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Research Article

EVALUATION OF ANTI-INFLAMMATORY ACTIVITY OF NOVEL SYNTHESIZED PHENYLPROPANOID ESTERS

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ABSTRACT

Inflammation plays an important role in the pathogenesis of various chronic and acute diseases. Our previous study showed that cimiracemate A (PubChem: CID 5315874) (**1**) possessed potent anti-inflammatory effect by suppressing LPS-induced TNF-α production in primary blood macrophage and inhibiting the 5-lipoxygenase enzymatic activity. In light of the biological activities of compound-**1**, we synthesize 3 analogues of compound-**1** and then screen their anti-inflammatory effects. The results showed that (E)-2-oxo-3-phenylpropyl 3-(3-hydroxy-4-methoxyphenyl)acrylate (**4**) was toxic to primary blood macrophage at concentration of 50 $\mu g.mL^{-1}$. (E)-3-(3,4-dihydroxyphenyl)-2-oxopropyl 3-(4-methoxyphenyl)acrylate (**3**) showed the highest suppressive effect on LPS-induced TNF-α production in primary blood macrophage when compared with compound-**1** and (E)-3-(3-hydroxyphenyl)-2-oxopropyl 3-(3,4-dihydroxyphenyl)acrylate (**2**). Compounds-**1** and -**3** showed similar inhibitive effects on LPS-induced mRNA levels of cytokines (TNF-α, IL-6, and IL-1β) and chemokines (IP-10, CCL-3, and CCL-5). Compound-**3** showed greater suppressive effect than compound-**1** on LPS-induced nitric oxide production in RAW264.7 and BV-2 cells. The result revealed that compound-**3** may have the potential to be further developed as a new class of anti-inflammatory therapeutic agent.

Keywords: Synthesis; phenylpropanoid esters; cimiracemate A; anti-inflammation; cytokines; chemokines; nitric oxide

INTRODUCTION

Inflammation plays an important role in the pathogenesis of various diseases including cardiovascular diseases (CVD) (Kofler, *et al.*, 2005), autoimmune diseases such as rheumatoid arthritis (McInnes, *et al.*, 2007), neurodegenerative diseases (Smith, *et al.*, 2012) and cancers (Seruga, *et al.*, 2008 and Taniguchi, *et al.*, 2014). These diseases are major contributors of morbidity, mortality and economic burden worldwide. According to the WHO, CVD and cancers killed nearly 17.7 million and 8.8 million people in 2015, respectively (WHO, 2015).

In response to injury and infection, human host mounts inflammatory responses against the pathogens and initiates the repair process (Tracey, *et al.*, 2002). During inflammation, a broad range of immune cells including macrophages, B lymphocytes, T lymphocytes and mast cells are recruited to the site of injury or infection and secreting a wide variety of inflammatory mediators to trigger a cascade of immune response. However, the dysregulation of the cytokines including interleukins (IL-1 and IL-6), tumor necrosis factor-α (TNF-α) and chemokines (IP-10, CCL-3, and CCL-5) have been implicated in various acute and chronic diseases (Tracey, *et al.*, 1993 and Tatemichi, *et al.*, 2010). These mediators were demonstrated to be involved in initiation of tumor growth (Aggarwal, *et al.*, 2006), cell proliferation (Woodworth, *et al.*, 1995), invasion (Tatemichi, *et al.*, 2010; Apte, *et al.*, 2006 and Montesano, *et al.*, 2005), and tumor metastasis (Apte, *et al.*, 2006; Egberts, *et al.*, 2008 and Yadav, *et al.*, 2011). These cytokines are not usually detectable in healthy individuals, but elevated serum and/or tissue levels are found in patients (Arvidson, *et al.*, 1994; Pavese, *et al.*, 2010; Levine, *et al.*, 1990; Khemka, *et al.*, 2014 and Ferrari, *et al.*, 2011) and correlated with the severity of diseases (Michalaki, *et al.*, 2004). Hence, novel targeted therapies directly inhibiting IL-1, IL-6, or TNF-α production have been developed as salvage treatment for refractory acute and chronic autoimmune diseases.

Nitric oxide (NO) is a free radical generated in mammalian cells and tissues. It serves as mediator in the regulation of physiological or even pathophysiological mechanisms involving cardiovascular, nervous and immunological systems (Aktan, *et al.*, 2004). Upon stimulation by oxidants, lipopolysaccharide (LPS), bacteria, viruses, and proinflammatory cytokines, NO is synthesized by iNOS. NO is cytotoxic upon further interaction with

superoxide anions to form peroxynitrite (ONOO−), which becomes the most reactive nitrogen species (RNS). Excess production of NO and RNS can damage DNA, lipids, proteins, and carbohydrates, leading to impairment of the cellular functions and enhancement of the inflammatory reactions.

Cimicifuga racemosa (CR), also known as black cohosh, is native to the Europeans and North Americans. In our recent study, an active compound, cimiracemate A (compound-**1**), was isolated from the herbal extract of CR. This compound showed anti-inflammatory effect by suppressing LPS-induced TNF-α production in primary blood macrophage (Yang, *et al.*, 2009). It is suggested that the anti-inflammatory activity of compound-**1** is due to the modulation of the signaling mitogen activated-protein kinase (MAPK) and transcription factor nuclear factor-κB (NF-κB) activities. Besides, compound-**1** is also an antioxidant that reduces the single-strand DNA damage induced by menadione in breast cancer cells in a dose dependent manner (Burdette, *et al.*, 2002).

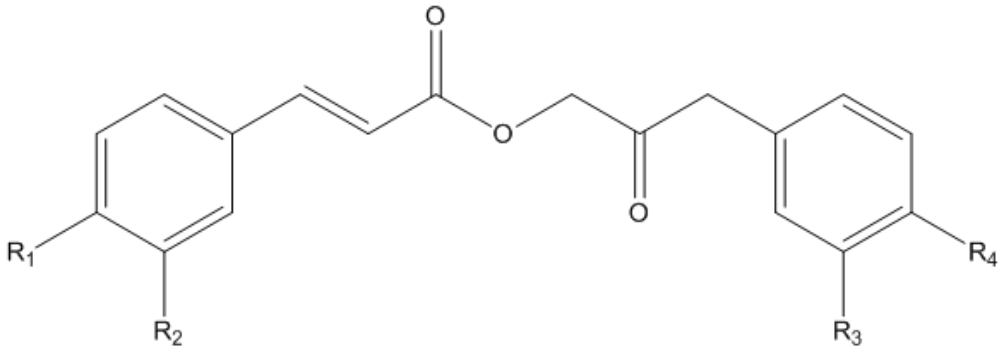
Based on our previous study, the content of compound-**1** in CR extract was around 5% (Lau, *et al.*, 2013). In light of the potent biological activities of compound-**1**, we aim (1) to synthesize compound-**1** and three new analogues of compound-**1** by chemical modification; and (2) to test their respective anti-inflammatory activities.

MATERIALS AND METHODS

Synthesis of compound-1

2-(3,4-dihydroxyphenyl)acetic acid (A-1) was dissolved in methanol (MeOH) containing 2.5% concentrated sulfuric acid and then stirred and refluxed for 3 h. The solvent was then evaporated under reduced pressure to afford an intermediate of A-2. A-2 was dissolved in acetone followed by the addition of potassium carbonate and allyl bromide (AllylBr). The mixture was then refluxed overnight. After cooling to room temperature, the mixture was filtered and the filtrate was concentrated under reduced pressure to afford A-3. A-3 was dissolved in MeOH and followed by the addition of 3M sodium dioxide (NaOH) dropwise at 0°C. The mixture was then stirred at room temperature overnight. After the reaction was

completed, the mixture was acidified with 3M hydrochloric acid (HCl) to pH=2-3 and the precipitate was filtered and washed with petroleum ether to afford A-4. A-4 was dissolved in anhydrous dichloromethane (CH_2Cl_2) and stirred at 0°C. A solution of Cobalt (II) chloride was added dropwise to the mixture, followed by a drop of dimethylformamide (DMF). The resulting mixture was allowed to stir for 3 h at room temperature. Then the solvent was evaporated to afford A-5. A-5 was dissolved in tetrahydrofuran (THF) and then trimethylsilyldiazomethane ($TMSCHN_2$) was added to the mixture dropwise. The mixture was stirred at room temperature overnight. THF was removed in vacuo and the residue was re-dissolved in THF (200 mL). Then hydrogen bromide (47% in H_2O) was added dropwise. The mixture was stirred at room temperature for 5 min and then added with aqueous sodium hydrogen carbonate ($NaHCO_3$) following the extraction with ethyl acetate (EtOAc) (100 mL x 3). The combined organic layer was washed with brine, dried over sodium sulfate (Na_2SO_4), filtered and evaporated. The crude product was purified by silica gel to give A-6.



Compounds	R_1	R_2	R_3	R_4
1	OCH_3	OH	OH	OH
2	OH	OH	OH	-
3	OCH_3	-	OH	OH
4	OCH_3	OH	-	-

Figure 1: Chemical structures of cimiracemate A (**1**) and its analogues (compounds-**2**, -**3**, and -**4**).

Isoferulic acid (A-7) (1-fold), AllylBr and potassium carbonate (K_2CO_3) were dissolved in acetone and then refluxed overnight. After cooling, the mixture was filtered and concentrated to afford A-8. A-8 was dissolved in 10-fold MeOH following the addition of aqueous $NaHCO_3$ (3.36M) and the mixture was refluxed overnight. After cooling, the mixture was

acidified by 3M HCl until pH reached 2-3. The mixture was filtered to afford A-9. A-9, potassium iodide (KI) and K_2CO_3 were dissolved in dimethylformamide (DMF) followed by addition of A-6. The mixture was stirred at room temperature overnight. The reaction mixture was partitioned with EtOAc (100 mL x 3) following washed with H_2O and brine and then dried over Na_2SO_4 to give A-10. A-10 and tetrakis(triphenylphosphine)palladium [$Pd(Ph_3P)_4$] were dissolved in THF. Morpholine was added. The reaction mixture was stirred at room temperature for 3 h. The mixture was acidified by 1 M HCl, extracted with EtOAc (60 mL x 3) and washed with brine. The organic layer was combined, dried over Na_2SO_4. The resulting extract was filtered, concentrated and purified by silica gel to give Cimiracemate A (Figure 1). The procedures were also summarized in Figure 2.

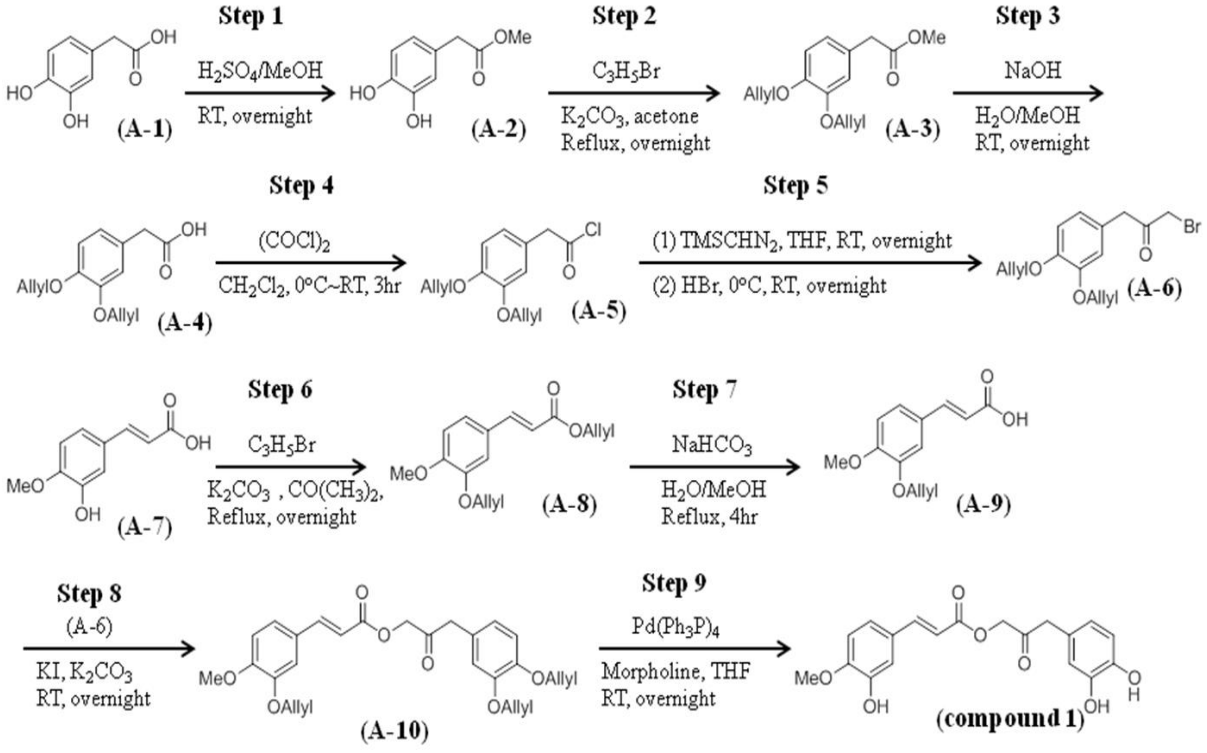


Figure 2: Synthetic pathways of compound-**1**.

Synthesis of compound-2

2-(3-hydroxyphenyl)acetic acid (A1-1), AllylBr and K_2CO_3 were dissolved in acetone and refluxed overnight. After cooling, the reaction mixture was filtered and concentrated to give

A1-2. A1-2 was dissolved in MeOH. Following the addition of aqueous 3M $NaHCO_3$, the mixture was refluxed for 4 h. After cooling, the mixture was acidified by 3M HCl to pH=2-3. A1-3 was obtained after filtration. A1-3 was dissolved in anhydrous CH_2Cl_2 and stirred at $0^{\circ}C$. A solution of $(COCl)_2$ was added to the mixture dropwise, followed by a drop of DMF. The mixture was allowed to stir for 3 h at room temperature. Then the solvent was removed to give A1-4. $TMSCHN_2$ was added to the solution of A1-4 in THF dropwise. The mixture was stirred at room temperature overnight. THF was removed in vacuo and the residue was dissolved in THF. Then HBr (47% in H_2O) was added dropwise to the mixture with stirring for 5 min at room temperature. Then, the aqueous $NaHCO_3$ was added and the resulting mixture was extracted with EtOAc for three times. The combined organic layer was washed with brine and dried over Na_2SO_4. The crude product was purified by silica gel column chromatography to give A1-5.

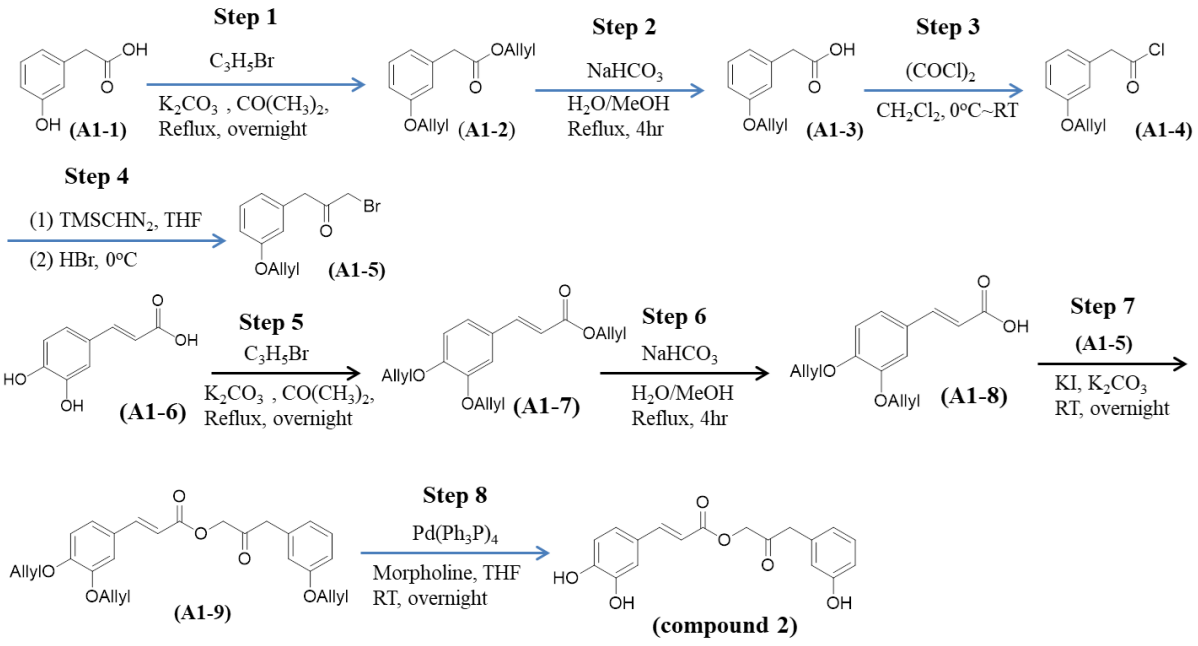


Figure 3: Synthetic pathways of compound-**2**.

2-(3,4-dihydroxyphenyl)acetic acid (A1-6), AllylBr and potassium carbonate (K_2CO_3) were dissolved in acetone and then refluxed overnight. After cooling, the mixture was filtered and concentrated to afford A1-7. A1-7 was dissolved in 10-fold MeOH following the addition of aqueous $NaHCO_3$ (3.36M) and the mixture was refluxed for 4 h. After cooling, the mixture

was acidified by 3M HCl until pH reached 2-3. The mixture was filtered to afford A1-8. A1-8, KI and K_2CO_3 were dissolved in dimethylformamide (DMF) followed by addition of A1-5. The mixture was stirred at room temperature overnight. The reaction mixture was partitioned with EtOAc (100 mL x 3) and then washed with H_2O and brine and then dried over Na_2SO_4 to give A1-9. A1-9 and tetrakis(triphenylphosphine)palladium [$Pd(Ph_3P)_4$] were dissolved in THF. Morpholine was added. The reaction mixture was stirred at room temperature overnight. The mixture was acidified by 1 M HCl, extracted with EtOAc (60 mL x 3) and washed with brine. The organic layer was combined, dried over Na_2SO_4. The resulting extract was filtered, concentrated and purified by silica gel column chromatography to give (E)-3-(3-hydroxyphenyl)-2-oxopropyl 3-(3,4-dihydroxyphenyl)acrylate (compound- **2**) (Figure 1). The procedures were also summarized in Figure 3.

Synthesis of compound-3

(E)-3-(4-methoxyphenyl)acrylic acid (A2-1), KI and K_2CO_3 were dissolved in dimethylformamide (DMF) followed by addition of A-6. The mixture was stirred at room temperature overnight. The reaction mixture was partitioned with EtOAc (100 mL x 3) following washed with H_2O and brine and then dried over Na_2SO_4 to give A2-2. A2-2 and tetrakis(triphenylphosphine)palladium [$Pd(Ph_3P)_4$] were dissolved in THF. Morpholine was added.

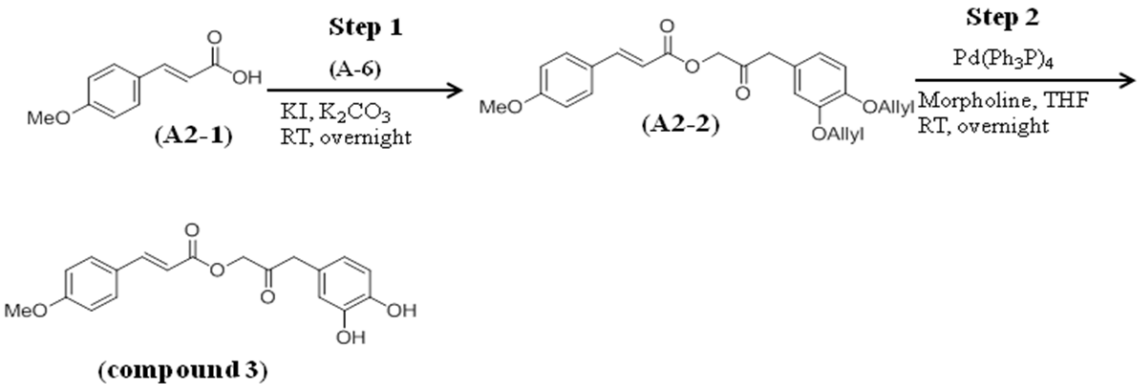


Figure 4: Synthetic pathways of compound-**3**.

The reaction mixture was stirred at room temperature for 3 h. The mixture was acidified by 1 M HCl, extracted with EtOAc (60 mL x 3) and washed with brine. The organic layer was

combined, dried over Na_2SO_4. The resulting extract was filtered, concentrated and purified by silica gel column chromatography to give (E)-3-(3,4-dihydroxyphenyl)-2-oxopropyl 3-(4-methoxyphenyl)acrylate (compound- **3**) (Figure 1). The procedures were also summarized in Figure 4.

Synthesis of compound-4

A3-1 was dissolved in anhydrous CH_2Cl_2 and stirred at 0°C. A solution of $(COCl)_2$ was added dropwise, followed by the addition of a drop of DMF. The mixture was allowed to stir for 3 h at room temperature. A3-2 was obtained after removing the solvents. $TMSCHN_2$ was added to a solution of A3-2 in THF dropwise. The mixture was stirred at room temperature overnight. THF was removed in vacuo and the residue was re-dissolved in THF. Then HBr (47% in H_2O) was added dropwise. The mixture was stirred at room temperature for 5 min. After adding aqueous $NaHCO_3$ to the mixture, it was extracted with EtOAc. The combined organic layer was washed with brine and dried over Na_2SO_4. A3-3 was obtained after purified by silica gel column chromatography. A-9, KI and K_2CO_3 were dissolved in DMF followed by the addition of A3-3. The mixture was stirred at room temperature overnight. The reaction mixture was participated with EtOAc and washed with H_2O and brine. After dried over Na_2SO_4, A3-4 was obtained. A3-4 and $Pd(Ph_3P)_4$ were dissolved in THF. Morpholine was added. The reaction mixture was stirred at room temperature overnight. The solvent was removed and followed by extracting with EtOAc. The organic layer was washed with brine and purified by silica gel column chromatography to give (E)-2-oxo-3-phenylpropyl 3-(3-hydroxy-4-methoxyphenyl)acrylate (compound-**4**) (Figure 1). The procedures were also summarized in Figure 5.

Structure identification

The structures of the resulting pure compounds were elucidated by using a Bruker 400 MHz NMR spectrometer, operating at 400 MHz for ^1H and at 100 MHz for ^{13}C NMR, using DMSO or $CDCl_3$ as the solvent. Shimadzu 2010 HPLC (Column: Sepax ODS 50 × 2.0 mm, 5 μm) or Agilent 1200 HPLC (Column: Shim-pack XR-ODS 30 × 3.0 mm, 2.2 μm) equipped with a quadrupole Mass Spectrometer (1956 MSD), which operated in ES (+) ionization mode.

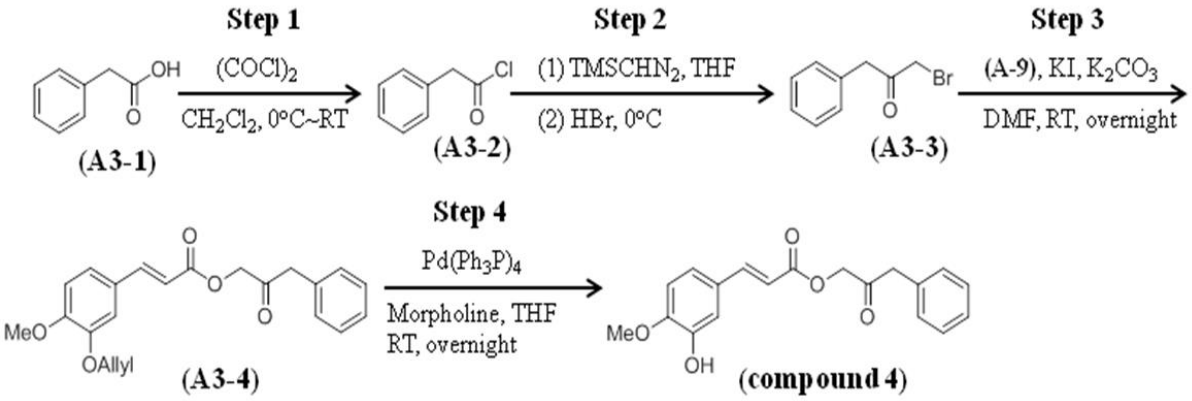


Figure 5: Synthetic pathways of compound-**4**.

Cell Culture and Primary Blood Macrophage Isolation

Human peripheral blood monocytic cells (PBMC) were isolated from the buffy coat of healthy donor blood supplied by Hong Kong Red Cross by Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ) density gradient centrifugation as described in our previous reports (Mackowiak, *et al.*, 2000). The PBMC were seeded onto tissue culture plates and incubated for another 14 days in order to differentiate the freshly isolated monocytic cells to primary blood macrophages (PBMac).

BV-2, a murine microglial cell line, was kindly provided by Prof. E Choi from Laboratory of Cell Death and Human Diseases, Korea University. RAW264.7, a murine macrophage cell line, was obtained from the American Type Culture Collection (Rockville, MD). Both cell lines were maintained in Dulbecco's modified Eagle's Minimum Essential Medium (DMEM) supplemented with 10% FBS and 1% Penicillin and Streptomycin (Invitrogen Life Technologies, Carlsbad, CA). The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2.

Isolation of RNA and Quantitative PCR (QPCR)

Total RNA from PBMac was extracted by TRIzol (Invitrogen). Reverse transcription of mRNA to cDNA was performed by SuperScript II system (Invitrogen) as per the

manufacturer's instruction. QPCR was performed according to the manufacturer's instructions by Applied Biosystems TaqMan Universal Master Mix. TNF-α, IL-6, IL-1β, IP-10, CCL-3 and CCL-5 TaqMan probes were purchased from Applied Biosystems (Foster City, CA), and GAPDH RNA was used as an internal control. Samples were allowed to run in duplicate in each QPCR assay.

Enzyme-Linked ImmunoSorbent Assay (ELISA)

Culture supernatants of the LPS-induced PBMac with or without treatment were collected and stored at -70°C. The TNF-α levels were determined by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Nitrite measurement

Nitrite levels in the culture media were determined by Griess reaction according to the manufacturer's instructions (Sigma Aldrich, St. Louis, MO). Fresh culture media were used as blanks and the nitrite levels were determined by a standard sodium nitrite curve.

Statistical Analysis

Differences between the means of the experimental groups were analyzed with the two-tailed Student's *t* test. A *p* value less than 0.05 and 0.01 were considered statistically significant.

RESULTS

Chemistry

The synthetic schemes for the preparation of compounds-**1**, **-2**, **-3** and **-4** are summarized in Figures 2-5. Compound-**1**: The NMR and MS spectroscopic data are similar with the data reported in our previous study (Yang, *et al.*, 2009). Compound-**2**: The ^{1}H NMR (400 MHz, DMSO) of compound-**2** showed signals at δ 9.649 (s, 1H, OH), 9.370 (s, 1H, OH), 9.175 (s, 1H, OH), 7.540-7.500 (d, 1H, CH), 7.132-7.020 (m, 3H, ArH), 6.784-6.763 (m, 1H, ArH),

6.649-6.631 (m, 3H, ArH), 6.357-6.317 (d, 1H, CH), 4.934 (s, 2H, CH_2), 3.729 (s, 2H, CH_2). Compound-**2** showed a $[M+H]^+$ ion peak at m/z 329.1 in its ESI-MS, consistent with the molecular formula $C_{18}H_{16}O_6$ (calc. 328.09). Compound-**3**: The ^1H NMR (400 MHz, DMSO) of compound **3** showed signals at δ 8.885-8.801 (m, 2H, 2OH), 7.719-7.627 (m, 3H, ArH), 7.002-6.9803 (m, 2H, ArH), 6.680-6.660 (d, 1H, CH), 6.612-6.538 (m, 2H, ArH), 6.491-6.466 (d, 1H, CH), 4.916 (s, 2H, CH_2), 3.809 (s, 3H, CH_3), 3.612 (s, 2H, CH_2). Compound-**3** showed a $[M+H]^+$ ion peak at m/z 343.2 in its ESI-MS, consistent with the molecular formula $C_{19}H_{18}O_6$ (calc. 342.11). Compound-**4**: The ^1H NMR (400 MHz, $CDCl_3$) of compound **4** showed signals at δ 7.699-7.660 (d, 1H, CH), 7.390-7.258 (m, 5H, ArH), 7.173-7.167 (m, 1H, ArH), 7.080-7.054 (m, 1H, ArH), 6.885-6.365 (m, 1H, ArH), 6.405-6.365 (d, 1H, CH), 5.654 (s, 1H, OH), 4.827 (s, 2H, CH_2), 3.957 (s, 3H, CH_3), 3.813 (s, 2H, CH_2). Compound-**4** showed a $[M+H]^+$ ion peak at m/z 327.1 in its ESI-MS, consistent with the molecular formula $C_{19}H_{18}O_5$ (calc. 326.12).

Cytotoxicity

The compounds were tested on primary blood macrophage (PBMac). PBMac were incubated with compounds-**1**, **-2**, **-3**, and **-4** at concentrations of 25 μg.mL^{-1}, 50 μg.mL^{-1}and 75 μg.mL^{-1} for 48 h. It was found that compounds-**1**, **-2**, and **-3** were not cytotoxic to the cells. However, significant morphological changes of PBMac were found after the treatment of compound-**4** at concentration of 50 μg.mL^{-1} for 48 h (data not shown).

Effects of compounds-1, -2, -3 and -4 on LPS-induced TNF-α protein production

The anti-inflammatory effect of compounds-**1**, **-2**, and **-3** on proinflammatory cytokine TNF-α production was examined. PBMac were incubated with compounds-**1**, **-2**, and **-3** for 18 h prior to the treatment of LPS at concentration of 10 ngmL^{-1} for another 24 h. The culture supernatants were collected and TNF-α protein levels were measured by ELISA. The results showed that compounds-**1**, **-2**, and **-3** suppressed LPS-induced TNF-α protein production. At the concentration of 50 μgmL^{-1}, compounds-**1**, **-2**, and **-3** suppressed the LPS-induced TNF-α protein production by 49.8 ± 19.2% (Figure 6A), 25.6 ± 15.0% (Figure 6B), 66.6 ± 10.2%

(Figure 6C), respectively. As compound-**4** was cytotoxic to PBMac at concentration of 50 µg.mL^{-1}, a lower concentration (25 µg.mL^{-1}) of compound-**4** was tested against LPS-induced TNF-α protein production. The result showed that compound-**4** did not significantly suppress LPS-induced TNF-α protein production (data not shown). In summary, compound-**3** showed the most prominent suppressive effect on LPS-induced TNF-α protein production. Furthermore, the suppressive effect on other cytokines and chemokines by compound-**1** and compound-**3** were studied.

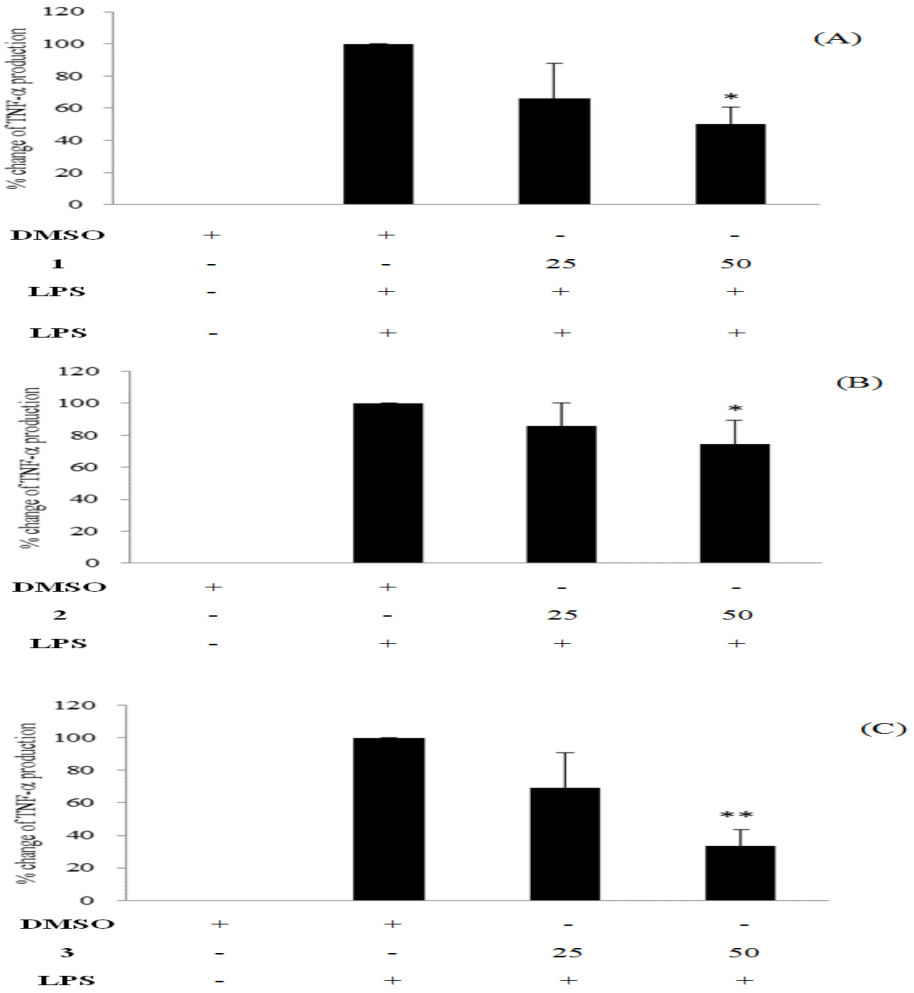


Figure 6: Inhibition of LPS-induced TNF-α production by compounds-1, -2, and -3. PBMac were incubated with 25 or 50 µg.mL-1 of compounds (A)-1, (B) -2 or (C) -3 for 18 h prior to the addition of 10 ngmL-1 LPS for another 24 h. The culture supernatants were collected and assayed for TNF-α by ELISA. The results shown were the mean values ± standard derivation

(S.D.) of at least three independent experiments, with cells obtained from different donors. * P<0.05, ** P<0.01 compared with DMSO + LPS control.

Effects of compounds-1 and 3 on LPS-induced cytokines and chemokines gene expressions

We further examined the effects of compounds-**1** and **-3** on gene expressions of other LPS-induced proinflammatory cytokines and chemokines. PBMac were incubated with compounds-**1** or **-3** for 18 h prior to the treatment of LPS at concentration of 10 ng.mL^{-1} for another 3 h. Quantitative PCR assays for the cytokines and chemokines were performed. The results indicated that compound-**3** (50 μg.mL^{-1}) suppressed not only LPS-induced TNF-α mRNA expression by 42.5 ± 19.0%, but also LPS-induced IL-6 and IL-1β mRNA expressions by 54.7 ± 28.3% and 40.6 ± 12.9%, respectively (Figure 7A). The percentages of suppressions were comparable to those exerted by compound-**1** (50 μg.mL^{-1}) on TNF-α mRNA (53.9 ± 8.7%), IL-6 mRNA (68.1 ± 15.6%) and IL-1β mRNA (47.6 ± 14.3%) production (Figure 7A). Similar percentages of TNF-α and IL-6 suppressions were found at lower concentration (25 μgmL^{-1}) of compounds-**1** and **-3** (Figure 7A).

Furthermore, compounds-**1** and **-3** showed similar potent suppression on LPS-induced IP-10 mRNA expression by 99.2 ± 1.1% and 99.4 ± 0.3%, respectively (Figure 7B). The suppressions were also shown in other chemokines including CCL-3 and CCL-5. At concentration of 50 μg.mL^{-1}, compound-**3** suppressed the LPS-induced CCL-3 mRNA expression by 59.3 ± 3.4% and CCL-5 mRNA expression by 76.6 ± 4.8% (Figure 7B). The percentages of suppressions were comparable to those exerted by compound-**1** (50 μg.mL^{-1}) on CCL-3 mRNA (49.2 ± 3.9%) and CCL-5 mRNA (74.6 ± 8.6%) expressions (Figure 7B). In summary, both compounds-**1** and **-3** suppressed the LPS-induced mRNA expression of proinflammatory cytokines TNF-α, IL-6 and IL-1β. In addition, both compounds down-regulated the LPS-induced mRNA expression of chemokines IP-10, CCL-3 and CCL-5.

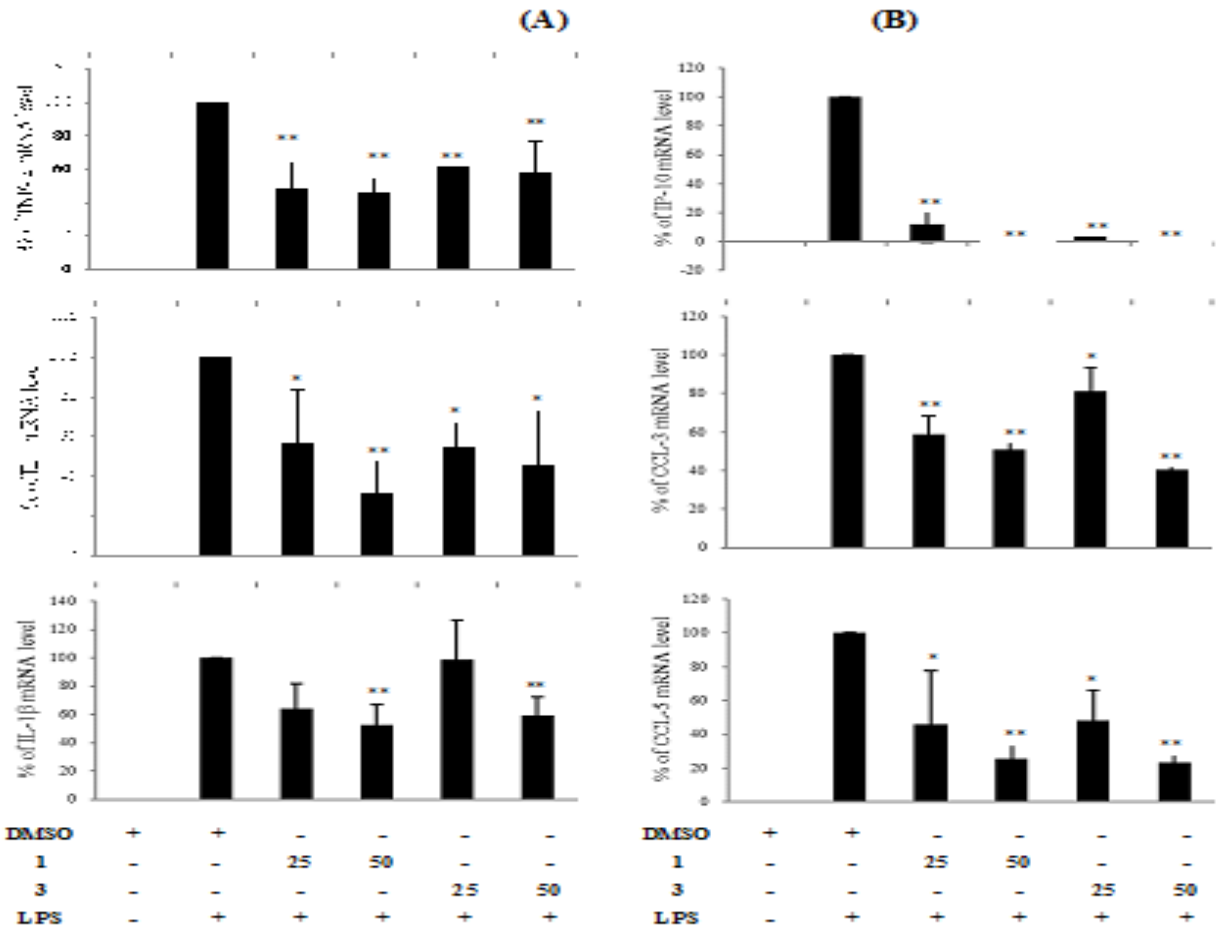


Figure 7: Inhibition of LPS-induced mRNA levels of (A) cytokines and (B) chemokines by compounds-1 and -3. PBMac were incubated with 25 or 50 μg.mL^{-1} of compounds-1 or -3 for 18 h prior to the addition of 10 ng.mL^{-1} LPS for another 3 h. Quantitative PCR assays for cytokines and chemokines were performed afterward. The results shown were the mean values ± standard derivation (S.D.) of at least three independent experiments, with cells obtained from different donors. * P<0.05, ** P<0.01 compared with DMSO + LPS control.

Effects of compounds-1 and -3 on LPS-induced nitric oxide production

The effect of compounds-**1** and **-3** on inflammatory mediator nitric oxide production was also examined. RAW264.7 and BV-2 cells were incubated with compounds-**1** or **-3** for 18 h prior to the treatment of LPS at concentration of 100 ng/mL for another 18 h. The culture

supernatants were collected and assayed for nitrite levels by Griess reagent. At concentration of 5 μg.mL^{-1}, compound-**3** suppressed LPS-induced nitrite level by 10.4 ± 0.4 μM in RAW264.7 (Figure 8A) and by 21.8 ± 1.8 μM in BV-2 cells (Figure 8B). Whereas compound-**1** at concentration of 5 μg mL^{-1} suppressed the nitrite level by 8.6 ± 0.3 μM in RAW264.7 (Figure 8A) and by 15.8 ± 4.4 μM in BV-2 cells (Figure 8B). The result indicated that compound-**3** showed greater suppressive effect than compound-**1** on LPS-induced nitric oxide production in RAW264.7 and BV-2 cells.

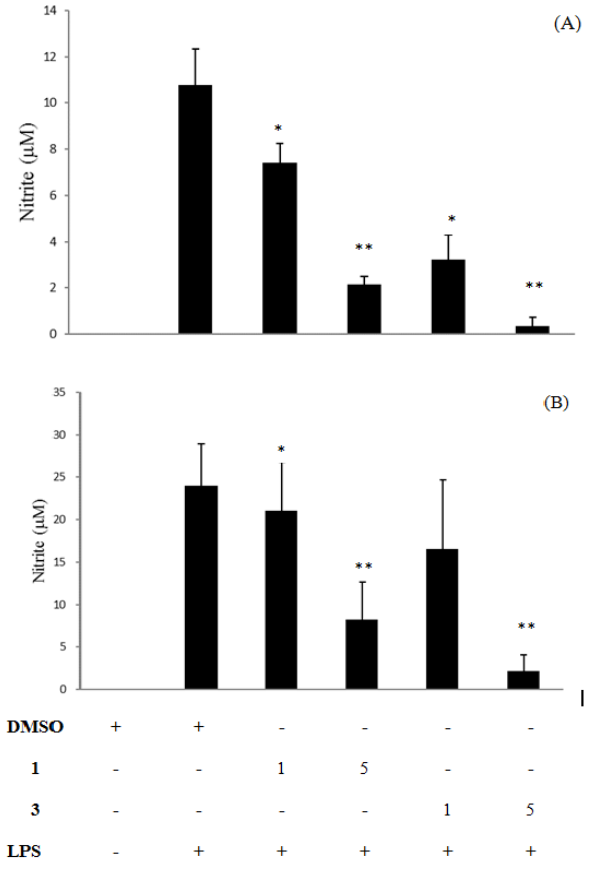


Figure 8: Inhibition of LPS-induced nitrite production by compounds-1 and -3. (A) RAW264.7 cells and (B) BV-2 cells were incubated with 1 or 5 μg.mL^{-1} of compounds-1 or -3 for 18 h prior to the addition of 100 ng.mL^{-1} LPS for another 18 h. The culture supernatants were collected and assayed for nitrite levels by Griess reagent. The results shown were the mean values ± standard derivation (S.D.) of at least three independent experiments. * P<0.05, ** P<0.01 compared with DMSO + LPS control.

DISCUSSION AND CONCLUSIONS

Herbal medicines have been used for the treatment of various diseases over a long period of time. Clinical, pharmacological, and chemical studies of these herbal medicines led to the discovery of novel drugs such as the active ingredient of aspirin, digoxin, morphine, taxenes and artemisinin compounds (DeSmet, *et al.*, 1997). These compounds are derived directly or indirectly from the plants. To cite aspirin as an example, the natural form of aspirin, salicylic acid, is associated with an awful taste and often induces gastric upset and vomiting. Chemical modification by adding an acetyl group to salicylic acid reduces its irritant properties (Mackowiak, *et al.,* 2000). Therefore, modification of important chemical compounds from natural products may help to discover novel medicines.

Plant-derived phenylpropanoids and their derivatives are among the most common biologically active components produced by plants. Their potential pharmacological effects include anti-oxidative, anti-tumor, anti-viral, anti-inflammatory and anti-bacterial activities (Dembitsky, *et al.,* 2005). Compound-**1** is a phenylpropanoid ester formed between isoferulic acid and 3-(3, 4-dihydroxylphenyl)-2-keto-propanol (Yang, *et al.,* 2009 and Chen, *et al.,* 2002). The presence of m-hydroxy or p-methoxy residues on cinnamic acid structure (isoferulic acid) have been shown to have potent bioactivities as an anti-inflammatory agent as well as an effective insulin inducer (Adisakwattana, *et al.,* 2008). This compound possesses an α, β-unsaturated carbonyl unit. Previous studies indicated that compounds with α, β-unsaturated carbonyl unit are not merely anti-oxidative, but rather directly connected to their Michael acceptor capable of activating Nrf2 through covalent modification of its repressor Keap1 (Dinkova-Kostova, *et al.,* 2001). One of the study indicated that 10-Gingerol was being the most potent among all the gingerols because of the presence of α, β-unsaturated carbonyl group in exhibiting the antioxidant and anti-inflammatory properties (Dugasani, *et al.*, 2010). Kim et al reported that compounds containing isoferulic acid showed higher anti-inflammatory activity than that containing caffeic acid (Sakai, *et al.,* 1999). In this study, we found that the anti-inflammatory effect of compound-**1** (containing isoferulic acid) was higher than that of compound-**2** (containing caffeic acid) which verifies Kim's observation.

The anti-inflammatory effect of compound-**1** was similar to that of compound-**3**. Compound-**1** consists of isoferulic acid whereas compound-**3** consists of *p*-methoxycinnamic acid. Isoferulic acid and *p*-methoxycinnamic acid have been found equally protective against CCl_4-induced hepatotoxicity and the cytoprotective action of these compounds has been associated with their antioxidative and chelating capacity (Lee, *et al.,* 2002). Furthermore, *p*-methoxycinnamic acid exhibits potent chemopreventive properties against 1, 2-dimethylhydrazine induced colon carcinogenesis in male albino wistar rats by modulating biotransforming bacterial enzymes and xenobiotic metabolizing enzymes (Gunasekaran, *et al.,* 2014). The anti-inflammatory effect of *p*-methoxycinnamic acid has not been demonstrated yet. In this study, we found that compounds consisted of isoferulic acid (Compound-**1**) and *p*-methoxycinnamic acid (Compound-**3**) showed similar inhibitory effects on cytokine and chemokine gene expressions in LPS-induced primary blood macrophages and they also showed similar nitrite suppressive effect in LPS stimulated RAW264.7 cells and BV-2 cells.

The involvement of intracellular signaling pathways including mitogen-activated protein kinases (MAPK) and the transcription factors nuclear factor kappa B (NF-κB) in the LPS-induced proinflammatory mediators' expressions are well documented (Davis, *et al.,* 1993 and Shakhov, *et al.,* 1990). Extracellular signal-regulated kinase-1/2 (ERK 1/2), p38 kinase and c-Jun N-terminal kinase (JNK) are the three MAPK subtypes known in human. Upon activation, MAPK transduces a variety of extracellular stimuli through a cascade of protein phosphorylations that lead to the activation of NF-κB. Then I-κB is dissociated from the NF-κB/I-κB cytosolic complex, leading to the translocation of p65NF-κB to the nucleus resulting in transcription of specific proinflammatory genes including IL-6 and TNF-α (Shakhov, *et al.,* 1990 and Brasier AR, *et al.,* 2010). Our previous study demonstrated that compound-**1** suppressed the LPS-induced TNF-α production in the primary blood macrophages which may be due to its inhibition of ERK1/2 phosphorylation and translocation of p65NF-κB to the nucleus (Yang, *et al.,* 2009). As compound-**1** and compound-**3** have similar structures and anti-inflammatory activities, they are likely to have similar effects on MAPK and NF-κB. However, the exact mechanism has to be further investigated.

Compound-**4** consists of isoferulic acid and 1-hydroxy-3-phenylpropan-2-one. This compound was found to be toxic to macrophages and did not show inhibitive effect on TNF-α production in LPS stimulated primary blood macrophages. The difference between compound-**1** and compound-**4** is that compound-**1** contains 3-(3, 4-dihydroxylphenyl)-2-keto-propanol whereas compound-**4** consists of 1-hydroxy-3-phenylpropan-2-one. Therefore, variation in the substitution pattern of the aromatic rings of polyphenols is crucial to anti-inflammatory and cytotoxic properties.

In general, compound-**1** is a potent anti-inflammatory compound. With the slightly structure modification of compound-**1**, we found that compound-**3** showed similar biological activity to compound-**1** with the replacement of isoferulic acid to *p*-methoxycinnamic acid. However, compound-**2** and compound-**4** only showed moderate anti-inflammatory effect and toxic effect, respectively. Therefore, compound-**1** and compound-**3** are candidates to be further developed as a new class of therapeutic agents against inflammatory diseases.

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