20 mg) was refluxed at 100°C in 7% HCl (40 ml) for 3 h. The reaction mixture was partitioned with $CHCl_3$-H_2O to give compound 1a. The structure of compound 1a was determined by comparison of its NMR spectral data with those of authentic sample reported previously (Seebacher *et al.*, 2003).

Alkaline hydrolysis

A solution of compound 1 (20 mg) was refluxed at 100°C in 5% NaOH (20 ml) for 2 h. The reaction mixture was adjusted to pH 6 with 1N HCl and then partitioned with *n*-BuOH to obtain compound 1b. This compound was identified by comparison of NMR spectral data with those of authentic sample reported previously (Nie *et al.*, 1984).

Sample preparation

All samples were dissolved in DMSO and further serial diluted with test medium at concentration from 0-60 μM before further studied.

Culture of macrophages

RAW 264.7 murine macrophage cells were grown at 37°C in RPMI 1640 medium supplemented with 10% FBS, 1% streptomycin/penicillin in a humidified atmosphere of 5% CO_2. Exponentially growing cells reaching 80% confluence were used in the experiments.

Measurement of nitrite formation and cell viability

Nitrite, as an indicator of NO synthesis, was measured in the cell supernatant by Griess reaction (Green *et al.*, 1982). The cells (0.2 ml, 3 x10^5 cells/ml) were placed in 24-well plates. After incubation for 24 h at 37°C, the cells were

treated with the test samples and co-incubated with LPS (final concentration of 100 ng/ml) for 24 h. The generated NO is readily oxidized to nitrite and further reacts with Griess reagent (1% sulfanilamide and 0.1% N-1 naphthylethylenediamine-dihydrochloride in 2.5% phosphoric acid) to form a purple azo derivative. Briefly, 100 µl of cell culture medium was mixed with 100 µl of Griess reagent and incubated at room temperature for 10 min. The absorbance at 550 nm was then measured in a microplate reader. The amount of nitrite in the samples was calculated using a standard curve of freshly prepared sodium nitrite in culture medium. To make sure that the determined NO is not resulted from the cell death, cell viability at the end of the experiment was confirmed by staining the cells with 0.01% neutral red dye for one hour or WST-8 assay (Okumura *et al.*, 2005).

The inhibitory and cytotoxic effects were expressed as IC_{50} (the concentration of a compound for 50% inhibition *in vitro*) and CC_{50} (the concentration of a compound for 50% cytotoxic *in vitro*)

Statistical analysis

Each experiment was performed in triplicate, and results are expressed as the mean±SD. The statistical analyses were performed using SPSS 11.0 for Windows. One-way ANOVA was performed to analyze differences among multiple means. *P*-values of <0.05 were considered statistically significant.

Results and discussion

The methanol (MeOH) extract of the whole plant of *A. philoxeroides* was fractionated with diethyl ether (Et_2O) and *n*-butanol (*n*-BuOH), successively. In *n*-BuOH fraction was further separated to isolate compound 1 as a major component yielding 0.04%. This compound was identified as 3-*O*-β-D-glucopyranosiduronic acid, 28-β-D-glucopyranosyl-oleanolate or chikusetsusaponins IVa by comparison of its NMR spectral data with those of the published data (Gohar *et al.*, 2002). Chikusetsusaponin IVa was previously reported to be isolated in several plants including rhizomes of *Panax japonicum* (Lin *et al.*, 1976), root of *Beta vulgaris* (Yoshikawa and Matsuda, 2000), and aerial parts of *Chenopodium ficifolium* (Gohar *et al.*, 2002). Moreover, the products from acid and alkaline hydrolysis of compound 1 were identified as oleanolic acid (compound 1a) and oleanolic acid-3-*O*-β-D-glucoronide (compound 1b), respectively (Figure 2).

The use of *A. philoxeroides* extract as an anti-inflammatory drug in traditional herbal medicine suggests that active compounds from the plant might be potential as anti-inflammation therapeutic agents. In this study, MeOH, Et_2O and *n*-BuOH fractions did not affect NO production in LPS-stimulated RAW 264.5 cells (data not shown). In contrast, compound 1 at concentrations ranging from 1 to 60 µM markedly inhibited NO production in LPS-stimulated RAW 264.7 cells in a concentration dependent manner (Figure 3), with IC_{50} value of 46.63 µM. The inhibitory effects observed in this study were not due to toxic effect of compound 1, since its cell viability was 98% and CC_{50} was more than 100 µM.

Previous data have revealed the potency of triterpenes and triterpenoidal saponins in the inhibition of NO production in LPS-stimulated RAW 264.7 cells (Suh *et al.*, 2007; Wang *et al.*, 2007). Glycyrrhizin having two glucuronic acid moieties at C-3 position has also affected NO

production (Jeong and Kim, 2002; Kondo and Takano, 1994). Therefore, the function at C-3 position of compound 1 might be important. In order to make clear at this point, compound 1 was hydrolyzed by both acid and alkaline conditions to give rise to compound 1a and 1b, respectively. The results showed that compound 1b exhibited inhibitory effect on NO production by LPS-stimulated RAW 264.7 cells with IC_{50} at 25.09 μM. However, the value was close to its CC_{50}, which was 59.35 μM. Compound 1a showed weak inhibition on NO production with IC_{50} > 100 μM and CC_{50} > 100 μM (Table 1). These results suggest that compound 1b, which having the substituents at C-3, might play an important role in the inhibitory effect on NO production in LPS-stimulated RAW 264.7 cells as oleanolic acid 3-*O*-β-D-glucopyranosyl (1-->3)-α-L-rhamnopyranosyl (1-->2)-α-L-arabinopyranoside from *Aralia elata* (Suh *et al.*, 2007) and kalopanaxsaponin A (hederagenin 3-*O*-[α-L-rhamnopyranosyl-(1-->2)-α-L-arabinopyranoside]) from *Akebia quinata* (Jung *et al.*, 2004). The sugar substituent at C-28 and C-3 as observed in compound 1, might be important for reduction of cytotoxicity and NO production. Therefore, the active compound identified from *A. philoxeroides* in this study might facilitate further studies on its anti-inflammatory activity.

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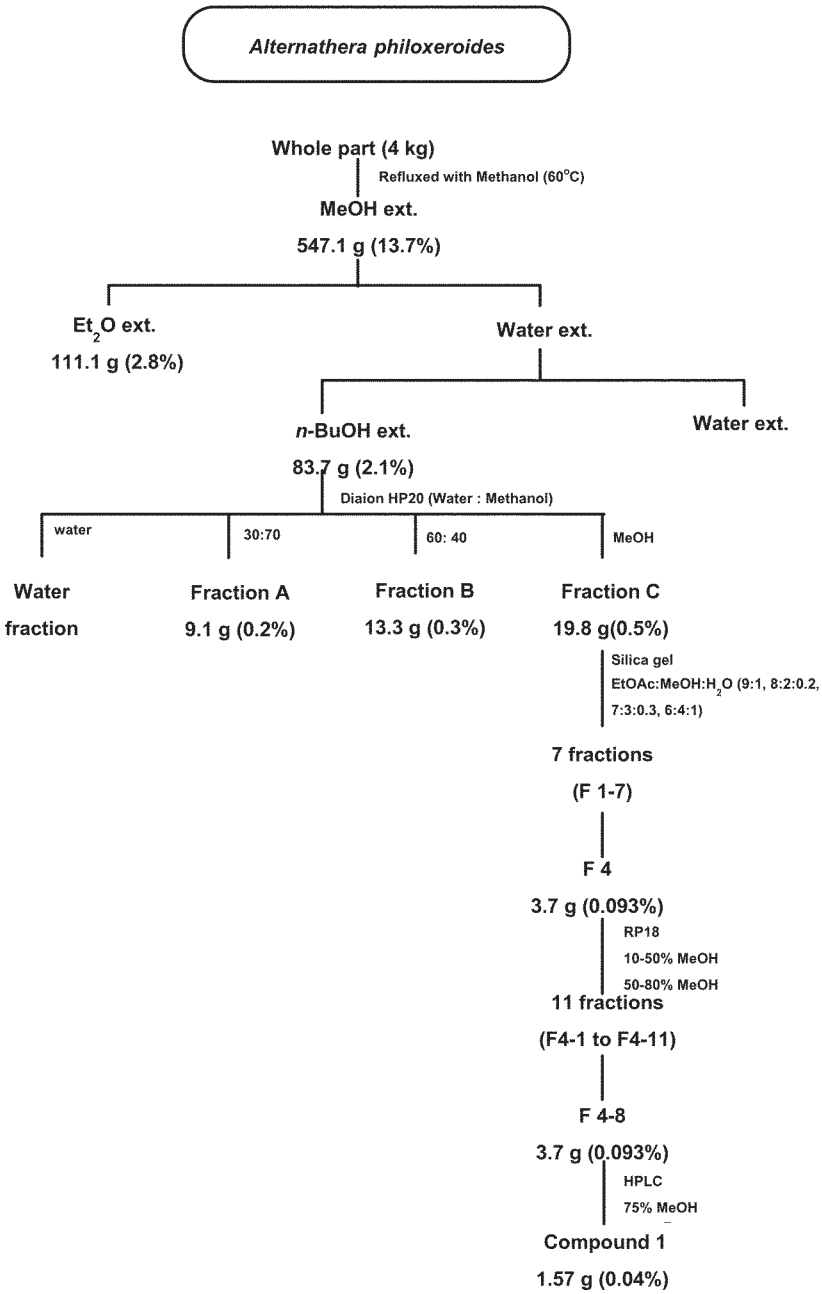
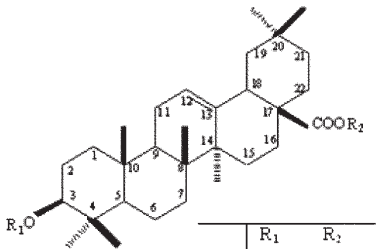


Figure 1 Extraction, isolation and purification of *A. philoxeroides*



	R_1	R_2
1	GlcA	Glu
1a	H	H
1b	GlcA	H

Figure 2 The structure of compound 1 and its derivatives.

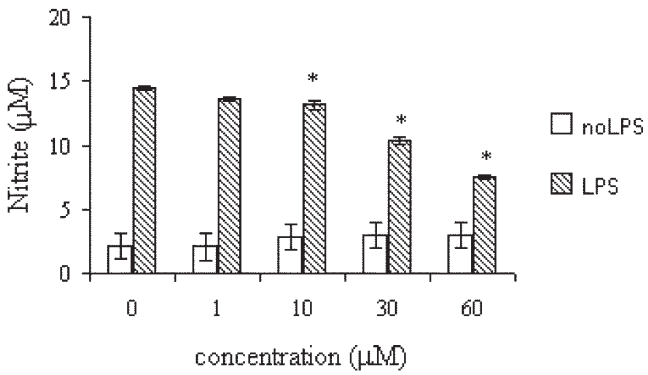


Figure 3 Effect of compound 1 on NO production in LPS-stimulated RAW 264.7 macrophage cells. *Statistically significant differences from control at $P < 0.05$.

Table 1 The cytotoxic and inhibitory effects of compound 1, 1a and 1b

	CC_{50} (μM)	IC_{50} (μM)
Compound 1	> 100	46.63
Compound 1a	> 100	> 100
Compound 1b	59.35	25.09