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

Chunjun Chu^{a,1}, Shi Yao^{a,1}, Jinglei Chen^a, Xiaochen Wei^a, Long Xia^a, Daofeng Chen^{b,*}, Jian Zhang^{a,*}

^aCollege of Pharmaceutical Science, Soochow University, Suzhou 215123, P. R. China

^bDepartment of Pharmacognosy, School of Pharmacy, Fudan University, Shanghai 201203, P. R. China

*Corresponding author. E-mail: jianzhang@suda.edu.cn (J. Zhang)

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-1 β (IL-1 β). 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.

Keywords:

Eupatorium lindleyanum DC.
Flavonoids fraction
Lipopolysaccharide
Acute lung injury
Complement
Pro-inflammatory mediators

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.

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 macroporous resin. The treated-extracts were segmented by a column loading-treated ODS (Cosmosil, 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 ($\Phi 4.6 \text{ mm} \times 250 \text{ mm}$, $5 \mu\text{m}$) 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 ($0.45 \mu\text{m}$) 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 $y = 52964x + 19.304$ ($r^2 = 0.9994$) and $y = 55161x + 210.79$ ($r^2 = 0.9998$), 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 VBS²⁺ (containing 0.5 mM Mg^{2+} and 0.15 mM Ca^{2+}). Samples were dissolved in VBS²⁺. 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 VBS²⁺; (2) 100% lysis: 200 μl EAs in 400 μl ultrapure water; (3) samples background: 200 μl dilution of each sample in 400 μl VBS²⁺. 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 (%) = $100 - 100 \times (\text{OD}_{\text{sample}} - \text{OD}_{\text{sample background}}) \div \text{OD}_{100\% \text{ lysis}}$.

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 1.0×10^8 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-1 β 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 (VBS²⁺) buffer contained 0.5 mmol/l Mg²⁺ and 0.15 mmol/l Ca²⁺. The veronal buffer saline (EGTA-VB) buffer contained 5 mmol/l Mg²⁺ 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, the animals were treated with DXM at 5 mg/kg and then treated by LPS). The doses of these drugs we chosen were on the basis of our preliminary experiments.

The negative control group and EUP-FLA-pretreated groups received an intragastric injection of EUP-FLA at given doses, each mouse was administered orally once per day for 7 days consecutively. The reference drug control group received DXM only on day 7. Mice from control groups and positive groups received the equal volume distilled water. On day 7, 2 h after EUP-FLA and DXM treatment, mice were slightly anesthetized with a 20% urethane (4 ml/kg) given intraperitoneally. Then, in experimental groups 2 mg/kg LPS was instilled intratracheally (i.t.) in 50 μ l NS to induce lung injury [12, 18-21]. Mice from control group and negative control group were only given a 50 μ l NS. It took about 5 ~ 6 min per mice to induce lung injury. Animals recovered quickly from the procedure with only mild discomfort. The mice charged by LPS depressed, breathed heavily and lost appetite. After charged by LPS the mice drank no

water during four hours, and drank less water gradually.

6 h after LPS challenge, half of the each group mice ($n = 10$) were sacrificed and the blood samples were collected (each one was approximately 1 ml). The right lung was used to collect BALF, which was lavaged three times with 0.8 ml of autoclaved NS [12, 20, 21]. The left lung was used to obtain the lung W/D ratio. 24 h after LPS challenge, the rest mice were sacrificed. The inferior lobe of right lung was used for histopathologic evaluation. The superior lobes of right lung were used to collect BALF. The left lung was homogenized using a homogenizer.

We measured NO and total protein in BALF, C3 and C3c in serum, obtained the lung W/D ratio at 6 h after LPS challenge. We measured TNF- α 、IL-6 and IL-1 β in BALF, MPO and SOD in lung tissue homogenate, and carried out histopathologic evaluation at 24 h after LPS challenge.

2.8. NO and protein analysis

BALF was centrifuged (4 °C, 1400 $\times$ g, 10 min), and its supernatants were stored at -80 °C for analysis of NO and protein levels. The content of NO and protein in the supernatants of the BALF (6 h) were measured by NO and BCA protein assay kits according to the manufacturer's instructions strictly.

2.9. Lung W/D ratio

Mice were sacrificed by exsanguination at 6 h after LPS challenge. The left lung was excised, blotted dry and weighed to obtain the “wet” weight, and then placed in an oven at 80 °C for 48 h to obtain the “dry” weight. The ratio of the wet lung to the dry lung was calculated to assess tissue edema.

2.10. C3 and C3c in serum analysis

At 6 h after LPS challenge, the blood of mice was collected. Blood samples were coagulated at room temperature for 10 min, and then centrifuged (4 °C, 1400 $\times$ g, 20 min), its supernatants (serum) were stored at -80 °C for subsequent analysis. The levels of C3 and C3c in serum were quantified by the sandwich ELISA kit, according to the manufacturer's instructions strictly.

2.11. Assays for SOD and MPO activities

At 24 h after LPS challenge, the left lungs were homogenized using a homogenizer, then they were prepared to 10% lung tissue homogenate. The tissue homogenate generated were assayed for MPO activity, which were measured by MPO determination kit using commercially available reagents according to the manufacturer's instructions. The homogenate was then centrifuged at 1400 $\times$ g for 10 min at 4 °C. The supernatants obtained were used for assay of SOD activity. SOD activity was expressed as units per milligram of protein.

2.12. Cytokine analysis

The levels of cytokine TNF- α 、IL-6 and IL-1 β in the supernatants of the BALF (24 h) were quantified in duplication by the sandwich ELISA kit using commercially available reagents, according to the manufacturer's instructions strictly. The intra and inter coefficient of variation for TNF- α assays is 4.80%, 6.60%, detection limit and detection range for TNF- α assays is 3.90 pg/ml and 7.80-500.00 pg/ml ($r^2 = 0.999$), the TNF- α ELISA kit does not react with CD40、CD40、Ligand、Fas、Fas Ligand、LIF、OPG、RANK、RANK-L、TNF- β 、TRAIL、TROY. The intra

and inter coefficient of variation for IL-1 β assays is 3.70%, 4.50%, detection limit and detection range for IL-1 β assays is 7.00 pg/ml and 31.20-2000.00 pg/ml ($r^2 = 0.999$), the IL-1 β ELISA kit does not react with IL-1 α 、IL-1Ra、IL-1RI、IL-1RII、IL-2、IL-3、IL-4、IL-5、IL-6、IL-7、IL-9、IL-10、IL-10Ra、IL-11、IL-11Ra、IL-12、IL-12 p40、IL-13、IL-15、IL-20、IL-23R. The intra and inter coefficient of variation for IL-6 assays is 6.50%, 7.10%, detection limit and detection range for IL-6 assays is 4.00 pg/ml and 7.80-500.00 pg/ml ($r^2 = 0.999$), the IL-6 ELISA kit does not react with CT-1、gp130、IL-6sR、IL-11、LIF、OSM.

2.13. Histological studies of lung

After mice were sacrificed, inferior lobe of right lung tissue was immediately harvested and fixed in 4% formaldehyde. Then lung tissue was dehydrated and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin (H&E) according to the regular staining method. Images were observed under an Olympus microscope at an original magnification of 400 $\times$ [12, 20, 21].

For the detection of complement deposits, the 5- μ m sections were deparaffinized, rehydrated, and incubated with rabbit anti-human C3c overnight at 4 $^{\circ}$ C. Slices were visualized using chromogenic substrate solution 3, 3'-diaminobenzidine (DAB). All slides were imaged with CoolPix 4500 camera (Nikon) matched on a CX21 microscope (Olympus) at an original magnification of 400 $\times$ [12, 20, 21].

2.14. Therapeutic effects of EUP-FLA and EUP-EtOH on LPS-induced ALI model

The mice were randomly divided into five groups ($n = 10$): control group, model (LPS, 2 mg/kg) group, EUP-FLA group (30 mg/kg), EUP-EtOH groups (30 mg/kg) and positive group (DXM, 5 mg/kg). Mice were slightly anesthetized with a 20% urethane (4 ml/kg) given intraperitoneally. Then, in experimental groups 2 mg/kg LPS was instilled intratracheally (i.t.) in 50 μ l NS to induce lung injury. Mice from control were given equal volume distilled water. 0.5 h after LPS challenge, mice from EUP-FLA group and EUP-EtOH groups were received an intragastric injection of EUP-FLA or EUP-EtOH at given doses, positive group received DXM, control group received distilled water. 6 h after LPS challenge, mice were sacrificed and the blood samples were collected for analysis of the levels of C3 and C3c. The right lung was used to collect BALF for analysis of protein levels and TNF- α . The inferior lobe of right lung tissue was stained for histological studies. The left lung was used to obtain the lung W/D ratio.

2.15. Statistical analysis

All values were expressed as mean $\pm$ S.D. To perform statistical analysis, one-way analysis of variance (ANOVA) was used. If any significant changes were found, post-hoc comparisons were performed using Fisher's PLSD. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. HPLC analysis of EUP-FLA

As show in Figure 1, the contents of nepetin and jaceosidin in EUP-FLA were 25.2% and 65.7%, respectively. The content of the two flavonoids in EUP-FLA was 90.9%.

Figure 1. HPLC analysis of EUP-FLA. The chromatographic peaks of the analyses were identified by comparing their retention times and UV spectra with those of the reference standards. The chromatograms were obtained at a wavelength of 365 nm. (A) 0 ~ 65 min; (B) 0 ~16 min.

3.2. Anti-complementary activity of EUP-FLA

As shown in Figure 2, the concentrations that resulted in 50% hemolysis inhibition (CP_{50}) were 0.009 ± 0.008 mg/ml for EUP-FLA and 0.063 ± 0.010 mg/ml for heparin, respectively. The concentrations that resulted in 50% hemolysis inhibition of ERs (AP_{50}) were 0.042 ± 0.010 mg/ml for EUP-FLA and 0.101 ± 0.012 mg/ml for heparin, respectively. Compared with heparin, EUP-FLA exhibited a strong inhibitory effect on the complement activation through both the classical and alternative pathway.

Figure 2. Anti-complementary activities of EUP-FLA. Results are expressed as hemolytic percentage (mean $\pm$ S.D., $n = 3$). (A) Inhibition of classical pathway-mediated hemolysis of EAs in 1: 60 diluted guinea pig serum in the presence of increasing amounts of EUP-FLA. (B) Inhibition of alternative pathway-mediated hemolysis of ERs in 1: 8 diluted NHS in the presence of increasing amounts of EUP-FLA.

3.3. Effects of EUP-FLA on lung W/D ratio, NO and total protein concentration in BALF of mice

As shown in Figure 3, the lung W/D ratio, NO and the total protein concentration in BALF were found to be significantly higher after LPS challenge compared with those of the control group ($P < 0.01$), while EUP-FLA (40 mg/kg) gavage itself did not cause significant changes.

Compared with vehicle treated ALI model group, EUP-FLA at 20 and 40 mg/kg markedly decreased the lung W/D ratio, NO and the total protein concentration in BALF ($P < 0.01$) (Figure 3A&3B&3C). DXM also significantly decreased the lung W/D ratio, NO and the total protein concentration.

Figure 3. Effects of EUP-FLA on the lung W/D ratio (A), NO (B) and total protein concentration (C) in BALF of mice. 2 h after EUP-FLA pretreatment on day 7, mice were slightly anesthetized, and then 2 mg/kg LPS was instilled intratracheally (i.t.) to induce lung injury. The lung W/D ratio was determined at 6 h after LPS challenge; the BALF were collected at 6 h following LPS challenge. NO and total protein concentration in the supernatant of BALF were determined by kits. Data expressed as means $\pm$ S.D. ($n = 10$); ^{##} $P < 0.01$ compared with control, ^{*} $P < 0.05$ and ^{**} $P < 0.01$ compared with vehicle treated ALI model group.

3.4. Effect of EUP-FLA on C3 and C3c production in serum of LPS-induced ALI

As shown in Figure 4, the levels of C3 and C3c were significantly higher after LPS challenge compared with those of the control group ($P < 0.01$), while EUP-FLA (40 mg/kg) gavage itself did not cause significant changes.

Compared with vehicle treated ALI model group, EUP-FLA at 20 and 40 mg/kg significantly decreased the level of C3 ($P < 0.01$) (Figure 4A), while it at 10, 20 and 40 mg/kg markedly decreased the level of C3c ($P < 0.01$) (Figure 4B). DXM also significantly decreased the level of C3 and C3c.

Figure 4. Effects of EUP-FLA on the level of C3 (A) and C3c (B) in serum of mice. 2 h after EUP-FLA pretreatment on day 7, mice were slightly anesthetized, and then 2 mg/kg LPS was instilled intratracheally (i.t.) to induce lung injury. The serum were collected at 6 h following LPS challenge. C3 and C3c concentration in serum was determined by mouse C3 and C3c ELISA kit. Data expressed as means $\pm$ S.D. ($n = 10$); $^{##} P < 0.01$ compared with control, $^{*} P < 0.05$ and $^{**} P < 0.01$ compared with vehicle treated ALI model group.

3.5. Effects of EUP-FLA on SOD and MPO activities in lung tissues of LPS-induced ALI

As shown in Figure 5, LPS challenge resulted in significant decreases of SOD activity and significant increases of MPO activity in the lungs compared with the control group ($P < 0.01$), while EUP-FLA (40 mg/kg) gavage itself did not cause significant changes.

EUP-FLA at 40 mg/kg decreased MPO activity ($P < 0.01$) (Figure 5A), while EUP-FLA at 20 and 40 mg/kg significantly increased SOD activity ($P < 0.01$) (Figure 5B). DXM also significantly increased SOD activity and decreased MPO activity.

Figure 5. Effects of EUP-FLA on MPO and SOD activities of lung tissue in mice. 2 h after EUP-FLA pretreatment on day 7, mice were slightly anesthetized, and then 2 mg/kg LPS was instilled intratracheally (i.t.) to induce lung injury. Lung homogenates were prepared at 24 h after LPS challenge. MPO and SOD activities were determined by kits. Data expressed as means $\pm$ S.D. ($n = 10$); $^{##} P < 0.01$ compared with control, $^{*} P < 0.05$ and $^{**} P < 0.01$ compared with vehicle treated ALI model group.

3.6. Effects of EUP-FLA on TNF- α , IL-6 and IL-1 β production in BALF

As shown in Figure 6, LPS significantly increased TNF- α , IL-6 and IL-1 β production compared with the control group ($P < 0.01$), while EUP-FLA (40 mg/kg) gavage itself did not cause significant changes.

Compared with vehicle treated ALI model group, EUP-FLA at 20 and 40 mg/kg significantly reduced the level of TNF- α and IL-6 in the BALF ($P < 0.01$) (Figure 6A&6B). EUP-FLA at 40 mg/kg markedly decreased the level of IL-1 β in the BALF of mice ($P < 0.01$) (Figure 6C). DXM also significantly decreased TNF- α , IL-6 and IL-1 β production.

Figure 6. Effects of EUP-FLA on production of inflammatory cytokine TNF- α , IL-6 and IL-1 β in BALF of mice. 2 h after EUP-FLA pretreatment on day 7, mice were slightly anesthetized, and then 2 mg/kg LPS was instilled intratracheally (i.t.) to induce lung injury. BALF was collected at 24 h following LPS challenge. The levels of TNF- α , IL-6 and IL-1 β in the supernatants of BALF were determined by mouse ELISA kits. Data expressed as means $\pm$ S.D. ($n = 10$); ^{##} $P < 0.01$ compared with control, * $P < 0.05$ and ** $P < 0.01$ compared with vehicle treated ALI model group.

3.7. Effect of EUP-FLA on lung histology

Histopathological changes of each group were observed by histochemical staining with H&E. Histopathological changes such as lung edema, increased alveolar wall thickness, inflammatory cells aggregation, pulmonary hemorrhage were observed in model group mice induced by LPS given intratracheally. As shown in Figure 7, these lesions were not apparent in control group stimulated with only NS. Pretreatment with EUP-FLA markedly ameliorated the pulmonary injury.

For vehicle treated ALI model group, immunohistochemistry of lung tissue sections showed a patchy dense immunoperoxidase indicative of complement deposition. Complement, appeared by bulk deposition, and was mainly deposited in lung tissue. In contrast, mice in control group had little complement deposition in lung tissue. Pretreatment with EUP-FLA markedly decreased complement deposition (Figure 7).

375
376

H&E

Complement

A

377
378

B

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380

C

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382

D

383
384

Figure 7. Hematoxylin and eosin-stained (H&E) (400 ×) and immunohistochemistry of lung tissue (400 ×). Lungs from each group were processed for histological evaluation at 24 h after LPS challenge: Section of control (A) and EUP-FLA (B) groups mice: normal lung tissue sections (H&E and complement). Section of a LPS-induced ALI model (C) group mouse: note increased alveolar wall thickness, inflammatory cells aggregation, pulmonary hemorrhage (H&E) and a patchy dense immunoperoxidase indicative of complement deposition (complement). Section from 10, 20 and 40 mg/kg EUP-FLA-pretreated (D, E, F, respectively) and DXM-treated (G) groups mouse: note mild alveolar wall thickness, reduced inflammatory cells aggregation, little pulmonary hemorrhage (H&E) and little complement deposition (complement).

3.8. Therapeutic effects of EUP-FLA and EUP-EtOH on the lung W/D ratio, total protein concentration and TNF- α , C3 and C3c in LPS-induced ALI model

As shown in Figure 8, LPS significantly increased the lung W/D ratio, total protein concentration and the level of TNF- α in BALF, the levels of C3 and C3c in serum compared with the control group ($P < 0.01$).

Compared with vehicle treated ALI model group, EUP-EtOH group and EUP-FLA group at

30 mg/kg significantly reduced the lung W/D ratio, total protein concentration and the levels of TNF- α , C3 and C3c ($P < 0.05$) (Figure 8), respectively. DXM also significantly decreased TNF- α , C3 and C3c production, the lung W/D ratio, total protein concentration ($P < 0.05$) (Figure 8).

Compared with EUP-EtOH, EUP-FLA markedly decreased the lung W/D ratio, total protein concentration and the level of TNF- α ($P < 0.01$) (Figure 8A&8B&8C), respectively. Compared with EUP-EtOH, EUP-FLA significantly decreased C3 and C3c production ($P < 0.05$) (Figure 8D&8E).

Figure 8. Therapeutic effects of EUP-FLA and EUP-EtOH on the lung W/D ratio, total protein concentration and TNF- α , C3 and C3c. 2 mg/kg LPS was instilled intratracheally (i.t.) in 50 μ l NS to induce lung injury. Mice from control were given equal volume distilled water. 0.5 h after LPS challenge, mice from EUP-FLA group and EUP-EtOH groups were received an intragastric injection of EUP-FLA or EUP-EtOH at 30 mg/kg, positive group received DXM, control group received distilled water. 6 h after LPS challenge, mice were sacrificed and the blood samples were collected for analysis of the levels of C3 and C3c. The right lung was used to collect BALF for analysis of protein levels and TNF- α . The left lung was used to obtain the lung W/D ratio. Data

expressed as means $\pm$ S.D. ($n = 10$); $^{##} P < 0.01$ compared with control, $^{*} P < 0.05$ and $^{**} P < 0.01$ compared with EUP-EtOH group. EUP-EtOH group, EUP-FLA group and positive group compared with vehicle treated ALI model, $P < 0.05$, respectively.

3.9. Therapeutic effects of EUP-FLA and EUP-EtOH on lung histology

As shown in Figure 9, the increased alveolar wall thickness, inflammatory cells aggregation, pulmonary hemorrhage (H&E) and the patchy dense immunoperoxidase indicative of complement deposition were observed in LPS-induced ALI model group. The mild alveolar wall thickness, reduced inflammatory cells aggregation, little pulmonary hemorrhage (H&E) and little complement deposition were observed in EUP-FLA, EUP-EtOH and DXM-treated groups mouse (Figure 9).

Figure 9. Therapeutic effects of EUP-FLA and EUP-EtOH on lung histology (400 ×). Lungs from each group were processed for histological evaluation at 6 h after LPS challenge: Section of control group mice: normal lung tissue sections (H&E and complement). Section of a

LPS-induced ALI model group mouse: note increased alveolar wall thickness, inflammatory cells aggregation, pulmonary hemorrhage (H&E) and a patchy dense immunoperoxidase indicative of complement deposition (complement). Section from 30mg/kg EUP-FLA, EUP-EtOH and LPS+DXM groups mouse: note mild alveolar wall thickness, reduced inflammatory cells aggregation, little pulmonary hemorrhage (H&E) and little complement deposition (complement).

4. Discussion

LPS can be employed in the investigation of ALI [22], which is the major component of the outer membranes of Gram-negative bacteria, acting as endotoxins, and eliciting strong inflammatory [23] and immune [24] response in animals. In our study, we established the animal model of direct ALI by i.t. instillation of LPS in mice [12, 18-21], and we used DXM as a positive control to evaluate the anti-inflammatory efficiency of EUP-FLA in LPS-induced ALI, which is the most frequently used anti-inflammatory drug in the clinical treatment of ALI/ARDS [25].

In our study on the protective effects of EUP-FLA on ALI induced by LPS, we selected two monitoring time points (6 h and 24 h after LPS challenge) because data were stable and significantly different from the control group in the two time points (Figure 3 in supplementary material) [12, 21]. After four hours charged by LPS, the levels of NO, protein and C3 raised quickly, then reached their peaks at 6 h. Data were significantly different from the control group, so we measured NO and total protein in BALF, C3 and C3c in serum at 6 h after LPS challenge. After two hours charged by LPS, the levels of TNF- α , IL-6 and IL-1 β raised quickly, then reached their peaks at 4 h or 8 h, but contents of TNF- α , IL-6 and IL-1 β were beyond the kit of detection, we measured TNF- α , IL-6 and IL-1 β in BALF at 24 h after LPS challenge in order to avoid human error. After two hours charged by LPS, the level of MPO raised quickly, then reached its peak at 12 h. After two hours charged by LPS, the level of SOD reduced rapidly, then hit its bottom at 12 h. The levels of MPO and SOD were stable and significantly different from the control group at 24 h, so we measured MPO and SOD in lung tissue homogenate at 24 h after LPS challenge. In addition, we obtained the lung W/D ratio at 6 h after LPS challenge and carried out histopathologic evaluation at 24 h after LPS challenge [12, 20, 21].

In ALI, neutrophils are the earliest immune cells to be recruited to the site of injury and express multiple cytotoxic products, such as reactive oxygen species, NO, and other cytotoxic and proinflammatory mediators [26]. These effects subsequently cause the intra-alveolar and interstitial edema, alveolar hemorrhage and overwhelming lung inflammation, which ideally imitate the pathophysiologic events observed in ALI patients [26]. MPO is an enzyme located mainly in the primary granules of neutrophils, and thus MPO activity in the parenchyma reflects the adhesion and margination of neutrophils in the lungs. SOD is an antioxidant enzyme that can scavenge superoxide free radical and it has been reported to be markedly decreased in LPS-induced ALI [27]. In our present investigation, we evaluated the lung wet-to-dry weight ratio to quantify the magnitude of pulmonary edema and measured the total protein content in the BALF to evaluate epithelial and endothelial permeability [28], which were the major characteristic of ALI [29]. At the same time, we measured the activities of SOD and MPO, and the productions of NO to evaluate whether EUP-FLA scavenged oxyradicals during the inflammatory response to LPS-induced ALI.

LPS can induce the production of several inflammatory cytokines, such as TNF- α , IL-6 and

IL-1 β which appears in the early phase of an inflammatory response and contributes to defense harmful stimuli upon bacterial invasion; however, excessive inflammatory reaction leads to tissue damage and manifestation of pathological states and the severity of lung injury [18, 30]. High levels of TNF- α , IL-6 and IL-1 β in the BALF have been noted in patients with ALI/ARDS [31]. We detected the inflammatory mediators in BALF at 24 h after LPS challenge, according the changes of inflammatory mediators in BALF and serum following intrapulmonary challenge with LPS in our previous studies [12, 21].

The complement system consists of more than 30 serum and cellular proteins. Activation of the complement system plays a key role in normal inflammatory response to injury but may cause substantial injury when activated inappropriately. The complement system links in three biochemical cascades, the classical, alternative and lectin complement pathways [32, 33]. C3 activation leads to the entry of the final common pathway resulting in the formation of the membrane attack complex (MAC, C5b-9) [34]. C3c is the stable conversion product of C3, which develops out of C3 within one hour at body temperature [35].

Complement activation contributes an important facet to the pathogenesis of ALI/ARDS. Complement 3 plays an important role in activating complement system of ALI. Animal model of ALI studies demonstrated alterations of complement 3 levels in ALI [5]. Pretreatment of wild type mice with humanized cobra venom factor, which inactivates C3, decreased polymorphonuclear neutrophil (PMN) in BAL cells and reduces C3 deposition in the lung [36]. Moreover, complement system and anaphylatoxin C5a are associated with full production of TNF- α and neutrophil aggregation [8]. C3, C5, C5a and C5aR may regulate the inflammatory response in a variety of inflammatory responses, such as ALI, ARDS, acute vascular injury after systemic activation of complement [5]. C3a has been shown to regulate IL-1 β and TNF- α gene expression and synthesis [37]. Elevated levels of C3a and C5a have been shown in patients at risk for ARDS, and inhibition of complement activation might be a potential therapeutic strategy [2-7]. We found that the levels of C3 and C3c increased significantly in serum at 6 h after LPS challenge, then immunohistochemistry of lung tissue sections showed abundantly complement deposition in vehicle treated ALI model group at 24 h. In our research, EUP-FLA exhibited the strong inhibitory effect on the complement activation through the classical pathway and the alternative pathway, the main compounds of EUP-FLA had anti-complement activities through the classical pathway (nepetin CP₅₀ = 0.071 $\pm$ 0.039 mg/ml and jaceosidin CP₅₀ = 0.037 $\pm$ 0.009 mg/ml). We thought EUP-FLA could protect the lung injury by decreasing the level of complement. We thought that inhibitors of complement may be potential adjunctive treatments for LPS-induced ALI.

In order to verify that EUP-FLA is the effective substances of EUP attenuating LPS-induced ALI, we carried out comparison of therapeutic effects of EUP-FLA and EUP-EtOH on LPS-induced ALI model. EUP-FLA and EUP-EtOH could attenuate ALI, respectively. Compared with EUP-EtOH group, EUP-FLA markedly decreased the lung W/D ratio, TNF- α and the total protein concentration in BALF, the levels of C3 and C3c in serum at the same dose. Therefore EUP-FLA was the effective substances of EUP attenuating LPS-induced ALI.

In conclusion, EUP-FLA attenuated LPS-induced lung injury, including reduction of lung W/D ratio, inhibition of protein level and NO over-production in BALF. In addition, EUP-FLA lowered TNF- α , IL-6 and IL-1 β level in BALF in ALI mice, increased SOD activity and inhibited MPO activity in lung tissue. Histological examination showed that EUP-FLA significantly ameliorated lung injury by improving lung morphology and decreasing complement deposition. Meantime,

EUP-FLA obviously reduced the levels of C3 and C3c in serum in ALI mice. The effects of EUP-FLA against ALI were related with the inhibition on the production of pro-inflammatory mediators and decreasing the level of complement and affecting the NO, SOD and MPO activity.

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References

- [1] M. Bakowitz, B. Bruns, M. McCunn, Acute lung injury and the acute respiratory distress syndrome in the injured patient, *Scandinavian Journal of Trauma, Resuscitation and Emergency Medicine* 20 (2012) 54.
- [2] P. A. Ward, Role of complement, chemokines, & regulatory cytokines in acute lung injury, *Ann. N.Y. Acad. Sci.* 796 (1) (1996) 104-112.
- [3] S.C. Makrides, Therapeutic inhibition of the complement system. *Pharmacological Reviews* 50 (1998) 59-87.
- [4] S.S. Asghar, M.C. Pasch, Therapeutic inhibition of the complement system Y2K update, *Frontiers in Bioscience* 5 (2000) E63-E81.
- [5] M. Bosmann, P.A. Ward, Role of C3, C5 and anaphylatoxin receptors in acute lung injury and in sepsis, *Advances in Experimental Medicine and Biology* 946 (2012) 147-159.
- [6] B.L. Garcia, K.X. Ramyar, D. Ricklin, J.D. Lambris, B.V. Geisbrecht, Advances in understanding the structure, function, and mechanism of the SCIN and Efb families of staphylococcal immune evasion proteins, *Advances in Experimental Medicine and Biology* 946 (2012) 113-133.
- [7] J.A. Weigelt, D.E. Chenoweth, K.R. Borman, J.F. Norcross, Complement and the severity of pulmonary failure, *The Journal of Trauma* 28 (1988) 1013-1019.
- [8] J. Hutchison, Neutrophils in sepsis, septic shock, and adult respiratory distress syndrome: how stiff is too stiff? *Critical Care Medicine* 28 (2000) 2659-2660.
- [9] An Editorial Committee (over 300 members) of the Administration Bureau of Traditional Chinese Medicine, *Chinese Materia Medica (Zhonghua Benchao)*. Shanghai Science & Technology Press 7 (1998) 839-841.
- [10] S.Q. Wu, L. Xia, S. Yao, H.Y. Han, J. Zhang, Advances on chemical constituents and pharmacological effects of *Eupatorium lindleyanum* DC., *China Pharmacy* 15 (2013) 1426-1428.
- [11] Z. Jiang, H. Yang., H.X. He, Y.D. Zhou, Protective Effects of *Eupatorium Lindleyanum* on Acute Lung Injury in Rats, *China Pharmacy* 27(2007) 2094-2096.
- [12] C.J. Chu, H.L. Ren, X.L. Li, L. Xia, J. Zhang, D.F. Chen, Protective effect of the extracts of *Eupatorium lindleyanum* on acute lung injury induced by LPS in mice, *West China Journal of Pharmaceutical Sciences* 30 (6) (2015) 653-656.
- [13] S.Q. Wu, Q. Sun, C.J. Chu, J. Zhang, Chemical constituents of *Eupatorium lindleyanum*, *China Journal of Chinese Materia Medica* 7 (2012) 937-940.
- [14] S.Q. Wu, N.Y. Xu, J. Zhang, S. Yao, C.J. Chu, Three new acyclic diterpenoids from *Eupatorium lindleyanum* DC, *Journal of Asian Natural Product Research* 14 (2012) 652-656.
- [15] S.Q. Wu, N.Y. Xu, Q. Sun, H.Y. Han, J. Zhang, Six New Sesquiterpenes from *Eupatorium lindleyanum*, *Helvetica Chimica Acta* 9 (2012) 1637-1644.
- [16] M.M. Mayer, E.A. Kabat, Complement and complement fixation, *Experimental Immunochemistry*. Springfield Publications (1961) 133-240.
- [17] J.P. Klerx, C.J. Beukelman, D.H. Van, J.M. Willers, Microassay for colorimetric estimation of complement activity in guinea pig, human and mouse serum, *Journal of*

- Immunological Methods 2 (1983) 215-220.
- [18] G.F. Chi, M.M. Wei, X.X. Xie, L.W. Soromou, F. Liu, S.H. Zhao, Suppression of MAPK and NF-kappa B pathways by Limonene contributes to attenuation of lipopolysaccharide-induced inflammatory responses in acute lung injury, *Inflammation* 36 (2013) 501-511.
- [19] X.M. Zhang, K.J. Song, H.Z. Xiong, H.Y. Li, X. Chu, X.M. Deng, Protective effect of florfenicol on acute lung injury induced by lipopolysaccharide in mice, *International Immunopharmacology* 9 (2009) 1525-1529.
- [20] J.Y. Xie, H.Y. Di, H. Li, X.Q. Cheng, Y.Y. Zhang, D.F. Chen, *Bupleurum chinense* DC polysaccharides attenuates lipopolysaccharide-induced acute lung injury in mice, *Phytomedicine* 19 (2012) 130-137.
- [21] C.J. Chu, N.Y. Xu, X.L. Li, L. Xia, J. Zhang, Z.T. Liang, Z.Z. Zhao, D.F. Chen, *Rabdosia japonica* var. *glaucoalyx* flavonoids fraction attenuates lipopolysaccharide-induced acute lung injury in mice, *Evidence-Based Complementary and Alternative Medicine* (2014) ID 894515, 12 pages.
- [22] Y. Wu, M. Singer, F. Thouron, M. Alaoui-El-Azher, L. Touqui, Effect of surfactant on pulmonary expression of type IIA PLA (2) in an animal model of acute lung injury, *American Journal of Physiology-Lung Cellular and Molecular Physiology* 282 (2002) L743-L750.
- [23] J. Saluk-Juszczak, B. Wachowicz, The proinflammatory activity of lipopolysaccharide, *Postepy Biochemii* 51 (2005) 280-287.
- [24] B. Pulendran, P. Kumar, C.W. Cutler, M. Mohamadzeadeh, T. Dyke, J. Banchereau, Lipopolysaccharides from distinct pathogens induce different classes of immune responses *in vivo*, *Journal of Immunology* 167 (2001) 5067-5076.
- [25] G.U. Meduri, E.A. Tolley, G.P. Chrousos, F. Stentz, Prolonged methylprednisolone treatment suppresses systemic inflammation in patients with unresolving acute respiratory distress syndrome: evidence for inadequate endogenous glucocorticoid secretion and inflammation-induced immune cell resistance to glucocorticoids, *American Journal of Respiratory and Critical Care Medicine* 165 (2002) 983-991.
- [26] H. Chen, C. Bai, X. Wang, The value of the lipopolysaccharide-induced acute lung injury model in respiratory medicine, *Expert Rev Respir Med*, 4 (2010) 773-783
- [27] H. Macarthur, T.C. Westfall, D.P. Riley, T.P. Misko, D. Salvemini, Inactivation of catecholamines by superoxide gives new insights on the pathogenesis of septic shock, *Proc Natl Acad Sci U S A*, 97 (2000) 9753-9758.
- [28] B.D. Beck, J.D. Brain, D.E. Bohannon, An *in vivo* hamster bioassay to assess the toxicity of particulates for the lungs, *Toxicology and Applied Pharmacology* 66 (1982) 9-29.
- [29] J. Yang, J.M. Qu, H. Summah, J. Zhang, Y.G. Zhu, H.N. Jiang, Protective effects of imipramine in murine endotoxin-induced acute lung injury, *European Journal of Pharmacology* 638 (2010) 128-133.
- [30] I.A. Giebelen, D.J. van Westerloo, G.J. LaRosa, A.F. de Vos, T. van der Poll, Local stimulation of alpha7 cholinergic receptors inhibits LPS-induced TNF-alpha release in the mouse lung, *Shock* 28 (2007) 700-703.
- [31] T. Minamino, I. Komuro, Regeneration of the endothelium as a novel therapeutic strategy for acute lung injury, *Journal of Clinical Investigation* 116 (2006) 2316-2319.
- [32] S. Thiel, T. VorupJensen, C.M. Stover, W. Schwaeble, S.B. Laursen, K. Poulsen, A.C. Willis, P. Eggleton, S. Hansen, U. Holmskov, K.B. Reid, J.C. Jensenius, A second serine protease associated with mannan-binding lectin that activates complement, *Nature* 386 (1997) 506-510.
- [33] S.C. Makrides, Therapeutic inhibition of the complement system, *Pharmacological Reviews* 50 (1998) 59-87.
- [34] R. Yanai, A. Thanos, K. Connor, Complement Involvement in Neovascular Ocular Diseases, *Advances in Experimental Medicine and Biology* 946 (2012) 161-183.
- [35] P. Garred, T. E. Mollnes, T. Lea, Quantification in enzymelinked immunosorbent assay of a C3 neopeptide expressed on activated human complement factor C3, *Scandinavian Journal of Immunology* 3 (1988) 329-335.
- [36] K. Takahashi, D. Saha, I. Shattino, V.I. Pavlov, G.L. Stahl, P. Finnegan, M.F.V. Melo, Complement 3 is involved with ventilator-induced lung injury, *International Immunopharmacology* 11 (2011) 2138-2143.

[37] N.M. Mervi, T. Anna-Maija, M. Seppo, Complement C3 Cleavage and Cytokines Interleukin-1 β and Tumor Necrosis Factor- α in Otitis Media With Effusion, The Laryngoscope 10 (2000)1745-1749.

Abbreviations

EUP	<i>Eupatorium lindleyanum</i> DC.
EUP-EtOH	the ethanol extract of <i>Eupatorium lindleyanum</i> DC.
EUP-DIT	the diterpenes fraction of <i>Eupatorium lindleyanum</i> DC.
EUP-FLA	the flavonoids fraction of <i>Eupatorium lindleyanum</i> DC.
EUP-SQT	the sesquiterpenes fraction of <i>Eupatorium lindleyanum</i> DC.
LPS	lipopolysaccharide
ALI	acute lung injury
BALF	the bronchoalveolar lavage fluid
C3	complement 3
C3c	complement 3c
ELISA	enzyme linked immunosorbent assay
Lung W/D ratio	lung wet-to-dry weight ratio
MPO	myeloperoxidase
SOD	superoxide dismutase
BCA	bicinchoninic acid
ARDS	acute respiratory distress syndrome
DXM	dexamethasone
NO	Nitric oxide
VBS	veronal buffer saline
EAs	sensitized erythrocytes
H&E	hematoxylin and eosin

Eupatorium lindleyanum DC (EUP)

the flavonoids fraction (EUP-FLA)

in vitro

in vivo

ALI model

lung

BALF

serum

lung tissue

lung histology

W/D ratio ↓

NO
Protein
TNF-α
IL-6
IL-1β ↓

C3
C3c ↓

SOD ↑
MPO ↓

H&E
C3c deposits ↓

Anti-complementary activity

the classical pathway

the alternative pathway