British Journal of Applied Science & Technology
4(29): 4201-4222, 2014

SCIENCEDOMAIN *international*
www.sciencedomain.org

Anti-Inflammatory Effects of New Flavonoids from *Streptomyces* sp. BT01 in Lipopolysaccharide-Stimulated RAW 264.7 Murine Macrophages via Inhibition of NF-KappaB Activation

Thongchai Taechowisan[1*], Srisakul Chanaphat[1], Wanwikar Ruensamran[2] and Waya S. Phutdhawong[2]

[1]Department of Microbiology, Faculty of Science, Silpakorn University, Nakorn Pathom 73000, Thailand.
[2]Department of Chemistry, Faculty of Science, Silpakorn University, Nakorn Pathom 73000, Thailand.

Authors' contributions

This work was carried out in collaboration between all authors. Author TT wrote the original concept, study design, managed the literature searches, conducted laboratory work and checked the data for validity and carried out the analyses of the study. Authors SC and WR performed the laboratory tests. Author WSP was involved monitoring advising and guiding the progression of the study, proof reading and editing the manuscripts. All authors read and approved the final manuscript.

Original Research Article

Received 16th June 2014
Accepted 24th July 2014
Published 7th August 2014

ABSTRACT

Most flavonoids are known to have anti-oxidant, anti-bacterial and analgesic properties. In this study, the new flavonoids, 7-methoxy-3,3',4',6-tetrahydroxyflavone (1) and 2',7-dihydroxy-4'5'-dimethoxyisoflavone (2) isolated from *Streptomyces* sp. BT01 inhibited the pro-inflammatory mediators including cytokines by blocking nuclear factor-kappaB (NF-κB) signalling in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. These flavonoids suppressed mRNA and protein expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in LPS-stimulated RAW 264.7 cells. The molecular mechanism was associated with the inhibition of NF-κB activation. These results suggest that these flavonoids have anti-inflammatory effects by suppressing expression of

**Corresponding author: E-mail: tewson84@hotmail.com;*

iNOS, COX-2 and cytokines by blocking the NF-κB signalling in LPS-stimulated RAW 264.7 cells.

Keywords: Anti-inflammatory effects; flavonoids; RAW 264.7; Streptomyces sp.

1. INTRODUCTION

Flavonoids are a family of substances whose members have many interesting biological properties including anticancer, antimicrobial, antiviral, antithrombotic, anti-inflammatory, and immunomodulatory activities [1,2,3,4]. Of these biological activities, the anti-inflammatory capacity of flavonoids has long been emphasized in oriental medicine. The excessive activation of macrophages can induce the production of several kinds of pro-inflammatory enzymes and cytokines. Pro-inflammatory enzymes, which are the mediators of inflammation, include inducible forms of nitric oxide synthase (iNOS) which makes nitric oxide (NO) from L-arginine. NO is involved in various biological processes, including inflammation [5,6]. Cyclooxygenase-2 (COX-2) is the enzyme that converts arachidonic acid to prostaglandins which are involved in inflammatory response. Pro-inflammatory cytokines, such as tumor necrotic factor (TNF)-α, interleukin (IL)-1β, and IL-6, are mainly produced in macrophages activated by Gram negative bacteria-derived lipopolysaccharide (LPS) [7]. TNF-α is thought to be one of the most important mediators of inflammatory diseases. It is elevated in some pathogenic conditions and possesses potential toxic effect that results in hypersensitivity reactions with chronic inflammation [8,9]. IL-1β is a multifunctional cytokine that is responsible for various processes including host defense, inflammation and response to injury. It is produced by many cell types, predominantly by macrophage [10,11]. IL-6 is a cytokine produced by a number of normal and transformed cells. It is believed to be an endogenous mediator of LPS-induced fever [12,13,14].

In recent years, people began to use natural product compounds from plants and microorganisms to prevent and treat inflammatory responses by inhibiting inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, and this has become an important area of investigation. During our recent investigations of the anti-inflammatory compounds, lansai C and lansai D were isolated from *Streptomyces* sp. SUC1 which had anti-inflammatory activity [15,16]. We report here the anti-inflammatory properties of two new flavonoids, 7-methoxy-3,3',4',6-tetrahydroxyflavone (1) and 2',7-dihydroxy-4'5'-dimethoxyisoflavone (2) by investigating their effects on the inhibition of production of NO, prostaglandin E_2 (PGE_2), TNF-α, IL-1β, and IL-6 and also expression of iNOS and COX-2 in LPS-activated macrophage RAW 264.7 cells. These two new flavonoids were found to have significant, dose-related inhibitory effects on LPS-induced NO, PGE_2, TNF-α, IL-1β, and IL-6 production.

2. MATERIALS AND METHODS

2.1 Microorganisms

Streptomyces sp. BT01 was isolated from the root tissues of *Boesenbergia rotunda* (L.) Mansf. by the surface-sterilization technique [17]. The characteristics of *Streptomyces* sp. BT01 were observed. For morphological characteristics, presence of aerial mycelium, spore mass colour, distinctive reverse colony colour, diffusible pigment, sporophore and sporechain morphology were recorded after 10 days incubation on International Streptomyces Project-2 (ISP-2) medium. Diaminopimelic acid isomers and sugars

from whole-cell extract were analysed for chemotaxonomic studies as report in a previous study [18].

2.2 Extraction and Purification of Active Compounds

A spore suspension of *Streptomyces* sp. BT01 was prepared in distilled water from cultures grown on International Streptomyces Project-4 (ISP-4) medium at 30 ^{o}C for 10 days. The suspension, 10^8 spores per 100 ml of liquid medium, was added to ISP-2 broth in each 500-ml Erlenmeyer flask. Cultures were kept on an orbital shaker at 30 ^{o}C for 48 h and used as seed stocks. For large production of culture filtrates, the strain BT01 was grown in a modified 3000 ml glass container containing 1500 ml of ISP-2 broth, and incubated in an orbital shaker for 5 days at the same condition. The 5-day-old cultures were filtrated by Whatman paper No. 1 under vacuum. The mycelial mats were washed with distilled water and separated by centrifugation at 8500 g for 20 min. The culture filtrate and mycelial mats of the strain BT01 were extracted three times with 1/3 volumes of ethyl acetate. This organic solvent was pooled and then taken to dryness under flash evaporation at 40ºC. The yield of dry material per litre was about 753 mg, which was dissolved in 10 ml of chloroform and fractionated on column chromatography (Merck silica gel 60, 35-70 mesh) with hexane, diethyl ether and methanol. The combined fractions eluted with 50% diethyl ether in hexane, 100% diethyl ether, and 5% methanol in diethyl ether (286 mg) were further separated by MPLC (400 x 40 mm column, Merck LiChroprep Si 60, 25-40 μm, UV-detection, 254 nm) to afford fraction A (57 mg) and fraction B (104 mg). Final purification of fraction A and B were achieved by prep TLC (Merck, Si gel 60, 0.5 mm; dichloromethane : diethyl ether = 75: 25) to give 16 mg of compound 1 from fraction A and 28.5 mg of compound 2 from fraction B.

2.3 Characterisation of the Compounds

The structures of purified compounds have been identified using NMR and mass spectral data. The melting point of the compounds was determined on a Buchi-540 melting point apparatus. Optical rotations were measured on a Perkin-Elmer 241 polarimeter, IR spectra on a Perkin-Elmer 1 spectrometer, ^{1}H and ^{13}C NMR spectra on a Bruker DRX 500 spectrometer, and EI-MS and FAB-MS respectively on a Hewlett-Packard 5989 B and a Finnigan/Thermo Quest Mat 95 XL mass spectrometer.

2.4 Anti-inflammatory Activity

Murine macrophage RAW 264.7 cell line obtained from American Type Culture Collection (ATCC, Maryland, USA), was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin G (100 IU/ml), streptomycin (100 µg/ml), and L-glutamine (2 mM) and incubated at 37ºC in a humidified atmosphere containing 5% CO_2. Cells ($1x10^6$/ml) were pre-incubated for 2 h with compound 1 or 2 (5, 10 and 20 µg/ml) and further cultured for 24 h (for pro-inflammatory cytokine, mediator and NO production) and 2, 6, 9, 12, and 24 h (for RT-PCR analysis) and 9 h (for protein expression) with LPS (1 µg/ml) in 6-well plates. Supernatants were removed at the allotted times and PGE_2, TNF-α, IL-1β, and IL-6 levels were quantified by immunoassay kits according to the manufacture's protocols (Assay Designs' Correlate-EIA^{TM}, Stressgen, USA), respectively, for Nitrite concentration in the supernatant, an indicator of NO production, was measured by a microplate assay method based on the Griess reaction [19], and the cells were used for RT-PCR analysis and protein expression.

2.5 Western Blot Analysis

Cellular proteins were extracted from control and compound 1 or 2 -treated RAW 264.7 cells. The washed cell pellets were resuspended in lysis buffer (50 mM HEPES pH 7.0 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonylfluoride, 0.5 mM dithiothreitol, 5 mM NaF, 0.5 mM Na orthovanadate) containing 5 µg/ml each of leupeptin and aprotinin and incubated for 15 min at 4ºC. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. Protein concentration was determined by BioRad protein assay reagent according to the manufactures instruction, 40-50 µg of cellular proteins from treated and untreated cell extracts were electroblotted onto nitrocellulose membrane following separation on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The immunoblot was incubated overnight with blocking solution (5% skimmed milk) at 4ºC, followed by incubation for 4 h with a 1:500 dilution of monoclonal anti-iNOS and COX-2 antibody (Santacruz, CA, USA). Blots were again washed two times with PBS and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Santacruz, CA, USA) for 1 h at room temperature. Blots were again washed three times in Tween 20/Tris-buffered saline (TTBS) and then developed by 3,3'-diaminobenzidine (DAB) (Sigma-Aldrich, USA) solution (0.2 M DAB, 2.5 mM $NiCl_2$) and H_2O_2 (final concentration of 0.002%) for 10 min at room temperature. After signal development, the nitrocellulose membrane was washed several times in water and dried overnight on blotting paper. The signal intensity of the specific proteins were evaluated by ImageJ and calculated as the relative amounts comparing with the LPS-treated group.

2.6 Cytotoxicity Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay was performed according to the method previously described [20]. MTT solution was added at a concentration of 50 µg/ml into each well, which also contain 5, 10, 20 and 40 µg/ml of compound 1 or 2. After 24 h of incubation at 37ºC, the medium was discarded and the formazan blue, which formed in the cells, was dissolved in 50 µl DMSO. The optical density values were measured at 570 nm using an AS10000 microplate reader (Hewlett-Packard spectracount, USA). The optical density of formazan formed in control (untreated) cells was taken as 100% of viability.

2.7 Preparation of Total mRNA and RT-PCR

RAW 264.7 cells were cultured in the presence of each compound in combination with LPS in 6-well plates and control for 6 h (for dose effect test) or at the allotted times (2, 6, 9, 12, and 24 h for time-course effect test). Total cellular RNA was isolated using the RNeasy mini kit (Qiagen) following the manufacturer's instructions. Total RNA (1 µg) was reverse-transcribed into cDNA using AccuPower RT-PCR Premix (Bioneer, Daejeon, Korea). The PCR primers used in this study were represented in Table 1. The β-actin gene was used as a constitutively expressed housekeeping gene for controls to determine the uniformity of the reverse transcriptions. PCR reactions were performed using Applied Biosystems 2720 thermal cycler (CA, USA) and an AccuPower RT-PCR Premix (Bioneer) according to the manufacturer's protocols. After amplification, products of the PCR reaction were separated on a 1% (w/v) tri-acetate/EDTA agarose gel, stained with 2% (w/v) ethidium bromide. The band was photographed under UV light using Kodak Image Station 440 (Kodak, Japan). The amount of mRNA was evaluated by ImageJ. The signal intensity of the specific mRNAs were

normalised by a comparison with that of β-actin and calculated as the relative amounts comparing with the LPS-treated group.

Table 1. Primers used in RT-PCR analysis

Gene[a]	Primer sequences (5' → 3')	PCR product size (bp)
TNF-α	TNF-α-f: TTGACCTCAGCGCTGAGTTG	364
	TNF-α-r: CCTGTAGCCCACGTCGTAGC	
IL-1β	IL-1β-f: CAGGATGAGGACATGAGCACC	447
	IL-1β-r: CTCTGCAGACTCAAACTCCAC	
IL-6	IL-6-f: GTACTCCAGAAGACCAGAGG	308
	IL-6-r: TGCTGGTGACAACCACGGCC	
iNOS	iNOS-f: CCCTTCCGAAGTTTCTGGCAGCAGC	496
	iNOS-r: GGCTGTCAGAGCCTCGTGGCTTTGG	
COX-2	COX-2-f: CCCAGAGCTCCTTTTCAACC	240
	COX-2-r: ATTTGGCACATTTCTTCCCC	
β-Actin	β-Actin-f: GTGGGCCGCCCTAGGCACCAG	603
	β-Actin-r: GGAGGAAGAGGATGCGGCAGT	

aTNF-α, Tumor necrosis factor-α; IL-1β, Interleukin-1β; IL-6, Interleukin-6; iNOS, Inducible nitric oxide synthase; COX-2, Cyclooxygenase-2

2.8 Determination of NF-κB Activation

To evaluate effects of compound 1 or 2 on the NF-κB activation, after incubation with LPS (1 μg/ml) in the presence or absence of the compounds (5, 10 and 20 μg/ml for dose effect test) for 0.5 h or at the allotted times (0.25, 0.5, 1 and 2 h for times-course effect test), RAW 264.7 cells (2 x 10^6 cells) were washed with ice-cold PBS/phosphatase inhibitor solution and were directly lysed with the complete lysis buffer at the time interval. Proteins were separated by centrifugation (12,000 g for 20 min at 4ºC) and stored at -80ºC until analysis. Activated p65 subunits of NF-κB were determined by Trans AM ELISA kit (Active Motif, CA, USA). Whole-cell extracts (containing 10 μg total protein) were used. Absorbance was measured at 450 nm with a reference wavelength of 620 nm with an AS10000 microplate reader (Hewlett-Packard spectracount, USA).

2.9 Statistical Analysis

All data are expressed as mean ± standard error of mean (S.E.M.) from at least three independent tests. The data were analyzed by the analysis of variance followed by the Duncan's multiple range test. All of the statistical analyses were performed by means of SPSS software with a probability level of $P < 0.05$ or 0.01 (SPSS for Windows, ver. 14.0; SPSS Inc., Chicago, IL, USA).

3. RESULTS

3.1 Isolation of the Compounds from *Streptomyces* sp. BT01

In the present study, 7-methoxy-3,3',4',6-tetrahydroxyflavone (1) and 2',7-dihydroxy-4'5'-dimethoxyisoflavone (2) were isolated from the ethyl acetate extracts from the culture of *Streptomyces* sp. BT01 by using silica gel column chromatography and thin-layer

chromatography. Their structures as indicated in Fig. 1 were elucidated in previous report [18]. Their spectral data were reported as follows:

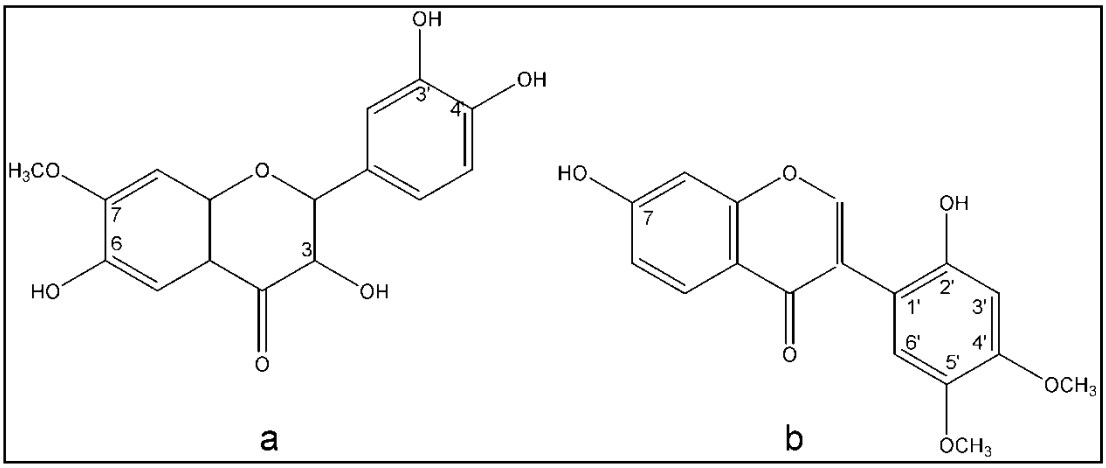


Fig. 1. Chemical structures of 7-methoxy-3,3',4',6-tetrahydroxyflavone (1)(a) and 2',7-dihydroxy-4'5'-dimethoxyisoflavone (2)(b).

Compound 1, identified by NMR and mass spectral data as 3,3',4',6-tetrahydroxy-7-methoxyflavone ($C_{16}H_{12}O_7$), was yellow crystals having: mp 315-317ºC (from methanol), UV: λ_{max} nm (log ε) = 239 (4.290), 257 sh (4.262), 349 (4.454). λ_{max} nm (+ $AlCl_3$) (log ε) = 235 (4.586), 276 (4.179), 360 (3.93), 431 (4.394). λ_{max} nm (+ $AlCl_3$/HCl) (log ε) = 228 sh (4.269), 267 (4.257), 357 sh (3.973), 419 (4.454). IR ν_{max} cm^{-1}: 3596, 3511, 3333, 3117, 1636, 1609, 1551, 1508, 1497, 1435, 1393, 1289, 1223, 1169, 1123, 1038. EI-MS *m/z*: 316 (M^+, 100%), 301 (22), 273 (35), 167 (13), 150 (16), 149 (24), 137 (39), 135 (21), 128 (14), 123 (16), 120 (13), 95 (18), 69 (42), 63 (17), 53 (31), 51 (26). HR-MS: $C_{16}H_{12}O_7$, found: 316.0580, calcd: 316.0588. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ: 3.91 (3H, s, 7-OCH_3), 6.86 (1H, d, *J*=8.4 Hz, H-5'), 7.20 (1H, s, 8-H), 7.29 (1H, s, 5-H), 7.54 (1H, dd, *J*=8.4, 2.2Hz, 6'-H), 7.70 (1H, d, *J*=2.2 Hz, 2'-H), 9.00 (1H, s, 3-OH), 9.24 (1H, s, 3'-OH), 9.53 (1H, s, 4'-OH), 9.72 (1H, s, 6-OH). ^{13}C-NMR (DMSO-d_6, 75.4 MHz) δ: 56.54 (7-OCH_3), 100.46 (C-8), 106.9 (C-5), 115.00 (C-10), 115.33 (C-2'), 115.86 (C-5'), 119.94 (C-6'), 123.01 (C-1'), 137.39 (C-3), 145.04 (C-6), 145.36 (C-3'), 145.45 (C-2), 147.48 (C-4'), 150.22 (C-9), 153.96 (C-7), 171.91 (C-4).

Compound 2, identified by NMR and mass spectral data as 2',7-dihydroxy-4',5'-dimethoxyisoflavone ($C_{17}H_{14}O_6$), was yellow crystals having: mp 237-239ºC (from methanol), UV: λ_{max} nm (log ε) = 248 sh (4.312), 264 sh (4.211), 301 (4.256). IR ν_{max} cm^{-1}: 3414, 2940, 1705, 1616, 1562, 1512, 1458, 1343, 1300, 1246, 1188, 1103. EI-MS *m/z*: 314 (M^+, 100%), 299 (85), 271 (19), 239 (22), 200 (20), 187 (28), 137 (30), 107 (24), 92 (22), 69 (57), 63 (36), 53 (25), 51 (33). HR-MS: $C_{17}H_{14}O_6$, found: 314.0799, calcd: 314.0730. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ: 3.66 (3H, s, 5'-OCH_3), 3.72 (3H, s, 4'-OCH_3), 6.52 (1H, s, 3'-H), 6.81 (1H, s, 6'-H), 6.87 (1H, d, *J*=2.1 Hz, 8-H), 6.92 (1H, dd, *J*=8.7, 2.1 Hz, 6-H), 7.94 (1H, d, *J*=8.7 Hz, 5-H), 8.22 (1H, s, 2-H), 8.99 (1H, s, 7-OH), 10.80 (1H, br, 2'-OH). ^{13}C-NMR (DMSO-d_6, 50 MHz) δ: 55.65 (4'-OCH_3), 56.66 (5'- OCH_3), 101.53 (C-3'), 102.27 (C-8), 110.08 (C-1'), 115.42 (C-6), 116.02 (C-6'), 116.70 (C-10), 121.64 (C-3), 127.44 (C-5), 141.70 (C-5'), 149.81 (C-2'), 149.90 (C-4'), 155.24 (C-9), 157.64 (C-9), 162.79 (C-7), 175.42 (C-4).

3.2 Effects of the Compounds on Cell Viability

Fig. 2 shows the cell viability at 5, 10, 20 and 40 µg/ml of compound 1 and 2. After 24 h of incubation, concentration of 40 µg/ml of the compound 1 significantly decreased cell viability to about 83% ($P < 0.05$), whereas concentration of the compounds ranging from 5 to 20 µg/ml did not exhibit any cytotoxic effect. Therefore, concentrations of the compounds were selected from 5 to 20 µg/ml for study on anti-inflammatory effects.

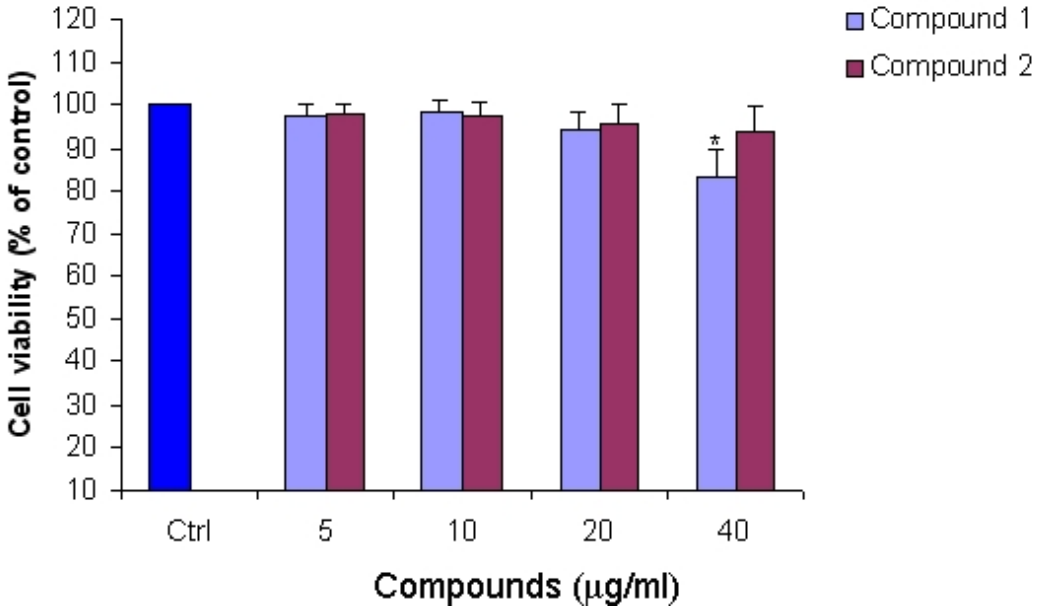
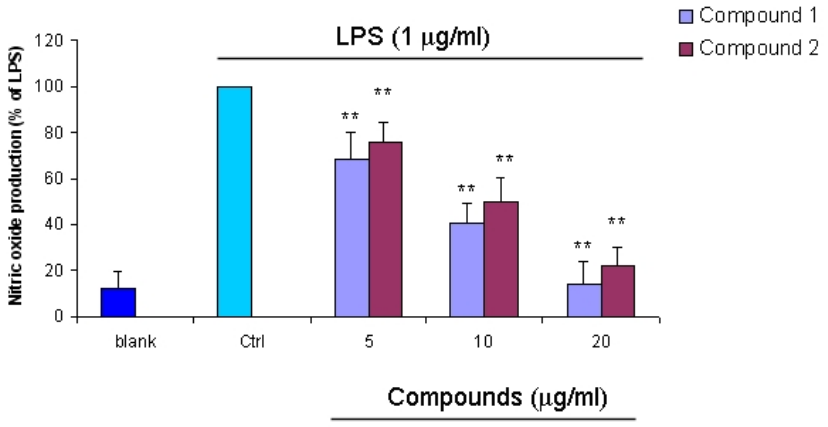


Fig. 2. Effect of the compound 1 and 2 on cell cytotoxicity. The RAW 264.7 cells were treated with indicated concentrations of the compounds (5-40 µg/ml) for 24 h, and the results are expressed by percentages of surviving cells over control cells using MTT assays

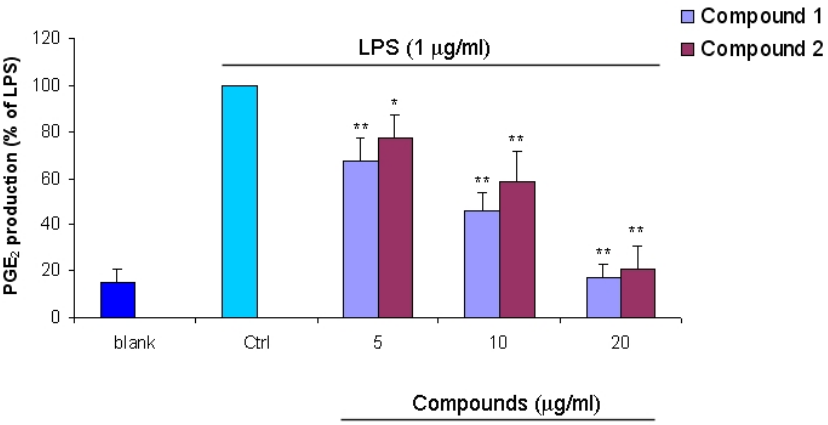
**P < 0.05 compared with the LPS-only treatment*

3.3 Effects of the Compounds on the Production of NO, PGE_2, TNF-α, IL-1β, and IL-6 in LPS-stimulated RAW 264.7 Cells

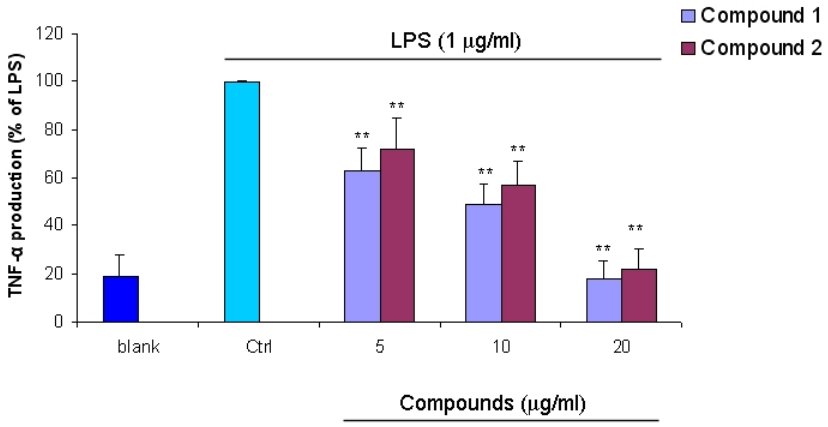
Fig. 3a, 3b, 3c, 3d, and 3e show the effects of compounds on NO, PGE_2, TNF-α, IL-1β, and IL-6 production in LPS-stimulated RAW 264.7 cells. ELISA assays were used for detection of the mediator and pro-inflammatory cytokines, and Greiss's reaction was used to estimate the NO generation. In the absence of LPS, very low amounts of NO, mediator and those pro-inflammatory cytokines were detected in the culture supernatants of RAW 264.7 cells. Upon stimulation with LPS (1 µg/ml), NO production was markedly increased (Fig. 3a). We also found significant increases of the mediator and pro-inflammatory cytokines in the presence of LPS-stimulation (Fig. 3b to 3e). However, pretreatment with the compound **1** and **2** for 2 h could significantly reduce the production of NO, PGE_2, TNF-α, IL-1β, and IL-6 in a dose-dependent manner (Fig. 3b to 3e).


Compound 1
Compound 2
LPS (1 μg/ml)
Nitric oxide production (% of LPS)
120
100
80
60
40
20
0
**
blank
Ctrl
5
10
20
Compounds (μg/ml)

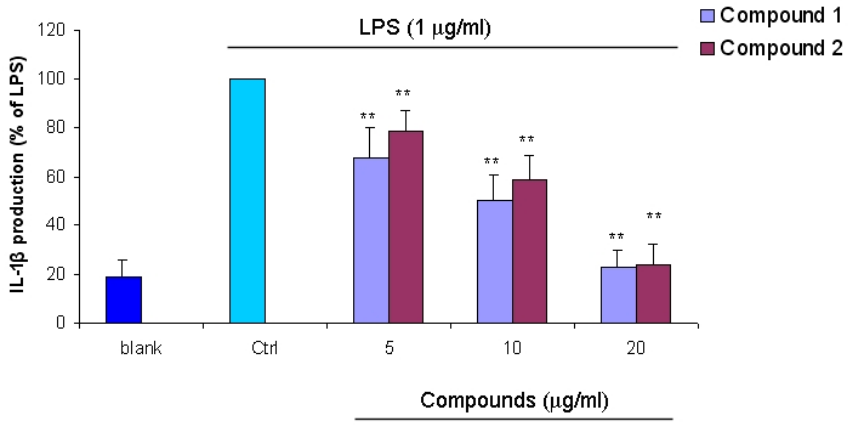
a


Compound 1
Compound 2
LPS (1 μg/ml)
PGE2 production (% of LPS)
120
100
80
60
40
20
0
*
**
blank
Ctrl
5
10
20
Compounds (μg/ml)

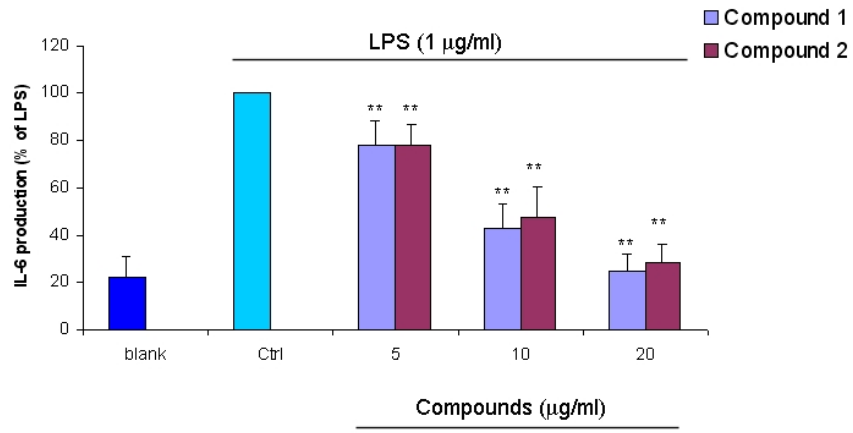
b


Compound 1
Compound 2
LPS (1 μg/ml)
TNF-α production (% of LPS)
120
100
80
60
40
20
0
**
blank
Ctrl
5
10
20
Compounds (μg/ml)

c



d



e

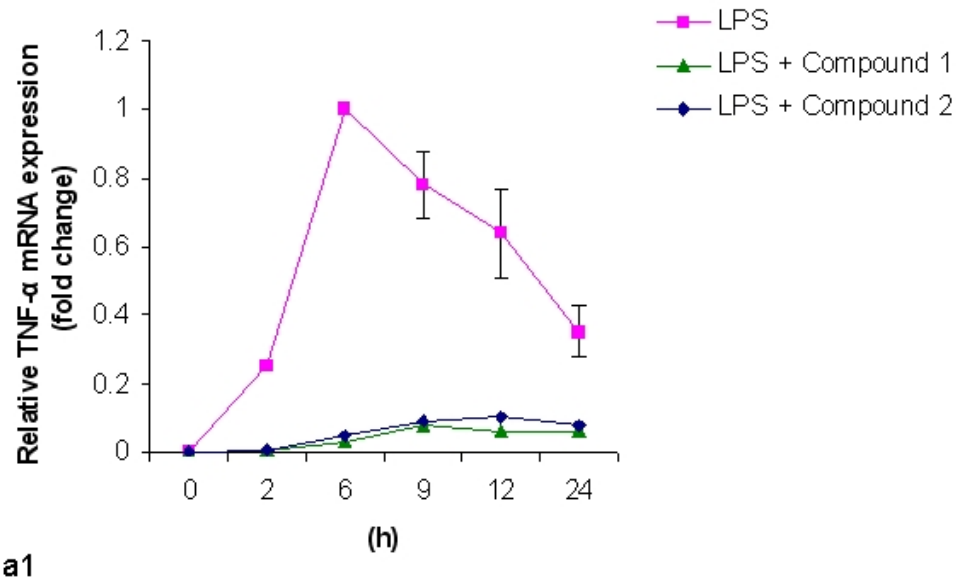
Fig. 3. Suppressive effects of the compound 1 and 2 on LPS-induced NO, PGE2, TNF-α, IL-1β, and IL-6 production in RAW 264.7 cells. Cells were pretreated with/without indicated concentrations of the compound 1 and 2 for 2 h then stimulated with LPS (1 μg/ml) for 24 h. Control values were obtained in the absence of LPS or the compounds. The values are presented as percentages of NO (a), PGE2 (b), TNF-α (c), IL-1β (d), and IL-6 (e) comparing with LPS-treated cells, respectively. The data were expressed as the means ± SDs for three independent experiments

**P < 0.05, **P < 0.01 compared with the LPS-only treatment*

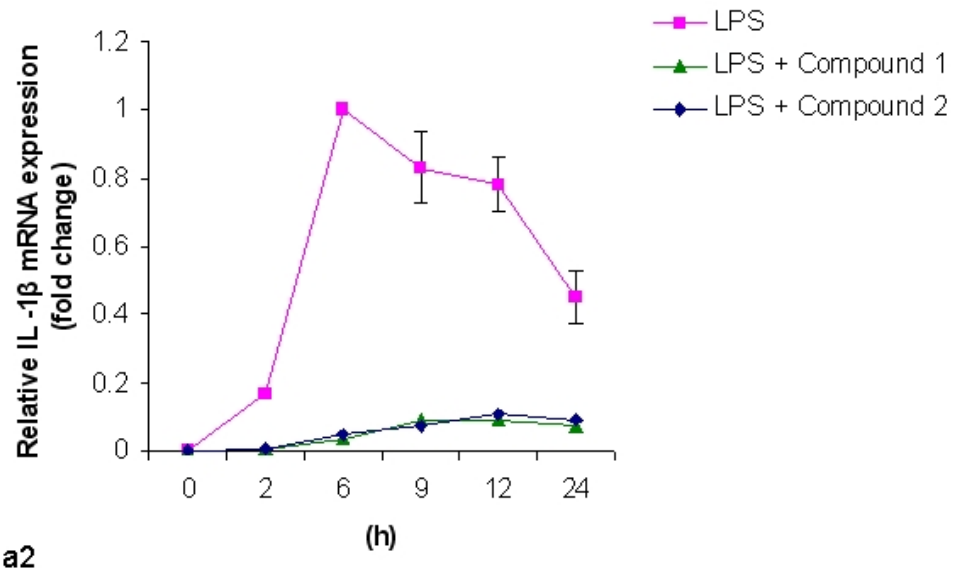
3.4 Effects of the Compounds on TNF-α, IL-1β, IL-6, iNOS and COX-2 mRNA Expression in LPS-stimulated RAW 264.7 Cells

Fig. 4 shows the effects of the compounds 1 and 2 on mRNA expression of TNF-α, IL-1β, IL-6, iNOS and COX-2 in LPS-stimulated RAW 264.7 cells. Time-and dose-effect of the compounds on the mRNA expression of TNF-α, IL-1β, IL-6, iNOS and COX-2 was measured in RAW 264.7 cells stimulated with LPS in the presence or absence of the compounds using RT-PCR. The mRNA expression reached a peak level at about 6 h for TNF-α, IL-1β, and IL-

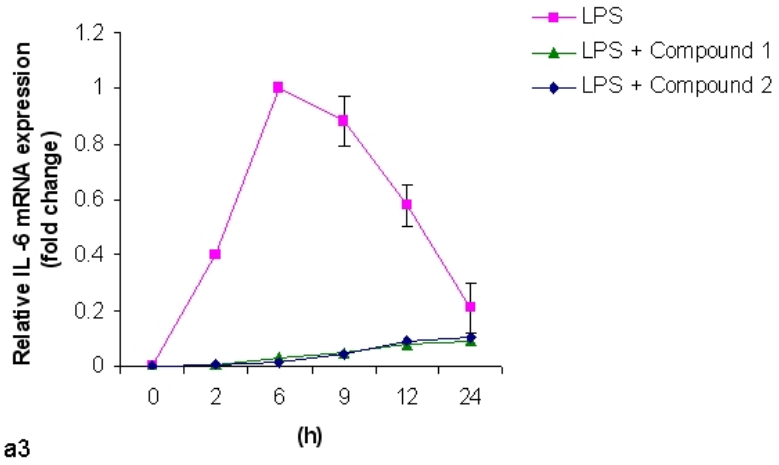
6, and 9 h for iNOS and COX-2. Pretreatment of the compound 1 and 2 at 20 µg/ml could block the expression of these mRNAs at all of indicated time points (Fig. 4a). We also observed that pretreatment of various concentrations of the compound 1 and 2 on the inhibition of LPS-induced mRNA levels of those five genes was a dose-dependent manner (Fig. 4b and Fig. 5).



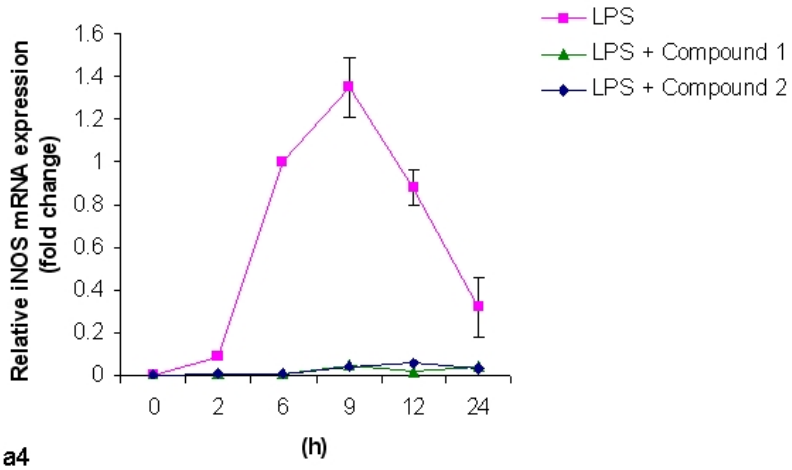
a1



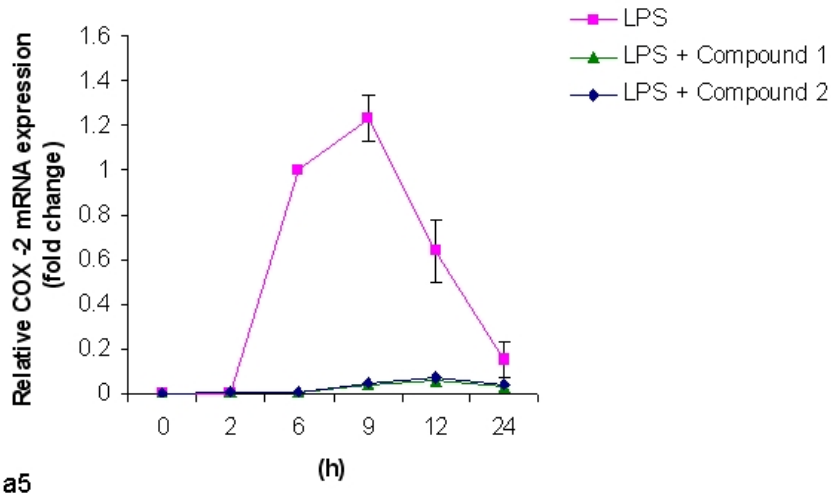
a2


LPS
LPS + Compound 1
LPS + Compound 2
Relative IL -6 mRNA expression (fold change)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
6
9
12
24
(h)

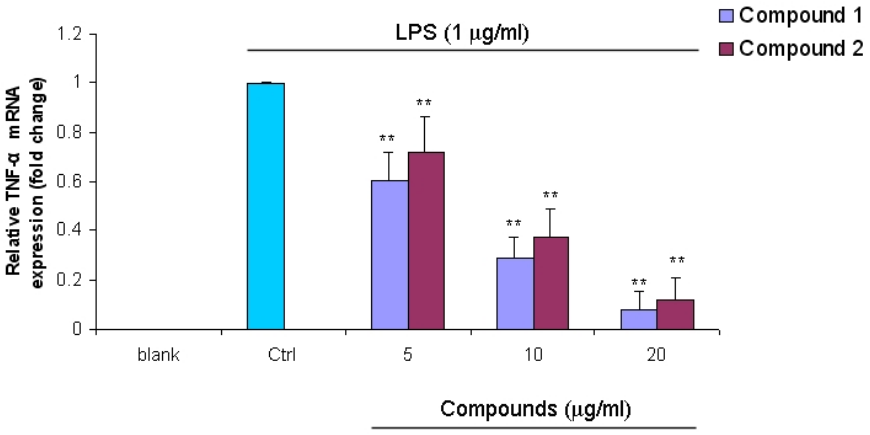
a3


LPS
LPS + Compound 1
LPS + Compound 2
Relative iNOS mRNA expression (fold change)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
6
9
12
24
(h)

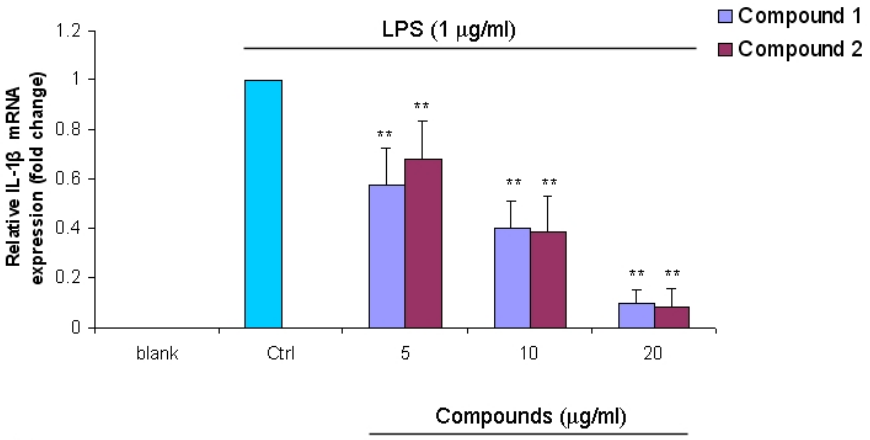
a4


LPS
LPS + Compound 1
LPS + Compound 2
Relative COX -2 mRNA expression (fold change)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
6
9
12
24
(h)

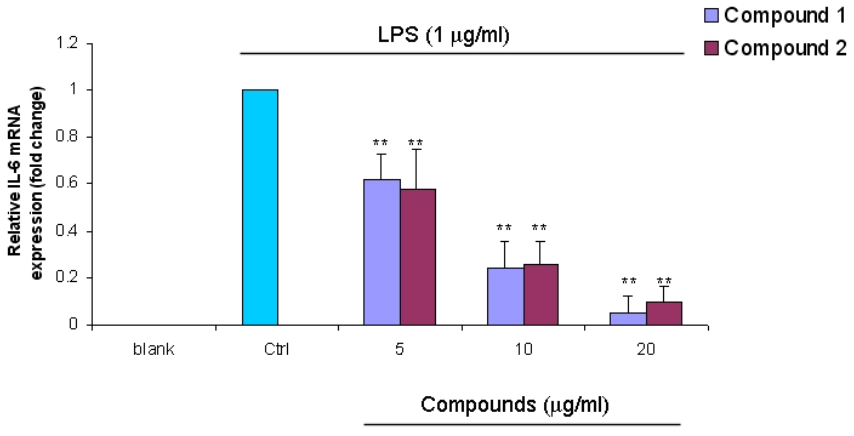
a5


Compound 1
Compound 2
LPS (1 μg/ml)
Relative TNF-α mRNA expression (fold change)
1.2
1
0.8
0.6
0.4
0.2
0
**
blank
Ctrl
5
10
20
Compounds (μg/ml)

b1


Compound 1
Compound 2
LPS (1 μg/ml)
Relative IL-1β mRNA expression (fold change)
1.2
1
0.8
0.6
0.4
0.2
0
**
blank
Ctrl
5
10
20
Compounds (μg/ml)

b2


Compound 1
Compound 2
LPS (1 μg/ml)
Relative IL-6 mRNA expression (fold change)
1.2
1
0.8
0.6
0.4
0.2
0
**
blank
Ctrl
5
10
20
Compounds (μg/ml)

b3

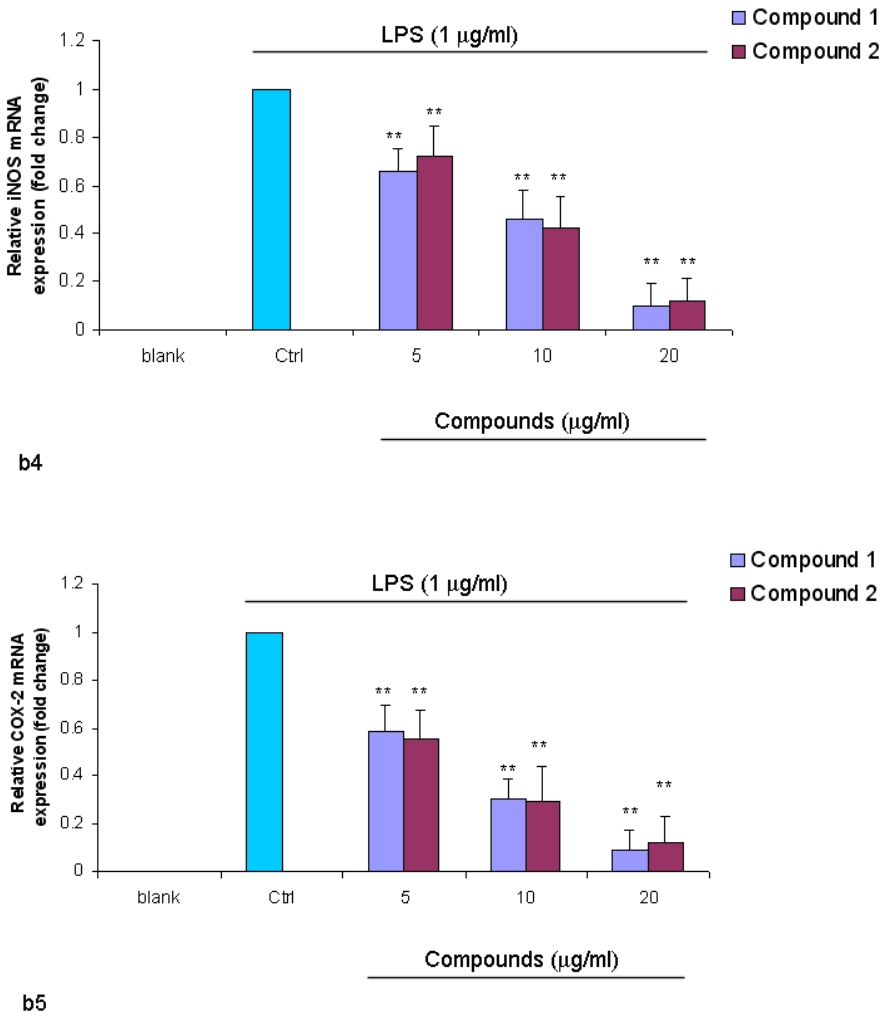


Fig. 4. Suppressive effects of the compound 1 and 2 on LPS-induced mRNA expression of TNF-α, IL-1β, IL-6, iNOS and COX-2 in RAW 264.7 cells. Time-course (a1-a5) and dose-dependent (b1-b5) inhibitory effect of the compound 1 and 2 were measured on LPS-induced TNF-α, IL-1β, IL-6, iNOS and COX-2 mRNA expression in RAW 264.7 cells were measured using RT-PCR. (a1-a5) Cells were pretreated with the compounds (20 μg/ml) or not for 2 h, then stimulated with LPS (1 μg/ml) at various time points. (b1-b5) Dose-effect relationship was measured after 6 h stimulation with LPS (1 μg/ml). Results were expressed as a target gene expressions ratio comparing with the LPS-treated group. The data were the means ± SDs for three independent experiments

***P < 0.01 compared with the LPS-only treatment*

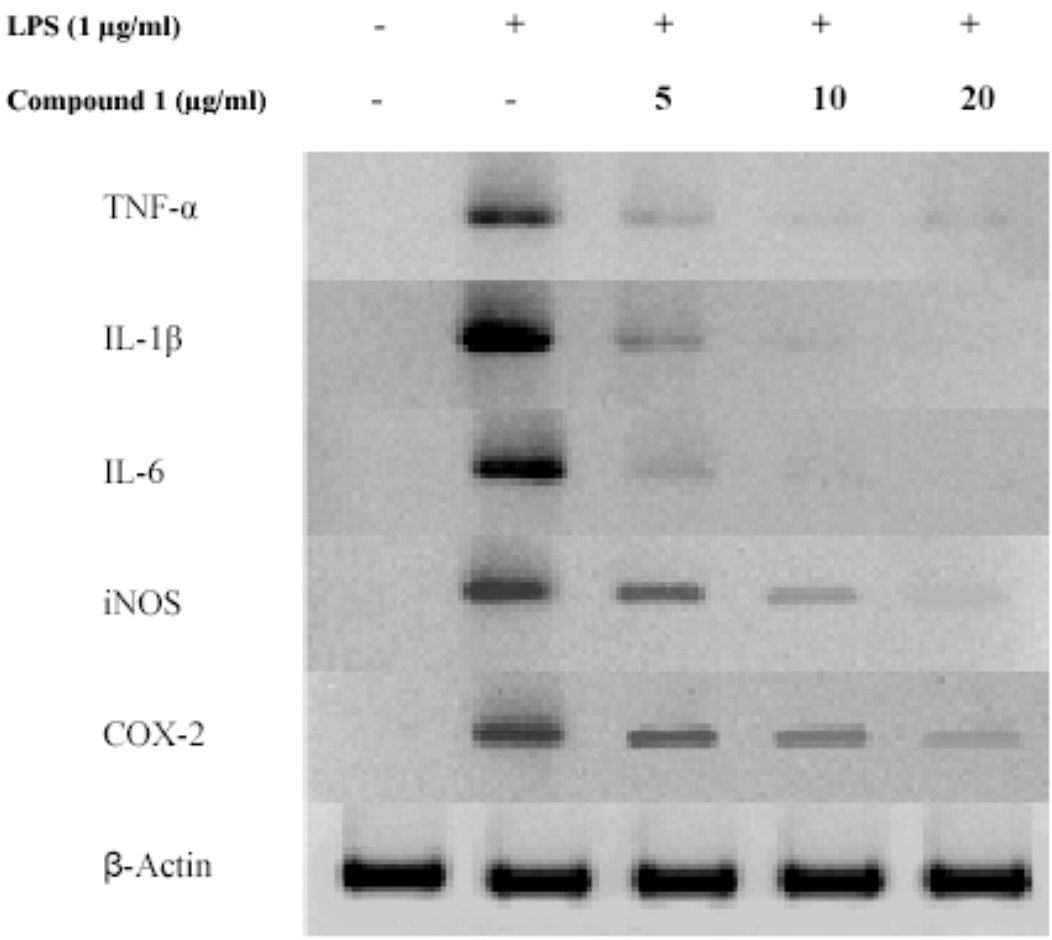
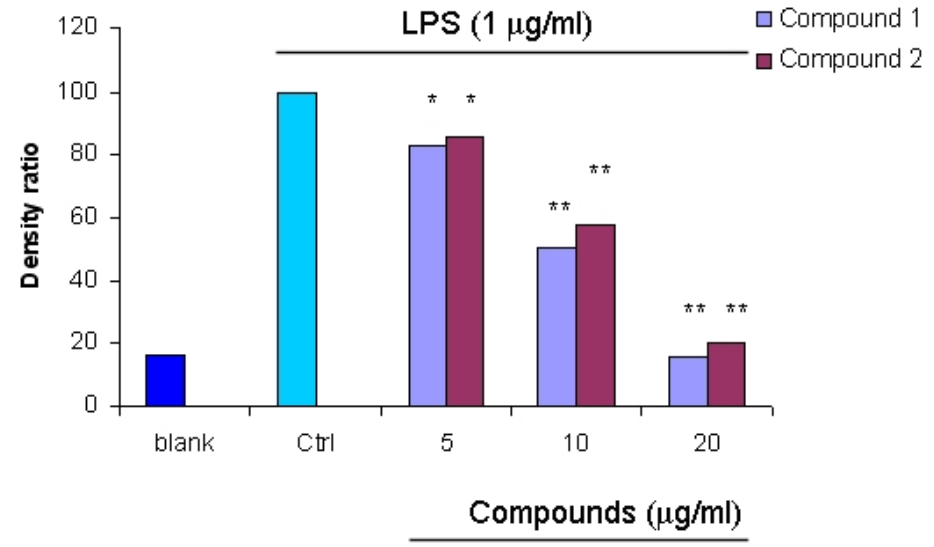


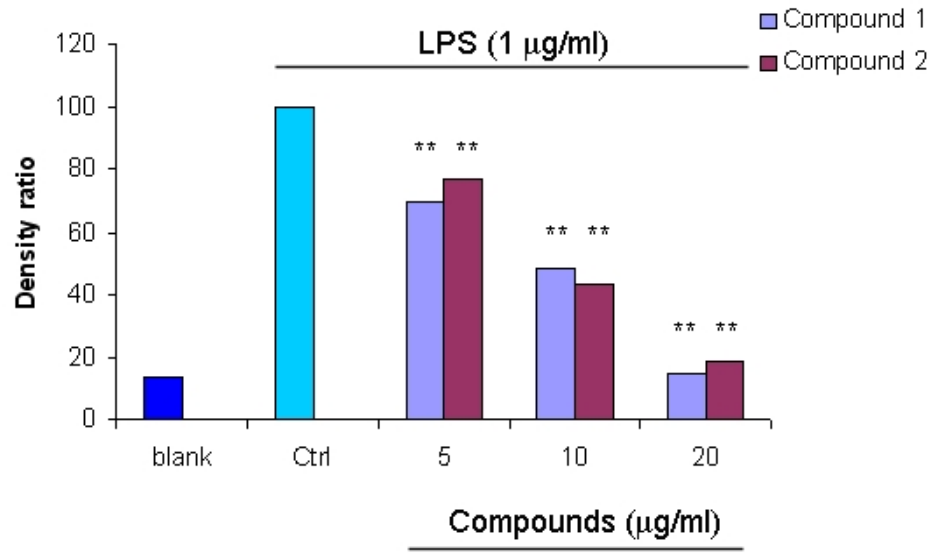
Fig. 5. Suppressive effects of compound 1on LPS-induced mRNA expression of proinflammatory cytokines, inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2) in RAW 264.7 cells. The cells were pretreated with different concentrations (5, 10, 20 μg/ml) of compound 1 for 2 h and then incubated with or without 1 μg/ml of LPS for 6 h, total mRNA was isolated, and the mRNA levels of proinflammatory cytokines, iNOS and COX-2 were examined by RT-PCR

3.5 Effects of the Compounds on iNOS and COX-2 Production in LPS-stimulated RAW 264.7 Cells

The iNOS and COX-2 productions of the compound 1 and 2 pretreatment in RAW 264.7 cells stimulated with LPS were performed by Western blot analysis. The relative intensity of iNOS and COX-2 bands were significantly increased upon LPS treatment and this induction was effectively inhibited in a dose-dependent manner by the compound 1 and 2 treatment (Fig. 6a and 6b). Similarly, in the case of mRNA expression of iNOS and COX-2. LPS-activated macrophages increased the protein expression of iNOS and COX-2 when compared to the untreated control group (Fig. 7).



a



b

Fig. 6. Suppressive effects of the compound 1 and 2 on LPS-induced protein expression of iNOS and COX-2 in RAW 264.7 cells. RAW 264.7 cells were pretreated with the compounds for 2 h and then stimulated with LPS (1 μg/ml) for 9 h. The cells were lysed, and the lysates were examined by Western blot for (a) iNOS and (b) COX-2. Results were expressed as a protein expressions ratio comparing with the LPS-treated group. The data were the means ± SDs for three independent experiments

**P < 0.05, **P < 0.01 compared with the LPS-only treatment*

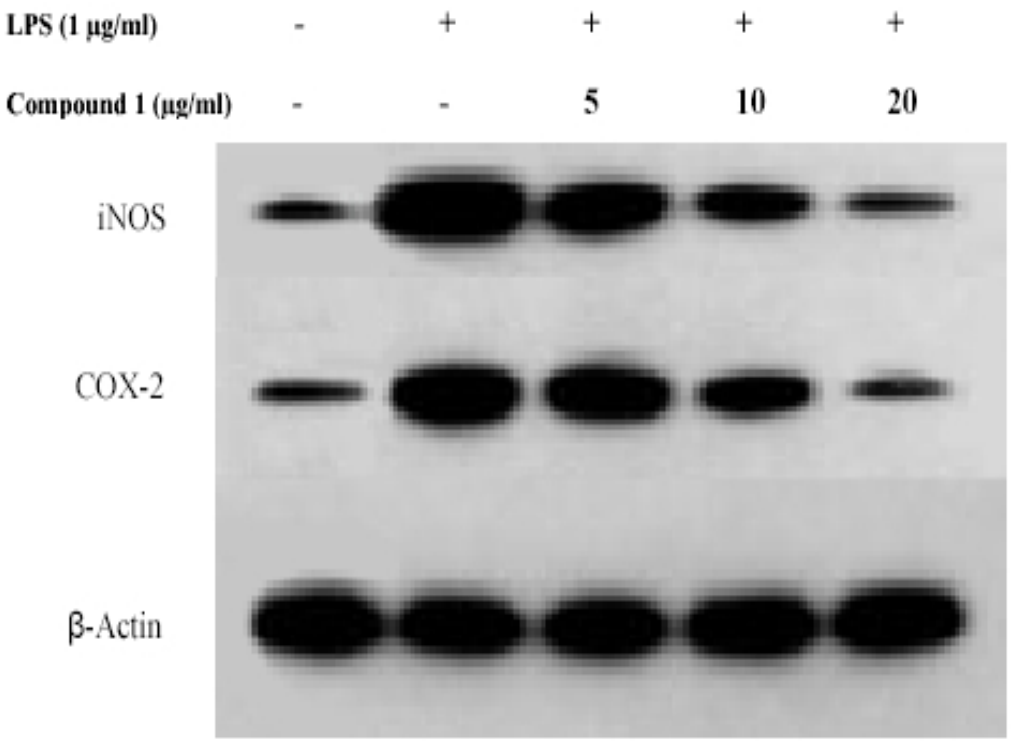


Fig. 7. Suppressive effects of compound 1 on LPS-induced protein expression of iNOS and COX-2 in RAW 264.7 cells. The cells were pretreated with different concentrations (5, 10, 20 μg/ml) of compound 1 for 2 h and then incubated with or without 1 μg/ml of LPS for 9 h. Protein samples were analyzed by Western blot with specific antibodies as described in materials and methods

3.6 Effects of the Compounds on NF-κB Activation in LPS-stimulated RAW 264.7 Cells

Only the LPS treatment significantly increased NF-κB as a transcription factor in RAW 264.7 cells, when compared to untreated LPS ($P < 0.01$). The compound 1 and 2 treatment with LPS significantly decreased the activation of NF-κB in a dose- and time-dependent manner ($P < 0.01$), when compared to only LPS treatment (Fig. 8a and 8b).

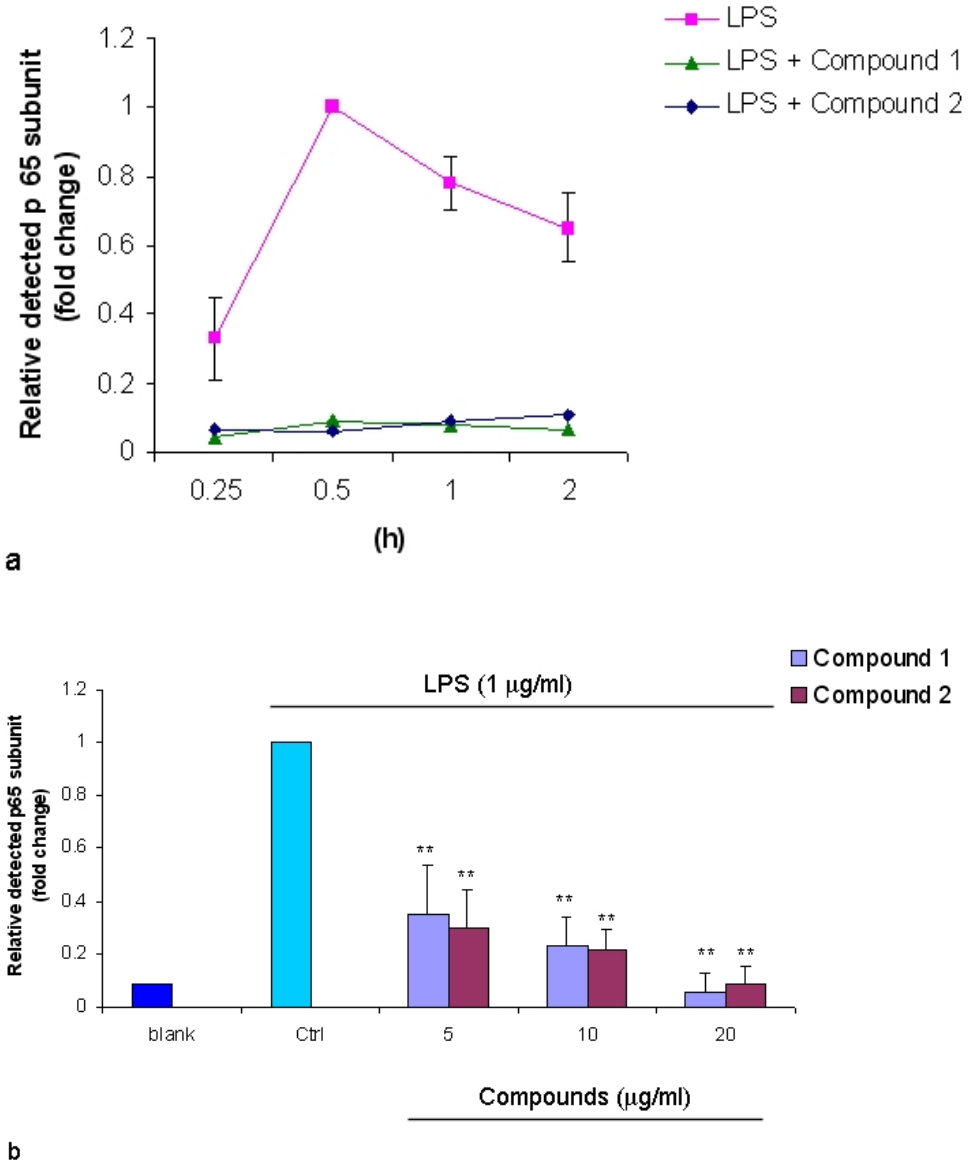


Fig. 8. Suppressive effects of the compound 1 and 2 on LPS-induced NF-κB activation in RAW 264.7 cells. Time-course (a) and dose-dependent (b) inhibitory effect of the compound 1 and 2 were measured on LPS-induced NF-κB activation in RAW 264.7 cells were measured using ELISA. (a) Cells were pretreated with the compounds (20 μg/ml) or not for 2 h, then stimulated with LPS (1 μg/ml) at various time points. (b) Dose-effect relationship was measured after 0.5 h stimulation with LPS (1 μg/ml). Results were expressed as a target gene expressions ratio comparing with the LPS-treated group. The data were the means ± SDs for three independent experiments

***P < 0.01 compared with the LPS-only treatment*

4. DISCUSSION

Previously, it was reported that the ethyl acetate extract of *Streptomyces* sp. BT01 culture possessed antibacterial activity [18]. The extract was purified by column chromatography and thin-layer chromatography. Two new flavonoids, 7-methoxy-3, 3',4',6-tetrahydroxyflavone (1) and 2',7-dihydroxy-4',5'-dimethoxyisoflavone (2) (Fig. 1) were obtained from the extracts. Flavonoids have biological activities including anti-inflammatory, anticancer, antimicrobial, antiviral, immunomodulatory, and antithrombotic activities [21]. In the present study the results demonstrated that the flavonoids isolated from *Streptomyces* sp. BT01 could inhibit inflammatory responses in LPS-induced RAW 264.7 macrophages.

It is known that macrophage plays an important role in the immune system as well as the inflammatory process. The activated macrophages can secrete a variety of inflammatory mediators and cytokines, including NO, PGE_2, TNF-α, IL-1β, and IL-6 [22]. However, chronic inflammation causes the increase of pro-inflammatory mediators and cytokines. These are active in the pathogenesis of various chronic inflammatory diseases such as multiple sclerosis, Parkinson's disease, Alzheimer's disease and colon cancer [23]. NO is a major product regulated by three distinct NOS isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). iNOS not only exists in healthy tissues, but also expressed after exposure to specific stimulants such as LPS and cytokines. The iNOS produces NO until the enzyme is decomposed [24]. Prostaglandins (PGs) play beneficial roles in almost every system and regulate different physiological processes including cell growth, ovulation, immunity, nerve growth and development and bone metabolism [25]. There are two major isoforms of cyclooxygenase; COX-1 and COX-2. COX-1 is expressed constitutively in many tissues and is associated with the synthesis of PGs involved in normal kidney and gastrointestinal function [26]. COX-2 is not detected in normal tissues, but is excessively induced by a variety of physiopathological conditions affecting tissues, such as growth factors, oncogenes, inflammatory stimuli and other ligands [27].

Many flavonoids for example; 4-methoxyhonokiol, poncirin genistein and apigenin inhibited LPS-induced expression of iNOS, COX-2 and cytokines through the inactivation of NF-κB in RAW 264.7 cells [28,29,30,31,32,33]. Thus, the regulation of iNOS and COX-2 is important in the inflammatory response. The present study examined the effect of the compound 1 and 2 on the expression of iNOS and COX-2 at the protein and mRNA levels. The results showed that the compound 1 and 2 dose-dependently suppressed the expression of iNOS and COX-2 at both the protein and mRNA levels in LPS-stimulated RAW 264.7 cells.

NF-κB is a major factor regulating the expression of inflammation-induced enzymes and cytokines such as iNOS, COX-2, TNF-α, IL-1β, and IL-6, which include the NF-κB binding sites in their promoters, and has attracted attention as a new target for treating inflammatory diseases [34,35,36]. Therefore, the suitable regulation of NF-κB may be beneficial in treating many inflammatory disorders. Earlier studies had demonstrated that various natural compounds including curcumin, green tea polyphenols, resveratrol and lactones inhibited NF-κB activation. Curcumin suppresses NOS by decreasing iKK and NF-κB activation in LPS-stimulated RAW 264.7 cells [37]. Green tea polyphenols and resveratrol inhibit NF-κB activation by suppressing IKK [38]. The extract from the root of *Panax notogingeng* inhibited LPS-induced inflammatory mediators, including iNOS and COX-2 by blocking I-κB degradation in the cytosol and the nuclear translocation of the NF-κB p65 subunit [39]. The present results showed that the compound 1 and 2 inhibited LPS-induced gene expression and overproduction of TNF-α, IL-1β, IL-6, iNOS and COX-2. The compound 1 and 2 significantly inhibited the activation of NF-κB in a dose and time-dependent manner, in

accordance with the suppressive effects on TNF-α, IL-1β, IL-6, iNOS and COX-2 production. The NF-κB plays a key role in the transcriptional up-regulation of the LPS-induced TNF-α, IL-1β, IL-6, iNOS and COX-2 [40,41,42]. These findings indicate that the suppression of NF-κB activation by the compound 1 and 2 is a possible mechanism of action for their anti-inflammatory activity.

In addition to cellular regulation affected by flavonoids, the various protein kinases such as protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) have been reported to be involved in signal transduction [21]. Through the inhibition of these enzymes, DNA-binding capacity of transcription factors such as NF-κB or activator protein-1 (AP-1) is regulated, and the expression rate of the gene target is controlled. Therefore, suppressing or inhibiting the activation of other inflammation-linked transcription factors. Signal transductions by these compounds should be studied further.

5. CONCLUSION

Our study confirmed the *in vitro* anti-inflammatory effects of two new flavonoids, 7-methoxy-3,3',4',6-tetrahydroxyflavone (1) and 2',7-dihydroxy-4'5'-dimethoxyisoflavone (2) isolated from *Streptomyces* sp. BT01. We also observed that these compounds could inhibit the production of NO, PGE_2, TNF-α, IL-1β, and IL-6 in LPS-stimulated RAW 264.7 cells. These compounds were able to regulate the mRNA expression of TNF-α, IL-1β, IL-6, iNOS and COX-2 in a time- and dose-dependent manner. These effects seem to be mediated by inhibiting the activation of NF-κB. We also found that these compounds could inhibit NF-κB activation in LPS-stimulated RAW 264.7 cells. These findings proved anti-inflammatory property of the new flavonoids isolated from *Streptomyces* sp. BT01. Hence, these flavonoids might be promising chemotherapeutic agents against inflammatory diseases.

ACKNOWLEDGEMENTS

This work was supported by Thailand Research Fund (Research grant No. RMU5480005) and Faculty of Science, Silpakorn University, Thailand.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Kim HP., Son KH, Chang HW, Kang SS. Anti-inflammatory plant flavonoids and cellular action mechanisms. J Pharmacol Sci. 2004;96:229-245.
2. Wietrzyk J, Grynkiewicz G, Opolski A. Phytoestrogens in cancer prevention and therapy - mechanisms of their biological activity. Anticancer Res. 2005;25:2357-2366.
3. Kao TH, Chen BH. Functional components in soybean cake and their effects on antioxidant activity. J Agri Food Chem. 2006;54:7544-7555.
4. Cushnie TP, Lamb AJ. Antimicrobial activity of flavonoids. Int J Antimicrob Agents. 2005;26:343-356.
5. Ialenti A, Ianaro A, Moncada S, Di Rosa M. Modulation of acute inflammation by endogenous nitric oxide. Eur J Pharmacol. 1992;211:177-182.

6. Stichtenoth DO, Frolich JC. Nitric oxide and inflammatory joint diseases. Br J Rheumatol. 1998;37:246-257.
7. Meng F, Lowell CA. Lipopolysaccharide (LPS)-induced macrophage activation and signal transduction in the absence of Src-family kinases Hck, Fgr, and Lyn. J Exp Med. 1997;185:1661-1670.
8. Morrison DC, Danner RL, Dinarello CA, et al. Bacterial endotoxins and pathogenesis of Gram-negative infections: Current status and future direction. Innate Immun. 1994;1:71-83.
9. Levine B, Kalman J, Mayer L, Fillit HM, Packer M. Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. N Engl J Med. 1990;323:236-241.
10. Kielian T, Bearden ED, Baldwin AC, Esen N. IL-1 and TNF-alpha play a pivotal role in the host immune response in a mouse model of *Staphylococcus aureus*-induced experimental brain abscess. J Neuropathol Exp Neurol. 2004;63:381-396.
11. Morrissey PJ, Charrier K. Treatment of mice with IL-1 before infection increases resistance to a lethal challenge with Salmonella typhimurium. The effect correlates with the resistance allele at the Ity locus. J Immunol. 1994;153:212-219.
12. Bartold PM, Haynes DR. Interleukin-6 production by human gingival fibroblasts. J Periodontal Res. 1991;26:339-345.
13. Cartmell T, Poole S, Turnbull AV, Rothwell NJ, Luheshi GN. Circulating interleukin-6 mediates the febrile response to localized inflammation in rats. J Physiol. 2000;526:653-661.
14. Lu XY, Wang ZC, Yang GZ. The relationships between IL-1, IL-6 and tumor. Guowai Yixue Shengli Bingli Kexue Yu Linchuang Fence. 1995;15:159-163.
15. Taechowisan T, Wanbanjob A, Tuntiwachwuttikul P, Liu J. Anti-inflammatory activity of lansais from endophyte *Streptomyces* sp. SUC1 in LPS-induced RAW 264.7 cells. Food Agri Immunol. 2009;20:67-77.
16. Taechowisan T, Wanbanjob A, Tuntiwachwuttikul P, Liu J. Anti-inflammatory effects of lansai C and D cause inhibition of STAT-1 and NF-κB activation in LPS-induced RAW 264.7 cells. Food Agri Immunol. 2010;21:57-64.
17. Taechowisan T, Peberdy JF, Lumyong S. Isolation of endophytic actinomycetes from selected plants and their antifungal activity. World J Microbiol Biotechnol. 2003;19:381-385.
18. Taechowisan T, Chanaphat S, Ruensamran W, Phutdhawong WS. Antibacterial activity of new flavonoids from *Streptomyces* sp. BT01; an endophyte in *Boesenbergia rotunda* (L.) Mansf. J App Pharm Sci. 2014;4:8-13.
19. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Anal Biochem. 1982;126:131-138.
20. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65:55-63.
21. García-Lafuente A, Guilamón E, Villares A, Rostagno MA, Martínez JA. Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. Inflamm Res. 2009;58:537-552.
22. Walsh LJ. Mast cells and oral inflammation. Crit Rev Oral Biol Med. 2003;14:188-198.
23. Heiss E, Herhaus C, Klimo K, Bartsch H, Gerhauser C. Nuclear factor kappaB is a molecular target for sulforaphane-mediated anti-inflammatory mechanisms. J Biol Chem. 2001;276:32008-32015.

24. MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. Annu Rev Immunol. 1997;15:323-350.
25. Gupta RA, Dubois RN. Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nat Rev Cancer. 2001;1:11-21.
26. Fletcher JR. Eicosanoids. Critical agents in the physiological process and cellular injury. Arch Surg. 1993;128:1192-1196.
27. Herschman HR. Prostaglandin synthase 2. Biochim Biophys Acta. 1996;1299:125-140.
28. Zhou HY, Shin EM, Guo LY, et al. Anti-inflammatory activity of 4-methoxyhonokiol is a function of the inhibition of iNOS and COX-2 expression in RAW 264.7 macrophages via NF-kappaB, JNK and p38 MAPK inactivation. Eur J Pharmacol. 2008;586:340-349.
29. Kim JB, Han AR, Park EY, et al. Inhibition of LPS-induced iNOS, COX-2 and cytokines expression by poncirin through the NF-kappaB inactivation in RAW 264.7 macrophage cells. Biol Pharm Bull. 2007;30:2345-2351.
30. Li Y, Sarkar FH. Inhibition of nuclear factor kappaB activation in PC3 cells by genistenin is mediated via Akt signalling pathway. Clin Cancer Res. 2002;8:2369-2377.
31. Davis JN, Kucuk O, Sarkar FH. Genistein inhibits NF-kappaB activation in prostate cancer cells. Nutr Cancer. 1999;35:167-174.
32. Rahman KW, Li Y, Sarkar FH. Inactivation of Akt and NF-kappaB play important roles during indole-3-carbinol-induced apoptosis in breast cancer cells. Nutr Cancer. 2004;48:84-94.
33. Shuka S, Gupta S. Suppression of constitutive and tumor necrosis factor alpha-induced nuclear factor (NF) –kappaB activation and induction of apoptosisby apigenin in human prostate carcinoma PC-3 cells: correlation with down-regulation of NF-kappaB-responsive genes. Clin Cancer Res. 2004;10:3169-3178.
34. Lawrence T, Gilroy DW, Colville-Nash PR, Willoughby DA. Possible new role for NF-kappaB in the resolution of inflammation. Nat Med. 2001;7:1291-1297.
35. Shan J, Fu J, Zhao Z, et al. Chlorogenic acid inhibits lipopolysaccharide-induced cyclooxygenase-2 expression in RAW 264.7 cells through suppressing NF-κB and JNK/AP-1 activation. Int Immunopharmacol. 2009;9:1042-1048.
36. Murad JM, Calvi SA, Soares AMVC, Bankova V, Sforcin JM. Effects of propolis from Brazil and Bulgaria on fungicidal activity of macrophages against *Paracoccidioides brasiliensis.* J Ethnopharmacol. 2002;79:331-334.
37. Pan MH, Lin-Shiau SY, Lin JK. Comparative studies on the suppression of nitric oxide synthase by curcumin and its hydrogenated metabolites through down-regulation of IkappaB kinase and NFkappaB activation in macrophages. Biochem Pharmacol. 2000;60:1665-1676.
38. Holmes-McNary M, Baldwin AS. Chemopreventive properties of trans-reveratrol are associated with inhibition of activation of the IkappaB kinase. Cancer Res. 2000;60:3477-3483.
39. Jung HW, Seo UK, Kim JH, Leem KH, Park YK. Flower extract of Panax notoginseng attenuates lipopolysaccharide-induced inflammatory response via blocking of NF-kappaB signalling pathway in murine macrophages. J Ethnopharmacol. 2009;122:313-319.

40. Cogswell JP, Godlevski MM, Wisely GB, Clay WC, Leesnitzer LM, Ways JP, Gray JG. NF-kappaB regulates IL-1 beta transcription through a consensus NF-kappaB binding site and a nonconsensus CRE-like site. J Immunol. 1994;153:712-723.

41. Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cell Signal. 2001;13:85-94.

42. Lim S, Kang KW, Park SY, et al. Inhibition of lipopolysaccharide-induced inducible nitric oxide synthase expression by a novel compound, mercaptopyrazine, through suppression of nuclear factor-kappaB binding to DNA. Biochem Pharmacol. 2004;68:719-728.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=621&id=5&aid=5662