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Mongolian Medicine Honghu-Qiqige Ethanol Extract Exhibits Anti-Inflammation Role in Arthritis

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Objective: To explore the therapeutic effects of Honghu-Qiqige on collagen-induced arthritis (CIA) rats and the pharmacological mechanism. **Methods:** We evaluated the anti-inflammatory effect of Honghu-Qiqige's ethanol extract on CIA, to our knowledge, for the first time. The level of pro-inflammatory cytokine was determined by an ELISA. The expression of TNF-α and IL-1β was analyzed by RT-PCR. Additionally, the expression level of NF-kBP65 was evaluated using the Western blot method. **Results:** Ethanol extract of Honghu-Qiqige significantly reduced the secretion of TNF-α and IL-1β in RAW264.7 cells stimulated by lipopolysaccharides. The expressions of IL-1β mRNA, TNF-α mRNA, and NF-kBP65 protein were inhibited in all dose ranges used (5, 20, and 50 μg/ml). The protective effect of the extract on RAW 264.7 macrophage inflammation induced by lipopolysaccharide was preliminarily confirmed, and the mechanism may be related to the NF-kBP65 pathway. The same results were obtained in vivo experiments. **Conclusion:** All these results suggest that Honghu-Qiqige ethanol extract has potential to develop drugs to treat arthritis.

Keywords: Medicine, Mongolian Traditional, Arthritis, Inflammation

Introduction

Osteoarthritis is the most common form of arthritis. It often affects joints of the body, especially in the knees, hips and fingers [1-4]. Patients with osteoarthritis typically have joint pain and swelling, which can seriously impede ambulation and activities of daily living and may be disabling for life, causing a substantial economic burden [5]. At present, clinical treatment drugs can effectively improve the symptoms of patients. But these drugs are accompanied by serious adverse reactions, such as gastrointestinal reactions [6]. Therefore, we urgently need

new drugs with fewer side effects and better therapeutic effects. In recent years, Chinese medicine has performed well in this regard.

The inflammatory response is an essential process mediated by a variety of inflammatory cytokines IL-1β, TNF-α, and related inflammatory mediators (such as NF-kB activation) and many biological markers of the inflammatory response [7, 8]. Inflammatory cells produce lots of cytokines and are involved in the pathogenesis of diseases [9]. Therefore, such pathways are helpful in studying anti-inflammatory mechanisms, treatment methods, and adjuvant treatment methods for many diseases. Lipopolysaccharide (LPS) is a critical component of the outer bacterial cell wall and is an endotoxin that is a potent trigger of inflammation [10, 11]. The LPS-stimulated RAW 264.7 cell model is one of the typical inflammation models used in vitro. LPS induces NF-kB activation through a signal transduction pathway, thereby inhibiting NF-kB phosphorylation and triggers fever, microbial invasion, and septic shock [12].

Adenophora is a genus of flowering plants in the family *Campanulaceae* which is endemic to Eastern Asia, especially Russia, Mongolia, and China. The root of *Adenophora* contains a variety of biologically active components, such as glycosides, terpenes, phenolic acids, coumarins, sterols, volatile oils, and mineral elements that could be expected to have important physiological effects in the treatment of gout, rheumatism, leprosy, and chronic inflammatory conditions like chronic disease and osteoarthritis [13]. Kim et al. demonstrated that 70% ethanol extract of *Adenophora triphylla* inhibited adipocyte differentiation in 3T3-L1 cells dose-dependently. Moreover, it inhibited the phosphorylation of IRS1, resulting in attenuated protein expression of C/EBPα, PPARγ, ap2, and FAS in 3T3-L1 cells [14]. In other research by Park et al. ethyl acetate fraction of *Adenophora triphylla* attenuated cancer cell migration by suppressing macrophage polarization toward the M2 phenotype [15]. In the study of Chun et al. a triterpenoid saponin from *Adenophora triphyllavar.japonica* effectively inhibited the growth of the human gastric cell line via the activation of caspases [16].

The Mongolian traditional medicine Honghu-Qiqige (HQE) is the dried root of *Adenophora stenanthiana* (Ledeb) Kitagawa, a plant belonging to the family *Campanulaceae*. It grows widely in China, mainly in the Inner Mongolian provinces of Qinghai, Hebei, Shanxi, and Gansu, as well as Russia, and Mongolia. The other names of HQE in China are *Lududorjshanba* and *Ludariyagan*. These are well documented in the Chinese Pharmacopoeia and commonly used in traditional Mongolian medicine as a substitute for the plant *Codonopsis* [17]. Compared to the above-mentioned studies of *Adenophora triphylla*, to our knowledge, there are no studies of the bioactive characteristics of *Adenophora stenanthiana* (Ledeb) Kitagawa. Therefore, this study's objective was to elucidate the inhibitory activity of the ethanol extract of HQE on inflammation of LPS-induced RAW 264.7 cells. We sought to determine HQE's effects on the inflammatory cytokines TNF-α and IL-1β, and measure mRNA gene expression of the TNF-α and IL-1β, and of NF-kBp65 protein.

Materials and Methods

Reagents

The HQE was obtained from Professor Bu he ba te, who identified it as the dried root of Adenophora stenanthina (Ledeb) Kitagawa. Reagents used were, ethylene diamine tetraacetic acid (EDTA) (E8040, Solarbio), tris-buffered saline (TTBS) (T1080, Solarbio), Dulbecco's Modified Eagle Medium (DMEM) (Beverly, MA, USA), DNase (Cat. No. 18067-016, GIBCOBRIL), Ethidium bromide (Nacalai, Cat. No. L8028), 3-[4,5-dimethylthiazol-2-yl]2,5-dipheyltetrazolium bromide (MTT) cell proliferation assays and lipopolysaccharide (LPS) from Sigma (USA), cDNA synthesis Kit (California, USA), IL-1β and TNF-α (RAW264.7 cells (Shanghai, China), cell culture medium (GNM-12700), tryptase (GMN-16400), and fetal bovine serum (FBS) (Shanghai, China), penicillin-streptomycin (Shanghai, China), TRIcol reagent (Cat. No. 15598, GIBCOBRL).

Study design

A total of 20 adult male Wistar rats, weighing approximately 200 to 250 ± 20 gram, were used for the experiment. They were divided randomly into 5 groups (4 animals in each group): control-non treatment, LPS, low-dose, medium-dose and high-dose groups. All animals were supplied with standard food during the experiment with access to water. Experimental procedures were conducted according to the regulations of the Animal Ethical Committee. Total serum collected at the time of sacrifice for the analysis of inflammatory cytokines.

Cell culture

The RAW 264.7 cells were grown in DMEM containing 12% FBS, penicillin (100 U/ml), and streptomycin (100 U/ml) at 37 °C and 5% CO_2 condition.

Preparation of Honghu-Qiqige's ethanol extract

We added a volume of 50% ethanol 15 times the volume of granulated HQE and then performed ultrasonic extraction three times for 30 min each time. The filtrates were then combined and concentrated by rotatory evaporation to the desired volume to achieve the desired concentration.

MTT test

We took RAW 264.7 cells in a logarithmic growth phase, digested them with 0.25% trypsin EDTA to make $1x10^5$ cells/mL single-cell suspension with DMEM containing 10% PBS, and put 100 µL per well into 96 well plates. There were six replicates per concentration. They were incubated at 37 °C in 5% CO_2 for 24 hours. HQE ethanol 5, 10, 20, 30, 50 mg/L extracts were added for 2 hours, and then LPS 1 mg/L was added to stimulate the culture. The cells were then cultivated for 24 h.

We discarded the supernatant and replaced it with serum-free DMEM 195 µL in each well, then MTT at 5 g/L was added. After incubation, culture medium was aspirated and 150 µL DMSO was added to the well, shook for 5 minutes, and then optical density (OD) value at 570nm was measured by microplate reader to detect the to reflect the cell viability. The percentage of cells surviving was calculated: Cell survival rate % = [1- (A blank control group-A drug group) / A blank control group)] × 100%.

Measurement of pro-inflammatory cytokines IL-1β and TNF-α

The RAW 264.7 cells scraped were adjusted to a concentration of 5×10^5 cells/mL and were seeded in a 24-well cell culture plate at a volume of 1 mL per well for one hour. One of three HQE concentrations (5 µg/mL, 20 µg/mL, or 50 µg/mL) was added to the drug-containing solution in the drug group. Each group was set up with four duplicate wells, cultured for 1 hour, and then added 1 mg/L of LPS to stimulate the cells (in the blank group, no drug or LPS treatment was added, only the same volume of PBS). After 24 h, cell supernatant centrifuged and 100 µL of this supernatant used for ELISA. We followed the instructions of the ELISA kit and then determined absorbance at 450 nm using a fully automatic microplate reader. The corresponding cytokines TNF-α and IL-6 concentrations were calculated from the standard curve according to the OD value.

Real-time PCR analysis

The total RNA of the cells was extracted with TRIcol reagent. The concentration of RNA was calculated by measuring the absorbance at 260 nm; RNA was pretreated with DNase. RT-PCR was added according to the kit. Proportions were performed with a total volume of 20 µL per tube; Clontech Laboratories provided human IL-1βand TNF-α primers (pruners). The reaction solution contained 5 µg RNA content of each tube. The first step of the RT-PCR reaction was to cool the solution, 50 ^{0}C × min, 94 ^{0}C × 2 min; the second step was cooling to 94 ^{0}C × 45 s, 60 ^{0}C× 45 s, 72 ^{0}C × 2 min, and 26 cycles are performed, respectively. In the third step, the solution was cooled to 72 ^{0}C × 7 min, and the reaction product was electrophoresed on 1% agar, stained with ethidium bromide, and finally photographed with a digital camera under UV light. The photos were stored in a computer, and the software KS400 provided by Microsoft was used to quantify the band density of IL-1β and TNF-α electrophoresis. We then calculated the relative ratio of the band density of IL-1β and TNF-α electrophoresis.

Western blot analysis

RAW264.7 cells with a concentration of 5×10^5 cells/mL were seeded on a 6-well cell culture dish for one day, and three-drug concentration gradients (5 µg/mL, 20 µg/mL, and 50 µg/mL) were added to each well. In the drug solution, four duplicate wells were set for each group, and LPS 1 mg/L was added to stimulate the cells after 1 hour (the blank group received no drugs or LPS, only the same volume of PBS). After 30 min, the supernatant was discarded, and the wells were washed three times with pre-chilled PBS. We then added the cell lysate and placed it in an ice bath for 30 min. It was then centrifuged at 13,000 rpm for 4 min at 4 ^{0}C, and the protein concentration was determined using the BCA protein concentration determination kit. After being separated by 10% sodium dodecyl sulfonate-polyacrylamide gel electrophoresis (SDS-PACE), it was transferred to a polyvinylidene fluoride (PVDF) membrane and blocked with 5% (w/v) skim milk. The primary antibody was then incubated overnight at 4 °C according to the instructions of the antibody. The membrane was washed three times with TTBS and incubated

in the secondary antibody for one hour. The membrane was again washed three times with TTBS, and the color developed according to the instructions for the ECL luminescence kit.

Statistical analysis

The Kruskal-Wallis test was carried out to compare the study groups. The Mann-Whitney U test was applied for multiple comparisons after the Bonferroni corrections. A critical p-value of < 0.05 was used. SPSS version 24 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses.

Ethical statement

The animal study was carried out following laboratory protocol. The study was approved by the Research Ethics Committee of Mongolian National University of Medical Sciences (No.2018/3-08). All efforts were made to minimize the number of animals used and their suffering.

Results

Effect of Honghu-Qiqige on RAW 264.7 cell viability

Before investigating, the cytotoxicity of HQE was measured first in RAW 264.7 cells using the MTT assay. As shown in Figure 1, HQE significantly reduced the viability at the concentrations of 30, 40, and 50 μg/mL, while 2.5, 5, 10, 20 μg/mL had minimal influence. Therefore, in all subsequent experiments, we used HQE concentrations, ranging from 2.5 to 20 μg/mL.

Honghu-Qiqige reduced LPS-induced secretions of TNF-α, IL-1β

RAW 264.7 cells were pre-treated with different concentrations of HQE (2.5, 5, 10, 20 μg/mL) and then stimulated with 1 μg/mL LPS for 24 hours to investigate the effect of HQE on inflammation. As shown in Figure 2, the levels of pro-inflammatory TNF-α and IL-1β were significantly increasing in LPS-stimulated cells compared with the control group, and the secretions were blocked with the HQE treatment. These results suggested that HQE reduced the LPS-induced secretions of TNF-α, IL-1β.

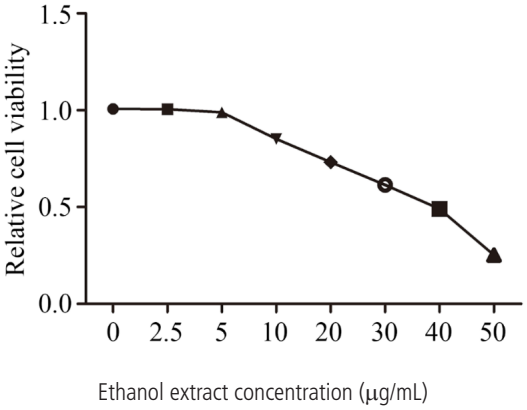

Figure 1. Effects of Honghu-Qiqige on cell viability of murine RAW264.7 cells. Cells were treated with 0, 2.5, 5, 10, 20, 30, 40, and 50 μg/mL Honghu-Qiqige ethanol extract for 24 h respectively. Cell viability was quantified spectrophotometrically by the MTT assay. The reported values are mean ± SD (n=4).

Effects of Honghu-Qiqige on the expression of NF-kBp65

RAW 264.7 cells were pre-treated with concentrations of HQE (2.5, 5, 10, 20 μg/mL) and stimulated with 1 μg/mL of LPS for 24 to determine the effects of HQE on the flows of pro-inflammatory NF-kBp65. As shown in Figure 3, LPS enhanced the expression of NF-kBp65 while HQE significantly inhibited the expression of NF-kBp65. These results demonstrate that HQE ethanol extract inhibited the production of NF-kBp65 effectively.

Table 1. The expression level ofNF-kBp65, IL-1βand TNF-α for Honghu-Qiqige (HQE) dose groups.

	Control	LPS	Low Dose HQE	Medium Dose HQE	High Dose HQE	*p-value
	n=4	n=4	n=4	n=4	n=4	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
NF-kBp65[a,b,c]	0.81 ± 0.16	1.84 ± 0.18	1.14 ± 0.04	1.03 ± 0.96	0.71 ± 0.14	0.029
IL-1β[d,e]	0.26 ± 0.12	0.93 ± 0.07	0.79 ± 0.09	0.49 ± 0.08	0.46 ± 0.07	0.014
TNF-α[f,g,h]	0.11 ± 0.01	0.87 ± 0.05	0.61 ± 0.04	0.43 ± 0.37	0.42 ± 0.09	0.006

*Kruskal-Wallis test; multiple comparisons: [a]control vs. LPS, $p < 0.021$; [b]control vs. medium dose, $p < 0.052$; [c]control vs. high dose, $p < 0.014$; [d]control vs. low dose, $p < 0.041$; [e]low dose vs. high dose, $p < 0.053$; [f]control vs. low dose, $p < 0.031$; [g]control vs. high dose, $p < 0.032$; [h]LPS vs. high dose, $p < 0.043$.

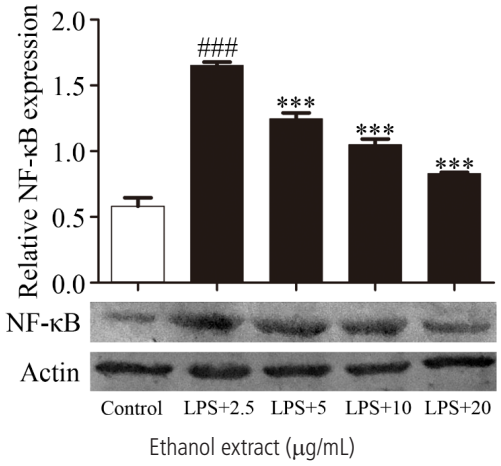

Figure 2. Effects of Honghu-Qiqige on the expression of NF-kBp65. The RAW264.7 cells were pretreated with Honghu-Qiqige for 2h followed by 1 μg/mL LPS. The expression of NF-kBp65 was detected by western blot. Data are represented as mean ± SD (n=4).

Effects on the mRNA expressions of IL-1β, and TNF-α

RAW 264.7 cells were pre-treated with different concentrations HQE (2.5, 5, 10, 20 μg/mL), and stimulation with 1 μg/mL LPS for 24 hours. As showed in Figure 4, LPS enhanced the expression of TNF-α and IL-1β while HQE significantly inhibited the expression of TNF-α and IL-1β. These results demonstrate that HQE inhibited the mRNA expression levels of TNF-α, IL-1β effectively.

Honghu-Qiqigereduce inflammation in *vivo*

To further investigate the effect of HQE on inflammation, we constructed a collagen-induced arthritis (CIA) model and explored the effect of HQE on inflammation. As shown in Figure 5, high doses of HQE also significantly reduced inflammatory cytokines IL-1β and TNF-α in the blood of the rat compared to the positive drug Codonopsis (Figure 5A and B). Further studies have shown that HQE can inhibit the expression of inflammatory cytokines IL-1β and TNF-α mRNA (Figure 5C and D). Besides, HQE can significantly inhibit the nucleation of transcription factor P65 (Figure 5E), which is consistent with the above results. All these results indicate that HQE can effectively inhibit inflammation.

Discussion

The genus *Adenophora* is in the family *Campanulaceae* in the major group Angiosperms. Adenophora is endemic to East Asia and well documented in the Chinese Pharmacopoeia, commonly used in traditional Mongolian medicine in place of the plant Codonopsis [13, 15].There are about 62 species in this genus, which have different morphological characteristics and biological activity. For example, Kim et al. demonstrated that *Adenophora remotiflora*, which is endemic to Korea and Japan, protects human skin keratinocytes against UV by regulating the anti-oxidative activity and inhibition of the MMP-1 protein [17, 18]. Moreover, research by Lee et al. showed that the root extract of the *Adenophora triphylla*var. *japonica* inhibited lipid accumulation in 3T3-L1 adipocytes. In high-fat-diet-induced obese mice experiments, there was a significant

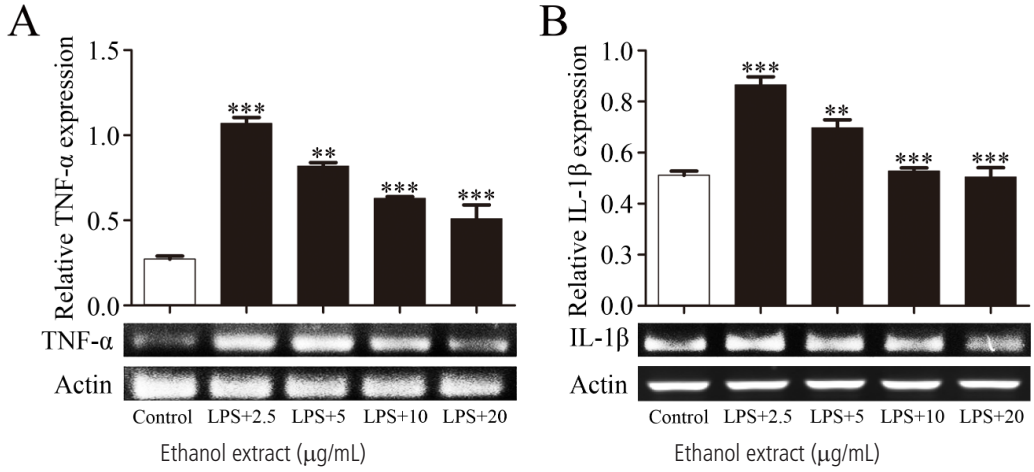

Figure 3. Effects on the mRNA expressions of IL-1β, and TNF-α. The RAW264.7 cells were pre-treated with Honghu-Qiqige for 2h followed by 1 μg/mL LPS for 24h. The expression of TNF-α and IL-1β was detected by PCR. Data are represented as mean ± SD (n=6).

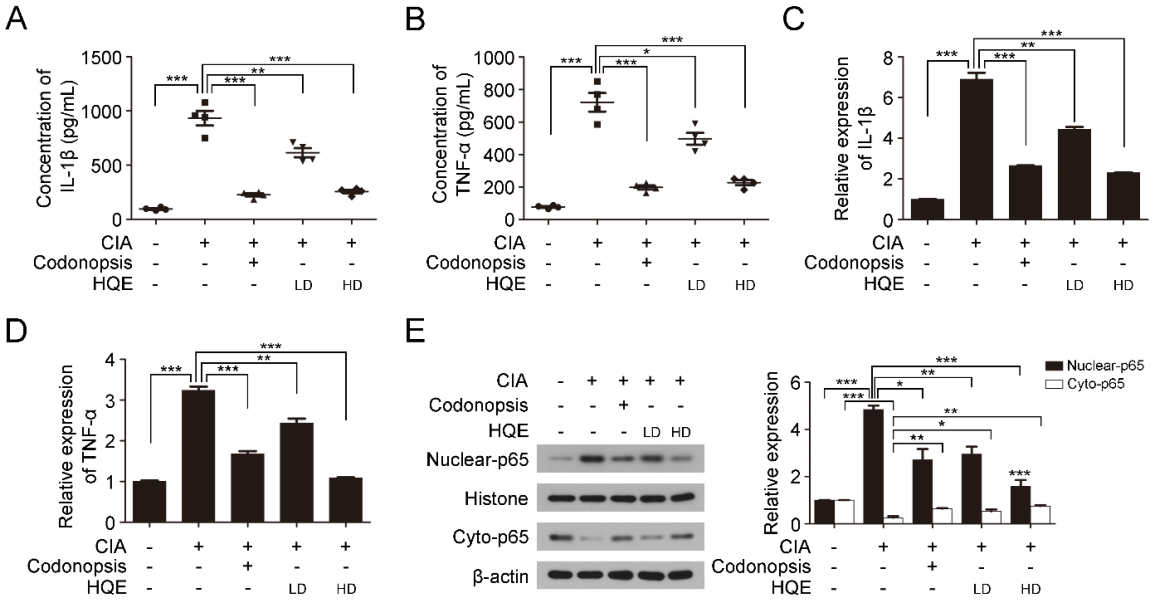

Figure 4. The effect of Honghu-Qiqigeon inflammation in vivo. The concentration of IL-1β and TNF-α were detected by ELISA. The expression of TNF-α and IL-1β was detected by QPCR. The nuclear translocation of p65 was detected by western blot. Data are represented as mean ± SD (n=6).

decrease in body weight gain, white adipose tissue and plasma triglyceride levels [19]. Zheng et al. likewise revealed that new phenolic glycosides and saponins purified from the root of *Adenophora triphylla*var. *japonica* were protective against obesity [20].

The Mongolian traditional medicine HQE is the dried root of *Adenophora stenanthiana* (Ledeb) Kitagawa. It widely grows in China, mainly in the north-eastern provinces of Inner Mongolian, such as Qinghai, Hebei, Shanxi, Gansu, as well as in Russia and Mongolia. Our study aimed to determine the anti-inflammatory activity of the original Mongolian plant specimen *Adenophora stenanthiana* (Ledeb) Kitagawa. We also note that, as far as we can tell, the biological and biochemical activity of HQE has not been previously studied. Our results show that, due to its content of triterpenes, known to be anti-inflammatory, the extract shows high anti-inflammatory activity.

Regarding the production of relative cytokines, different studies have focused on a variety of various cytokines. According to reports, IL-1β is a relatively pro-inflammatory cytokine [21]. Compared with other research regarding Chinese herbal medicine extracts, HQE showed significant inhibition of IL-1β and TNF-α.

Activation of NF-kB leads to excessive production of pro-inflammatory cytokines IL-1β and TNF-α and plays a non-negligible role in inflammatory diseases [22]. We have assessed their relative protein and mRNA expression further. Using different doses of HQE on LPS-induced RAW 264.7 cells, and all concentrations exhibited significant inhibitive effects on the production of NF-kBp65 protein and mRNA expressions of IL-1β and TNF-α. This implies that HQE showed substantial inhibitory effects on TNF-α and IL-1β production compared to previous studies [23-25].

A limitation of this study is that due to the complexity of the inflammatory response, it is not clear how the properties of HQE affect the treatment of gout, rheumatism, and arthritis diseases. Based on these results, we will direct further research investigating the biomedical importance of ethanol extracts of HQE for NF-kB activation as well as other signal transduction mechanisms. Moreover, we will need to determine the exact active compound of the extract that is against inflammation.

Conclusion

In this study, the extract of HQE significantly inhibited the typical cytokines, reduced levels of inflammation-related mRNA and protein. More importantly, it demonstrated the molecular mechanism controlling anti-inflammatory properties through the

NF-kB pathway and proved its protective effect on inflammatory cells. We propose that the ethanol extract of HQE is a strong candidate for the development of anti-inflammatory drugs.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

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