Anti-atherosclerotic effect of herbal extracts in N(G)-nitro-L-arginine methyl ester-treated rats

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Received: 22 July 2019 / Accepted: 19 August 2019 / Published Online: 30 September 2019
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Abstract This study aimed to evaluate the anti-atherosclerotic and anti-hypertensive effects of six different plant extracts using a N(G)-nitro-L-arginine-methyl ester (L-NAME)-induced rat model of hypertension. All extracts were administered orally for six weeks. At the end of the study period blood pressure, blood flow, aortic histopathology, and hepatic endothelial nitric oxide synthase (eNOS) expression were measured. Subsequently, we also measured the levels of intracellular reactive oxygen species, nitric oxide (NO), and anti-inflammatory cytokines in vitro. Based on these screening results, we selected extracts of Cinnamomum cassia (C. cassia) and Salvia miltiorrhiza (S. miltiorrhiza) for further evaluation. C. cassia and S. miltiorrhiza extracts ameliorated hypertension and atherosclerosis in L-NAME-treated rats in a dose-dependent manner. In addition, a mixture of C. cassia and S. miltiorrhiza had an additive effect to reduce blood pressure, increase blood flow, and normalize aortic tissue. This mixture demonstrated anti-oxidative and anti-inflammatory activities in vitro. In conclusion, although further analysis of the therapeutic mechanism is required, the anti-hypertensive and anti-atherosclerotic effects of this mixture are likely mediated by increased eNOS expression, and its anti-oxidative and anti-inflammatory activities.

Keywords Anti-oxidation · Atherosclerosis · Hypertension · Inflammation

Introduction

Hypertension has become one of the major causes of early death worldwide over recent decades. A recent World Health Organization report estimates there are one million people affected, and hypertension contributes to approximately 12.8% of annual mortality around the world [1]. Hypertension is diagnosed in more than 90-95% of these patients. In most cases, it is caused by endothelial defects involving nitric oxide (NO) depletion [2]. High blood pressure is one of the most important factors predisposing toward diseases, including heart failure, artherosclerosis, myocardial infarction, and stroke. A lack of NO is a key defect in the development of hypertension and cardiovascular diseases [3]. Specifically, a reduction in NO synthesis contributes to the pathogenesis of high blood pressure, coronary artery disease, myocardial infarction, diabetes, and stroke. Treatment of rats or mice with N(G)-nitro-L-arginine-methyl ester (L-NAME) is a commonly used method for modeling high blood pressure in humans [4-6]. L-NAME-treated animals demonstrate reduced endothelial nitric oxide synthase (eNOS) expression in arteries, and a consequent reduction in plasma NO, which results in narrower blood vessels and higher blood pressure. Thus, NO production is one of the most important factors in regulating cardiovascular function and homeostasis [5,7].

There are connections between the hypertension induced by L-NAME and physiological stress markers. Specifically, a large dose of L-NAME (60 mg/kg/100 mL, water/day) increases the levels of vascular superoxide, malondialdehyde, and plasma protein carbonyls [6,7]. These have been demonstrated to contribute to the etiology of hypertension in animals and humans, along with higher concentrations of reactive oxygen species (ROS) [7]. Oxidative stress mechanisms have been well documented by in vitro and in
vivo studies of L-NAME-treated rats [8-12], involving the production of large quantities of ROS. Furthermore, damage to vascular tissue in L-NAME-treated rats results in high ROS concentrations and greater secretion of proinflammatory cytokines [8-12].

Asians frequently use herbal remedies as alternative medicines, because of their lower cost, perceived efficacy, and tolerability [13]. Several medicinal plant extracts have been used to prevent degenerative diseases. *Cinnamomum cassia* (C. cassia) and *Salvia miltiorrhiza* (S. miltiorrhiza) are frequently used in Chinese traditional medicines and are routinely used to treat hypertensive diseases [14]. However, there have been no studies of using these herbs in combination. In the present study, we aimed to determine whether a combination of *C. cassia* and *S. miltiorrhiza* extracts can improve blood flow and vasodilation, and reduce inflammation, using in vitro and in vivo models.

**Materials and Methods**

**Plant extract preparation**

A series of candidate plants (*C. cassia*, *Crataegus pinnatifida* Bunge, *Eucommia ulmoides*, *Morus nigra* L. fruit, *Prunella vulgaris*, and *S. miltiorrhiza*) were purchased from Kyungdong oriental herb market in Korea and a medical herb market in China. The voucher specimens (EDU001-EDU019) are stored at Chungbuk National University, Korea. Plants were extracted with ethanol and/or water as reported by Yang et al. [15], and the content of marker compound in various extracts were determined by spectrometric method or HPLC method (data not shown).

*C. cassia* bark was extracted three times in 100% ethanol for 2 h. The extract of the aerial parts was not less than 5% polyphenol. After filtration, the extract was vacuum-concentrated to dryness. *Crataegus pinnatifida* (C. pinnatifida) Bunge fruit extract was extracted in 70% ethanol, contained 5% procyanidins, and concentrated to dryness using a spray dry system. *Eucommia ulmoides* (E. ulmoides) bark was extracted in 70% ethanol, contained 2.5% chlorogenic acid, and concentrated to dryness using a spray dry system. *Morus nigra* (M. nigra) L. fruit was extracted in 70% ethanol, contained 25% anthocyanins, and concentrated to dryness using a spray dry system. *Prunella vulgaris* (P. vulgaris) was extracted in water three times for 2 h, with the extract containing 2% flavone, and concentrated to dryness using a vacuum system. *S. miltiorrhiza* root was extracted in 70% ethanol, contained 9.5% salvianolic acid B, and concentrated to dryness using a spray dry system.

**Animals**

Healthy adult male Wistar rats were procured from the Dooyeol biotech Lab, South Korea, maintained at constant temperature (23-25°C) and under a 12 h light/12 h dark cycle, and fed a standard chow diet. After an acclimation period of seven days, the rats were allocated to specific treatment groups based on body weight, using a randomized block design. The animal study was approved by the Ethics Committee for Animal Experimentation of Chungbuk National University (Permit Number: CBNUR-909-15, Korea).

**Design of the in vivo screening study**

The rats were allocated to eight experimental groups. The normal control group received sterilized drinking water, and the same water was administered intragastrically for six consecutive weeks. The L-NAME hypertensive control group was administered L-NAME (60 mg/kg/100 mL, water/day) in sterilized drinking water, and the same water was administered intragastrically for six consecutive weeks. The treatment groups were administered L-NAME in sterilized drinking water, and each plant extract (300 mg/kgbw per day) was administered intragastrically for six consecutive weeks. No treatment-related mortality or morbidity was observed during the experimental period. The rats’ body weights were measured on the initial and final days of the treatment period. On the day of sacrifice, blood flow rate was measured according to the manufacturer’s instructions (Flo-C1; Omegawave, Inc., Tokyo, Japan).

**Measurement of blood pressure**

The rats’ blood pressure was measured, noninvasively and without anesthesia, with an indirect tail cuff method (Harvard Apparatus, Millis, MA, USA). The rats were restrained on a warming plate and acclimated for 5 min before the cuff was fitted. Blood pressure was measured twice during the experimental period.

**Estimation of blood flow in the carotid artery**

The untreated control, L-NAME control, and extract-treated animals were anesthetized with urethane (Sigma-Aldrich, St. Louis, MO, USA); then the carotid artery was exposed. Blood flow was then measured using laser Doppler flowmetry (Flo-C1; Omegawave, Inc.) and analyzed using Chart 5 software (eDAQ Pty Ltd, NSW, Australia).

**Sampling**

After six weeks of treatment, the rats were anesthetized with urethane, and blood samples were collected from the caudal vena cava. The collected blood samples were centrifuged at 3,000 rpm for 15 min to separate sera for biochemical analyses. Afterwards, liver samples were collected and stored at 80 until analyzed.

**Histopathologic analysis**

After euthanasia, the ascending aorta was collected from each rat and fixed in 10% formaldehyde solution. The tissues were fixed, processed, and paraffin-embedded, then sectioned at 5 μm thickness, stained with hematoxylin and eosin (H&E), and examined using a light microscope [3]. Image analysis was performed using analySIS® TS auto (Olympus Soft Imaging System, Münster,
Germany) to quantify the aortic diameter and vessel wall thickness.

**Design of the confirmatory study**

Based on the screening study results, we selected two plant extracts (of *C. cassia* and *S. miltiorrhiza*) for further study. Rats were allocated to seven groups of 10 rats each. Hypertension was induced using L-NAME, as described above, and the rats were treated once daily for six weeks with extracts of *C. cassia* (150 mg/kg bw per day), *S. miltiorrhiza* (150 mg/kg bw per day), or mixtures of *C. cassia* (50%) and *S. miltiorrhiza* (50%) (150, 300, or 600 mg/kg bw per day). On the day of sacrifice, blood pressure and blood flow rate were measured according to the manufacturers’ instructions.

**Western blot analysis**

Total NOS activity was determined in crude liver homogenates using a commercial kit. Briefly, equal amounts of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with blocking buffer for 1 h at 4 °C and then incubated with anti-eNOS antibody (Abcam, Cambridge, UK) overnight at 4 °C. They were then washed four times for 10 min in TBST buffer, incubated with secondary antibody for 2 h at room temperature, and then washed again four times for 10 min in TBST. eNOS expression was normalized to that of β-actin, using the antibody supplied by the manufacturer. Bound antibodies were identified by chemiluminescence.

**Nitric oxide release assay**

HUVEC cells were seeded in 96-well plates at 3×10^4 cells/well and incubated for 24 h. The medium was then removed, and the cells were pretreated for 30 min, and then they were incubated with 500 μM H_2O_2 for 4 h. The total NO content was then measured in the test medium using an NO assay kit (R&D systems, MN, USA).

**Intracellular ROS production**

HUVEC cells were seeded in 96-well black plates at 3×10^4 cells/well and incubated for 24 h. After the medium was removed, the cells were washed once with assay buffer, treated with 25 μM of 2', 7'-dichlorofluorescein, and then incubated for 45 min in a 37 °C incubator. After the reaction, the sample was washed once with assay buffer and pretreated for 30 min; then, after the addition of 500 μM H_2O_2 for 1 h, absorption was measured using excitation and emission wavelengths of 485/530 nm.

**Enzyme-linked immunosorbent assay (ELISA)**

RAW264.7 cells were seeded in 96-well plates at 1×10^5 cells/well and incubated for 24 h. After removal of the medium and pretreatment for 1 h, lipopolysaccharide (1 μg/mL) was added for 24 h. Pro-inflammatory cytokine concentrations were then measured in the media using ELISA assays (R&D systems) per the manufacturer’s instructions.

**Statistics**

Statistical analyses of the data were performed using Student’s *t*-test. Values are stated as the mean ± SD.

**Results**

**Body weight during the in vivo screening study**

To confirm the extracts’ safety, we monitored the rats’ body weight, mortality, and clinical signs. All of the rats survived until their scheduled necropsy, and no gross abnormalities were observed during the study that could be related to the plant extracts. The mean body weights of the rats were recorded on day 0 and, as shown in Fig. 1A, the rats were assigned to groups to minimize body weight differences. At the end of the experiment, there were no statistically significant differences in body weight among the experimental groups.

**Blood flow in the carotid artery**

L-NAME treatment is known to induce hypertension and harden arteries, which reduces the blood flow. As expected, blood flow was reduced by L-NAME treatment (Fig. 1B). However, there were increases in blood flow after administration of the crude plant extracts prepared from *C. cassia*, *C. pinnatifida*, *E. ulmoides*, *M. nigra*, *P. vulgaris*, and *S. miltiorrhiza* for six weeks. Of these six extracts, treatment with the *C. cassia* extract had the largest effect on blood flow, near-normalizing it. Although its effect was variable, *S. miltiorrhiza* extract had the second-largest effect.

**Effect of *C. cassia* and *S. miltiorrhiza* extracts on rat body weight in the confirmatory study**

Based on the results of the screening studies, we selected two plant extracts (of *C. cassia* and *S. miltiorrhiza*) for further study. In the preliminary experiment, we observed no gross abnormalities or mortality after treatment with 300 mg/kg bw of the extracts (Fig. 1). Therefore, we increased the dose to a maximum of 600 mg/kg bw for the follow-up experiment, but even at the highest dose, no gross abnormalities or mortality were observed during the study period. The rats’ mean body weights on the day of sacrifice were similar among the groups (Fig. 2A).

**Effect of *C. cassia* and *S. miltiorrhiza* extracts on blood pressure**

Oral administration of L-NAME induced significant increases in systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) compared to the normotensive control rats (Table 1; 44, 51, and 41%, respectively). High blood pressures were reduced by treatment with *C. cassia* and *S. miltiorrhiza* extracts, either singly or in combination. Moreover, the combination extracts administered at 600 mg/kg bw reduced these parameters in a dose-dependent manner (by 24, 30, and 26%, respectively) compared to the L-NAME group.
Effect of *C. cassia* and *S. miltiorrhiza* extracts on blood flow

To confirm the positive effects of *C. cassia* and *S. miltiorrhiza* extracts on blood flow, the extracts were administered to rats, either individually or in combination. As shown in Fig. 2(B), blood flow was reduced by L-NAME treatment, but this deleterious effect was ameliorated by treatment with *C. cassia* and *S. miltiorrhiza* extracts, either singly or in combination. This finding indicates that the plant extracts cause vasodilation of the carotid artery.

Effect of *C. cassia* and *S. miltiorrhiza* extracts on the blood vessels of L-NAME-treated animals

To further determine the effects of *C. cassia* and *S. miltiorrhiza* extracts on blood vessels, we analyzed the wall thickness of the ascending aorta histopathologically. Ascending aortas were prepared as 5 μm-thick sections and then stained with H&E. As shown in Fig. 3, the ascending aortae of L-NAME-treated rats had almost three-fold thicker walls than those of control rats, indicating hypertrophy. However, the thickness of the aorta walls was significantly reduced by treatment with *C. cassia* or *S. miltiorrhiza* extracts. Rats treated with extracts of a single plant had aortic walls that were still two-fold thicker than those of the control group, but treatment with a mixture of *C. cassia* and *S. miltiorrhiza* extracts had a larger effect, which was not dose-dependent. Other vascular histologic changes, like as connective tissue layer of adventitia, were also observed in the L-NAME-treated rats, which

**Table 1 Changes of blood pressure in L-NAME treated rats**

| Variables  | Normal | L-NAME | L-NAME + C. *pinnatifida* | L-NAME + S. *ulmoides* | L-NAME + Mixture 150 mg/kgbw | L-NAME + Mixture 300 mg/kgbw | L-NAME + Mixture 600 mg/kgbw |
|------------|--------|--------|---------------------------|------------------------|-----------------------------|-----------------------------|-----------------------------|
| SBP        | 92±5.68 | 164±4.98 | 158±3.56                  | 151±6.98               | 148±8.13                    | 131±3.54                    | 124±7.39                   |
| DBP        | 65±8.64 | 132±8.23 | 129±3.81                  | 128±6.81               | 115±2.52                    | 118±4.82                    | 92±6.15                     |
| MAP        | 89±3.23 | 152±6.12 | 147±2.82                  | 148±5.37               | 137±2.92                    | 129±8.17                    | 113±7.13                   |

Data are presented as mean ± SD, (n = 5, p < 0.05). Statistical significance (*p < 0.05, Student’s t-test) as compared with L-NAME group. Systolic Blood Pressure (SBP); Diastolic Blood Pressure (DBP); Mean Arterial Pressure (MAP)
were ameliorated in rats treated with plant extracts (Fig. 3).

**Effect of C. cassia and S. miltiorrhiza on eNOS protein expression**

L-NAME is an inhibitor of NOS activity, both in vivo and in vitro; therefore, L-NAME induces hypertension and damage in vascular tissues because of a reduction in NO synthesis [6,7]. The eNOS protein expression was confirmed by western blotting of liver tissue lysates. The eNOS expression in liver lysates was significantly lower in L-NAME-treated rats than in control rats, but the plant extracts significantly ameliorated this reduction in eNOS, except for the 150 mg/kgbw mixture (Fig. 4). The highest eNOS expression levels were found in rats that were administered the combination of plant extracts at 600 mg/kgbw.

**Intracellular ROS production**

ROS provokes an inflammatory reaction by inducing oxidative damage in vascular endothelial cells and ultimately induces vascular wall dysfunction. To determine whether ROS production is inhibited by the plant extracts, oxidative stress was induced in vascular endothelial cells (HUVEC cells) by treatment with H$_2$O$_2$, and the cells were then treated with the C. cassia and S. miltiorrhiza extracts. As shown in Fig. 5A, H$_2$O$_2$ treatment significantly increased ROS production, but this effect was ameliorated by treatment with either single extracts or mixtures of C. cassia and S. miltiorrhiza. The largest reduction in ROS production was induced by the highest concentration of extracts in a 1:1 mixture of C. cassia and S. miltiorrhiza extracts.

**Nitric oxide release**

To further clarify the anti-oxidative action of the plant extract mixture, we compared the amounts of NO produced by HUVEC cells in vitro after each treatment. NO mediates blood vessel relaxation and permeability; therefore, we measured NO production after inducing oxidative stress by treatment with H$_2$O$_2$. As shown in Fig. 5B, the cells’ release of NO was reduced by treatment with H$_2$O$_2$, but this reduction was limited by treatment with a mixture of C. cassia and S. miltiorrhiza extracts.

**Effect of C. cassia and S. miltiorrhiza on the production of proinflammatory cytokines**

Damage to blood vessels induces inflammation, which involves inflammatory cells, including macrophages and dendritic cells. Activated macrophages secrete pro-inflammatory cytokines, such as...
as tumor necrosis factor (TNF-α), interleukin (IL)-1β, and IL-6. These cytokines are important initiators of the inflammatory response and play a role in developing a variety of inflammatory diseases [16]. Therefore, the murine macrophage cell line, Raw 264.7, was pretreated with the plant extracts and then stimulated with lipopolysaccharide (LPS). As shown in Fig. 6, TNF-α, IL-6, and IL-1β production was significantly increased following 1 μg/mL LPS stimulation, but their concentrations were reduced by pretreatment with *C. cassia* and *S. miltiorrhiza* extracts. The mixture of *C. cassia* and *S. miltiorrhiza* extracts reduced TNF-α and IL-6 production, which may be beneficial for the vasodilatory and antihypertensive properties of the artery.

### Discussion

Although there are still few effective treatments for hypertension, there are several types of natural plant extracts that have anti-hypertensive effects [14]. In this study, we have used L-NAME to induce vascular disease in rats. This model has provided significant information regarding the etiology, pathogenesis, and sequelae of such disease, and also provides a means for testing the efficacy of candidate therapeutic compounds. This model is also useful as a model of atherosclerosis, because the reduction in NO production induced by L-NAME results in hyperlipidemia [17]. In the present study, we used this model to screen six plant extracts for their anti-atherosclerotic and anti-hypertensive effects, and then selected extracts of *C. cassia* and *S. miltiorrhiza* for further study.

*C. cassia* and *S. miltiorrhiza* are commonly used as Chinese herbal medicines to treat cardiovascular diseases. They have been shown to have pharmaceutical effects in the cardiovascular system: anti-thrombotic effects, cardioprotective effects, anti-hypertensive effects, and anti-atherosclerotic effects. They also maintain the microcirculation in the brain and heart [18,19]. Both extracts have been shown to promote blood flow and improve the circulation in the heart [18,19]. Improvements in blood flow were also shown using laser Doppler blood flowmetry *in vivo* in the present study. L-NAME-treated rats had lower blood flow (8.9 mL/sec) than control rats, but when treated with *C. cassia* (26.87 mL/sec) and *S. miltiorrhiza* (23.42 mL/sec) alone, or with a combination at low (17.18 mL/sec), intermediate (23.13 mL/sec), or high (23.65 mL/sec) concentrations, there were substantial improvements in blood flow, which returned it to near normal (21.87 mL/sec).

According to a previous report, vascular fibrosis, which is characterized by lumen diameter reduction and thickening of the coronary artery wall, can be attributed to degradation of the adventitia and inhibition of matrix degradation [19]. The analysis of arterial tissue in a vascular disease model is typically performed by microscopic examination of the intima, media, and adventitia [20]. In the present study, aortic vessel layer remodeling was classified in all six experimental groups. The control group was characterized by a clear separation of the three layers, whereas L-NAME-treated rat aortae showed significant degradation of the intima and adventitia. However, remodeling of the intima (in which endothelial cells are in direct contact with the blood) and adventitia was observed in all the treatment groups. Furthermore, the aortae of L-NAME-treated rats were thicker in cross-section than those of control rats, indicating hypertrophy. However, the thickness was significantly reduced in rats treated with *C. cassia* and *S. miltiorrhiza* extracts.

In the present study, the plant extracts ameliorated the L-NAME-induced hypertrophy of the aortic wall, even at the lowest doses, which was likely mediated by an increased nitric oxide synthase expression. It is known that eNOS expression is an
important mediator of physiologic vasodilatation in rats, and in our in vivo study, L-NAME reduced both blood flow and the expression of eNOS, both of which were restored by treatment with C. cassia or S. miltiorrhiza extracts. This is consistent with an indirect mechanism, depending on NO synthesis and release from endothelial cells, mediating blood vessel relaxation [21]. We have also demonstrated that C. cassia and S. miltiorrhiza extracts increase NO production by endothelial cells in vitro. C. cassia and S. miltiorrhiza extracts mitigated ROS production by oxidatively damaged HUVEC cells. ROS play a role as second messengers in many immune responses [22] and as cytolytic molecules involved in killing pathogens [23, 24]. Oxidative stress is considered a key component of the pathogenesis of atherosclerosis [25]. The production of large amounts of ROS leads to vasoconstriction, vascular remodeling, inflammation, and fibrosis [26]. An inflammatory reaction occurs secondary to oxidative damage to vascular endothelial cells and ultimately induces dysfunction of the vascular wall.

Numerous studies have shown that oxidative stress leads to production of proinflammatory cytokines [27], thereby linking ROS with inflammation and vascular endothelial dysfunction. Inflammation is the immune system’s response to infection, injury, or irritation, and involves the production of pro-inflammatory mediators, which increase or suppress the production of other inflammatory mediators, such as NO, cyclooxygenase, and prostaglandins [28]. Macrophages are the most common cell type used to screen potential anti-inflammatory compounds because they are at the center of the inflammatory response and initiate the secretion of a cytokine cascade. Activated macrophages secrete proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, which are essential for the inflammatory response and are involved in many inflammatory diseases [29]. Anti-inflammatory effects C. cassia and S. miltiorrhiza extracts have already been

![Fig. 4. Effect of C. cassia and S. miltiorrhiza extracts on eNOS protein expression. The expression of eNOS was confirmed by western blot with the extract of liver tissue. The data shown are representative of three independent experiments](image)

![Fig. 5. Effect of C. cassia and S. miltiorrhiza extracts on ROS (A) and NO (B) production from HUVEC cells. The data from three independent experiments, each of which was performed in triplicate, are indicated as mean ± SD. Statistical significance (***p < 0.001, Student’s t-test) as compared with H2O2 control](image)
demonstrated in the treatment of cardiovascular diseases [29]. In the present study, we have also identified anti-inflammatory properties of ethanolic plant extracts of *C. cassia* and *S. miltiorrhiza*, alone and mixed, because they suppressed TNF-α, IL-1β, and IL-6 production by LPS-stimulated macrophages. The anti-inflammatory properties of *C. cassia* and *S. miltiorrhiza* shown in the *in vitro* experiment appeared to be similar to those of other compounds, such as cinnamic aldehyde [29], which can be derived from *C. cassia*. We also attempted to demonstrate the effects of the extracts on TNF-α, IL-1β, and IL-6 production *in vivo*, but the cytokine concentrations were too low to measure.

In this study, we have demonstrated additive effects of a mixture of *C. cassia* and *S. miltiorrhiza* extracts in L-NAME-treated hypertensive rats. This mixture causes a moderate reduction in blood pressure, and ameliorates reduced blood flow and abnormal aortic histology induced by L-NAME. Although further studies are required to identify the mechanism involved, we present evidence that the anti-hypertensive and anti-atherosclerotic effects of this mixture may be caused by upregulation of eNOS expression, and anti-oxidative and anti-inflammatory activities.

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![Fig. 6. Effect of *C. cassia* and *S. miltiorrhiza* on the production of proinflammatory cytokines. Proinflammatory cytokines were measured by ELISA assay after sampling the culture supernatants. The data from three independent experiments, each of which was performed in triplicate, are indicated as mean ± SD. Statistical significance (*p <0.05; **p <0.01, Student's t-test) as compared with LPS control](image)
