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Research Article
Extract of *Scabiosa comosa* Exhibits an Anti-Inflammatory Effect on Carrageenan and Lipopolysaccharide-Induced Acute Inflammation in Rats

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Abstract

Background and Objective: The herb *Scabiosa comosa* (SC) is a herbal medicine used in both Mongolian and Chinese traditional medicine for liver disease. The previous studies show the presence of flavonoids and other high anti-oxidant compounds which may exert anti-inflammatory effects. This study is devoted to evaluating the anti-inflammatory effect of the aerial part of *Scabiosa comosa* (SC) based on their bioactive compound contained and *in vivo* model. **Materials and Methods:** Bioactive compound estimation is based on a total phenolic compound and flavonoid content, according to Folin-Ciocalteu reagent and aluminium trichloride reagent methods, respectively. *In vivo* experiments, acute pulmonary inflammation induced by lipopolysaccharide (LPS) and carrageenan-induced paw edema models were used to investigate the anti-inflammatory effect of SC. **Results:** The total phenolic compound and content were 626.4±1.4 μg EGA mg^{-1} and flavonoid content was 3.3±0.3 μg EQ mg^{-1}. The SC significantly reduced the volume of the hind paw after the injection of carrageenan at 120 min. The SC showed fewer histopathological changes such as haemorrhage, neutrophil infiltration and alveolar edema after the injection of LPS. In addition, SC considerably reduced the plasma levels of pro-inflammatory cytokine (TNF-α, IL-6 and IL-1β) while upregulating the plasma level of anti-inflammatory cytokine (IL-10). **Conclusion:** Overall, the study defines SC as a potential anti-inflammatory agent against LPS and carrageenan-induced inflammation.

Key words: *Scabiosa comosa*, paw edema, acute inflammation, Mongolian herbal medicine, inflammation, flavonoids, phenolic acid, pharmacological activities

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The herb *Scabiosa comosa* Fischer ex Roemer and Schultes (SC) is a perennial plant with basal leaf rosettes and leafy stems, one of the two sub-endemic species of plants grown in Mongolia which belongs to the genus *Scabiosa*. This biologically diverse genus comprises 62 officially documented species native to temperate regions of Europe, Asia and Southern Africa[1,2]. From the perspective of folk medicine, *Scabiosa* species are constantly considered potential therapeutic agents throughout history. Furthermore, the name *Scabiosa* emanated from its traditional usage as medicine to treat "Scabies" which is characterized by its severe itchiness and a pimple-like rash [3]. Preceding works of literature mainly stated medicinal application of *Scabiosa* is vastly contributed by its biologically active compounds such as iridoids, pentacyclic triterpenoids and polyphenolic compounds. These compounds were mostly found in this species of plants and yield beneficial therapeutic properties such as antioxidant, anti-fibrotic, anti-inflammatory, anti-HCV, anti-amnesic, antitumor and antibacterial activity[2,4-10]. In the case of SC, the inflorescence of SC used along with *Scabiosa tschilliensis*, 'Lanpenhua' in Chinese, was traditionally used to treat liver disease in Mongolian folk medicine[8]. Also, used as a remedy for headache, fever, cough, liver heat, pulmonary heat and throat heat[4].

Polyphenols are plant-derived secondary metabolites that constitute a broad range of compounds ubiquitously presented in angiosperms. The distinction between their chemical structure and moiety forms five main classes: Flavonoids, phenolic acid, tannins, stilbenes and lignans[11,12]. Most polyphenols are more emphasized due to their beneficial therapeutic agents against various diseases. In particular, flavonoids were much more pronounced as compared to their counterparts due to their biological (pharmacological) activities, pro-oxidant, antioxidant, anti-cholinesterase, antibacterial, antifungal, lipid mediator and xanthine oxidase modulating activity[13]. Even if the insufficient number of studies showed a direct relation between chemical structure and pharmacological activities, few studies have stated that the flavonoid class does not define the mechanism of action, but the hydroxyl group substituted the carbon atom at positions 5 and 7 of A ring and position 4 of B ring is a critical characteristic for determining their antiphlogistic and antioxidant mechanism of action[14].

During the inflammation, various types of mediators secreted from different sources of cells participated in the communication and interaction between the cells such as irritant recognition and recruiting cells to inflammatory foci. Tumour Necrosis Factor-Alpha (TNF-α) is one of the critical mediators classified as pro-inflammatory cytokine and plays a critical role in nuclear factor kappa-light-chain enhancer of activated B cell (NF-κB) activation of inflammation and activate Stress-Activated Protein Kinases (SAPKs)[15]. The TNF-α is secreted from macrophages and other cells during Pathogen-Associated Molecular Patterns (PAMPs) in acute inflammation. Despite the beneficial role in host defense mechanism, elevated level of TNF-α causes systematic inflammatory response syndrome and multiple organ failure and disseminates intravascular coagulation followed by induction of septic shock[16]. Preceding studies on flavonoids mentioned the anti-inflammatory mechanism of action, regulating intracellular pathways (through modulating NF-κB, mitogen-activated protein Kinase (MAPk) and arachidonic pathways), expression of the pro-inflammatory cytokine and interacting with reactive oxygen species (ROS)[17]. In this vein, different types of flavonoids have been widely investigated in the form of plant extract and independent forms. For instance, quercetin inactivates the NF-κB pathway by blocking the nuclear translocation of p50 and p65, subunits of NF-κB, resulting in the reduction of TNF-α, IL-1, IL-6 and IL-8[18].

The related works on SC revealed 36 bioactive compounds, mainly flavonoids, phenylpropanoids and iridoids. The presence of derivatives of di-caffeoylquinic acid and chlorogenic acid in the inflorescence is responsible for its high radical scavenging and anti-HCV activity[4]. As mentioned above we postulated SC may exert an anti-inflammatory effect regarding its high amount of bioactive compounds and high antioxidants.

In this study, the bioactive compound of SC was quantified in terms of total phenolic compound content and flavonoid content. In addition, the anti-inflammatory effect of SC was investigated *in vivo* models of LPS-induced acute pulmonary inflammation and carrageenan-induced hind paw edema in rats.

MATERIALS AND METHODS

Plant materials and extraction: The aerial parts of SC were collected from Tsenkhermandal sum of Khentii Province in July, 2019. The plant was identified by Dr. Sh. Dariimaa of the National University of Mongolia. To obtain the crude extract, the collected aerial parts were air-dried at room temperature and ground to a coarse powder. Then extracted with ethanol (96%), evaporated with a rotary vacuum evaporator and air-dried indirectly from sunlight. The crude extract was solubilized in distilled water immediately before animal administration. The voucher specimen (*S. comosa*) was deposited in the Laboratory of Plant Biotechnology, School of Engineering and Applied Sciences, National University of Mongolia.

Experimental animals: The animal experiment and experimental protocol were approved by the Animal Care and Use Committee, Mongolian University of Life Sciences. A total of 100 male Wistar rats (200-250 g) aged between 8-10 weeks old were used in this study and were obtained from the Institute of Traditional Medicine and Technology, Ulaanbaatar. All animals were kept under controlled conditions (25±2°C and 12 hrs light/dark cycle) and fed on a regular sterile chow diet and free access to water. The 20 rats were used in the carrageenan-induced paw edema model (n = 5 in each group) and the remaining 80 rats (n = 20 in each group) were used in the LPS-induced acute inflammation model.

Chemicals and reagents: Lipopolysaccharide (*Escherichia coli* 055: B5 endotoxin), λ-carrageenan, Folin-Ciocalteu reagent, quercetin ($C_{15}H_{10}O_7$), sodium carbonate (Na_2CO_3), aluminium trichloride ($AlCl_3$) and gallic acid ($C_7H_6O_5$) were purchased from Sigma Aldrich Co. (USA). The measurement of plasma levels of cytokines determined commercially available Enzyme-Linked Immune Sorbent Assay (ELISA) kits from MLBIO Co. (China).

Chemical assessment of bioactive compounds

Total phenolic content: The total phenolic content estimation of plant extract was based on the Folin-Ciocalteu method[19]. The test sample was prepared at a concentration of 0.1 g mL^{-1}. Five hundred microliters of sample were added to 2.5 mL of Folin-Ciocalteu reagent (previously prepared by diluting 10-times with deionized water). Then the mixture was allowed to stand and react for 4 min. Subsequently, 2 mL of sodium carbonate solution and 20 mL of deionized water were added to the test mixture and vigorously shaken. After incubation at room temperature for 2 hrs in darkness, the absorbance was recorded at 765 nm using a UV-Vis spectrophotometer (UV-160, Shimazu, Japan). The calibration curve was obtained with gallic acid (5-200 μg mL^{-1}). The total phenolic content of the test sample was expressed in microgram equivalents of gallic acid per milligram of extract (μg GA/mg of extract).

Total flavonoid content: The trichloro aluminum method was used to quantification of the total flavonoid content in plant extract[20]. An equal volume of aluminium chloride ($AlCl_3$) at a concentration of 2% and plant sample solution were mixed in which both solutions were dissolved in methanol. After the mixture was vigorously shaken and incubated for 10 min at room temperature, the absorbance of the sample was measured at 430 nm using a UV-Vis spectrophotometer (UV-160, Shimazu, Japan). The calibration curve to estimate the concentration of flavonoids in plant extract was obtained with quercetin (0-100 μg mL^{-1}). The result was expressed in microgram equivalents of quercetin per milligram of extract (μg EQ/mg of extract).

Carrageenan-induced paw edema: The experimental animals were separated randomly into four groups, each containing five rats[21]. In the normal group rats non-treated with carrageenan were chosen to make a comparison between the hind paw volume of non-treated and treated animals. Before the administration, all group received their testing treatments by orally corresponding to their groups, consecutively 5 days. The control group, received sterile saline by oral gavage (10 mL kg^{-1}). In the diclofenac and SC group, diclofenac (25 mg kg^{-1}) and SC extract (190 mg kg^{-1}) were given by oral gavage as pretreatment, respectively. The paw edema was induced with 1% carrageenan in all the groups after 1 hr of oral administration. The paw volume was measured before and after carrageenan injection at 30, 60, 120, 180 and 240 min, using a plethysmometer (Ugo Basile Co., Italy). After the last paw measurement, rats were euthanized with an excessive dose of ketamine hydrochloride.

LPS-induced acute pulmonary inflammation: The experimental animals were randomized into four groups, each containing twenty rats. Before the administration, all groups received their testing treatments by orally corresponding to their groups, consecutively 5 days. In the control group, sterile saline was received by oral gavage (10 mL kg^{-1}). In the diclofenac and SC group, diclofenac (25 mg kg^{-1}) and SC extract (190 mg kg^{-1}) were given by oral gavage as pretreatment, respectively. One hour later oral administration, acute pulmonary inflammation was induced by an LPS 7.5 mg kg^{-1} (dissolved in a 0.9% saline) intravenous injection via rats' tail vein. The plasma levels of cytokines (Tumour Necrosis Factor α (TNF-α), Interleukin 1β (IL-1β), Interleukin 6 (IL-6) and Interleukin 10 (IL-10)) were measured with Enzyme-Linked Immune Sorbent Assay (ELISA) kits at 1, 3, 6, 9 and 12 hrs after the injection. Before the blood samples were collected by cardiac puncture, rats were anaesthetized with an injection of ketamine hydrochloride (80-90 mg kg^{-1})[22].

Histopathological analysis: Rats were euthanized (n = 3 for all groups, respectively) for collecting lung samples after the 12 hrs of LPS injection. The acquired lung tissues were fixed in 10% formalin solution, washed in running tap water, dehydrated in ethanol, xylene and paraffin solutions and then embedded in paraffin. The paraffin blocks were sliced with 3-5 µm, stained with hematoxylin and eosin (HE) and observed by light microscope (Nikon Eclipse, Japan). Histopathological changes were graded for the degree of intra-alveolar edema, intra-alveolar haemorrhage, atelectasis and neutrophil infiltration using grades 0-4 (0: Absent, 1-2: Very few, 3: Few and 4: Moderate) with a maximum score of 12[23].

Data analysis: All experiments were performed with at least triplicate samples and repeated three times. The data were reported as the Mean±SD. The statistical comparison between the groups was performed using two-way ANOVA followed by Tukey's multiple comparison test. A $p<0.05$ value was considered statistically significant.

RESULTS

Chemical assessment of bioactive compounds: Based on preceding works of literature, ethanol was chosen as the extracting solvent considering plants of the genus *Scabiosa* have rich phenolic content which is more soluble in polar organic solvents due to the presence of a hydroxyl group. The assessment revealed the presence of total phenolic compound and content was 626.4±1.4 µg EGA mg^{-1} and flavonoid content was 3.3±0.3 µg EQ mg^{-1}.

Carrageenan-induced paw edema: From the result, injection of carrageenan in the hind paw leads to an increase in the volume of the hind paw in all groups. The volume amounts form two distinctive time-dependent phases after the injection which are initiated with a relatively rapid early phase (0-60 min) and followed by a late phase (60-240 min). Throughout both phases, the control group remains the highest volume among the other groups and reached its peak in the late phase (120 min). Whereas, orally administrated with SC (190 mg kg^{-1}) and diclofenac (50 mg kg^{-1}) groups were shown a relatively different pattern in which both significantly reduced the volume of the hind paw and reached their minimum at 120 and 240 min, respectively (Fig. 1).

LPS-induced acute inflammation: The sudden increase in plasma level of cytokines (TNF-α, IL-1β, IL-6 and IL-10) was observed in the control group after the injection of LPS at 1 hr. In particular, proinflammatory cytokines (TNF-α, IL-1β and IL-6) reached their peak at 6 hrs while anti-inflammatory cytokine (IL-10) gradually increased and reached its peak at 9 hrs after the LPS injection. Despite the increasing levels of TNF-α at 9 hrs in the SC group, TNF-α, IL-β and IL-6 levels of diclofenac and SC-treated groups showed relatively steady levels compared with the control group over time. The anti-inflammatory cytokine IL-10 showed constantly increasing traits over time whereas, SC and diclofenac groups increase significantly within 3 hrs. Subsequently, IL-10 levels were considerably decreased (Fig. 2a-d).

Histopathological analysis: In addition, the pulmonary histological score (Fig. 3) illustrated the difference between treated and non-treated groups in which the score of the

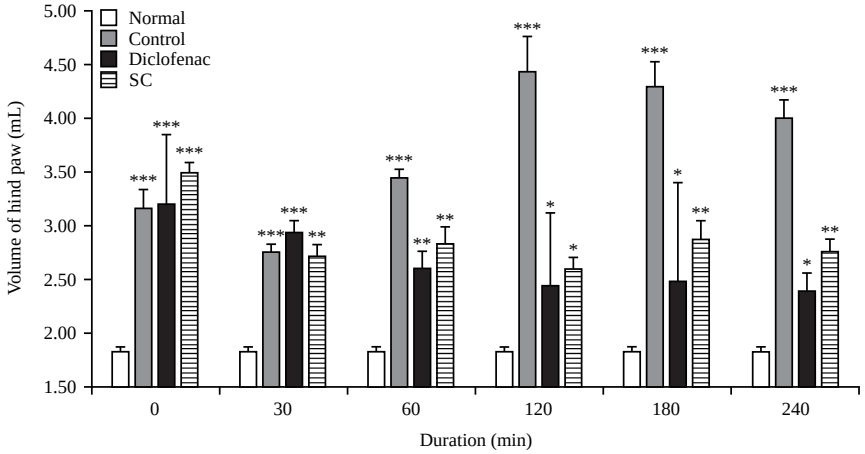

Fig. 1: Volume of the hind paw in the groups along the time after carrageenan injection (n = 5)

Oral administration of SC (190 mg kg^{-1}) and diclofenac (25 mg kg^{-1}) considerably inhibited carrageenan-induced paw edema. Values expressed as Mean±SD of 5 rats in the groups, *p<0.05, **p<0.01 and ***p<0.001 compared with non-carrageenan treated normal group

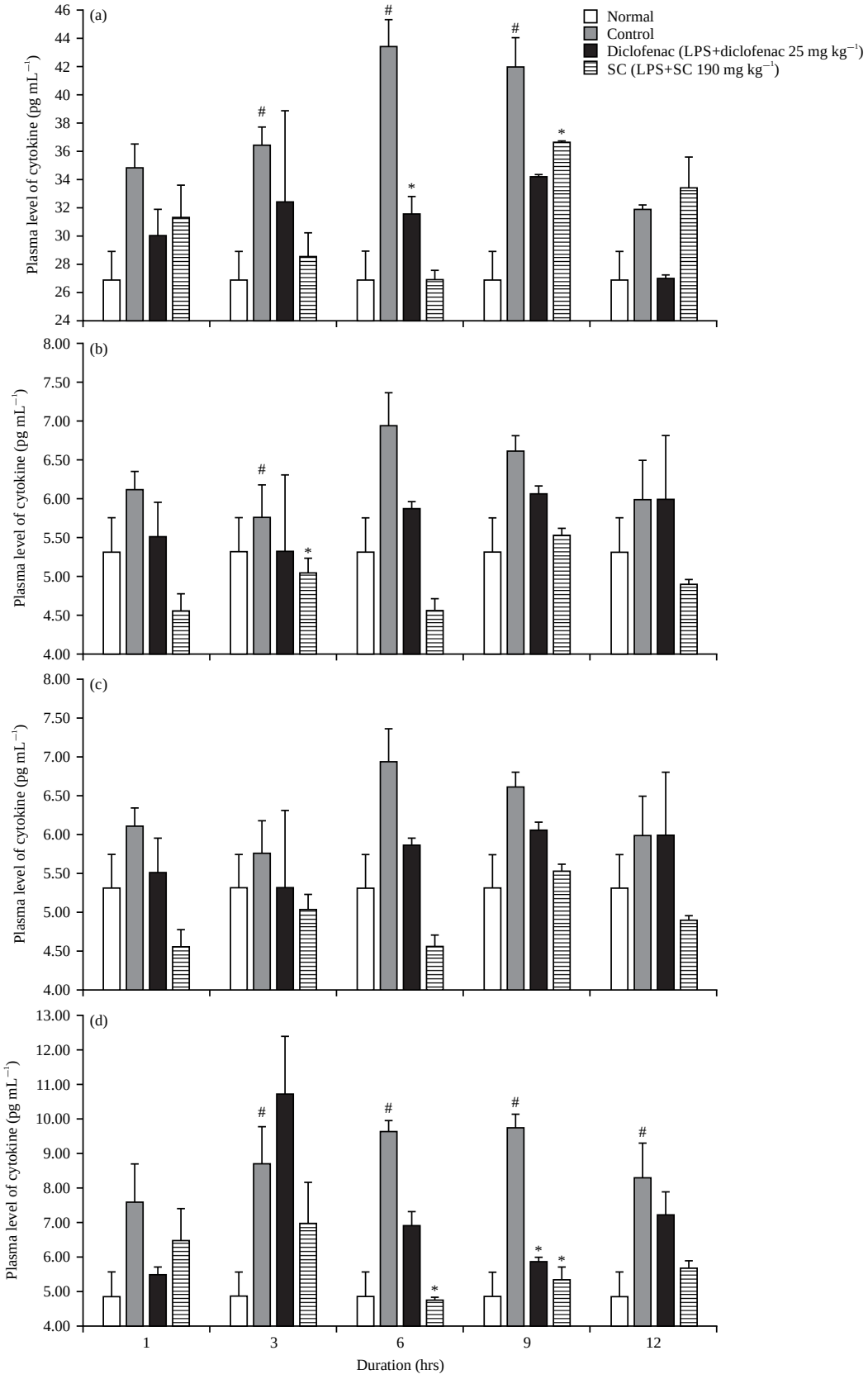

Fig. 2(a-d): Plasma level of the cytokine after injection of LPS, (a) TNF-α, (b) IL-1β, (c) IL-6 and (d) IL-10

#p<0.05 compared with the normal group and *p<0.05 compared with the control group by two-way ANOVA followed by Tukey's *post hoc* Test

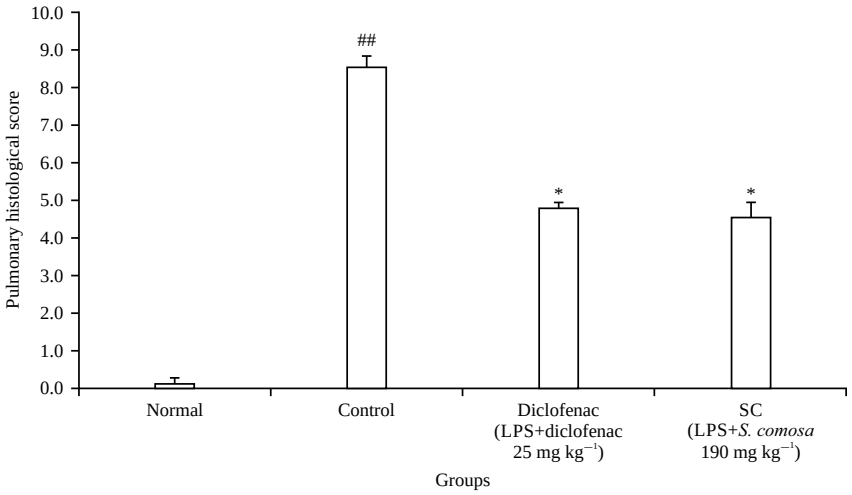

Fig. 3: Pulmonary histological score in different groups

##p<0.05 compared with normal group, *p<0.05 compared with control group by two-way ANOVA followed by Tukey's *post hoc* Test. Histopathological changes were graded for the degree of intra-alveolar edema, intra-alveolar hemorrhage, atelectasis and neutrophil infiltration

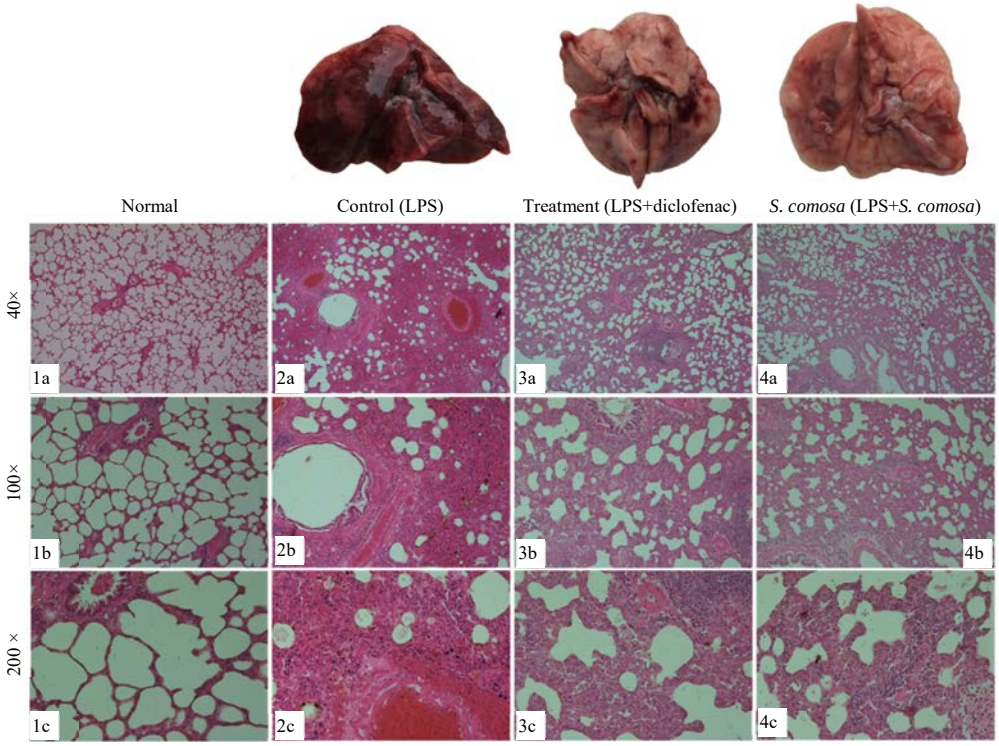

Fig. 4: Histopathological changes showed significant difference between LPS treated and non-treated animals. LPS treated groups (2a-4c) have increased alveolar wall thickness compared to non-treated group (1a-1c). Amidst these groups, less alveolar edema, neutrophil infiltration, alveolar haemorrhage and atelectasis were observed in group treated with diclofenac (3a-3c) and *S. comosa* (4a-4c)

Hematoxylin and Eosin stain (x40, x100, x200 magnification)

diclofenac and SC groups was considerably lower (p<0.05) compared to that of the control group. Histopathological assessment vividly demonstrates the difference between LPS-treated and non-treated animal lungs (Fig. 41a-4c). The healthy group shows the structure of normal lung tissue and clear pulmonary alveoli (1a-1c) whereas, histopathological changes of LPS injection defined its overwhelming alveolar haemorrhage, severe neutrophil

infiltration, alveolar edema, atelectasis and increased alveolar wall thickness (2a-2c). Meanwhile, diclofenac (25 mg kg^{-1}) and SC (190 mg kg^{-1}) groups relatively maintained their lung structure with less alveolar haemorrhage, neutrophil infiltration, alveolar wall thickness and atelectasis (3a-3c, 4a-4c).

DISCUSSION

Herein antiphlogistic effect of SC was investigated using LPS-induced acute pulmonary inflammation and carrageenan-induced hind paw edema corresponding with their considerable amount of polyphenolic compound. The total phenolic compound was assessed according to the Folin-Ciocalteu method, which equals 626.4±1.4 μg EGA mg^{-1}, which is considered a high amount compared with same genus plants but, flavonoid content was 3.3±0.3 μg EQ mg^{-1}, which considered less[6,24,25].

In the carrageenan-induced model, injection of irritant forms a biphasic reaction dependent on the time after injection in rats. The former phase is initiated by the injection of an irritant and continues for an hour which is regulated by the secretion of chemical mediators such as serotonin and histamine due to the carrageenan. Subsequently, carrageenan promotes the production of prostaglandins, proteases and lysosomes resulting in the accelerated formation of edema forming the latter phase which begins at the end of the first hour and persists through the 3rd or 4th hrs[26]. The SC-treated group showed less volume of hind paw compared to the control group but the result didn't produce less volume than the diclofenac group in the former phase. After 2 hrs, the paw volume of treated and non-treated groups differed significantly and SC treated group showed inhibiting effects as much as diclofenac compared with the control group. In addition, flavonoids inhibit Cyclooxygenase-1 (COX-1), COX-2 and 5-Lipoxygenase (5-LO) resulting in the reduction of prostaglandin, thromboxane and leukotrienes, respectively[27]. Thus, it may underlie the inhibition action mechanism of carrageenan-induced paw edema in SC-treated rats.

During the LPS administration, irritant recognized by Toll-Like Receptor 4 (TLR4) triggers activation of an intracellular signalling pathway involving NF-κB, leading to the upregulation of many genes responsible for the secretion of inflammatory mediators including TNF-α, IL-1β and IL-6[17]. The TNF-α, IL-1β and IL-6 are addressed as pro-inflammatory cytokines which are involved in the process of pathological pain. Further, excessive inflammatory cytokines in circulation lead to disruption and epithelial integrity, the influx of neutrophils into the interstitial tissue and bronchioalveolar space, interstitial edema and leakage of protein into the alveolar space[28]. In addition, the subsequent local activation of neutrophils and macrophages leads to tissue damage via the release of cytotoxic and immune cell-activating agents such as cytokines, cationic polypeptides, free radicals and proteinases[29]. In our case, SC treated group shows less level of pro-inflammatory cytokine (TNF-α, IL-1β and IL-6) than the diclofenac-treated group. Despite the increasing level of TNF-α at 9 and 12 hrs, SC substantially suppressed the plasma levels of pro-inflammatory cytokine throughout the experiment.

In addition, anti-inflammatory cytokines are considered important mediators during the inflammation process which regulates the production and function of pro-inflammatory cytokines at multiple levels. Major anti-inflammatory cytokine includes IL-1 receptor antagonist, IL-4, IL-10, IL-11 and IL-13. Among all the anti-inflammatory cytokines, IL-10 is considered significant due to its inhibiting activity against TNF-α, IL-1 and IL-6[16]. Further, IL-10 and IL-13, T-cell-derived cytokines appear to be the main immunomodulatory and anti-inflammatory gene products[30]. The imbalance between pro-inflammatory and anti-inflammatory cytokines may lead to endothelial dysfunction and leakage syndrome, which is associated with hypertension, edema and organ dysfunctions[31]. The related studies on flavonoids described that quercetin suppressed the pro-inflammatory cytokine TNF-α, IL-1β and IL-6 while upregulating the anti-inflammatory cytokine IL-10 may partly underlie the protective effect of quercetin against LPS-induced lethal endotoxemia and acute lung injury[31]. Likewise, this pattern was also observed in our experiment. The plasma level of IL-10 was increased overall every in group 3 hrs after injection. Subsequently, declining levels were observed in both SC and diclofenac groups while the control group peaked at 9 hrs. But, at 12 hrs, the level of the SC group was elevated while the level of which in the control group was declining. Pathologically, SC treated group showed less histopathological changes such as mild neutrophil infiltration, alveolar haemorrhage, alveolar wall thickness and atelectasis compared to the control group. To sum up, our findings define SC as a potential anti-inflammatory agent against LPS-induced acute pulmonary inflammation and carrageenan-induced paw edema.

CONCLUSION

This study demonstrated that the pretreatment of SC extract in rats exhibited fewer pathological changes in lung tissue such as intra-alveolar haemorrhage, neutrophil infiltration, interstitial edema and atelectasis. Also,

pretreatment of SC extract reduced plasma levels of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) while upregulating anti-inflammatory cytokine (IL-10) compared to the untreated one in the LPS-induced model. In addition, SC extract significantly reduced the volume of paw edema in the carrageenan-induced model. Overall, results suggested that SC could be used in inflammation treatment as a prominent natural medical plant source.

SIGNIFICANCE STATEMENT

In our findings, we aim to pursue the significance of herbal medicine used in both Traditional Mongolian and Chinese medicine. The herb *Scabiosa comosa* is used solely along with *Scabiosa tschiliensis* for liver diseases due to their antioxidant, anti-inflammatory, analgesic and other beneficial effects. The previous literature on this species mainly indicates such therapeutic effects are highly dependent on their considerable number of polyphenolic compounds, the mainly flavonoids in their inflorescences. In this research, we revealed the presence of a high amount of phenolic compound and the capability of pro-inflammatory cytokine inhibition effect in the rat model. In addition, SC maintained the lung structure and showed fewer histopathological changes during acute pulmonary inflammation. Thus, we conclude SC is a beneficial natural herbal source of anti-inflammatory agents.

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