

Efficacy of *Lagopsis supina* to promote blood circulation, remove blood stasis, and block inflammation in a rat model of traumatic blood stasis

Tingting Xu^{1†}, Xiumei Wang^{1,3†}, Zhongwei He², Li Yang^{1*},
Rongrui Wei¹, Guoyue Zhong¹, Junwei He^{1*}

¹Jiangxi University of Chinese Medicine, Nanchang, China, ²School of Information Technology, Jiangxi University of Finance and Economics, Nanchang, China, ³College of Mongolian Medicine and Pharmacy, Inner Mongolia University for Nationalities, Tongliao, China

Lagopsis supina (Steph) IK. Gal. was a traditional Chinese medicinal plant for promoting blood circulation and removing blood stasis (PBCRBS), anti-inflammatory and diuresis with little scientific validation. The aims of this study were first to evaluate the PBCRBS and anti-inflammatory effects of *L. supina* in a rat model of traumatic blood stasis (TBS). We demonstrated that an ethanolic extract of *L. supina* (LS, 460 mg/kg/d) possessed significant PBCRBS and remarkable inhibitory effect on inflammation cytokines, which were associated with renovated amount of the injured muscle fibers, alleviate the degree of the damaged tissue edema, decreased the number of inflammatory cells, increased the number of the capillary hyperplasia by hematoxylin and eosin (HE), as well as remarkably down-regulated ($p < 0.05$ or $p < 0.01$) the levels of inflammation cytokines, including TNF- α , IL-6, IL-8, and IL-10 by ELISA. This finding provide a pharmacological basis and partial interpretation for the clinical application of *L. supina*, which has therapeutic properties for blood stasis syndrome (BSS) and inflammation-related diseases.

Keywords: *Lagopsis supina*. Promoting blood circulation and removing blood stasis (PBCRBS). Anti-inflammatory. Traumatic blood stasis (TBS).

INTRODUCTION

Traditional Chinese medicines (TCMs), one of the oldest medical systems of health care in China and other Asian countries, have been used to treat a variety of human diseases for many centuries due to their therapeutic efficacy, little side effects and broad applications (He *et al.*, 2016; Pang *et al.*, 2016; Li *et al.*, 2017; Qiu *et al.*, 2017; Zhou *et al.*, 2018). In recent years, TCMs have been recognized as ideal examples of complementary and alternative medicines and are typically composed of multiple components that have

multiple disease targets. TCMs increasingly attract attention from researchers worldwide. Blood stasis syndrome (BSS) is a serious pathological syndrome that can be treated with TCMs and Korean medicine (Dang *et al.*, 2015; He *et al.*, 2019; Jung *et al.*, 2018; Liu *et al.*, 2012). BSS is defined as a state in which blood circulation is turbulent or stagnant occurring as a result of traumatic injury, cardiovascular disease, and other diseases (Dang *et al.*, 2015; Jung *et al.*, 2018; Liu *et al.*, 2012). Many TCM formulas or extracts have been used to treat BSS and have achieved good clinical outcomes *via* promoting blood circulation and removing blood stasis (PBCRBS). Such outcomes include an inhibition of platelet aggregation, the promotion of the hemostasis release reaction, and other salutary outcomes (Dang *et al.*, 2015; Li *et al.*, 2009; Liu *et al.*, 2012; Zhang *et al.*, 2010). However, the lack of scientific evidence regarding the ability of TCMs to treat disease may lead to an underestimation

*Correspondence: J. W. He. Jiangxi University of Traditional Chinese Medicine. Nanchang, 330004, China. Phone: +86-791-87118873. Fax: +86-791-87118873. E-mail: hjwjin2008@163.com. ORCID: 0000-0001-6376-3505. L. Yang. Jiangxi University of Traditional Chinese Medicine. Nanchang, 330004, China. Phone: +86-791-87118658. Fax: +86-791-87118658. E-mail: yangli07971@163.com. †These authors contributed equally to this work.

of their value in modern medicine. Furthermore, the clinical application and quality control of TCMs still faces enormous challenges. To the best of our knowledge, a pattern or syndrome that reflects multi-system changes is an ideal candidate for TCM treatment. Therefore, the establishment of a whole animal model that reflects a certain functional state or a certain syndrome is of great importance in the pharmacological evaluation of TCMs. According to TCM theory, the rat of traumatic blood stasis (TBS) model was a simulation of the BSS in human patients (Tian *et al.*, 2006; Yan *et al.*, 2004). Moreover, inflammatory cytokines, such as IL-6, IL-8, TNF, IL-1 β , and NOS2, are involved in the processes by which TCMs act on PBCRBS according to experimental researches and network pharmacology analysis (Dang *et al.*, 2015; Lv *et al.*, 2015; Song *et al.*, 2015). Accordingly, TBS rat model has been used to evaluate efficacy for PBCRBS and anti-inflammatory activity of TCMs or prescriptions, such as *Salvia miltiorrhiza* Bunge (Dong *et al.*, 2013, 2014), arisaema plants (Wang *et al.*, 2017), *Asarum sieboldii* Miq. (Bai *et al.*, 2014), *Pinellia ternata* (Lv *et al.*, 2013), and Huo-xue-cu-yu capsules (Xu *et al.*, 2013).

Lagopsis supina (Steph) IK. Gal. is a perennial herbaceous plant species of the Labiatae family and widely distributed in northeast Asia. It was first described in the authoritative medical book of ancient China “Shennong’s Herbal Classics (Shen Nong Ben Cao Jing)” and is known to induce PBCRBS, block inflammation, and promote diuresis. The whole plants of *L. supina*, known as “Xiazhicao (夏至草)” in Chinese, has been used in TCM, specifically in Han, Tibetan, and Mongolian medicines (State Administration of Traditional Chinese Medicine 1999; Jia *et al.*, 2016). Previous phytochemical studies on *L. supina* have identified the presence of diterpenoids (Li *et al.*, 2014), flavonoid glycosides (Zhang *et al.*, 2015a), phenylethanoid glycosides (Zhang *et al.*, 2015b), and monoterpenes (Zhang *et al.*, 2015b). It has been suggested that *L. supina* improves blood and lymph microcirculation (Zhang *et al.*, 2004), cardioprotective (Liang *et al.*, 2008), antioxidative (Zhang *et al.*, 2008), and antiviral (Wang *et al.*, 2014) effects. However, the efficacy of *L. supina* for PBCRBS and for the inhibition of inflammation has not yet been studied in detail. Therefore, the purpose of the present study is to evaluate the efficacy for PBCRBS

of *L. supina* in the TBS rat model and to evaluate the capacity of *L. supina* to inhibit inflammatory cytokines.

MATERIAL AND METHODS

Materials and reagents

L. supina whole plants were collected in Keerqin District, Tongliao City, Inner Mongolia, China in June 2016 having been identified by one of the authors (Guoyue Zhong). A voucher specimen (no. XZC201606) was deposited at the Research Center of Natural Resources of Chinese Medicinal Materials and Ethnic Medicine, Jiangxi University of Chinese Medicine, Nanchang, China. The preparation of the crude extract was conducted according to our previously method (He *et al.*, 2019): briefly, the air-dried and powdered whole plants (38 kg) were exhaustively extracted using 95% EtOH (300 L \times 3) and subsequently 50% EtOH (300 L \times 3) by maceration at room temperature for seven days. After filtration, combination, and solvent evaporation, an ethanol crude extract of *L. supina* (LS, 8.7 kg, the yield was 23 %) was obtained. The crude ethanol extract was resuspended in 0.3% sodium carboxymethyl cellulose (CMC-Na) for subsequent intragastric administration and kept at -20°C until use.

Enzyme-linked immunosorbent assay (ELISA) kits for TNF- α , IL-1 β , IL-4, IL-6, IL-8, and IL-10 were purchased from Nanjing SenBeiJia Biological Technology Co., Ltd. (Nanjing, China). All of the other chemicals used in this study were of analytical reagent grade.

Animals and sample collection

All animal experimental protocols were reviewed and approved by the Animal Care and Research Committee of Jiangxi University of Traditional Chinese Medicine. Male Sprague-Dawley rats (weighing 180–220 g) were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China). Rats were housed in polyethylene boxes with free access to autoclaved water and food under a controlled room temperature (23 \pm 2)°C with 12 h light and dark cycles. All of the experiments were carried out in adherence with the guidelines of the Institutional Animal

Care and Use Committee of China and were approved by the Animal Care and Research Committee of Jiangxi University of Traditional Chinese Medicine.

After a week of adaptive feeding, 30 rats were randomly assigned to the sham operated group (control group), traumatic injury group (TBS model group) and LS-treated group (TBS + LS group, referred to as LS group), with 10 rats in each group. The TBS model group and LS group animals were established according to the method of TBS model published previously (Dong et al., 2013, 2014). Briefly, rats were fixed on a wooden board, and a 500 g weight was dropped from a height of 58 cm, hitting the midsection of the right hind limb (potential energy was approximately 2.86 Joule (J) and the striking area was 19.2 cm²). Unilateral claudication without obvious skin damage, bleeding, or obvious fracture confirmed the successful procedure.

According to clinical TCM practice the dosage of LS for adults (60 kg/person) is 9–30 g/kg/day (State Administration of Traditional Chinese Medicine, 1999). For rats, this dosage is 2 g raw herb/kg/day (equivalent 460 mg crude extract/kg/day) after adjusting for the difference in body surface area. Rats of LS group were orally administered crude drug (LS, 460 mg/kg) once daily for 7 successive days. The control group and TBS group received an equivalent amount of 0.3% CMC-Na. Each rat was housed individually in a metabolic cage, and the cumulative urine output was determined at daily intervals for 7 successive days and stored at -20°C for the examination of TNF- α , IL-1 β , IL-4, IL-6, IL-8, and IL-10 levels. At the end of the experimental period, 4 mL of blood was collected in vacuum tubes from the abdominal aorta, allowed to clot on ice, and subsequently subjected to centrifugation at 3000 rpm at 4°C for 10 min. All experiments were completed within 3 h after blood collection. Then, all of the rats were killed by cervical dislocation. Injured muscle tissue and kidney tissue were dissected for histopathological evaluation within 3 h.

Biochemical analysis

The serum and urine levels of TNF- α , IL-1 β , IL-4, IL-6, IL-8, and IL-10 were detected using ELISA kits, according to the manufacturer's instructions.

Histopathological analysis

Injured muscle and kidney tissue were fixed in 4% (w/v) paraformaldehyde over 24 h for histopathological examination (Liu *et al.*, 2018). Following graded ethanol dehydration, xylene and liquid paraffin series, specimens were embedded in paraffin wax. 4 μ m sections were obtained from paraffin embedded specimens. Subsequently, sections were deparaffinized, rehydrated and stained with hematoxylin and eosin (HE). Mounted sections were examined using a BX53 microscope (Olympus Corporation, Japan).

Statistical analysis

Data was analyzed using SPSS Statistics V17.0 software and represented as mean \pm standard deviation (SD). One-way analysis of variance (ANOVA) was used for multi-group comparisons following Tukey's test. $p < 0.05$ was considered to be statistically significant.

RESULTS

LS improves the behavior of TBS rats

The behavior of the TBS rats was slightly depressed as compared to control rats, and claudication was present. Additionally, the TBS rats exhibited reduced food intake and symptoms in the injured muscles, such as swelling, purple skin, and hypokinesia. In the LS group, the swelling in the injured muscles was significantly reduced. Furthermore, food intake and activity in LS group rats were similar to that of the control rats.

Biochemical results

The levels of TNF- α , IL-6, and IL-8 in serum were significantly increased in TBS group rats compared to control group rats ($p < 0.01$, Figure 1). However, the levels of IL-1 β , IL-4, and IL-10 in serum were no significant changes in TBS group rats compared to control group rats ($p < 0.01$, Figure 1). Following LS treatment, these high serum levels of TNF- α , IL-6, and IL-8 were decreased remarkably ($p < 0.01$).

As shown in Figure 2, the ELISA results revealed that the levels of TNF- α , IL-6, IL-8, and IL-10 in urine from TBS group rats were significantly upregulated compared to control group rats ($p < 0.01$). Moreover, the levels of IL-1 β and IL-4 in serum were no significant changes in TBS group rats compared to control group rats ($p < 0.01$, Figure 2). Conversely, the LS treatment group rats had significantly lower levels of TNF- α , IL-6, IL-8, and IL-10 in urine than the TBS group rats ($p < 0.01$ or $p < 0.05$).

Histopathological results

Muscle tissue

In the control group, muscle tissue exhibited a normal appearance and morphology by light microscopy (Figure 3A). However, muscles of TBS group rats showed significant tissue damage, including muscle fibers rearrangement (black arrow), inflammatory cell infiltration (yellow arrow), blood stasis (green arrow),

and edema (blue circle, Figure 3B). Treatment with LS improved these histopathological measures of tissue injury, including restoration of the injured muscle fibers, the alleviation of tissue edema, a decrease in inflammatory cell infiltration, and by the promotion of capillary hyperplasia (Figure 3C).

Kidney tissue

Control group rats exhibited normal kidney appearance by light microscopy (Figure 4A). Glomerular atrophy (blue circle), renal tubular epithelial cell swelling and deformation (blue arrow), capillary congestion (green arrow), and inflammatory cell infiltration (yellow arrow) were observed in TBS group rats (by arrows, Figure 4B). In contrast, treatment with LS improved the histopathological measures of renal injury, including recovery of the damaged glomerulus and renal tubules, the alleviation of capillary congestion, and by a decrease in inflammatory cell infiltration (Figure 4C).

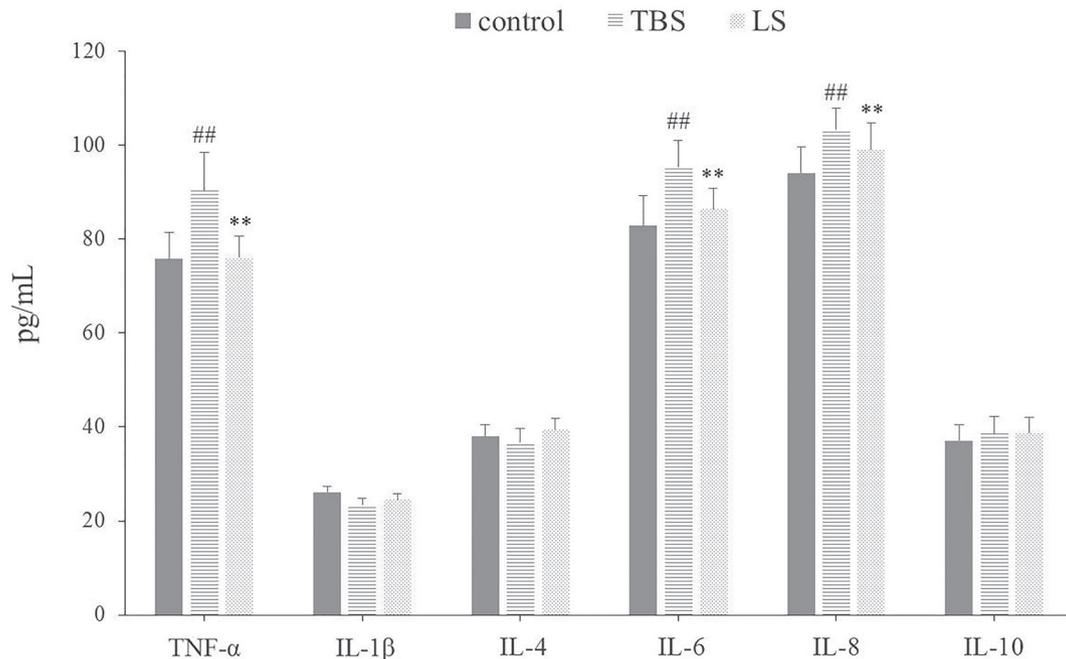


FIGURE 1 - The levels of TNF- α , IL-1 β , IL-4, IL-6, IL-8 and IL-10 from rat serum (pg/mL). Data shown are mean \pm SD (n = 10 per group). ^{##} $p < 0.01$ vs control group, ^{**} $p < 0.01$ vs TBS group.

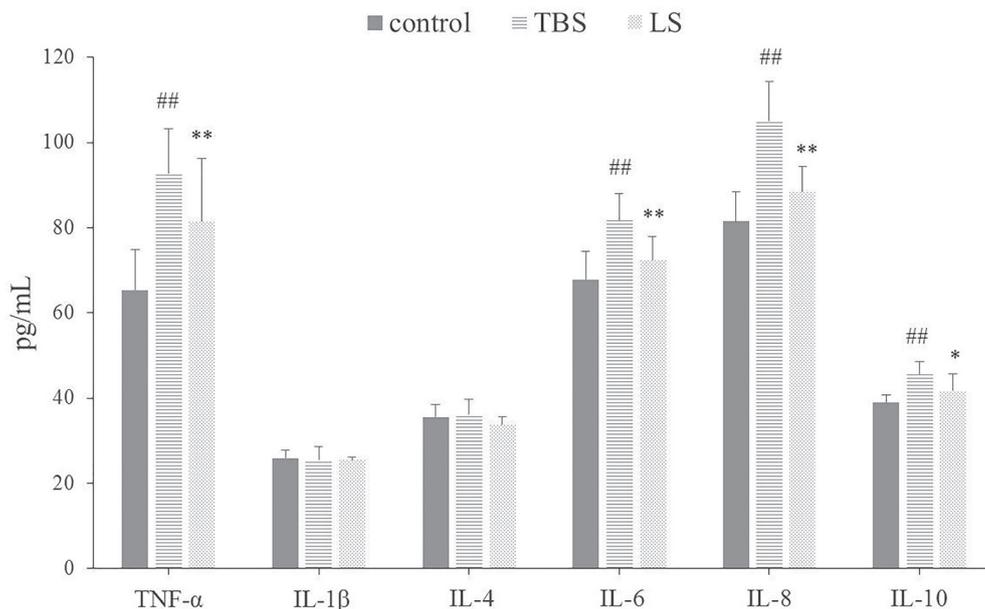


FIGURE 2 - The levels of TNF- α , IL-1 β , IL-4, IL-6, IL-8, and IL-10 from rat urine (pg/mL). Data shown are mean \pm SD (n = 10 per group). ^{##}*p* < 0.01 vs control group, ^{**}*p* < 0.01 and ^{*}*p* < 0.05 vs TBS group.

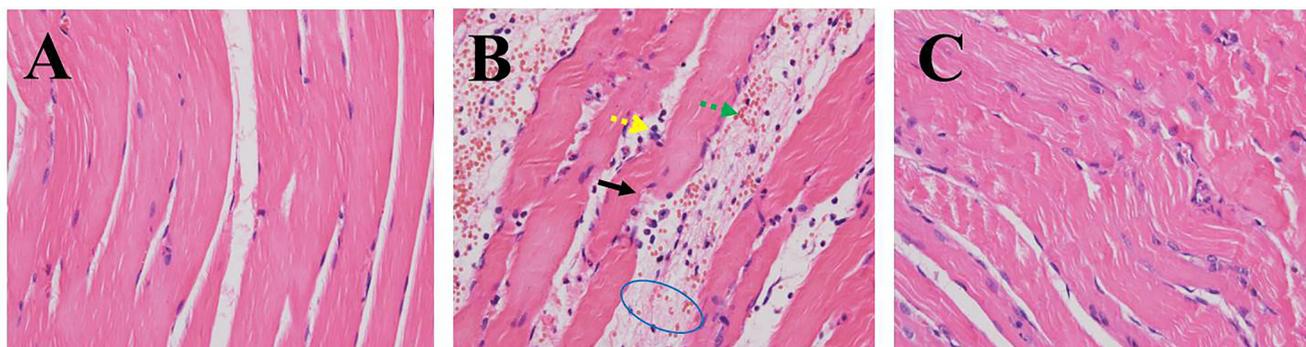


FIGURE 3 - Effect of LS on injured muscle in the TBS rats by HE staining (400 \times magnification, black arrow-muscle fiber, yellow arrow-inflammatory cell, green arrow-blood stasis, blue circle-edema). (A) Control group; (B) TBS group; and (C) LS group.

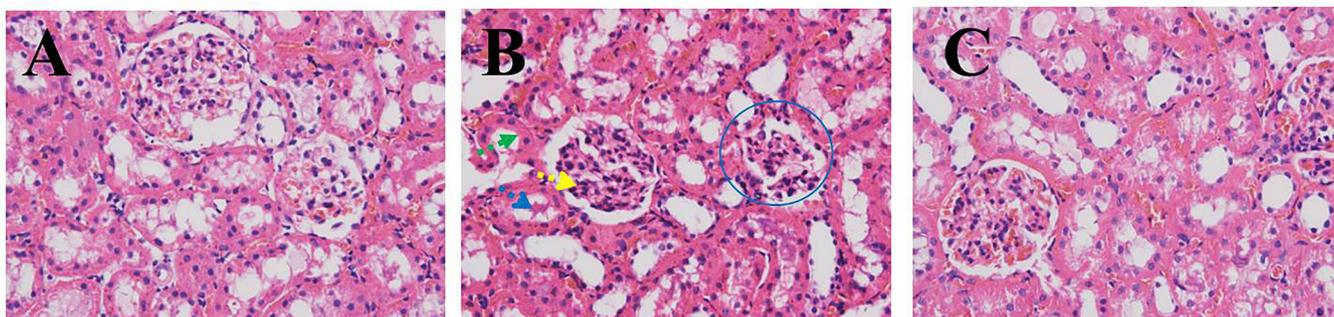


FIGURE 4 - Effect of LS on renal damage in the TBS rats by HE staining (400 \times magnification, blue circle-glomerular atrophy, blue arrow-renal tubular epithelial cell, green arrow-capillary, yellow arrow-inflammatory cell). (A) control group; (B) TBS group; and (C) LS group.

DISCUSSION

Although some studies have indicated that the crude extract of *L. supina* might improve blood and lymph microcirculation in a rat model of acute blood stasis induced by chemical drugs, such as Dextran 500 (Zhang *et al.*, 2004), it cannot fully reflect the efficacy of PBCRBS. BSS is a result of a series of pathological changes caused by blood stasis and can lead to edema, tissue degeneration, and inflammation (Dong *et al.*, 2013, 2014; Kim *et al.*, 2018). In this experiment, we observed that muscles of BSS model group (TBS group) rats showed significant tissue damage, including muscle fibers rearrangement, inflammatory cell infiltration, blood stasis, and edema compared with those of the model group. Moreover, glomerular atrophy, renal tubular epithelial cell swelling and deformation, capillary congestion, and inflammatory cell infiltration were observed in TBS group rats compared with the model group. Treatment with LS improved these histopathological measures of tissue injury, edema, and inflammation. These results indicate that LS could remarkably improve the pathological changes of BSS in a rat model of TBS.

Inflammation is a very important and common regulated process of the host defence system. Moreover, many factors including physical stress, chemical stress, and microbiological toxins can cause chronic inflammatory diseases, such as inflammatory bowel disease, blood stasis disease, arthritis and hyperlipidemia (Song *et al.*, 2015; Zhai *et al.*, 20016). Inflammatory cytokines, such as IL-6, IL-8, TNF, IL-1 β , and NOS2, are involved in the processes by which TCMs act on PBCRBS according to experimental researches and network pharmacology analysis (Dang *et al.*, 2015; Lv *et al.*, 2015; Song *et al.*, 2015). Therefore, it is very important to assess the efficacy of *L. supina* for anti-inflammatory activity in the TBS rat model. However, the anti-inflammatory activity of *L. supina* has only been reported by one study (Li *et al.*, 2014), which showed that four diterpenoids isolated from *L. supina* moderately inhibited lipopolysaccharide (LPS) induced nitric oxide production by BV-2 microglial cells. In the present study, this is the first reported notable anti-inflammatory effect by an ethanolic extract of *L. supina*, which probably was through downregulated the levels of

the inflammatory cytokines TNF- α , IL-6, IL-8 and IL-10 in a rat model of TBS.

CONCLUSIONS

To summarize our findings, this is the first study to investigate the effects of *L. supina* on PBCRBS and anti-inflammatory properties in a TBS rat model. Based on our results, we conclude that *L. supina* carries out its PBCRBS and anti-inflammatory effect mainly by improve the pathological changes of BSS and downregulated the levels of the inflammatory cytokines (TNF- α , IL-6, IL-8, and IL-10) in a rat model of TBS, respectively. These finding suggest that LS may warrant further evaluation as a possible agent for the treatment of BSS and inflammatory diseases. Further work is needed to interpret the precise active components of LS and the underlying molecular mechanisms by which they exert their effects.

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