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Original Research Article

In vivo anti-inflammatory activity of *Liquidambar formosana* Hance infructescence extract

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Abstract

Purpose: To evaluate the anti-inflammatory activity of *Liquidambar formosana* Hance infructescence (*Liquidambaris fructus*, ELF) in vivo, and clarify its underlying mechanisms.

Methods: The in vivo anti-inflammatory activity of ELF was examined by xylene-induced ear swelling test in mice as well as carrageenan-induced paw edema method in rats. The levels of inflammatory cytokines (TNF- α , IL-1 β , IL-6 and IL-10) in serum were measured by enzyme-linked immunosorbent assay (ELISA), while the expressions of COX-2, iNOS and NF- κ B p65 in paw tissue of rats were evaluated by western blot.

Results: After ELF treatment, the levels of TNF- α ($p < 0.001$), IL-1 β ($p < 0.001$) and IL-6 ($p < 0.001$) in serum decreased and the levels of anti-inflammatory cytokine IL-10 increased ($p < 0.01$). In addition, ELF treatment resulted in decrease of COX-2 ($p < 0.01$), iNOS ($p < 0.01$) and NF- κ B p65 ($p < 0.01$) expressions in Wistar rats.

Conclusion: The results reveal that ELF possesses significant anti-inflammatory effect in vivo. The anti-inflammatory activity is associated with the levels of TNF- α , IL-1 β , IL-6 and IL-10 in serum. Furthermore, the suppression of NF- κ B p65, iNOS and COX-2 is linked to its anti-inflammatory effect. These results provide a rationale for the use of *Liquidambaris fructus* in inflammatory disease in traditional medicine.

Keywords: Anti-inflammatory activity, *Liquidambaris fructus*, Cytokines, Ear swelling test, Paw edema

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INTRODUCTION

As is well-known that inflammation is part of the complex biological response of body tissues to harmful stimuli. The function of inflammation is to eliminate invading pathogens and to initiate healing process, but while it is uncontrolled, over production of inflammatory mediators can lead to cellular injury and may even result in several diseases [1, 2]. During inflammatory process, inflammatory mediators such as NO, PGE₂, TNF- α , IL-1 β , IL-6 and COX-2 are highly produced, all of which have been implicated in the pathogenesis of tissue injury [3].

Non-steroidal anti-inflammatory drugs (NSAIDs) are usually used for the treatment of inflammatory diseases. However, numerous side effects, such as gastrointestinal disorders, immunodeficiency and nephrotoxicity limited the wide use of these drugs [4]. Traditional Chinese medicine may be another choice for the treatment of inflammatory diseases.

Liquidambar formosana Hance belongs to the family Hamamelidaceae. The infructescence of *Liquidambar formosana* Hance, named *Liquidambaris fructus* (Lulutong in Chinese), has

long been used to treat rheumatism and inflammatory diseases in traditional Chinese medicine [5]. But so far, few reports on the anti-inflammatory activities of *Liquidambaris fructus* have been published.

In our study, we administered ELF *in vivo* and found that ELF has anti-inflammatory effect. This study may contribute to find the pharmacological basis of the use of *Liquidambaris fructus* in traditional Chinese medicine to treat rheumatism and inflammatory diseases.

EXPERIMENTAL

Animals

Male Balb/c mice (18 ~ 22 g weight) and Wistar rats (180 ~ 220 g weight) were purchased from the Center for Disease Prevention and Control in Hubei province, China (no. SCXK (Hubei) 2008-0005). Animals were housed in plastic cages, maintained at 22 ± 2 °C and 45 ~ 65 % humidity with alternating 12 h light-dark cycle and given free access to both food (standard rat chow) and water. They were bred for 1 week to adapt to the environment before the experiments.

All experiments were approved by the Committee on the Ethics of Animal Experiments of Wuhan First Hospital (no. WHYY14021) and followed the guidelines of 'Principles of Laboratory Animal Care' (World Health Organization (WHO) Chronicle, 1985) [6].

Plant materials

The infructescence of *Liquidambar formosana Hance* was purchased from Hubei Tianji Chinese Herbal Medicine Co., Ltd (China), authenticated by Prof Changgong Zhang (Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China) according to Chinese Pharmacopoeia [5]. A voucher specimen (no. *L. f* 2013-0621) have been deposited in Department of Pharmacy, Wuhan First Hospital, Wuhan, Hubei, PR China.

Chemicals

Carrageenan and indomethacin were purchased from Sigma Chemical Co. (USA). TNF- α , IL-1 β , IL-6, IL-10 ELISA kits were purchased from Neobioscience Technology Company (China). Antibodies of COX-2, iNOS and NF- κ B p65 were purchased from Boster Biotechnology Co., Ltd (China) and Beijing Biosynthesis Biotechnology Co., Ltd (China) respectively. All other chemicals were of analytical grade and purchased locally.

Preparation of the ELF

The air dried *Liquidambaris fructus* were shattered to a fine powder (2.5 kg) and extracted with 95 % ethanol (1: 3 w/v) for 1 week at room temperature three times. The filtrates were combined and concentrated under vacuum at 40 °C to afford 57.0 g of crude extract, which was then suspended in 2 L water and successively partitioned with ethyl acetate 3 times (2 L each). The ethyl acetate phase was dried under vacuum to yield dried powder (32.3 g). The dried powder was stored in a refrigerator at 4 °C.

Acute toxicity test

In the acute toxicity study of ELF, Balb/c mice were randomly divided into five groups (n = 6): the control group, the 0.250 g/kg ELF group, the 0.500 g/kg ELF group, the 1.000 g/kg ELF group and the 2.000 g/kg ELF group. The control group received normal saline orally. The rest groups were treated with 0.250, 0.500, 1.000 and 2.000 g/kg ELF by oral route. Mortality, general behavioral changes, neurological, signs of toxicity and hazardous symptoms of the mice were observed continuously for a period of 14 days [7].

Xylene-induced mice ear swelling

Xylene-induced mouse ear swelling test is a commonly used method to evaluate acute inflammation [8]. The mice were randomly divided into five groups (n = 8) and treated orally with distilled water (10 mL/kg), ELF (75, 150 and 300 mg/kg) or indomethacin (10 mg/kg) respectively 30 minutes before induction of swelling by application of xylene (0.02 mL) to the inner surface of the left ear. 30 minutes later, the animals were sacrificed by cervical dislocation. Round pieces (diameter of 8 mm) of both ears of the mice were cut off and their weights were measured. Quality difference between the left and right ear of each animal was calculated and inhibition ratio (H) was calculated as in Eq 1 [9].

$$H (\%) = \{(V_c - V_t / V_c)\} 100 \dots \dots \dots (1)$$

where V_c represents the weight difference between right and left ear in control groups and V_t represents the weight difference between right and left ear in indomethacin or ELF treated groups.

Carrageenan-induced rat paw edema assay

The *in vivo* anti-inflammatory activity of ELF was evaluated by the carrageenan-induced paw edema test in male Wistar rats (180 ~ 220 g).

The animals were randomly divided into six groups (n = 8). Edema was induced by injection of 100 μ L carrageenan (0.1 % in sterile saline) into the sub-plantar tissue of the right hind paw while the control group received the same volume of saline [10]. 60 minutes before carrageenan injection, the animals were treated with indomethacin (10 mg/kg), ELF (50, 100 and 200 mg/kg) or normal amount of saline by oral route. Paw thickness was measured at 0, 1, 2, 3, 4, 5 hours after carrageenan injection using a caliper ruler and inhibition ratio was calculated as in Eq 2. Then the rats were sacrificed after narcotized, and the blood were collected via cardiac puncture. The serum was obtained after centrifuged at 5000 rpm for 5 min and then stored at - 20 °C for TNF- α , IL-6, IL-1 β and IL-10 assay. The carrageenan induced paws were dissected, followed by stored at - 80 °C. Then the tissue lysate was prepared in RIPA for the western blot analysis of COX-2, iNOS, NF- κ B p65. The tissues were also fixed in 10 % buffered formalin for histological assessment.

$$\text{Inhibition (\%)} = \{(V_c - V_t / V_c)\} 100 \dots\dots\dots (2)$$

where V_c represents mean edema volume in the control group and V_t represents mean edema volume in groups treated with indomethacin or ELF.

Measurement of serum TNF- α , IL-6, IL-1 β and IL-10 by ELISA

The levels of TNF- α , IL-6, IL-1 β and IL-10 in serum were measured by ELISA kits according to the manufacturers' instructions.

Histological assessment

The paw tissues were fixed in 10 % buffered formalin, dehydrated in a gradient ethanol series (70 ~ 100 %), and then embedded in paraffin. Thereafter, tissues were cut into 5 μ m sections, and then mounted on clean glass slides and dried overnight at 37 °C. Sections were cleared, hydrated, and stained with haematoxylin and eosin (HE) according to the manufacturers' instructions. Then the histopathological changes in all tissue sections were examined with an inverted biological microscope (XSP-18CE, Changfang, China).

Western blot analysis of COX-2, iNOS and NF- κ B p65

The paw tissues were homogenized in PBS buffer (pH = 7.2) and centrifuged at 5000 rpm for 5 min to obtain supernatant (stored at - 20 °C). Total protein was extracted with a RIPA solution

(radio immunoprecipitation assay buffer) at - 20 °C overnight. Protein from the supernatant was resolved by 12 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the protein was transferred onto polyvinylidene fluoride membranes. After blocked with 5 % non-fat dried milk-TBST buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl and 1 % (v/v) Tween 20), the membranes were incubated with primary antibodies overnight at 4 °C. Then the membranes were washed three times with TBST buffer at room temperature. After washing, the membranes were incubated with a 1: 2000 dilution of horseradish peroxidase conjugated anti-mouse IgG secondary antibody for 1 h at room temperature. After washing three times, the membranes were used for protein detection by enhanced chemiluminescent assay. The results were quantified by Molecular Imaging Software (Offic 600F, EPSON, Japan; Quantity One 4.62, BIO-RAD, USA).

Statistical analysis

All data are expressed as mean \pm SEM. Statistical significance was assessed by one-way analysis of variance (ANOVA), followed by Dunnet's t-test or Student-Newman-Kuals test. $P < 0.05$ was considered statistically significant. The analysis was performed using SPSS software, version 18.0.

RESULTS

Acute toxicity of ELF

None of the mice showed mortality or overt signs of toxicity after 14 days' treatment. The results suggest that ELF has a low toxicity profile.

Effects of ELF on Xylene-induced ear swelling

Ear swelling was obvious at 30 min after wipe of xylene. The data described in Table 1 showed that ELF suppressed xylene-induced ear swelling in mice with inhibition rate of 60.67 % and 63.56 % at the doses of 150 and 300 mg/kg respectively. Indomethacin (10 mg/kg) also showed significant inhibitory effect.

Effect of ELF on carrageenan-induced rats paw edema

The inflammatory response of carrageenan-induced paw edema test was quantified by increment in paw thickness 0, 1, 2, 3, 4 and 5 h after carrageenan injection. ELF exhibited a dose-dependent suppression of carrageenan-induced paw edema. The most obvious inhibition

activity was exhibited at doses of 200 mg/kg (Figure 1) with the inhibition rate of 50.8 % while the inhibition rate of indomethacin was 56.4 %.

Table 1: Effect of ELF on xylene-induced ear swelling in mice

Group	Dose (mg/kg)	Ear swelling (mg)	Inhibition (%)
Control	1% CMC-Na	6.23 ± 0.63	
ELF	75	4.02 ± 0.52*	35.47
	150	3.53 ± 0.47**	43.34
	300	2.45 ± 0.39***	60.67
Indo	10	2.27 ± 0.40***	63.56

Data are presented as mean ± SEM (n = 8). *p < 0.05, **p < 0.01, ***p < 0.001, compared with the control group

Effect of ELF on serum levels of TNF- α , IL-6, IL-1 β and IL-10

Pro-inflammatory cytokines TNF- α , IL-6, IL-1 β and anti-inflammatory cytokine IL-10 have central roles in the process of inflammation. Therefore, levels of TNF- α , IL-6, IL-1 β and IL-10 in serum after treatment with ELF were detected by ELISA. As shown in Figure 2, the levels of TNF- α , IL-6, IL-1 β in serum were decreased while the level of IL-10 was increased after ELF treatment in a dose-dependent manner. The results suggested that obvious inflammation response was induced in the model group (carrageenan treated group) and ELF could regulate the release of inflammatory mediators and therefore relieve the inflammatory response in carrageenan-induced rats.

Effect of ELF on histopathological changes

Immune cellular infiltration was obviously observed in the paw tissue in the model group. On the contrary, rats treated with ELF and indomethacin showed a reduction in

inflammatory responses and slightly inflammatory cell infiltration (Figure 3), which demonstrated that administration of ELF directly reduce the inflammation in disease.

Effect of ELF on COX-2, iNOS and NF- κ B p65 expressions in paw tissue

The expression levels of iNOS, COX-2 and NF- κ B p65 in the paw tissue of carrageenan-induced rats were measured by western blot analysis. The results (Figure 4) showed that expressions of COX-2 and NF- κ B p65 in the model group were significantly increased compared to the control group, while the expression levels in ELF groups decreased with a dose-dependent manner compared to the model group. Meanwhile, the expression level of iNOS was increased in the model group, and ELF could decrease the expression of iNOS.

DISCUSSION

Many plants used in traditional Chinese medicine exhibit different pharmacological properties, thus they may offer potential as therapeutic agents. However, lack of scientific evidence of their mechanism of action limited the wide use of traditional Chinese medicines. The infructescence of *Liquidambar formosana Hance* recorded in Chinese pharmacopoeia, has been used for the treatment of various diseases such as rheumatism and inflammatory diseases [5].

Thus, in the present study we administrated ELF *in vivo*, and testify their potential anti-inflammatory activity by xylene-induced ear swelling test and carrageenan induced rat paw edema method. The results suggested that ELF can significantly suppress the formation of xylene-induced ear swelling.

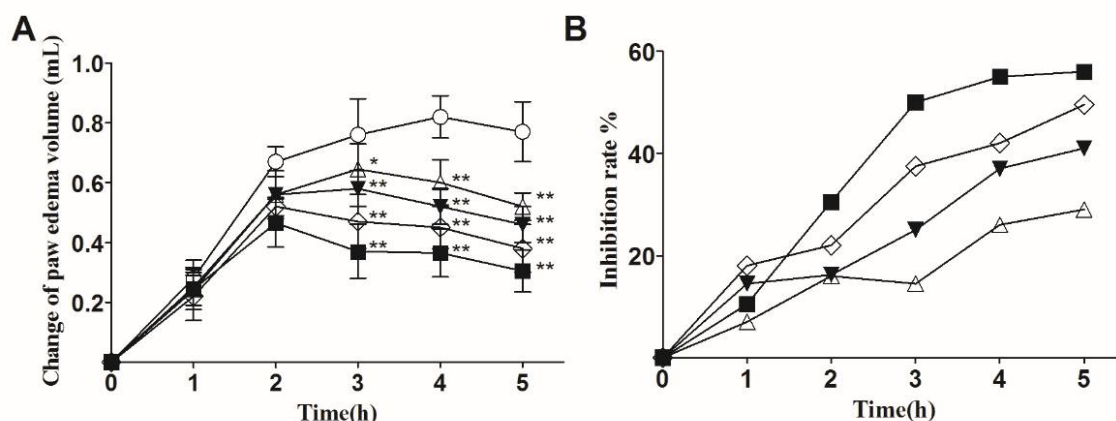


Figure 1: Effect of ELF and indomethacin on carrageenan-induced paw edema in rats. (A) change of paw edema; (B) edema inhibition rate. Data are presented as mean ± SEM (n = 8). *p < 0.05, **p < 0.01. ○: model group, ■: indomethacin group, △: 50 mg/kg ELF group, ▼: 100 mg/kg ELF group, ◇: 200 mg/kg ELF group

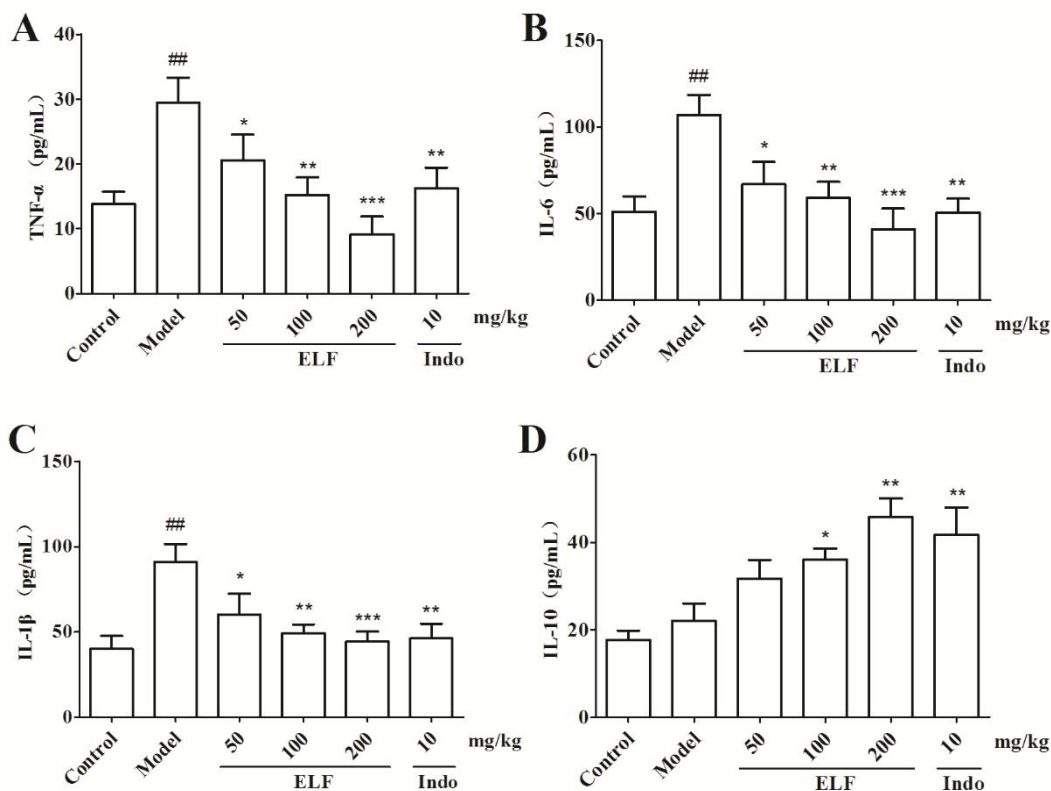


Figure 2: Effect of ELF on TNF- α (A), IL-6 (B), IL-1 β (C) and IL-10 (D) production in serum of carrageenan-induced rats. The animals were treated with different concentrations (50, 100, 200 mg/kg) of ELF followed by stimulation with 100 μ L carrageenan (0.1 % in saline). The values are presented as mean \pm SEM (n = 8); ^{##}p < 0.01 compared with control group; ^{*}p < 0.05, ^{**}p < 0.01 and ^{***}p < 0.001 compared with model group

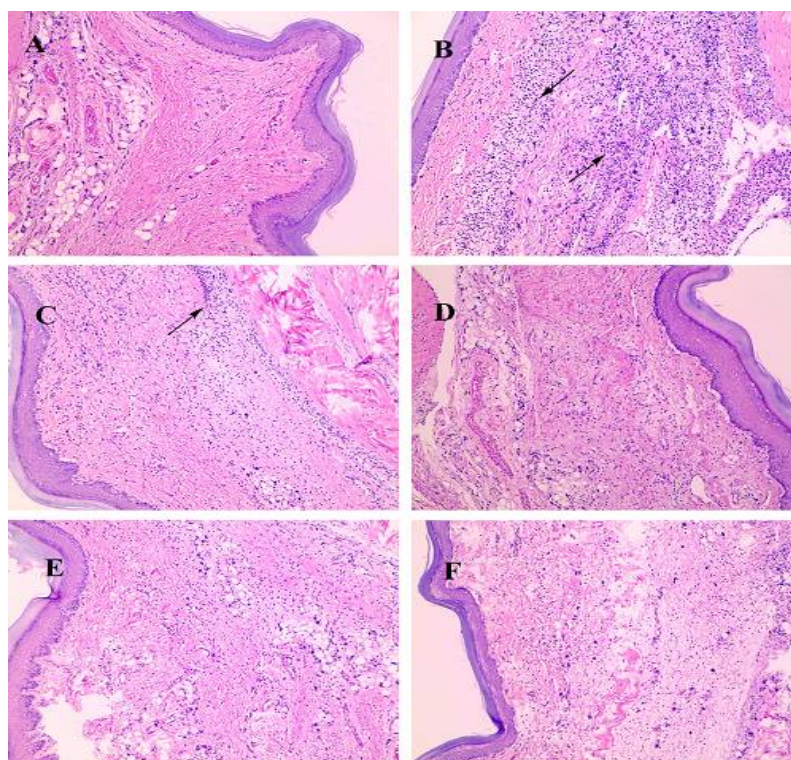


Figure 3: Histological appearance of the paws 5 h after injection of carrageenan (100 \times). Heavy infiltration of inflammatory cells can be observed. (A): control group, (B): model group, (C): 50 mg/kg ELF group, (D): 100 mg/kg ELF group, (E): 200 mg/kg ELF group, (F): 10 mg/kg indomethacin group

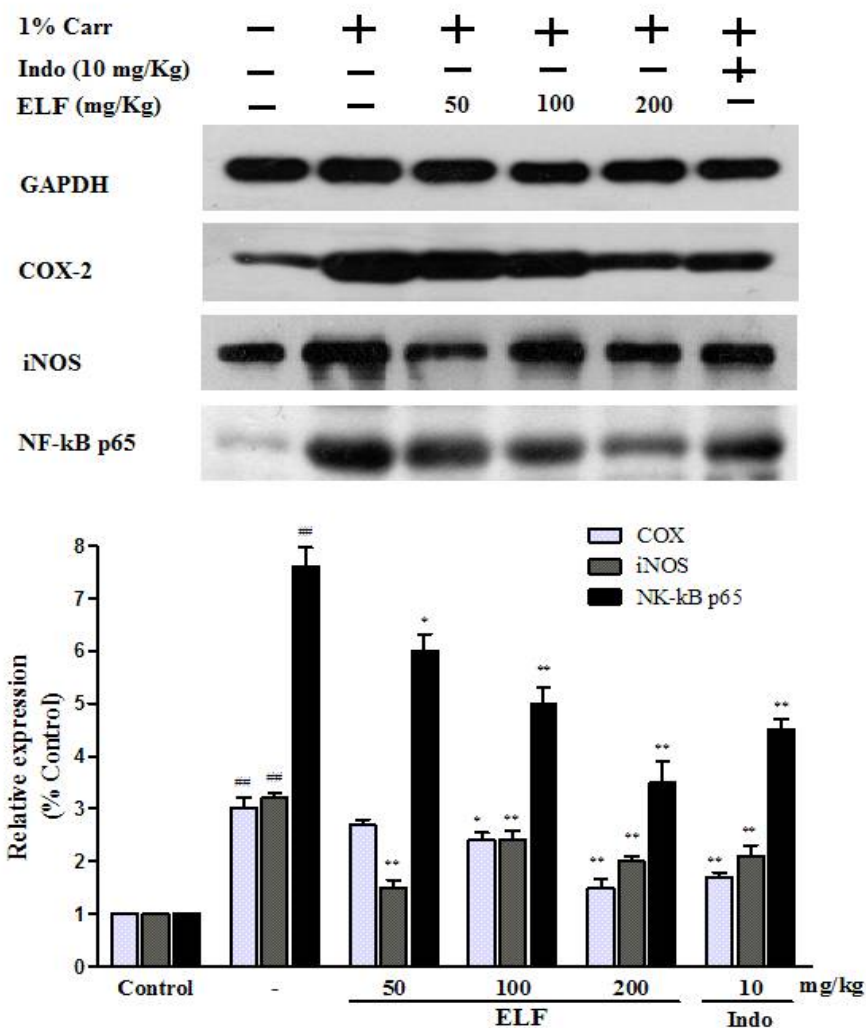


Figure 4: Effect of ELF on the expressions of iNOS, COX-2 and NF-kB p65 in carrageenan-induced paw tissue. The values are presented as means \pm SEM (n = 8); ^{##} $p < 0.001$ compared with the control group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ compared with the model group

The inhibition effect of 300 mg/kg ELF was comparable to that of 10 mg/kg indomethacin. In the carrageenan-induced paw edema experiment, ELF at doses of 100 and 200 mg/kg showed a long-lasting effect of inhibition of swelling during 5 hours. The immune cell infiltration was significantly reduced in the paw tissue of the mice treated with ELF. The results indicated that ELF exhibited significant anti-inflammatory activity. The anti-inflammatory activity is possibly associated with the changes of multiple inflammatory chemical mediators [11,12].

A complex cytokine network is involved in inflammation process, and this network is comprised of positive and negative feedback loops that enhance or suppress the inflammatory response [13]. During the process of inflammation, the release of large amounts of the pro-inflammatory cytokines, such as IL-1 β , IL-6 and TNF- α , could lead to the aggravation of

inflammatory diseases [14, 15]. On the contrary, anti-inflammatory cytokines such as IL-10 have been shown to inhibit expressions of pro-inflammatory cytokines [16]. Previous reports have demonstrated that IL-10 could suppress the inflammatory cytokines IL-1, TNF- α , IL-6, IL-8, and IL-12, as well as inhibit the synthesis of nitric oxide, gelatinase, and collagenase [17]. Therefore, increased IL-10 and inhibition of pro-inflammatory cytokines have been proposed to be a useful approach to treat inflammatory diseases [18]. In the present study, the anti-inflammatory effect of ELF in the carrageenan-induced paw edema experiment appears to result from the regulation of key cytokines in inflammatory pathogenesis, such as decreasing the levels of TNF- α , IL-6, IL-1 β and increasing the level of IL-10 in serum.

NF- κ B is a critical factor for the expression of various pro-inflammatory cytokines, such as TNF- α , IL-6 and IL-1 β [19]. Suppression of NF-

κB can lead to the reduction of pro-inflammatory cytokines, thus modulate the inflammatory process. Inflammation mediator NO is produced by iNOS and involved in many biological functions. Overexpression of iNOS is associated with inflammatory responses and serious disorders such as septic shock and rheumatoid arthritis [20]. Our study showed that the levels of COX-2, iNOS and NF-κB p65 expressions were significantly decreased in ELF and indomethacin treated groups. Moreover, the expressions of NF-κB p65 and COX-2 were down-regulated in a dose-dependent manner.

CONCLUSION

The findings indicate that ELF exhibits anti-inflammatory activity in xylene-induced mice ear swelling test and carrageenan-induced rat paw edema experiment, and ELF can regulate the levels of TNF-α, IL-1β, IL-6 and IL-10 in serum of carrageenan treated rats. The anti-inflammatory effect is associated with suppression of NF-κB p65, iNOS and COX-2. These results may suggest that ELF may be useful in the treatment to treat inflammatory disease in future.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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