

Total flavonoids of *Hedyotis diffusa* Willd inhibit inflammatory responses in LPS-activated macrophages via suppression of the NF- κ B and MAPK signaling pathways

YUNLONG CHEN^{1,2}, YANYAN LIN³, YACHAN LI¹ and CANDONG LI¹

¹Research Base of TCM Syndrome, Fujian University of Traditional Chinese Medicine, Fuzhou, Fujian 350108;

²Department of Traditional Chinese Medicine, Sanming First Hospital Affiliated to Fujian Medical University;

³Department of Nephrology, Sanming Integrative Medicine Hospital Affiliated to Fujian University of Traditional Chinese Medicine, Sanming, Fujian 365000, P.R. China

Received December 17, 2014; Accepted October 29, 2015

DOI: 10.3892/etm.2015.2963

Abstract. Nuclear factor κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) signaling pathways play a central role in inflammatory responses. Total flavonoids of *Hedyotis diffusa* Willd (TFHDW) are active compounds derived from *Hedyotis diffusa* Willd, which has been long used in Chinese traditional medicine for the treatment of various inflammatory diseases, including ulcerative colitis and bronchitis; however, the precise mechanisms underlying the effects of TFHDW are largely unknown. In the present study, the anti-inflammatory effect of TFHDW was evaluated and the underlying molecular mechanisms were investigated in an *in vitro* inflammatory model comprising lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. The results indicated that TFHDW inhibited the inflammatory response as it significantly reduced the LPS-induced expression of pro-inflammatory nitric oxide, tumor necrosis factor (TNF)- α , interleukin (IL)-6 and IL-1 β in a concentration-dependent manner, without causing cytotoxicity. In addition, the mRNA expression of inducible nitric oxide synthase, TNF- α , IL-6 and IL-1 β was suppressed by treatment with TFHDW in LPS-stimulated RAW 264.7 cells. Moreover, TFHDW

treatment significantly inhibited the LPS-induced activation of NF- κ B via the suppression of inhibitor of κ B (I κ B) phosphorylation, and reduced the phosphorylation of MAPK signaling molecules (p38, c-Jun N-terminal protein kinase and extracellular signal-regulated kinase 1/2), which resulted in the inhibition of cytokine expression. These findings suggest that TFHDW exerted anti-inflammatory activity via suppression of the NF- κ B and MAPK signaling pathways.

Introduction

Inflammation is an orchestrated biological process, induced by tissue injury or microbial infection, which protects the body from these inflammatory stimuli. However, persistent or excessive inflammation is associated with a variety of pathological conditions, including rheumatoid arthritis, bacterial sepsis and skin inflammation (1,2). Macrophages play a key role in the host defense against noxious substances and are involved in numerous inflammatory diseases (3). The activation of macrophages by inflammatory stimuli can generate reactive oxygen species, such as H₂O₂ and superoxide, and induce the expression of various genes such as interleukin (IL)-6 and tumor necrosis factor (TNF)- α , in addition to other inflammatory mediators, including nitric oxide (NO) and prostaglandin E2 (PGE2), which are synthesized by inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, respectively. Inflammatory cytokines and mediators contribute to the pathogenesis of numerous inflammation-associated human diseases (4). Lipopolysaccharide (LPS) from gram-negative bacteria induces inflammation and is frequently used to stimulate macrophages in order to study inflammation and the mechanisms of action of potential anti-inflammatory agents.

The anti-inflammatory actions of various phytochemicals have been found to be mediated through suppression of the NF- κ B pathway (5). NF- κ B is a key regulator of a various genes involved in immune and inflammatory responses (6). In resting cells, NF- κ B is complexed with the inhibitor of κ B (I κ B) protein in the cytoplasm, and is thereby inactivated. When cells receive pathological stimuli, I κ B kinase phosphorylates I κ B, causing it to break away from NF- κ B. The

Correspondence to: Dr Candong Li, Research Base of TCM Syndrome, Fujian University of Traditional Chinese Medicine, 1 Huatuo Road, Minhou Shangjie, Fuzhou, Fujian 350108, P.R. China
E-mail: fjzylcd@126.com

Abbreviations: HDW, *Hedyotis diffusa* Willd; TFHDW, total flavones of HDW; LPS, lipopolysaccharide; NO, nitric oxide; iNOS, inducible NO synthase; IL-1 β , interleukin-1 β ; TNF- α , tumor necrosis factor- α ; NF- κ B, nuclear factor- κ B; I κ B α , inhibitor of κ B α ; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2

Key words: traditional Chinese medicine, *Hedyotis diffusa* Willd, NF- κ B, MAPK, inflammation

uncomplexed NF- κ B then translocates to the nucleus, where it binds to DNA and activates the transcription of various genes including iNOS, COX-2, IL-6 and TNF- α (7). Therefore, NF- κ B is regarded as a target molecule for anti-inflammatory drug development (8).

In addition to activating NF- κ B, LPS also activates mitogen-activated protein kinases (MAPKs) (9). There are three major subgroups of MAPK, namely extracellular signal-regulated kinase (ERK), c-Jun N-terminal protein kinase (JNK) and p38. These kinases play key roles in the regulation of numerous cellular functions, including cell survival, apoptosis and cellular responses to inflammation (10).

Hedyotis diffusa Willd (HDW), which is a herb of the Rubiaceae family, has a long history of use in Chinese medicine, and is widely distributed in northeast Asia. According to the theories of traditional Chinese medicine (TCM), it has heat-clearing, detoxification, blood circulation promoting and blood stasis eliminating effects (11,12). Pharmacological studies have shown that it contains compounds with anticancer, anti-inflammatory, antibacterial and immunomodulatory activities, which include flavonoids, anthraquinones, hemiterpenes, polyphenols, organic acids and polysaccharides (13-16).

The anti-inflammatory effects of total flavonoids of HDW (TFHDW) have been investigated in various inflammatory models, including ulcerative colitis induced by dextran sulfate sodium in mice (17,18), ear edema induced by dimethylbenzene in mice, granuloma pouch induced by turpentine in rats, and paw edema caused by egg white in rats (19). However, few studies on its anti-inflammatory activity *in vitro* and its mechanisms have been carried out. In the present study, the cell-based anti-inflammatory activities and the mechanisms of action of TFHDW in LPS-stimulated RAW 264.7 macrophages were investigated. In order to elucidate the mechanisms underlying the anti-inflammatory actions, the effect of TFHDW on the expression of iNOS, TNF- α , IL-6 and IL-1 β at the mRNA and protein levels, as well as on NF- κ B and MAPK signaling pathways were also studied.

Materials and methods

Materials and reagents. Fetal bovine serum (FBS), RPMI-1640, penicillin, streptomycin, 0.05% (w/v) trypsin-ethylenediamine tetraacetic acid (EDTA) and phosphate-buffered saline (PBS) were HyClone products (GE Healthcare, Logan, UT, USA). Cytokine (IL-6, TNF- α and IL-1 β) enzyme-linked immunosorbent (ELISA) kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). TRIzol reagent and SuperScript II reverse transcriptase were Invitrogen products (Thermo Fisher Scientific, Waltham, MA, USA). Anti-phosphorylated I κ B α (anti-p-I κ B α ; #2859), anti-I κ B α (#4812), anti-NF- κ B p65 (#6956), anti-p-NF- κ B p65 (#3033) and anti- β -actin monoclonal mouse or rabbit (#4970) antibodies and anti-rabbit IgG (#7074) and anti-mouse IgG (#7076) horseradish peroxidase (HRP)-conjugated secondary antibodies were from Cell Signaling Technology (Danvers, MA, USA). Bio-Plex phosphoprotein assay kits were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). All other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of TFHDW from *Hedyotis diffusa* Willd. Dried plant materials of *Hedyotis diffusa* Willd. were purchased from Guo Yi Tang Chinese Herbal Medicine Store (Fujian, China). The original herb was identified as *Hedyotis diffusa* Willd by Dr Wei Xu at the Department of Pharmacology, Fujian University of Traditional Chinese Medicine (Fuzhou, China). The material was coarsely ground prior to extraction. A total of 300 g of the material was extracted three times with 80% ethanol for 3 h at 50°C. The fluid was filtered through a filter with a 1-mm pore-size. The filtrate was then evaporated, and the crude extract was isolated using a column containing AB-8 macroporous adsorption resin (Cangzhou Bon Chemical Co., Ltd., Hebei, China) with the application of 80% aqueous ethanol to elute the flavonoids. The ethanol solvent was then evaporated using a rotary evaporator (Model RE-2000; Shanghai Yarong Biochemical Instrument Factory, Shanghai, China).

Cell culture and treatment. Cells of the RAW 264.7 mouse monocyte-macrophage cell line (American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI-1640 supplemented with FBS (10%), 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells were plated in 96-, 24- and 6-well plates at densities of 8x10⁴, 1x10⁵ and 5x10⁵ cells/well. When cell treatments were conducted, the cells were incubated in serum-free medium for 4 h, then treated with LPS (1 μ g/ml) and/or TFHDW for 24 h (for ELISA), or for 12 h [for reverse transcription-polymerase chain reaction (RT-PCR)] or for 20 min (for western blotting) for the detection of protein or mRNA expression.

Cytotoxicity assay. RAW 264.7 cells were grown in 96-well plates at a density of 8x10⁴ cells/ml. TFHDW was added at various concentrations (0, 25, 50, 100, 200, 400 and 800 μ g/ml). A methyl thiazolyl tetrazolium assay (MTT) assay was used to measure the viability of the cells. Briefly, after 24 h incubation with or without TFHDW, MTT solution (0.05 mg/ml) was added and the cells were incubated for another 4 h at 37°C. Then, the supernatant was removed and 100 μ l dimethylsulfoxide was added to dissolve the formazan. The absorbance of the cells was measured using a microplate reader (ELx800; BioTek, Winooski, VT, USA) at wavelength of 570 nm. The control group, which consisted of untreated cells, was considered to comprise 100% viable cells. Results are expressed as a percentage of viable cells compared with the control group.

Determination of NO production. The release of NO by iNOS is one of the major factors contributing to the inflammatory process (20). The production of nitrite, a metabolite of NO, was assessed by the Griess reaction. Cells were plated in 96-well plates (8x10⁴ cells/ml) and treated with LPS (1 μ g/ml) in the presence or absence of TFHDW (50, 100 and 200 μ g/ml). After incubation for 24 h, suspended media were collected for measurement of the nitrite concentrations using the Griess reaction. This involved taking a 50- μ l aliquot of the culture supernatant, mixing it with an equal volume of Griess reagent [0.1% N-(1-naphthyl)-ethylenediamine, 1% sulfanilamide in 5% phosphoric acid] and incubating the mixture at room temperature (RT) for 10 min. The absorbance at 540 nm

was measured using a microplate absorbance reader. The concentration of nitrite was determined from a sodium nitrite standard curve.

Measurement of inflammatory cytokines. RAW 264.7 cells were plated in a 24-well cell culture plate (1×10^5 cells/ml) and incubated with TFHDW (50, 100 and 200 $\mu\text{g/ml}$) in the presence or absence of LPS (1 $\mu\text{g/ml}$) for 24 h. A 1-ml volume of culture-medium supernatant was then collected for measurement of the levels of IL-6, TNF- α and IL-1 β using the relevant ELISA kit according to the manufacturer's instructions.

RT-PCR. Total RNA was extracted from the cells using TRIzol reagent following the manufacturer's protocol. The purity and integrity of the RNA were assessed using a NanoDrop spectrophotometer (ND-2000C; Thermo Fisher Scientific). Subsequently, first-strand cDNA synthesis was performed with 2 μg total RNA using SuperScript II reverse transcriptase kit (Fermentas; Thermo Fisher Scientific) according to the manufacturer's protocol. The obtained cDNA was used to determine the mRNA levels of TNF- α , IL-6, IL-1 β and iNOS using a DreamTaq Green PCR Master Mix (2X) PCR kit (Fermentas; Thermo Fisher Scientific). The primers used were as follows: TNF- α forward, 5'-CTCAAGGACAACAGC CAGTTC-3' and reverse, 5'-GGCACTAAGGGCTCAGT CAG-3'; IL-6 forward, 5'-GGATACCACCCACAACAGACC-3' and reverse, 5'-AATCAGAATTGCCATTGCAC-3'; IL-1 β forward, 5'-ATCACTCATTGTGGCTGTGG-3' and reverse, 5'-GTCGTTGCTTGGTTCTCCT-3'; iNOS forward, 5'-CAG ATCGAGCCCTGGAAGAC-3' and reverse, 5'-CTGGTCCAT GCAGACAACCT-3'; and GAPDH forward, 5'-CACTCACGG CAAATTCAACGGCA-3' and reverse, 5'-GACTCCACGACA TACTCAGCAC-3'. The PCR cycling reaction was performed using an S1000 thermocycler (Bio-Rad Laboratories, Inc.). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Western blot analysis. Total cells were harvested after treatment, washed twice with ice-cold PBS, and gently lysed in radioimmunoprecipitation assay buffer containing phosSTOP phosphatase inhibitor cocktail and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Lysates were centrifuged for 15 min at 12,000 \times g to obtain a supernatant for further analysis. The protein concentration of the lysate was measured using a bicinchoninic acid (BCA) quantification assay (Pierce, Rockford, IL, USA). Proteins (50 μg) were separated by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (0.45- μm pore size IPVH00010; Millipore, Billerica, MA, USA). The membranes were incubated with primary antibody overnight at 4°C. The primary antibodies were monoclonal antibodies targeting I κ B α , p-I κ B α , NF- κ B p65, p-NF- κ B p65 and β -actin (1:1,000) diluted in immunoblot buffer (TBS containing 0.05% Tween-20 and 5% non-fat dry milk). Following washing with TBS and Tween 20 three times, membranes were incubated with the secondary HRP-conjugated anti-mouse (or rabbit) IgG antibody (1:1,000) for 1 h at RT. After washing, the blots were detected with Clarity ECL Western Blotting Substrate

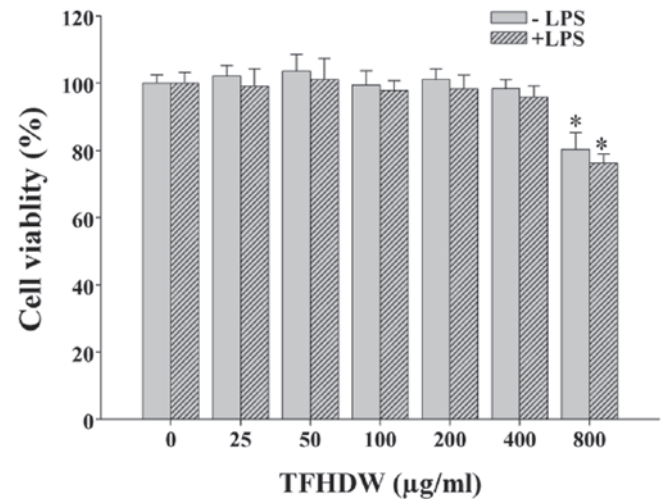


Figure 1. Effect of TFHDW on the viability of RAW 264.7 cells. RAW 264.7 cells were treated with LPS in the presence and absence of TFHDW at concentrations of 0-800 $\mu\text{g/ml}$ for 24 h. Cell viability was determined by MTT assay. The data were normalized to the viability of the untreated control cells. Each value indicates the mean \pm standard deviation from at least three independent experiments. * $P < 0.05$ vs. control group. TFHDW, total flavonoids of *Hedyotis diffusa* Willd; LPS, lipopolysaccharide; MTT, methyl thiazolyl tetrazolium assay.

(Bio-Rad Laboratories, Inc.) for 1 min using a camera with the ChemiDoc XRS⁺ System (Bio-Rad Laboratories, Inc.). The pixel intensities of the immunoreactive bands were quantified using the percentage adjusted volume feature of Image Lab software (Bio-Rad Laboratories, Inc.). β -actin served as an internal control.

Bio-Plex phosphoprotein assay. A bead-based multiplex assay for phosphoproteins (Bio-Plex Phosphoprotein assay) was used to detect p-ERK1/2, p-JNK and p-p38. Cells were lysed using a lysis kit (Bio-Rad Laboratories, Inc.) and were then centrifuged at 15,000 \times g for 15 min. Protein concentrations were determined by BCA protein assay. Then, 25 μl protein extract and 25 μl testing assay buffer were transferred into a 96-well filter plate coated with antibodies against p-ERK1/2, p-JNK and p-p38 and the plate was incubated overnight on a platform shaker at room temperature. Following a series of washes to remove the unbound proteins, a mixture of biotinylated detection antibodies, each specific for a different epitope, was added to the reaction. Streptavidin-phycoerythrin was then added to bind to the biotinylated detection antibodies. Data acquisition and analysis was conducted using the Bio-Plex 200 suspension array system (Bio-Rad Laboratories, Inc.). The total proteins for ERK1/2, JNK and p38 were also quantified using the Bio-Plex total protein assay kit (Bio-Rad Laboratories, Inc.). The phosphorylation level was expressed as the ratio of phosphoprotein to total protein.

Statistical analysis. Data are expressed as mean \pm standard deviation. One-way analysis of variance was used when comparing the data obtained under different experimental conditions. *In vitro* experiments were conducted in triplicates; representative results are shown. A P-value of < 0.05 was considered to indicate a statistically significant result.

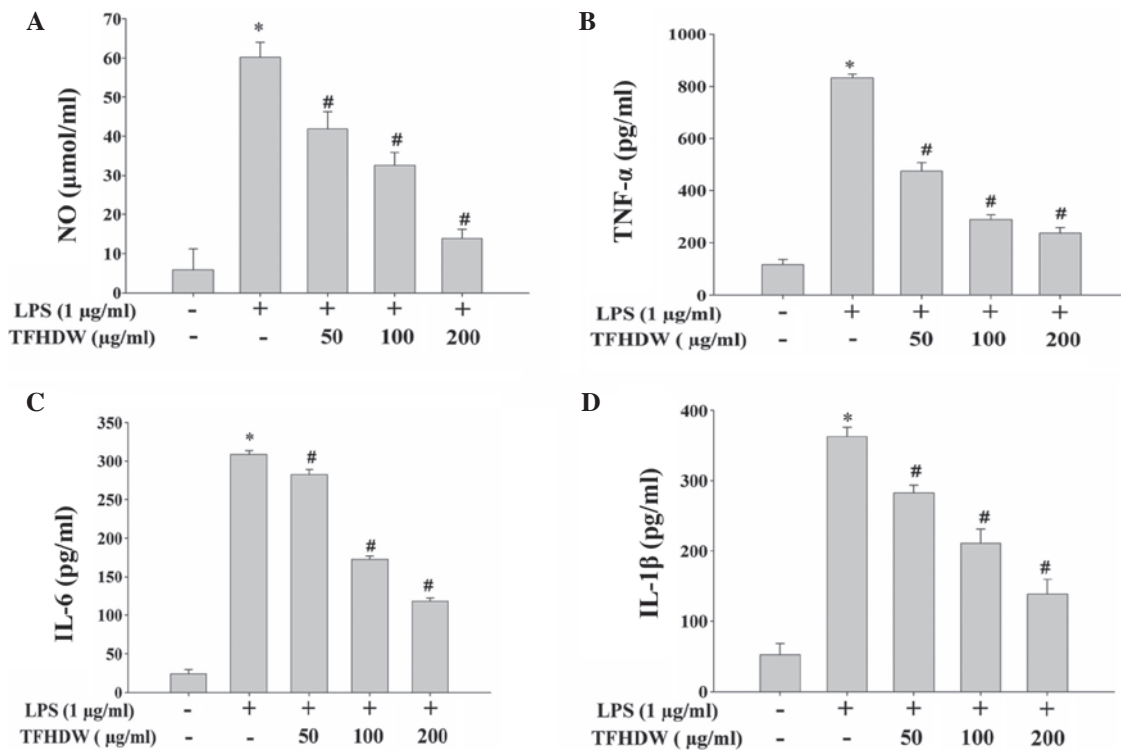


Figure 2. Effect of TFHDW treatment on the release of pro-inflammatory factors in LPS-stimulated RAW 264.7 cells. Cells were treated with LPS and/or the indicated concentrations of TFHDW. (A) Content of NO was measured using the Griess reaction. The secretion levels of TNF- α (B), IL-6 (C) and IL-1 β (D) were measured using respective ELISA kits. Each value indicates the mean \pm standard deviation from three independent experiments. * $P < 0.05$ vs. untreated control cells; # $P < 0.05$ vs. cells treated with LPS alone. TFHDW, total flavonoids of *Hedyotis diffusa* Willd; LPS, lipopolysaccharide; NO, nitric oxide; TNF, tumor necrosis factor; IL, interleukin; ELISA, enzyme-linked immunosorbent assay.

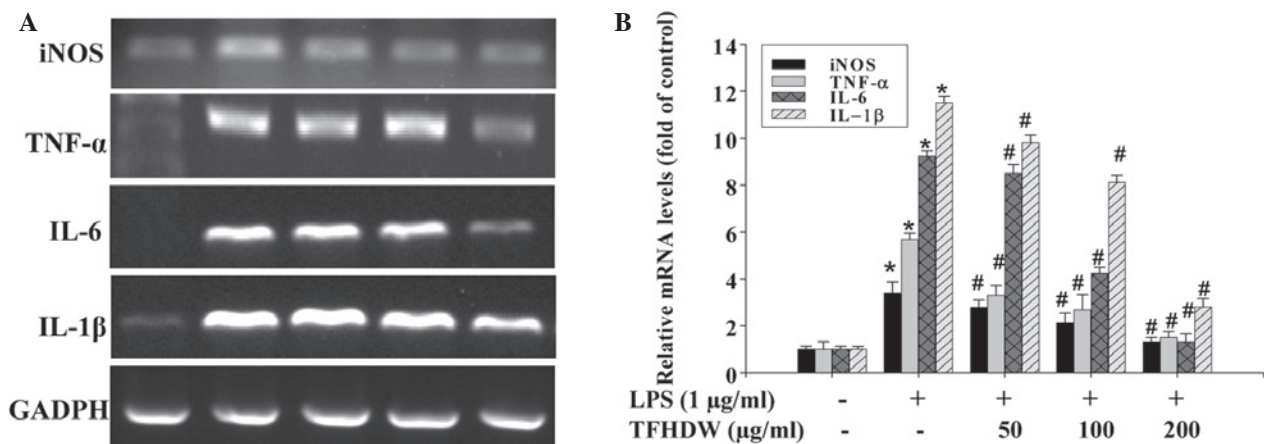


Figure 3. Effect of TFHDW on the transcription of pro-inflammatory factors in LPS-stimulated RAW 264.7 cells. (A) The mRNA expression of iNOS, TNF- α , IL-6 and IL-1 β was determined by reverse transcription-polymerase chain reaction. GAPDH was used as the internal control. Images are representatives of three independent experiments. (B) Densitometric analysis. The data were normalized to the mean mRNA expression of the untreated control. * $P < 0.05$ vs. untreated control cells; # $P < 0.05$ vs. cells treated with LPS alone. TFHDW, total flavonoids of *Hedyotis diffusa* Willd; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; TNF, tumor necrosis factor; IL, interleukin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Results

TFHDW did not exhibit cytotoxicity against RAW 264.7 cells.

RAW 264.7 cells were treated with various concentrations of TFHDW for 24 h, and the viability and cytotoxicity were determined by MTT assay. TFHDW did not exhibit cytotoxicity to RAW 264.7 cells in the absence and presence of LPS at concentrations of 50, 100 and 200 $\mu\text{g/ml}$, and these concentrations were used in the following experiments. However, when

the concentrations reached 800 $\mu\text{g/ml}$, TFHDW appeared to inhibit cell viability (Fig. 1).

TFHDW inhibits the LPS-induced inflammatory response in RAW 264.7 cells. The effect of TFHDW on LPS-induced inflammation in RAW 264.7 cells was evaluated by measuring the production of NO and pro-inflammatory cytokines (TNF- α , IL-6 and IL-1 β). As shown in Fig. 2, stimulation with LPS for 24 h significantly induced the release of NO, TNF- α ,

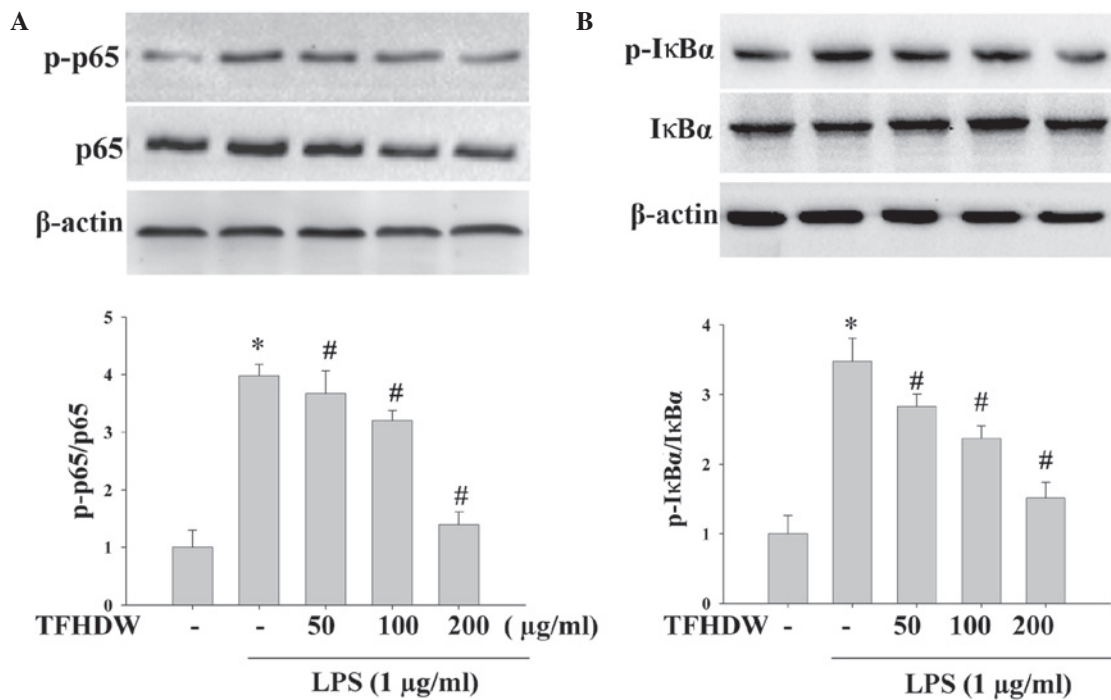


Figure 4. NF- κ B pathway is involved in the inflammatory effect of TFHDW in LPS-induced RAW 264.7 cells. Cells were treated with LPS alone or in the presence of different concentrations of TFHDW for 24 h. The levels of proteins associated with NF- κ B pathway were measured by western blotting analysis. Representative images for (A) p-p65 and p65 and (B) p-I κ B α were from one of three repeated experiments. The expression of all proteins was quantified by densitometry analysis. Data shown re the mean \pm standard deviation. *P<0.05 vs. untreated control cells; #P<0.05 vs. cells treated with LPS alone. NF- κ B, nuclear factor- κ B; TFHDW, total flavonoids of *Hedyotis diffusa* Willd; LPS, lipopolysaccharide; p, phospho; I κ B α , inhibitor of κ B α .

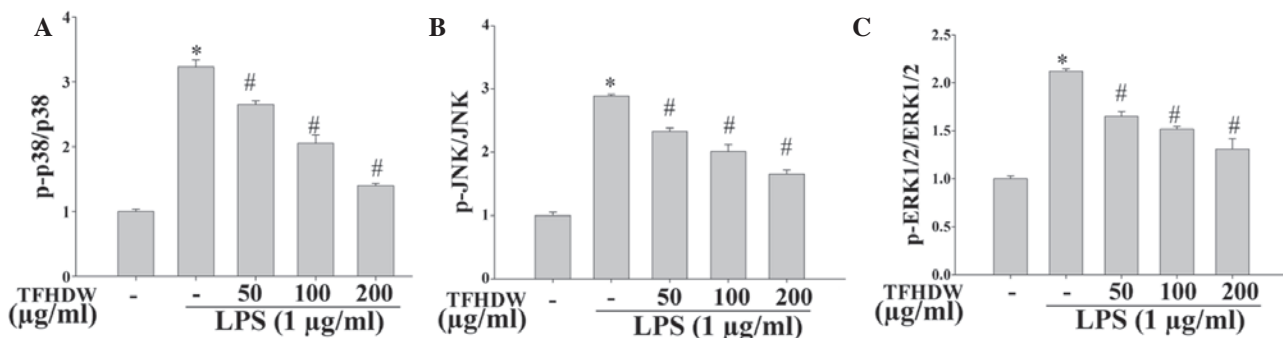


Figure 5. Effects of TFHDW on LPS-induced phosphorylation of MAPKs in RAW 264.7 cells. The phosphorylation levels of (A) p38, (B) JNK and (C) ERK 1/2 in RAW 264.7 cells were determined by Bio-Plex phosphoprotein assay. The data were normalized to the phosphorylation level within controls and represented as fold of control. Data were shown as the mean \pm SD. *P<0.05 vs. untreated control cells; #P<0.05 vs. cells treated with LPS alone. TFHDW, total flavonoids of *Hedyotis diffusa* Willd; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal protein kinase; ERK, extracellular signal-regulated kinase; p, phospho.

IL-6 and IL-1 β , indicating that an inflammatory response was induced in the RAW 264.7 cells. However, the LPS-induced release of inflammatory mediators was significantly inhibited by TFHDW treatment in a concentration-dependent manner. To further verify these observations, the effect of TFHDW on the mRNA expression of these pro-inflammatory factors was determined. As shown in Fig. 3, stimulation with LPS significantly and concentration-dependently increased the mRNA expression levels of iNOS, TNF- α , IL-6 and IL-1 β in RAW 264.7 cells and these increases were markedly suppressed by TFHDW treatment.

TFHDW prevents LPS-induced activation of the NF- κ B pathway in RAW 264.7 cells. NF- κ B is an important upstream

transcription factor inducing the expression of iNOS, TNF- α , IL-6 and IL-1 β mRNA following stimulation by LPS. As p65 is the functional subunit of NF- κ B activated by LPS in macrophages, the phosphorylation of p65 was analyzed by western blot analysis. As shown in Fig. 4, treatment with LPS significantly increased the phosphorylation of p65 in the RAW 264.7 cells; However, pretreatment with TFHDW notably inhibited this excessive phosphorylation. During the p65 phosphorylation process, phosphorylation of I κ B α is essential to release NF- κ B from the NF- κ B/I κ B α complex. Therefore, the effect of TFHDW on the LPS-induced phosphorylation of I κ B α was also investigated. The results showed that TFHDW strongly suppressed the phosphorylation of I κ B α , suggesting that TFHDW inhibited NF- κ B

activation and the phosphorylation of p65 by reducing the phosphorylation of I κ B α (Fig. 4).

TFHDW suppresses the phosphorylation of p38, JNK and ERK 1/2. In order to investigate whether the anti-inflammatory effects of TFHDW are mediated via MAPK signaling pathways, the phosphorylation of the MAPK signaling molecules p38, JNK and ERK 1/2 was analyzed using a Bio-Plex phosphoprotein assay. The results suggest that LPS significantly induced the phosphorylation of p38, JNK and ERK 1/2; however, the phosphorylation levels of the MAPK isoforms were markedly decreased in the cells treated with TFHDW and LPS compared with the cells treated with LPS alone. These results indicate that TFHDW effectively blocks MAPK signaling pathways in activated macrophages (Fig. 5).

Discussion

Inflammation is a biological protective response to tissue injury or microbial invasion capable of causing cell injury, under which pro-inflammatory mediators are released (21). Inflammatory factors are important elements in the chronic inflammation associated with diseases such as arteriosclerosis, obesity, diabetes, neurodegenerative diseases and cancer. Steroidal and non-steroidal anti-inflammatory drugs are currently used to treat acute inflammation. However, these drugs are not entirely successful in curing chronic inflammatory disorders, and often have side effects. Therefore, the identification of new, safer and more effective anti-inflammatory compounds is necessary (22). TCM, which plays an important role in primary health care in China, involves the use of extracts of various plants for the treatment of pathological disturbances, including acute and chronic inflammation. Flavonoids, which are an active constituent of TCM have been found to have notable biological properties, including anticancer, antimicrobial, antiviral, anti-inflammatory, immunomodulatory and antithrombotic activities (23-25). TFHDW, the flavonoid compounds of the traditional Chinese herb HDW, has been demonstrated to be effective for the treatment of inflammation *in vivo* (17-19). The purpose of the present study was to further investigate the anti-inflammatory effects of TFHDW on pro-inflammatory mediators (NO, TNF- α , IL-6, IL-1 β) and pro-inflammatory enzyme (iNOS) expression in LPS-activated RAW 264.7 cells and to explore the mechanism. It was found that TFHDW inhibited LPS-induced iNOS, TNF- α , IL-6 and IL-1 β expression at the protein and transcription levels, confirming that TFHDW exhibits an anti-inflammatory effect.

The NF- κ B signaling pathway is an important LPS-activated pathway. NF- κ B is present as a complex with I κ B located in the cytoplasm in unstimulated cells. When stimulated, I κ B is phosphorylated, ubiquitinated and rapidly degraded, which releases the p65 subunit of NF- κ B from I κ B, resulting in p65 translocation into the nucleus where it regulates gene transcription (26,27). To explore the mechanism of NF- κ B inactivation by TFHDW, the effects of the extract on the LPS-induced phosphorylation of p65 and I κ B were examined by western blotting. The levels of phosphorylation were increased upon exposure to LPS, and treatment with TFHDW reduced the phosphorylation of p65 and I κ B without affecting the total cellular levels of these proteins.

Inhibition of the MAPK pathway is also known to disrupt the synthesis of proinflammatory mediators (28). Several studies have shown that MAPKs are involved in the activation of NF- κ B (28-30). The effects of TFHDW on the LPS-induced phosphorylation of p38, JNK and ERK1/2 were examined in the present study in order to further elucidate its anti-inflammatory mechanism. TFHDW inhibited the phosphorylation of p38, JNK and ERK1/2 in LPS-stimulated cells in a concentration-dependent manner. The attenuation of MAPK activation may be a mechanism by which TFHDW reduces cytokine production. The results of the current study suggest that the anti-inflammatory activity of TFHDW is mediated by the inhibition of iNOS, TNF- α , IL-6 and IL-1 β expression via the downregulation of the NF- κ B and MAPK signaling pathways.

In conclusion, in the present study, it was demonstrated that TFHDW negatively modulates the production of NO and cytokines (TNF- α , IL-6 and IL-1 β) in LPS-treated RAW 264.7 cells in a concentration-dependent manner. It was also found that TFHDW blocks the activation of NF- κ B and MAPK signaling pathways. These data suggest that TFHDW has notable anti-inflammatory activity and is worthy of further development as a herbal remedy for the treatment of various inflammatory diseases.

Acknowledgements

This study was supported as Project No. 81173203 by the National Natural Science Foundation of China.

References

- Dinarello CA: Proinflammatory and anti-inflammatory cytokines as mediators in the pathogenesis of septic shock. *Chest* 112 (Suppl 6): S321-S329, 1997.
- Palladino MA, Bahjat FR, Theodorakis EA and Moldawer LL: Anti-TNF- α therapies: The next generation. *Natl Rev Drug Discov* 2: 736-746, 2003.
- Pierce GF: Macrophages: Important physiologic and pathologic sources of polypeptide growth factors. *Am J Respir Cell Mol Biol* 2: 233-234, 1990.
- Lee SJ, Bai SK, Lee KS, Namkoong S, Na HJ, Ha KS, Han JA, Yim SV, Chang K, Kwon YG, *et al*: Astaxanthin inhibits nitric oxide production and inflammatory gene expression by suppressing I (κ)B kinase dependent NF- κ B activation. *Mol Cells* 16: 97-105, 2003.
- Kundu JK and Surh YJ: Breaking the relay in deregulated cellular signal transduction as a rationale for chemo prevention with anti-inflammatory phytochemicals. *Mutat Res* 591: 123-146, 2005.
- Xie QW, Kashiwabara Y and Nathan C: Role of transcription factor NF- κ B/Rel in induction of nitric oxide synthase. *J Biol Chem* 269: 4705-4708, 1994.
- Rice NR and Ernst MK: In vivo control of NF- κ B activation by I κ B α . *EMBO J* 12: 4685-4695, 1993.
- Makarov SS: NF- κ B as a therapeutic target in chronic inflammation: Recent advances. *Mol Med Today* 6: 441-448, 2000.
- Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K and Cobb MH: Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions. *Endocr Rev* 22: 153-183, 2001.
- Cargnello M and Roux PP: Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev* 75: 50-83, 2011.
- Wu YG and Song LR (eds): *Zhong Hua Ben Cao*. Shanghai Science and Technology Press, Shanghai, pp1530-1533, 1998 (In Chinese).
- Cai QY, Lin JM, Wei L, Zhang L, Wang L, Zhan Y, Zeng J, Xu W, Shen A, Hong Z and Peng J: *Hedyotis diffusa* willd inhibits colorectal cancer growth in vivo via inhibition of STAT3 signaling pathway. *Int J Mol Sci* 13: 6117-6128, 2012.

13. Zhang YY and Luo JB: Analysis of the chemical constituents of *Hedyotis diffusa*. *Nan Fang Yi Ke Da Xue Xue Bao* 28: 127-128, 2008 (In Chinese).
14. Lee HZ, Bau DT, Kuo CL, Tsai RY, Chen YC and Chang YH: Clarification of the phenotypic characteristics and anti-tumor activity of *Hedyotis diffusa*. *Am J Chin Med* 39: 201-213, 2011.
15. Huang W, Li Y and Jiang J: Chemical constituents from *Hedyotis diffusa*. *Zhongguo Zhong Yao Za Zhi* 34: 712-714, 2009 (In Chinese).
16. Wang YL, Zhang Y, Fang M, Li QJ, Jiang Q and Ming L: Immunomodulatory effects of total flavonoids of *Oldenlandia diffusa* Willd. *Zhongguo Yao Li Xue Tong Bao* 21: 444-447, 2005 (In Chinese).
17. Luo SY, Zhong ZG and Zhou L: Experimental study of the total flavonoids of *Oldenlandia diffusa* on ulcerative colitis in the rats. *Zhongguo Yi Yuan Yao Xue Za Zhi* 31: 437-440, 2011 (In Chinese).
18. Luo SY, Le Z, Lv XH and Zhong ZG: Study on effect of total flavonoids of *Oldenlandia diffusa* on ulcerative colitis and its immunological mechanism. *Zhongguo Zhong Yao Za Zhi* 39: 896-900, 2014 (In Chinese).
19. Wang YL, Zhang Y, Fang M, Li QJ, Jiang Q and Ming L: Anti-inflammatory and antibacterial effects of total flavones of *Oldenlandia diffusa* Willd. *Zhongguo Yao Li Xue Tong Bao* 21: 348-350, 2005 (In Chinese).
20. Korhonen R, Lahti A, Kankaanranta H and Moilanen E: Nitric oxide production and signaling in inflammation. *Curr Drug Targets Inflamm Allergy* 4: 471-479, 2005.
21. Ferrero-Miliani L, Nielsen OH, Andersen PS and Girardin SE: Chronic inflammation: Importance of NOD2 and NALP3 in interleukin-1beta generation. *Clin Exp Immunol* 147: 227-235, 2007.
22. Yoon JH and Baek SJ: Molecular targets of dietary polyphenols with anti-inflammatory properties. *Yonsei Med J* 46: 585-596, 2005.
23. Robak J and Gryglewski RJ: Bioactivity of flavonoids. *Pol J Pharmacol* 48: 555-564, 1996.
24. Russo A, Acquaviva R, Campisi A, Sorrenti V, Di Giacomo C, Virgata G, Barcellona ML and Vanella A: Bioflavonoids as anti-radicals, antioxidants and DNA cleavage protectors. *Cell Biol Toxicol* 16: 91-98, 2000.
25. Havsteen BH: The biochemistry and medical significance of the flavonoids. *Pharmacol Ther* 96: 67-202, 2002.
26. de las Heras B and Hortelano S: Molecular basis of the anti-inflammatory effects of terpenoids. *Inflamm Allergy Drug Targets* 8: 28-39, 2009.
27. Israf DA, Khaizurin TA, Syahida A, Lajis NH and Khozirah S: Cardamonin inhibits COX and iNOS expression via inhibition of p65NF-kappaB nuclear translocation and Ikappa-B phosphorylation in RAW 264.7 macrophage cells. *Mol Immunol* 44: 673-679, 2007.
28. Zhou HY, Shin EM, Guo LY, Youn UJ, Bae K, Kang SS, Zou LB and Kim YS: Anti-inflammatory activity of 4-methoxyhonokiol is a function of the inhibition of iNOS and COX-2 expression in RAW 264.7 macrophages via NF-kappaB, JNK and p38 MAPK inactivation. *Eur J Pharmacol* 586: 340-349, 2008.
29. Kim HG, Yoon DH, Lee WH, Han SK, Shrestha B, Kim CH, Lim MH, Chang W, Lim S, Choi S, *et al*: *Phellinus linteus* inhibits inflammatory mediators by suppressing redox-based NF-kappaB and MAPKs activation in lipopolysaccharide-induced RAW 264.7 macrophage. *J Ethnopharmacol* 114: 307-315, 2007.
30. Jung WK, Choi I, Lee DY, Yea SS, Choi YH, Kim MM, Park SG, Seo SK, Lee SW, Lee CM, *et al*: Caffeic acid phenethyl ester protects mice from lethal endotoxin shock and inhibits lipopolysaccharide-induced cyclooxygenase-2 and inducible nitric oxide synthase expression in RAW 264.7 macrophages via the p38/ERK and NF-kappaB pathways. *Int J Biochem Cell Biol* 40: 2572-2582, 2008.