

Serotonins of safflower seeds play a key role in anti-inflammatory effect in lipopolysaccharide-stimulated RAW 264.7 macrophages

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Abstract Safflower (*Carthamus tinctorius*) seeds are well-known traditional oriental medicines that have long been used for the remedies of blood stasis and bone formation in east Asia. In this study, ethyl acetate (EtOAc) was used for extraction of the main chemical compounds from *C. tinctorius* seeds. Four major compounds were identified, acacetin, cosmosiin, N-feruloyl serotonin and N-(*p*-coumaroyl) serotonin. Each compound was evaluated for its inhibitory activity against the inflammatory process of macrophages. All compounds significantly inhibited production of lipopolysaccharide (LPS)-stimulated nitric oxide (NO) and pro-inflammatory cytokines. The protein levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were dramatically decreased by serotonin in a dose-dependent manner in LPS-stimulated RAW 264.7 macrophages. These results suggest that serotonin derivatives from safflower seeds may reduce inflammation-related diseases.

Keywords Anti-inflammatory, LPS-stimulated RAW 264.7 macrophages, Safflower seeds, Serotonins

Introduction

The flowers and leaves of safflower (*Carthamus tinctorius* L.), which belongs to chrysanthemum family, have long been used to treat stroke, gynecological diseases, coronary heart disease, angina pectoris and hypertension in traditional Chinese

medicines (Han et al. 2009; Li et al. 2009; Mandade et al. 2012). Additionally, *C. tinctorius* seeds, which are rich in unsaturated fatty acid and α -linoleic acid, are widely used for the treatment of blood stasis, bone formation, and osteoporosis and prevention of rheumatism in Korea (Kim 1998; Seo et al. 2000).

Inflammation is a defense mechanism against harmful microorganisms, but the persistence of this state is related to many adult diseases. Although inflammatory mediators like nitric oxide (NO), prostaglandin E₂ (PGE₂), interleukins (IL-1 β , IL-6), and tumor necrosis factors- α (TNF- α) are crucial in a defense mechanism, over expression of these mediators may provoke chronic diseases (Delgado et al., 2003; Kubes and McCafferty, 2000). Lipopolysaccharide (LPS) treated macrophages are often used to investigate the suppression of inflammation of many different samples. LPS can induce inflammatory mediators in order to imitate the real conditions in RAW 264.7 macrophages (Hewett and Roth 1993; Watson et al. 1999). During LPS-stimulation, inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) can produce NO and PGE₂, respectively (Abdelrahman et al. 2005; Makarov 2000). Inhibited expression of iNOS and COX-2 is thoroughly for its potential to prevent inflammatory diseases and cancer (Surh and Na 2008). Although various biological activities of *C. tinctorius* flowers and leaves have been reported (Sakamura et al. 1978), there have been few investigations of their inhibitory activity with respect to inflammation. Roh et al. report is pretty much the only research paper about a whitening effect using safflower (*C. tinctorius* L.) seeds (Roh et al. 2004). We previously reported that the partially purified ethyl acetate (EtOAc) soluble fraction of *C. tinctorius* seeds showed inhibition of cytokine, iNOS and COX-2 expression (Kim et al. 2013). Further, we purified and identified four major chemicals from seed extract and explored their anti-inflammatory activity in LPS-stimulated RAW264.7 macrophages *in vitro*.

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Materials and Methods

Seed extraction and chemical identification

Safflowers (*C. tinctorius* L.) were grown by contract farming, and seeds were purchased in 2011 from Humanherb Co. Five kilograms of air-dried *C. tinctorius* seeds were extracted with n-hexane for purification of bioactive compounds using the protocol described by Kim et al. (Kim et al. 2012). After removing the n-hexane layer, the aqueous solution was extracted three times with 70% ethanol (EtOH) under reflux for 4 hours. The EtOH extract was then filtered and exhaustively concentrated to give an EtOH extract (615 g). After sequential purification with various solvents, final fractions of n-hexane, EtOAc, and n-BuOH were obtained 15.8 g, 288.6 g, and 182.9 g respectively. The EtOAc fraction (200 g) was then applied to silica gel (7.5 X 50 cm) and eluted with CHCl₃ : MeOH (10 : 1) to separate into six fractions (Fr.1-6). Fr.1 and Fr. 5 were combined and crystallized from cold methanol to yield (1) acacetin (7.74 mg) and (2) cosmosin (4.76 mg). Fr. 4 was subjected to further chromatography on a silica gel column (4 X 30 cm) and then eluted with CHCl₃ : MeOH (5 : 1) to afford five sub-fractions (Fr.4.1-Fr.4.5), after which (3) N-feruloyl serotonin (6.13 mg) was crystallized from Fr. 4.2. Further chromatography of Fr.4.3 was conducted on a silica gel column (4 X 30 cm) and then eluted with CHCl₃ : MeOH (10 : 1) to yield (4) N-(*p*-coumaroyl) serotonin (15.33 mg). Analytical TLC was performed on pre-coated silica gel 60 F254 plates or RP-18 F254. For column chromatography, silica gel Kiesel gel 60 was used. All silica gels were purchased from Merck (Merck, Readington, NJ, USA). After re-confirming the chemicals by liquid chromatography (LC), the chemical structures were analyzed by ¹³C-NMR spectroscopy using a 500MHz FT-NMR spectrometer (Jeol, ToKyo, Japan).

Cell culture and cell viability

Mouse RAW 264.7 macrophages were purchased from the Korean Cell Line Bank (<http://www.atcc.org/>). Cells were grown at 37°C under a humidified atmosphere (5% CO₂) in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum, 100 IU/mL penicillin G, and 100 mg/L streptomycin (Gibco BRL, Grand Island, NY, USA). Cell viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells (3 X 10⁴ in 200 µL/medium) were treated with 20 µL of each extract and then incubated for 24 hours at 37°C. MTT reagent (0.5 mg/L) (Sigma, St. Louis, MO, USA) was then added to the medium, after which the

samples were incubated for an additional 4 hours. Following removal of the medium, MTT-formazan was dissolved in 200 µL/mL dimethylsulfoxide (DMSO). The extent of the reduction of MTT to formazan was quantified by measuring the absorbance at 540 nm using a microplate reader (Sunrise, Tecan, Salzburg, Austria).

Anti-inflammation assay

NO and PGE₂ production were measured for analysis of the inflammatory inhibitory effect of *C. tinctorius* seed extracts. NO liberated from RAW 264.7 macrophages was measured using a Griess assay according to the method described by Kim et al. (Kim et al. 2013). The medium was subsequently mixed with an equal volume of Griess reagent (Sigma, St. Louis, MO, USA), after which the amount of NO was quantified by measuring the absorbance at 540 nm using a microplate reader and comparing the values to a sodium nitrite standard curve. The concentration of PGE₂ and cytokines in the supernatant of macrophage cultures was determined by enzyme-linked immuno sorbent assay (ELISA) using an antibody from R&D Systems according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Western blot analysis

After the extracts were prepared at concentrations of 6.25, 12.5, or 25 µg/L, treated RAW 264.7 macrophages were incubated for 18 hours at 37°C. The cells were subsequently harvested by washing with PBS, lysed with 100 µL of RIPA buffer (pH 7.4) composed of 50 mM Tris, 0.1% SDS, 50 mM NaCl, 1% NP-40, 1 mM PMSF, 10 µg/L of aprotinin and leupeptin, and then centrifuged at 12,000 rpm for 20 minutes. The protein content in the supernatant was quantified using a BioRad protein assay kit (BioRad, Hercules, CA, USA). Briefly, 20 µg of proteins were analyzed by 10% SDS-PAGE, then transferred onto PVDF membranes using a Trans-Blot apparatus (BioRad, Hercules, CA, USA). Following transfer, the membrane was blocked with 5% nonfat dried milk (w/v) in TBS-T for 1 hour at RT. After six washes, the membrane was incubated with primary iNOS and COX-2 rabbit polyclonal antibodies from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA, US), then diluted (1 : 1,000) with TBS-T for 12 hours at 4°C. After a strong wash, samples were incubated with mouse anti-rabbit IgG HRP (1 : 1,000) for 2 hours at RT. The samples were then washed again, after which proteins were detected using an Enhanced Chemiluminescence kit (Amersham Bioscience, Little Chalfont, UK). The detection reagent was poured onto the membrane and incubated for 1

minute, after which the band density was quantified with a LAS4000 image analyzer (Fujifilm Life Science, Tokyo, Japan).

Statistical analysis

Analysis of variance was performed using SPSS (SPSS Inc., Chicago, IL, USA). All data are expressed as the means \pm SD. Differences among means were determined by Tukey's range test (*t*-test) with $p < 0.05$ and $p < 0.01$ considered to indicate significance.

Results and Discussion

Identification of four major chemical compounds

We previously demonstrated that the EtOAc fraction of *Carthamus tinctorius* seeds generated the highest inhibitory effect of inflammation among various extraction buffers, including 70% EtOH, n-hexane, ethyl acetate, and butyl alcohol (Kim et al. 2013). In this study, partially purified

EtOAc seed extract was further fractionated and major peaks were isolated using a modified method described by Kim et al. (2012). The four major compounds were identified as acacetin, cosmosiin, N-feruloyl serotonin and N-(*p*-coumaroyl) serotonin by ^{13}C labelled NMR (Fig. 1A and 1B). The main peaks and chemical structures were similar to those identified in former studies (Sakamuta et al. 1980).

NO and PGE₂ levels were decreased by treatment with each major compound

When animals get infected, inflammation is started as the first reaction of the immune system by cytokines, NO and PGE₂. Kim et al. (2013) reported that crude extract of *C. tinctorius* seeds decreased inflammation in macrophages (Kim et al. 2013). In this study, four compounds (acacetin, cosmosiin, N-feruloyl serotonin and N-(*p*-coumaroyl) serotonin) were purified and identified, after which each compound was evaluated for its ability to modulate the regulatory mechanism of NO, cytokines, PGE₂, iNOS and COX-2 in LPS-activated RAW264.7 cells. To investigate key anti-inflammatory chemicals among major compounds, a colorimetric MTT assay was used for cell toxicity. The results showed that the four compounds had little or no effect on cell viability (data not shown). LPS was added in mouse RAW 264.7 macrophages to produce cytokines such as TNF- α , IL-1 β , and IL-6. The levels of pro-inflammatory cytokines were significantly reduced by all compounds (1) – (4). N-feruloyl serotonin (Fig. 1C) showed the most inhibitory effect of TNF- α , IL-1 β , and IL-6, with IC₅₀ values of 20.7 \pm 1.62, 12.8 \pm 2.15 and 11.1 \pm 0.44 μM ($p < 0.05$, $p < 0.001$), when compared with pyrrolidine dithiocarbamate (PDTC) as a positive control (IC₅₀=27.3 \pm 0.51, 21.7 \pm 2.17 and

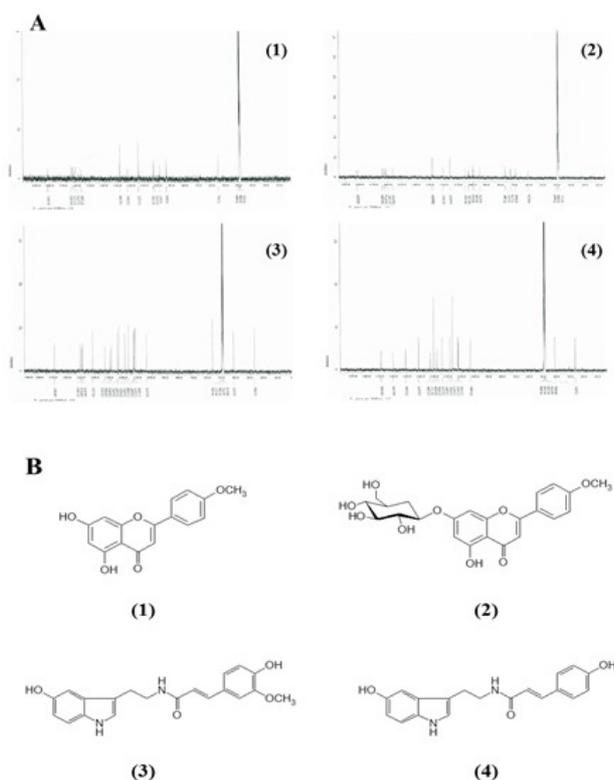


Fig. 1 Chemical structures of major four compounds from the ethyl acetate extract of *C. tinctorius* seeds. (A) NMR data showing the highest peak from ^{13}C labelled major chemicals (B) chemical structures of (1) acacetin, (2) cosmosiin, (3) N-feruloyl serotonin and (4) N-(*p*-coumaroyl) serotonin

Table 1 Effect of Four Major Compounds from Ethyl Acetate Extract of *C. tinctorius* Seeds on TNF- α , IL-1 β , and IL-6 Production in RAW 264.7 Macrophages

Compound	IC ₅₀ (μM) ^Z		
	TNF- α	IL-1 β	IL-6
Acacetin	35.2 \pm 3.11	19.6 \pm 2.51	21.1 \pm 6.14
Cosmosiin	32.4 \pm 0.54	18.4 \pm 0.48	17.1 \pm 1.81
N-feruloyl serotonin	20.7 \pm 1.62	12.8 \pm 2.15	11.1 \pm 0.44
N-(<i>p</i> -coumaroyl) serotonin	27.3 \pm 0.84	16.8 \pm 3.16	17.7 \pm 2.14
PDTC	23.3 \pm 0.51	21.7 \pm 2.17	21.1 \pm 1.69

^ZIC₅₀ values were determined by regression analysis and expressed as the mean \pm SD of three separate experiments. PDTC was used as a positive control. Production of TNF- α , IL-1 β , and IL-6 in various concentrations of major compounds was measured, and the 50% of inhibition concentration (IC₅₀) was calculated by the formula. $\text{IC}_{50} = [\text{I}]/[\text{v}_0/\text{v}_i - 1]$, where v_0 is the control reaction rate without inhibitor and v_i is the rate with inhibitor at concentration [I].

21.1±1.69 μM) (Table 1). The two serotonin derivatives (Fig. 1C and 1D) highly suppressed expression of inflammatory mediators especially cytokines when compared to other flavonoids (Fig. 1A and 1B). From the results, the location of the feruloyl group may influence inhibition of pro-inflammatory cytokines. Especially the two serotonin derivatives, N-feruloyl serotonin and N-(*p*-coumaroyl) serotonin, significantly suppressed the production of pro-inflammatory cytokines.

NO, a crucial secondary messenger, is synthesized from L-arginine (Palmer et al. 1988). In the inflammatory mechanism, NO and PGE₂ are produced by iNOS (Xie and Nathan 1994) and COX-2, respectively (Tannenbaum et al. 1996). Inhibitions of NO and PGE₂ production were also investigated in LPS-stimulated macrophages. In Figure 2, PDTC at 25 μM inhibited NO and PGE₂ by 54%, 60%, 55% and 75%, respectively. NO and PGE₂ levels were significantly decreased by the compounds, whereas only LPS was treated as a negative control caused increased NO and PGE₂ production (Fig. 2). Therefore, we investigated more on the relationship between the compounds and iNOS and COX-2 expression.

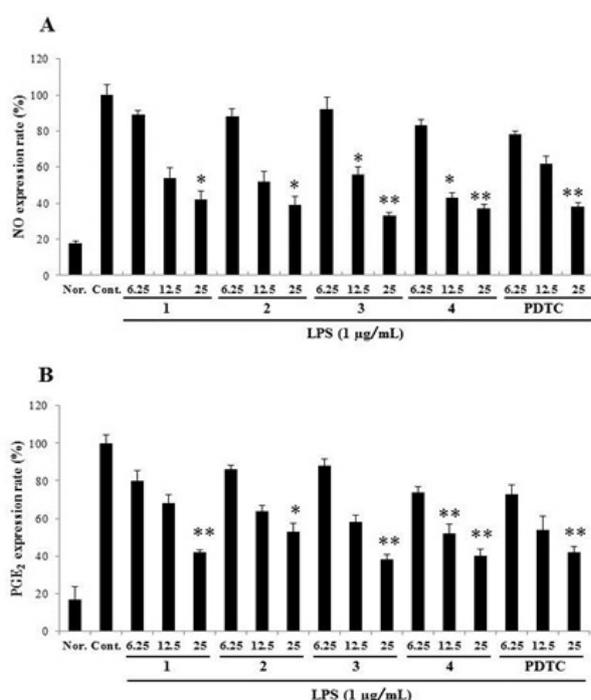


Fig. 2 Effects of four major active compounds of *C. tinctorius* seeds on NO (A) and PGE₂ (B) production by LPS-stimulated RAW 264.7 macrophages. 3 X 10⁵ cells were treated with various concentrations (6.25, 12.5 and 25 μM) of each major compound after treatment with 1 μg/mL LPS. PDTC was used as a positive control. ‘Nor.’ indicates normal RAW cells without any treatment. ‘Cont.’ represents cells used as negative controls after LPS induction. Values are the mean ± SD of three separate experiments. * *p* < 0.05, ** *p* < 0.01 indicates a significant difference compared to LPS alone

The level of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) was suppressed by serotonins of *C. tinctorius* seeds.

The level of iNOS and COX-2 following treatment with the four major compounds was evaluated by Western blot analysis (Fig. 3 and Fig. 4). The iNOS protein level decreased significantly in LPS-stimulated RAW 264.7 macrophages in response to each tested concentration (Fig. 3). A similar decrease was found in COX-2 protein with N-feruloyl serotonin and N-(*p*-coumaroyl) serotonin inducing the greatest reduction (Fig. 4). These results suggest that phenylpropanoid-conjugated serotonins (Fig. 1C and 1D) and *O*-methylated flavones (Fig. 1A and 1B) may defend macrophages from inflammation through suppression of iNOS and COX-2 expression.

Serotonins are found in many plants, as well as in animals and insect venom (Kang et al. 2009). Plant seeds produce serotonins to eliminate accumulated ammonia (Schröder et al. 1999). In addition, serotonins produced by plants may facilitate the removal of seeds from the digestive tract. Moreover, many laxatives are made with seed and fruit extracts containing serotonins. Walnut and hickory have high levels of serotonins (25–400 mg/kg), while pineapples, bananas, kiwis, and tomatoes contain about 3–30 mg/kg (Feldman and Lee 1985). In the present study, *C. tinctorius* seeds were found to contain 4.3 mg/kg serotonin.

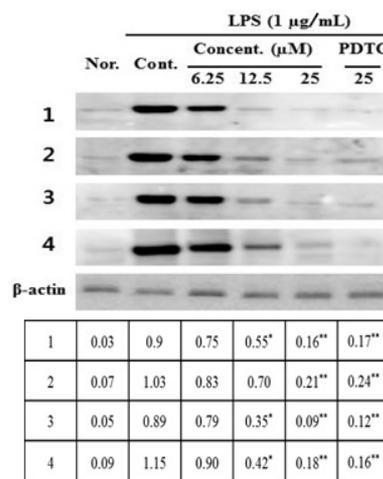


Fig. 3 Changes in iNOS expression level after treatment with four major seed compounds in LPS-stimulated RAW 264.7 macrophages. Cells were treated with compounds after LPS stimulation (1 μg/mL) and the production of iNOS was then quantified by Western blot analysis (upper). Numbers 1–4 indicate four major compounds (1. acacetin, 2. cosmosiin, 3. N-feruloyl serotonin and 4. N-(*p*-coumaroyl) serotonin). Beta-actin was used as a comparative control. The table shows protein levels normalized by β-actin using densitometry. **p* < 0.05, ** *p* < 0.01 indicates a significant difference compared to the negative control (Cont.)

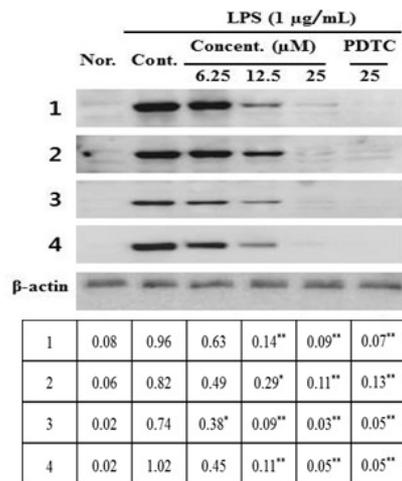


Fig. 4 Effects of four major compounds from *C. tinctorius* seeds on LPS-stimulated COX-2 expression in RAW 264.7 macrophages. Cells were treated with compounds 1-4 for 1 hour after 24 hours of LPS stimulation (1 μg/mL). The level of COX-2 was analyzed by Western blot (upper). Numbers 1-4 indicate four major compounds (1. acacetin, 2. cosmosiin, 3. N-feruloyl serotonin and 4. N-(*p*-coumaroyl) serotonin). β-actin was used as an internal control. The table shows protein levels normalized by β-actin using densitometry. * $p < 0.05$, ** $p < 0.01$ indicates a significant difference compared to the negative control (Cont.)

Flowers of safflowers have been used as the traditional remedies of stroke and coronary heart disease in China. However, Korean prefers to use their seeds for the treatments of bone formation, osteoporosis, and prevention of rheumatism. In this study, main active compounds of *C. tinctorius* seeds were identified especially feruloyl serotonins as major chemicals. Serotonins are commonly known for prevention of ammonia accumulation in plants. Apart from that, we found that serotonins isolated from *C. tinctorius* seeds (4.3 mg/kg) shows outstanding anti-inflammatory effect in a cell model. In particular, N-feruloyl serotonin and N-(*p*-coumaroyl) serotonin clearly inhibited inflammatory mediators such as NO and PGE₂ via reduction of iNOS and COX-2 in LPS-stimulated macrophages. It is plausible to say that these major serotonin derivatives from *C. tinctorius* seeds have important anti-inflammatory activity and therefore have the potential for pharmaceutical applications.

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