Hepatoprotective effect of *Alhagi sparsifolia* against Alcoholic Liver injury in mice

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Overconsumption of alcohol leads to alcoholic liver disease (ALD). Natural compounds have been investigated previously for their hepatoprotective activities against liver injury. This study investigated the protective effect of *Alhagi sparsifolia* on ALD. Alcohol was administered to mice for three consecutive days; either alone or in combination with *Alhagi sparsifolia* extract (150, 300, 600 mg/kg). Serum aspartate aminotransferase and alanine transaminase as biomarkers of liver injury, the content of malonaldehyde, hydrogen peroxide (H_2O_2) and glutathione which indicated the redox status of liver and the antioxidant enzyme activity of super oxide dismutase were detected, respectively. Moreover, the expression of protein cytochrome P450 2E1 (CYP2E1) the key enzyme of alcohol metabolism, and also tested by western blot experiment. Subsequently, the mRNA levels of inflammatory factors including TNF- α and TLR4 was determined real-time PCR. Results showed that *Alhagi sparsifolia* significantly alleviated alcohol-induced liver injury by reducing serum ALT and AST, inhibiting MDA and H_2O_2 content, increasing SOD, and GSH level in the liver ($P< 0.05$). In addition, the *Alhagi sparsifolia* treatment inhibited the expression of CYP2E1 ($P< 0.05$). The results suggest that *Alhagi sparsifolia* could be a promising natural substance for ameliorating acute alcohol-induced oxidative stress and hepatic injury.

Keywords: Alcoholic liver disease. *Alhagi sparsifolia*. Hepatoprotective effect. Antioxidantion. CYP2E1.

INTRODUCTION

Alcoholic liver disease (ALD) is one of the most common causes of liver injury, accounting for 3.8% of all global deaths and 4.6% of global disability-adjusted life-years attributable to alcohol (Rehm*et al.*, 2009). Consistent, excessive alcohol consumption has long been identified as an important risk factor for the development of liver disease (Yip, Burt, 2006). Although the precise mechanisms and factors responsible for liver injury are not completely understood, many pathways have been suggested in which oxidative stress plays a key role in alcohol-induced liver injury (Cederbaum, 2001;

Arteel, 2003). The liver is the main organ responsible for metabolizing alcohol in the body, mainly through alcohol dehydrogenase, aldehyde dehydrogenase and cytochrome P450 (CYP2E1) (Agarwal, 2001). Among the CYP450 family, cytochrome 2E1 (CYP2E1) has been identified as a key microsomal enzyme in alcoholic liver injury, since it is highly inducible with high catalytic activity against alcohol, which catalyzes the oxidation of exogenous and endogenous compounds, and it plays a significant role in the metabolism of alcohol by the liver. Thus, it is particularly relevant to the development of ALD caused by the generation of alcohol-induced reactive oxygen species (ROS) (Wang, 2010), which leads to lipid peroxidation in liver cells, and changes of alcohol metabolizing enzymes (Charles *et al.*, 1985). CYP2E1 has a specific metabolic effect on xenobiotics; alcohol itself is a strong inducer of CYP2El enzyme activity and expression (Takahashi *et al.*, 1993). In addition, alcohol use results in the production of inflammatory cytokines such as

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tumor necrosis factor-α (TNF-α), which contributes to hepatocellular damage (Petrasek *et al*., 2011). Previously, a large number of medicinal plants have been investigated for hepatoprotective effects against alcohol-induced liver damage.

Alhagi sparsifolia (*Ci Tang in* Chinese) is also a well famous herb among the herbal plants and this belongs to aAlhagi genus and it is a sweet, yellow-brown substance secreted by *Alhagi sparsifolia* leaves. In Compendium of Materia Medica, *Alhagi sparsifolia* is recorded as top grade, nontoxic, antipyretic, and antidote herbal drug (Jian *et al*., 2014). *Alhagi sparsifolia* is used as a diaphoretic, diuretic, expectorant, and treatment forulcers (Kulieva, Shasvarov, 1972) and liver disorders (Shaker, Mahmoud, Mnaa, 2010; Aidouri, Alessa, 2010) *Alhagi sparsifolia* is rich in biologically active phytochemicals such as polysaccharides, phenolics, flavonoids and alkaloids along with different essential minerals, proteins and lipids (Muhammad *et al*., 2015).Many reports have indicated that polysaccharide has antioxidant activity and can prevent carbon tetrachloride and rifampicin-induced liver injury (Dong *et al.*, 2014; Jayakumar, Sakthivel, Thomas, 2008). The extracts of the leaves and flowers of *Alhagi sparsifolia* possess high antioxidant activity (Laghari*et al.*, 2012). Research has shown that *Alhagi sparsifolia* have strong antioxidant property and a good free radical scavenging capacity (Jian *et al.*, 2014).

Regardless, this herbal plant has limited number of studies as hepatoprotective. So this study was conducted to focus on its novel effects of *Alhagi sparsifolia*on alcohol-induced liver injury and to explore its potential mechanism.

MATERIAL AND METHODS

Plant extract preparation

Alhagi sparsifolia (collected from the Turpan basin) was procured from the Xinjing Uyghur Autonomous Region Uyghur Medicine Hospital Xinjiang, China in June 2016. In a round bottom flask, 10 g of *Alhagi sparsifolia* was boiled with 200 mL of distilled water for 2h. After boiling, the supernatant was separated and placed into a fresh container and the remaining pellets were again boiled with 100 mL of distilled water for 1.5 h. The supernatants obtained after each boiling were subsequently mixed together and concentrated in a rotary vacuum evaporator at -65 °C. The concentrated extract was lyophilized and stored at 4 °C until further study.

Estimation of total polysaccharides in *Alhagi sparsifolia* extract

The phenol-sulfuric acid method was employed to estimate the total polysaccharide content (Jian *et al*., 2016). In brief *Alhagi sparsifolia* extract (0.4 mL), 5% phenol reagent (1 mL), distilled water (1.6 mL), and H_2SO_4 (5 mL) were blended in a test tube (10 mL). After a thorough mixing, the mixture was diluted with distilled water to 10 mL and maintained without disturbance for 10 min at room temperature. The optical density of the mixture was determined at 490 nm using a spectrophotometer. The total polysaccharide content was calculated on the basis of the standard curve of glucose and expressed as milligrams of glucose equivalents per gram of dried *Alhagi sparsifolia* extract.

Animal experiment

All animal experimental protocols were reviewed and approved by the Animal Ethical Committee of Northwest A&F University. Female Kun-Ming mice (6weeks old, 28 ± 2 g b. w.) were obtained from the Experimental Animal Center of the Fourth Military Medical University (Xi'an, China). All animals were kept under standard laboratory conditions, and the animals had free access to feed and water throughout the experiment. In total, 56 experimental mice were randomly assigned into 8 groups of 7 mice in each group (n=7). The reported protocol (Jiang *et al*., 2016) was exploited to modeling alcohol induced liver injury. Group 1 was the normal control group (NC group). Group 2 was the negative control group treated with 50% alcohol (10 mL/kg, ALC group). Groups 3, 4, and 5 were the intervention groups treated with alcohol (10 mL/kg) along with low, middle, and high (AH_L+ALC, AH_M+ALC, and AH_M+ALC group) concentrations of *Alhagi sparsifolia*(150, 300, and 600 mg/kg, b. w, respectively). Group 6 was the group with a high dose of *Alhagi sparsifolia* (600 mg/kg, AH_H group). Groups 7 and 8 were the positive control groups treated with both alcohol (10 mL/kg) and silymarin and sodium salicylate (300 mg/kg, SL+ALC and SS+ALC group), respectively. After treatment, the experimental animals fasted overnight, weighed and the mice were anesthetized with chloral hydrate to peritoneal venous collect blood serum. Then the animals were euthanized and dissected to collect liver tissue. The serum was separated from the blood by centrifugation at 2,500 rpm for 10 min at 4 °C and stored at -80 °C for further analysis. The liver was divided into three parts: 0.3 g for tissue homogenate, 0.4 g for microsomal protein analysis, and 0.1 g for

mRNA levels detection. All of the samples were stored at -80 °C.

Determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in serum

The enzymatic activities of ALT and AST in serum were determined spectrophotometrically using commercial diagnostic kits (Jiancheng, Inc, Nanjing, China).

Estimation of hepatic malondialdehyde(MDA), hydrogen peroxide(H_2O_2), superoxide dismutase(SOD), and glutathione(GSH)

The frozen liver tissue samples were thawed and homogenized in ice-cold phosphate buffered saline. The homogenate was centrifuged at 3,000 rpm for 10 min at 4 °C, and the supernatants were subjected to assays to estimate the MDA, oxidative stress products H_2O_2, SOD, and GSH levels using commercially available assay kits (Jiancheng, Inc., Nanjing, China). The Bradford protein assay was used to determine the protein concentrations in tissue homogenates using bovine serum albumin as the standard (Tiangen Biotech, Beijing, China).

Analysis of gene expression by real-time quantitative polymerase chain reaction (PCR)

Total mRNA was extracted from the liver tissue samples using an RNA extraction kit (Tiangen Biotech, China), and reverse transcribed into cDNA using HiScript™ RT Super Mix for real-time quantitative PCR (Vazyme Biotech, China). The aliquots of cDNAs were amplified using specific primers (Table I). Real-time quantity PCR was performed on an iQ5 real-time quantitative PCR detection system (Bio-Rad, USA) with a SYBR green master mix (Vazyme Biotech, China). ß-actin was used as an internal control to normalize the target gene transcript levels. The relative expression of mRNA was expressed as the ratio of the target gene to the control gene using the following formula; $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct$=control $(Ct_{Target}-Ct_{ß-actin})$ – treatment $(Ct_{Target}-Ct_{ß-actin})$.

Western blot analysis

The microsomal preparation was conducted according to the method described by Jiang *et al*. (2016), and the Bradford protein assay was used to estimate the protein content using bovine serum albumin as the standard (Tiangen Biotech, Beijing, China). Proteins were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore Corporation, Boston, ME, USA). TBST containing 5% skimmed milk powder was used for blocking. The membranes were incubated overnight at 4 °C with primary antibodies against CYP2EI (1:800) (Boster, Wuhan, China) and glyceraldehyde phosphate dehydrogenase (GAPDH, 1: 1,000) (Tianjin Sungene Biotech, Tianjin, China). The blots were incubated with a 1: 1,000 dilution of secondary antibodies conjugated horseradish peroxidase (Tianjin Sungene Biotech, Tianjin, China) for 2 h at room temperature. Protein bands were visualized by ECL reaction (Genshare Biological, Xi'an, China). The protein levels were quantified using the Gel-Pro Analyzer software (Media Cybernetics, Washington, MD, USA) and normalized to GAPDH.

Statistical analysis

All data were presented as the mean ± standard (SD). One-way ANOVA was used to assess the significant difference in the respective controls in all the experiments using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Values of $p < 0.05$ were considered statistically significant.

TABLE I - Primers for real-time quantitative PCR

Gene (accession number)	Primer sequences (5'-3')	Product (bp)
TNF-α (X55152)	F: ATTGAGCACAGGCATAAAGAT	715
	R: TCTAGTTCTGGTTTTGAACAGTG	
TLR4 (G12481)	F: GCACTGTTCTTCTCCTGCC	293
	R: GTTTCCTGTCAGTATCAAG	
β-Actin (U20114)	F: GGCTCCCAGCACCATGAA	185
	R: GCCACCGATCCACACAGAGT	

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RESULTS

Total polysaccharide assay

A linear relation (y=0.177x-0.032 [R^2=0.992]) was observed for the values of standard glucose. The total polysaccharide content in the extract was (718.1 ± 20.78 mg/g).

Effect of *Alhagi sparsifolia* against alcohol-induced hepatic dysfunction

Serum AST and ALT activities were significantly increased in the alcohol-treated group compared with the control group, confirming the hepatotoxicity of alcohol (Figure 1). In the group that received alcohol following pretreatment with *Alhagi sparsifolia* (600, 300 and 150 mg/kg), serum ALT and AST activities were significantly reduced compared with alcohol group, indicating that *Alhagi sparsifolia* protected against alcohol hepatotoxicity.

Alcohol-induced oxidative stress in the liver

The activities of SOD were decreased in the ALC group (Figure 2C), while they increased in the AH_L, AH_M and AH_H +ALC groups. The levels of H_2O_2 (Figure 2B) and MDA (Fig. 2A) were increased in the ALC group with the GSH depletion (Figure 2D). In the AH_L, AH_M and AH_H +AL groups, *Alhagi sparsifolia* increased the level of GSH and decreased the levels of H_2O_2 and MDA.

Alhagi sparsifolia inhibited alcohol-induced TNF-α and TLR4 mRNA expression levels in mice liver

Alcohol overdose markedly increased mRNA expressions level including TLR4 and TNF-α (Figure 3), which decreased significantly in the group pre-treated with *Alhagi sparsifolia* in a dose-dependent manner, suggesting that *Alhagi sparsifolia* mediated its anti-inflammatory effect at the transcriptional level.

CYP2E1 protein expression

Our data showed that the CYP2E1 protein expressions clearly increased in the alcohol group ($p < 0.01$). The CYP2E1 level in the *Alhagi sparsifolia* (L)/(M)-alcohol-treated group was similar to that of the control group ($p < 0.01$), and that in the AH_H and AH_H alcohol-treated group significantly decreased in comparison with the control (Figure 4).

DISCUSSION

Alhagi sparsifolia has been investigated with various objectives to utilize its biological properties for therapeutic applications. Previous study has illustrated that, *Alhagi sparsifolia* could attenuate carbon tetrachloride induced liver damage and drug induced acute liver failure. It has been speculated that the anti-oxidant properties of *Alhagi sparsifolia* might be responsible for its hepatoprotective effects (Muhammad *et al*., 2015).
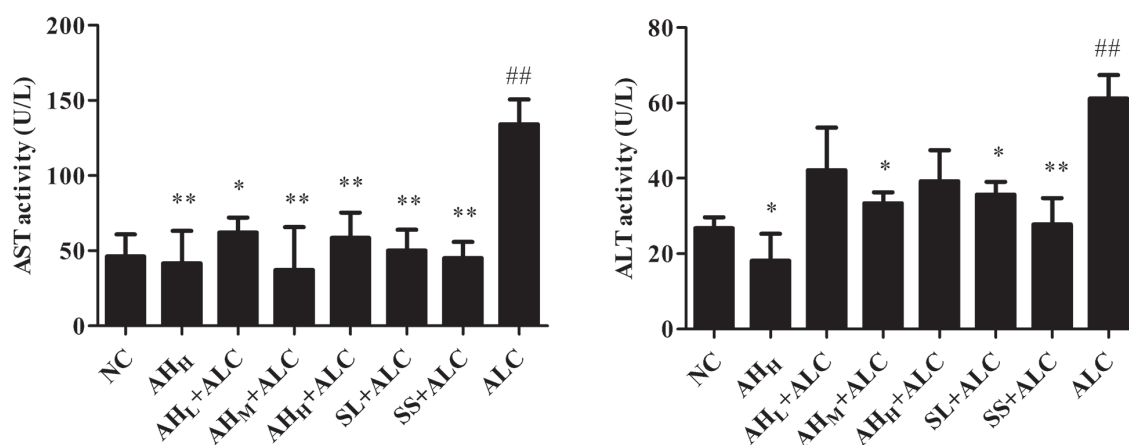

FIGURE 1 - Effects of *Alhagi sparsifolia* on Serum levels of AST and ALT. Notes: Each value represents as mean ± SD (n=7). ##$p < 0.01$ Denotes significant difference compared to NC group; *$p < 0.05$ and **$p < 0.01$ denotes significant difference to alcohol group. NC group, normal control group; AH_H group, treated with 600 mg/kg of *Alhagi sparsifolia*; AH_L+ALC group, treated with 150 mg/kg of *Alhagi sparsifolia* and alcohol (10 mL/kg); AH_M + ALC group, treated with 300 mg/kg of *Alhagi sparsifolia* and alcohol (10 mL/kg); AH_H + alcohol group, treated with 600 mg/kg of *Alhagi sparsifolia* and alcohol (10 mL/kg); SL + ALC group, treated with 300 mg/kg of silymarin and alcohol (10 mL/kg); SS + ALC group, treated with 300 mg/kg of sodium salicylate and alcohol (10 mL/kg), and ALC group, treated with 50% alcohol (10 mL/kg).
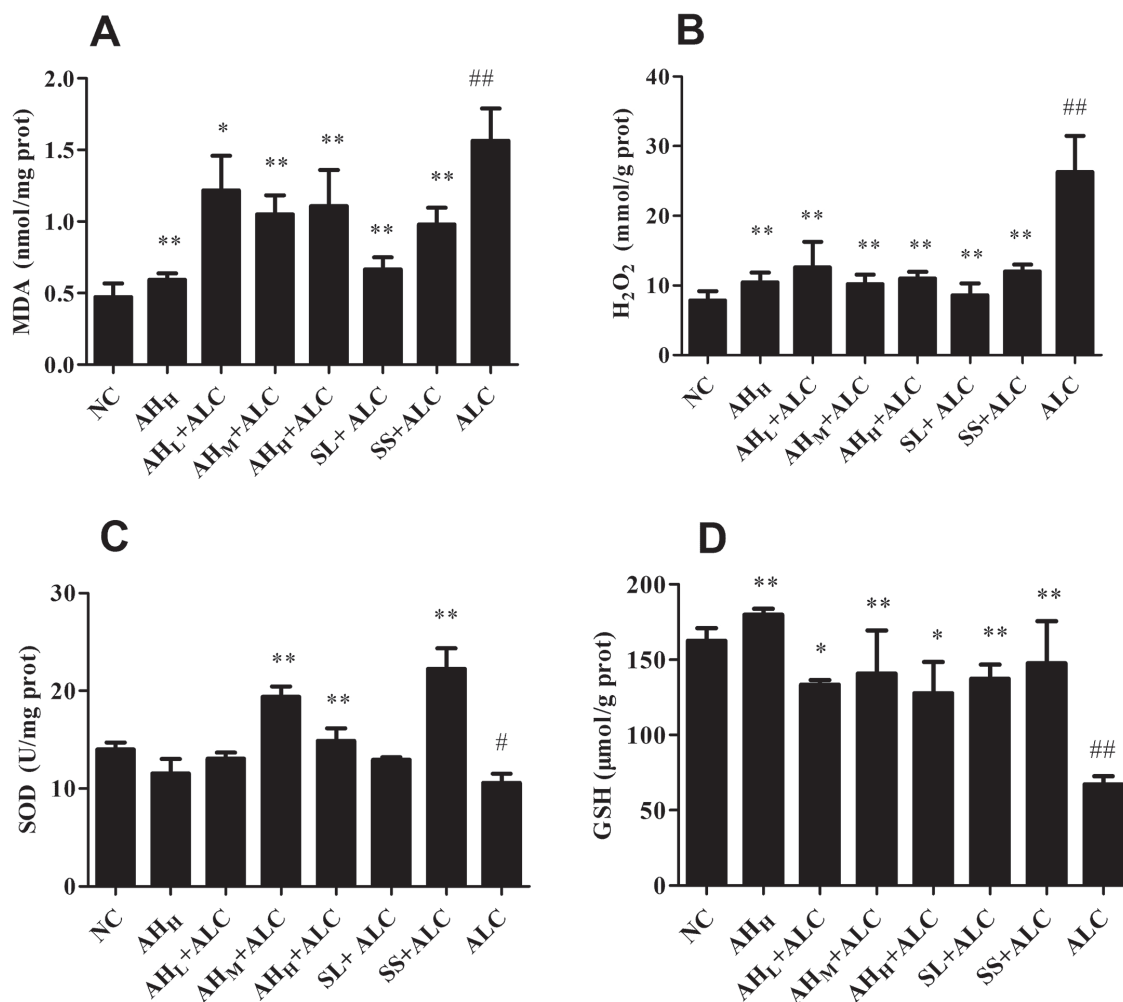
FIGURE 2 - Effect of *Alhagi sparsifolia* on alcohol-induced oxidative stress and lipid peroxidation. Notes: Each value represents as mean $\pm$ SD (n=7). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ Denotes significant difference compared to NC group; $^{*}p < 0.05$ and $^{**}p < 0.01$ denotes significant difference to alcohol group. NC group, normal control group; AH_H group, treated with 600 mg/kg of *Alhagi sparsifolia*; AH_L+ALC group, treated with 150 mg/kg of *Alhagi sparsifolia* and alcohol (10 mL/kg); AH_M + ALC group, treated with 300 mg/kg of *Alhagi sparsifolia* and alcohol (10 mL/kg); AH_H + alcohol group, treated with 600 mg/kg of *Alhagi sparsifolia* and alcohol (10 mL/kg); SL + ALC group, treated with 300 mg/kg of silymarin and alcohol (10 mL/kg); SS + ALC group, treated with 300 mg/kg of sodium salicylate and alcohol (10 mL/kg), and ALC group, treated with 50% alcohol (10 mL/kg).

ALD leads to cellular enzyme leakage into the serum (AST and ALT) because of increased membrane permeability, cellular damage and/or necrosis of hepatocytes (Mir, Sarwat, 2011). Serum AST and ALT are the simple and well-accepted biomarkers for hepatic dysfunction. The AST and ALT levels in serum, confirm that the alcohol-induced model has been successfully constructed in mice (Lambert *et al*., 2003). The results revealed the potential of *Alhagi sparsifolia* in alcohol intervention.

Oxidative stress and lipid peroxidation play a key role in the occurrence and development of alcoholic liver injury. Alcohol oxidation in the liver produces too many oxidative stress ROS products, such as OH^-, O_2, H_2O_2,

and hydroxyethyl radicals. Lipid peroxidation generates a number of degradation products, including MDA, which causes cell membrane destruction and DNA damage (Girotti, 1998). In the current study, alcohol-induced toxicity increased the MDA and H_2O_2 levels in liver tissue in comparison with the NC group. The treatment with *Alhagi sparsifolia* significantly decreased the MDA and H_2O_2 levels in comparison with the negative control group. During alcohol metabolism, the depletion of the major non-enzymatic antioxidant GSH and the enzymatic antioxidant SOD acting as a preventive antioxidant by protecting cells against oxidative stress are the first line of defense against oxidative injury (Hou, Qin, Ren, 2010). Our study found that, the SOD and GSH levels decreased
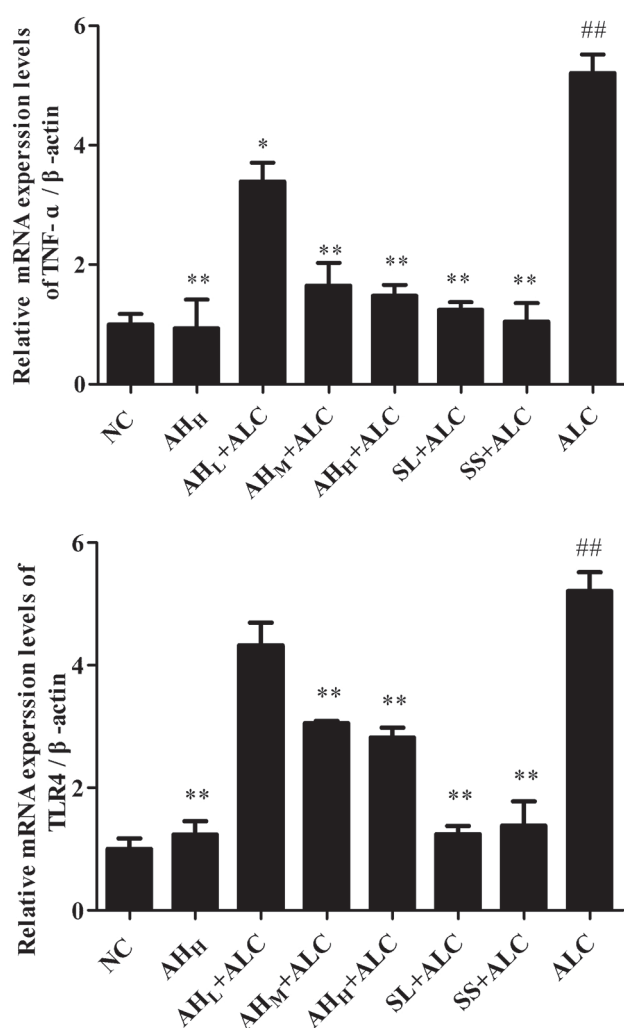
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FIGURE 4 - Effects of *Alhagi sparsifolia* on Inhibited CYP2E1 Expression. Notes: The expression of CYP2E1 was detected by western blot analysis. Each value represents as mean ± SD (n=7). $^{##}p < 0.01$ Denotes significant difference compared to NC group; $^{**}p < 0.01$ denotes significant difference to alcohol group. NC group, normal control group; AH_H group, treated with 600 mg/kg of *Alhagi sparsifolia*; AH_L+ALC group, treated with 150 mg/kg of *Alhagi sparsifolia* and alcohol (10 mL/kg); AH_M + ALC group, treated with 300 mg/kg of *Alhagi sparsifolia* and alcohol (10 mL/kg); AH_H + alcohol group, treated with 600 mg/kg of *Alhagi sparsifolia* and alcohol (10 mL/kg); SL + ALC group, treated with 300 mg/kg of silymarin and alcohol (10 mL/kg); SS + ALC group, treated with 300 mg/kg of sodium salicylate and alcohol (10 mL/kg), and ALC group, treated with 50% alcohol (10 mL/kg).

FIGURE 3 - Relative mRNA levels of TNF-α and TLR4. Notes: $^{*}p < 0.05$ and $^{**}p<0.01$ compared with the APAP group, $^{#}p < 0.05$ and $^{##}p < 0.01$ compared with the control group. NC group, normal control group; AH_H group, treated with 600 mg/kg of *Alhagi sparsifolia*; AH_L+ALC group, treated with 150 mg/kg of *Alhagi sparsifolia* and alcohol (10 mL/kg); AH_M + ALC group, treated with 300 mg/kg of *Alhagi sparsifolia* and alcohol (10 mL/kg); AH_H + alcohol group, treated with 600 mg/kg of *Alhagi sparsifolia* and alcohol (10 mL/kg); SL + ALC group, treated with 300 mg/kg of silymarin and alcohol (10 mL/kg); SS + ALC group, treated with 300 mg/kg of sodium salicylate and alcohol (10 mL/kg), and ALC group, treated with 50% alcohol (10 mL/kg).

in the alcohol-treated group, the administration of *Alhagi sparsifolia* restored the GSH levels and SOD activities similar to those in the control group.

The TLR family has been given much attention for a long time. It recognizes pathogen-associated molecular patterns and initiates inflammatory responses (Luo *et al.*, 2016), gives the TLR4 pathway to promote the release of
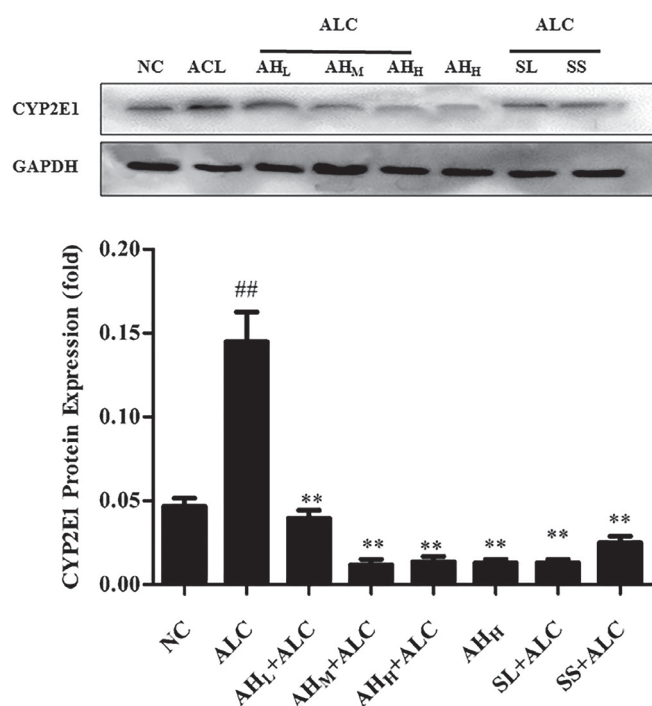
TNF-α, and increases pro-inflammatory cytokines and inflammation (Duan*et al.*, 2014). Our study demonstrated that the TNF-α and TLR4 mRNA expressions significantly increased after *Alhagi sparsifolia* was administered to the alcohol group. The *Alhagi sparsifolia* intervention significantly reduced the TNF-α and TLR4 mRNA expressions levels (Figure 4), thus indicating that *Alhagi sparsifolia*could could accelerate alcohol metabolism.

CYP2E1 has been reported as a major contributor to alcohol-induced oxidative stress and alcohol-induced liver injury. It is particularly related to ALD through the alcohol-induced ROS generation (Lu, Cederbaum, 2008). The expression and activity of CYP2E1 are increased by acute and chronic alcohol intake, and they catalyze the

conversion from alcohol to acetaldehyde, subsequently causing ROS over-production (Jimenezlopez, Cederbaum, 2005). The absence of CYP2E1 in knockout mice significantly lowered alcohol-induced oxidative stress and reduced lipid accumulation in the liver (Wang, 2010). Previous reports suggested that the great number of natural products and their active substances could inhibit CYP2E1. In the present study, the treatment of AH_M (300 mg/kg) remarkably reduced the alcohol induced CYP2E1 expression. These findings demonstrate the protective effect of *Alhagi sparsifolia* alcoholic livery injury by reducing oxidative stress by down-regulating the CYP2E1 expression.

CONCLUSION

Alhagi sparsifolia significantly ameliorated acute alcohol liver injury by enhancing antioxidative defenses. *Alhagi sparsifolia* could be an effective therapeutic agent for alcohol liver injury by inhibiting the activity and expression of CYP2E1 and inhibiting oxidative stress.

ACKNOWLEDGMENT

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