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## Original Article

# Functional components in *Scutellaria barbata* D. Don with anti-inflammatory activity on RAW 264.7 cells

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## ABSTRACT

The objectives of this study were to determine the variety and amount of various functional components in *Scutellaria barbata* D. Don as well as study their anti-inflammatory activity on RAW 264.7 cells. Both ethanol and ethyl acetate extracts were shown to contain the functional components including phenolics, flavonoids, chlorophylls, and carotenoids, with the former mainly composed of phenolics and flavonoids, and the latter of carotenoids and chlorophylls. Both extracts could significantly inhibit ( $p < 0.05$ ) the production of lipopolysaccharide-induced nitric oxide, prostaglandin  $E_2$ , interleukin-6, and interleukin- $1\beta$ , as well as the expressions of phosphor extracellular signal-regulated kinase and phosphor-c-Jun N-terminal kinase (p-JNK), but failed to retard tumor necrosis factor- $\alpha$  expression. Both ethanol and ethyl acetate extracts had a dose-dependent anti-inflammatory activity on RAW 264.7 cells. Furthermore, the anti-inflammatory efficiency can be varied for both ethanol and ethyl acetate extracts, which can be attributed to the presence of different varieties and amounts of functional components, as mentioned above. This finding suggested that *S. Barbata* extract may be used as an anti-inflammatory agent for possible future biomedical application.

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## 1. Introduction

Ban-Zhi-Lian, a traditional Chinese herb with the scientific name *Scutellaria barbata* D. Don or *Scutellaria rivularis* Wall, belongs to the Labiatae species [1]. *S. barbata* is widely grown in Asian countries such as Taiwan, China, Korea, and Japan, and

has been reported to possess vital biological activities, including antibacterial [2,3], anticancer [1], and anti-inflammatory activities [4].

Inflammation, a kind of innate immunity, represents an important protective function of humans against invasion of foreign substances such as bacteria and virus. After invasion, the body immunity function can be induced for subsequent

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activation of macrophages to resist foreign pathogens and ameliorate damage to human health [5]. However, prolonged inflammation can elevate oxidative stress *in vivo*, leading to an increase in the incidence of chronic diseases such as cancer and cardiovascular disease. In addition, during inflammation, many proinflammatory factors such as nitric oxide (NO), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), and interleukins (IL) can be released by macrophages and neutrocytes, after which both can migrate to the inflammatory site to kill microorganisms and endotoxins. However, the excessive secretion can cause damage to tissues [5]. Among the various proinflammatory factors, NO plays a key role in the various forms of physiology and pathology. Physiology can regulate nerve conduction, immune function, inflammation, and artery smooth muscle relaxation [6,7], while pathology can be associated with incidence of atherosclerosis, chronic inflammation and cancer [8].

In several earlier studies, *S. barbata* was shown to exhibit anti-inflammatory activity; however, the functional components and underlying mechanism remained uncertain [9,10]. *S. barbata* has been reported to contain flavonoids, phenolic acids, terpenoids, carotenoids, chlorophylls, polysaccharides, essential oils, alkaloids, organic acids, and trace elements [1,11]. However, only the compositions of flavonoids, phenolic acids, carotenoids, terpenoids, polysaccharides, and essential oils were determined [1,12]. Thus, some other vital functional components such as chlorophylls need to be determined as well. In a previous study, we developed a high-performance liquid chromatography–photodiode array detector–mass spectrometry–atmospheric pressure chemical ionization (HPLC–DAD–MS–APCI) method for separation and quantitation of various carotenoids in *S. barbata*, and a total of 18 carotenoids were identified with all-*trans*-lutein being present in the most abundant amount [13].

The objectives of this study were to determine total flavonoids, total phenolics, carotenoids, and chlorophylls in both ethanol and ethyl acetate extracts of *S. barbata*. In addition, the anti-inflammatory activity of these functional components toward RAW 264.7 cells was investigated.

## 2. Methods

### 2.1. Materials

A total amount of 3 kg *S. barbata* was procured from a local Chinese drug store and subjected to cleaning, freeze drying (–40°C, 60 mTorr), grounding into powder, pouring into several separate bags, sealing under vacuum, and storing at –20°C until use.

### 2.2. Chemicals and reagents

Carotenoid standards, including all-*trans*-zeaxanthin and all-*trans*- $\beta$ -carotene, were purchased from Sigma-Aldrich Co. (St Louis, MO, USA), while all-*trans*-lutein was obtained from Fluka Chemical Co. (Buchs, Switzerland) and 9- or 9'-*cis*-neoxanthin from ChromaDex (Irvine, CA, USA). Internal standard all-*trans*- $\beta$ -apo-8'-carotenal was also from Fluka Chemical Co. Anhydrous sodium sulfate was from Nacalai Tesque Co.

(Kyoto, Japan). Chlorophyll standards including chlorophyll a, chlorophyll b, and internal standard zinc-phthalocyanine were from Sigma-Aldrich Co. Silica gel 60 thin-layer chromatography plates (0.5 mm thickness) were from Merck Co. (Darmstadt, Germany); 95% ethanol was from Taiwan Tobacco and Wine Monopoly Bureau (Tainan, Taiwan). Deionized water was made using a Milli-Q water purification system from Millipore Co. (Bedford, MA, USA). Solvents including ethanol, ethyl acetate, chloroform, hexane, acetone, toluene, methanol, acetonitrile, and methylene chloride were from Merck Co. Macrophage cell RAW 264.7 was from Bioresource Collection and Research Center (Hsinchu, Taiwan). Reagents for cell culture were from Invitrogen Co. (Carlsbad, CA, USA), Hyclone Co. (Logan, UT, USA), and Sigma-Aldrich Co. Lipopolysaccharide (LPS; from *Escherichia coli* O26:B6) was also from Sigma-Aldrich Co. BCA protein assay reagent kits and NE-PER nuclear and cytoplasmic extraction reagents were from Pierce Co. (Belvidere, IL, USA). The prostaglandin E<sub>2</sub> enzyme immunoassay (PGE<sub>2</sub> EIA) kit was from Bnzo Life Sciences Co. (Farmingdale, NY, USA). The DuoSet enzyme-linked immunosorbent assay (ELISA) kits for cytokine determination were from R&D Systems Co. (Minneapolis, MN, USA). Western blotting reagents were from Cell Signaling Co. (Danvers, MA, USA), BD Biosciences Co. (San Jose, CA, USA), and Anaspec Co. (Fremont, CA, USA).

### 2.3. Instrumentation

The HPLC instrument is composed of an Agilent G1311A pump, a G1316A column temperature controller, a G1315B photodiode array detector, and a 6130 quadrupole mass spectrometer with multimode ion source (APCI and electrospray ionization; Agilent Co., Palo Alto, CA, USA). The Beckman DU640 spectrophotometer was from Beckman Co. (Fullerton, CA, USA). The N-1000 rotary evaporator was from Eyela Co. (Tokyo, Japan). The Sorvall RC5C high-speed centrifuge was from Du Pont Co. (Wilmington, DL, USA). The DC400H sonicator was from Chuan-Hua Co. (Taipei, Taiwan). The V-U shaker was from Hsiang-Tai Co. (Taipei, Taiwan). A YMC C<sub>30</sub> polymeric column (250 × 4.6 mm<sup>2</sup> ID, 5  $\mu$ m particle size) used to separate carotenoids was from YMC Co. (Kyoto, Japan). A HyPURITY C<sub>18</sub> column (150 × 4.6 mm<sup>2</sup> ID, 5  $\mu$ m particle size) used to separate chlorophylls was from Thermo-Keystone Co. (Bellefonte, CA, USA). The freeze dryer (FD24) was from Gin-Ming Co. (Taipei, Taiwan). The VersaMax ELISA microplate reader was from Molecular Devices Co. (Sunnyvale, CA, USA).

### 2.4. Preparation of *S. barbata* extracts

*S. barbata* powder (96 g) was poured into 12 centrifuged flasks separately with 8 g each, followed by addition of 160 mL ethanol or ethyl acetate to six flasks each. Then the solution was sonicated at 25°C for 10 minutes, shaken for 20 minutes, and centrifuged at 15,400g for 20 minutes. The supernatant was then collected and filtered through a No. 1 filter paper. After evaporation to dryness under vacuum, the residue was weighed. All the residues were combined, and 2.9082 g powder was obtained from the ethanol extract, while 1.4399 g powder was obtained from the ethyl acetate extract. Next, both powder from ethanol extracts and that from ethyl acetate extracts

were dissolved in ethanol and diluted to 25 mL and 20 mL, respectively, for storage at  $-20^{\circ}\text{C}$  for anti-inflammatory experiment with the doses controlled at 50  $\mu\text{g}/\text{mL}$ , 100  $\mu\text{g}/\text{mL}$ , and 200  $\mu\text{g}/\text{mL}$ .

## 2.5. Analysis of functional components in *S. barbata*

### 2.5.1. Total phenolic compounds and flavonoids

Total phenolic compounds were determined using the Folin–Ciocalteu reagent according a method based on Kao et al's [14] study and expressed as gallic acid equivalents. In addition, a method as described by Kao et al [14] was used to determine total flavonoids in both extracts, which were expressed as quercetin equivalents.

### 2.5.2. Carotenoids

A method based on Liu et al's [13] study was used for carotenoid analysis in *S. barbata* extract. In brief, 1 g of freeze-dried *S. barbata* powder was mixed with 30 mL of hexane/ethanol/acetone/toluene (10:6:7:7, v/v/v/v) in a 100 mL volumetric flask, after which the mixture was shaken at room temperature for 1 hour. Then 2 mL of 40% methanolic KOH solution was added for saponification for 16 hours in the dark under nitrogen, after which 15 mL of hexane was added and shaken for 10 minutes. Then 15 mL of 10% anhydrous sodium sulfate solution was added and shaken vigorously for 1 minute, followed by standing at room temperature until separation into two layers. The upper layer containing carotenoids was collected, and the residue was repeatedly extracted with 15 mL of hexane four times. All the supernatants were pooled, evaporated to dryness, dissolved in 5 mL of methylene chloride, and filtered through a 0.22  $\mu\text{m}$  membrane filter, and 20  $\mu\text{L}$  was injected for HPLC–DAD–MS–APCI analysis.

Identification was performed by comparing retention time, absorption spectra, and mass spectra of unknown peaks with reference standards. However, in the absence of commercially available carotenoid standards, the identification was carried out by comparing absorption spectra and mass spectra of unknown peaks with those reported in the literature. The violaxanthin standard was prepared from spinach by thin-layer chromatography using the same method as described by Liu et al [13]. For quantitation, an internal standard, all-trans- $\beta$ -apo-8'-carotenal, was used; the standard curves of various concentrations of all-trans forms of lutein and  $\beta$ -carotene as well as violaxanthin and 9- or 9'-cis-neoxanthin were prepared by plotting concentration ratio against area ratio to obtain the regression equations and correlation coefficient ( $r$ ). However, the cis form and epoxy form of carotenoids were quantified based on the standard curves of their corresponding all-trans forms [13].

### 2.5.3. Chlorophylls

A method based on Kao et al's [15] study was modified to determine the various chlorophylls in *S. barbata* extract. Briefly, 0.5 g sample powder of *S. barbata* was mixed with 30 mL of hexane/acetone/toluene/ethanol (10:7:7:6, v/v/v/v) in a flask, after which the mixture was shaken for 1 hour and 5 mL hexane was added to it and shaken for 10 minutes. Then 15 mL of 10% anhydrous sodium sulfate solution (w/v) was added, followed by shaking for 1 minute and centrifuging at

1000g for 1 minute. The supernatant was collected, and 15 mL of hexane was added for repeated extraction three to five times until colorless. All the supernatants were pooled, and 1 mL of *S. barbata* extract was evaporated to dryness under  $\text{N}_2$  and dissolved in 2 mL acetone. After filtration through a 0.22  $\mu\text{m}$  membrane filter, a 20  $\mu\text{L}$  sample was injected for HPLC–DAD–MS–APCI analysis. A HyPURITY  $\text{C}_{18}$  column and a gradient mobile phase of (A) methanol/dimethylformamide (97:3, v/v), (B) acetonitrile, and (C) acetone were used, with the initial ratio at 65% A, 30% B, and 5% C, changed to 60% A, 30% B, and 10% C in 8 minutes, 90% A and 10% C in 13 minutes, 60% A and 40% C in 15 minutes, maintained at 60% A and 40% C for 7 minutes and returned to original ratio in 25 minutes. A total of 12 chlorophylls were separated within 23 minutes with detection at 660 nm and at a flow rate of 1 mL/min. An internal standard zinc-phthalocyanine was used for quantitation, and the standard curves of both chlorophyll a and chlorophyll b were prepared by plotting concentration ratio against area ratio. Both chlorophyll a and chlorophyll b as well as their derivatives were quantified based on the regression equations.

## 2.6. Anti-inflammatory activity of *S. barbata* extract

### 2.6.1. Cell culture

RAW 264.7 cells were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100  $\mu\text{g}/\text{mL}$  streptomycin, and incubated in an incubator containing 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . The medium was replaced every 2 days for subculture for < 20 times.

### 2.6.2. Supernatant collection

Initially,  $2 \times 10^5$  cells per well were seeded onto 24-well plates and cultured for 24 hours, after which the medium was replaced with various doses of *S. barbata* extracts and 1  $\mu\text{g}/\text{mL}$  of LPS was added to induce inflammation. After successive incubation for 24 hours, the supernatant was collected for analysis of proinflammatory cytokines including NO,  $\text{PGE}_2$ , interleukin (IL)-1 $\beta$ , IL-6, and TNF- $\alpha$ . The culture medium without LPS was used as normal treatment, while that with LPS (1  $\mu\text{g}/\text{mL}$ ) was used as control treatment.

### 2.6.3. Determination of NO concentration

Seven concentrations of  $\text{NaNO}_2$  (100 $\mu\text{M}$ , 80 $\mu\text{M}$ , 60 $\mu\text{M}$ , 40 $\mu\text{M}$ , 20 $\mu\text{M}$ , 10 $\mu\text{M}$ , and 5 $\mu\text{M}$ ) were prepared, and 100  $\mu\text{L}$  each was collected and mixed with the cell supernatants in a 96-well plate; 100  $\mu\text{L}$  of phosphoric acid solution (5%) was used as blank. Then 100  $\mu\text{L}$  of Griess reagent was added and reacted in the dark for 5 minutes, after which the absorbance was measured at 540 nm with an ELISA reader. The  $\text{NaNO}_2$  standard curve was prepared by plotting concentration against absorbance, and the regression equation as well as correlation coefficient ( $\gamma$ ) was obtained for quantitation of  $\text{NO}_2^-$  in sample extracts.

### 2.6.4. Determination of $\text{PGE}_2$ and cytokine secretion

$\text{PGE}_2$  and cytokine levels were determined by an ELISA according to manufacturer's instructions. The concentration of  $\text{PGE}_2$  was determined using a  $\text{PGE}_2$  EIA kit following a standard procedure. Likewise, concentrations of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  were measured using a DuoSet ELISA development kit following the standard procedure.

### 2.6.5. Western blotting analysis

Proteins associated with inflammation, such as nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), phosphor extracellular signal-regulated kinase (p-ERK), phosphor-c-Jun N-terminal kinase (p-JNK), and nuclear factor-kappa B (NF-κB), were determined based on a method as described by Kao et al [14]. Initially, cells ( $2 \times 10^6$ /plate) were replaced with fresh medium containing 1 μg/mL of LPS and 200 μg/mL of each *S. barbata* extract. The incubation time of 24 hours was adopted for iNOS and COX-2 determination, while 1 hour was adopted for NF-κB and 30 minutes for p-ERK and p-JNK measurements. Then proteins were collected from cells, and the expressions of iNOS, COX-2, NF-κB, p-ERK, and p-JNK were analyzed with a Western blotting technique. In brief, the pre-made electrophoretic gel with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was placed in a tank and 10 μg of hydrolyzed cell protein sample was added, followed by separation of protein hydrolysate under 80 V for 2–3 hours and transfer of protein onto polyvinylidene difluoride (PVDF) membrane under 100 V at 4°C for overnight. Then the PVDF membrane was collected, soaked in blocking buffer (5% skim milk in Tris-buffered saline (TBS) buffer solution), and mixed gently for 1–2.5 hours; Tris-buffered saline-Tween 20 mixture (TBST) was added three times for 10 minutes each to remove the unattached protein. Next, the diluted primary antibodies including iNOS, COX-2, NF-κB, p-ERK, p-JNK, and β-actin were added separately and reacted at 4°C overnight. Likewise, TBST was added three times, and the secondary antibody was added to conjugate horse-radish peroxidase, followed by reaction at room temperature for 1 hour, addition of TBST for washing three times, addition of enhanced chemiluminescence (ECL) reagent for luminal oxidation to produce chemiluminescence, and pressing with X-ray film for image expression.

### 2.7. Statistical analysis

All the analyses were performed in duplicate, and the data were subjected to analysis of variance and Duncan's multiple range test for significance in mean comparison ( $p < 0.05$ ) by using SAS (SAS Institute, Inc., Cary, NC, USA).

## 3. Results

### 3.1. Functional components in *S. barbata*

Table 1 shows the various functional components in both ethanol and ethyl acetate extracts, including phenolics,

flavonoids, chlorophylls, and carotenoids. The ethanol extract was shown to contain a higher level of total phenolics and total flavonoids, while the ethyl acetate extract had a higher content of chlorophylls and carotenoids. In addition, the *S. barbata* extract may contain polysaccharides, glycosides, saponins, tannins, alkaloid, amino acid, terpenoids, trace elements, essential oil, and steroids [1,11,12]. The total amount of functional components in the ethanol extract was about twice higher than that in the ethyl acetate extract.

Amounts of various chlorophylls, carotenoids, and their derivatives in *S. barbata* extracts are shown in Table 2. The ethanol extract was found to contain hydroxychlorophyll b, chlorophyll b, chlorophyll b', chlorophyll a, chlorophyll a', pheophytin b, hydroxypheophytin a, hydroxypheophytin a', pheophytin a, and pheophytin a', in which pheophytin a, chlorophyll b, pheophytin a', and chlorophyll b' constituted the largest portion. The ethyl acetate extract contained the same chlorophylls and derivatives as shown above, but two more chlorophyll derivatives including 15-OH-lactone chlorophyll a and hydroxychlorophyll a were also present. In addition, chlorophyll a, chlorophyll b, chlorophyll a', pheophytin a, and chlorophyll b' accounted for the largest portion in the ethyl acetate extract (Table 2). The ethyl acetate extract contained a higher amount of total chlorophylls than the ethanol extract, which can be attributed to lower polarity of the former. Table 3 shows the contents of various carotenoids

**Table 2 – Contents of chlorophylls (mg/g)<sup>a</sup> in *Scutellaria barbata* extracts.<sup>b</sup>**

		EtOH	EtAc
1	Hydroxychlorophyll b	0.15±0.00 <sup>b</sup>	0.43±0.01 <sup>a</sup>
2	Chlorophyll b	4.73±0.02 <sup>b</sup>	6.77±0.11 <sup>a</sup>
3	Chlorophyll b'	1.14±0.00 <sup>b</sup>	1.56±0.03 <sup>a</sup>
4	15-OH-lactone chlorophyll a	ND <sup>b</sup>	0.52±0.00 <sup>a</sup>
5	Hydroxychlorophyll a	ND <sup>b</sup>	0.67±0.00 <sup>a</sup>
6	Chlorophyll a	0.63±0.00 <sup>b</sup>	20.80±0.36 <sup>a</sup>
7	Chlorophyll a'	0.37±0.00 <sup>b</sup>	2.57±0.05 <sup>a</sup>
8	Pheophytin b	0.59±0.00 <sup>a</sup>	0.15±0.00 <sup>b</sup>
9	Hydroxypheophytin a	0.40±0.00 <sup>a</sup>	0.37±0.00 <sup>a</sup>
10	Hydroxypheophytin a'	0.37±0.00 <sup>a</sup>	0.35±0.00 <sup>a</sup>
11	Pheophytin a	7.04±0.04 <sup>a</sup>	1.92±0.03 <sup>b</sup>
12	Pheophytin a'	1.55±0.01 <sup>a</sup>	0.60±0.01 <sup>b</sup>
	Total	16.97±0.07 <sup>b</sup>	36.71±0.60 <sup>a</sup>

EtAc = ethyl acetate extract; EtOH = ethanol extract; ND = not detected.

<sup>a</sup> Average of duplicate analyses ± standard deviation.

<sup>b</sup> Data with different superscript letters (a and b) in the same row are significantly different at  $p < 0.05$ .

**Table 1 – Contents of functional components (mg/g)<sup>a</sup> in ethanol and ethyl acetate extracts of *Scutellaria barbata*.<sup>b</sup>**

Extracts	Total phenolics <sup>c</sup>	Total flavonoids <sup>d</sup>	Chlorophylls	Carotenoids	Total
EtOH	25.72±0.52 <sup>a</sup>	70.77±0.78 <sup>a</sup>	16.97±0.07 <sup>b</sup>	1.90±0.02 <sup>b</sup>	115.36±1.39 <sup>a</sup>
EtAc	12.77±0.31 <sup>b</sup>	12.17±0.66 <sup>b</sup>	36.71±0.60 <sup>a</sup>	3.78±0.06 <sup>a</sup>	65.34±1.63 <sup>b</sup>

EtAc = ethyl acetate extract; EtOH = ethanol extract.

<sup>a</sup> Average of duplicate analyses ± standard deviation.

<sup>b</sup> Data with different superscript letters (a and b) in the same column are significantly different at  $p < 0.05$ .

<sup>c</sup> Data expressed as mg/g of gallic acid equivalent.

<sup>d</sup> Data expressed as mg/g of quercetin equivalent.

**Table 3 – Contents of carotenoids (mg/g)<sup>a</sup> in *Scutellaria barbata* extracts.<sup>b</sup>**

		EtOH	EtAc
1	All-trans-violaxanthin	0.03±0.00 <sup>b</sup>	0.18±0.01 <sup>a</sup>
2	9- or 9'-cis-Neoxanthin	ND <sup>b</sup>	0.04±0.00 <sup>a</sup>
3	Luteoxanthin	0.02±0.00 <sup>b</sup>	0.12±0.00 <sup>a</sup>
4	13- or 13'-cis-Lutein	0.03±0.00 <sup>a</sup>	0.09±0.00 <sup>a</sup>
5	13- or 13'-cis-Lutein	0.05±0.00 <sup>a</sup>	0.05±0.00 <sup>a</sup>
6	All-trans-lutein	1.60±0.02 <sup>b</sup>	2.68±0.03 <sup>a</sup>
7	All-trans-zeaxanthin	0.01±0.00 <sup>a</sup>	0.01±0.00 <sup>a</sup>
8	9- or 9'-cis-Lutein	0.01±0.00 <sup>a</sup>	0.04±0.00 <sup>a</sup>
9	β-Carotene-5,6-epoxide	ND <sup>b</sup>	0.02±0.00 <sup>a</sup>
10	β-Carotene-5,8-epoxide	ND <sup>b</sup>	0.02±0.00 <sup>a</sup>
11	15- or 15'-cis-β-Carotene	0.01±0.00 <sup>a</sup>	0.04±0.01 <sup>a</sup>
12	cis-β-Carotene	ND <sup>a</sup>	0.01±0.00 <sup>a</sup>
13	All-trans-β-carotene	0.10±0.00 <sup>b</sup>	0.36±0.01 <sup>a</sup>
14	9- or 9'-cis-β-Carotene	0.04±0.00 <sup>b</sup>	0.12±0.00 <sup>a</sup>
	Total	1.90±0.02 <sup>b</sup>	3.78±0.06 <sup>a</sup>

EtAc = ethyl acetate extract; EtOH = ethanol extract; ND = not detected.

<sup>a</sup> Average of duplicate analyses ± standard deviation.

<sup>b</sup> Data with different superscript letters (a and b) in the same row are significantly different at  $p < 0.05$ .

and their cis isomers in *S. barbata* extracts. The ethanol extract was found to contain all-trans-violaxanthin, luteoxanthin, 13- or 13'-cis-lutein, all-trans-lutein, all-trans-zeaxanthin, 9- or 9'-cis-lutein, 15- or 15'-cis-β-carotene, all-trans-β-carotene, and 9- or 9'-cis-β-carotene. In addition to these carotenoids, four more carotenoids including 9- or 9'-cis-neoxanthin, β-carotene-5,6-epoxide, β-carotene-5,8-epoxide, and cis-β-carotene were present in the ethyl acetate extract. Similar to total chlorophyll content, the ethyl acetate extract contained a higher level of total carotenoids than the ethanol extract, which can be due to lower polarity of the former.

### 3.2. Anti-inflammatory activity of *S. barbata* extract

#### 3.2.1. Effect on NO production and expression of iNOS and NF-κB

In our pre-experiment, the LPS-induced RAW 264.7 cell viability as affected by the *S. barbata* extracts at various doses (50–1000 ppm) was determined by MTT assay, and the outcome showed that high cell viability (> 90%) was attained for ethanol and ethyl acetate extracts at < 500 μg/mL and < 200 μg/mL, respectively. Thus, three doses (50 μg/mL, 100 μg/mL, and 200 μg/mL) were selected for subsequent experiments. Only a low level of 0.49 μM NO was produced for normal treatment without LPS stimulation (Figure 1A). However, after treatment with 1 μg/mL LPS for 24 hours, the NO level rose pronouncedly to 17.06 μM. Following further treatment with *S. barbata* extracts, the NO level showed a dose-dependent decline for both ethanol and ethyl acetate extracts, with the latter possessing a higher inhibition effect than the former. As the ethyl acetate extract contained a higher amount of carotenoids and chlorophylls than the ethanol extract, both functional components should play a vital role in retarding NO production. Similarly, no expression of iNOS was shown for normal treatment without LPS stimulation (Figure 1B). However, after treatment with LPS for 24 hours, the iNOS expression increased greatly (Figure 1B). In addition, the iNOS expression decreased greatly for both

ethanol and ethyl acetate extracts at 200 μg/mL, with the latter exhibiting better inhibition efficiency than the former, which may be accounted for by the presence of higher levels of carotenoids and chlorophylls.

Following LPS-induced stimulation of cells, a series of signal transduction can occur for Iκ-Bα kinase activation and NF-κB dissociation into nucleus for conjugation with the κB site of promoter or enhancer, leading to transcription of target genes. Moreover, many studies have demonstrated that the NF-κB-regulated genes include proinflammatory factors such as iNOS. Thus, the expressions of both iNOS and NF-κB were measured, with the subunit being p65. A similar trend was observed for the NF-κB expression, but the ethanol extract showed a higher inhibition effect than the ethyl acetate extract, although there was no significant difference ( $p > 0.05$ ) between normal treatment and ethyl acetate extract (Figure 1C).

#### 3.2.2. Effect on PGE<sub>2</sub> production and COX-2 expression

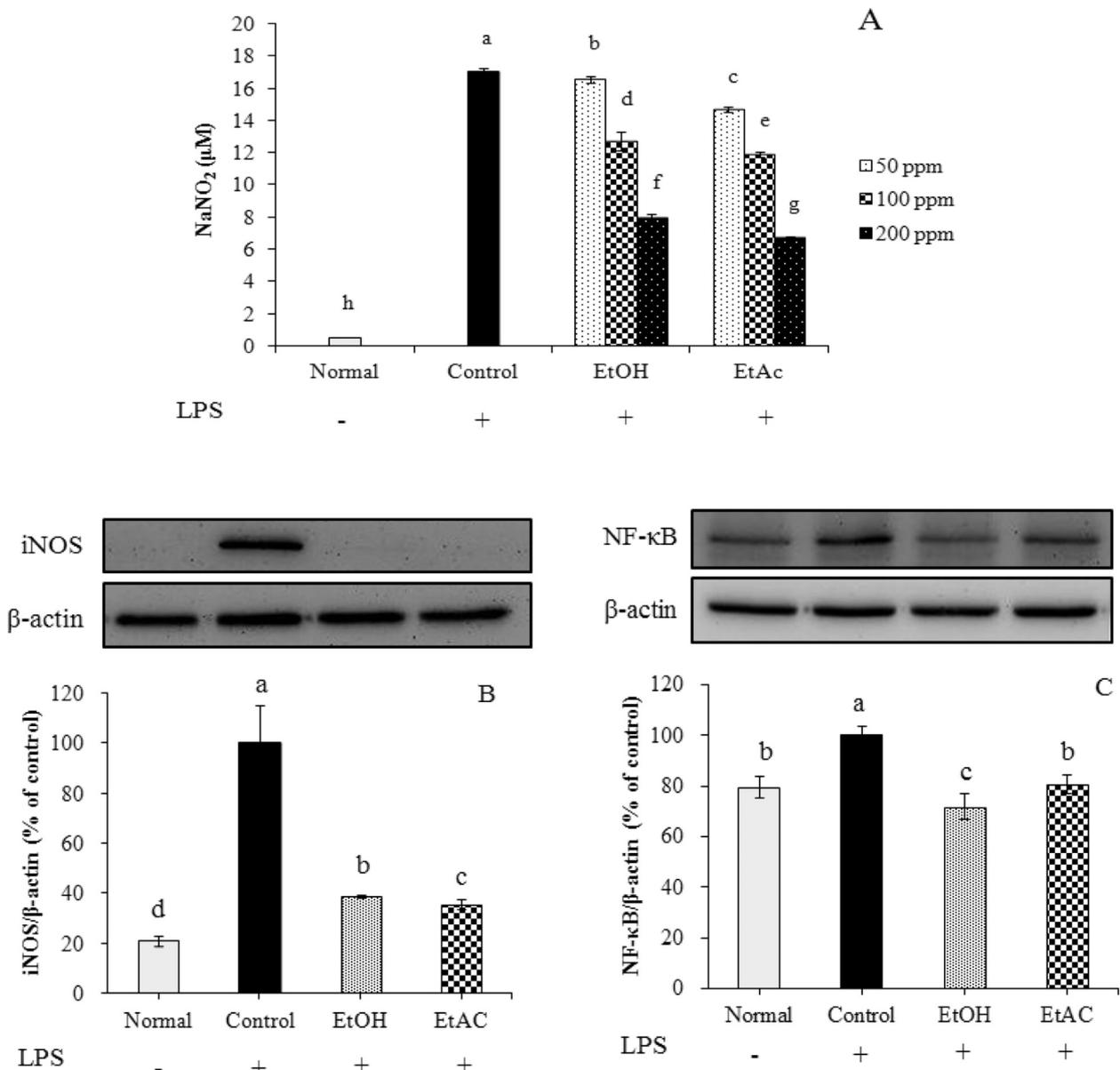
Figure 2 shows the effect of both ethanol and ethyl acetate extracts on PGE<sub>2</sub> production and COX-2 expression. After treatment of RAW 264.7 cells with 1 μg/mL LPS for 24 hours (control treatment), PGE<sub>2</sub> production was significantly higher ( $p < 0.05$ ) than that without 1 μg/mL LPS (Figure 2A). However, both ethanol and ethyl acetate extracts followed a dose-dependent decrease in PGE<sub>2</sub> production. In addition, the higher the dose, the better the inhibition effect. By comparison at the same dose (100 μg/mL and 200 μg/mL), lower PGE<sub>2</sub> production was shown for the ethanol extract than the ethyl acetate extract. Likewise, both ethanol and ethyl acetate extracts at the same dose (200 μg/mL) could inhibit COX-2 expression significantly, with the former possessing a higher retardation effect (Figure 2B).

#### 3.2.3. Effect on IL-6 and IL-1β production as well as p-ERK expression

Figure 3 shows the effect of both ethanol and ethyl acetate extracts on the production of IL-6 and IL-1β. Large amounts of IL-6 (10.32 ng/mL) and IL-1β (207 pg/mL) were produced after treatment of RAW 264.7 cells with 1 μg/mL LPS, which were significantly higher ( $p < 0.05$ ) than those produced without LPS treatment (Figure 3A and 3B). Moreover, both ethanol and ethyl acetate extracts showed a dose-dependent decline in both IL-6 and IL-1β production. Comparatively, both extracts at the same dose showed only a slight difference in inhibiting IL-6 and IL-1β production. As the regulation of IL-1β and IL-6 can be closely associated with the activation route of ERKs [16], the effect of *S. barbata* extracts on p-ERK expression was investigated. As shown in Figure 3C, the p-ERK expression rose pronouncedly after treatment of RAW 264.7 cells with LPS for 30 minutes. However, after treatment with both ethanol and ethyl acetate extracts at 200 μg/mL, the p-ERK expression was reduced significantly ( $p < 0.05$ ), with the latter showing a more pronounced effect than the former. It may be postulated that both *S. barbata* extracts were effective in inhibiting IL-β and IL-6 production through the ERK-activated pathway.

#### 3.2.4. Effect on TNF-α production and p-JNK expression

The effect of *S. barbata* extracts on TNF-α production and p-JNK expression is shown in Figure 4. A large TNF-α production

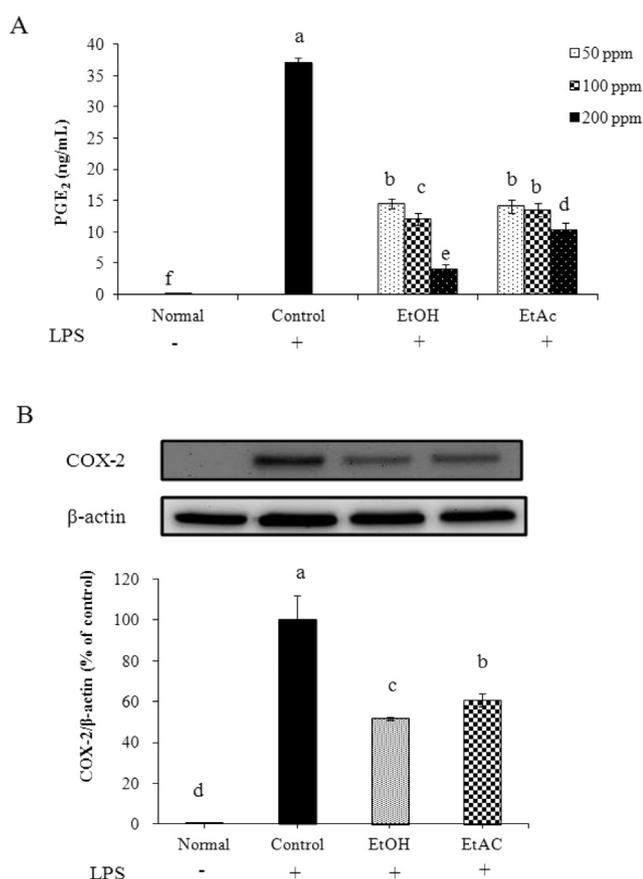


**Figure 1 – (A) Production of NO and expressions of (B) iNOS and (C) NF-κB in LPS-stimulated RAW 264.7 cells as affected by *Scutellaria barbata* extracts. Data with different letters (a–h) are significantly different at  $p < 0.05$ . EtAc = ethyl acetate extract; EtOH = ethanol extract; iNOS = nitric oxide synthase; LPS = lipopolysaccharide; NF-κB = nuclear factor-kappa B.**

and p-JNK expression was found after treatment of RAW 264.7 cells with LPS. Compared with control, only a slight difference in TNF- $\alpha$  production was observed for both ethanol and ethyl acetate extracts at the same dose (Figure 4A). However, for the p-JNK expression, both ethanol and ethyl acetate extracts at 200  $\mu\text{g}/\text{mL}$  showed a significantly lower level ( $p < 0.05$ ) than control, while there was no significant difference ( $p > 0.05$ ) between ethanol and ethyl acetate extracts (Figure 4B). This outcome implied that both extracts could reduce LPS-induced p-JNK expression of RAW 264.7 cells significantly ( $p < 0.05$ ).

#### 4. Discussion

Most of the literature reports focused on determination of flavonoids, including scutellarin, hesperidin, naringenin, luteolin, apigenin, baicalin, and wogonin [4,12,17], while the other functional components were less explored in *S. barbata* extracts. In our previous study, we developed an HPLC–DAD–MS–APCI method for determination of 18 carotenoids in *S. barbata* extracts [13]. In this study, we further identified and quantified 12 chlorophylls and their



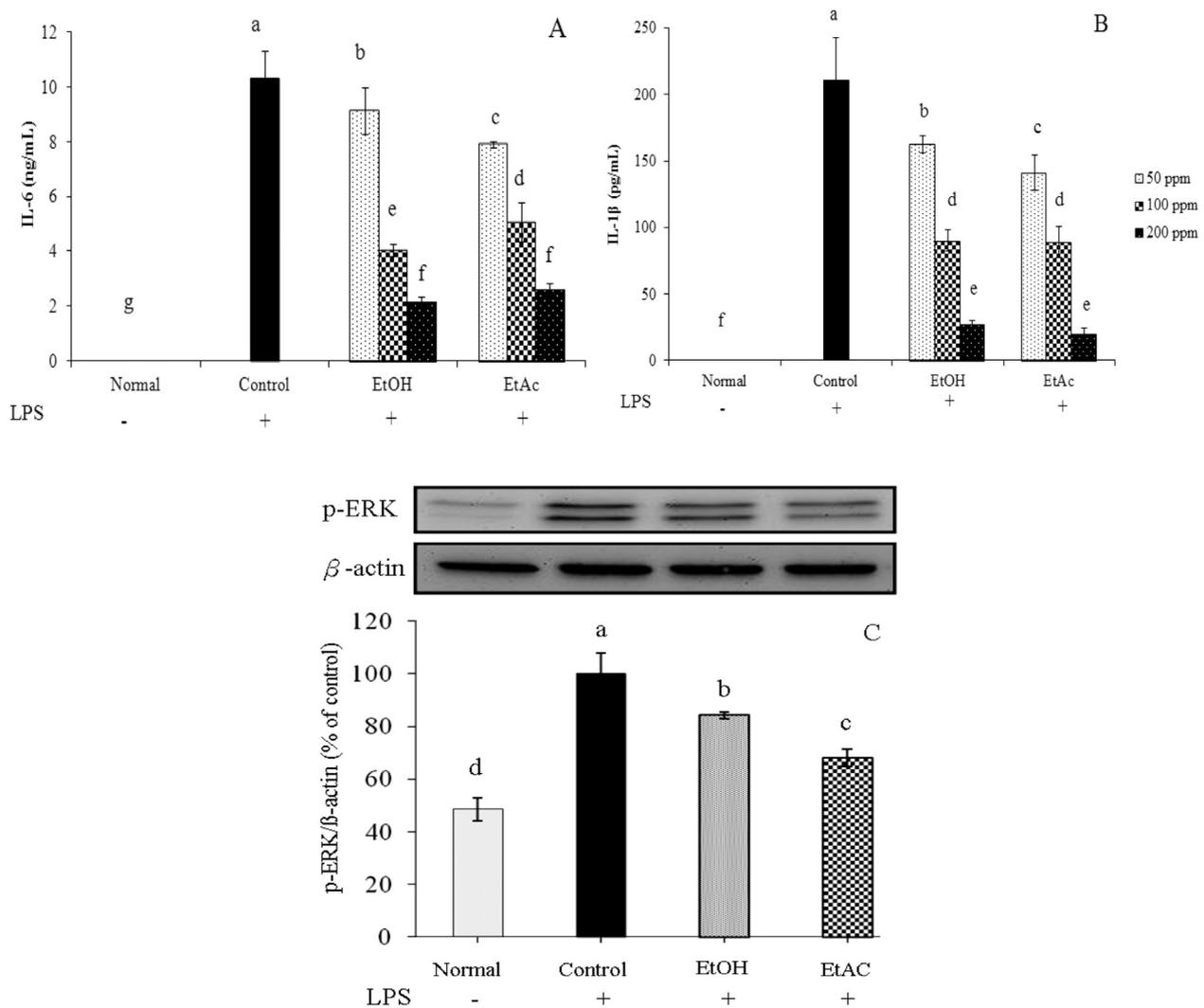
**Figure 2 – (A) Production of PGE<sub>2</sub> and (B) expression of COX-2 in LPS-stimulated RAW 264.7 cells as affected by *Scutellaria barbata* extracts. Data with different letters (a–f) are significantly different at  $p < 0.05$ . COX-2 = cyclooxygenase-2; EtAc = ethyl acetate extract; EtOH = ethanol extract; LPS = lipopolysaccharide; PGE<sub>2</sub> = prostaglandin E<sub>2</sub>.**

derivatives in *S. barbata* extracts, thereby providing more detailed information about the variety and amount of functional components. In addition, we demonstrated that the solvent variety can affect extraction efficiency and composition of functional components, as evident from the presence of more carotenoids and chlorophylls in the ethyl acetate extract, as well as more flavonoids and phenolic acids in the ethanol extract (Table 1). A similar outcome was reported by Lou et al [18], showing that a higher level of total phenolics was present in calamondin peel. Likewise, most of the phenolic compounds in *S. barbata* extracts were found to be hydrophilic. In another study, Kalidindi et al [19] used chloroform or methanol to extract functional components from *Annona squamosa* Linn. leaves, and reported that glycosides, saponins, tannins, flavonoids, phenols, alkaloids, carbohydrates, amino acids, and steroids were present in the extract, some of which were efficient in regulation of immune function. In a similar study, Lin and Shieh [9] compared the effect of *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and water extracts of *S. barbata* on anti-inflammatory activity of carrageenan-induced paw edema in rats, and reported that the chloroform extract was the

most efficient, with baicalin being more effective than baicalein or wogonin. More recently, Chen and Zhang [10] extracted 81 Chinese herbs with 70% ethanol and studied their anti-inflammatory activity on LPS- and interferon gamma-induced RAW 264.7 cells, and reported that the ethanol extract could inhibit 50% NO production. However, both studies did not investigate the anti-inflammation mechanism and determine the variety and amount of functional components. Our study demonstrated that both ethanol and ethyl acetate extracts could decrease the expressions of iNOS, COX-2, p-ERK, and p-JNK through inhibition of NF- $\kappa$ B activity for a subsequent decrease of secretion of proinflammatory mediators, including NO, PGE<sub>2</sub>, IL-1 $\beta$ , and IL-6. Nevertheless, no significant decline ( $p > 0.05$ ) in TNF- $\alpha$  expression was observed for both ethanol and ethyl acetate extracts when compared with the control, probably because of the presence of some other functional components that were not determined in this study. Miles et al [20] conducted an experiment by diluting and stimulating human whole-blood cultures with LPS in the presence of phenolics and flavonoids, including vanillic, *p*-coumaric, syringic, homovanillic, and caffeic acids; kaempferol; oleuropein glycoside; and tyrosol, at a concentration range of  $10^{-7}$ – $10^{-4}$ M. Result showed that all the polyphenols failed to inhibit TNF- $\alpha$  expression. Likewise, Yeh et al [21] reported that a low dose of  $\beta$ -carotene ( $2\mu\text{M}$ ) failed to retard the LPS-induced expression of RAW 264.7 cells. On the contrary, a higher dose of  $\beta$ -carotene ( $20\mu\text{M}$ ) could raise TNF- $\alpha$  expression. However, in our study, both ethanol and ethyl acetate extracts showed a dose-dependent anti-inflammatory activity on RAW 264.7 cells. Nevertheless, the anti-inflammatory efficiency can be varied for both ethanol and ethyl acetate extracts, which can be attributed to the presence of different varieties and amounts of functional components as mentioned above.

The anti-inflammatory activity of phenolic acids, flavonoids, chlorophylls, and carotenoids has been well documented. For instance, flavonoids can retard signal transduction of mitogen-activated protein kinase or NF- $\kappa$ B expression for further reduction of proinflammatory cytokine expression. In addition, flavonoids can decrease expressions of COX-2, iNOS, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, as well as reduce PGE<sub>2</sub> production through inhibition of arachidonic acid production from PLA 2 and expression of both COX-1 and COX-2 [22]. The same phenomenon was observed in several other studies [23–25]. In addition to flavonoid extracts, the effect of flavonoid standards on anti-inflammation was extensively studied. In a study dealing with the effect of edaravone and scutellarin on anti-inflammation in activated microglia in experimentally induced ischemia injury in rats and in BV-2 microglia, Yuan et al [26] reported that the production of iNOS, NO, and reactive oxygen species could be inhibited. Likewise, Pyungwi-San, a traditional Korean herb, was shown to inhibit LPS-induced inflammation of RAW 264.7 cells through retardation of PGE<sub>2</sub> production, which could be due to the presence of liquiritin, hesperidin, and glycyrrhizin [27].

In addition to flavonoid standards, chlorophyll a ( $2.5$ – $10\mu\text{g/mL}$ ) was found to be effective in inhibiting NO production caused by LPS-induced inflammation of RAW 264.7 cells [28]. The mechanism was associated with the inhibition of NF- $\kappa$ B activity and decrease of iNOS expression.

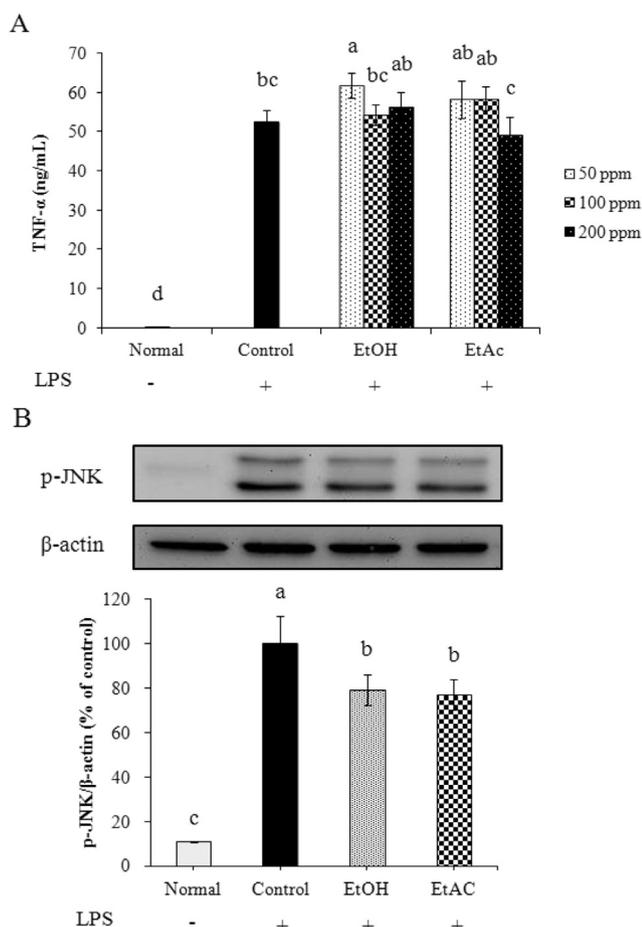


**Figure 3 – Production of (A) IL-6 and (B) IL-1 $\beta$ , as well as (C) expression of p-ERK in LPS-stimulated RAW 264.7 cells as affected by *Scutellaria barbata* extracts. Data with different letters (a–g) are significantly different at  $p < 0.05$ . EtAc = ethyl acetate extract; EtOH = ethanol extract; IL = interleukin; LPS = lipopolysaccharide; p-ERK = phosphor extracellular signal-regulated kinase.**

Moreover, the inhibition followed a dose-dependent response [28]. In another study, Subramaniam et al [29] illustrated that chlorophyll a, chlorophyll b, pheophytin a, and pheophytin b possessed anti-inflammatory activity both *in vitro* and *in vivo*. The former *in vitro* experiment involved LPS-induced inflammation of human placenta kidney cells, with chlorophyll a being more effective in inhibiting TNF- $\alpha$  expression than chlorophyll b. The latter *in vivo* experiment dealt with carrageenan-induced paw edema of Swiss albino mice through smearing of chlorophyll a, chlorophyll b, pheophytin a, and pheophytin b (all dissolved in coconut oil) onto mice feet. The outcome showed that chlorophyll a was more efficient in inhibiting inflammation than pheophytin a. The effect of various chlorophylls isolated from the algae *Saccharina japonica* on anti-inflammation of RAW 264.7 cells was studied by Islam et al [30], reporting that only pheophorbide a and pheophytin a could inhibit iNOS expression and NO production. In a recent study, Park et al [31] further demonstrated that both pheophytin a and chlorophyll a could significantly

reduce ( $p < 0.05$ ) the levels of NO, TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and chemokines including macrophage inhibitory protein-1 $\alpha$ , macrophage chemoattractant protein-1, and interferon gamma-inducible protein-10 in BV2 cells stimulated with LPS and interferon gamma.

For carotenoids,  $\beta$ -carotene was efficient in inhibiting the production of both arachidonic acid and PGE $_2$  [32]. Similarly, Obgami et al [33] reported that carotenoids (12.5  $\mu$ m and 25  $\mu$ m) could inhibit LPS-induced inflammation of RAW 264.7 cells through a decrease in PGE $_2$  production. In addition,  $\beta$ -carotene could reduce iNOS expression and NO level in LPS-induced inflammation of RAW 264.7 cells [34]. In a later study, Yang et al [35] evaluated the effect of *Dunaliella salina* alga, rich in all-trans- $\beta$ -carotene and 9- or 9'-cis- $\beta$ -carotene, on LPS-induced inflammation of RAW 264.7 cells, and reported that all the expressions of IL-1 $\beta$ , IL-6 TNF- $\alpha$ , NO, PGE $_2$ , COX-2, and iNOS followed a dose-dependent decline. Taken together, all the results shown above are in agreement with our findings in this study.



**Figure 4 – (A) Production of TNF- $\alpha$  and (B) expression of p-JNK protein expression in LPS-stimulated RAW 264.7 cells as affected by *Scutellaria barbata* extracts. Data with different letters (a–d) are significantly different at  $p < 0.05$ . EtAc = ethyl acetate extract; EtOH = ethanol extract; LPS = lipopolysaccharide; TNF- $\alpha$  = tumor necrosis factor- $\alpha$ .**

## 5. Conclusion

In conclusion, both ethanol and ethyl acetate extracts contained functional components including flavonoids, phenolic acids, chlorophylls, and carotenoids, with the former containing more flavonoids and phenolic acids, and the latter containing more chlorophylls and carotenoids. Both extracts possessed anti-inflammatory activity toward RAW 264.7 cells through inhibition of NF- $\kappa$ B activity for reduction in the expressions of iNOS, COX-2, p-ERK, and p-JNK, as well as in the production of NO, PGE<sub>2</sub>, IL-1 $\beta$ , and IL-6. This finding suggested that the *S. barbata* extract may be used as an anti-inflammatory agent for possible future biomedical applications.

## Conflicts of interest

The authors declare that there are no potential conflicts of interest.

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