

# Anti-inflammatory effects of alcohol extract from *Saussurea medusa* Maxim against lipopolysaccharides-induced acute lung injury mice

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## Abstract

**OBJECTIVE:** To investigate the anti-inflammatory effects of alcohol extract from *Saussurea medusa* Maxim on acute lung injury of mice.

**METHODS:** Animals were randomly divided six groups (n=15) including normal group, model group, control group (Dex group), low-dose group of SM, middle-dose group of SM and high-dose group of SM. Ear oedema was induced by xylene and oedematous response was measured. Leukocyte, TNF- $\alpha$  and COX-2 level of blood was determined. Histological changes of lung were examined by hematoxylin and eosin.

**RESULTS:** Compared with that of normal group, levels of right ear oedema decreased 46.17% and 50.92% in middle-dose group of SM and high-dose group of SM, respectively. Compared with that of model group, quantity of leukocyte reduced 47.32% ( $P<0.05$ ), 56.20% ( $P<0.05$ ) and 49.47% ( $P<0.05$ ) at 1h, 6h and 12h in the high-dose group of SM, respectively. In the high-dose group of SM, the level of TNF- $\alpha$  reduced 85.47% ( $P<0.05$ ) and 73.74% ( $P<0.05$ ) at 6 h and 12 h compared with that of model group, respectively. In the high-dose group of SM, the level of COX-2 reduced 37.69% ( $P<0.05$ ) at 12 h in contrasted with that of model group. Injury of lungs were attenuated by different-dose of alcohol extract from *S. medusa* treatment.

**CONCLUSION:** The alcohol extract from *S. medusa* can play a protecting-role against lung injury in acute lung injury mice maybe through decreasing leukocyte, TNF- $\alpha$  and COX-2 level and enhancing the anti-inflammatory effects.

## Keywords

*Saussurea medusa*; Alcohol extract; Acute lung injury of mice; Anti-inflammatory effects

## 1. INTRODUCTION

Acute lung injury (ALI) is a major health problem for children and elderly population characterized by activation of the pulmonary endothelium, disruption of the endothelial and alveolar epithelial barriers, and increase of microvascular permeability. 1 Lung, a crucial airway for pathogens into the body, is constantly exposed to a large number of microorganisms which can cause acute inflammation and induce ALI. 2 Lipopolysaccharides (LPS) presented in cell wall of gram-negative bacterium can produce experimental ALI model. 3, 4 In this process, pro-inflammatory cytokines

and anti-inflammatory cytokines are involved into lung injury induced LPS, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1, IL-2, IL-6, IL-8, IL-4, IL-10 and interferon- $\gamma$ .<sup>5,6,7</sup>

Tibetan medicine is widely used in the area of the historical Tibet, which is distributed in the several provinces in western China.<sup>8</sup> A number of herbal materials are mainly constitution of profile of Tibetan pharmacology.<sup>9</sup> The plants are ordered according to their indication and classified according to their sensory qualities including of taste, texture and colour. In a formula of disease treatment, more than three and up to twenty herbal materials are used, and even seven up to seventy and more. Each herbal ingredient is already a chemically complex mixture of substances, the whole formula is a pharmacological entity with complex characterizations. Without single active substance prevails, the whole mixture consisted by multi-component nature can be regarded as a pharmacologically active unit and exhibit a distinct pleiotropic activity signature. With characterizations of respect of relatively safe and effective alternatives to allopathic drugs, single herbal materials of Tibetan medicine have also been gained great attention.

Snow lotus, a famous traditional Chinese medicine derived from dried area of species of the genus *Saussurea*, has been widely used in inflammatory diseases. *Saussurea laniceps* Hand.-Mazz. (SL), *S. medusa* Maxim. (SM) and *S. involucmicea* Sch. Bip.<sup>10</sup> (SI) are authentic sources of the snow lotus medicine and showed special medicinal qualities and clinical effects. It has been reported that *S. laniceps* is recommended as one of the most potential herbs with its outstanding anti-inflammatory and anti-nociceptive capacities.<sup>11</sup> *S. involucmicea* can display anti-fatigue, anti-inflammatory, and anti-tumor effects and it is able to eliminate free radicals.<sup>12</sup> *S. medusa* is mainly distributed in the Qinghai-Tibet plateau at heights of 3500–5300m and prescribed for the treatment of pain in Tibetan folk medicine.

Compared with that of *S. laniceps* and *S. involucmicea*, the studies of *S. medusa* are very limited. Take into consideration of great interest to shed light of mysterious veil of Tibetan Medicine, the current study was performed to investigate the effects of ethanol extract of *S. medusa* on the ALI of mice.

## 2. METHODS AND MATERIALS

### 2.1. Plant material collection and extraction

*S. medusa* Maxim were collected from Qilian Mountain in Qinghai of China, and was identified and authenticated by Lijuan Mei (Northwest Institute of Plateau Biology, Chinese Academy of Sciences). The *S. medusa* Maxim (1.0 kg) were ground to a fine powder and equally divided into 4 fractions, followed by successive extraction at room temperature with ethanol. Briefly, filtration and evaporation of extracts of *S. medusa* Maxim were performed under reduced pressure at 45°C, followed by lyophilization, and stored at 4°C until use. Finally, the solid form of the extract was dissolved with deionized water for use in experiments. Lipopolysaccharides (LPS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dexamethasone (Dex), an positive control drug was purchased from Kerui Pharmaceutical Co., Ltd. (Chongqing, China). Methanol and acetonitrile of chromatography grade were purchased from Aladdin Reagent Co.(Shanghai, China). Commercial kits of TNF- $\alpha$  and COX-2 were purchased from Thermo Fisher scientific (Shanghai, China).

### 2.2. Animals and treatment

BALB/c mice weighing 18–22g were provided by Experimental Animal Center of Hubei province (Certification Number SCXK2016-0575, China). All animals were fed with standard rodent diet and water, and housed at a room temperature of 23±2°C with a 12h light/dark cycle. The experimental study was performed following approval from the Pingdingshan University Animal Care and Ethics Committee. All experimental and surgical procedures were conducted by the Pingdingshan University Surgical Application and Research Center. Animals culture were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### 2.3. Analysis of oedematous response

The xylene-induced ear oedema test was carried out according to the previous study<sup>[13, 14]</sup>. Fifty mice were randomly divided into five groups of ten animals each group, including normal group, control group (Dex group), low-dose group of SM, middle-dose group of SM and high-dose group of SM. Animals in the low-dose group of SM, middle-dose group of SM and high-dose group of SM were respectively received SM solution at a concentration of 20 mg/kg, 40 mg/kg and 80 mg/kg, in Dex group with Dex at 3 mg/kg. Mice in normal group was perfused similar volume distilled water only. After one week, each mice was treated with 0.03 mL of xylene to the anterior and the posterior surface of the right ear. The left ear was used as a control. One hour later, mice were sacrificed under ether anaesthesia and both ears were removed. Circular sections were taken using a cork borer (7 mm in diameter) and weighed. Oedematous response was

measured as the weight difference between the two ear plugs.

#### 2.4. Measurement of leukocyte, TNF- $\alpha$ and COX-2 level

Animals were randomly divided six groups ( $n=15$ ) including normal group, model group, control group (Dex group), low-dose group of SM, middle-dose group of SM and high-dose group of SM. Base on previous study, animals in the low-dose group of SM, middle-dose group of SM and high-dose group of SM were respectively received SM solution at a concentration of 20 mg/kg, 40 mg/kg and 80 mg/kg, in Dex group with Dex at 3 mg/kg. Mice in normal and model control groups were administrated with similar volume of distilled water, respectively. After one week, Except normal group, mice in other groups were treated a single intratracheal instillation with 4 mg/kg LPS. Lung and blood were collected at 0, 1, 6 and 12 h after LPS. Divide the blood into 3 eppendorf tubes. The leukocyte quantity was determined by flow cytometry. The levels of TNF- $\alpha$  and COX-2 in mice serum samples were measured by an enzyme linked immunosorbent assay (ELISA) according to the kit's instructions.

#### 2.5. Histological study

The lung tissue of mice were removed after their sacrifice and fixed in 15% paraformaldehyde for 3 days. Then the tissues were embedded in paraffin after dehydration to carry out the lung histopathology testing. 5  $\mu$ m thick of sections were mounted on glass slides, and stained with hematoxylin and eosin (H&E).

#### 2.6. Statistical analysis

All the indexes were detected 3 times for parallel examination and the results were expressed as mean $\pm$ SD. The results were analyzed by one-way analysis of variance (ANOVA) followed by a Tukey's test for multiple comparisons, with the level of significance chosen as  $P<0.05$ .

### 3. RESULTS

#### 3.1. Effects of alcohol extract from *Saussurea medusa* on the ear oedema

Compared with that of normal group, administration of Dex could result in a significant inhibitory effect on the auricle swelling of mice induced by xylene. Compared with that of normal group, levels of right ear oedema decreased 46.17% and 50.92% in middle-dose group of SM and high-dose group of SM, respectively (Fig. 1).

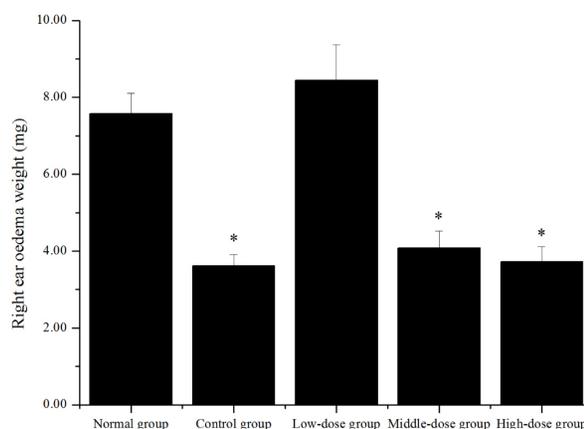


Figure 1. Effects of alcohol extract from *Saussurea medusa* on the ear oedema

#### 3.2. Histopathologic analysis

Histopathological examination of lung sections showed normal cellular architecture with distinct alveolar cell at different times in the normal group (Fig. 2). After LPS Administration, the lung sections exhibited a high degree of damage characterized by focal hemorrhage, distortion, and alveolar thickening. In contrast, injury of lungs were attenuated by Dex treatment and different-dose of alcohol extract from *S. medusa* treatment. Furthermore, obvious protecting role against LPS injury was observed in control group and high-dose group of SM.

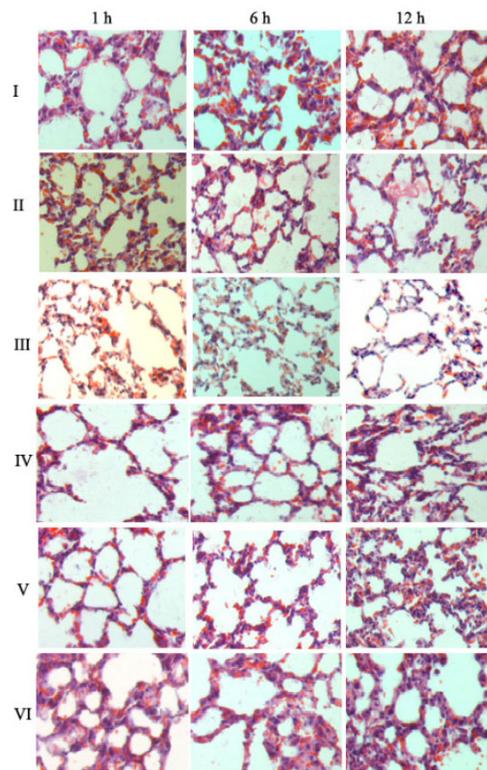


Figure 2. Histopathological examination of lung sections

### 3.3. Effects of alcohol extract from *S. medusa* on the leukocyte counts

Compared with that of normal group, the quantity of leukocyte was significantly increase ( $P<0.05$ ) in model group at 6h and 12h (Table 1). Administration of Dex and different-dose of alcohol extract from *S. medusa* could result in a decline of leukocyte quantity compared with that of model group. In the high-dose group of SM, quantity of leukocyte reduced 47.32% ( $P<0.05$ ), 56.20% ( $P<0.05$ ) and 49.47% ( $P<0.05$ ) at 1h, 6h and 12h compared with that of model group, respectively.

Table 1. Effects of alcohol extract of *Saussurea medusa* on quantity of leukocyte ( $\times 10^9/L, \bar{x} \pm s$ )

Groups	0h	1h	6h	12h
Normal group	2.18±0.24	2.20±0.17	2.96±0.35	2.51±0.21
Model group	2.18±0.24	2.43±0.25	3.95±0.41 <sup>a</sup>	3.78±0.36 <sup>a</sup>
Control group	2.18±0.24	1.35±0.14 <sup>ab</sup>	1.92±0.23 <sup>ab</sup>	1.06±0.13 <sup>ab</sup>
Low-dose group	2.18±0.24	1.86±0.22 <sup>b</sup>	1.80±0.20 <sup>ab</sup>	2.33±0.25 <sup>b</sup>
Middle-dose group	2.18±0.24	1.56±0.17 <sup>ab</sup>	1.86±0.21 <sup>ab</sup>	2.25±0.24 <sup>b</sup>
High-dose group	2.18±0.24	1.28±0.15 <sup>ab</sup>	1.73±0.20 <sup>ab</sup>	1.91±0.20 <sup>ab</sup>

Note: <sup>a</sup> $P<0.05$  vs at normal control group. <sup>b</sup> $P<0.05$  vs at model group.

### 3.4. Effects of alcohol extract from *S. medusa* on the level of TNF- $\alpha$ and COX-2

Compared with that of normal group, the level of TNF- $\alpha$  was significantly increase ( $P<0.05$ ) in model group at 6 h and 12 h (Table 2). Treatments of Dex and different-dose of alcohol extracts from *S. medusa* could result in a down-regulation of TNF- $\alpha$  level. In addition, down-regulations of TNF- $\alpha$  level showed a dose dependent matter in MS treated groups. In the high-dose group of SM, the level of TNF- $\alpha$  reduced 85.47% ( $P<0.05$ ) and 73.74% ( $P<0.05$ ) at 6 h and 12

h compared with that of model group, respectively.

**Table 2. Effects of alcohol extract of *Saussurea medusa* on level of TNF- $\alpha$  (pg/ml,  $\bar{x} \pm s$ )**

Groups	0h	1h	6h	12h
Normal group	136 $\pm$ 7.52	140.12 $\pm$ 5.19	123.87 $\pm$ 9.74	121.98 $\pm$ 13.24
Model group	136 $\pm$ 7.52	142.48 $\pm$ 10.44	668.96 $\pm$ 36.73 <sup>a</sup>	278.87 $\pm$ 18.98 <sup>a</sup>
Control group	136 $\pm$ 7.52	116.07 $\pm$ 12.30	87.87 $\pm$ 5.86 <sup>a</sup>	46.25 $\pm$ 9.17 <sup>ab</sup>
Low-dose group	136 $\pm$ 7.52	120.92 $\pm$ 9.82	399.74 $\pm$ 22.65 <sup>ab</sup>	191.02 $\pm$ 20.99 <sup>ab</sup>
Middle-dose group	136 $\pm$ 7.52	132.98 $\pm$ 9.75	331.20 $\pm$ 32.70 <sup>ab</sup>	177.89 $\pm$ 16.77 <sup>ab</sup>
High-dose group	136 $\pm$ 7.52	130.28 $\pm$ 8.22	312.72 $\pm$ 33.74 <sup>ab</sup>	152.11 $\pm$ 15.22 <sup>ab</sup>

Note: <sup>a</sup> $P$ <0.05 vs at normal control group. <sup>b</sup> $P$ <0.05 vs at model group.

### 3.5. Effects of alcohol extract from *S. medusa* on the level of COX-2

Compared with that of normal group, the level of COX-2 was significantly increase ( $P$ <0.05) in model group at 6 h and 12 h (Table 3). Administrations of Dex different-dose of alcohol extract from *S. medusa* could result in a decline of COX-2 level. In the high-dose group of SM, the level of COX-2 reduced 37.69% ( $P$ <0.05) at 12 h in contrasted with that of model group.

**Table 3. Effects of alcohol extract of *Saussurea medusa* on level of COX-2 (pg/ml,  $\bar{x} \pm s$ )**

Groups	0h	1h	6h	12h
Normal group	86.42 $\pm$ 5.25	89.57 $\pm$ 4.31	85.80 $\pm$ 5.63	84.12 $\pm$ 2.05
Model group	86.42 $\pm$ 5.25	85.86 $\pm$ 8.14	158.24 $\pm$ 17.35 <sup>a</sup>	106.29 $\pm$ 13.52 <sup>a</sup>
Control group	86.42 $\pm$ 5.25	84.14 $\pm$ 5.47	108.62 $\pm$ 14.32 <sup>ab</sup>	85.79 $\pm$ 9.63 <sup>b</sup>
Low-dose group	86.42 $\pm$ 5.25	89.24 $\pm$ 7.68	159.81 $\pm$ 16.57 <sup>a</sup>	81.62 $\pm$ 10.02 <sup>b</sup>
Middle-dose group	86.42 $\pm$ 5.25	76.92 $\pm$ 6.14	153.49 $\pm$ 14.68 <sup>a</sup>	84.34 $\pm$ 9.56 <sup>b</sup>
High-dose group	86.42 $\pm$ 5.25	85.93 $\pm$ 5.42	141.64 $\pm$ 14.24 <sup>a</sup>	66.23 $\pm$ 7.55 <sup>b</sup>

Note: <sup>a</sup> $P$ <0.05 vs at normal control group. <sup>b</sup> $P$ <0.05 vs at model group.

## 4. DISCUSSION

In the current study, oral administration of ethanol extract of *S. medusa* showed a significant systemic and topical anti-inflammatory, analgesic effects on ALI mice. Treatment of LPS can result an increase of generation and release of proinflammatory mediators such as histamine and serotonin. It is an inflammatory response and also considered an immune response that can promote vasodilatation and increase permeability of blood vessel.<sup>13, 14</sup> Therefore, instant irritation is often occurred in the mouse ear during LPS treatment. Generally, the ear oedema is widely used to screen and investigate the anti-inflammatory activity of drugs in the acute phase of inflammation.<sup>14</sup> Considering here, the ethanol extract of *S. medusa* may play an improving role against analgesic effects of LPS treatment in the ALI mice.

Treatment of ethanol extract of *S. medusa* Maxim.(SM) could result in a significant increase of leukocyte quantity compared with that of control group, suggesting *S. medusa* extract exert as a protecting-role against LPS induced ALI of mice. In the LPS-induced acute lung injury, severe thrombocytopenia can cause haemorrhages and decreased survival of alveolar cells. In this process, platelets play an important role in pulmonary function by maintaining vascular integrity. Platelet are activated via soluble factors and physical interaction facilitated by a variety of receptors of leukocytes.<sup>15,16</sup> Few receptors are involved into the direct interactions between platelet and leukocytes. In LPS-induced ALI, quantity of platelets are decrease by 90% and reach to the threshold level for maintaining basic haemostasis that should result in a damage of leukocytes recruitment into the alveolar compartment, tissue injury and vascular leakage.<sup>17</sup> Meanwhile,

leukocyte recruitment towards the sites of injury function as a player of defense against invading pathogens and support the inflammatory responses. Therefore, leukocyte recruitment and extravasation to sites of inflammation of platelet-leukocyte interactions contribute to promote leukocyte release of neutrophil extracellular traps and pro-inflammatory mediators, phagocytosis, oxidative burst, that in turn may weaken inflammation with some pathological conditions. 18

Compared with that of model group, the plasma level of TNF- $\alpha$  was significantly decreased In the ethanol extract of *S. medusa* treated group, suggesting the protecting-role of *S. medusa* extract on the ALI mice is associated with down-regulation of pro-inflammatory cytokines expression. Macrophages are an important source of TNF- $\alpha$  and IL-1 $\beta$  secretion. Several studies have demonstrated TNF- $\alpha$  plays a key role in the development of ALI by stimulating chemotaxis and recruiting neutrophils.<sup>19</sup> Meanwhile, TNF- $\alpha$  functions as an important player in inducing apoptosis in alveolar of lung and microvascular endothelial cells of lung. <sup>20</sup> Injury of the alveolar epithelium and vascular endothelium is generally considered as an marker of pathogenesis of ALI. The high expression of TNF- $\alpha$  is regarded as a direct manifestation of cellular injury. Alveolar macrophages are involved into inflammation of ALI.<sup>21</sup> Once activated, macrophages produce a variety of inflammatory cytokines such as TNF- $\alpha$ , monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), and reactive oxygen species (ROS).<sup>22</sup> Therefore, down-regulation of TNF- $\alpha$  expression derived from ethanol extract of *S. medusa* treated group is contribute to attenuate the injury of lung.

Administration of *S. medusa* extract could result in a remarkable decrease of COX-2 expression which reveal pathway of NF- $\kappa$ B is likely one target acted by *S. medusa* extract. The progress of lung inflammation is coordinated by the activation, expression, and secretion of numerous pro-inflammatory mediators from the lung parenchyma and vascular cells, such as cytokines, leukotriene, inflammation and adhesion molecules. Intracellular pathway is blocked by chemopreventive agent that is now considered as a novel scenario of treatment for the ALI. COX-2, an inducible enzyme for the synthesis of PGE<sub>2</sub>, is generated via the NF- $\kappa$ B signal pathway. COX-2 is involved into activated inflammatory response by increasing vasodilation, vascular permeability and edema in ALI.<sup>23, 24</sup> The modulation of COX-2 involved into signaling pathway has been considered a new approach for preventing oxidative stress, inflammation, and toxicity. It has demonstrated activated NF- $\kappa$ B transactivate COX-2 expression, hence its inhibition is a valuable marker for down-regulation of various inflammatory diseases. <sup>25</sup> Multiple targets should be acted by ethanol extract of *S. medusa* because different components should be isolated form ethanol extract. Therefore, we postulate that down-regulation of COX-2 expression is associated with inhibition of NF- $\kappa$ B. *S. medusa*, an important member of Tibetan Medicine, its highlights and targets acted should be further elucidated in the future.

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