

Anti-allergic inflammatory components from *Sanguisorba officinalis* L.Xiang Dong Su^{a,d}, Rui Hong Guo^{b,d}, Hong Xu Li^a, Jin Yeul Ma^c, Young Ran Kim^{b,*}, Young Ho Kim^{a,*}, Seo Young Yang^{a,*}^a College of Pharmacy, Chungnam National University, Daejeon 34134, Republic of Korea^b College of Pharmacy and Research Institute of Drug Development, Chonnam National University, Gwangju 61186, Republic of Korea^c Korean Medicine Application Center, Korea Institute of Oriental Medicine, Daegu 41062, Republic of Korea

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ABSTRACT

Sanguisorba officinalis L. was well known as a traditional herbal medicine to treat inflammation and allergic skin diseases. The aim of this research was to identify compounds with anti-allergic inflammatory property. Twenty-five compounds (**1–25**) were isolated from *S. officinalis* including two new compounds (**1** and **8**), and their chemical structures were identified by NMR and ESIMS analysis. Consequently, the anti-allergic inflammatory activities of these isolates were investigated by inhibiting β-hexosaminidase and IL-4 production in PMA/A23187-stimulated RBL-2H3 cells. Compounds **6**, **8**, **13**, **17–18** and **25** significantly inhibited β-hexosaminidase release and IL-4 production. Additionally, compounds **8**, **17** and **25** effectively suppressed the activation of NF-κB and NF-κB p65 translocation into the nucleus. Anti-inflammatory effects of isolated compounds were evaluated in LPS-stimulated RAW264.7 macrophages, and they showed dramatic inhibition on LPS-induced overproduction of nitric oxide (NO) and TNF-α. Consistently, the protein levels of iNOS and COX-2 were remarkably decreased by the single compounds **8**, **13** and **25**. These results showed that compounds **8**, **13** and **25** from *S. officinalis* may have a therapeutic potential for allergic inflammatory diseases.

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Sanguisorba officinalis L., a member of sub-family Rosoideae and family Rosaceae, is widely distributed in Asia, Western Europe and North America. Previous studies have reported that an ethanol extract of *S. officinalis* significantly inhibited LPS-stimulated nitric oxide (NO) and prostaglandin E₂ (PGE₂) production in RAW264.7 cells and played important roles in the inhibition of methicillin-resistant *Staphylococcus aureus*.^{1,2} Methanol extract of *S. officinalis* exhibited clear antiviral activities.³ Aqueous extract of *S. officinalis* root on immediate-type allergic reactions was investigated through *in vivo* and *in vitro* tests.⁴ However, the anti-allergic inflammatory effects of some single compounds isolated from *S. officinalis* have not been demonstrated.

An allergy occurs in individuals who have formed allergen-specific IgE antibodies following exposure and sensitization to specific allergens, such as pollen, dust, chemicals, variant proteins, or ultraviolet radiation.^{5,6} With the allergy patients increasing annually, approximately 10–20% of people in the world are affected by allergies.⁷ Previous studies have reported that high

serum IgE levels induce the activation of mast cells and cause an allergic reaction. Mast cells aggregate high-affinity IgE receptors (FcεRI) on their surfaces, which is important to the pro-inflammatory allergic response.^{8–10} When an IgE-antigen binds with FcεRI, the receptor is activated, and a variety of biologically active mediators are released, causing allergic reactions, including the release of β-hexosaminidase, a common degranulation marker, histamine and inflammatory cytokines.¹¹ IL-4 is a critical cytokine for switching B cell antibody production to IgE and mast cell development.¹² RBL-2H3 cells originated from rat basophilic leukemia are a mast cell line and have been widely used to study IgE-FcεRI interactions and degranulation. Furthermore, RBL-2H3 cells are a useful model for *in vitro* screening of anti-allergy drug candidates.⁷

Inflammation is the first response of the immune system to infection or irritation. Lipopolysaccharide (LPS) is a principle component of the outer membrane of Gram-negative bacteria, which can activate the inflammatory cells and stimulate the production of inflammatory mediators such as nitric oxide (NO), prostaglandin E₂ (PGE₂), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6.¹³ NF-κB is a ubiquitously expressed family of transcription factors controlling the expression of pro-inflammatory mediators involved in inflammation.¹⁴

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In our efforts to search for natural components with anti-allergy property, two new compounds (**1** and **8**) with twenty-three known compounds (**2–7**, **9–25**) were isolated from *S. officinalis* (Fig. 1). Herein, we describe the isolation and structural determination of two new compounds, and twenty-three known compounds. Afterwards, we investigated the anti-allergic inflammatory effects of all isolated compounds.

Compound **1**¹⁵ was isolated as white needles. The molecular formula was established as C₁₅H₂₀O₁₀ by a quasi-molecular ion peak [M+Na]⁺ at *m/z* 383.0962 (calcd. 383.0949) in the HR-ESI-MS spectrum. The IR spectrum showed characteristic absorption bands of hydroxyl groups (3318 cm⁻¹), a carbonyl group (1696 cm⁻¹), an aromatic moiety (1603 cm⁻¹, 1514 cm⁻¹, 1408 cm⁻¹) and an ether group (1230 cm⁻¹). The ¹H NMR spectrum of compound **1** (Table 1) revealed signals of two aromatic protons at δ_H 7.45 (d, *J* = 1.86 Hz, H-2) and 7.24 (d, *J* = 1.86 Hz, H-6), two methyl groups at δ_H 3.81 (s, 5-OMe) and 3.78 (s, 7-OMe), and one β -glucopyranosyl moiety from an anomeric proton at δ_H 4.73 (d, *J* = 7.26 Hz, H-1'). The ¹³C NMR spectrum of compound **1** (Table 1) showed fifteen carbon signals, which indicated the presence of one sugar unit along with nine carbon signals for an aglycone moiety. By further interpretation of HSQC spectra, nine signals were assigned to two methoxy signals at δ_C 56.0 (5-OMe) and 52.0 (7-OMe), two methine signals at δ_C 111.6 (C-2) and 107.8 (C-6), and five quaternary carbons at δ_C 166.0 (C-7), 147.9 (C-5), 145.3 (C-3), 141.5 (C-4) and 119.5 (C-1), while signals of the sugar unit

were assigned at δ_C 102.7 (C-1'), 73.4 (C-2'), 75.9 (C-3'), 69.7 (C-4'), 77.3 (C-5') and 60.6 (C-6'). Additionally, HMBC correlations (Fig. 2) of δ_H 7.45 (H-2) and 7.24 (H-6) to δ_C 166.0 (C-7) indicated a carbonyl group attached to C-1. The correlations of δ_H 7.24 (H-6) and 3.81 (5-OMe) to δ_C 147.9 (C-5) confirmed the linkage position of a methoxy group to C-5. The key correlation from δ_H 4.73 (H-1') to δ_C 145.3 (C-3) indicated that glucose unit was linked at C-3 position. After acid hydrolysis of compound **1**, the sugar unit was determined to be D-glucose by GC analysis. The larger coupling constant (7.26 Hz) of the anomeric proton indicated the β -configuration of the glucosyl moiety. Thus, the structure of compound **1** was determined to be methyl 3-(β -D-glucopyranosyloxy)-4-hydroxy-5-methoxybenzoate.

Compound **8**¹⁵ was isolated as a yellowish viscous liquid with its molecular formula to be determined as C₂₁H₄₀O₁₁ based on an HR-ESI-MS peak at *m/z* 491.2459 [M+Na]⁺ (calcd for 491.2463). The IR spectrum showed typical absorption bands for hydroxyl groups (3368 cm⁻¹), and a distinctive absorption band for an ether group (1039 cm⁻¹). Acid hydrolysis of compound **8** gave D-glucose and L-arabinose. The ¹H and ¹³C NMR spectrum of compound **8** (Table 1) showed signals of a monoterpene moiety, which contained three methyl groups at δ_H 1.17 (s, H-8 and H-9) and 0.92 (d, *J* = 6.60 Hz, H-10); δ_C 29.4 (C-8), 29.3 (C-9) and 20.2 (C-10), four methylene groups at δ_H 1.68 (m, H-2a) and 1.43 (m, H-2b), 1.33 (m, H-4a) and 1.13 (m, H-4b), 1.42 (m, H-5a) and 1.31 (m, H-5b), 1.44 (m, H-6a) and 1.41 (m, H-6b); δ_C 38.0 (C-2), 38.9 (C-4), 22.8 (C-5),

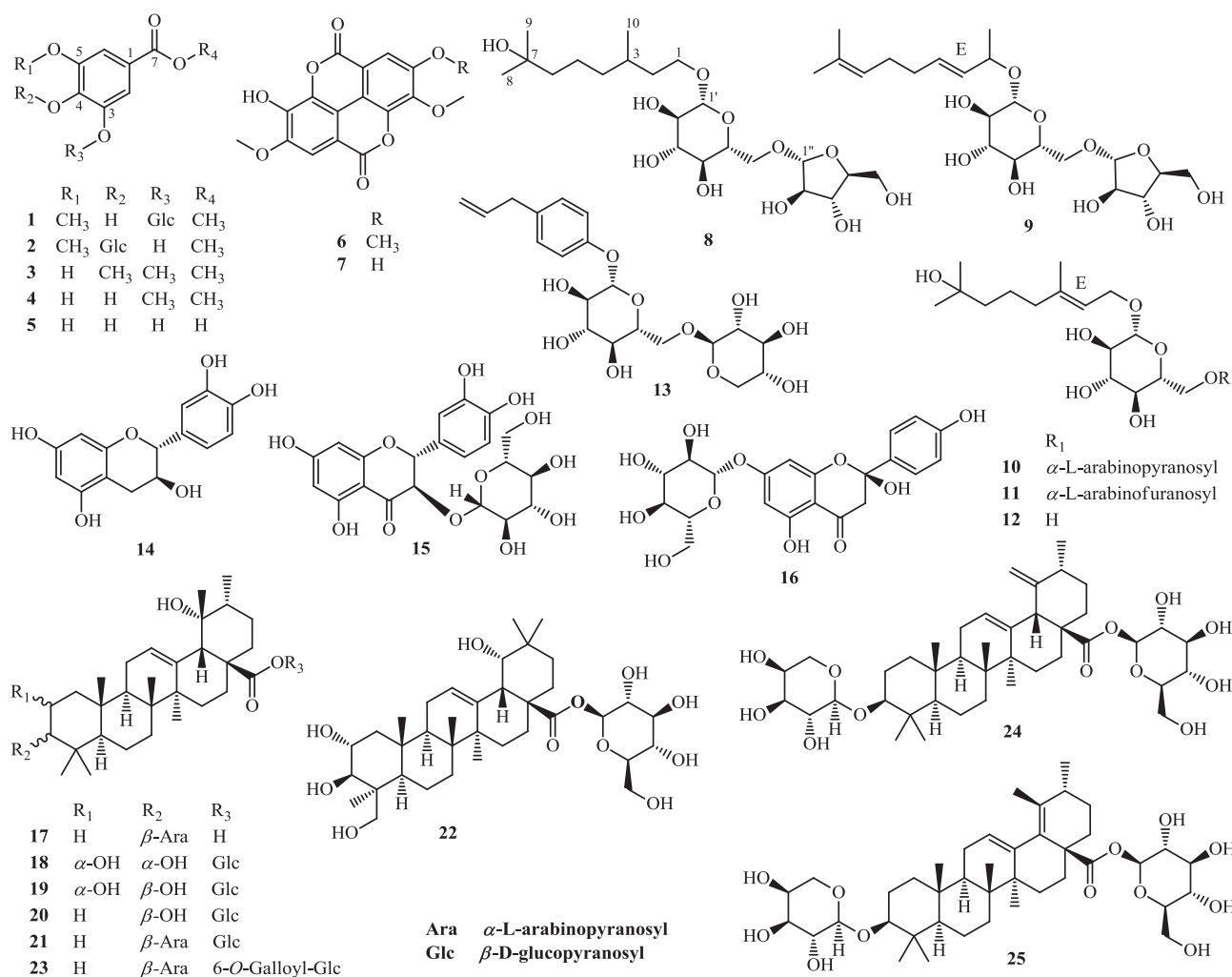


Fig. 1. Structures of compounds **1–25** from *S. officinalis*.

Table 1
The ^1H and ^{13}C NMR spectroscopic data of compounds **1** and **8**.

Position	1 ^a		Position	8 ^b	
	$\delta_{\text{C}}^{\text{d}}$	$\delta_{\text{H}}^{\text{c}}$ (J in Hz)		$\delta_{\text{C}}^{\text{d}}$	$\delta_{\text{H}}^{\text{c}}$ (J in Hz)
1	119.5		1	69.5	3.55 dt (9.42, 7.14) 3.94 m ^e
2	111.6	7.45 d (1.86)	2	38.0	1.68 m ^e , 1.43 m ^e
3	145.3		3	30.9	1.62 m ^e
4	141.5		4	38.9	1.33 m ^e , 1.13 m ^e
5	147.9		5	22.8	1.42 m ^e , 1.31 m ^e
6	107.8	7.24 d (1.86)	6	45.1	1.44 m ^e , 1.41 m ^e
7	166.0		7	71.6	
5-OMe	56.0	3.81 s	8	29.4	1.17 s
7-OMe	52.0	3.78 s	9	29.3	1.17 s
1'	102.7	4.73 d (7.26)	10	20.2	0.92 d (6.60)
2'	73.4	3.31 m ^e	1'	104.6	4.25 d (7.86)
3'	75.9	3.30 m ^e	2'	75.2	3.16 dd (9.12, 7.86)
4'	69.7	3.20 m ^e	3'	78.1	3.35 t (9.12)
5'	77.3	3.29 m ^e	4'	72.0	3.27 t (9.12)
6'	60.6	3.69 d (10.32), 3.51 m ^e	5'	76.7	3.44 m ^e
			6'	68.2	4.02 dd (11.22, 2.40) 3.61 dd (11.22, 6.00) 4.96 d (1.26)
			1''	110.0	4.96 d (1.26)
			2''	83.2	4.00 dd (3.24, 1.26)
			3''	79.0	3.82 dd (5.88, 3.24)
			4''	86.0	3.97 dt (5.88, 3.48)
			5''	63.2	3.73 dd (11.88, 3.48) 3.63 dd (11.88, 3.48)

Assignments were done by HMQC and HMBC experiments; J values (Hz) are in parentheses.

- ^a Measured in DMSO d_6 .
^b Measured in methanol d_4 .
^c 600 MHz.
^d 150 MHz.
^e Overlapped.

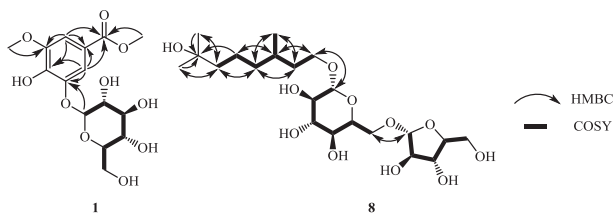


Fig. 2. The key ^1H – ^1H COSY and HMBC correlations of compounds **1** and **8**.

and 45.1 (C-6); a methine group at δ_{H} 1.62 (m, H-3); δ_{C} 30.9 (C-3), an oxygenated methylene at δ_{H} 3.55 (dt, $J = 9.42, 7.14$ Hz, H-1a) and 3.94 (m, H-1b); δ_{C} 69.5 (C-1) and an oxygenated quaternary carbon at δ_{C} 71.6 (C-7). The ^1H – ^1H COSY spectrum (Fig. 2), correlations between the oxygenated methylene group (H-1) and methylene group (H-2), together with the spin system from H-2 through a methine group H-3 to H-6 and continuing to H-10, established the segment of $-\text{OCH}_2(1)-\text{CH}_2-\text{CH}(3)-[\text{CH}_3(10)]-\text{CH}_2(4)-\text{CH}_2(5)-\text{CH}_2(6)$. Furthermore, the HMBC correlations (Fig. 2) of δ_{H} 0.92 (H-10) to δ_{C} 38.0 (C-2) and 38.9 (C-4) indicated a methyl group was located at C-3, while the correlations between δ_{H} 1.17 (H-8/H-9) to δ_{C} 71.6 (C-7) and 45.1 (C-6) implied that two methyl groups were attached to C-7. Based on both 1D-NMR and 2D-NMR data, the monoterpene moiety was identified as hydroxycitronellol.³¹ The coupling constant of the anomeric proton at δ_{H} 4.25 (d, $J = 7.86$ Hz, H-1') indicated the β -configuration of glucosyl moiety. The HMBC correlations of δ_{H} 4.25 (H-1') to δ_{C} 69.5 (C-1) and δ_{H} 3.55 (H-1a) and 3.94 (H-1b) to δ_{C} 104.6 (C-1') confirmed the glucose was connected to C-1 of the aglycone. Moreover, the HMBC correlations of δ_{H} 4.02 (H-6'a) and 3.61 (H-6'b) to δ_{C} 110.0 (C-1''), and δ_{H} 4.96 (H-1'') to δ_{C} 68.2 (C-6') implied the arabinose was linked to C-6' of the glucose, which was further confirmed by the downfield

shift by about 5.0 ppm of the signal of δ_{C} 68.2 (C-6'). The coupling constant of the anomeric proton at δ_{H} 4.96 (d, $J = 1.26$ Hz, H-1''), as well as signal at δ_{C} 86.0 (C-4'') and 83.2 (C-2'') indicated that arabinose moiety was an α -arabinofuranose structure.³¹ Therefore, the structure of compound **8** was elucidated as 7-hydroxy-3,7-dimethyloctyl-6-O- α -L-arabinofuranosyl- β -D-glucopyranoside.

Together with the two new compounds (**1** and **8**), twenty-three other compounds were identified as methyl 4-(β -D-glucopyranosyloxy)-3-hydroxy-5-methoxybenzoate (**2**),¹⁶ methyl 3,4-O-dimethylgallate (**3**),¹⁷ methyl 3-methoxy-4,5-dihydroxybenzoate (**4**),¹⁷ gallic acid (**5**),¹⁸ 3,3',4-tri-O-methylellagic acid (**6**),¹⁹ 3,4'-di-O-methylellagic acid (**7**),¹⁹ geranyl 6-O- α -L-arabinofuranosyl- β -D-glucopyranoside (**9**),²⁰ rhodiolide E (**10**),²¹ (2E)-7-hydroxy-3,7-dimethyl-2-octenyl 6-O- α -L-arabinofuranosyl- β -D-glucopyranoside (**11**),²² (2E)-7-hydroxy-3,7-dimethyl-2-octenyl- β -D-glucopyranoside (**12**),²³ miyaginidin (**13**),²⁴ (+)-catechin (**14**),¹⁸ taxifolin 3-O- β -D-glucopyranoside (**15**),²⁵ (2S)-hydroxynaringenin-7-O- β -D-glucopyranoside (**16**),²⁶ ziyuglycoside II (**17**),¹⁹ kaji-ichigoside F₁ (**18**),²⁷ rosamultin (**19**),²⁷ 28-O- β -D-glucopyranosyl pomolic acid (**20**),¹⁹ ziyuglycoside I (**21**),¹⁹ sericoside (**22**),²⁸ 3 β -[(α -L-arabinopyranosyl)oxy]-19 α -hydroxyurs-12-en-28-oic acid 28-(6-O-galloyl- β -D-glucopyranosyl) ester (**23**),²⁹ 3 β -[(α -L-arabinopyranosyl)oxy]urs-12,19(29)-dien-28-oic acid β -D-glucopyranosyl ester (**24**),²⁹ 3 β -[(α -L-arabinopyranosyl)oxy]urs-12,18-dien-28-oic acid β -D-glucopyranosyl ester (**25**).³⁰ Their structures were elucidated by spectroscopic data, chemical evidence, and comparisons with previous reports (Fig. 1).

To study the anti-allergic inflammatory activity, the effects of isolated compounds on cell viability were evaluated by MTS to ensure that the decreased level of mast cell granules and IL-4 production in RBL-2H3 cells was not due to the cell death in each condition. Treatment with test samples for 24 h at the concentrations of 20 μM and 5 μM produced no significant cytotoxic effects except compound **6**, which showed the significant cytotoxic to RBL-2H3

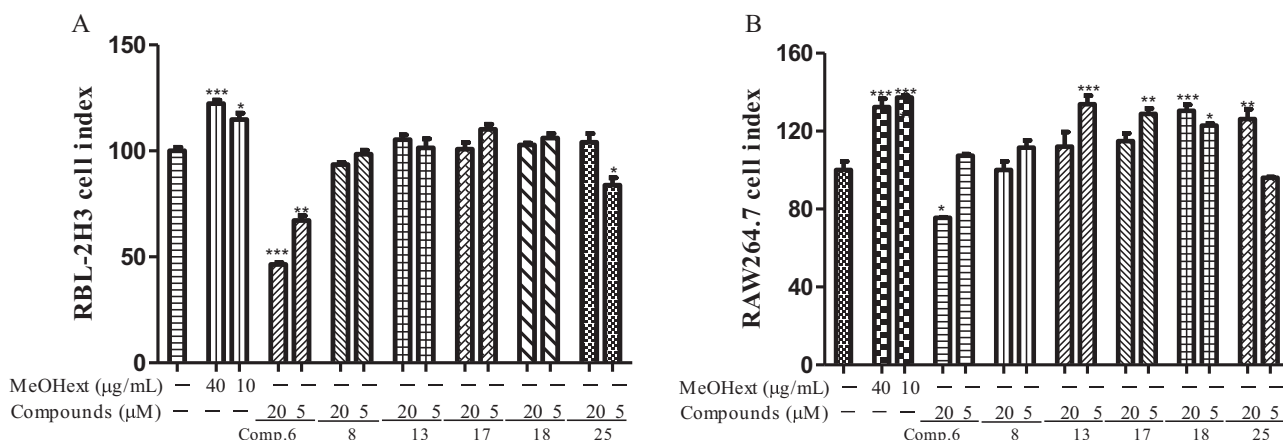


Fig. 3. Effect on the viability of RBL-2H3 cells and RAW264.7 cells; (A) RBL-2H3 cells seeded into 96-well plate overnight were treated with samples for 24 h, and the cell viability was tested by MTS assays. (B) RAW264.7 cells were treated with samples for 24 h, and cell viability was assayed by MTS assays. The data are representative of three independent experiments and expressed as mean $\pm$ SEM (* P < 0.05; ** P < 0.01; *** P < 0.001 versus with control group).

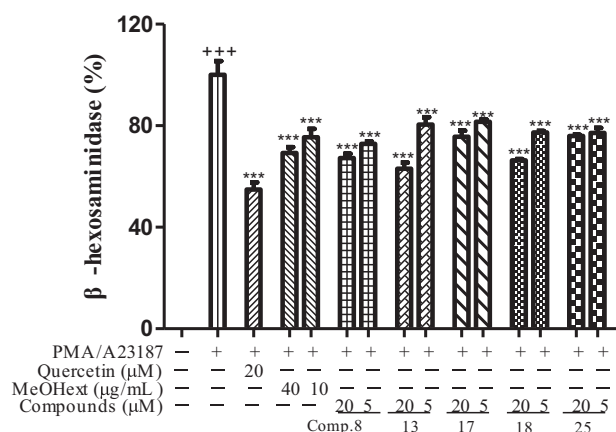


Fig. 4. Effect on β -hexosaminidase secretion in RBL-2H3 cells; RBL-2H3 cells incubated into 48-well plate overnight were pretreated with samples for 1 h prior to stimulation with 30 ng/mL of PMA plus 350 ng/mL of A23187 for 1 h. The supernatants were collected for the detection of β -hexosaminidase. Experiments were conducted in triplicate and expressed as mean $\pm$ SEM (*** P < 0.001 versus with PMA/A23187 group).

cells as compared with the control (Fig. 3A). Test samples also showed no cellular toxicity against RAW264.7 cells except compound 6 which showed little toxicity to cells (Fig. 3B).

Mast cells contain a large amount of cytoplasmic granules, which activation caused the process of degranulation.³² The enzyme β -hexosaminidase is stored in the secretory granules of mast cells and is released along with chemical mediators when mast cells are stimulated. The release of β -hexosaminidase can be used to quantify the extent of degranulation.³³ Twenty-five isolated compounds were tested on the β -hexosaminidase assay to determine whether these compounds regulate the degranulation of mast cells. Results showed that methanol extract and five compounds (8, 13, 17–18 and 25) significantly reduced the β -hexosaminidase level released from the PMA/A23187-stimulated RBL-2H3 cells (Fig. 4).

Modulation of inflammatory cytokines from mast cells is one of the key indicators of reduced allergic symptoms.³⁴ We examined whether five compounds (8, 13, 17–18 and 25) could regulate pro-inflammatory cytokines IL-4 in RBL-2H3 cells. Treatment with samples dose-dependently blocked IL-4 secretion induced by DNP-BSA or PMA/A23187 in RBL-2H3 cells (Fig. 5A and B).

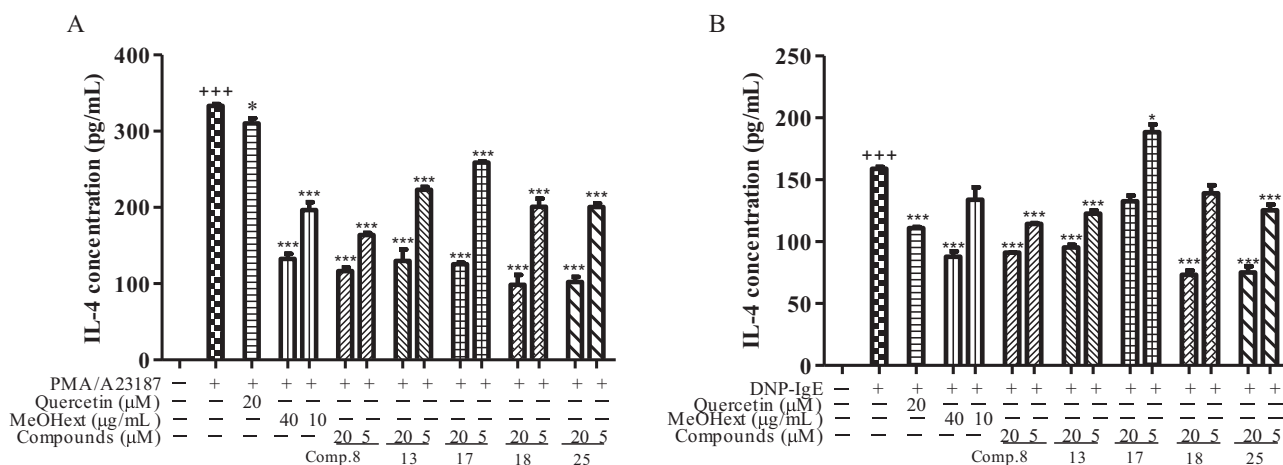


Fig. 5. Effect on IL-4 production in RBL-2H3 cells; (A) RBL-2H3 cells incubated into 48-well plate overnight were pretreated with samples for 2 h prior to stimulation with 30 ng/mL of PMA plus 350 ng/mL of A23187 for 18 h. The supernatants were collected and IL-4 cytokine was tested by using ELISA kit. Experiments were conducted in triplicate and expressed as mean $\pm$ SEM (*** P < 0.001 versus with PMA/A23187 group). (B) RBL-2H3 cells were sensitized with IgE overnight at 37 °C, followed by pretreatment with drug for 2 h before stimulation with 1 μg/mL DNP-BSA for 18 h. The supernatants were analyzed for IL-4 production (* P < 0.05; ** P < 0.01; *** P < 0.001 versus with DNP-BSA group).

NF- κ B is a transcription factor related to inflammation. The effects on NF- κ B activation was tested in 293T cells. PMA increased NF- κ B transcription, which was decreased by methanol extract, and compounds (**8**, **17** and **25**) pretreatment in a dose-dependent manner. Methanol extract (40 μ g/mL) and compounds **8**, **17** and **25** (20 μ M) showed the significant reduction on the activation of NF- κ B transcription (Fig. 6A). Additionally, RAW264.7 cells were pretreated with samples or Bay11-7082 (20 μ M) for 2 h prior to addition of LPS (1 μ g/mL). The effect of methanol extract, and compounds (**8**, **13**, **17**–**18** and **25**) on LPS-induced NF- κ B pathway was tested. LPS caused the translocation of NF- κ B from the cytosol to the nucleus, which was inhibited by the methanol extract, and compounds (**8**, **17** and **25**) (Fig. 6B).

RAW264.7 cells were pretreated with samples for 2 h prior to addition of LPS (500 ng/mL). TNF- α cytokine was assayed using ELISA kit. Methanol extract (40 μ g/mL) and compounds **8**, **13**, **17**–**18** and **25** (20 μ M) decreased the production of TNF- α in LPS-activated RAW264.7 cells, especially compounds **8** and **17** significantly decreased the production of TNF- α (Fig. 7A). NO production in LPS-activated RAW264.7 cells was also decreased by the treatment of

samples, especially the methanol extract (40 μ g/mL) and compounds **8**, **13**, **17** and **25** (20 μ M) showed the significant decrease on NO production (Fig. 7B).

Pro-inflammatory enzymes, such as iNOS and COX-2, cause inflammation. Thus, we also examined whether isolated compounds could affect iNOS and COX-2 protein expressions by Western blot analysis. RAW264.7 cells were pretreated with test samples or celecoxib (20 μ M) for 2 h prior to addition of LPS (200 ng/mL). Stimulation of the RAW264.7 cells with LPS resulted in accumulation of iNOS and COX-2 proteins, as determined by Western blot analysis. Compounds **8**, **13** and **25** significantly reduced the levels of iNOS protein at the concentration of 20 μ M. Celecoxib (20 μ M), methanol extract (40 μ g/mL), compounds **8**, **13** and **25** (20 μ M) reduced the levels of COX-2 protein expression in LPS-activated RAW264.7 cells (Fig. 8).

Modern research has shown that *Sanguisorba* plants exhibit a wide range of pharmacological activities, such as hemostatic, anti-inflammatory, anti-allergy, antibacterial, anti-oxidant, hypoglycemic, neuroprotective and anticancer effects.³⁵ The present study showed that methanol extract and single compounds **6**, **8**,

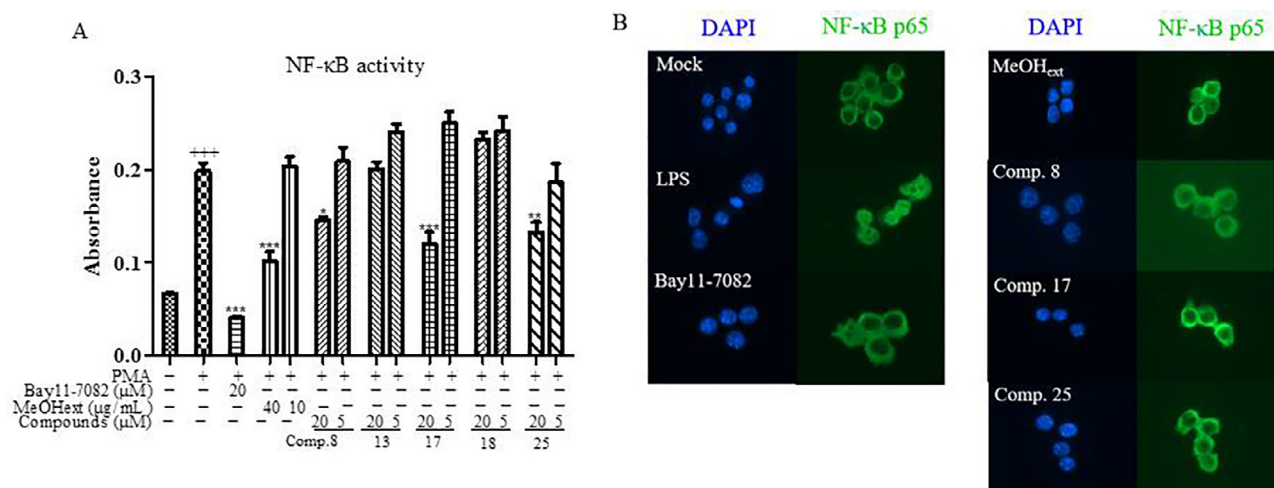


Fig. 6. Effect on NF- κ B transcription and translocation; (A) Effect on PMA-induced NF- κ B activation in 293T cells. The 293T cells were transfected with a reporter plasmid, pNF- κ B-SEAP. Experiments were conducted in triplicate and expressed as mean $\pm$ SEM ($^{*}P < 0.01$; $^{***}P < 0.001$ versus with PMA group). (B) Effect on NF- κ B translocation into nucleus. RAW264.7 cells in eight-well glass chamber plate were pre-incubated with samples or Bay 11-7082 for 2 h prior to addition of LPS (1 μ g/mL). The cells were immunostained with a polyclonal anti- NF- κ B p65 antibody and Alexa Fluor 488-conjugated 2nd antibody. Bay 11-7082 was used as a control for the NF- κ B inhibitor.

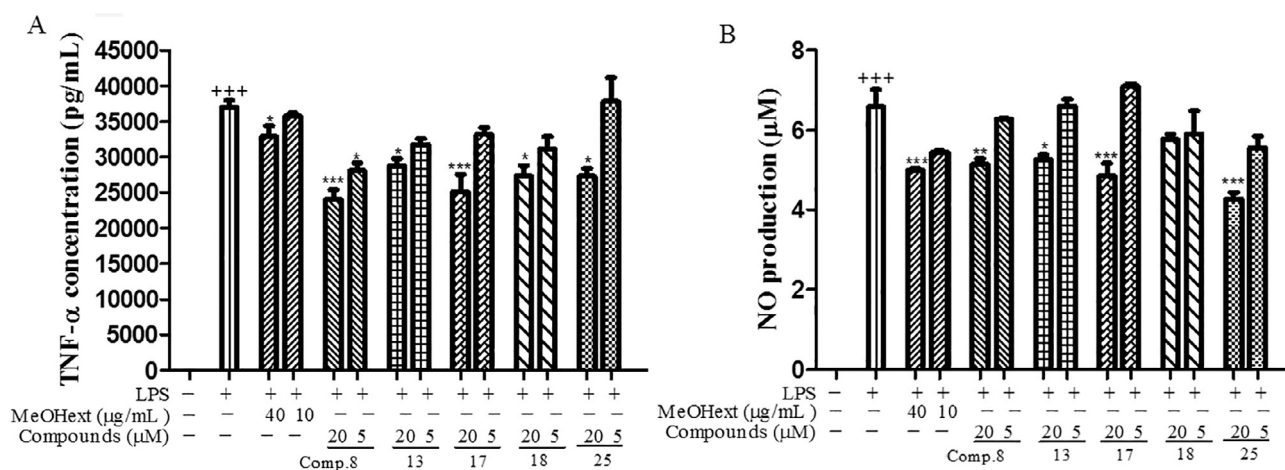


Fig. 7. Effect on the production of inflammatory mediators in LPS-activated RAW264.7 cells; RAW264.7 cells were pretreated with samples for 2 h prior to addition of LPS for 24 h. TNF- α in the supernatants was measured by ELISA (A) and NO in the supernatants was detected by Griess reagent (B). Experiments were conducted in triplicate and expressed as mean $\pm$ SEM ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus with LPS group).

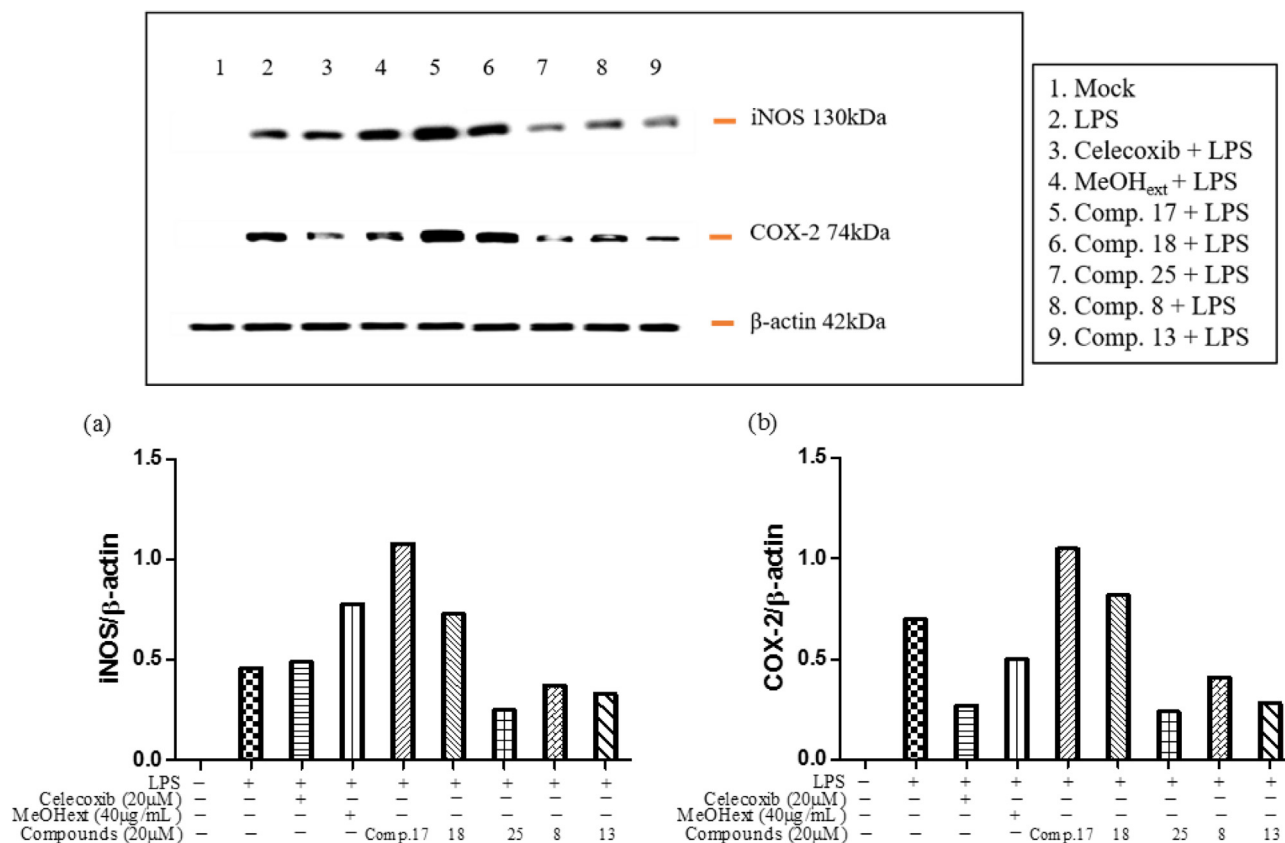


Fig. 8. Effect on the expression of COX-2 and iNOS in LPS-activated RAW264.7 cells; RAW264.7 cells were pretreated with samples for 2 h, and then LPS was added to the cells for 18 h. The protein levels of iNOS and COX-2 were detected by Western blot analysis. Celecoxib was used as a control for the COX-2 inhibitor, β-actin was used as the control protein.

13, 17–18 and **25** isolated from *S. officinalis* profoundly affected PMA plus A23187-induced systemic allergic reaction and anti-DNP IgE-induced local allergic reaction. Methanol extract and single compounds **6, 8, 13, 17–18** and **25** inhibited PMA plus A23187-mediated β-hexosaminidase release and PMA plus A23187 or anti-DNP IgE-mediated cytokine IL-4 production. But Compound **6** showed the cytotoxicity to RBL-2H3 cells after 24 h treatment, that's why compound **6** looked like having the effects on the reduction of β-hexosaminidase release and cytokine IL-4 production. During the pathogenesis of allergic disease, IL-4 is crucial for the induction of IgE synthesis and mast cell development.¹² Therefore, we simply speculate that anaphylactic degranulation of mast cells is suppressed by methanol extract and single compounds **8, 13, 17–18** and **25**.

NF-κB participates in regulating the expression of cytokines that are involved in the inflammatory response.³⁶ Inhibition of this signaling pathway may explain the potent activity of isolated compounds as suppressors of inflammatory cytokines. In this study, methanol extract and single compounds **8, 17** and **25** significantly suppressed the activation of NF-κB P65 and NF-κB translocation into the nucleus (Fig. 6), indicating that NF-κB pathway played a significant role in the anti-inflammatory effects of isolated compounds.

Macrophages play an important role in the specific and non-specific immune responses during the inflammation process. After macrophages are activated by LPS, large amounts of the inflammatory mediators will be released.³⁷ Therefore, LPS induced macrophages have usually been used for assessing the anti-inflammatory effects of various agents. We investigated the effects

of isolated compounds on the inflammatory mediators of LPS-stimulated RAW264.7 macrophage cells. We found that pretreatment with tested compounds significantly inhibited the production of NO and TNF-α induced by LPS (Fig. 7). NO is a free radical produced from L-arginine by nitric oxide synthases (NOS), the high level of NO might cause inflammatory damage to a target tissue during an infection.^{38–40} Therefore, the regulation of NO release via inhibiting iNOS is helpful to alleviate the inflammatory destruction. COX-2 is an inducible isoform of cyclooxygenase, and mainly exerts its important role in the inflammation.^{41,42} We also found that compounds **8, 13** and **25** significantly decreased iNOS and COX-2 expression levels in RAW264.7 cells induced by LPS (Fig. 8).

In summary, our findings demonstrated that *S. officinalis* especially compounds **8, 13** and **25** are potential therapeutic candidates for anti-allergic inflammatory drugs.

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A. Supplementary data

Supplementary data (¹H, ¹³C NMR, HMQC, HMBC, COSY, and HR-ESI-MS spectra of compounds **1** and **8**; general experimental procedures, bio-assay) associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.bmcl.2018.04.033>.

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15. *Physical and spectroscopic data of new compounds: Methyl 3-(β-D-glucopyranosyloxy)-4-hydroxy-5-methoxybenzoate (1):* White needles; $[\alpha]_{D}^{20}$ -40.6° ($c = 0.1$, MeOH); IR (KBr): $\nu_{\max}$ 3318, 1696, 1603, 1514, 1408, 1230 cm^{-1} ; ^1H NMR (DMSO- d_6 , 600 MHz) and ^{13}C NMR data (DMSO- d_6 , 150 MHz), see Table 1; HR-ESI-MS: m/z 383.0962 $[\text{M}+\text{Na}]^+$ (calcd. for 383.0949, $\text{C}_{15}\text{H}_{20}\text{O}_{10}\text{Na}$). *7-Hydroxy-3,7-dimethyloctyl-6-O-α-L-arabinofuranosyl-β-D-glucopyranoside (8):* Yellowish viscous liquid; $[\alpha]_{D}^{20}$ -79.0° ($c = 0.1$, MeOH); IR (KBr): $\nu_{\max}$ 3368, 1039 cm^{-1} ; ^1H NMR (CD_3OD , 600 MHz) and ^{13}C NMR data (CD_3OD , 150 MHz), see Table 1; HR-ESI-MS: m/z 491.2459 $[\text{M}+\text{Na}]^+$ (calcd. for 491.2463, $\text{C}_{21}\text{H}_{40}\text{O}_{11}\text{Na}$).
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Supplementary material

Anti-allergic inflammatory components from *Sanguisorba officinalis* L.

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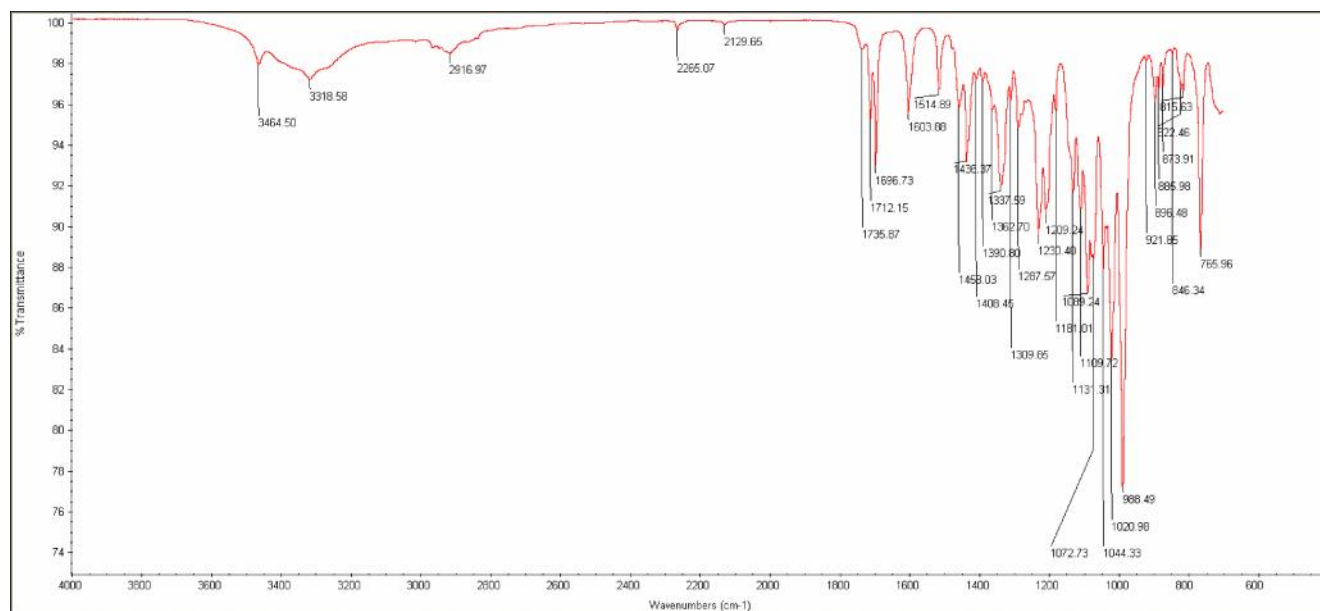


Figure S1. IR spectrum of compound **1**

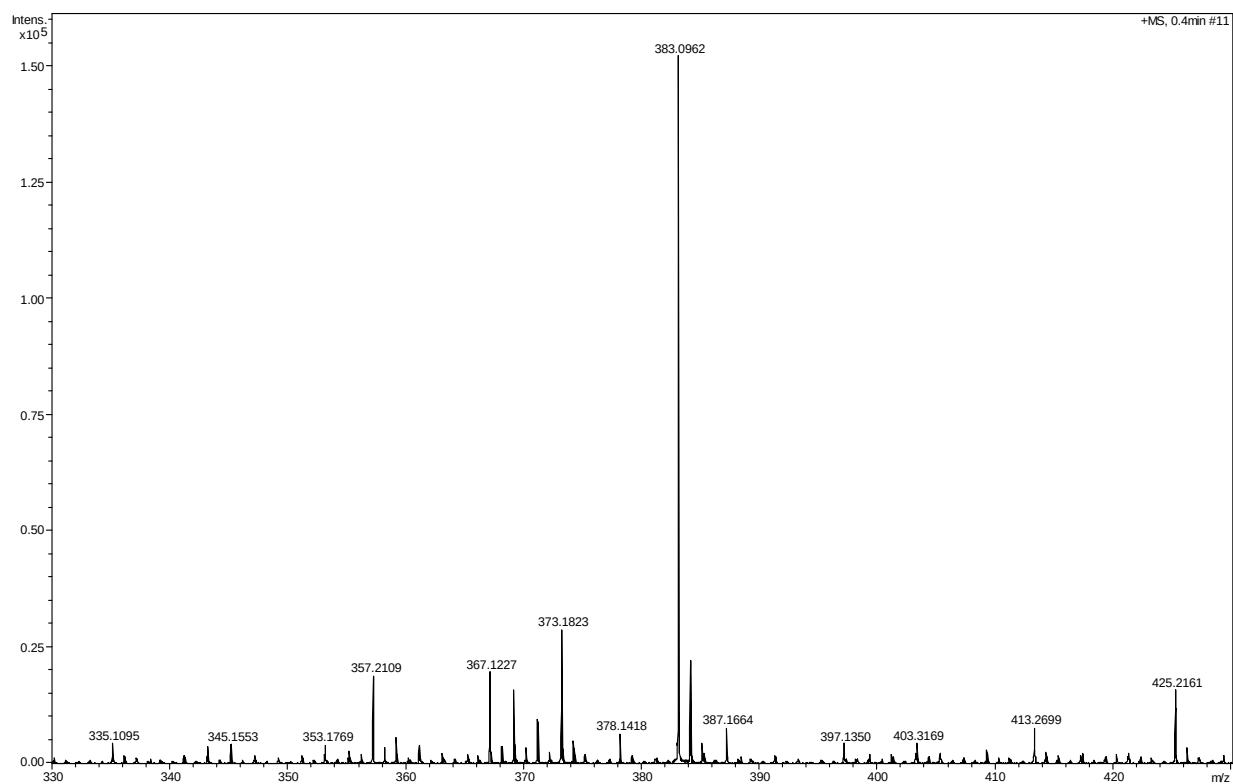


Figure S2. HR-ESI-MS spectrum of compound **1**

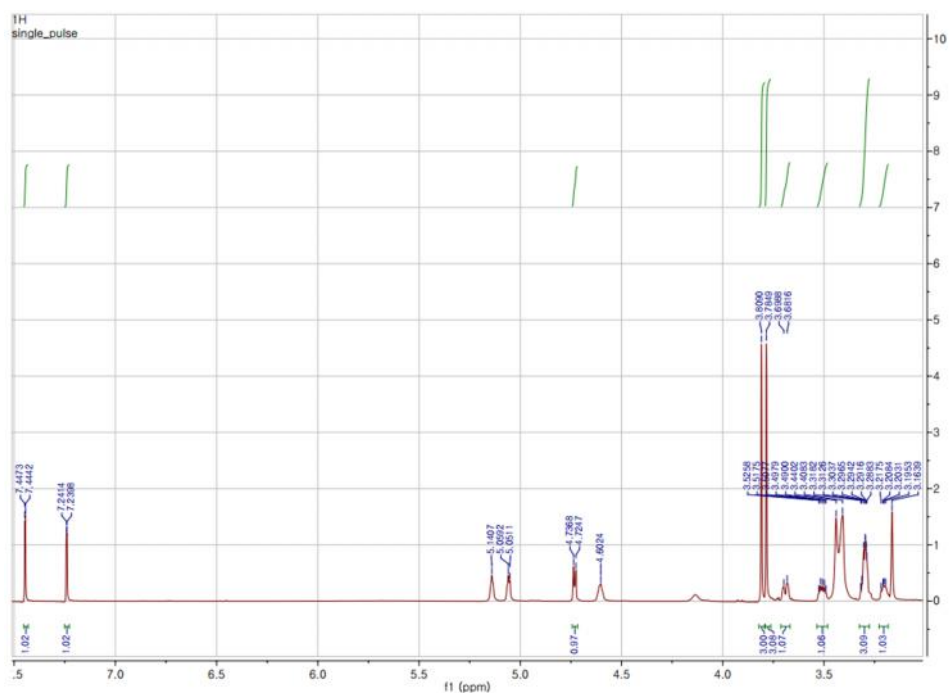


Figure S3. ¹H-NMR spectrum of compound **1** in DMSO-*d*₆ (600 MHz)

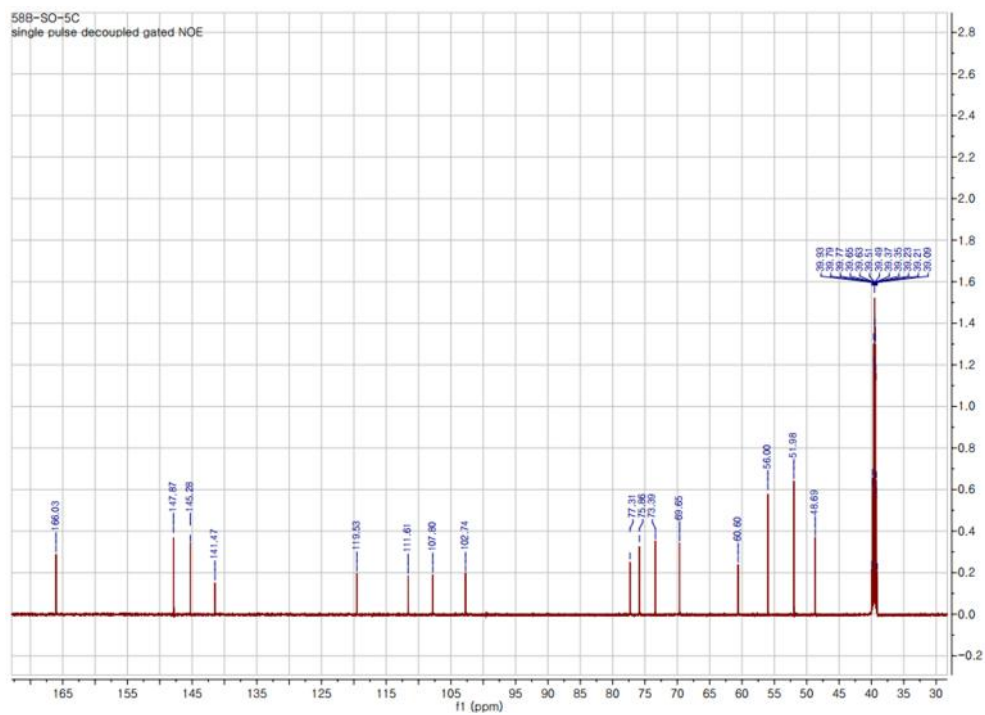


Figure S4. ¹³C-NMR spectrum of compound **1** in DMSO-*d*₆ (150 MHz)

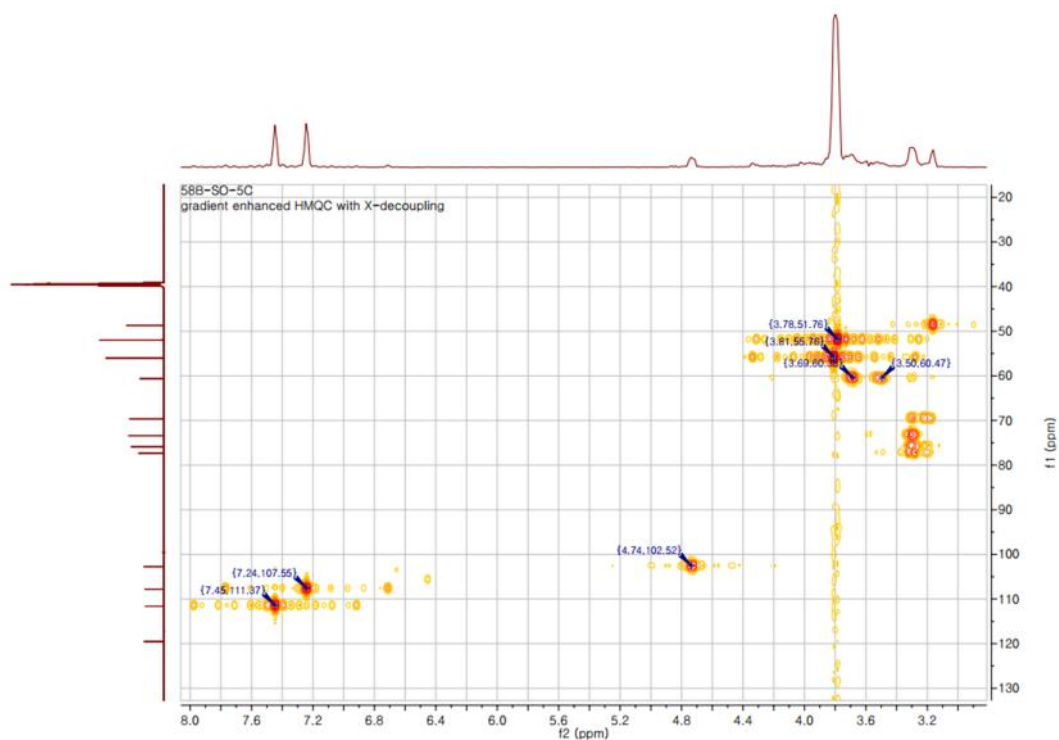


Figure S5. HSQC spectrum of compound **1** DMSO-*d*₆ (600 MHz)

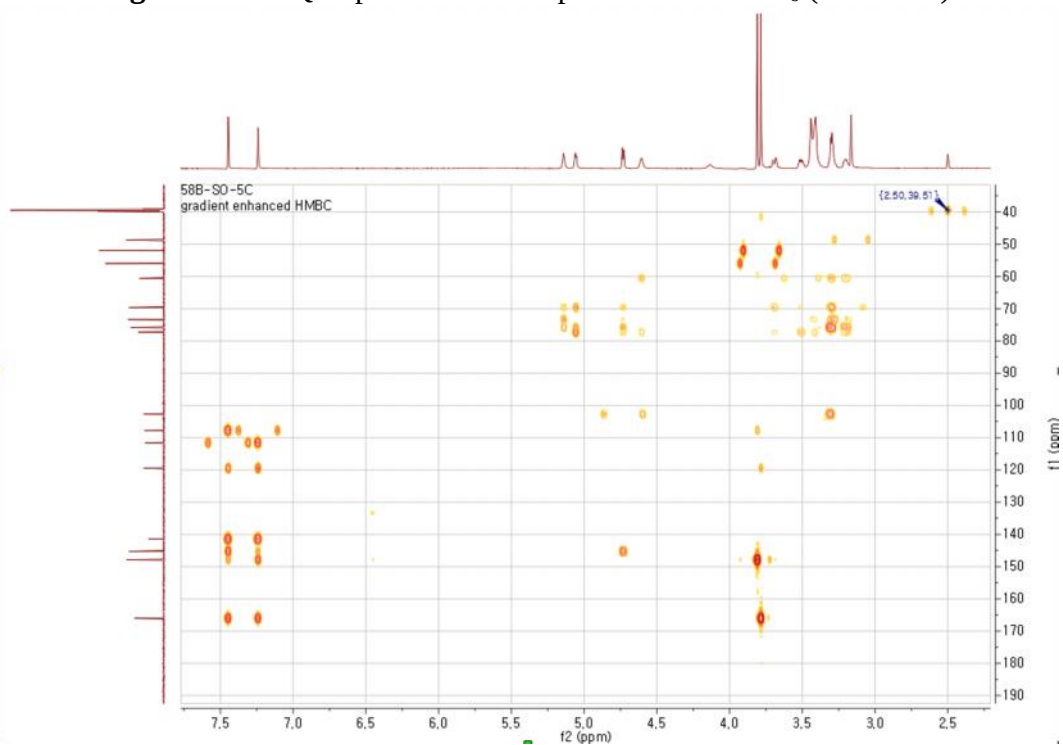


Figure S6. HMBC spectrum of compound **1** in DMSO-*d*₆ (600 MHz)

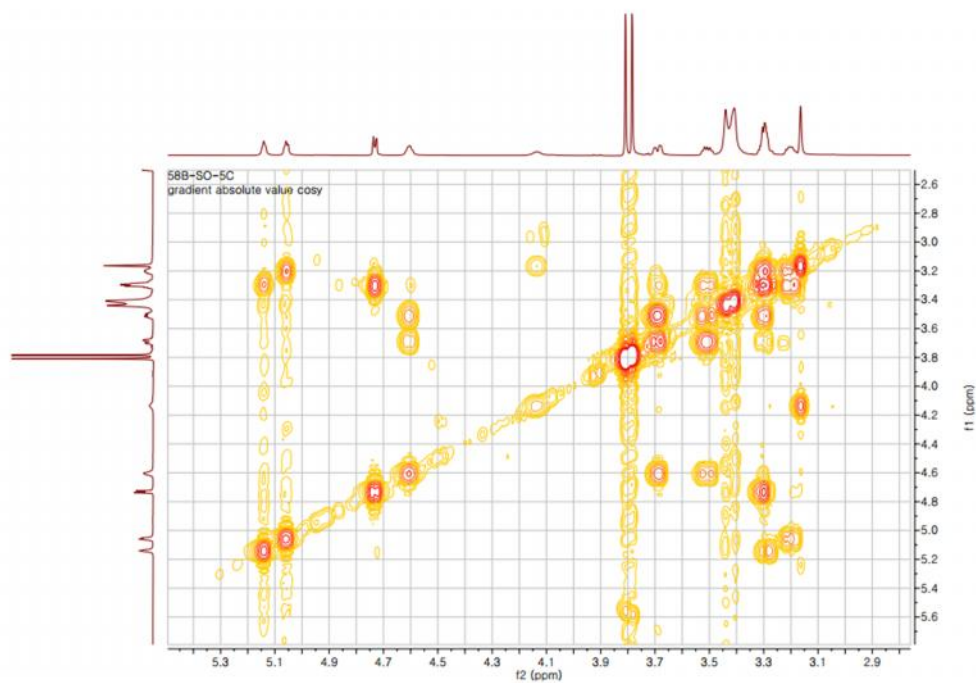


Figure S7. COSY spectrum of compound **1** in DMSO- d_6 (600 MHz)

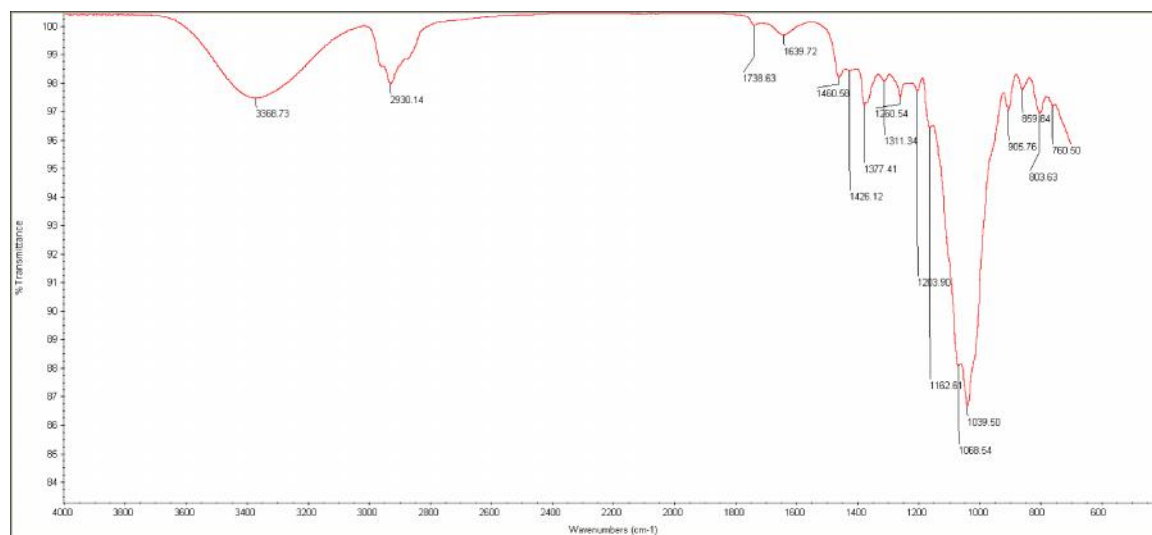


Figure S8. IR spectrum of compound **8**

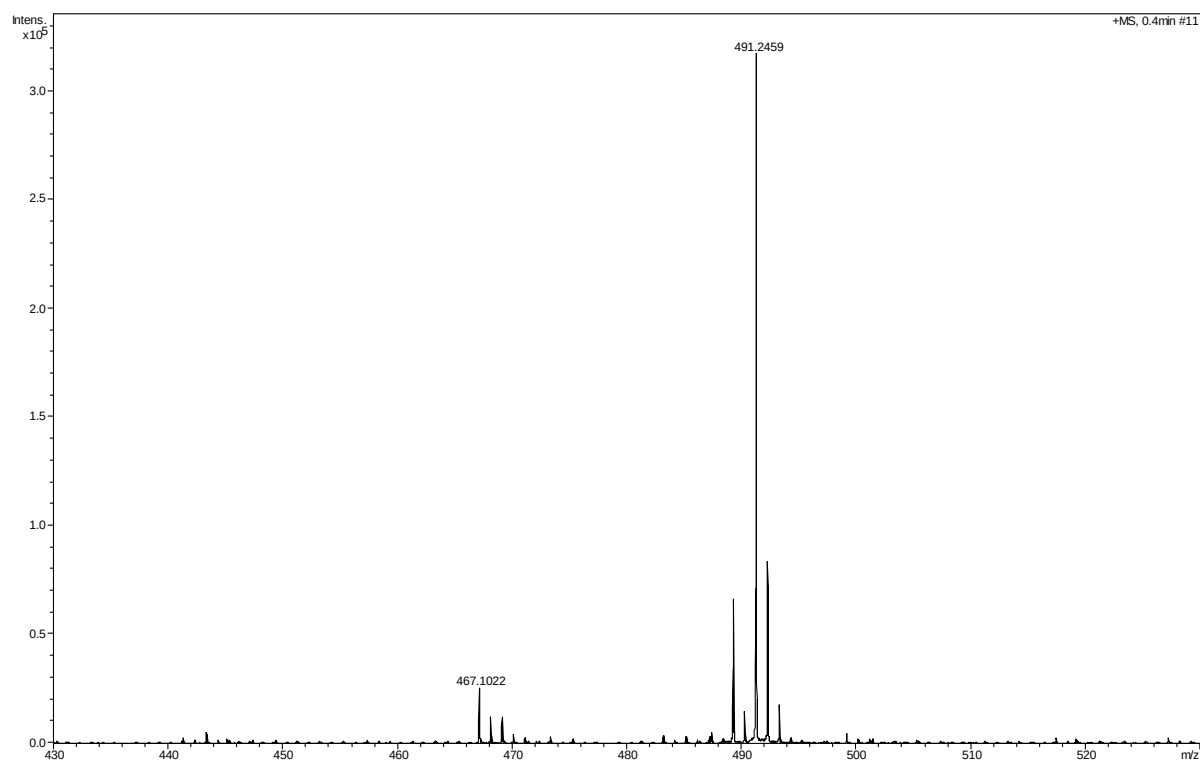


Figure S9. HR-ESI-MS spectrum of compound **8**



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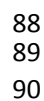


Figure S11. ^{13}C -NMR spectrum of compound **8** in Methanol- d_4 (150 MHz)

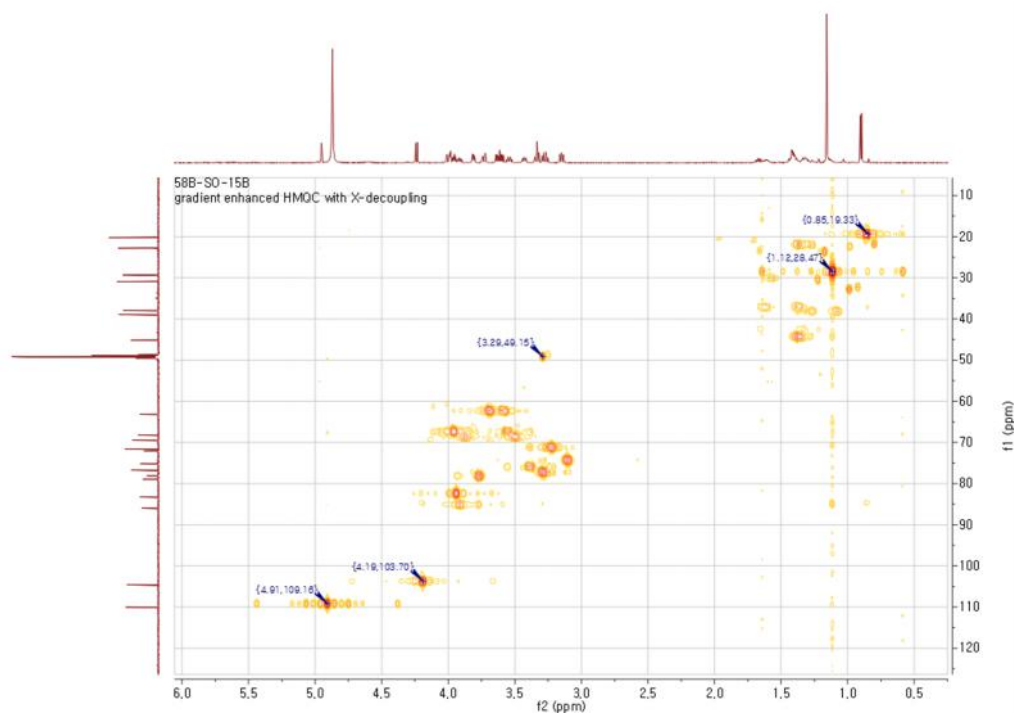


Figure S12. HSQC spectrum of compound **8** in Methanol- d_4 (600 MHz)

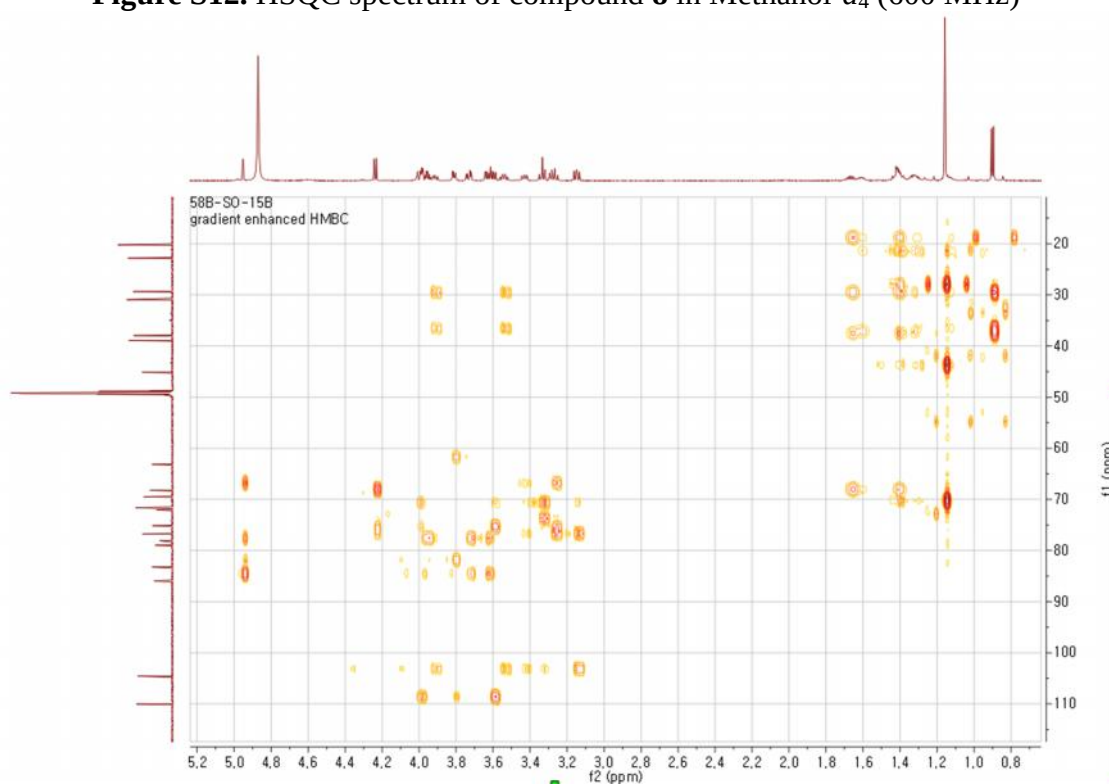


Figure S13. HMBC spectrum of compound **8** in Methanol- d_4 (600 MHz)

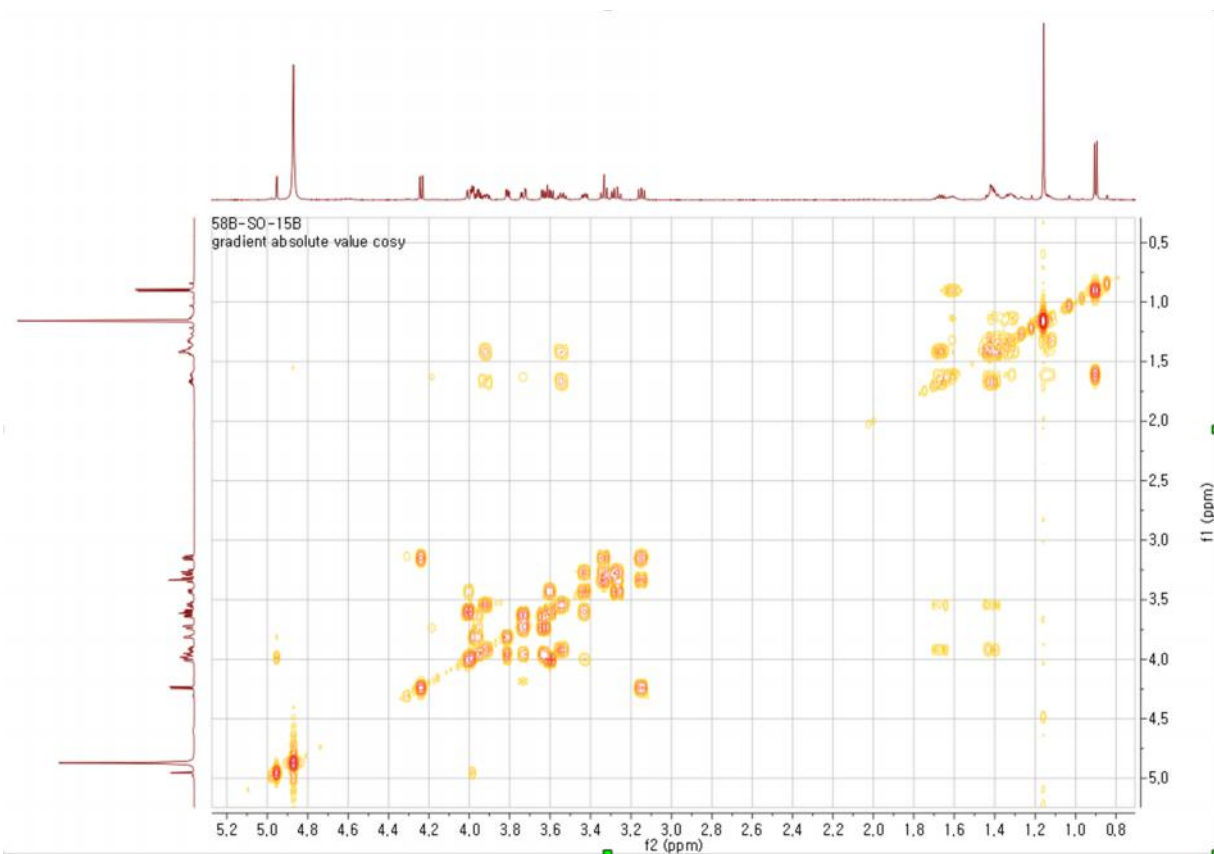


Figure S14. COSY spectrum of compound **8** in Methanol- d_4 (600 MHz)

General experimental procedures

Column chromatography (CC) was performed by using silica gel (SiO₂; 70-230, 230-400 μm particles size; *Fuji Silysia Chemical Ltd., Kasugai, Japan*) and YMC RP-18 resins (75 μm, *Fuji Silysia Chemical Ltd., Kasugai, Japan*). Thin-layer chromatography (TLC) was performed by using pre-coated silica gel 60 F₂₅₄ and reversed phase (RP)-18 F_{254S} plates (*Merck, Darmstadt, Germany*). The NMR spectra were recorded by using a JEOL ECA 600 spectrometer (¹H, 600 MHz; ¹³C, 150 MHz; JEOL Ltd, Tokyo, Japan), δ in ppm relative to Me₄Si as an internal standard and *J* in Hz. High-resolution electrospray ionization mass spectra (HR-ESI-MS) were obtained using an Agilent 6530 accurate-mass quantitation-time of flight-liquid Chromatography/mass spectrometry (Q-TOF LC/MS). The FT-IR spectra were conducted by using Nicolet 380 FT-IR spectrometer; KBr pellets; $\tilde{\nu}$ in cm⁻¹.

Plant material

Dried roots of *S. officinalis* were provided by Korean Medicine Application Center, Korea Institute of Oriental Medicine, and taxonomically identified by one of the authors (Prof. Young Ho Kim). A voucher specimen (CNU 14115) was deposited at Herbarium of the College of Pharmacy, Chungnam National University.

Extraction and isolation

Dried crushed roots (2.4 kg) of *S. officinalis* were extracted with MeOH (8.0 L × 4 times) under reflux. The MeOH extract (1.0 kg) was suspended in water and partitioned with EtOAc and *n*-BuOH, yielding EtOAc (178.0 g) and *n*-BuOH (141.5 g) fractions.

The EtOAc fraction (178.0 g) was subjected to silica gel (5.0 × 30 cm) column chromatography

136 with a gradient of CHCl₃-MeOH-H₂O (10:1:0, 7:1:0.1, 5:1:0.1, 3:1:0.1, 1:1:0.1, 1:2:0.1, 1:4:0.1;
137 4.0 L for each steps) to give 12 fractions (Fr. 1A-1L). Fraction 1A (775.3 mg) was separated
138 using silica gel (1.0 × 60 cm) column chromatography with a gradient of CHCl₃-EtOAc (30:1,
139 20:1, 10:1; 1.0 L for each steps) to obtain compound **6** (68.0 mg). Fraction 1C (2.2 g) was
140 isolated using a silica gel (2.0 × 80 cm) column chromatography with a gradient of CHCl₃-
141 acetone (25:1, 20:1, 15:1, 5:1, 1:1; 2.5 L for each steps) to give compounds **4** (70.0 mg), **7** (10.0
142 mg) and **17** (8.0 mg). Fraction 1G (1.9 g) was separated using YMC (2.0 × 80 cm) column
143 chromatography with a MeOH-H₂O (3:1, 5:1; 2.0 L for each steps) elution solvent to obtain
144 compounds **5** (120.0 mg), **14** (100.0 mg), **18** (10.0 mg), **19** (7.0 mg) and **20** (20.0 mg). Fraction
145 1I (25.0 g) was subjected to silica gel (2.5 × 40 cm) column chromatography with a gradient of
146 CHCl₃-MeOH-H₂O (10:1:0, 7:1:0.1, 5:1:0.1, 1:1:0.1; 3.0 L for each steps) to give compounds **21**
147 (2.8 g), **22** (11.0 mg), **24** (20.0 mg) and **25** (20.0 mg). Fraction 1J (8.0 g) was subjected to silica
148 gel (2.0 × 80 cm) column chromatography with a gradient of CHCl₃-MeOH-H₂O (5:1:0.1,
149 3:1:0.1, 1:1:0.1; 1.5 L for each steps) to give compound **23** (16.0 mg).

150 The *n*-BuOH fraction (141.5 g) was subjected to silica gel (5 × 30 cm) column chromatography
151 with a gradient of CHCl₃-MeOH-H₂O (6:1:0.1, 3:1:0.1, 2:1:0.1, 1:1:0.1; 6.0 L for each steps) to
152 give 8 fractions (Fr. 2A-2H). Fraction 2A (865.0 mg) was separated using YMC (1.0 × 80 cm)
153 column chromatography with a MeOH-H₂O (1:2, 1:1; 1.0 L for each steps) elution solvent to
154 obtain compound **3** (50.0 mg). Fraction 2C (14.0 g) was subjected to silica gel (2.0 × 60 cm)
155 column chromatography with a gradient of CHCl₃-MeOH (15:1, 10:1, 5:1, 2:1, 1:1; 1.0 L for
156 each steps) to give compounds **1** (5.4 mg) and **2** (4.0 mg). Fraction 2G (3.0 g) was separated
157 using YMC (2.0 × 80 cm) column chromatography with a MeOH-H₂O (1:4, 1:2, 1:1; 1.0 L for
158 each steps) elution solvent to yield compound **9** (100.0 mg). The fraction 2E (18.0 g) was

separated using silica gel (2.5 × 60 cm) column chromatography with a gradient of CHCl₃-MeOH-H₂O (5:1:0, 3:1:0.1, 1:1:0.1, 1.0 L for each steps) to give compounds **8** (50.0 mg), **10** (28.0 mg), **11** (300.0 mg), **12** (34.0 mg), **13** (60.0 mg), **15** (20.0 mg) and **16** (18.0 mg).

Acid Hydrolysis - Compounds **1** (2.0 mg) and **8** (2.0 mg) was dissolved in 1N HCl (dioxane/H₂O 1:1, v/v, 1 ml) and then heated to 80° in a water bath for 3 hours. The cooled mixture was diluted with H₂O (4 ml) and extracted with AcOEt (3 x 5 ml). The aq. layer was thoroughly dried under N₂ after neutralization with Ag₂CO₃. The residues were dissolved in 0.1 ml of dry pyridine, and then L-cysteine methyl ester hydrochloride in pyridine (0.06M, 0.1 ml) was added to the solns. The mixture heated at 60° for 1.5 h. The dried product were partitioned with hexane and H₂O (0.1 ml each), and the org. layer was analyzed by GC (column: *SPB-1*, 0.25 mm x 30 m; detector, FID; detector temp., 300°; column temp., 210°; injector temp., 270°; carrier gas, He, 2 ml/min). The monosaccharides were confirmed as D-glucose derivative and L-arabinofuranose derivative by comparisons of the retention time (*t_R* 5.31 min and *t_R* 14.60 min) with authentic sugar derivatives.

Chemicals and reagents

The rat basophilic leukemia cell line RBL-2H3 was purchased from the Korean Cell Line Bank (Seoul, Korea). RBL-2H3 cells were cultured in Minimum Essential Medium (MEM) (Welgene, Korea) supplemented with 1% penicillin-streptomycin and 10% FBS (Gibco BRL, Rockville, MD, USA) under an atmosphere of 5% CO₂ in a humidified 37 °C incubator. Calcium ionophore A23187 and phorbol 12-myristate13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RAW264.7 macrophage cells, IGEL b4 cells and 293 T cells were supplied

from American Type Culture Collection (Manassas, VA, USA) and were cultured in Dulbecco Modified Eagle's medium (DMEM) with 10% FBS and 1% penicillin-streptomycin. Quercetin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Albumin from bovine serum, 2,4-dinitrophenylated (DNP-BSA) was purchased from Invitrogen by Thermo Fisher Scientific (Eugene, OR, USA).

Measurement of cell viability

RBL-2H3 and RAW264.7 cells were seeded in 96 well plates (SPL life Sciences Co., Pocheon, Korea) at 1×10^4 cells/well. The cells were treated with samples for 24 h. Cell proliferation was assayed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega, Madison, WI, USA) according to manufacturer's instructions. The absorbance was read with a microplate reader (BioTek, Winooski, VT, USA) at 490 nm.

β -hexosaminidase release activity from RBL-2H3 cells

β -hexosaminidase has frequently been used as an indicator for monitoring degranulation by mast cell.⁵ RBL-2H3 cells were incubated into 48-well plate (3×10^5 cells/mL) in MEM with 10 % FBS overnight. The cells were washed 2 times with siraganian buffer (pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 25 mM PIPES, 40 mM NaOH), and then incubated with siraganian buffer containing 5.6 mM glucose, 1 mM CaCl_2 , and 0.1% BSA. Cells were then pretreated with samples respectively for 2 h prior to stimulation with 30 ng/mL of PMA and 350 ng/mL of A23187 for 1 h at 37 °C and stopped on ice for 5 min, and the cell supernatants collected by centrifugation (25 μ l) were incubated with 25 μ l of 1 mM 4-nitrophenyl-N-acetyl- β -D-glucosaminide (Sigma, St Louis, MO, USA) in 0.1 M sodium citrate (pH 4.5) at 37 °C for 1 h.

The reaction was terminated with 200 μ l/well of carbonate buffer containing 0.1 M Na_2CO_3 and 0.1 M NaHCO_3 (pH 10), and then the absorbance was measured at 405 nm.

Rat IL-4 secretion assay from RBL-2H3 cells

To measure the IL-4 concentrations in the culture media, RBL-2H3 cells were incubated into 48-well plate (2×10^5 cells/mL) in MEM with 10 % FBS and IgE anti-DNP a 37 °C overnight. After washing 2 times with MEM, the cells were pretreated with samples respectively for 2 h and then treated with DNP-BSA (1 μ g/ml) for 18 h at 37 °C. The cell-free supernatants from each group were collected and IL-4 concentrations were measured using ELISA kit (R&D, Minneapolis, MN, USA) according to the manufacturer's instruction.

In addition, RBL-2H3 cells were incubated into 48-well plate in MEM with 10 % FBS overnight and pretreated with samples for 2 h. The cells were stimulated with 30 ng/mL of PMA and 350 ng/mL of A23187 for 1 h. The supernatants were collected for the measurement of IL-4 levels.

DNA transfection and NF- κ B reporter assay

293T cells were seeded into poly-D-lysine hydrobromide (Sigma, MO, USA) coated 96 well plate at 1×10^4 cells/well, and then transient transfection of a reporter plasmid pNF- κ B-SEAP (Clontech Laboratories, Palo Alto, CA, USA) was performed for the cells using the HilyMax for 4 h. After transfection, the cell medium was replaced with fresh DMEM overnight. The cells were treated with test samples for 2 h prior to stimulation with 3 ng/mL of PMA (Sigma, St. Louis, MO, USA) overnight. The supernatants were incubated with Quanti-Blue (Invitrogen, Carlsbad, MA, USA) for 1 h, and the absorbance was read at 630 nm with an ELISA microplate reader (BioTek, Winooski, VT, USA).

NF- κ B staining

RAW264.7 cells were plated into an 8-well glass chamber plate (Thermo Fisher Scientific, USA) at 2×10^4 cells/well overnight. The cells were pretreated with test samples for 2 h prior to stimulation with 1 μ g/mL of LPS for 2 h, and then fixed using 4 % paraformaldehyde (Molecular Probes, Inc., Eugene, OR, USA) and permeabilized with 0.1 % triton for 15 min. The NF- κ B p65 protein was detected by immunostaining using a polyclonal-anti-NF- κ B p65 antibody (Invitrogen, Carlsbad, MA, USA) and Alexa Fluor 488-conjugated anti-rabbit IgG antibody (Molecular Probes, Invitrogen, MA, USA). The cells were mounted by using Prolong gold antifade reagent with DAPI (Molecular Probes). Bay 11-7082 (Sigma, MO, USA) was used as the control for the NF- κ B inhibitors. Fluorescence images were acquired by fluorescence microscopy with a digital camera (DXMI1200C, Nikon).

Measurement of pro-inflammatory cytokines production

RAW264.7 cells were seeded into 48-well plates (SPL Life Sciences, Pocheon, Korea) at 1×10^5 cells/well and treated with test samples for 2 h before stimulation with 500 ng/mL of LPS. The supernatants of cultured medium were collected after incubation for 24 h to measure the TNF- α (R&D system, Minneapolis, MN, USA) using an ELISA kit according to manufacturer's instructions.

Nitric oxide analysis

RAW264.7 cells were plated into 48-well plates (SPL Life Sciences Co., Pocheon, Korea) at 1×10^5 cells/well and treated with samples for 2 h before stimulation with 500 ng/mL of LPS. After

24 h of incubation, the level of nitric oxide (NO) production in the culture supernatant was measured using Griess Reagent (1% sulfanilamide in 5% H₃PO₄, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride) and incubated for 30 min at room temperature. The absorbance was read at 570 nm using an ELISA microplate reader (BioTek, Winooski, VT, USA).

Western blot analysis of COX-2 and iNOS

RAW264.7 cells were cultured in a 6 well plate at 1×10^6 cells/well overnight. The cells were treated with samples or celecoxib (20 μ M) (Sigma Co., MO, USA) for 2 h prior to stimulation with 200 ng/mL of LPS (Sigma Co., MO, USA) for 18 h. Equal amounts of protein extracts (30 μ g) in a lysis buffer were subjected to 10% SDS-PAGE analysis and then electro-transferred onto polyvinylidene fluoride membranes (PVDF) (Millipore, Bedford, MA, USA). Western blot analysis was conducted using the above mentioned methods with polyclonal antibodies specific to COX-2 (1:1000, Cell Signaling Tech., Danvers, MA, USA), iNOS (1:1000, Santa Cruz, CA, USA), or β -actin (1:1000, Santa Cruz, MA, USA).

Statistical Analysis

All results are presented as the means $\pm$ SEM. Statistical comparisons were evaluated using Student's *t*-test with a *P* value < 0.05 considered statistically significant. All experiments were repeated at least three times, and the data were obtained at least in triplicate. The results are shown from representative experiments.