Life Sciences 231 (2019) 116519

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Phytochemical profile of hot water extract of *Glechoma hederacea* and its antioxidant, and anti-inflammatory activities

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ARTICLE INFO

Keywords:
Glechoma hederacea
Rosmarinic acid
Antioxidant
Anti-inflammatory
RAW264.7 macrophages

ABSTRACT

Glechoma hederacea belongs to the Labiatae family and has many biological activities. The objective of this study was to evaluate the chemical composition, antioxidant and anti-inflammatory activities of a hot water extract of *G. hederacea* (HWG). Our results indicated that rosmarinic acid, chlorogenic acid, caffeic acid, rutin, genistin, and ferulic acid were the most abundant phytochemicals in HWG. The free radical scavenging capacity of HWG in cell-free systems was evaluated by using the α,α-diphenyl-β-picrylhydrazyl (DPPH) and β-carotene bleaching assays. The antioxidant and anti-inflammatory activities of HWG were determined *in vitro* in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages. The results demonstrated that DAPI staining, the comet assay, and DNA fragmentation showed that HWG prevented LPS-induced DNA damage in RAW264.7 macrophages, reduced the content of LPS-induced nitric oxide (NO) and malondialdehyde (MDA), increased GSH levels, and regulated antioxidant enzyme activities. We also demonstrated that HWG significantly decreased the LPS-induced mRNA expression of iNOS, COX-2, TNF-α, IL-6, and IL-1β in RAW264.7 macrophages, and reduced the LPS-induced protein expression of iNOS and COX-2 in RAW264.7 macrophages. These results show that HWG and its main components possess potent antioxidant and anti-inflammatory properties.

1. Introduction

Oxidative stress is the imbalance between the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and their clearance by the antioxidants. ROS, such as superoxide (O_2^-), and reactive nitrogen species (RNS), such as nitric oxide (NO), are produced spontaneously from activated cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), to form large amounts of peroxynitrite ($ONOO^-$), which is also an effective nitrating agent that reacts with lipids, proteins, carbohydrates, and deoxyribonucleic acids. The prolonged generation of ROS caused by inflammatory mediators can induce oxidative DNA damage [1]. Oxidative stress may result from either an increase in ROS production, a decrease in the activity of antioxidant enzymes (glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT)), or a decrease in the levels of non-enzymatic antioxidants such as glutathione (GSH). Malondialdehyde (MDA), an indicator of lipid peroxidation, is frequently measured to determine the level of oxidative damage in cells [2]. ROS have an important role in lipopolysaccharide (LPS)-induced toxicity through the activation of the nuclear factor-kappa B (NF-κB) pathway, which induces the gene expression of inflammatory mediators [1,3].

Oxidative stress and inflammation are both responsible for the pathogenesis and development of various chronic diseases, such as cancer, hepatic diseases, and autoimmune diseases [4]. One of the LPS-induced pathways involves NF-κB, which participates in the production of several constitutive cytokines such as TNF-α, IL-6, IL-1β, and inflammation-related gene expression of the iNOS and COX-2 genes [5].

Glechoma hederacea is a traditional Chinese herbal medicine belonging to the Labiatae family. It was been previously reported that *G. hederacea* var. *longituba* exhibited inhibition of IFN-γ/LPS-induced NO, IL-12p70, and TNF-α production in mouse peritoneal macrophages [6]. We reported that hot water extracts of *G. hederacea* possesses antioxidant activity owing to the presence of polyphenolic compounds [7,8]. Supporting evidence indicated that *G. hederacea* extracts possess various biological activities, including depigmentation, anti-melanogenic, anti-tumor, antioxidative, hepatoprotective, and anti-inflammatory activity [9–13]. Therefore, the aim of the present study was to investigate the *in vitro* antioxidant and anti-inflammatory effects of

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https://doi.org/10.1016/j.lfs.2019.05.075

Available online 30 May 2019
0024-3205/ © 2019 Published by Elsevier Inc.

hot water extract of *G. hederacea*, and to assess the ability of this extract to combat LPS-induced oxidative stress and inflammation.

2. Material and methods

2.1. Chemicals and reagents

β-Carotene, linoleic acid, Tween 80, butylated hydroxy anisole (BHA), dimethyl sulfoxide (DMSO), monobromobimane (MbBr), phenylmethylsulfonyl fluoride (PMSF), 4′,6-diamidino-2-phenylindole (DAPI), lipopolysaccharide (LPS; from *Escherichia coli*, serotype O111: B4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Griess reagent (1 g/kg sulfanilamide and 0.1 g/kg N-1-naphthylethylene diamidedihydrochloride in 2.5 g/kg H_3PO_4), sodium nitrite, pyrogallol, nitroblue tetrazolium (NBT), bovine serum albumin (BSA), ethidium bromide, dithiothreitol (DTT), agarose, and other required chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin, trypsin-EDTA, deoxynucleotide triphosphate (dNTP), oligo(dT), and Taq DNA polymerase were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). All reagents used were of at least reagent grade. Deionized distilled water (ddH_2O), used for the preparation of solutions and buffers, was purified by using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Preparation of G. hederacea extracts

Naturally grown *G. hederacea* was obtained from Taichung City, Taiwan. A voucher specimen was identified by Dr. Bing-Shiunn Chen and deposited in the Department of Horticulture, National Chung Hsing University, Taichung City, Taiwan (No. NCHU-2016-001). The *G. hederacea* extracts were prepared in accordance with our previously reported procedures [8].

2.3. High-performance liquid chromatography (HPLC) analysis

To determine the polyphenolic compounds in *G. hederacea* extracts, HPLC analysis was performed in accordance with our previously described method, with modifications [14]. According to the plot of the peak-area ratio (y) *vs.* concentration (x), the regression equations of the constituents and their correlation coefficients (r) were quantification was performed with external standards for which the standard curves had an r^2 value of ≥ 0.9999.

2.4. Measurement of DPPH radical-scavenging activity

The DPPH radical-scavenging activity of HWG was estimated in accordance with our previously reported procedures [15].

2.5. Determination of antioxidant activity by using β-carotene bleaching assay

Antioxidant activity was evaluated by using the β-carotene bleaching method [16]. To determine the absorbances of all samples were performed in accordance with our previously reported procedures [13].

2.6. Determination of cell viability

The RAW 264.7 murine macrophage cell line was obtained from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). The cells were cultured in DMEM supplemented with 10 g/kg FBS, 2 mM L-glutamine, and 1 g/kg penicillin-streptomycin. RAW 264.7 cells were plated onto 12-well plates at a density of 3×10^5 cells/ml/well. The cells were treated 100, 200, and 400 μg/ml HWG alone or co-treated with LPS (1 μg/ml) and incubated at 37 °C for 20 h. After incubation, was performed in accordance with our previously described method [13].

2.7. DAPI, comet and DNA fragmentation assay

4′,6-Diamidino-2-phenylindole (DAPI) staining was conducted to detect apoptotic changes in RAW 264.7 cells due to HWG. And DNA damage in RAW 264.7 cells was detected by using the comet assay, which used the single-cell gel electrophoresis method described by Chao et al. [17] RAW 264.7 cells were incubated with various concentrations of HWG and/or LPS for 16 h, and were performed in accordance with our previously reported procedures [13].

2.8. Measurement of lipid peroxide (MDA) and reduced glutathione (GSH) levels, and glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) activities

RAW 264.7 cells were incubated with various concentrations of HWG and/or LPS for 16 h, harvested, and then sonicated with phosphate buffer (pH 6.8) containing 1 mM PMSF to obtain cell homogenates. To determine the MDA, GSH, GPx, SOD and CAT were performed in accordance with our previously reported procedures [13].

2.9. Determination of NO concentration

Nitrite was measured as an indicator of NO production after treatment of HWG and/or LPS for 16 h. To determine the NO was performed in accordance with our previously described method [13].

2.10. Measurement of mRNA expression of iNOS, COX-2, TNF-α, IL-6, and IL-1β

Total RNA was extracted using the Qiagen RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA). Reverse transcription-polymerase chain reaction (RT-PCR) was applied to evaluate the mRNA expression of iNOS, COX-2, TNF-α, IL-6, and IL-1β. PCR primers for iNOS, COX-2, TNF-α, β-actin and the following PCR conditions were performed in accordance with our previously reported procedures [13]. PCR primers for IL-6 and IL-1β used as an internal control for each RT-PCR, contained the following sequences: IL-6 sense (5′-GAG GAT ACC ACT CCC AAC AGA-3′) and antisense (5′-AAG TGC ATC ATC GTT GTT CAT ACA-3′); IL-1β sense (5′-TGC AGA GTT CCC CAA CTG GTA CAT C-3′) and antisense (5′-GTG CTG CCT AAT GTC CCC TTG AAT C-3′). The following PCR conditions were applied: IL-6 (94 °C, 60 s; 57 °C, 60 s; 72 °C, 60 s) for 30 cycles; IL-1β (94 °C, 60 s; 60 °C, 60 s; 72 °C, 60 s) for 30 cycles. Rat β-actin was used as a control for cDNA loading and the relative expressions of iNOS, COX-2, TNF-α, IL-6, and IL-1β were calculated. Photographs and scans were analyzed by using the BioSpectrum® imaging system (UVP, Cambridge, United Kingdom).

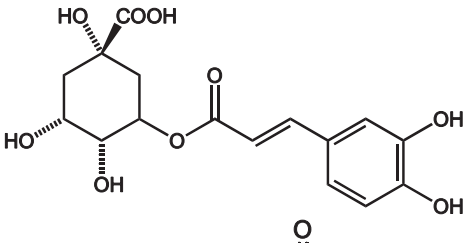
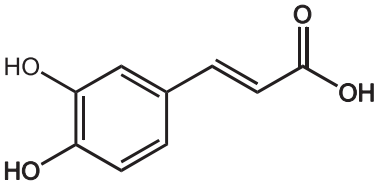
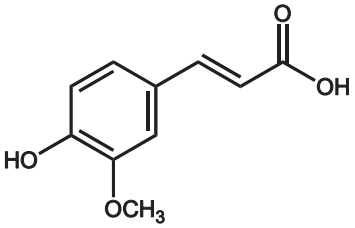
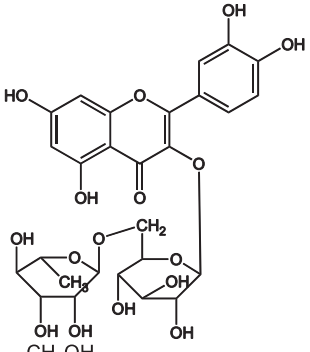
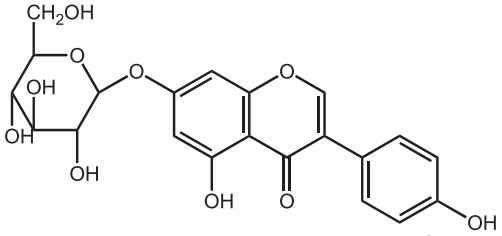
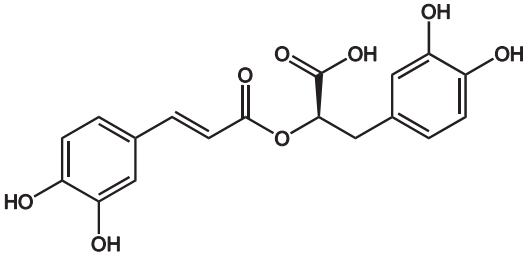
2.11. Western blot analyses

Total proteins were extracted from cells and the concentrations were determined by using a bicinchoninic acid (BCA) protein assay kit (Pierce). The samples were separated by SDS-PAGE electrophoresis and then transferred to PVDF membranes. The blots were incubated overnight at 4 °C with the primary antibodies, including iNOS, COX-2, and β-actin, and then incubated for 1 h further with the secondary antibody. The band intensities were measured by using ImageJ software.

2.12. Statistical analysis

All assays were conducted a minimum of three times with three different sample preparations. All data are expressed as the mean ± standard deviation (SD). Analysis of variance (ANOVA) was performed by using SPSS software (version 16.0; SPSS Inc., USA). One-way

Table 1
The standards content of hot water extract of Glechoma hederacea (HWG) extracts.

Compound	Retention time (min)	Amount in HWG (mg/100g)	Structures
Gallic acid	4.83	-	
Catechin	13.3	-	
Chlorogenic acid	13.97	**249.86 ± 18.33**	
Caffeic acid	15.90	**181.75 ± 8.28**	
Daidzin	21.29	14.10 ± 4.71	
Ferulic acid	24.11	**67.01 ± 5.71**	
Rutin	26.00	**93.60 ± 15.65**	
Genistin	27.95	**90.39 ± 6.92**	
Rosmarinic acid	32.92	**1397.90 ± 62.35**	
Quercetin	40.71	-	
Genistein	46.87	1.97 ± 1.56	
Flavone	55.12	-	

Each value represents mean ± S.D. (n = 3).

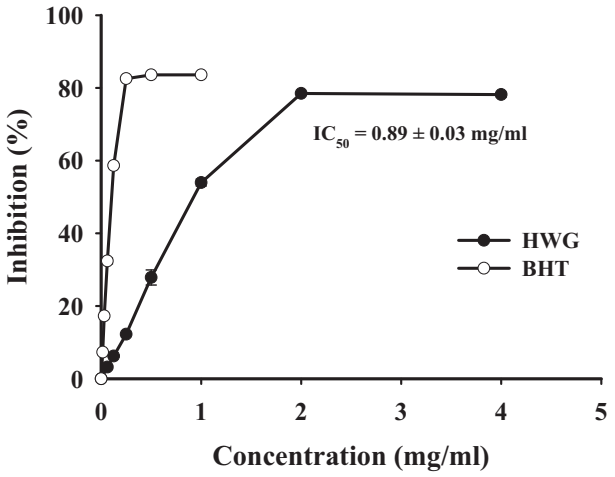


Fig. 1. The scavenging effect of HWG and BHT on DPPH radical. Each value represents the mean ± S.D. (n = 3).

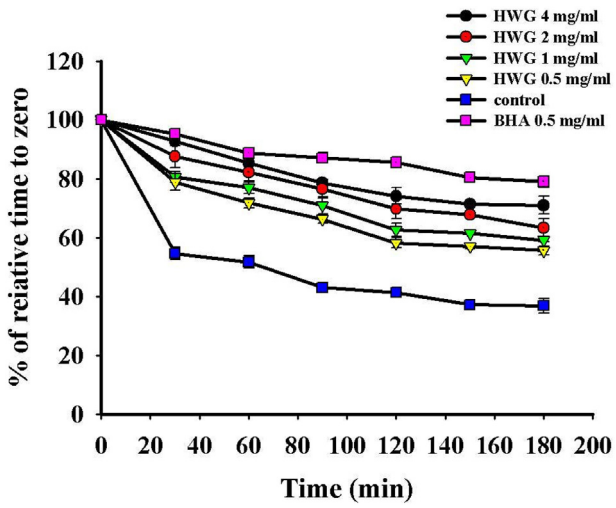


Fig. 2. The antioxidant activity of HWG assessed by using the β-carotene/linoleic acid bleaching method. The data are the mean ± S.D. (n = 3).

ANOVA and Scheffe's method were used to determine the differences between the means, with differences of $P < 0.05$ considered statistically significant.

3. Results

3.1. Chemical profile and phytochemical content

A total of 12 peaks were detected in the HPLC chromatogram of the infusion extract. The concentrations of the phenolic compounds in HWG are shown in Table 1. The study identified several polyphenolic compounds, including rosmarinic acid (1397.90 ± 62.35 mg/100 g extract), chlorogenic acid (249.86 ± 18.33 mg/100 g extract), caffeic acid (181.75 ± 8.28 mg/100 g extract), rutin (93.60 ± 15.65 mg/100 g extract), genistin (90.39 ± 6.92 mg/100 g extract), ferulic acid (67.01 ± 5.71 mg/100 g extract), daidzin, genistein, gallic acid, catechin, quercetin, and flavone.

3.2. DPPH scavenging radical of HWG

The DPPH radical is a stable free radical that has been used extensively as a tool for the estimation of the free radical scavenging activities of antioxidants. Fig. 1 shows the dose-response curve for the radical-scavenging activity of HWG and the commercial antioxidant, BHT. At a dosage of 0.0625–4 mg/ml, HWG resulted in 4.52%–83% scavenging of the DPPH radical. A comparison with the commercial antioxidant showed that the concentrations needed to obtain 80% DPPH radical scavenging activity for the HWG and BHT were 1 and 0.25 mg/ml, respectively. The results of the DPPH scavenging activity showed that the IC_{50} values for HWG and BHT were 0.89 ± 0.03 mg/ml and 0.10 ± 0.01 mg/ml, respectively.

3.3. Antioxidant activity of HWG

The percentage antioxidant activity of HWG in the β-carotene/linoleic acid bleaching method was calculated. In the absence of an antioxidant, β-carotene undergoes rapid decolorization because it is attacked by free linoleic acid radicals. The results are shown in Fig. 2. Further, 0.5–4 mg/ml HWG can markedly slow down the decrease in the absorbance at 470 nm between 0 and 180 min. The percentage of oxidized β-carotene was 46.02%–70.31% for the different concentrations of HWG, and 33.32% for BHA.

3.4. Effects of HWG on apoptosis, LPS-induced oxidative damage in LPS-stimulated RAW 264.7 cells

DAPI staining was used to detect any apoptotic changes induced by HWG in RAW 264.7 cells. As shown in Fig. 3A, fluorescence intensity increased (87.08 ± 2.43) in LPS-stimulated RAW 264.7 cells. However, 100, 200, and 400 μg/ml HWG reduced cellular DAPI fluorescence intensity levels in a dose-dependent manner (54.80 ± 2.71, 36.56 ± 2.48, and 19.60 ± 2.41, respectively). The comet assay, which detects breaks in the single and double stranded naked supercoiled DNA, was used to evaluate the DNA damage induced by chemical oxidants. The comet moment was increased (24.63 ± 2.82) in LPS-stimulated RAW 264.7 cells. However, HWG reduced cellular comet moment in a dose-dependent manner ($P < 0.05$) (Fig. 3B). The LPS-induced DNA damage of HWG-treated cells was analyzed by using a DNA fragmentation assay to examine to confirm whether HWG protected cells from inflammation. Treatment of the cells with all tested concentrations of HWG resulted in lower appearance on the DNA ladder than treatment with LPS (Fig. 3C).

3.5. Effect of HWG on MDA production, GSH levels, and antioxidant enzyme activities in LPS-stimulated RAW 264.7 cells

MDA levels increased (31 ± 0.68 nmol/mg protein) in LPS-stimulated RAW 264.7 cells. However, HWG reduced cellular MDA levels in a dose-dependent manner (Fig. 4A). The GSH/GSSG ratio was considered to be a marker of oxidative stress. Our results indicated that the GSH levels were reduced in LPS treated RAW 264.7 cells (6.03 ± 0.35 nmol/mg protein). However, the increase in the concentration of HWG significantly increased cellular GSH levels (Fig. 4B); although they did not return to the basal level, our results showed that HWG could decrease LPS-induced oxidative stress in RAW 264.7 cells. For further evaluation of the antioxidant activity of HWG, we determined the activity of important cellular antioxidant enzymes. The activity of the investigated antioxidant enzymes was found to increase in LPS-treated cells. In addition, they decreased the levels in LPS stimulated RAW 264.7 cells co-treated with HWG (Fig. 4C–E).

3.6. Effect of HWG on cell viability, NO production, iNOS, COX-2 mRNA expression and iNOS, COX-2 protein expression in LPS-stimulated RAW 264.7 macrophages

In Fig. 5A, it was shown that after treatment of RAW 264.7 cells with HWG, cell viabilities in all groups were > 98% ($P < 0.05$). LPS

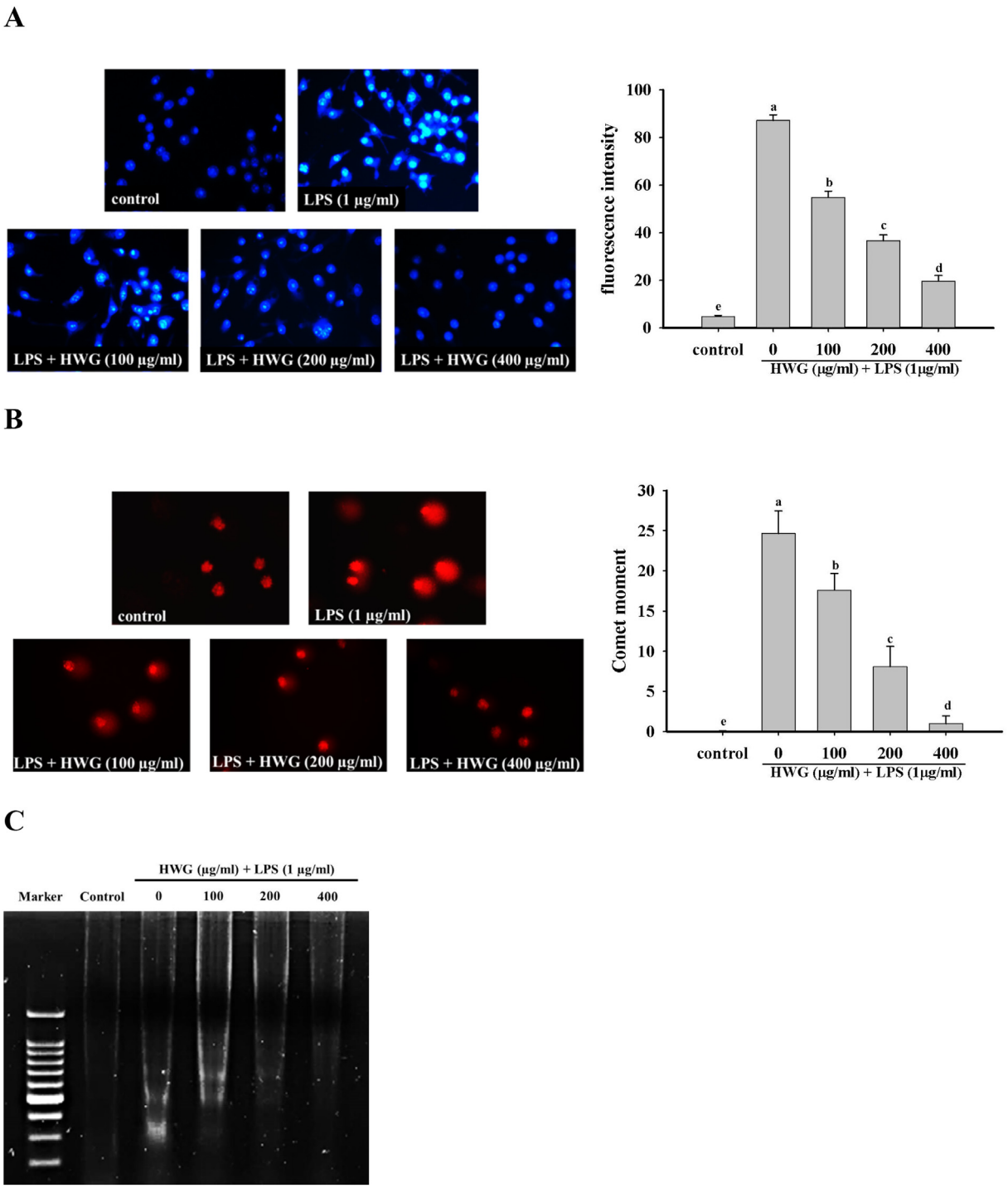


Fig. 3. The effects of HWG on DNA damage in LPS (1 μg/mL) stimulated RAW 264.7 macrophages determined by using DAPI staining and fluorescence microscopy (200×) and presented as the fluorescence intensity (A); the comet assay and fluorescence microscopy and presented as the comet moment (B); DNA fragmentation (C). Different letters (a–e) denote significant differences between groups ($P < 0.05$). The arrow indicates the apoptosis phenomenon in DNA fragments.

significantly increased the concentration of NO (39.21 ± 0.12 μM), which is a key indicator of inflammation (Fig. 5B); however, HWG inhibited nitrite accumulation in a dose-dependent manner. We also used RT-PCR to examine the effects of HWG on gene transcription. The quantification of these results is presented in Fig. 5C. iNOS mRNA expression was increased 5.22 (± 0.24)-fold in LPS-stimulated RAW 264.7 cells; however, HWG reduced cellular iNOS mRNA expression in a dose-dependent manner. COX-2 mRNA expression ratio increased by 3.19 (± 0.02)-fold in LPS-stimulated RAW 264.7 cells, and a dose-dependent decreased in COX-2 mRNA expression was also found (Fig. 5D). Similar patterns were found for the protein expression. iNOS protein expression (2.96 (± 0.07)-fold) and COX-2 protein expression (18.77 (± 0.75)-fold) were increased by in LPS-stimulated RAW 264.7 cells. In contrast, co-treatment with HWG reduced iNOS and COX-2 protein expression in a dose-dependent manner ($P < 0.05$) (Fig. 5E–F).

3.7. HWG inhibits TNF-α, IL-6, and IL-1β mRNA expression in LPS-stimulated RAW 264.7 macrophages

The mRNA expression of TNF-α (1.66 ± 0.04), IL-6

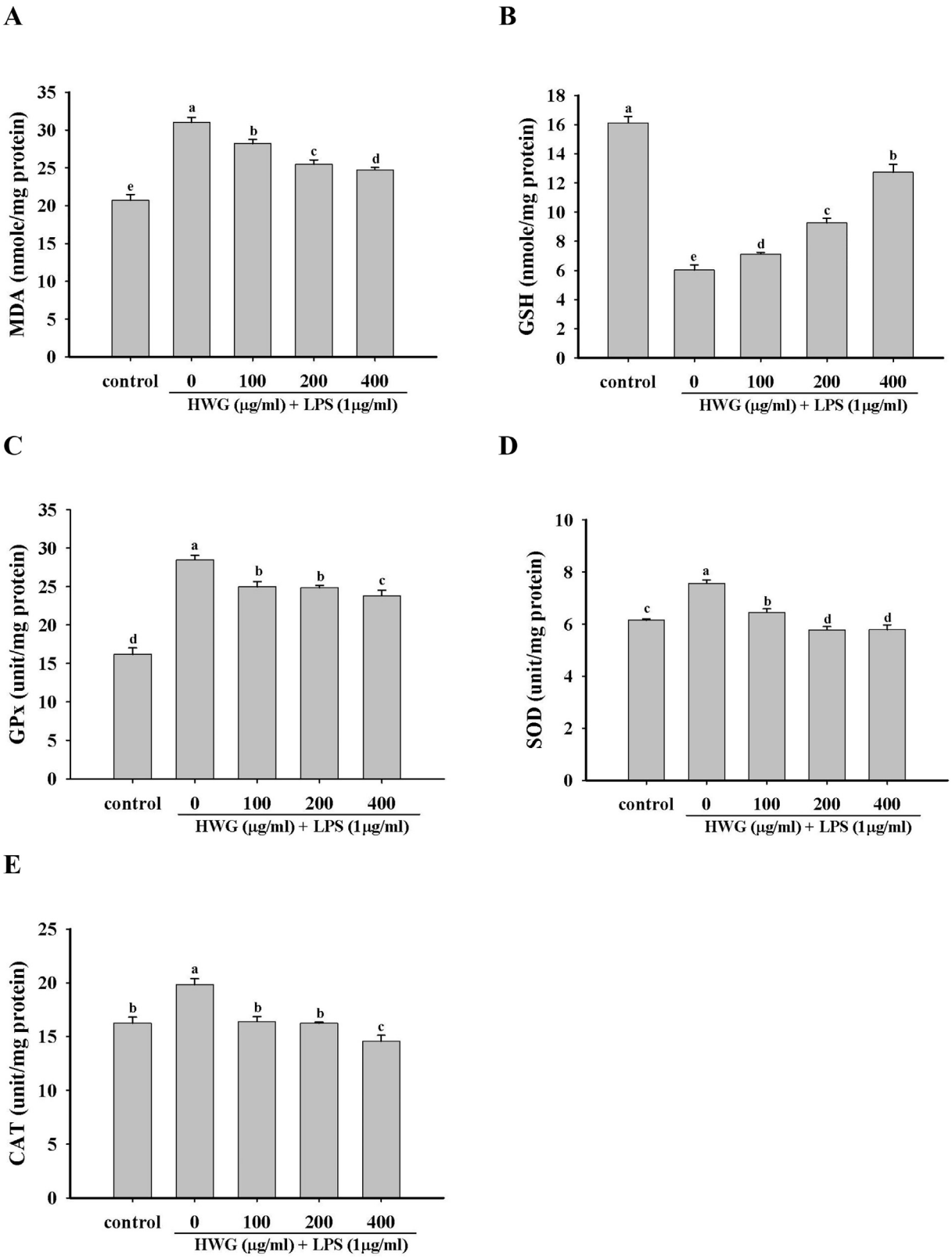


Fig. 4. The effects of HWG on the MDA (A) and GSH (B) levels, and the activities of GPX (C), SOD (D), and CAT (E) in LPS stimulated RAW 264.7 macrophages. The data are the mean ± S.D. (n = 3). Different letters (a–e) denote significant differences between groups ($P < 0.05$).

(11.76 ± 0.28), and IL-1β (5.17 ± 0.41) was increased significantly in RAW 264.7 cells after LPS stimulation. LPS was able to induce a significant increase of mRNA expression of TNF-α, IL-6, and IL-1β in RAW 264.7 cells compared with the control group ($P < 0.05$). HWG significantly inhibited the mRNA expression of TNF-α (Fig. 6A), IL-6 (Fig. 6B), and IL-1β (Fig. 6C). In this study, we showed that HWG suppressed the production of NO through blocking iNOS and COX-2 expression, and TNF-α, IL-6 and IL-1β. These data indicate that HWG

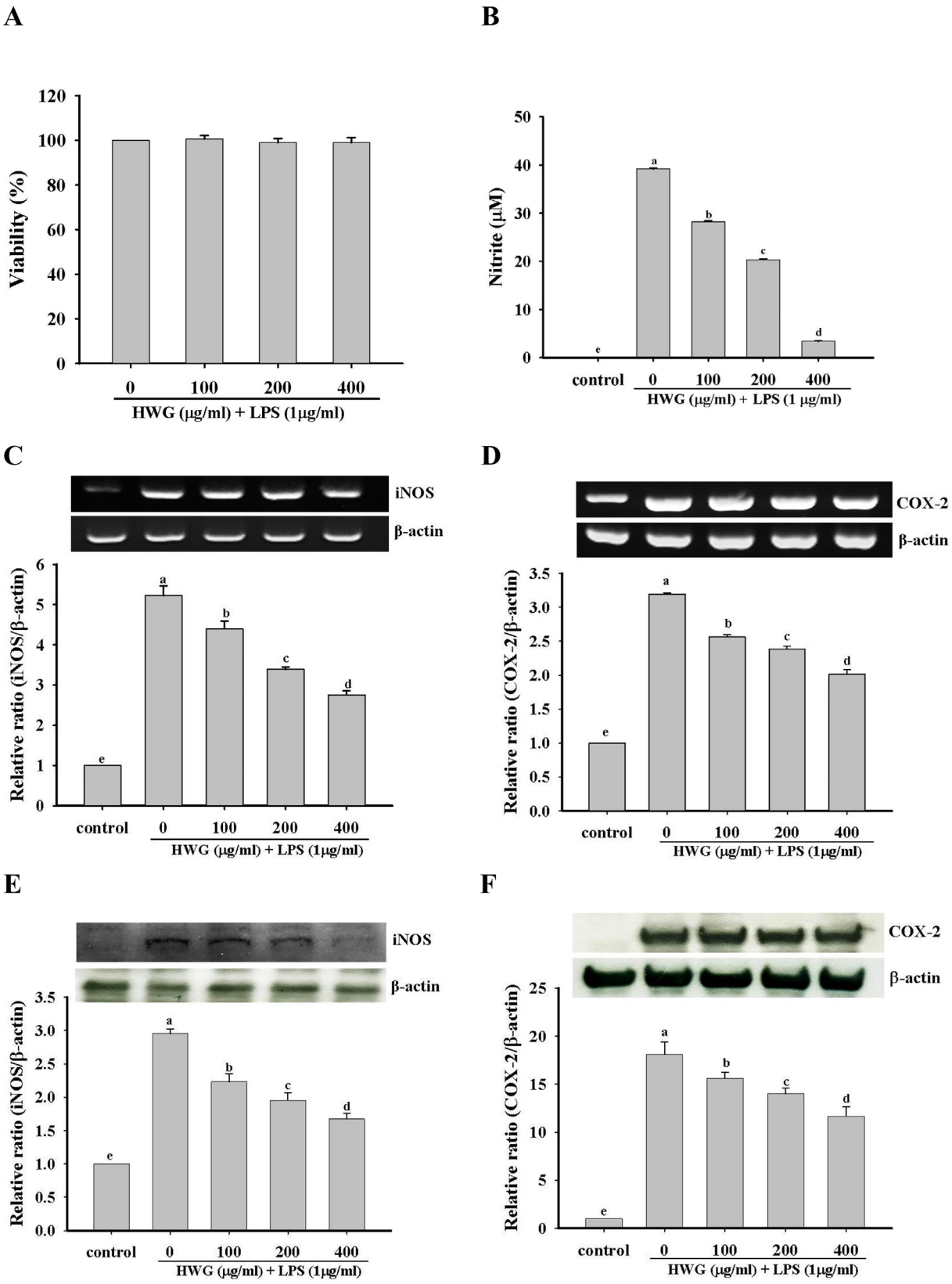


Fig. 5. The effects of HWG on cell viability, production of NO, and the expression of iNOS and COX-2 in RAW 264.7 macrophages stimulated by LPS. (A) Cell viability, (B) NO production. The mRNA (C, D) and protein (E, F) expression of iNOS and COX-2 was measured by RT-PCR and western blot analysis, respectively. The data are the mean ± S.D. of three independent experiments. Different letters (a–e) denote significant differences between groups ($P < 0.05$).

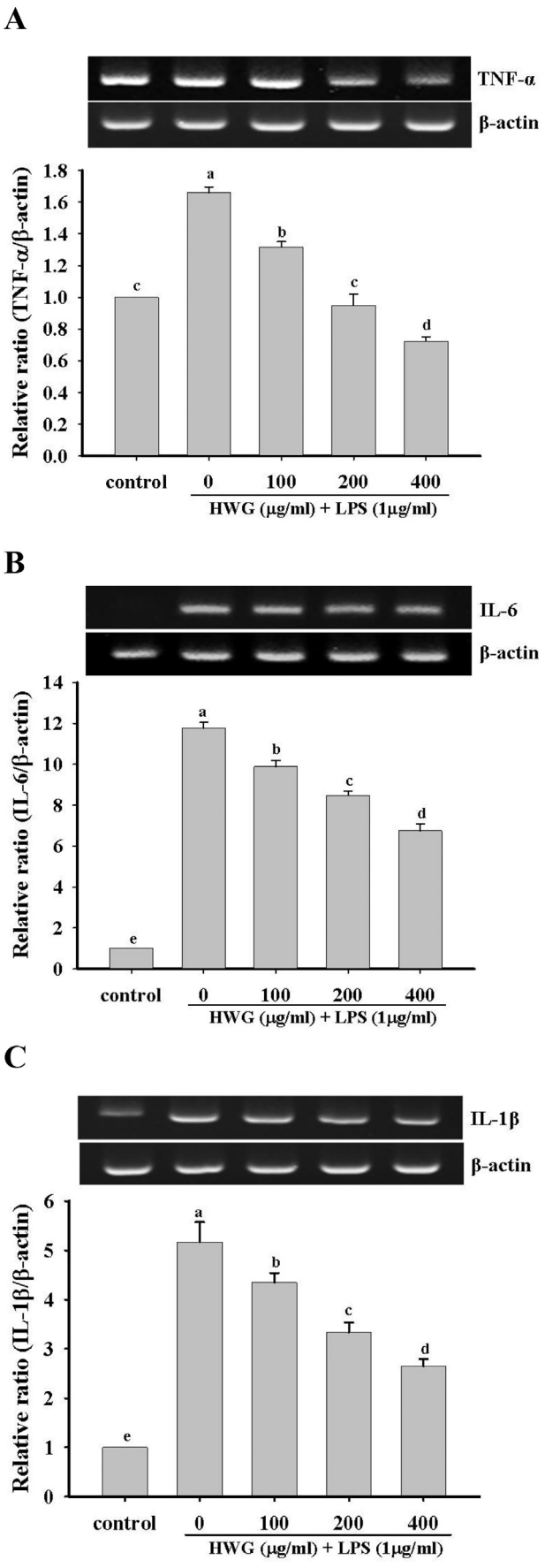


Fig. 6. The effects of HWG on mRNA expression of TNF-α (A), IL-6 (B), and IL-1β (C) in LPS stimulated RAW 264.7 macrophages. The mRNA expression of target genes was determined by using RT-PCR. The data are the mean ± S.D. (n = 3) of three independent experiments. Letters (a–e) denote significant differences between groups ($P < 0.05$).

may exert anti-inflammatory activity. Some of the general mechanisms involved in the anti-inflammatory activity are blocking pro-inflammatory cytokines, ROS scavenging mechanisms and inhibiting the NF-κB, iNOS and COX-2 pathways.

4. Discussion

Studies suggest that flavonoids, plant polyphenolic compound derivatives from natural origin, have a wide range of putative biological activities. Herb plants have traditionally been used to prevent or treat various human diseases. Several compounds such as ursolic acid, oleanolic acid, and rosmarinic acid have been identified in *G. hederacea* [7]. Rosmarinic acid and caffeic acid are common water-soluble phenolic compounds found mainly in plants of the Lamiaceae family [18]. Chizzola et al. [19] reported that rosmarinic acid influenced DPPH activity. In particular, it was shown that the antiradical activity of rosmarinic acid and of other caffeic acid derivatives was higher than that exerted by flavonoids [20].

Mitochondria regulate caspase activation through a process called mitochondrial outer membrane permeabilization. Co-treatment with HWG clearly suppressed LPS-induced oxidative damage and DNA damage in RAW 264.7 cells in a dose-dependent manner. Polyphenols, particularly flavonoids, are natural antioxidant substances with powerful ROS scavenging activities that strongly reduced the risk of oxidative stress [21].

The degree of lipid peroxidation was assayed with the level of MDA used as a measure of LPS-induced cellular oxidative stress. HWG reduced cellular MDA levels. GSH, a tripeptide composed of glutamate, exerted critical roles as antioxidants plays an important role in the protection of tissues from peroxidative attack and is perceived as a first-line defense against the ROS attack. SOD, as one of the most significant intracellular antioxidant enzymes, could function as a ROS scavenger. In addition, certain polyphenolic compounds are able to increase GSH level and the antioxidant enzyme activities [22]. Therefore, we hypothesized that HWG can increased the activities of cellular SOD, GPX, and CAT to suppress LPS-induced oxidative stress in RAW 264.7 cells.

We have demonstrated that HWG acts as a NO inhibitor through the inhibition of iNOS expression. The results of the study demonstrated that HWG exhibits significant inhibitory effects on the expression of pro-inflammatory mediators, such as NO, iNOS, and COX-2. NO is an important cellular signaling molecule that has been implicated in various pathological and physiological processes, including non-specific host defense, vasodilation, and acute or chronic inflammation [23]. Thus, the reduction of NO production by HWG was attributable to the inhibition of iNOS mRNA and protein expression.

Our study demonstrated that HWG suppressed the LPS-induced mRNA expression of TNF-α, IL-6, and IL-1β in RAW 264.7 cells. HWG not only decreased the production of the pro-inflammatory mediators from LPS, but also normalized their secretion and expression. This may be related to the presence of the polyphenolic compounds. Polyphenols can be divided into two groups: flavonoids and non-flavonoids. Polyphenol chemical structures share a basic polyphenolic structure with a single phenol ring, including phenolic acids and phenolic alcohols. These active bioactive compounds have many pharmacological effects, including antioxidant, anti-inflammatory, and anticancer properties [24,25].

In our study, we evaluated the hot water extracts of *G. hederacea* by HPLC using and detected a number of phenolic compounds. In structure-activity relationships, the phenolic compound rosmarinic acid is an

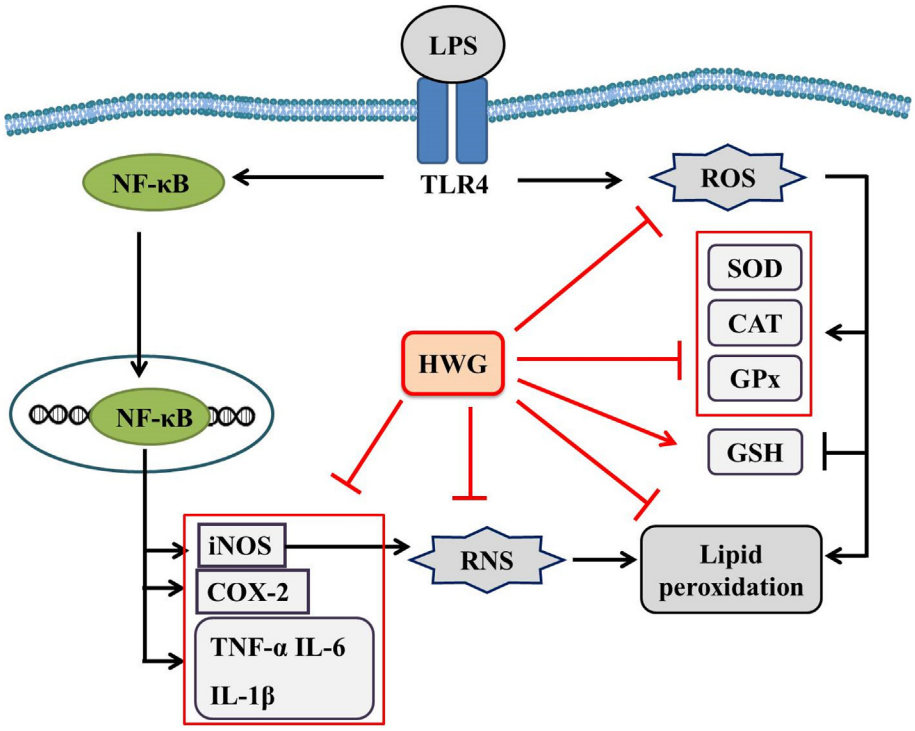


Fig. 7. The potential antioxidant and anti-inflammatory mechanisms of HWG in LPS-induced RAW 264.7 macrophages. Accumulation of reactive oxygen species (ROS) and nitrogen species is generally concurrent with inflammatory responses, which are preceded by upregulation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2). The activation of Toll-like receptor 4 (TLR4) with LPS leads to NF-κB activation. Their downstream signaling pathways are activated, which is associated with an increase in the production of TNF-α, IL-6, and IL-1β. In contrast, HWG decreased LPS-induced DNA damage and enhanced GSH content in LPS-induced RAW 264.7 macrophages.

ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. This compound is mainly present in Labiatae herbs. Rosemary extract and its bioactive components inhibited inflammatory responses stimulated by LPS through the inhibition of NO and TNF-α [26,27]. In addition, the hepatoprotective [11] and antineurodegenerative [28] effects of rosemary extract and rosmarinic acid have been explored. It is suggested that rosmarinic acid exerts a key response to inflammatory and oxidative markers, including NO, COX-2, IL-1β, PGE_2, and MMP2 [29].

Chlorogenic acid is a polyphenol abundant in a variety of plant foods, such as coffee bean and apple, and is formed by the esterification of caffeic and quinic acids. The findings from *in vivo* and *in vitro* studies have indicated that chlorogenic acid possesses a variety of biological functions, including antioxidant, anticarcinogenic and hepatoprotective activities [30]. Moreover, protect against LPS-induced acute lung injury in mice and septic shock in C57BL/6 mice [31,32]. On the other hand, chlorogenic acid also blocked the transcription of NF-κB [33].

Caffeic acid (3,4-dihydroxycinnamic acid), containing both phenolic and acrylic functional groups, is found in garlic, fruits, grains, coffee, and herbs. In macrophages, caffeic acid was shown to downregulate the LPS induced elevation of iNOS, COX-2, and TNF-α expression *via* the inhibition of the phosphorylation of mitogen-activated protein kinases (MAPKs), JNK1/2, NF-κB, and STAT-3 [34,35]. Caffeic acid was also shown to inhibit IKKβ-kinase activity and rescued C57BL/6J mice after LPS-injection or CLP-induced sepsis [36].

Rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rutinoside), which is a glycone of quercetin (also known as quercetin-3-rutinoside), is found in apple peels, potatoes, tomatoes, carrots, oranges, grapefruits, and lemons. The flavonol rutin is generated by the glycosylation of quercetin, and can be metabolized to quercetin by hydrolases present in the oral and intestinal microflora [37]. Rutin is also known to have strong anti-inflammatory [38,39], antioxidant [40] and cardiovascular protective [41] effects.

In soy and soy products, 95%–99% of genistein exists in the conjugated form genistin (glycoside). Soy isoflavones can remove free radicals, preventing aging, cardiovascular diseases, and cancer [42,43]. Both the *in vivo* and *in vitro* administration of genistin and genistein suppressed LPS-induced pro-inflammatory cytokine production [44,45], cardioprotective effects [46].

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is a strong natural antioxidant that acts *via* its phenolic nucleus and extended side chain conjugation, and forms a resonance-stabilized phenoxyradical, which accounts for its potent antioxidant potential. Ferulic acid was first isolated from a commercial resin in 1866 and chemically synthesized in 1925 [47]. Ferulic acid, inhibited LPS-induced ROS production, nitrite accumulation, iNOS protein expression [48,49]. As expected, rosmarinic acid, chlorogenic acid, caffeic acid, rutin, genistin and ferulic acid, which are known anti-oxidant, anti-inflammatory flavonoids and non-flavonoids, were identified in HWG (Table 1), strongly suggesting that the anti-oxidant, anti-inflammatory activities of HWG is attributed to the anti-inflammatory agents rosmarinic acid, chlorogenic acid, caffeic acid, rutin, genistin and ferulic acid.

5. Conclusions

In conclusion, we demonstrated that HWG improved functionality in LPS-induced cell inflammation through the reduction of ROS levels and the downregulation of pro-inflammatory genes expression. This may be related to the presence of polyphenolic compounds in this extracts, as these have been reported to inhibit NF-κB expression in LPS-stimulated macrophages. A schematic representation of the mechanism of HWG action is presented in Fig. 7. Collectively, our data revealed that HWG exerted strong antioxidant and anti-inflammatory activities.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgments

The authors are grateful for financial support from the Ministry of Science and Technology of the People's Republic of China (NSC102-2313-B-126-004-MY3, MOST 107-2635-B-424-001).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://

doi.org/10.1016/j.lfs.2019.05.075.

References

[1] G. Kallapura, N.R. Pumford, X. Hernandez-Velasco, B.M. Hargis, G. Tellez, Mechanisms involved in lipopolysaccharide derived ROS and RNS oxidative stress and septic shock, J. Microbiol. Res. Rev. 2 (1) (2014) 6–11.

[2] M. Ponzoni, F. Pastorino, D.D. Paolo, P. Perri, C. Brignole, Targeting macrophages as a potential therapeutic intervention: impact on inflammatory diseases and cancer, Int. J. Mol. Sci. 19 (7) (2018) 1953.

[3] W.W. Chao, Y.H. Kuo, B.F. Lin, Anti-inflammatory activity of new compounds from *Andrographis paniculata* by NF-κB trans-activation inhibition, J. Agric. Food Chem. 58 (4) (2010) 2505–2512.

[4] A. Sánchez, A. Calpena, B. Clares, Evaluating the oxidative stress in inflammation: role of melatonin, Int. J. Mol. Sci. 16 (8) (2015) 16981–17004.

[5] Q. Zhang, M.J. Lenardo, D. Baltimore, 30 years of NF-kappa B: a blossoming of relevance to human pathobiology, Cell 168 (1–2) (2017) 37–57.

[6] H.J. An, H.J. Jeong, J.Y. Um, H.M. Kim, S.H. Hong, *Glechoma hederacea* inhibits inflammatory mediator release in IFN-γ and LPS-stimulated mouse peritoneal macrophages, J. Ethnopharmacol. 106 (3) (2006) 418–424.

[7] J. Kim, S. Song, I. Lee, Y. Kim, I. Yoo, I. Yuoo, K. Bae, Anti-inflammatory activity of constituents from *Glechoma hederacea* var. *longituba*, Bioorg. Med. Chem. Lett. 21 (11) (2011) 3483–3487.

[8] S.T. Chou, Y.R. Chan, Y.C. Chung, Studies on the antimutagenicity and antioxidant activity of the hot water extract of *Glechoma hederacea*, J. Food Drug Anal. 20 (3) (2012) 637–645.

[9] M. Milovanovic, D. Zivkovic, B. Vucelic-Radovic, Antioxidant effects of *Glechoma hederacea* as a food additive, Nat. Prod. Commun. 5 (1) (2010) 61–63.

[10] Z. Qiao, Y. Koizumi, M. Zhang, M. Natsui, M.J. Flores, L. Gao, K. Yusa, S. Koyota, T. Sugiyama, Anti-melanogenesis effect of *Glechoma hederacea* L. extract on B16 murine melanoma cells, Biosci. Biotechnol. Biochem. 76 (10) (2012) 1877–1883.

[11] S.Y. Lin, Y.Y. Wang, W.Y. Chen, S.L. Liao, S.T. Chou, C.P. Yang, C.J. Chen, Hepatoprotective activities of rosmarinic acid against extrahepatic cholestasis in rats, Food Chem. Toxicol. 108 (2017) 214–223.

[12] Y.Y. Wang, S.Y. Lin, W.Y. Chen, S.L. Liao, C.C. Wu, P.H. Pan, S.T. Chou, C.J. Chen, *Glechoma hederacea* extracts attenuate cholestatic liver injury in a bile duct-ligated rat model, J. Ethnopharmacol. 204 (2017) 58–66.

[13] S.T. Chou, C.C. Lai, C.P. Lai, W.W. Chao, Chemical composition, antioxidant, anti-melanogenic and anti-inflammatory activities of *Glechoma hederacea* (Lamiaceae) essential oil, Ind. Crop. Prod. 122 (2018) 675–685.

[14] T.K. Kao, Y.C. Ou, S.L. Raung, W.Y. Chen, Y.J. Yen, C.Y. Lai, S.T. Chou, C.J. Chen, *Graptopetalum paraguayense* E. Walther leaf extracts protect against brain injury in ischemic rats, Am. J. Chin. Med. 38 (3) (2010) 495–516.

[15] W.W. Chao, Y.C. Chung, I.P. Shih, H.Y. Wang, S.T. Chou, C.K. Hsu, Red bean extract inhibits lipopolysaccharide-induced inflammation and H_2O_2-induced oxidative stress in RAW 264.7 macrophages, J. Med. Food 18 (7) (2015) 724–730.

[16] A.A. Elzaawely, T.D. Xuan, S. Tawata, Antioxidant and antibacterial activities of *Rumex japonicus* HOUTT. aerial parts, Biol. Pharm. Bull. 28 (12) (2005) 2225–2230.

[17] W.W. Chao, C.C. Su, H.Y. Peng, S.T. Chou, *Melaleuca quinquenervia* essential oil inhibits α-melanocyte-stimulating hormone-induced melanin production and oxidative stress in B16 melanoma cells, Phytomedicine 34 (2017) 191–201.

[18] A. Khojasteh, M.H. Mirjalili, D. Hidalgo, P. Corchete, J. Palazon, New trends in biotechnological production of rosmarinic acid, Biotechnol. Lett. 36 (12) (2014) 2393–2406.

[19] R. Chizzola, H. Michitsch, C. Franz, Antioxidative properties of *Thymus vulgaris* leaves: comparison of different extracts and essential oil chemotypes, J. Agric. Food Chem. 56 (16) (2008) 6897–6904.

[20] S. Pacifico, S. Galasso, S. Piccolella, N. Kretschmer, S.P. Pan, S. Marciano, Seasonal variation in phenolic composition and antioxidant and anti-inflammatory activities of *Calamintha nepeta* (L.) Savi, Food Res. Int. 69 (2015) 121–132.

[21] M. Kassim, M. Achoui, M.R. Mustafa, M.A. Mohd, K.M. Yusoff, Ellagic acid, phenolic acid, and flavonoids in Malaysian honey extracts demonstrate *in vitro* anti-inflammatory activity, Nutr. Res. 30 (9) (2010) 650–659.

[22] H. Agarwal, A. Nakara, V.K. Shanmugam, Anti-inflammatory mechanism of various metal and metal oxide nanoparticles synthesized using plant extracts: a review, Biomed. Pharmacother. 109 (2019) 2561–2572.

[23] W.W. Chao, Y.H. Kuo, W.C. Li, B.F. Lin, The production of nitric oxide and prostaglandin E_2 in peritoneal macrophages is inhibited by *Morus alba*, *Angelica sinensis* and *Andrographis paniculata* ethyl acetate fraction extracts, J. Ethnopharmacol. 122 (2009) 68–75.

[24] C.Y. Chen, C.L. Kao, C.M. Liu, The cancer prevention, anti-inflammatory and anti-oxidation of bioactive phytochemicals targeting the TLR4 signaling pathway, Int. J. Mol. Sci. 19 (9) (2018) 2729.

[25] A. Serrano, G. Ros, G. Nieto, Bioactive compounds and extracts from traditional herbs and their potential anti-inflammatory health effects, Medicines 5 (3) (2018) 76.

[26] N. Huang, C. Hauck, M.Y. Yum, L. Rizshsky, M.P. Widrlechner, J.A. McCoy, P.A. Murphy, P.M. Dixon, B.J. Nikolau, D.F. Birt, Rosmarinic acid in *Prunella vulgaris* ethanol extract inhibits lipopolysaccharide-induced prostaglandin E2 and nitric oxide in RAW 264.7 mouse macrophages, J. Agric. Food Chem. 57 (22) (2009) 10579–10589.

[27] Y. So, S.Y. Lee, A.R. Han, J.B. Kim, H.G. Jeong, C.H. Jin, Rosmarinic acid methyl ester inhibits LPS-induced NO production via suppression of MyD88-dependent and -independent pathways and induction of HO-1 in RAW 264.7 cells, Molecules 21 (8) (2016) 1083.

[28] Y. Shan, D.D. Wang, Y.X. Xu, C. Wang, L. Cao, Y.S. Liu, C.Q. Zhu, Aging as a precipitating factor in chronic restraint stress-induced Tau aggregation pathology, and the protective effects of rosmarinic acid, J. Alzheimers Dis. 49 (3) (2016) 829–844.

[29] M.G. Rahbardar, B. Amin, S. Mehri, S.J. Mirnajafi-Zadeh, H. Hosseinzadeh, Rosmarinic acid attenuates development and existing pain in a rat model of neuropathic pain: an evidence of anti-oxidative and anti-inflammatory effects, Phytomedicine 40 (2018) 59–67.

[30] D. Cheng, H. Li, J. Zhou, S. Wang, Chlorogenic acid relieves lead-induced cognitive impairments and hepato-renal damage via regulating the dysbiosis of the gut microbiota in mice, Food Funct. 10 (2) (2019) 681–690.

[31] X. Zhang, H. Huang, T.T. Yang, Y. Ye, J.H. Shan, Z.M. Yin, L. Luo, Chlorogenic acid protects mice against lipopolysaccharide-induced acute lung injury, Injury 41 (7) (2010) 746–752.

[32] S.H. Park, S.I. Baek, J. Yun, S. Lee, D.Y. Yoon, J.K. Jung, S.H. Jung, B.Y. Hwang, J.T. Hong, S.B. Han, IRAK4 as a molecular target in the amelioration of innate immunity-related endotoxic shock and acute liver injury by chlorogenic acid, J. Immunol. 194 (3) (2015) 1122–1130.

[33] L. Bao, J. Li, D. Zha, L. Zhang, P. Gao, T. Yao, X. Wu, Chlorogenic acid prevents diabetic nephropathy by inhibiting oxidative stress and inflammation through modulation of the Nrf2/HO-1 and NF-κB pathways, Int. Immunopharmacol. 54 (2018) 245–253.

[34] M.C. Búfalo, I. Ferreira, G. Costa, V. Francisco, J. Liberal, M.T. Cruz, M.C. Lopes, M.T. Batista, J.M. Sforcin, Propolis and its constituent caffeic acid suppress LPS-stimulated pro-inflammatory response by blocking NF-κB and MAPK activation in macrophages, J. Ethnopharmacol. 149 (1) (2013) 84–92.

[35] M. Lu, Y. Dai, M. Xu, C. Zhang, Y. Ma, P. Gao, M. Teng, K. Jiao, G. Huang, J. Zhang, Y. Yang, Z. Chu, The attenuation of 14-3-3ζ is involved in the caffeic acid-blocked lipopolysaccharide-stimulated inflammatory response in RAW264.7 macrophages, Inflammation 40 (5) (2017) 1753–1760.

[36] J.H. Choi, S.H. Park, J.K. Jung, W.J. Cho, B. Ahn, C.Y. Yun, Y.P. Choi, J.H. Yeo, H. Lee, J.T. Hong, Caffeic acid cyclohexylamide rescues lethal inflammation in septic mice through inhibition of Ikappa b kinase in innate immune process, Sci. Rep. 7 (2017) 41180.

[37] I.A. Macdonald, J.A. Mader, R.G. Bussard, The role of rutin and quercitrin in stimulating flavonol glycosidase activity by cultured cell-free microbial preparations of human feces and saliva, Mutat. Res. 122 (2) (1983) 95–102.

[38] R. Gautam, M. Singh, S. Gautam, J.K. Rawat, S.A. Saraf, G. Kaithwas, Rutin attenuates intestinal toxicity induced by Methotrexate linked with anti-oxidative and anti-inflammatory effects, BMC Complement. Altern. Med. 16 (2016) 99.

[39] H.L. Song, X. Zhang, W.Z. Wang, R.H. Liu, K. Zhao, M.Y. Liu, W.M. Gong, B. Ning, Neuroprotective mechanisms of rutin for spinal cord injury through anti-oxidation and anti-inflammation and inhibition of p38 mitogen activated protein kinase pathway, Neural Regen. Res. 13 (1) (2018) 128–134.

[40] A. Shahid, R. Ali, N. Ali, S.K. Hasan, S. Rashid, F. Majed, S. Sultana, Attenuation of genotoxicity, oxidative stress, apoptosis and inflammation by rutin in benzo(a) pyrene exposed lungs of mice: plausible role of NF-kappaB, TNF-alpha and Bcl-2, J. Complement. Integr. Med. 13 (1) (2016) 17–29.

[41] L. Xianchu, Z. Lan, L. Ming, M. Yanzh, Protective effects of rutin on lipopolysaccharide induced heart injury in mice, J. Toxicol. Sci. 43 (5) (2018) 329–337.

[42] D.C. Vitale, C. Piazza, B. Melilli, F. Drago, S. Salomone, Isoflavones: estrogenic activity, biological effect and bioavailability, Eur. J. Drug Metab. Pharmacokinet. 38 (1) (2013) 15–25.

[43] C.M. Chiang, D.S. Wang, T.S. Chang, Improving free radical scavenging activity of soy isoflavone glycosides daidzin and genistin by 3′-hydroxylation using recombinant *Escherichia coli*, Molecules 21 (12) (2016) 1723.

[44] J.H. Zhao, Y. Arao, S.J. Sun, A. Kikuchi, F. Kayama, Oral administration of soy-derived genistin suppresses lipopolysaccharide-induced acute liver inflammation but does not induce thymic atrophy in the rat, Life Sci. 78 (8) (2006) 812–819.

[45] H.J. Lee, S.M. Lim, D.B. Ko, J.J. Jeong, Y.H. Hwang, D.H. Kim, Soyasapogenol B and genistein attenuate lipopolysaccharide-induced memory impairment in mice by the modulation of NF-κB mediated BDNF expression, J. Agric. Food Chem. 65 (32) (2017) 6877–6885.

[46] M. Gu, A.B. Zhang, J. Jin, Y. Cui, N. Zhang, Z.P. Che, Y. Wang, J. Zhan, W.J. Tu, Cardioprotective effects of genistin in rat myocardial ischemia-reperfusion injury studies by regulation of P2X7/NF-κB pathway, Evid. Based Complement. Alternat. Med. (2016) (2016) 5381290.

[47] Z. Zhao, M.H. Moghadasian, Chemistry, natural sources, dietary intake and pharmacokinetic properties of ferulic acid: a review, Food Chem. 109 (4) (2008) 691–702.

[48] D. Ronchetti, F. Impagnatiello, M. Guzzetta, L. Gasparini, M. Borgatti, R. Gambari, E. Ongini, Modulation of iNOS expression by a nitric oxide-releasing derivative of the natural antioxidant ferulic acid in activated RAW 264.7 macrophages, Eur. J. Pharmacol. 532 (1–2) (2006) 162–169.

[49] N. Lampiasi, G. Montana, The molecular events behind ferulic acid mediated modulation of IL-6 expression in LPS-activated Raw 264.7 cells, Immunobiology 221 (3) (2016) 486–493.