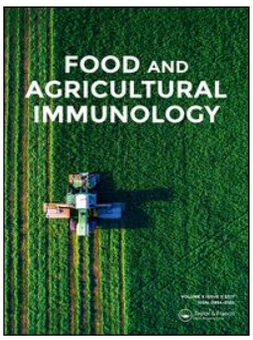

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# Salvia miltiorrhiza polysaccharides protect against lipopolysaccharide-induced liver injury by regulating NF-κb and Nrf2 pathway in mice

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**ABSTRACT**

The current study was conducted to investigate the hepatoprotective effects of Salvia miltiorrhiza polysaccharides (SMP) against lipopolysaccharide (LPS) induced liver injury. The mice were treated with SMP first and LPS later. Results showed that SMPs significantly reduced the activities of serum alanine aminotransferase (ALT), aspartate transaminase (AST), total bilirubin (TBIL), malondialdehyde (MDA) and contents of inflammation factors and mRNA expressions in LPS induced liver injury mice. However, SMP significantly increased the contents of glutathione (GSH), Superoxide dismutase (SOD) and Total antioxidant capacity (T-AOC). SMP also downregulated the expressions of p-p65, p-IκBα, inducible nitricoxide synthase (iNOS) and improved the expression of Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and Heme Oxygenase-1 (HO-1). The study indicates SMP protects the liver via attenuating inflammatory reactions and antioxidant effects.

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**KEYWORDS**
Immunological liver injury; inflammatory factor; antioxidant effects; Salvia miltiorrhiza polysaccharides (SMPs)

## 1. Introduction

Liver injury is a clinical multiple pathological status. Drugs, toxin and autoimmune responses can cause liver damage, its long-term presence often leads to liver fibrosis, and even cirrhosis and hepatocellular carcinoma (Sugiyama, Sato, Shimizu, Ando, & Takeuchi, 2010). There are a lot of Gram-negative bacteria in the intestinal tract which will give out lipopolysaccharide (LPS). LPS is a major component of the outer membrane that plays a key role in host–pathogen interactions with the innate immune system. The classical LPS molecule has a tripartite structure comprising (i) lipid A, the hydrophobic moiety that anchors LPS to the outer leaflet of the outer membrane; (ii) core oligosaccharide (herein core), which together with lipid A, contributes to maintain the integrity of the

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outer membrane; and (iii) O antigen polysaccharide or O antigen, which is connected to the core and consists of a polymer made of repeating oligosaccharide units in direct contact with the external milieu (Whitfield & Trent, 2014). LPS is also the main factor that the gram-negative bacteria triggers the host's immune responses, leading to the body injury (Nolan, 2010). The liver is the primary organ that intestinal endotoxin challenges and is also a common target organ for immune injury. In severe trauma and infection stresses, great amount of intestinal LPS enters blood circulation, and then causes rapidly increasing concentration of LPS in the blood to aggravate liver damage (Magdaleno, Blajszczak, & Nieto, 2017). Experimental studies have shown that LPS causes liver damage through Th1 cytokines such as TNF-α and IFN-γ. Studies on alcoholic early liver injury model in mice suggest that plasma endotoxin can activate immune cells, promote releases of TNF-α, IFN-γ, IL-6 and increase expressions of other inflammatory cytokines, leading to liver damage (Li et al., 2014; Uesugi, Froh, Arteel, Bradford, & Thurman, 2001). LPS can not only increase liver cirrhosis and alcoholic liver injury, LPS itself can also lead to liver injury. However, the mechanism by which endotoxin activates Th1 cytokine-induced signal transduction pathways and subsequent mechanisms of tissue damage is unclear.

There is a close relationship between Chinese herb and food. In fact, many kinds of food are regarded as Chinese herbs under the guidance of traditional Chinese medicine (TVM) theory, and a large number of Chinese herbs are also used as food in people's daily life (Liu, Li, Xu, & Zhu, 2016). Nowadays, with the continuous improvement of people's awareness of health, more and more Chinese medicines have been developed into functional food (Liu, Wen, Kan, & Jin, 2015). The development of those functional foods is mainly based on traditional Chinese medicine pharmacology (Zou, 2016). Through the research of the modern food science and technology, the mechanism of action, active ingredients, utilization methods and toxic and side effects of traditional Chinese medicine are precisely studied and utilized, which makes the health products of traditional Chinese medicine can play a more effective role. Salvia miltiorrhiza, as a traditional Chinese medicine, has also been developed into a variety of health productions for heart disease, diabetes and so on (Zhang, 2010). Salvia miltiorrhiza polysaccharide as a polysaccharide mixture extracted from Salvia miltiorrhiza, its own characteristics are extremely suitable for the development of health food. Therefore, exploring the pharmacological effects of Salvia miltiorrhiza polysaccharides is helpful for the development of health food.

In the pharmacological and clinical studies, the compounds of Salvia miltiorrhiza, tanshinone and salvianolic acid were found to be strong antioxidants and potent free radical scavengers (Zhang, Bao, Wu, Rosen, & Ho, 1990; Zhao, Xiang, Ye, Yuan, & Guo, 2006). These compounds could improve blood circulation, reduce the area of cerebral infarct, and inhibit the renin angiotensin system (Lao et al., 2003; Wang et al., 2017; Zhao et al., 2008). And other modern pharmacological studies have found that some effective components of Salvia miltiorrhiza has anti-inflammatory and immunomodulation effects (Ma, Zhang, Lou, Sun, & Ji, 2016; Maione et al., 2015; Xie, Ma, Lou, Zhu, & Sun, 2014). Research has shown that Salvia miltiorrhiza polysaccharide (SMP) has protective effect against immunological liver injury (Song et al., 2008). However, the mitigation mechanism of SMP on immune liver injury is not clear so far. Some studies have shown that the mechanism of other ingredients of Chinese Herbal medicine in alleviating

immunological liver injury is closely related to their anti-inflammatory and immunomodulatory effects (Hu et al., 2014; Sun et al., 2010; Zhong, Chiou, Pan, & Shahidi, 2012). In this research, we aimed to explore whether SMP alleviates immune liver injury through regulating inflammation and antioxidant. And the effects of SMP on NF-κB pathway and Nrf2 pathway were studied. Further explored was the mechanism of SMP in alleviating immune liver injury with an aim to provide the basis for further development and clinical application of herbal polysaccharides.

## 2. Materials and methods

### 2.1. Drugs and reagents

SMP (purity ≥95%) were purchased from Hunan Sunaccord Biological Technical Co. Ltd (Hunan, China). LPS was purchased from Sigma (St. Louis, MO, USA). Commercial kits used for ALT, AST, TBIL, MDA, GSH, SOD and T-AOC were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Commercial kits used for determining TNF-α, IL-6, IL-1β, and IL-17A were obtained from R&D Systems (Minneapolis, MN, USA). Other chemicals used in these experiments of analytical grade were from commercial sources.

### 2.2. Animals

Kunming mice (18–22 g), 6–8 weeks old, were purchased from the SPF (Beijing, China) Biotechnology Co, Ltd (NO SCXK(Jing)2016-0002). They were allowed feed and water *ad libitum*. The mice were housed in plastic cages and maintained under standard conditions (12 h light/12 h dark cycle; 25 ± 3°C; 35–60% relative humidity). The experimental protocals were approved by the Animal Welfare Committee of Agricultural University of Hebei. (Permission number: AUH-2018008).

### 2.3. Study design

One hundred Kunming mice were randomly divided into five groups ($n = 20$). Group A is the control group. Group B is the LPS model group. Group C is LPS + 500 mg/kg SMP group. Group D is the LPS + 250 mg/kg SMP group and group E is LPS + 125 mg/kg SMP group. Mice in groups C, D and E, were given different doses SMP daily via gavage for 7 days consecutively. Mice in groups A and B were given an equal volume of normal saline (NS). Seven days later, each mouse in groups B, C, D, E was given 250 μg of LPS in 0.2 ml NS via lateral tail vein injection. Mice in group A were given an equal volume of NS. Two hours and six hours after the injection of LPS, ten mice at a time were weighed and euthanized by 33% carbon dioxide and sacrificed to collect blood sample from mice orbital vein. Livers were immediately collected. Mice livers were partly fixed in neutralized formalin for H&E staining and analyzed for pathological changes. The remaining livers were stored at −80°C for other analysis.

### 2.4. Liver function and cytokines assay

Plasma was separated after centrifugation at 300 g for 5 min. ALT AST and TBIL levels in serum and MDA, GSH, SOD, T-AOC contents in liver tissues were measured by

Semiautomated clinical chemistry analyser (Microlab 300, The Netherlands). The concentrations of TNF-α, IL-6, IL-1β, and IL-17A were detected using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D system, USA).

### 2.5. Histopathology assay

Liver tissues were harvested at 6 h after LPS administration intravenously. Liver samples were fixed in 4% buffered paraformaldehyde for at least 24 h. Sections of 5 ìm on slides were deparaffinized in xylene, rehydrated in degrading concentrations of ethanol, and stained with hematoxylin and eosin (H&E).

### 2.6. RNA isolation and real-time PCR analysis

Total RNA was isolated from the homogenate of the liver with Trizol reagent (Invitrogen, USA) at 8 h after LPS challenge. Cellular RNA was treated with DNase I and then primed with a dT oligonucleotide and reversely transcribed with Superscript II. For real-time assays, PCR reactions were prepared in SYBR Green PCR Master Mix. DNA targets were amplified and analyzed with a Chromo Real-Time PCR Detection System (Bio-Rad Life Sciences). The murine primer sequences are shown as follows. Mouse TNF-α (Forward, F): 5-GGGCTACAGGCTTGTCACTCG-3 and (Reverse, R): 5-ACTCCAGGCGGTGCCTATGTC-3, Mouse IL-6 (F) 5′-CTGATGCTGGTGACAACCAC-3′ and (R): 5′-TCCACGATTTCCCAGAGAAC-3′, and Mouse IL-1β (F): 5-TCCAGTTAAGG- AGCCCTTTTAGACC-3 and (R): 5-TGAAATCATCCCTGCGAGCCTAT-3, Mouse IL-17A (F): 5-CCTCAAACTTGGCAATACTCA-3 and (R): 5-CTCAAGTGGCATAGATGTGGA-3, Mouse iNOS (F): 5-GGACCCAGTGCCCTGCTTT-3 and (R): 5-GAAAGCCCTCTACAGTGACATC-3; GAPDH (F): 5-AGAGTGGGAGTTGCTGTTG-3 and (R): 5-GCCTTCCGTGTTCCTACC-3. Total RNA was treated with DNase I to eliminate genomic DNA contamination, followed by synthesis of the first-strand using reverse transcription system. Reverse transcription was carried out as follows: 42°C for 60 min, 70°C for 10 min, and first-strand cDNA was stored at −20°C. Real-time PCR was performed in a 20 μL of reaction solution containing SYBR Premix Ex Taq, primers, and cDNAs. The cycles for PCR were as follows: 95°C for 2 min, 40 cycles of 95°C for 15 s, 58°C for 20 s, and 72°C for 20 s. Melting curves were determined by heat-denaturing PCR products over a 35°C temperature gradient at 0.5°C/sec from 65 to 99.5°C. GAPDH was used as an internal control (Cao, Zou, Zhu, Fan, & Li, 2013; Tu, Han, Liu, & Zhang, 2011). The relative amount of mRNA was determined using the $2^{-\Delta\Delta CT}$ technique as described previously (Livak & Schmittgen, 2001). The levels of mRNA were expressed as fold-changes after normalization to GAPDH.

### 2.7. Western-blotting analysis for protein expressions of NF-κB and Nrf2 pathway

Livers were carefully excised and homogenized into lysis buffer (Thermo, Waltham, MA, USA) to yield a homogenate. After centrifugation (12,000 g for 10 min) at 4°C, protein concentration was detected by Bradford protein assay kit (Thermo, Waltham, MA, USA) with bovine serum albumin as standard. Equal amounts of protein extracts were

separated discontinuously onto 10% polyacrylamide gels (Life Technologies, Carlsbad, CA, USA) and transferred to nitrocellulose membranes (Life Technologies, Carlsbad, CA, USA). After blockade of nonspecific binding sites, membranes were incubated with various antibodies against phospho-IκBα, phospho-NF-κB p65 (Cell Signaling Technology, Danvers, MA, USA) and iNOS, Nrf2, HO-1 (R&D Systems, Minneapolis, MN, USA) for 6 h at room temperature. Membranes were developed by chemiluminescence using an Amersham prime ECL Plus detection system (GE Healthcare Life, fairfield, CT, USA).

### 2.8. Statistical analysis

Data were recorded using the Excel database and further analyzed using SPSS19.0 software (IBM corporation, Armonk, NY, USA). Differences in parameters were analyzed by analysis of variance (ANOVA). The significance levels were $P < 0.05$(*) or $P < 0.01$(**).

## 3. Results

### 3.1. Effects of SMP on pathological changes in immunological liver injury mice

Of the control group, histological architecture of the liver was clear. The cytoplasm was deeply stained, and the inflammatory cells were not infiltrated in the portal area. The central meridian hepatocytes were swollen and degenerated, necrotic of the model group (Figure 1B). A lot of inflammatory cells were infiltrated, while SMP group showed less damage of liver cells, indicating that the herbal ingredient alleviated the injury (Figure 1C–E).

### 3.2. Effects of SMP on serum activities of ALT, AST and TBIL content in immunological liver injury mice

Immunological liver injury induced by LPS provoked a significant increment of ALT, AST and TBIL contents in the LPS group as compared to the control group at two and eight hours ($P < 0.01$) (Figure 2a–c) This indicated that the immunological liver injury model in mice was successfully duplicated. Results showed that treatment with SMP significantly restored ALT and AST activities ($P < 0.01$ or $P < 0.05$) (Figure 2a,b) and reduced TBIL content ($P < 0.01$) (Figure 2c).

### 3.3. The anti-inflammatory effect of SMP on LPS induced hepatitis

LPS-induced hepatitis is associated with the production of various proinflammatory cytokines. After LPS administration, the levels of proinflammatory cytokines such as TNF-á, IL-6, IL-1â and IL-17A were elevated in the plasma ($P < 0.01$). In the SMP pretreatment group, these inflammatory cytokines mentioned above decreased dramatically at 2 and 8 h after LPS administration ($P < 0.01$) (Figure 3a–d).

Subsequently the effects of SMP on expressions of TNF-α, IL-6, IL-1β and IL-17A mRNA in liver were investigated at 6 h after LPS administration intravenously. The results showed that levels of TNF-α, IL-6, IL-1β and IL-17A mRNA significantly increased

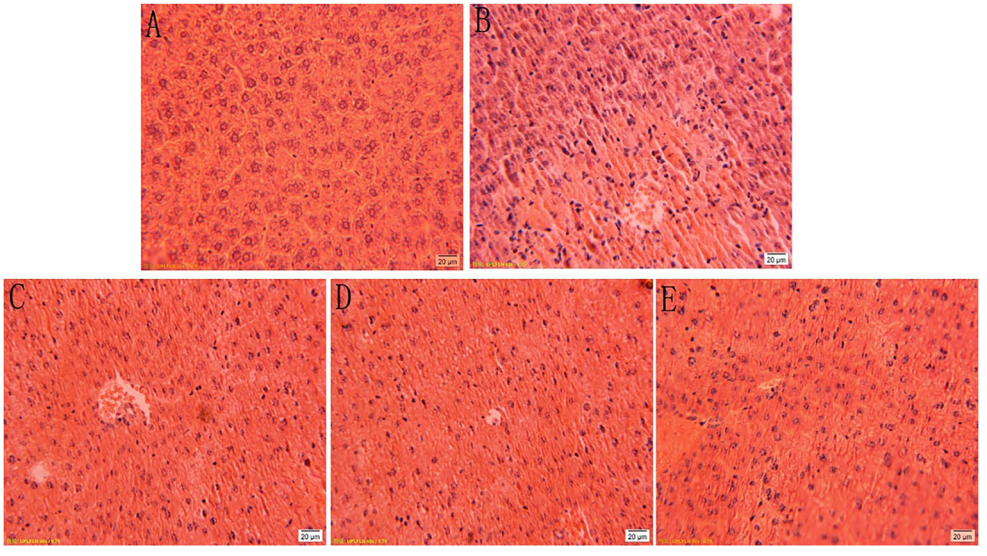


**Figure 1.** Histopathological changes of mouse liver. The sections were stained with H&E (400 X). (A) the Control group, histological structure of the liver was clear. (B) LPS (10 mg/kg) group, the central meridian hepatocytes were swollen and degenerated. (C) LPS (10 mg/kg) + high-dose (500 mg/kg) SMP, (D) LPS (10 mg/kg) + high-dose (250 mg/kg) SMP, (E) LPS (10 mg/kg) + high-dose (125 mg/kg) SMP, liver cells were less damaged.

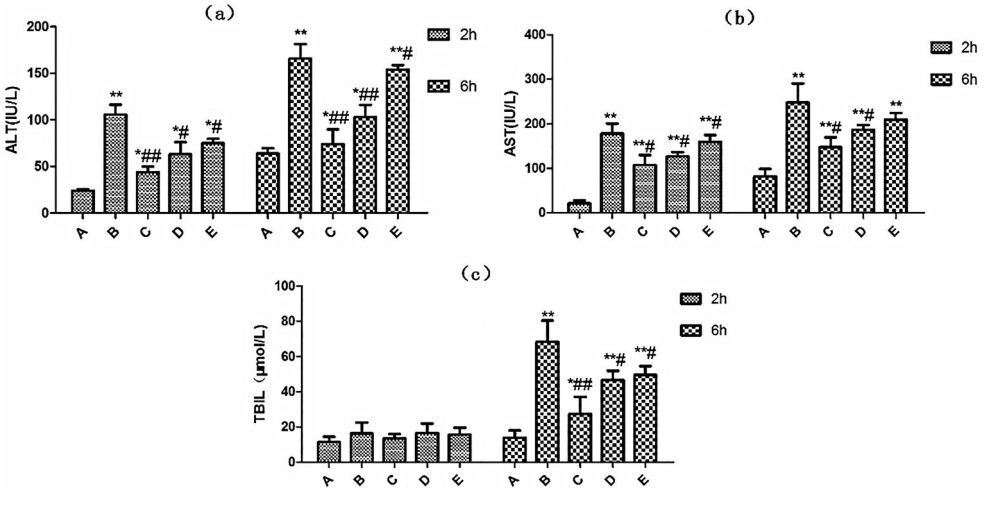


**Figure 2.** Treatment with SMP reduced immunological liver injury in mice. Serum levels of ALT (a), AST (b) and TBIL (c) were analyzed as a measure of hepatocellular injury at two hours and six hours. Group A is Control group, Group B is LPS (10 mg/kg) group, Group C is LPS (10 mg/kg) + high-dose (500 mg/kg) SMP. Group D is LPS (10 mg/kg) + middle-dose (250 mg/kg) SMP and group E is LPS (10 mg/kg) + low-dose (125 m mg/kg) SMP. Data represented mean ± S.D.; $n = 10$ mice per group. $^{**}P < 0.01$, compared with group A. $^{*}P < 0.05$, compared with group A. $^{\#\#}P < 0.01$, compared with group B. $^{\#}P < 0.05$, compared with group B.

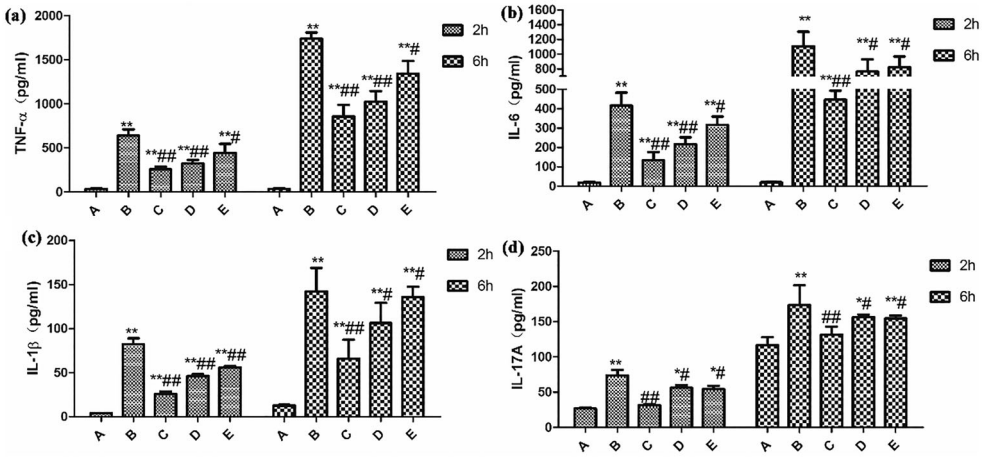


**Figure 3.** Treatment with SMP reduced immunological liver injury in mice. Serum levels of TNF-á(a), IL-6(b), IL-1â (c) and IL-17A (d) were analyzed as a measure of hepatocellular injury. Group A is Control group, Group B is LPS (10 mg/kg) group, Group C is LPS (10 mg/kg) + high-dose (500 mg/kg) SMP. Group D is LPS (10 mg/kg) + middle-dose (250 mg/kg) SMP and group E is LPS (10 mg/kg) + low-dose (125 m mg/kg) SMP. Data represented mean ± S.D.; $n = 10$ mice per group. $^{**}P < 0.01$, compared with group A. $^{*}P < 0.05$, compared with group A. $^{\#\#}P < 0.01$, compared with group B. $^{\#}P < 0.05$, compa with red group B.

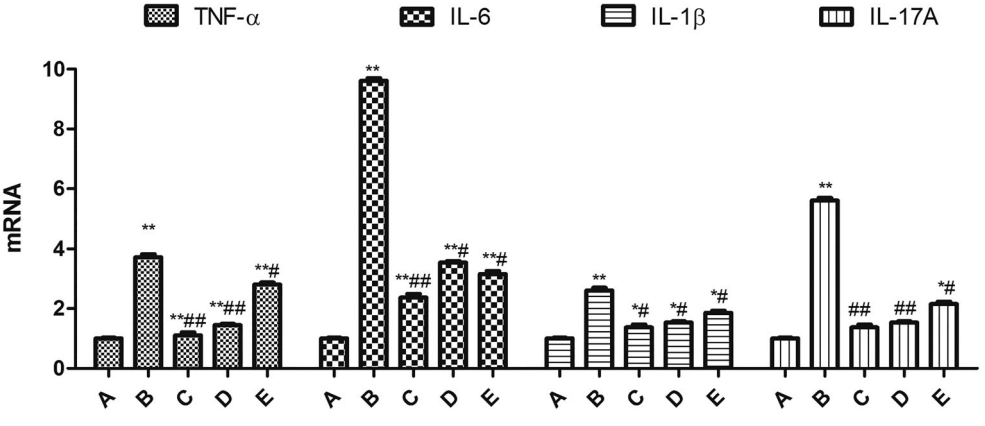


**Figure 4.** Treatment with SMP reduced immunological liver injury. The expressions of TNF-á, IL-6, IL-1â and IL-17A mRNA in hepatic tissue were analyzed as a measure of hepatocellular injury. Group A is Control group, Group B is LPS (10 mg/kg) group, Group C is LPS (10 mg/kg) + high-dose (500 mg/kg) SMP. Group D is LPS (10 mg/kg) + middle-dose (250 mg/kg) SMP and group E is LPS (10 mg/kg) + low-dose (125 m mg/kg) SMP. Data represented mean ± S.D.; $n = 10$ mice per group. $^{**}P < 0.01$, compared with group A. $^{*}P < 0.05$, compared with group A. $^{\#\#}P < 0.01$, compared with group B. $^{\#}P < 0.05$, compared with group B.

in LPS model group compared to the control group ($P < 0.01$). Pretreatment with SMP significantly reduced the mRNA levels of those cytokines in the liver tissue ($P < 0.01$ or $P < 0.05$) (Figure 4).

### 3.4. SMP pretreatment suppressed phosphorylation of IκBα and p65

The production of proinflammatory cytokines, including TNF-α and IL-6, is largely regulated by the activation of transcription factors such as NF-κB. We then explored whether SMP inhibits NF-κB activation *in vivo*, which is revealed by the phosphorylation of IκBα and p65. Our results revealed that SMP pretreatment inhibited phosphorylation of IκBα and p65 compared with LPS model group ($P < 0.01$) (Figure 5).

### 3.5. SMP pretreatment suppressed NO and iNOS expression

NO is one of the important factors to promote liver damage, and iNOS is a synthase of NO, its expression directly determines the amount of NO secretion, is an important indicator of immunological liver injury.so we investigated the effects of SMP on secretions of NO in serum and expressions of iNOS in liver tissue, we found that SMP pretreatment significantly decreased NO secretion in serum ($p < 0.01$) (Figure 6a) and significantly reduced the mRNA and protein expressions of iNOS in liver tissue ($p < 0.01$) (Figure 6b).

### 3.6. The antioxidant effect of SMP on LPS induced hepatitis

We found that when LPS was injected into mice, the content of MDA in liver tissue was significantly increased while the contents of GSH, SOD and T-AOC were significantly decreased ($P < 0.01$). Compared with LPS model group, pretreatment with SMP could

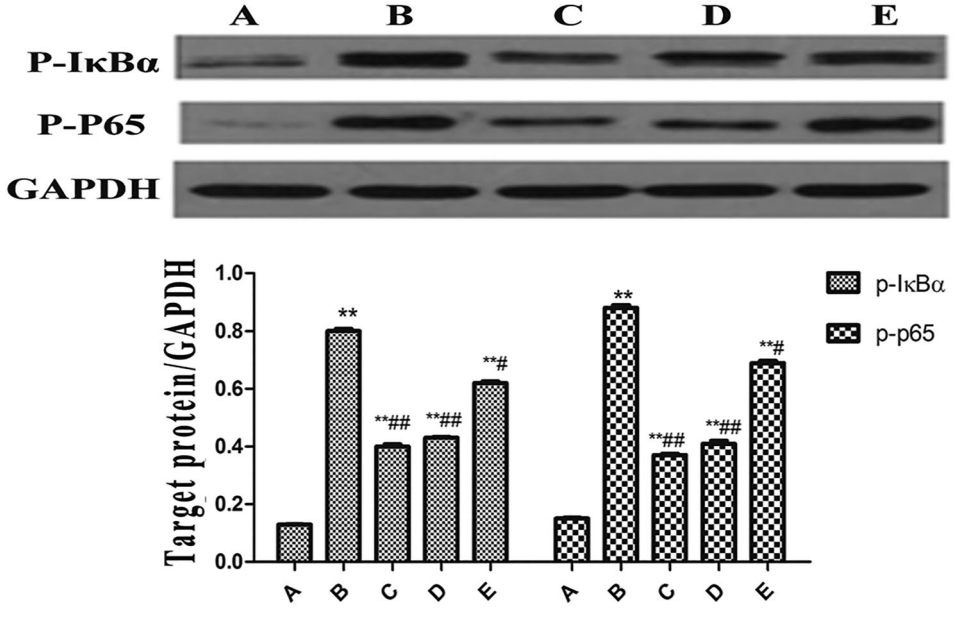


**Figure 5.** Effects of SMP on p-p65 and p-lêBá protein levels in hepatic tissue. Group A is Control group, Group B is LPS (10 mg/kg) group, Group C is LPS (10 mg/kg) + high-dose (500 mg/kg) SMP. Group D is LPS (10 mg/kg) + middle-dose (250 mg/kg) SMP and group E is LPS (10 mg/kg) + low-dose (125 m mg/kg) SMP. Data represented mean ± S.D.; $n = 10$ mice per group. $^{**}P < 0.01$, compared with group A. $^{*}P < 0.05$, compared with group A. $^{\#\#}P < 0.01$, compared with group B. $^{\#}P < 0.05$, compared with group B.

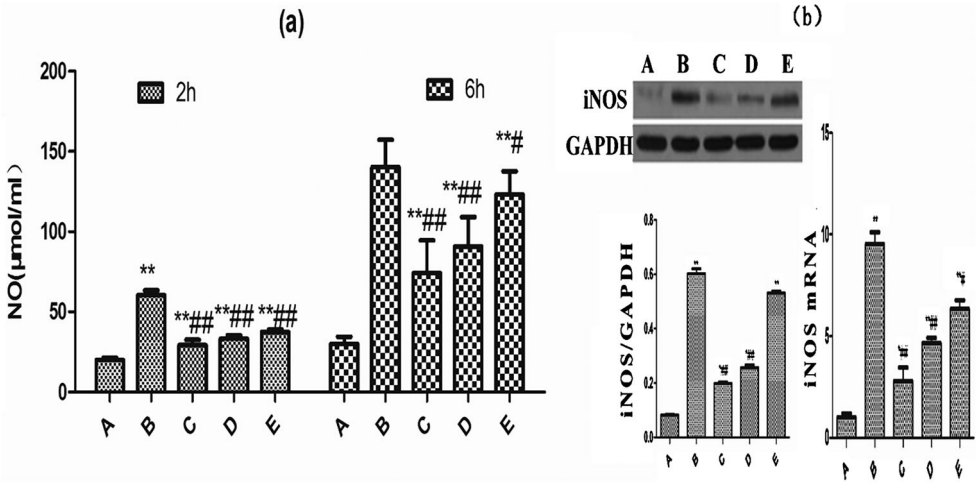


**Figure 6.** Effects of SMP on NO secretion amount in mice serum at two hours and six hours after LPS administration intravenously (a) and iNOS expressions in mice hepatic tissue at six hours after LPS administration (b). Group A is Control group, Group B is LPS (10 mg/kg) group, Group C is LPS (10 mg/kg) + high-dose (500 mg/kg) SMP. Group D is LPS (10 mg/kg) + middle-dose (250 mg/kg) SMP and group E is LPS (10 mg/kg) + low-dose (125 m mg/kg) SMP. Data represented mean ± SD; $n = 10$ mice per group. $^{**}P < 0.01$, compared with group A. $^{*}P < 0.05$, compared with group A. $^{\#\#}P < 0.01$, compared with group B. $^{\#}P < 0.05$, compared with group B.

significantly inhibit the increase of MDA content in liver tissue and the decrease of GSH, SOD and T-AOC content ($P < 0.05$ or $P < 0.01$) (Figure 7). Nrf2 is an important transcription factor that regulates the cellular oxidative stress responses and is also the central regulator in maintaining the intracellular redox homeostasis. In our study, we also found that LPS significantly decreased the proteins of Nrf2 and HO-1 ($P < 0.05$). However, different doses of SMP could significantly alleviate the decrease of Nrf2 and HO-1 induced by LPS ($P < 0.05$) (Figure 8).

## 4. Discussions

Hepatitis, caused by virus infection and some certain drugs as well as autoimmune factors, is an increasingly frequent health issue. Despite advances in medical science and understanding of pathogenesis of hepatitis, effective therapeutic approaches remain unresolved. In the current study, we investigated the protective effects of SMP on LPS-induced hepatitis.

Salvia miltiorrhiza polysaccharide is a homogeneous polysaccharide mixture extracted and purified from Salvia miltiorrhiza. It is composed of five different monosaccharides: mannose, rhamnose, arabinose, glucose and galactose. The total sugar content of Salvia miltiorrhiza polysaccharide is more than 90%, which contains a small amount of uric acid and does not contain protein and nucleic acid. A research has reported that the molecular weight of Salvia miltiorrhiza polysaccharide is about 69 KDa (Liu et al., 2013). Current studies have proved that SMP has protective effects on insulin resistance, antioxidants (Jiang et al., 2014; Wenlin, Zhu, Zhang, Yang, & Zhou, 2012), immune regulation

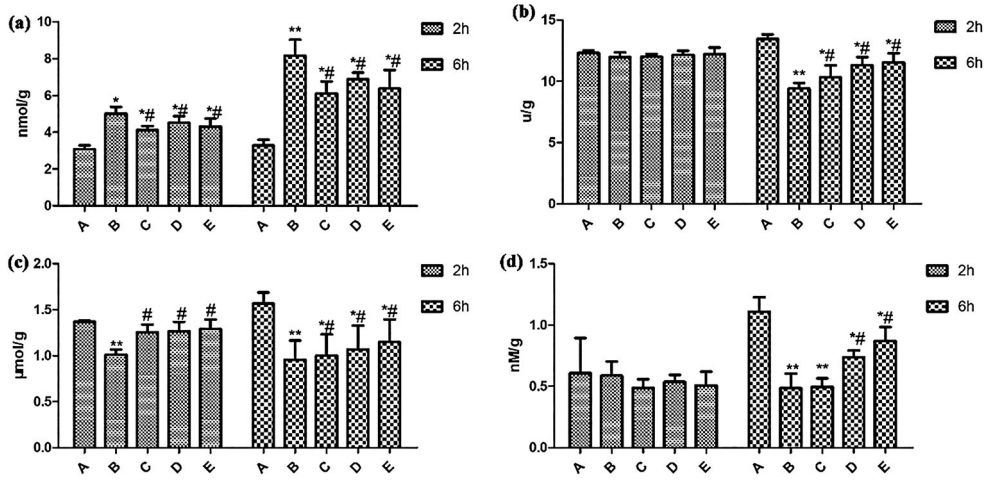


**Figure 7.** Treatment with SMP reduced immunological liver injury. levels of MDA (a), SOD (b), GSH (c) and T-AOC (d) in hepatic tissue were analyzed as a measure of hepatocellular injury. Group A is Control group, Group B is LPS (10 mg/kg) group, Group C is LPS (10 mg/kg) + high-dose (500 mg/kg) SMP. Group D is LPS (10 mg/kg) + middle-dose (250 mg/kg) SMP and group E is LPS (10 mg/kg) + low-dose (125 m mg/kg) SMP. Data represented mean ± S.D; $n = 10$ mice per group. $^{**}P < 0.01$, compared with group A. $^{*}P < 0.05$, compared with group A. $\#\#P < 0.01$, compared with group B. $\#P < 0.05$, compared with group B.

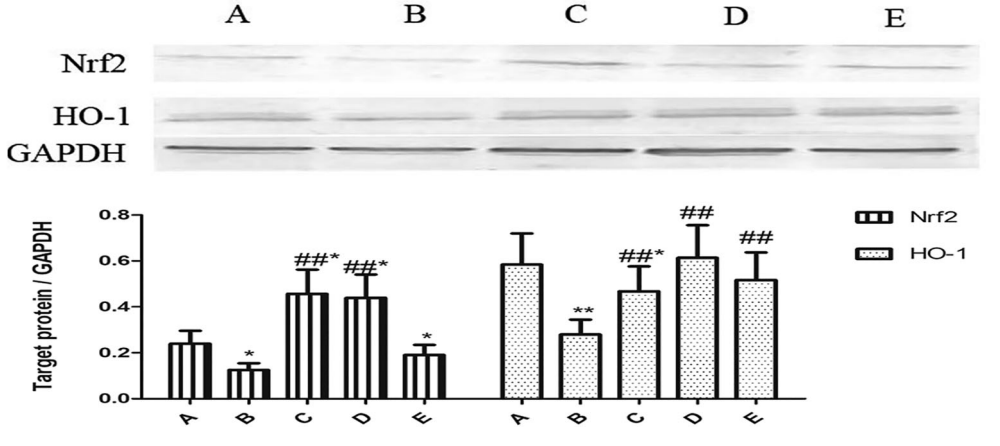


**Figure 8.** Effects of SMP on Nrf2 and HO-1 protein levels in hepatic tissue. Group A is Control group, Group B is LPS (10 mg/kg) group, Group C is LPS (10 mg/kg) + high-dose (500 mg/kg) SMP. Group D is LPS (10 mg/kg) + middle-dose (250 mg/kg) SMP and group E is LPS (10 mg/kg) + low-dose (125 m mg/kg) SMP. Data represented mean ± S.D.; $n = 10$ mice per group. $^{**}P < 0.01$, compared with group A. $^{*}P < 0.05$, compared with group A. $\#\#P < 0.01$, compared with group B. $\#\ P < 0.05$, compared with group B.

(Revajová, Pistl, Levkut, Marcin, & Levkutová, 2010), anti-tumor (Liu et al., 2013; Wang et al., 2014), cardioprotective (Geng, Huang, Song, & Song, 2015; Song, Huang, Zhao, & Song, 2013) and hepatoprotective properties (Song et al., 2008). Our results also show that Salvia miltiorrhiza polysaccharides can antagonize immune liver injury due to their

immunomodulatory and antioxidant effects. Salvia miltiorrhiza polysaccharide can be used to develop a Chinese medicine health product due to its liver protection effect.

In our present study, SMP pretreatment showed a protective effect on LPS-induced hepatitis, reduced the levels of ALT and AST in plasma as well as the severity of hepatic necrosis. The potential mechanisms of its beneficial effects involve in inhibiting inflammatory responses and infiltration of lymphocytes. So, activated immune system has been found to play an important role in the development of liver diseases (Luster, Simeonova, Gallucci, Matheson, & Yucesoy, 2000).

TNF-α released from inflammatory cells is considered essential for understanding in detail the mechanism of liver injury (de la Mata et al., 1990; Memon et al., 2019). TNF-α, which is thought to be a common early effector molecule in liver injury, is a pleiotropic pro-inflammatory cytokine produced chiefly by activated macrophages. A lot of pro-inflammatory mediators including NO, IL-1, IL-6, IL-8 and SIL-2R are stimulated by TNF-α which has direct cytotoxic effects (Dong et al., 2016; Muntane et al., 2000; Nadler et al., 2001; Simeonova et al., 2001). Stimulation of these pro-inflammatory mediators is important for inflammation and consequent liver damage. IFN-ã can not only directly affect the liver parenchymal cells through the signalling pathway, but also exacerbate the liver injury by promoting the expression of TNF-á in KC cells (Sun, Tian, Kulkarni, & Gao, 2004). IL-6 is an inflammatory factor that increases liver tissue damage (Abdollahi, Shoar, Nayyeri, & Shariat, 2012). In our study, SMP inhibited the secretions of TNF-α, IFN-γ, IL-6 and expressions of TNF-α, IFN-γ mRNA in the liver. The effect of SMP on TNF-α was the most obvious, indicating that to decrease TNF-α expression by SMP is one of the most important mechanisms in alleviating immunological liver injury.

IL-17 is derived from activated T cells and induces inflammatory responses in cells. It plays a key role in liver damage caused by drug-induced inflammation. Furuya et al. (2015) studied the role of IL-17A in the inflammatory response of liver injury by establishing three LPS/GalN-induced liver injury models in mice: It was found that IL-17A has a pro-inflammatory response in liver injury (Liao et al., 2016; Yano et al., 2012). In our experiments, SMP can significantly inhibit the IL-17 content in the serum and the transcription of IL-17A mRNA in liver tissue of mice with liver injury, thus achieving the effect of alleviating liver injury in mice.

NO participates in the regulation of the physiological processes of the body and the immune response, while excessive production of NO leads to tissue damage and inflammatory response. NO *in vivo* is produced by L-arginine (L-Arg) catalyzed by nitric oxide synthase (NOS). NOS can be divided into structural (eNOS) and inducible (iNOS). eNOS produces only a small amount of NO, plays a normal physiological role. Pathogenic microbial infection and tissue damage can induce iNOS expression, promote a large amount of NO production. NO has dual function of anti-inflammatory and inflammatory (Welsh, Grassia, Botha, Sattar, & Maffia, 2017). A small amount of NO inhibits the inflammatory activity by inhibiting the adhesion of neutrophils and endothelial cells (Zhou & Zhu, 2009). On the contrary, in the process of the inflammatory disease, excessive NO through the activation of NF-êB induced TNF-á, IL-6 and other proinflammatory cytokines promotes inflammatory response. These cytokines can activate iNOS, promote the body to produce more NO. NO and cytokine secretion can be sustained, so that the inflammatory responses become more durable and more intense (Medzhitov, 2001).

Our study showed that SMP decreased LPS-induced NO secretion and inhibited iNOS mRNA transcription and protein expression to achieve the effect of liver protection.

The lipopolysaccharide in the cell wall activates the nuclear transcription factor (NF-κB) signal transduction pathway and the corresponding transcription factor, which mediates the expression of inflammatory mediators such as TNF-α and further releases a large number of cell attack factors such as NO, leading to the body producing oxidative stress and inflammatory response (Cao et al., 2011). Under the stress conditions, IκB can be phosphorylated by IκB protein kinase, further degraded. The released κBp65 translocates into the nucleus, binding to the κBp65 on the DNA. The above reaction generates TNF-α mRNA and participate in the body's inflammatory responses (Kang et al., 2014; Kim et al., 2012). Our results indicate SMP pretreatment suppresses IκBα kinase and p65 phosphorylation in LPS-induced hepatitis, which in turn inhibites the activation of NF-κB. These results suggest that the attenuation of the severity of LPS-induced hepatitis by SMP pretreatment is mediated by suppression of inflammatory responses through down-regulating phosphorylation of NF-κB activity partly.

LPS can induce oxidative stress responses in tissues (Awad et al., 2011; Su et al., 2014), which is also partly responsible for liver injury. Our study found that SMP has a good alleviating effect on oxidative stress of liver tissue induced by LPS, and this effect is achieved by regulating Nrf2 pathway. Nrf2 is an important transcription factor that regulates the cellular oxidative stress responses and is also the central regulator in maintaining the intracellular redox homeostasis (Kensler, Wakabayash, & Biswal, 2007; Kobayashi &

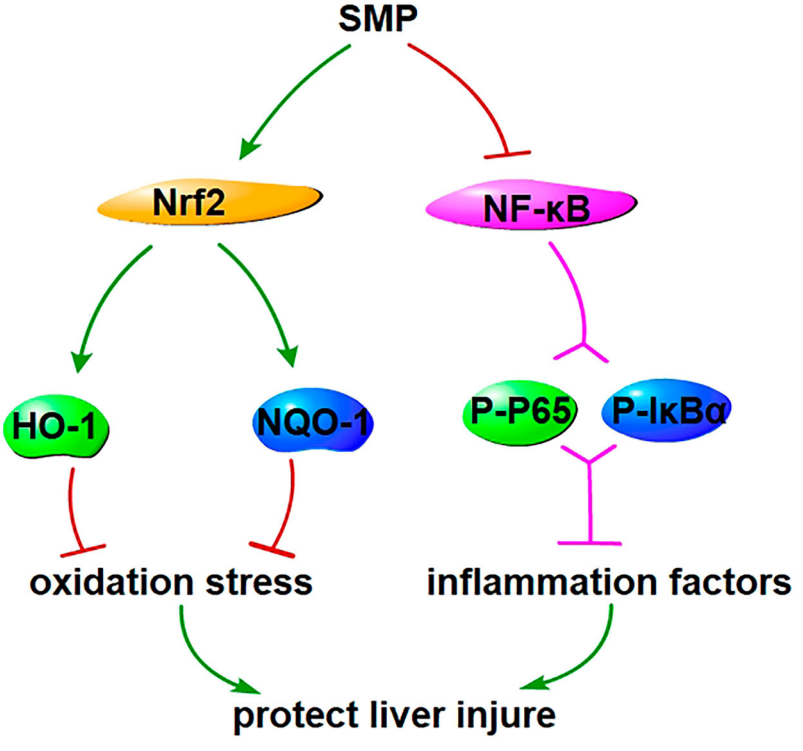


**Figure 9.** Mechanisms of Salvia miltiorrhiza polysaccharide in relieving immune liver injury.

Yamamoto, 2005). Through the induction and regulation of the constitutive and inducible expression of a series of antioxidant proteins, Nrf2 can reduce the cell damage caused by reactive oxygen species and electrophiles, keep the cells in a stable state and maintain the redox dynamic equilibrium (Song et al., 2018). SMP can increase the expression of Nrf2 protein in liver tissue, and then attenuates the contents of MDA in liver. In addition, HO-1 is an important antioxidant enzyme, which can resist peroxide, peroxynitrite, hydroxyl and superoxide free radicals (Gonzalez-Burgos, Carretero, & Gomez-Serranillos, 2012). HO-1 gene and protein expression can be up-regulated after oxidative stress and cell injury, while Nrf2 can directly regulate the activity of HO-1 promoter, When exposed to various toxic substances in vitro, including diethyl maleate, paraquat and cadmium chloride can induce HO-1 gene and protein expression (Klaassen & Reisman, 2010). SMP can significantly increase the expression of HO-1 in liver tissue, thereby alleviating liver oxidative damage.

## 5. Conclusion

SMP can not only inhibit the infiltration of inflammatory factors into the liver by inhibiting the phosphorylation of p65 and IκBα in the NF-kappa B pathway, but also promote the expression of Nrf2, HO-1 and NQO-1 proteins in the Nrf2 pathway to reduce the oxidative stress of the liver, thereby protecting the liver from injury (Figure 9).

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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## References

Abdollahi, A., Shoar, S., Nayyeri, F., & Shariat, M. (2012). Diagnostic value of simultaneous measurement of procalcitonin, interleukin-6 and hs-CRP in prediction of early-onset neonatal sepsis. *Mediterranean Journal of Hematology and Infectious Diseases*, *4*(1), e2012028.

Awad, N., Khatib, N., Ginsberg, Y., Weiner, Z., Maravi, N., Thaler, I., … Beloosesky, R. (2011). N-acetyl-cysteine (NAC) attenuates LPS-induced maternal and amniotic fluid oxidative stress and

inflammatory responses in the preterm gestation. *American Journal of Obstetrics and Gynecology*, *204*(5), 450.e15–20.
Cao, W., Zhang, W., Liu, J., Wang, Y., Peng, X., Lu, D., … Wang, H. (2011). Paeoniflorin improves survival in LPS-challenged mice through the suppression of TNF-alpha and IL-1beta release and augmentation of IL-10 production. *International Immunopharmacology*, *11*(2), 172–178.
Cao, L. J., Zou, Y., Zhu, J., Fan, X., & Li, J. (2013). Ginsenoside Rg1 attenuates concanavalin A-induced hepatitis in mice through inhibition of cytokine secretion and lymphocyte infiltration. *Molecular and Cellular Biochemistry*, *380*(1–2), 203–210.
de la Mata, M., Meager, A., Rolando, N., Daniels, H. M., Nouri-Aria, K. T., Goka, A. K., … Williams, R. (1990). Tumour necrosis factor production in fulminant hepatic failure: Relation to aetiology and superimposed microbial infection. *Clinical and Experimental Immunology*, *82*(3), 479–484.
Dong, Y., Liu, Y., Kou, X., Jing, Y., Sun, K., Sheng, D., … Wei, L. (2016). The protective or damaging effect of tumor necrosis factor-alpha in acute liver injury is concentration-dependent. *Cell and Bioscience*, *6*, 8.
Furuya, S., Kono, H., Hara, M., Hirayama, K., Sun, C., & Fujii, H. (2015). Interleukin 17A plays a role in lipopolysaccharide/D-galactosamine-induced fulminant hepatic injury in mice. *Journal of Surgical Research*, *199*(2), 487–493.
Geng, Z. H., Huang, L., Song, M. B., & Song, Y. M. (2015). Cardiovascular effects in vitro of a polysaccharide from Salvia miltiorrhiza. *Carbohydrate Polymers*, *121*, 241–247.
Gonzalez-Burgos, E., Carretero, M. E., & Gomez-Serranillos, M. P. (2012). Diterpenoids isolated from Sideritis species protect astrocytes against oxidative stress via Nrf2. *Journal of Natural Products*, *75*(10), 1750–1758.
Hu, B. J., Zou, Y., Liu, S. S., Wang, J., Zhu, J. L., Li, J. B., … Deng, X. M. (2014). Salidroside attenuates concanavalin A-induced hepatitis via modulating cytokines secretion and lymphocyte migration in mice. *Mediators of Inflammation*, *2014*, 314081.
Jiang, Y. Y., Wang, L., Zhang, L., Wang, T., Yu, L., Ding, C. B., … Zhou, Y. H. (2014). Characterization, antioxidant and antitumor activities of polysaccharides from Salvia miltiorrhiza Bunge. *International Journal of Biological Macromolecules*, *70*(7016), 92–99.
Kang, S. M., More, S. V., Park, J. Y., Kim, B. W., In, P. J., Yoon, S. H., & Choi, D. K. (2014). A novel synthetic HTB derivative, BECT inhibits lipopolysaccharide-mediated inflammatory response by suppressing the p38 MAPK/JNK and NF-kappaB activation pathways. *Pharmacological Reports*, *66*(3), 471–479.
Kensler, T. W., Wakabayash, N., & Biswal, S. (2007). Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annual Review of Pharmacology and Toxicology*, *47*, 89–116.
Kim, I. T., Ryu, S., Shin, J. S., Choi, J. H., Park, H. J., & Lee, K. T. (2012). Euscaphic acid isolated from roots of Rosa rugosa inhibits LPS-induced inflammatory responses via TLR4-mediated NF-kappaB inactivation in RAW 264.7 macrophages. *Journal of Cellular Biochemistry*, *113*(6), 1936–1946.
Klaassen, C. D., & Reisman, S. A. (2010). Nrf2 the rescue: Effects of the antioxidative/electrophilic response on the liver. *Toxicology and Applied Pharmacology*, *244*(1), 57–65.
Kobayashi, M., & Yamamoto, M. (2005). Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxidants & Redox Signaling*, *7*(3-4), 385–394.
Lao, C. J., Lin, J. G., Kuo, J. S., Chiang, S. Y., Chen, S. C., Liao, E. T., & Hsieh, C. L. (2003). Effect of Salvia Miltiorrhiza Bunge on cerebral infarct in ischemia-reperfusion injured rats. *American Journal of Chinese Medicine*, *31*(2), 191–200.
Li, Y., Wang, X. Y., Wei, Z. T., Mao, H. J., Gao, M., Liu, Y. P., … Wang, X. Y. (2014). Pretreatment with wortmannin alleviates lipopolysaccharide/D-galactosamine-induced acute liver injury. *Biochemical and Biophysical Research Communications*, *455*(3–4), 234–240.
Liao, C. C., Day, Y. J., Lee, H. C., Liou, J. T., Chou, A. H., & Liu, F. C. (2016). Baicalin attenuates IL-17-mediated acetaminophen-induced liver injury in a mouse model. *Plos One*, *11*(11), e0166856.
Liu, L., Jia, J., Zeng, G., Zhao, Y., Qi, X., He, C., … Li, Z. (2013). Studies on immunoregulatory and anti-tumor activities of a polysaccharide from Salvia miltiorrhiza Bunge. *Carbohydrate Polymers*, *92*(1), 479–483.

Liu, D., Li, Y., Xu, Y., & Zhu, Y. (2016). Anti-aging traditional Chinese medicine: Potential mechanisms involving AMPK pathway and calorie restriction based on "medicine-food homology" theory. *China Journal of Chinese Materia Medica*, *41*(6), 1144–1151 (in chinese).

Liu, J., Wen, X. Y., Kan, J., & Jin, C. H. (2015). Structural characterization of two water-soluble polysaccharides from black soybean (Glycine max (L.) Merr.). *Journal of Agricultural and Food Chemistry*, *63*(1), 225–234.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods*, *25*(4), 402–408.

Luster, M. I., Simeonova, P. P., Gallucci, R. M., Matheson, J. M., & Yucesoy, B. (2000). Immunotoxicology: Role of inflammation in chemical-induced hepatotoxicity. *International Journal of Immunopharmacology*, *22*(12), 1143–1147.

Ma, S. L., Zhang, D. W., Lou, H. X., Sun, L. R., & Ji, J. B. (2016). Evaluation of the anti-inflammatory activities of tanshinones isolated from Salvia miltiorrhiza var. alba roots in THP-1 macrophages. *Journal of Ethnopharmacology*, *188*, 193–199.

Magdaleno, F., Blajszczak, C. C., & Nieto, N. (2017). Key events participating in the pathogenesis of alcoholic liver disease. *Biomolecules*, *7*(1), 9.

Maione, F., Cantone, V., Chini, M. G., De Feo, V., Mascolo, N., & Bifulco, G. (2015). Molecular mechanism of tanshinone IIA and cryptotanshinone in platelet anti-aggregating effects: An integrated study of pharmacology and computational analysis. *Fitoterapia*, *100*, 174–178.

Medzhitov, R. (2001). Toll-like receptors and innate immunity. *Nature Reviews Immunology*, *1*(2), 135–145.

Memon, M. A., Wang, Y., Xu, T. L., Ma, N. N., Zhang, H. M., Roy, A. C., … Shen, X. Z. (2019). Lipopolysaccharide induces oxidative stress by triggering MAPK and Nrf2 signalling pathways in mammary glands of dairy cows fed a high-concentrate diet. *Microbial Pathogenesis*, *128*, 268–275.

Muntane, J., Rodriguez, F. J., Segado, O., Quintero, A., Lozano, J. M., Siendones, E., … Mino, G. (2000). TNF-alpha dependent production of inducible nitric oxide is involved in PGE(1) protection against acute liver injury. *Gut*, *47*(4), 553–562.

Nadler, E. P., Dickinson, E. C., Beer-Stolz, D., Alber, S. M., Watkins, S. C., Pratt, D. W., & Ford, H. R. (2001). Scavenging nitric oxide reduces hepatocellular injury after endotoxin challenge. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *281*(1), G173–G181.

Nolan, J. P. (2010). The role of intestinal endotoxin in liver injury: A long and evolving history. *Hepatology*, *52*(5), 1829–1835.

Revajová, V., Pistl, J., Levkut, M., Marcin, A., & Levkutová, M. (2010). Influence of oregano and salvia extracts on lymphocyte subpopulation and functional activity of blood phagocytes and lymphocytes in chickens. *Food and Agricultural Immunology*, *21*(4), 307–316.

Simeonova, P. P., Gallucci, R. M., Hulderman, T., Wilson, R., Kommineni, C., Rao, M., & Luster, M. I. (2001). The role of tumor necrosis factor-alpha in liver toxicity, inflammation, and fibrosis induced by carbon tetrachloride. *toxicology and Applied Pharmacology*, *177*(2), 112–120.

Song, M., Huang, L., Zhao, G., & Song, Y. (2013). Beneficial effects of a polysaccharide from Salvia miltiorrhiza on myocardial ischemia–reperfusion injury in rats. *Carbohydrate Polymers*, *98*, 1631–1636.

Song, Y. H., Liu, Q., Lv, Z. P., Chen, Y. Y., Zhou, Y. C., & Sun, X. G. (2008). Protection of a polysaccharide from Salvia miltiorrhiza, a Chinese medicinal herb, against immunological liver injury in mice. *International Journal of Biological Macromolecules*, *43*(2), 170–175.

Song, J., Zhang, W., Wang, J., Yang, H., Zhao, X., Zhou, Q., … Du, G. (2018). Activation of Nrf2 signaling by salvianolic acid C attenuates NFkappaB mediated inflammatory response both in vivo and in vitro. *International Immunopharmacology*, *63*, 299–310.

Su, Z. Q., Mo, Z. Z., Liao, J. B., Feng, X. X., Liang, Y. Z., Zhang, X., … Lai, X. P. (2014). Usnic acid protects LPS-induced acute lung injury in mice through attenuating inflammatory responses and oxidative stress. *International Immunopharmacology*, *22*(2), 371–378.

Sugiyama, A., Sato, A., Shimizu, H., Ando, K., & Takeuchi, T. (2010). PEGylated lactoferrin enhances its hepatoprotective effects on acute liver injury induced by D-galactosamine and lipopolysaccharide in rats. *Journal of Veterinary Medical Science*, *72*(2), 173–180.

Sun, R., Tian, Z. G., Kulkarni, S., & Gao, B. (2004). IL-6 prevents T cell-mediated hepatitis via inhibition of NKT cells in CD4(+) T cell- and STAT3-dependent manners. *The Journal of Immunology*, *172*(9), 5648–5655.

Sun, L., Zhu, B., Li, D., Wang, L., Dong, X., Murata, Y., … Dong, Y. (2010). Purification and bioactivity of a sulphated polysaccharide conjugate from viscera of abalone\\r *Haliotis discus hannai*\\r Ino. *Food and Agricultural Immunology*, *21*(1), 15–26.

Tu, C. T., Han, B., Liu, H. C., & Zhang, S. C. (2011). Curcumin protects mice against concanavalin A-induced hepatitis by inhibiting intrahepatic intercellular adhesion molecule-1 (ICAM-1) and CXCL10 expression. *Molecular and Cellular Biochemistry*, *358*(1–2), 53–60.

Uesugi, T., Froh, M., Arteel, G. E., Bradford, B. A. U., & Thurman, R. G. (2001). Toll-like receptor 4 is involved in the mechanism of early alcohol-induced liver injury in mice. *Hepatology*, *34*(1), 101–108.

Wang, F. J., He, Q. S., Wang, J. X., Yuan, Q., Guo, H., Chai, L. J., … Zhang, Y. (2017). Neuroprotective effect of salvianolate lyophilized injection against cerebral ischemia in type 1 diabetic rats. *BMC Complementary and Alternative Medicine*, *17*, 258.

Wang, N., Yang, J., Lu, J., Qiao, Q., Wu, T., Du, X., … He, X. (2014). A polysaccharide from Salvia miltiorrhiza Bunge improves immune function in gastric cancer rats. *Carbohydrate Polymers*, *111*(20), 47–55.

Welsh, P., Grassia, G., Botha, S., Sattar, N., & Maffia, P. (2017). Targeting inflammation to reduce cardiovascular disease risk: A realistic clinical prospect? *British Journal of Pharmacology*, *174* (22), 3898–3913.

Wenlin, W. U., Zhu, Y., Zhang, L. I., Yang, R., & Zhou, Y. (2012). Extraction, preliminary structural characterization, and antioxidant activities of polysaccharides from Salvia miltiorrhiza Bunge. *Carbohydrate Polymers*, *87*(2), 1348–1353.

Whitfield, C., & Trent, M. S. (2014). Biosynthesis and export of bacterial lipopolysaccharides. *Annual Review of Biochemistry*, *83*, 99–128.

Xie, T. T., Ma, S. L., Lou, H. X., Zhu, R. X., & Sun, L. R. (2014). Two novel abietane norditerpenoids with anti-inflammatory properties from the roots of Salvia miltiorrhiza var. alba. *Tetrahedron Letters*, *55*(51), 7106–7109.

Yano, A., Higuchi, S., Tsuneyama, K., Fukami, T., Nakajima, M., & Yokoi, T. (2012). Involvement of immune-related factors in diclofenac-induced acute liver injury in mice. *Toxicology*, *293*(1–3), 107–114.

Zhang, Y. Y. (2010). Determination of three Tanshinones in Salviae miltiorrhizae health tea by HPLC. *Medicinal Plant*, *9*, 47–48.

Zhang, K. Q., Bao, Y., Wu, P., Rosen, R. T., & Ho, C. T. (1990). Antioxidative components of tanshen (Salvia miltiorrhiza Bung). *Journal of Agricultural and Food Chemistry*, *38*(5), 1194–1197.

Zhao, G. R., Xiang, Z. J., Ye, T. X., Yuan, Y. J., & Guo, Z. X. (2006). Antioxidant activities of Salvia miltiorrhiza and Panax notoginseng. *Food Chemistry*, *99*(4), 767–774.

Zhao, G. R., Zhang, H. M., Ye, T. X., Xiang, Z. J., Yuan, Y. J., Guo, Z. X., & Zhao, L. B. (2008). Characterization of the radical scavenging and antioxidant activities of danshensu and salvianolic acid B. *Food and Chemical Toxicology*, *46*(1), 73–81.

Zhong, Y., Chiou, Y. S., Pan, M. H., & Shahidi, F. (2012). Anti-inflammatory activity of lipophilic epigallocatechin gallate (EGCG) derivatives in LPS-stimulated murine macrophages. *Food Chemistry*, *134*(2), 742–748.

Zhou, L., & Zhu, D. Y. (2009). Neuronal nitric oxide synthase: Structure, subcellular localization, regulation, and clinical implications. *Nitric Oxide-Biology and Chemistry*, *20*(4), 223–230.

Zou, P. (2016). Traditional Chinese medicine, food therapy, and hypertension control: A narrative review of Chinese literature. *The American Journal of Chinese Medicine*, *44*(08), 1579–1594.