Bioorganic & Medicinal Chemistry Letters 19 (2009) 4389–4393

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Phenolic glycosides from *Alangium salviifolium* leaves with inhibitory activity on LPS-induced NO, PGE_2, and TNF-α production

Tran Manh Hung [a,e], Nguyen Hai Dang [b], Jin Cheol Kim [c], Jae Sue Choi [d], Hyeong Kyu Lee [e], Byung-Sun Min [a,*]

[a] College of Pharmacy, Catholic University of Daegu, Gyeongsan 712–702, Republic of Korea
[b] Institute of Natural Product Chemistry, Vietnamese Academy of Science and Technology, 18A Hoang Quoc Viet, Caugiay, Hanoi, Viet Nam
[c] Korea Research Institute of Chemical Technology, Daejeon 305-600, Republic of Korea
[d] Faculty of Food Science and Biotechnology, Pukyoung National University, Busan 608-737, Republic of Korea
[e] Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Republic of Korea

ARTICLE INFO

Article history:
Received 15 April 2009
Revised 4 May 2009
Accepted 16 May 2009
Available online 24 May 2009

Keywords:
Alangium salviifolium
Alangiaceae
Phenolic glycosides
Anti-inflammatory

ABSTRACT

Three new phenolic glycosides, salviifosides A–C (**1**–**3**), and three known compounds salicin (**4**), kaempferol (**5**), and kaempferol 3-*O*-β-D-glucopyranoside (**6**) were isolated from the leaves of *Alangium salviifolium* (L.f.) Wangerin (Alangiaceae). The structures of the new metabolites were determined on the basic of spectroscopic analyses including two dimensional NMR. The anti-inflammatory activities of new compounds (**1**–**3**) were investigated on lipopolysaccharide (LPS)-induced murine macrophage cells line, RAW 264.7. Salviifoside B (**2**) potentially inhibits the productions of nitric oxide (NO), prostaglandin E_2 (PGE_2), and tumor necrosis factor-α (TNF-α).

Inflammation is the response of vascularized living tissue to local injury. Chronic inflammations and infections lead to the upregulation of a series of enzymes and signaling proteins in affected tissues and cells. Among these pro-inflammatory enzymes, the inducible forms of nitric oxide synthase (NOS), and cyclooxygenase (COX), which are responsible for increasing the levels of NO and prostaglandins (PGs), respectively, are known to be involved in the pathogenesis of many chronic diseases including multiple sclerosis, Parkinson's, Alzheimer's diseases, and colon cancer.[1,2] NO is produced by iNOS in macrophages, hepatocytes, and renal cells, under the stimulation of lipopolysaccharide (LPS), tumor necrosis factor-alpha (TNF-α), interleukin-1 or interferon-g,[3] meanwhile, COX is the enzyme that converts arachidonic acid to PGs. Like NOS, COX has been found to exist in two isoforms and one of these, COX-2, is an inducible form which is responsible for the production of large amounts of pro-inflammatory PGs at the inflammatory site.[4] Furthermore, TNF-α is one of the most important pro-inflammatory cytokines and is mainly produced by monocytes and macrophages. It is secreted during the early phase of acute and chronic inflammatory diseases such as asthma, rheumatoid arthritis, septic shock and other allergic diseases, as well as the activation of T cells.[5]

During a screening procedure on higher plants to find novel candidates as anti-inflammatory agents, the 70% EtOH extract of *Alangium salviifolium* (L.f.) Wangerin (Alangiaceae) was shown to exhibit considerable inhibitory activity. *A. salviifolium* is a medicinal plant which has been traditionally used for tonic and treatment of hemorrhoid. This plant showed promising antimicrobial activity.[6] The root and root bark are claimed to be effective in helmintiasis, skin diseases, piles, dysentery, inflammations, hypertension, snake bite, and eczema.[7a] Leaves were used as poultice to reduce rheumatic pains, stem in vomiting and diarrhea, analgesic, and anti-inflammatory properties.[7b] Previous work on this species resulted in the isolation of alkaloids, sterols, fatty acids, and chromones.[7a] In our present study, six compounds including three new phenolic glycosides (**1**–**3**) were isolated. This paper describes the isolation, elucidates the structures of new compounds and evaluates their anti-inflammatory activity on LPS-induced RAW 264 cells.

Repeated chromatography of the EtOAc-soluble fraction of the 70% EtOH extract of *A. salviifolium* on silica gel, YMC gel, Sephadex LH-20, and C_{18} columns led to the isolation of six compounds (**1**–**6**).[8] Three of them are known compounds, and identified as salicin (**4**),[9] kempferol (**5**), and kaempferol-3-*O*-β-D-glucopyranoside (**6**),[10] (Fig. 1) by comparison of physicochemical (mp, $[\alpha]_D$) and spectroscopic (^1H and ^{13}C NMR) data with published values.

Salviifoside A (**1**) was obtained as colorless needles, and its negative HR-ESI-MS showed a $[M]^-$ ion at *m*/*z* 405.1238, which established that the molecular formula is $C_{20}H_{22}O_9$. The IR spectrum

* Corresponding author. Tel.: +82 53 850 3613; fax: +82 53 850 3602.
E-mail address: bsmin@cu.ac.kr (B.-S. Min).

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doi:10.1016/j.bmcl.2009.05.070

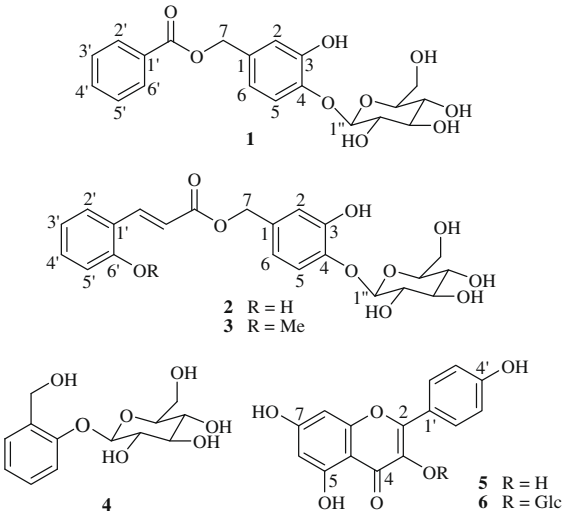

Figure 1. Chemical structures of isolated compounds.

indicated the presence of a phenolic hydroxy group at 3320 cm^{-1}. In the UV spectrum, λ_{max} values at 230 and 288 nm were observed, indicating the presence of aromatic groups.[11a] A typical ABX spin system at δ 6.79 (1H, d, J = 2.8 Hz, H-2), 7.04 (1H, d, J = 9.2 Hz, H-5), and 6.68 (1H, dd, J = 2.8, 9.2 Hz, H-6) was used to identify a 3,4-dihydroxybenzyl moiety in the ^{1}H NMR spectrum. A pair of proton signals at δ 5.01 (1H, d, J = 9.2 Hz, H-7a) and 5.30 (1H, d, J = 9.2 Hz, H-7b) was evidence for an oxygenated methylene group. In addition, another set of peaks appearing between δ 7.56 and 8.01 was attributed to aromatic protons of a benzoyl group moiety. Furthermore, the ^{1}H NMR spectrum of **1** showed the presence of signals corresponding to an anomeric proton of a sugar moiety appeared at δ 4.47 (1H, d, J = 6.8 Hz, H-1″). The ^{13}C NMR and DEPT spectrum of **1** showed 20 carbon signals in the molecule. Among them, six signals at δ 102.6, 73.4, 77.0, 69.8, 76.6, and 60.8 belonged to a glucose unit, six signals appearing between δ 128.8 and 133.4 belonged to a monosubstituted benzoyl group, a signal at δ 61.6 belonged to an oxymethylene group, and the other belonged to a trisubstituted benzyl group. These data suggested that compound **1** is a monoglucoside of 3,4-dihydroxyphenyl.[12]

Table 1
^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectral data of compounds **1**–**3** in DMSO (δ ppm)

Position	1		2		3	
	δ_H[a]	δ_c	δ_H	δ_c	δ_H	δ_c
1		126.6		127.8		125.2
2	6.79 (d, 2.8)	114.5	7.03 (d, 2.0)	115.3	7.34 (d, 2.0)	115.4
3		148.7		146.9		147.9
4		152.4		149.7		149.3
5	7.04 (d, 9.2)	115.7	6.77 (d, 8.0)	115.1	7.15 (d, 8.0)	114.4
6	6.68 (dd, 2.8, 9.2)	117.7	6.95 (dd, 2.0, 8.0)	116.6	7.12 (dd, 1.6, 8.0)	114.9
7	5.01 (d, 12.0)	61.6	5.42 (d, 12.8)	62.7	5.30 (d, 12.8)	60.7
	5.30 (d, 12.0)		5.30 (d, 12.8)		5.27 (d, 12.8)	
1′		129.7		130.5		125.6
2′	8.01 (dd, 1.6, 6.8)	128.8	6.95 (dd, 2.9, 8.0)	127.3	7.03 (br t, 7.2)	128.7
3′	7.56 (br t, 8.0)	129.2	7.06 (br t, 8.0)	123.6	7.14 (dd, 1.6, 8.0)	123.2
4′	7.69 (m)	133.4	7.30 (td, 2.0, 8.0)	130.7	7.28 (td, 1.6, 8.0)	129.2
5′	7.56 (br t, 8.0)	129.2	7.37 (dd, 2.0, 8.0)	121.3	7.34 (dd, 1.8, 8.0)	121.7
6′	8.01 (dd, 1.6, 6.8)	128.8		157.1		155.1
7′			7.58 (d, 16.4)	147.3	7.60 (d, 16.0)	145.3
8′			6.30 (d, 16.4)	116.8	6.55 (d, 16.0)	115.4
Glc						
1″	4.47 (d, 6.8)	102.6	4.98 (d, 7.3)	103.5	4.87 (d, 7.2)	101.0
2″	3.13–3.26 (m)	73.4	3.40–3.53 (m)	75.1	3.16–3.34 (m)	73.3
3″		77.0		78.1		77.0
4″		69.8		71.4		69.7
5″		76.6		78.3		76.5
6″	3.69 (dd, 2.0, 12.2)	60.8	3.90 (dd, 2.0, 12.4)	62.7	3.72 (dd, 2.0, 12.4)	60.7
	2.47 (m)		3.71 (m)		3.48 (m)	
C=O		165.9		169.9		169.6
OCH_3					3.81 (s)	55.7

[A] Mult, J in Hz.

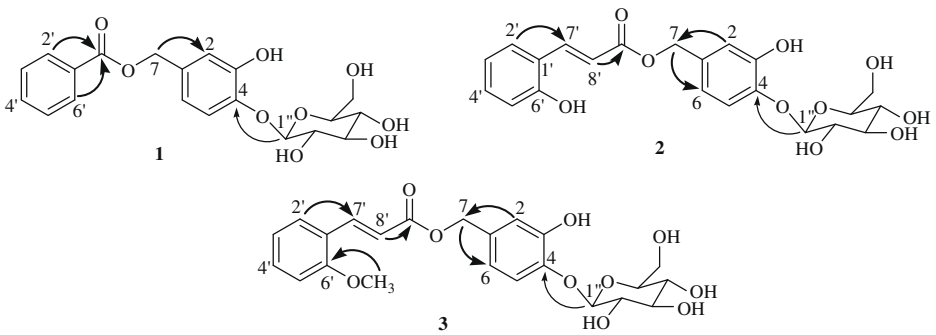

Figure 2. Selected HMBC correlations of **1**–**3**.

All ^{1}H and ^{13}C NMR signal assignments of **1** (Table 1) were confirmed by the present study from the HMQC and HMBC spectra (Fig. 2). The sugar was assigned as glucopyranose on the basis of NMR data and the R_f value compared with authentic glucose after enzymatic (naringinase) hydrolysis of **1**.[13a,13b] The absolute configuration was determined to be D-glucose by gas chromatography (GC).[14] The $J_{H,H}$ value (6.8 Hz) of the anomeric proton (H-1″) indicated that glucose was linked via a β-linkage. In addition, the position of the glucose linkage in **1** was established at the C-4 hydroxyl group of the 3,4-dihydroxybenzyl moiety by the HMBC technique (Fig. 2). Thus, the structure of the **1** was established as benzoyloxymethyl-3-hydroxy-phenyl-4-β-D-glucopyranoside, named salviifoside A.

Salviifoside B (**2**) was isolated as amorphous powder, with a composition of $C_{22}H_{24}O_{10}$, as determined on the basis of the peak at m/z 447.1365 $[M]^-$ in the negative HR-ESI-MS.[11b] It showed UV maxima at 216 and 327 nm and IR bands at 3350, 1700, and 1456 cm^{-1}. The ^{1}H NMR spectrum revealed proton signals at δ 7.03 (1H, d, J = 2.0 Hz, H-2), 6.77 (dd, J = 8.0 Hz, H-5), and 6.95 (d, J = 2.0, 8.0 Hz, H-6), indicating the presence of a 1,3,4-trisubstituted

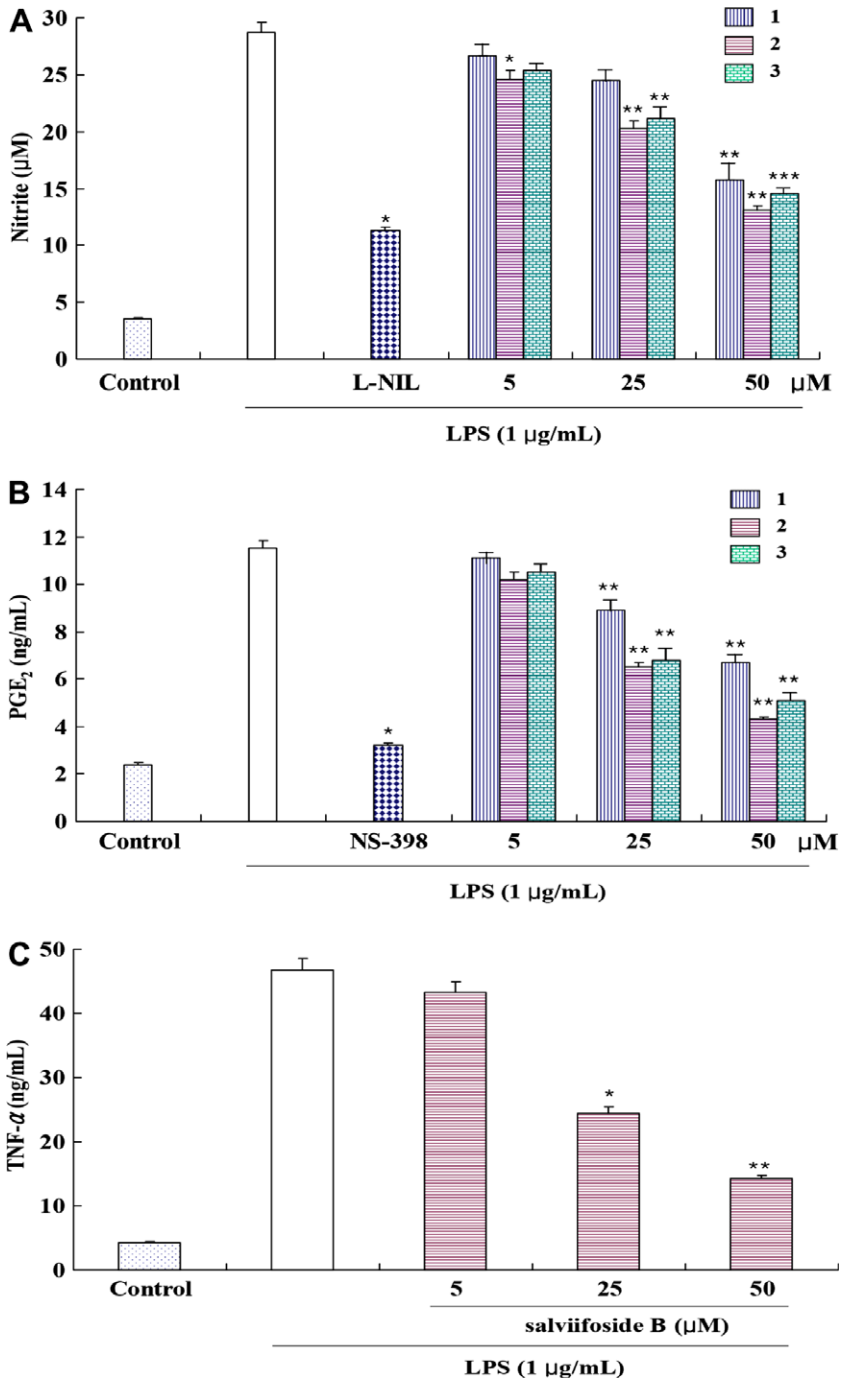

Figure 3. Effects of compounds **1**–**3** on NO (A), PGE_2 (B), and (C) effect of **2** on TNF-α production in RAW 264.7 cells. The cells were pretreated for 1 h with isolated compounds with different concentrations (5, 25, and 50 μM), and then LPS (1 μg/mL) was added and the cells were incubated for 24 h. Control values were obtained in the absence of LPS or tested compounds, the blank bar values were obtained in the presence of LPS. (A) L-N6-(1-iminoethyl)lysine (L-NIL, 10 μM) was used as a positive control. (B) NS-398 (10 μM) was used as a positive control. The values represent the means ± S.E.M. from three independent experiments. $^{*}p$ <0.05, $^{**}p$ <0.001 versus LPS-treated group. The significance of the difference between the treated groups was evaluated using the Student's t-test.

phenolic ring. The signals of four aromatic protons at δ 6.95 (1H, dd, J = 2.9, 8.0 Hz, H-2′), 7.06 (1H, br t, J = 8.0 Hz, H-3′), 7.30 (1H, td, J = 2.0, 8.0 Hz, H-4′), and 7.37 (1H, dd, 2.0, 8.0 Hz, H-5′) indicated the presence of a disubstituted benzene ring.[12,15] The signals of two *trans*-olefinic protons at δ 6.30 (1H, d, J = 16.4 Hz, H-8′) and 7.58 (1H, d, J = 16.4 Hz, H-7′), as well as a pair of oxygenated methylene protons at δ 5.30 and 5.42 (each H, d, J = 12.8 Hz, H-7) were also observed. In addition, the signals arising at δ 4.98 (1H, d, J = 7.3, H-1″), 3.40–3.53 (each H, m, H-2″-5″) and 3.90 (1H, dd, J = 2.0, 12.4 Hz, H-6″a) and 3.71 (1H, m, H-6″b) indicated the presence of a β-glucose unit. The ^{13}C NMR and DEPT spectrum of **2** showed 22 signals including oxygenated aromatic carbons, oxygenated methylene carbon, carbonyl carbon, together with six signals of a glucose unit. These ^{13}C NMR resonances are similar to those of **1**, the difference between two compounds is that compound **2** has the signals of two *trans*-olefinic carbons at δ 147.3 (C-7′) and 116.8 (C-8′). A quaternary carbon signal at δ 157.1 indicated the additional one hydroxyl group on aromatic ring. In combination with signals in ^{1}H NMR spectra, these observations suggested the presence of a 1,6-disubstituted benzene ring, which was further confirmed by the ^{1}H–1H COSY couplings between H-2′/H-3′, H-3′/H-4′, and H-4′/H-5′ and by the relevant ^{13}C–1H long-range correlations observed in the HMBC spectrum (Fig. 2). In addition, the absolute configuration was determined to be D-glucose by GC after enzymatic hydrolysis, and the position of the glucose linkage in **2** was also established at the C-4 of the 3,4-dihydroxybenzyl moiety by the HMBC technique. Thus, structure of compound **2** was assigned as 3-(2-hydroxyphenyl)-acryloxymethyl-3-hydroxy-phenyl-4-β-D-glucopyranoside, and it was named salviifoside B.

Compound **3**, salviifoside C, was obtained as a white amorphous powder. The negative HR-ESI-MS spectrum showed the $[M]^-$ peak at m/z 461.1514, which established a molecular formula of $C_{23}H_{26}O_{10}$.[11c] The ^{1}H and ^{13}C NMR spectra of **3** also showed seven and six characteristic signals of a sugar moiety in the region ranging from δ 3.16 to 4.87 and from δ 60.7 to 101.0, respectively. The sugar was identified as glucose and its β-glycosidic linkage was revealed on the basis of the large vicinal coupling constant at δ 4.87 (1H, d, J = 7.2 Hz, H-1″). The linkage position of glucose was confirmed by HMBC correlation between H-1″ and C-4 (Fig. 2). The spectral feature demonstrated its close similarity to compound **2**, except for the addition of a methoxy group at δ_H 3.81 (3H, s) and δ_C 55.7. The attachment of methoxy group at C-6′ was confirm by the correlation signal between δ_H 3.81 (–OCH_3) and δ_c 155.1 (C-6′) in HMBC spectrum (Fig. 2). Thus, compound **3** was deduced to be 3-(2-methoxyphenyl)-acryloxymethyl-3-hydroxy-phenyl-4-β-D-glucopyranoside, named salviifoside C.

The cytotoxic effects of salviifoside A, B, and C (**1**–**3**) were evaluated in the presence or absence of LPS using the MTT assay,[16] and these compounds did not affect the cell viability of RAW 264.7 cells in either the presence or absence of LPS even at a dose of 50 μM after a period of 24 h (data not shown). The amount of produced NO was determined by the amount of nitrite, a stable metabolite of NO. To assess the effects of salviifosides A, B, and C on the LPS-induced NO production in RAW 264.7 cells, cell culture medium was harvested and the production of nitrite was measured using the Griess reaction.[17a] During incubation time of 24 h, RAW 264.7 macrophage produced 3.5 ± 0.07 μM nitrite in the resting state. After LPS (1 μg/mL) stimulation, NO production increased dramatically to 28.72 ± 1.31 μM nitrite after 24 h. Salviifosides A, B, and C reduced the NO production 24 h after LPS stimulation in a dose-dependent manner (Fig. 3A). L-NIL, a positive inhibitor, significantly inhibited LPS-induced NO production (11.3 ± 0.5 μM) at the concentration of 10 μM.

To examine whether the tested compounds could inhibit PGE_2 production in the same manipulation, the cells were pre-incubated with compounds for 1 h and then activated with 1 μg/mL LPS for 24 h.[17b] The RAW 264.7 macrophage produced 2.4 ± 0.15 ng/mL PGE_2 in the resting state, however, after LPS (1 μg/mL) stimulation, PGE_2 production increased to 11.5 ± 0.36 ng/mL PGE_2. In this experiment, NS-398 (10 μM), a COX-2 enzyme inhibitor was used as a positive control, decreased PGE_2 production to 3.2 ± 0.18 ng/mL. As shown in Figure 3B, the tested compounds (**1**–**3**) significantly inhibited the production of PGE_2 in a dose-dependent manner.

Since salviifoside B (**2**) was the most potent inhibitor of the pro-inflammatory mediators among the tested compounds, we further investigated the effect of salviifosides B on the LPS-induced TNF-α release using an enzyme immunoassay.[17b] Pre-treatment of the cells with salviifoside B at the concentration of 5, 25, and 50 μM for 1 h decreased the TNF-α production to 43.2 ± 4.5, 24.4 ± 2.8, and 11.2 ± 1.0 ng/mL, respectively, in the comparison with the production of cells in resting state (4.3 ± 0.05 ng/mL) and cells with additional LPS state (46.7 ± 4.8 ng/mL) (Fig. 3C).

A. salviifolium has been traditionally used for treatment of inflammation. Our results showed that salviifosides A–C from *A. salviifolium* suppressed the productions NO, PGE_2, and TNF-α in LPS-stimulated RAW 267.4 cells. Thus, it is possible to demonstrate that isolated phenolic glycosides might be important anti-inflammatory constituent of this plant.

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8. The leaves of *Alangium salviifolium* were collected in Tuyen Quang province, North of Vietnam, in July 2007 and identified by Professor Pham Thanh Ky, Department of Pharmacognosy, Hanoi College of Pharmacy. A voucher specimen (HN-0901) was deposited in the herbarium of the Hanoi College of Pharmacy. The leaves (1.2 kg) were extracted with 3 L of 70% EtOH, three times. The 70% EtOH extract was combined and concentrated to yield a residue which was suspended in water and then successively partitioned with hexane, EtOAc, and BuOH. The EtOAc–soluble fraction (11.8 g) was separated by silica gel column chromatography using a gradient of hexane–EtOAc (from 30:1 to 5:1), then EtOAc–MeOH (from 20:1 to 1:1), to yield ten fractions (E1–E10) according to their TLC profiles. Fraction E6 (1.2 g) was chromatographed over silica gel column using a gradient of EtOAc–MeOH (from 15:1 to 5:1), to yield five subfractions E6.1–E6.5. The E6.2 fraction was further purified by semi preparative HPLC [RS Tech Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size); mobile phase MeOH–H_2O (65:35); flow rate 2 mL/min; UV detection at 230 nm] to obtain compounds **1** (11.7 mg; t_R = 29.5 min). The E6.4 fraction was separated by reversed-phase C_{18} (RP-18) column chromatography using a stepwise gradient of MeOH–H_2O (from 1:1 to 1:0 for each step), to afford ten subfractions (E5.1–E5.10). Fraction E5.3 was purified by Sephadex LH-20 using MeOH–H_2O (4:1) to obtain compounds **5** (6.0 mg) and **6** (12.7 mg). Fraction E7 (0.8 g) was chromatographed over silica gel column using a gradient of $CHCl_3$–MeOH (from 30:1 to 5:1), to yield five subfractions E7.1–E7.5. Repeated chromatography E7.2 on a C_{18} column eluted with MeOH–H_2O (1.5:1), compound **4** (14.1 mg) was obtained from the collected subfraction E7.2.1. Fraction E8 was purified by a C_{18} column eluted with MeOH–H_2O (1:1) to yield five subfractions E8.1–E8.5. The E8.2 fraction was further purified by semi preparative HPLC [RS Tech Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size); mobile phase MeOH–H_2O (70:30); flow rate 2 mL/min; UV detection at 230 nm] to obtain compounds **2** (11.2 mg; t_R = 18.9 min) and **3** (10.4 mg; t_R = 23.7 min).
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11. Physical and spectroscopic data of new compounds: (a) compound **1** (salviifoside A): white amorphous powder; $[\alpha]_D^{22}$ −18.5 (*c* 0.2, MeOH); UV λ_{max} (MeOH) nm (ε): 230 (4.20), 288 (3.87); IR (KBr) cm^{-1}: 3320, 2895, 2730,

1700, 1610, 1490; HR-ESI-MS *m*/*z* 405.1238 $[M]^-$ (calcd for $C_{20}H_{22}O_9$, 405.1240), for ^1H and ^{13}C NMR spectral data, see Table 1; (b) compound **2** (salviifoside B): white amorphous powder; $[\alpha]_{22}^{D}$ −15.8 (*c* 0.2, MeOH); UV λ_{max} (MeOH) nm (ε): 216 (4.25), 327 (4.05); IR (KBr) cm^{-1}: 3350, 1700, 1605, 1540, 1456, 1200 cm^{-1}; HR-ESI-MS *m*/*z* 447.1365 $[M]^-$ (calcd for $C_{22}H_{24}O_{10}$, 447.1385); for ^1H and ^{13}C NMR spectral data, see Table 1; (c) compound **3** (salviifoside C): white amorphous powder; $[\alpha]_{22}^{D}$ −15.2 (*c* 0.2, MeOH); UV λ_{max} (MeOH) nm (ε): 220 (4.17), 325 (4.30); IR (KBr) cm^{-1}: 3385, 1690, 1625, 1560, 1515, 1290 cm^{-1}; HR-ESI-MS *m*/*z* 461.1514 $[M]^-$ (calcd for $C_{23}H_{26}O_{10}$, 461.1542); for ^1H and ^{13}C NMR spectral data, see Table 1.

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13. (a) Naringinase (100 mg, from *Penicillium decumbens*) was added to a suspension of **1**, **2** and **3** (5 mg) in 50 mM acetate buffer (pH 5.5), and the mixture was stirred at 37 °C for 5 h. The reaction mixture was extracted with EtOAc (10 mL × 3), and the water layer was checked by silica gel TLC (EtOAc–MeOH–H_2O–AcOH, 65:20:15:15). The spot on the TLC plate was visualized by an anisaldehyde–H_2SO_4 reagent. The configuration of glucose was determined by a GC method, and the sugar derivative thus obtained showed a retention time of 21.30 min, identical with that of authentic D-glucose:; (b) Min, B. S.; Na, M. K.; Oh, S. R.; Ahn, K. S.; Jeong, G. S.; Li, G.; Lee, S. K.; Joung, H.; Lee, H. K. *J. Nat. Prod.* **2004**, *67*, 1980.
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17. (a) The nitrite, which accumulated in the culture medium, was measured as an indicator of NO production by means of the Griess reaction. Briefly, 100 mL of cell culture medium (without phenol red) was mixed with an equal volume of Griess reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamine–HCl), incubated at room temperature for 10 min, and then the absorbance was measured at 550 nm using a microplate reader. Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was obtained by means of the $NaNO_2$ serial dilution standard curve and the nitrite production was measured; (b) The PGE_2 and TNF-α levels in the macrophage culture medium were quantified using EIA kits according to the manufacturer's instructions.