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Eupatorium lindleyanum DC. flavonoids fraction attenuates lipopolysaccharide-induced acute lung injury in mice

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ABSTRACT

Eupatorium lindleyanum DC., "Ye-Ma-Zhui" called by local residents in China, showed anti-inflammatory activity and is used to treat tracheitis. We had isolated and identified the flavonoids, diterpenoids and sesquiterpenes compounds from the herb. In the present study, we evaluated the protective effects of the flavonoids fraction of *E. lindleyanum* (EUP-FLA) on lipopolysaccharide (LPS)-induced acute lung injury (ALI) in mice and the possible underlying mechanisms of action. EUP-FLA could significantly decrease lung wet-to-dry weight (W/D) ratio, nitric oxide (NO) and protein concentration in BALF, lower myeloperoxidase (MPO) activity, increase superoxide dismutase (SOD) activity and down-regulate the levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β). Additionally, EUP-FLA attenuated lung histopathological changes and significantly reduced complement deposition with decreasing the levels of Complement 3 (C3) and Complement 3c (C3c) in serum. These results demonstrated that EUP-FLA may attenuate LPS-induced ALI *via* reducing productions of pro-inflammatory mediators, decreasing the level of complement and affecting the NO, SOD and MPO activity.

1. Introduction

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS) continue to be the major cause of mortality in adult and pediatric critical care medicine. In the early presentation, the histology demonstrates mark diffuse alveolar damage, accumulation of neutrophils, vasodilation, endothelial cell damage, and pulmonary edema, secondary to increase vascular permeability [1]. The lung injury is associated with a systematic inflammatory response. Complement activation also contributes an important facet to the pathogenesis of ALI/ARDS. C3 is involved with lung injury and inhibition of complement activation might be a potential therapeutic strategy [2–6]. Elevated levels of C3a and C5a have been shown in patients at risk for ARDS [7]. Moreover, complement system and anaphylatoxin C5a are associated with full production of TNF-α and neutrophil aggregation [8].

Eupatorium lindleyanum DC., called "Ye-Ma-Zhui" by local residents, is the authentic herb of Jiangsu Province in China. The whole plant showed anti-inflammatory activity, which is used for the treatment of cough and tracheitis [9]. Ye-Ma-Zhui Syrup produced by Jiangsu Ange Pharmaceutical Corporation is used for the treatment of chronic bronchitis, cough and phlegm. In previous studies, sesquiterpenes, triterpenoids and flavonoids were isolated from this plant [10]. *Eupatorium lindleyanum* had protective effect on acute lung injury in rat induced by intravenous injection of oleic acid [11].

In our studies, the ethanol extract (EUP-EtOH) [12], the trichloromethane extract and the ethyl acetate extract of Ye-Ma-Zhui could attenuate LPS-induced ALI. The main compounds of trichloromethane extract were the flavonoids and diterpenoids analyzed by TLC. The main compounds of ethyl acetate extract were the sesquiterpenes and flavonoids analyzed by HPLC. Moreover we isolated and identified the flavonoids [13], diterpenoids [14] and sesquiterpenes [15] compounds, such as eupatrin, jaceosidin, nepetin, eupalinolide F, eupalinolide G and 3-(hydroxymethyl)-1, 13, 15-trihydroxy-7, 11, 15-trimethyl-2, 6, 10-hexadecatrien-14-yl acetate. We prepared the flavonoids (EUP-FLA), diterpenoids (EUP-DIT) and sesquiterpenes (EUP-SQT) fraction, respectively. And we found EUP-FLA and EUP-SQT could attenuate LPS-induced ALI, EUP-DIT had not protective effect on LPS-induced ALI (Table 1 in Supplementary material), we presumed that EUP-FLA or EUP-SQT were the effective substances of EUP attenuating LPS-induced ALI.

In this paper we investigated protective effects of the flavonoids fraction of *Eupatorium lindleyanum* DC. (EUP-FLA) on ALI induced by LPS in mice and tried to find its possible mechanism.

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2. Materials and methods

2.1. Plant materials and preparation of EUP-FLA

Eupatorium lindleyanum DC. was purchased from Wangdian, Xuyi, Jiangsu Province of China, in October of 2012. The plant material was authenticated by Prof. Daofeng Chen, Department of Pharmacognosy, School of Pharmacy, Fudan University, Shanghai, China, and the voucher specimen (NO. DFC-YMZ2012101201) was deposited at the Herbarium of Materia Medica, Fudan University, Shanghai, China. The extraction and purification of EUP-FLA were carried out according to our preliminary experiments. The dried leaves and stems of *Eupatorium lindleyanum* DC. (350 g) were extracted under reflux with 80% EtOH (3500 ml) for 2 h, repeated twice. After filtration, the combined 80% EtOH extracts were evaporated to dryness under vacuum at 60 °C. The extracts were treated by petroleum ether and AB-8 macroreticular resin. The treated-extracts were segmented by a column loading-treated ODS (Cosmsil, Japan), and then eluted with 30% MeOH, 50% MeOH and 70% MeOH. 70% MeOH were treated by a column loading-treated polyamide (Taizhou Luqiao Sijia biochemical plastic factory, Zhejiang, China), and then eluted with 70% MeOH. EUP-FLA in the column was eluted with 90% MeOH, and the eluting solution was evaporated to dryness at 60 °C (0.384 g).

2.2. High performance liquid chromatography (HPLC) analysis of EUP-FLA

An Agilent (America) HPLC system, consisting of a binary pump, an online degasser and a diode array detector (DAD) was used to determine the contents of nepetin and jaceosidin. For chromatographic analysis, a YMC-PACK ODS-A (Φ4.6 mm × 250 mm, 5 μm) column was used at room temperature. The mobile phase was a mixture of acetonitrile (A) and water (B), both containing 0.1% formic acid, using a gradient elution (0 min: 36% A, 15 min: 36% A, 30 min: 90% A, 65 min: 90% A); the flow rate was 1.0 ml/min; the wavelength was 365 nm. All solutions were filtered through a membrane filter (0.45 μm) prior to HPLC analysis, and the injection volume was 20 μl. The retention times of nepetin and jaceosidin were 7.442 min and 13.179 min, respectively. The chromatographic peaks were identified by comparing their retention times and UV spectra with those of the reference standards. Two experimental points were employed for establishing a calibration curve. The regression lines for nepetin and jaceosidin were $y = 52{,}964x + 19.304$ ($r^2 = 0.9994$) and $y = 55{,}161x + 210.79$ ($r^2 = 0.9998$), respectively, where y is the peak area of analyte, and x is the injection quantity of analyte.

2.3. Anti-complementary activity through the classical pathway

Based on Mayer's modified method [16], sensitized erythrocytes (EAs) were prepared by incubation of 2% sheep erythrocytes (4.0×10^8 cells/ml) with rabbit anti-sheep erythrocyte antibody (1:1000) in VBS^{2+} (containing 0.5 mM Mg^{2+} and 0.15 mM Ca^{2+}). Samples were dissolved in VBS^{2+}. Guinea pig serum was used as the complement source. The 1:60 diluted guinea pig serum was chosen to give submaximal lysis in the absence of complement inhibitors. In brief, various dilutions of tested samples (200 μl) were mixed with 200 μl of Guinea pig serum, and 200 μl of EAs was added, then the mixture was incubated at 37 °C for 30 min. The different assay controls were incubated in the same conditions: (1) vehicle control: 200 μl EAs in 400 μl VBS^{2+}; (2) 100% lysis: 200 μl EAs in 400 μl ultrapure water; (3) samples background: 200 μl dilution of each sample in 400 μl VBS^{2+}. The reacted mixture was centrifuged immediately at 4 °C after incubation. Optical density of the supernatant was measured at 405 nm on well scan (Labsystems Dragon). Results were indicated in percentage of hemolytic inhibition. Inhibition of lysis (%) = $100–100 \times (OD_{sample} - OD_{sample\ background}) \div OD_{100\%\ lysis}$.

2.4. Anti- complementary activity through the alternative pathway

According to the method of Klerx [17], each sample was dissolved in EGTA-VB, and various dilutions of sample were made. Each sample (150 μl) was mixed with 1:8 diluted NHS (150 μl), then 200 μl rabbit erythrocytes (ERs 1.0 × 108 cells/ml) was added. The mixture was incubated at 37 °C for 30 min. Cell lysis was determined as described in the section on anti-complementary activity through the alternative pathway.

2.5. Animals

Male Balb/c mice, about 20 – 22 g, were purchased from the Center of Experimental Animals Soochow University (Suzhou, Jiangsu, China). The mice were kept in a specific pathogen free condition and received food and water *ad libitum*. Laboratory temperature was 24 ± 1 °C, and relative humidity was 40 – 80%. Before experimentation, the mice were allowed to adapt to the experimental environment for a minimum of 3 days. The experimental protocols shown in this study were approved by the Animal Ethical Committee of School of Pharmacy at Soochow University.

2.6. Reagents

LPS (*Escherichia coli* 055:B5) was purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). Dexamethasone (DXM) acetate tablets (No. H33020822) were purchased from Zhejiang Xianju Pharmaceutical Co., Ltd. (Hangzhou, Zhejiang, China). Mouse TNF-α, IL-6 and IL-1β ELISA kits (No. B1007233) were purchased from Shanghai Chuanfu Biotechnology Co., Ltd. (Shanghai, China). Mouse C3 and C3c ELISA kits (No. 20131001A) were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). NO and bicinchoninic acid (BCA) protein assay kit, MPO and SOD determination kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Polyclonal rabbit anti-human C3c complement (RS-0367R) was purchased from Shanghai Ruiqi Biological Technology Co., Ltd. (Shanghai, China). Sheep erythrocytes were collected in Alsevers' solution. Anti-sheep erythrocyte antibody was obtained from rabbit antiserum and kindly provided by Prof. Yunyi Zhang (Department of Pharmacology, School of Pharmacy, Fudan University, Shanghai, China). Rabbit erythrocytes were obtained from the ear vein of New Zealand white rabbits. Normal human serum (NHS) was obtained from healthy male donors (average age of 20 years). The isotonic veronal-buffered saline (VBS^{2+}) buffer contained 0.5 mmol/l Mg^{2+} and 0.15 mmol/l Ca^{2+}. The veronal buffer saline (EGTA-VB) buffer contained 5 mmol/l Mg^{2+} and 8 mmol/l EGTA. Heparin (sodium salt, 160 IU/mg) was purchased from Shanghai Aizite Biotech Co. Ltd. The solvents, acetonitrile and methanol were of HPLC grade from E. Merck (Darmstadt, Germany). Water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). AB-8 macroporous adsorption resins were purchased from Baoen Adsorption-material Technology Co., Ltd. (Cangzhou, Hebei, China). The purities of nepetin and jaceosidin were above 98%. All other reagents were of the highest quality available.

2.7. Establishment of the ALI model and preventive regimen [12,18–21]

EUP-FLA was ground and suspended in distilled water containing 0.5% sodium carboxymethyl cellulose (CMC-Na) for administration to mice.

The mice were randomly divided into seven groups (each group, $n = 20$): control group, negative control group (EUP-FLA group, the animals were treated only with EUP-FLA at 40 mg/kg), positive control group (LPS group, the animals were treated only with LPS at 2 mg/kg), EUP-FLA-pretreated groups (LPS + EUP-FLA group, the animals were treated with EUP-FLA at 10, 20 and 40 mg/kg and then treated by LPS, respectively) and reference drug control group (LPS + DXM group,