

ORIGINAL ARTICLE

Inhibitory Effects of Emodin, Thymol, and Astragalin on *Leptospira interrogans*-Induced Inflammatory Response in the Uterine and Endometrium Epithelial Cells of Mice

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Abstract—Leptospirosis is a systemic infection that causes, among others, acute kidney injury, acute liver disease, muscle pain, vasculitis, bleeding disorders, and reproductive loss. In an effort to reduce uterine inflammatory responses induced by *Leptospira*, we evaluated the anti-inflammation effects of emodin, thymol, and astragalin in a mouse model. Our results showed that treatment with emodin, thymol, and astragalin alleviated uterine inflammation induced by leptospira infection *via* suppression of pro-inflammatory cytokine expression and prevented tissue damage. Furthermore, we used primary endometrium epithelial cells to show that treatment with these chemicals inhibited the expression of pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 using enzyme-linked immunosorbent assay and quantitative polymerase chain reaction. Western blot results showed that these chemicals suppressed the phosphorylation of p38, p65, extracellular signal-regulated kinase, and c-Jun N-terminal kinase. These results indicate that treatment with emodin, thymol, and astragalin suppressed inflammatory response by regulating NF- κ B and mitogen-activated protein kinase signaling pathways in leptospira-infected uterine and endometrium epithelial cells of mice.

KEY WORDS: leptospira; inflammatory; emodin; thymol; astragaline.

INTRODUCTION

Leptospirosis is a zoonotic disease of global importance, with worldwide distribution, especially in tropical areas [1, 2]. Rodents, as typical reservoir animals, asymptotically carry leptospira in their kidneys and shed leptospire in the

urine, thereby contaminating the environment [3]. Humans and animals can be infected by contact with contaminated water and soil by means of skin abrasions and damaged mucous membranes. Upon infection, leptospira disseminate rapidly *via* the bloodstream and invade the host tissues and organs [4]. Sensitive animals have typical clinical symptoms such as jaundice, fever, renal and hepatic insufficiency, pulmonary hemorrhage, and reproductive failure [5]. Endometritis as one of the most important reproductive diseases can cause infertility and even death, incurring huge economic losses in the animal husbandry industry.

Leptospira-induced uterine inflammatory and reproductive failure has been found in many domestic animals [6, 7]. Infection of the uterus by pathogens is always followed by serious inflammatory reactions during endometritis. Additionally, severe leptospirosis induces a “cytokine storm” during the early stage of infection, and the overproduction of IL-10 and IL-6

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cytokines may be implicated in the mechanism of severe leptospirosis with a high fatality rate [8]. Thus, the targeted reduction of the cytokine response may help guide new therapeutic approaches to reduce morbidity and mortality as a consequence of severe leptospirosis. However, there are very few reports on the mechanism of reproductive disorders caused by leptospirosis and the use of anti-inflammatory agents in the clinical treatment of leptospirosis.

Traditional Chinese medicine has been gaining more attention in the search for and characterization of new anti-inflammatory treatments. Our laboratory recently screened traditional Chinese herbs for the ability to inhibit inflammation, especially emodin, thymol, and astragalin [9–11]. Emodin (Fig. 1a) is an anthraquinone derivative from the rhizome of *Rheum palmatum* that is widely used as a laxative in traditional Chinese medicine [12]. Thymol (Fig. 1b) is a natural monoterpene phenol primarily found in thyme, oregano, and tangerine peel [10]. Astragalin (Fig. 1c), a flavonoid isolated from the leaves of persimmon or *Rosa agrestis*, has been used for treating many diseases in traditional Chinese medicine for many years [13]. It has been reported that emodin, thymol, and astragalin possess a number of similar biological properties, such as elimination of reactive oxygen species and reduction of inflammation [14].

The presence of leptospires in uterine infections of ruminants is difficult to eliminate with the treatment by antibiotics alone. Therapy with immunostimulants could be helpful in the control of these infections. Although the anti-inflammatory effect of emodin, thymol, and astragalin has been evaluated in previous studies, their inhibitory effects on leptospirosis-induced inflammatory response in uterine and endometrium epithelial cells are not clear. In this study, we evaluated the anti-inflammatory effect of emodin, thymol, and astragalin in uterine and endometrium epithelial cells infected by leptospirosis with the aim of revealing a

potential mechanism of reproductive failure induced by leptospirosis.

MATERIALS AND METHODS

Animals and Bacterial Strains

Female BALB/c mice (8 to 10 weeks old) were purchased from Norman Bethune University of Medical Science of Jilin University. The mice were housed in a dedicated room at 24 ± 1 °C and 40–60% humidity. All animal experiments followed the regulations for the Administration of Affairs Concerning Experimental Animals in China. The protocol was approved by the Committee on the Ethics of Animal Experiments of the First Norman Bethune Hospital of Jilin University, China ((2013) clinical trial (2013–121)). *Leptospira interrogans* serovar Autumnalis strain Lin 4 (56606) was used for infection. The strain was cultivated at 29 °C in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium. The hamsters were used to maintain the pathogenicity of leptospirosis.

In Vivo Experiment

All female mice were randomly divided into six groups ($n = 8$) as follows: the control group, the leptospirosis-infected group, the leptospirosis + dexamethasone group (5 mg/kg), the leptospirosis + emodin group (4 mg/kg), the leptospirosis + thymol group (20 mg/kg), and the leptospirosis + astragalin group (50 mg/kg). Emodin (purity $\geq 98\%$ HPLC), thymol (purity $\geq 98\%$ HPLC), and astragalin (purity $\geq 98\%$ HPLC) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Jilin, China). The model of leptospirosis-induced uterine inflammation in mice followed protocols established in a previous study in dogs, with some modifications [6]. Briefly, mice were pretreated with emodin, thymol, astragalin,

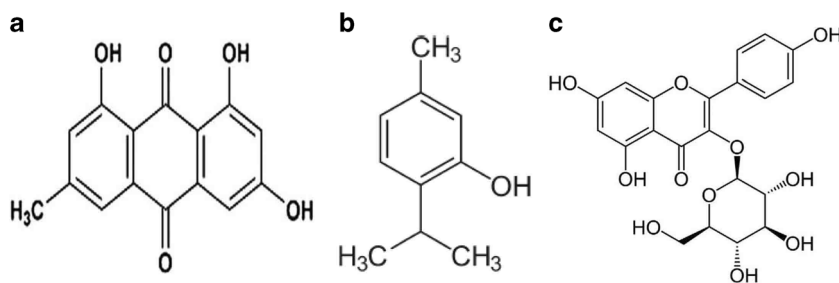


Fig. 1. Chemical structure of emodin (a), thymol (b), and astragalin (c).

and dexamethasone intraperitoneally for 1 h before intrauterine infusion with 10^8 heat-killed leptospire. Mice were retreated with the corresponding pre-treatment solution after 12 and 24 h. The dosage of parenteral solution was based on the results of a previous study [11, 15, 16]. The uteruses of the mice in each group were collected 48 h after leptospira infection.

In Vitro Experiment

The uterine epithelium of mice was isolated as previously described [17]. All cultures were maintained in 5% CO_2 at 37 °C. The cells were identified by Keratin-18 monoclonal antibody (Shanghai, China) and then seeded into 24-well dishes with 2×10^5 cells per well. After 24 h, cells were pretreated with emodin (40 $\mu\text{g/ml}$), thymol (40 $\mu\text{g/ml}$), and astragalol (100 $\mu\text{g/ml}$) for 1 h, followed by leptospira infection at an MOI of 100 leptospira per cell for 24 h. Subsequently, the cells and the supernatant were checked by ELISA, quantitative polymerase chain reaction (qPCR), and Western blot. Each *in vitro* experiment was repeated three times. The anti-inflammatory activities of these drugs (emodin, thymol, and astragalol) were improved in a dose-dependent manner in LPS-induced inflammation response in mouse mammary epithelial cells. We chose the most effective dose of these drugs (40 $\mu\text{g/mL}$ for emodin and thymol, 100 $\mu\text{g/mL}$ for astragalol) to investigate whether these drugs also applied to leptospira-induced inflammation response in endometrium epithelial cells.

CCK-8 Analysis for Cell Viability

A CCK-8 assay was performed as previously described [18] to measure the toxicity of emodin, thymol, or astragalol on uterine epithelium cells. Briefly, uterine epithelium cells were plated at a density of 1×10^4 cells/well in 96-well plates and incubated at 37 °C, 5% CO_2 for 4 h followed by treatment with emodin (40 $\mu\text{g/mL}$), thymol (40 $\mu\text{g/mL}$), and astragalol (100 $\mu\text{g/mL}$) for 25 h. Then, 10 μL of CCK-8 solutions (Dojindo Laboratories, Japan) was added to each well and incubated for 3 h in the dark. The optical density in the wells was measured at 450 nm. The cells cultured in medium alone and medium without cells served as negative controls.

Histological Analysis

Uterine thin sections were made from formalin-fixed paraffin-embedded tissues. The tissue sections

Table 1. The Primers of TNF- α , IL-1 β , and IL-6

Target name	Sequences (5' → 3')
TNF- α	Forward: 5'-TCCCAGACCCTCACACTCAGA-3' Reverse: 5'-TAGTCGGGGCCAGCCTTGT-3'
IL-1 β	Forward: 5'-TCCAGGATGAGGACATGAGCAC-3' Reverse: 5'-GAACGTACACACCAGCAGGTTA-3'
IL-6	Forward: 5'-CCACTTCACAAGTCGGAGGCTTA-3' Reverse: 5'-GCAAGTGCATCATCGTTGTTCATAC-3'
β -Actin	Forward: 5'-GACCCTGAAGTACCCCATTTG-3' Reverse: 5'-GGTCATCTTTTCACGGTTGG-3'

were dehydrated in graded alcohol and embedded in paraffin prior to staining with hematoxylin and eosin (H&E). The tissue sections were examined with a microscope (Olympus, Japan) at the magnification of $\times 400$.

Enzyme-Linked Immunosorbent Assays

Cells from five treatment groups were cultured for 18 h after leptospira infection. The culture supernatant was collected and tested for TNF- α , IL-1 β , and IL-6 with ELISA kits (Biolegend, USA) following the manufacturer's instructions. The levels of TNF- α , IL-1 β , and IL-6 in uterine tissues were also checked using ELISA. Each sample was analyzed in triplicate.

qPCR Assays

The total RNA from the uterine tissue samples and endometrium epithelial cells from each group were extracted using TRIzol reagent (Invitrogen, China) according to the manufacturer's instructions [19]. The

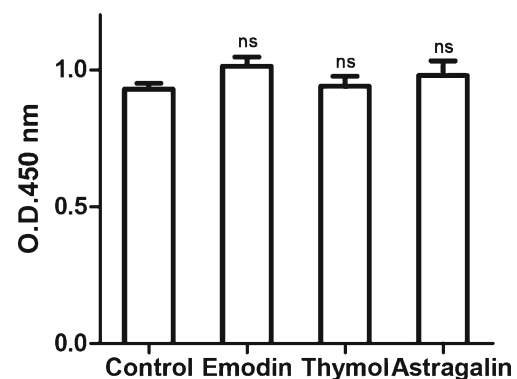


Fig. 2. Effects of emodin, thymol, and astragalol on cell viability. Cells were cultured with emodin (40 $\mu\text{g/mL}$), thymol (40 $\mu\text{g/mL}$), and astragalol (100 $\mu\text{g/mL}$) for 25 h. The cell viability was determined by a CCK-8 assay. Each column shows the mean \pm SEM of three independent experiments.

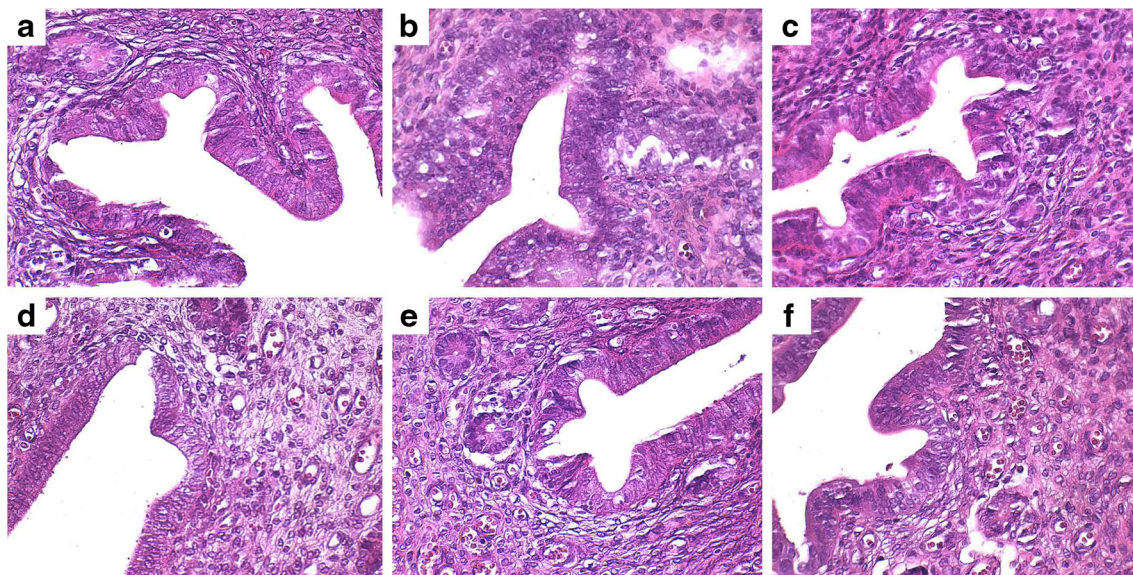


Fig. 3. Histopathology of uterine tissue infected with leptospira. The uteruses of the mice in each group ($n = 8$) were collected 48 h after leptospira infection. **a** Control group. **b** Leptospira group. **c** Leptospira + 5 mg/kg dexamethasone group. **d** Leptospira + 4 mg/kg emodin group. **e** Leptospira + 20 mg/kg thymol group. **f** Leptospira + 50 mg/kg astragalin group. Magnification, $\times 400$.

total RNA was reverse-transcribed into first-strand complementary DNA (cDNA) using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The primers used to amplify specific genes are listed in Table 1. Each sample was analyzed in triplicate using an ABI Prism™ 7500 Sequence Detector (Applied Biosystems, USA) and the SYBR Green Plus reagent kit (Roche Applied Science, Germany). The cycling parameters were an initial denaturation step at 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Using the $2^{-\Delta\Delta CT}$ method, the level of target gene expression was normalized to the housekeeping gene, β -actin.

Western Blot Analysis

The total protein was extracted from each group of the endometrium epithelial cells using the Mammalian Protein Extraction Reagent (Thermo Scientific, USA). The total protein concentration was determined by the bicinchoninic acid (BCA) method. The proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using Tris–HCl precast gels and then transferred onto a polyvinylidene difluoride

membrane. After blocking with 5% skim milk, and sequential incubation with the specific primary antibody and secondary antibody, the membrane was developed using the Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, USA).

Statistical Analysis

Statistical analyses were performed using the SPSS software package (SPSS Inc., USA). The significance was determined using Student's *t* test with a significance level of $p < 0.05$. All values are expressed as the mean \pm SEM.

RESULTS

Effect of Emodin, Thymol, and Astragalin on Cell Viability

The potential cytotoxicities of emodin (40 $\mu\text{g/mL}$), thymol (40 $\mu\text{g/mL}$), and astragalin (100 $\mu\text{g/mL}$) were evaluated by a CCK-8 assay. The data shows that there was no observable toxicity of any of the treatments on

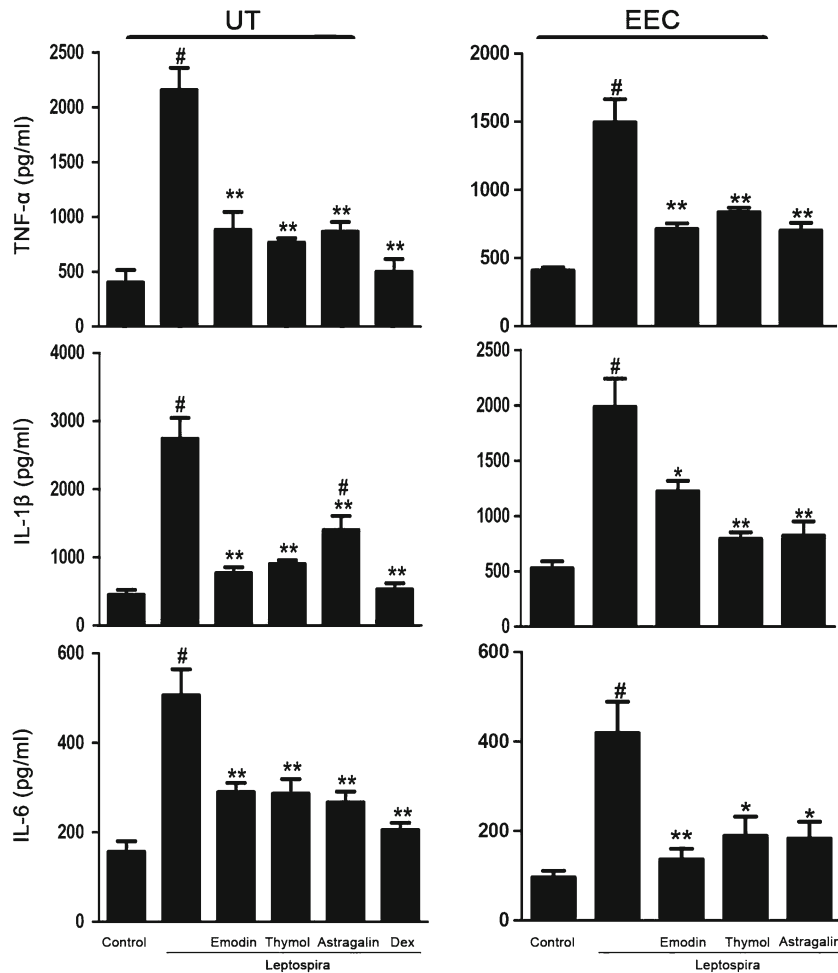


Fig. 4. Effects of emodin, thymol, and astragalol on the production of TNF- α , IL-1 β , and IL-6 in uterine tissues (UT) and endometrium epithelial cells (EEC) of mice. The protein levels of TNF- α , IL-1 β , and IL-6 in UT and EEC were examined by ELISA. The values presented are the means \pm SEM of three independent experiments. Differences between mean values were assessed by Student's *t* test. #*p* < 0.05 vs. control group; **p* < 0.05, ***p* < 0.01 vs. leptospira group.

uterine epithelium cells after incubating for 25 h (Fig. 2). Thus, in this study, the effects of these drugs can be attributed to the anti-inflammatory properties, and the mechanisms are credible and meaningful on uterine epithelium cells.

Histopathological Findings

The pathological changes of the uterine tissues were analyzed by staining with H&E. In the uninfected group, no other uterine pathological changes were found; the whole uterine muscle layer and stroma cells were regularly arranged (Fig. 3a). In contrast,

leptosira-infected group showed the irregular epithelial cells and lots of inflammatory cell infiltration in endometrial stroma (Fig. 3b). However, these histopathological changes were significantly attenuated by the administration of dexamethasone (Fig. 3c) or emodin, thymol, and astragalol (Fig. 3d-f).

Emodin, Thymol, and Astragalol Reduce the Release of Cytokines

Cytokines such as TNF- α , IL-1 β , and IL-6 play important roles in inflammation. To determine whether emodin, astragalol, and thymol have an effect on the

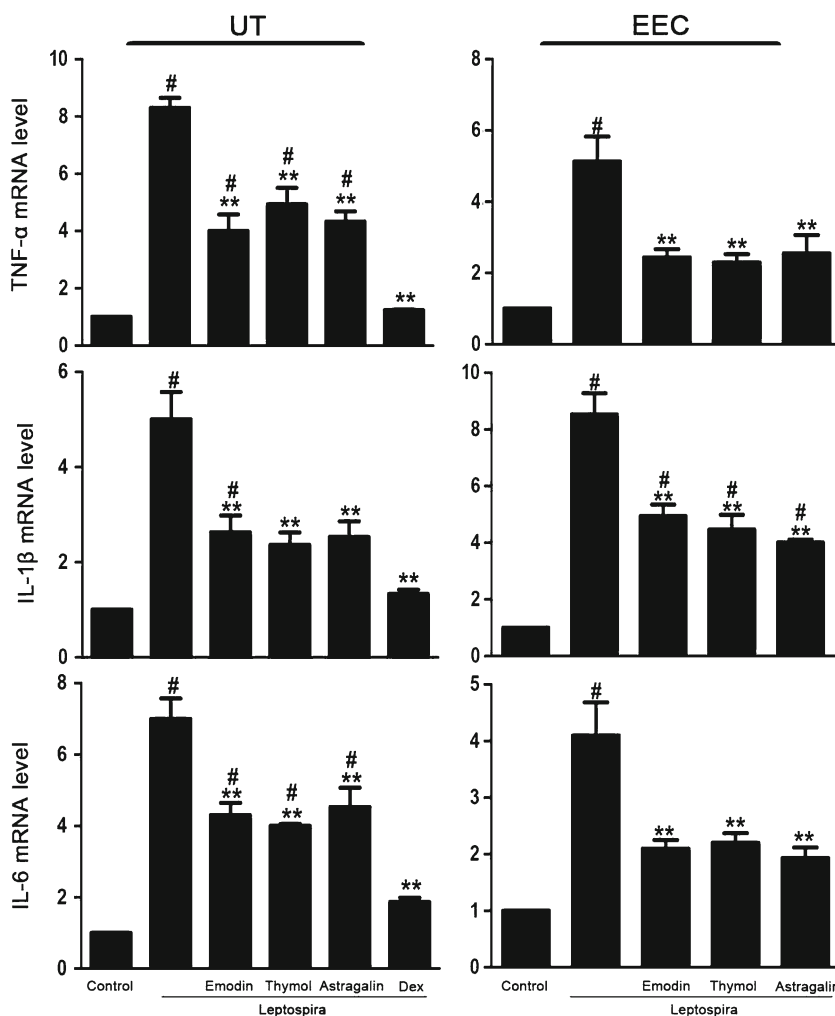


Fig. 5. Effects of emodin, thymol, and astragalol on the gene expression of TNF- α , IL-1 β , and IL-6 in uterine tissues (UT) and endometrium epithelial cells (EEC) of mice. The mRNA levels of TNF- α , IL-1 β , and IL-6 in UT and EEC were examined by qPCR. The results were normalized to the expression of the housekeeping gene β -actin. # $p < 0.05$ vs. control group; * $p < 0.05$, ** $p < 0.01$ vs. leptospira group.

expression of inflammatory cytokines in leptospira-infected endometrium epithelial cells, the level of TNF- α , IL-1 β , and IL-6 was measured using ELISA. The levels of TNF- α , IL-1 β , and IL-6 were higher in the untreated leptospira group compared to the control group (Fig. 4). We observed that the levels of all cytokines, in response to treatment with emodin, thymol, and astragalol, significantly declined compared with the untreated leptospira group in both the uterine tissue (UT) samples and the endometrium epithelial cells (EEC).

Emodin, Thymol, and Astragalol Decrease the Gene Expression of Cytokines

TNF- α , IL-1 β , and IL-6 messenger RNA (mRNA) levels in the uterine tissue samples and EEC were measured by RT-PCR. The mRNA levels of TNF- α , IL-1 β , and IL-6 in the untreated leptospira group were higher compared to the control group (Fig. 5). Dramatic reductions in the levels of the TNF- α , IL-1 β , and IL-6 mRNA were found in the groups treated with emodin, thymol, and astragalol compared with the untreated leptospira group.

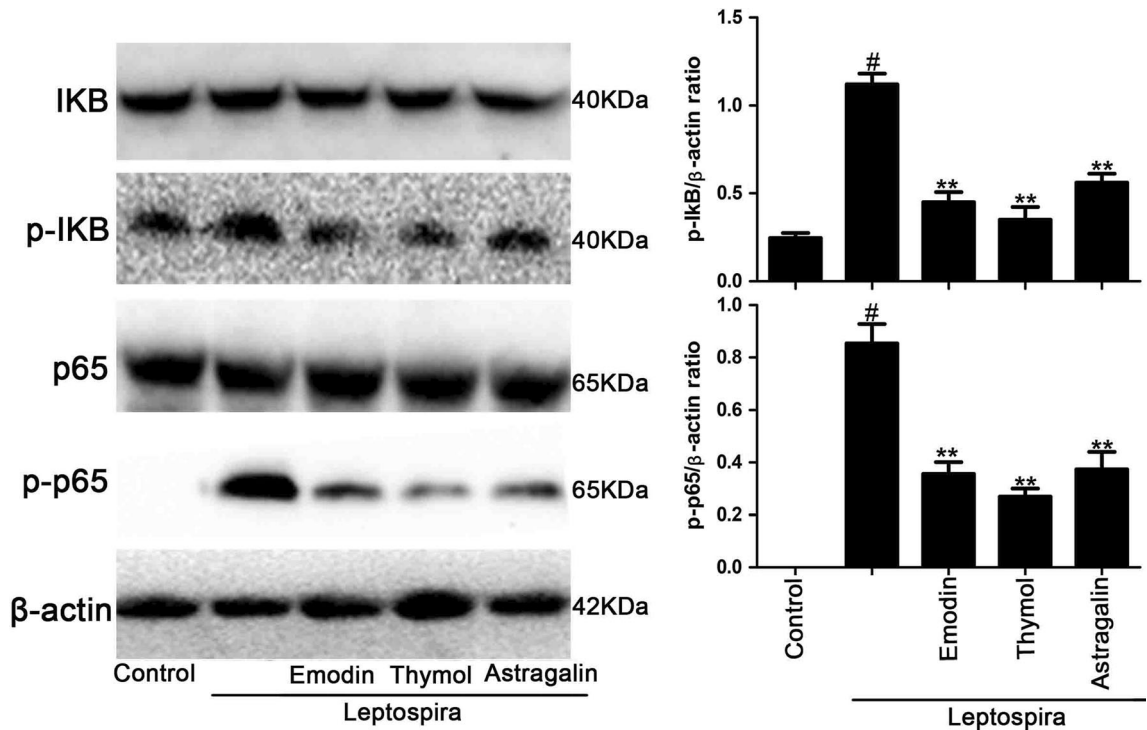


Fig. 6. Effects of emodin, thymol, and astragalalin on the NF- κ B signaling pathway. I κ B, p-I κ B, p65, and p-p65 proteins were measured by Western blot with specific antibodies. β -actin was included as a control. Each column shows the mean \pm SEM of three independent experiments. Differences between mean values were assessed by Student's *t* test. #*p* < 0.05 vs. control group; **p* < 0.05, ***p* < 0.01 vs. leptospira group.

These results were in accordance with the ELISA results (Figs. 4 and 5).

Effect of Emodin, Thymol, and Astragalalin on the Activation of NF- κ B and MAPK Signaling Pathways

The phosphorylation levels of the I κ B and p65 proteins of the NF- κ B pathway were higher in the leptospira-infected group relative to the control (Fig. 6). In contrast, the effects of emodin, astragalalin, and thymol treatment on the NF- κ B signaling pathway were all comparable, with reduced phosphorylation levels of I κ B and p65 compared to the untreated leptospira group (Fig. 6). In addition, emodin, thymol, and astragalalin also influenced the MAPK signal transduction pathway by inhibition of p38, extracellular signal-regulated kinase (ERK), and c-Jun NH2-terminal kinase (JNK) (Fig. 7). These results show that emodin, thymol, and astragalalin suppress the activation of NF- κ B and MAPK signaling induced by leptospira.

DISCUSSION

Reproductive failure, one of the main clinical symptoms of leptospirosis, causes systemic health problems in animals [20] and significant economic loss. Previous reports have shown that the balance of the uterine microenvironment is a prerequisite for pregnancy, which is closely associated with the regulation of cytokines [21, 22].

Typically, low levels of TNF- α function to maintain homeostasis and the estrous cycle of endometrial tissue [23]. Excessive expression of TNF- α causes adverse effects on the fetus and may even lead to spontaneous abortion [24]. IL-1 β can promote adhesion between human chorionic trophoblast cells and EEC, stimulate the expansion of trophoblasts, and stimulate embryo implantation [25]. Reduced IL-6 expression may hinder embryo implantation and is detrimental to pregnancy [26, 27]. In addition, IL-6 can bring about spontaneous abortion by regulating the differentiation

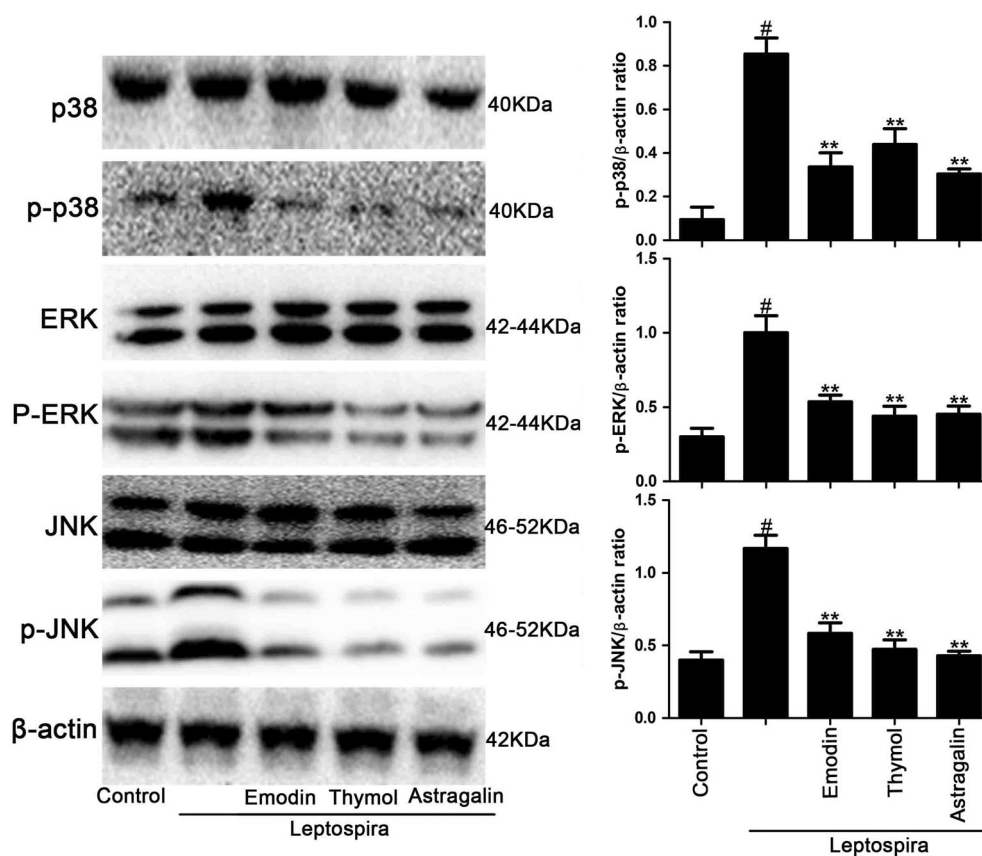


Fig. 7. Effects of emodin, thymol, and astragalin on the MAPK signaling pathway. p38, p-p38, ERK, p-ERK, JNK, and p-JNK proteins were measured by Western blot with specific antibodies. β -actin was included as a control. Each column shows the mean \pm SEM of three independent experiments. The differences between mean values were assessed by Student's *t* test. #*p* < 0.05 vs. control group; **p* < 0.05, ***p* < 0.01 vs. leptospira group.

of T helper 17 cells [28]. Some studies have also shown that excessive IL-1 β and IL-6 have a negative impact on pregnancy and post-partum maternal or offspring health [29].

The infection of the uterus by leptospira would likely be harmful to pregnancy, as increased levels of pro-inflammatory cytokines would destroy the balance of the uterine microenvironment [8, 30]. Therefore, the inhibition of the inflammatory cytokines may represent a novel treatment strategy for uterine inflammation resulting from leptospira infection.

Toll-like receptors (TLRs) acting as pattern recognition receptors (PRRs) can recognize the pathogen-associated molecular patterns (PAMPs) [31]. Leptospira can induce pro-inflammatory cytokines through both the TLR2- and TLR4-dependent c-Jun N-terminal kinase (JNK) and nuclear factor- κ B (NF- κ B) pathways [32]. It is reported that leptospira can activate the intracellular NLRP3 inflammasome through the downregulation of the

Na/K-ATPase pump [33]. Our results also showed that leptospira could induce inflammatory reaction by NF- κ B and MAPK signaling pathways in endometrium epithelial cells. Therefore, finding the drugs that inhibit these pathways may help treat leptospira-induced inflammatory response. Our previous study showed that using TLR2 agonist Pam3CSK4 improved IL-10 level and alleviates the pathology 1 of leptospirosis in hamster [34].

Our laboratory has previously confirmed the anti-inflammatory effect of Chinese herbs on mice. All the drugs, emodin, thymol, and astragalin, could ameliorate lipopolysaccharide-induced mastitis in mice [10, 11, 15], though their structures were dissimilar. In this report, we chose emodin, thymol, and astragalin to examine their effects on leptospira-induced inflammation in uterus. The experimental results showed that emodin, thymol, and astragaline can reduce the expression of TNF- α , IL-1 β , and IL-6 following leptospira infection by inhibiting NF-

κ B and MAPK signaling pathways. A possible explanation for the anti-inflammatory effects of these three traditional Chinese medicines is their regulation of the NF- κ B and MAPK signaling pathways by reducing p38, ERK, and JNK physiological levels. Based on the data presented, we believe that emodin, thymol, and astragalins could be potential drugs for uterine inflammation therapy and to rebalance the uterine microenvironment in cases of leptospirosis infection.

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COMPLIANCE WITH ETHICAL STANDARDS

All animal experiments followed the regulations for the Administration of Affairs Concerning Experimental Animals in China. The protocol was approved by the Committee on the Ethics of Animal Experiments of the First Norman Bethune Hospital of Jilin University, China ((2013) clinical trial (2013–121)).

Conflict of Interest. The authors declare that they have no conflict of interest.

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