Pharmacogn. Mag.
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ORIGINAL ARTICLE

Involvement of Toll-like Receptor 2/Myeloid Differentiation Factor 88/Nuclear Factor kappa B/NLR Family Pyrin Domain-containing 3 Signaling Pathways in the Hepatoprotective Effect of *Lagotis brachystachys* in Rats with Alcoholic Liver Disease

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Submitted: 12-Dec-2019 Revised: 09-Jan-2020 Accepted: 17-Mar-2020 Published: 20-Oct-2020

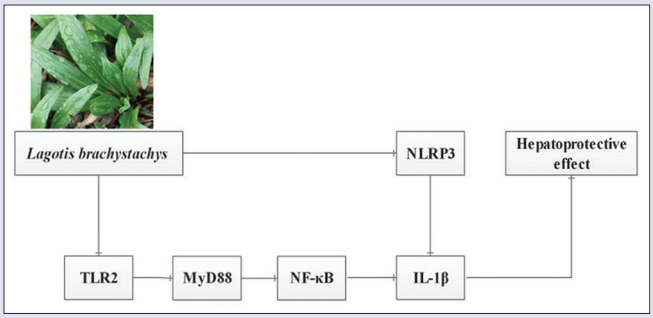
ABSTRACT

Background: Excessive consumption of alcohol is ranked as one of the leading causes of death from alcoholic liver disease (ALD). Our previous study demonstrated that the whole extracts of *Lagotis brachystachys* decreased acute hepatic injury in rats. However, the compounds of the extracts that are responsible for the hepatoprotective activity and their underlying mechanism are still unknown. **Objectives:** The objective of the study was to evaluate the hepatoprotective effect of whole extracts of *L. brachystachys* against chronic alcohol-induced ALD in rats. **Materials and Methods:** Different polar compounds of ethanolic extract from *L. brachystachys* were orally administered to rats that underwent 8 weeks of alcohol exposure. **Results:** The histological evaluation of rat liver showed that the rats exposed to 30% and 50% ethanolic extracts of *L. brachystachys* had significantly less formation of lipid droplets and showed less inflammatory infiltration than that of control rats with ALD. The extracts also inhibited alcohol-induced elevation of serum lipid peroxidation levels. In addition, *L. brachystachys* restored the levels of antioxidants and inhibited the alcohol-induced activation of the hepatic Toll-like receptor 2 (TLR2)/myeloid differentiation factor 88 (MyD88)/nuclear factor kappa B (NF-κB)/NLR family pyrin domain-containing 3 (NLRP3) signaling pathway, thereby decreasing the release of interleukin (IL)-1β. **Conclusion:** Our data revealed that *L. brachystachys* showed hepatoprotective effect against chronic alcohol-induced hepatic injury by decreasing the levels of lipid peroxidation and oxidative stress and by inhibiting the inflammatory processes. The extracts decreased the release of IL-1β via inactivation of the hepatic TLR2/MyD88/NF-κB/NLRP3 signaling pathway.

Key words: Alcoholic liver disease, *Lagotis brachystachys*, lipid peroxidation, oxidative stress, TLR2

SUMMARY

- *Lagotis brachystachys* protects against chronic alcohol-induced hepatic injury
- *L. brachystachys* decreases lipid peroxidation and oxidative stress
- *L. brachystachys* inhibits the hepatic Toll-like receptor 2/myeloid differentiation factor 88/nuclear factor kappa B/NLR family pyrin domain-containing 3 signaling pathway.



Abbreviations used: ALD: Alcoholic liver disease; TG: Triglyceride; TC: Total cholesterol; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GSH: Glutathione peroxidase; SOD: Superoxide dismutase; TLR2: Toll-like receptor 2; NLRP3: NLR family pyrin domain containing 3; MyD88: Myeloid differentiation factor 88; NF-κB: Nuclear factor kappa B.

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DOI: 10.4103/pm.pm_557_19

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INTRODUCTION

Alcoholic liver disease (ALD) is a type of chronic progressive hepatic disease caused due to the long-term unrestrained consumption of alcohol. It is the leading cause of hepatic-related morbidity and mortality worldwide. During the development of ALD, the disease is first characterized by simple steatosis and alcoholic steatohepatitis.[1] However, if alcohol is consumed more frequently, ALD may advance to the stages of fibrosis and cirrhosis and finally may lead to hepatocellular carcinoma.[2] In the United States, liver cirrhosis caused due to the excess consumption of alcohol is the 12th leading cause of death, accounting for 1.1% of all deaths. Typically, countries with high levels of alcohol consumption, such as Spain, France, and Italy, have higher mortality

Cite this article as: Zhu JX, Shan JL, Ouyang X, Yang HY, Wei RR, Zeng JX, *et al*. Involvement of toll-like receptor 2/myeloid differentiation factor 88/nuclear factor kappa B/NLR family pyrin domain-containing 3 signaling pathways in the hepatoprotective effect of *Lagotis brachystachys* in rats with alcoholic liver disease. Phcog Mag 2020;16:518-23.

rates. In countries where alcohol consumption is low (e.g., Iceland, New Zealand, and Norway), the mortality rate is relatively low.[3] In China, hepatic injury due to alcohol intake is increasing annually, and ALD has become the second leading cause of hepatic disease after viral hepatitis.[4] Unfortunately, despite the serious social impact and disease burden of ALD,[5] there has been no milestone progress in the control or treatment of ALD, and so far, the United States Food and Drug Administration has not approved any drugs or therapies to treat ALD.[6] The usual strategies of ALD intervention in the clinical setting include abstinence, natural and synthetic steroids, nutritional supplements, liver transplantation, and/or herbal remedies.

Lagotis brachystachys Maxim is a traditional Tibetan medicine. It is primarily used to alleviate inflammation. Our previous studies have shown that the whole extract of *L. brachystachys* exhibited the hepatoprotective effect on acute hepatic injury in mice caused due to alcohol consumption.[7] However, the components of the extract that are responsible for this action and their underlying mechanism in alleviating alcohol-induced chronic hepatic injury are still unknown. Considering that chronic inflammation is positively correlated with the development of ALD,[8] the anti-inflammatory activity involved in the hepatoprotective effects of *L. brachystachys* was evaluated in this study.

MATERIALS AND METHODS

Animals

Specific pathogen-free Sprague–Dawley rats (weighing about 180–220 g, male) were purchased from Hunan Slake Jingda Experimental Animal Co., Ltd. (Changsha, China). The animal license number was SCXK (Xiang) 2016-0002. The animals had free access to drinking water and food. All animals were adapted to the laboratory environment for 1 week before the formal experiment. All the procedures were approved by the Jiangxi University of Traditional Chinese Medicine. All institutional and national guidelines for the care and use of laboratory animals were followed.

Herbal medicine and preparation of the extracts

L. brachystachys Maxim was purchased from the Chengdu medicinal materials market and identified by Prof. Zhongguo Yue of the Jiangxi University of Traditional Chinese Medicine. The voucher specimen (voucher no. 20171126018) was deposited in the Research Center of Natural Resources of Chinese Medicinal Materials and Ethnic Medicine. Dried *L. brachystachys* (4.7 kg) were powdered and then extracted thrice with 75% ethanol (47 L) for 2 h under reflux. Then, the extract was concentrated and dried under reduced pressure. The dried extract was then dissolved in a small amount of water and was subjected to column chromatography on a macroporous resin D101 using water, 30% ethanol, 50% ethanol, and 95% ethanol as solvents. The different polar parts were then collected. The yield of the different polar parts was 30.7% for water, 4.64% for 30% ethanol, 3.50% for 50% ethanol, and 0.93% for 95% ethanol.

Reagents

The positive drug polyene phosphatidylcholine (Essentiale forte) was purchased from Sanofi Aventis (Paris, France). The commercial kits for triglyceride (TG), total cholesterol (TC), alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutathione peroxidase (GSH), and superoxide dismutase (SOD) were purchased from Nanjing Jiancheng (Nanjing, China). A rat interleukin (IL)-1β ELISA kit was purchased from Neobioscience (Shenzhen, China). Toll-like receptor 2 (TLR2) and NLR family pyrin domain-containing 3 (NLRP3) primary antibodies were purchased from Bioss (Beijing, China). Myeloid differentiation factor 88 (MyD88) and nuclear factor kappa B (NF-κB) primary antibodies were purchased from Proteintech (Wuhan, China). The β-actin primary antibody was purchased from GeneTex (Irvine, USA).

Drug administration

In total, 110 rats were randomly divided into 11 groups (n = 10) as follows: control group, ALD group, polyene phosphatidylcholine (95 mg/kg) group, 30% ethanol groups (0.5 g/kg and 2 g/kg), 50% ethanol groups (0.5 g/kg and 2 g/kg), 95% ethanol groups (0.5 g/kg and 2 g/kg), and water groups (0.5 g/kg and 2 g/kg). The dose of different polar parts for oral administration was finally multiplied by their respective yields [Table 1]. The rats except those in the control group were administered alcohol for 8 consecutive weeks.[9] During alcohol administration, drugs were orally administered at a rate of 10 mL/kg body weight.

Serum and tissue collection

After the last administration, the rats were anesthetized with 10% chloral hydrate (0.35 mL/kg). Blood samples were collected from the abdominal aorta and separated in a refrigerated centrifuge at 4°C for 10 min at 3000 g. The serum was stored at −20°C until assays were performed. The liver was quickly separated on the ice platform and was fixed in 10% formaldehyde fixative solution. The other parts of the liver were placed in EP Tubes® and stored at −80°C.

Biochemical assays

Serum levels of TC, TG, ALT, and AST, as well as hepatic tissue-specific GSH and SOD levels were measured with the microenzyme method according to the kit's instructions. Serum IL-1β levels were detected by the ELISA technique according to the kit's instructions.

Western blot

Liver samples were homogenized in a lysis buffer and left on ice for 20 min. The supernatant was obtained by centrifugation at 10,000 ×*g*. Then, the concentration of protein sample was measured using a bicinchoninic acid kit. The samples were prepared according to the concentration and subjected to conventional loading electrophoresis. Then, the gel was transferred to a polyvinylidenefluoride membrane in a transfer tank. Subsequently, the membrane was blocked by incubating the membrane in 5% skim milk for 1 h at room temperature. After blocking, the membrane was incubated with TLR2 (1:2000), MyD88 (1:2000), NF-κB (1:2000), NLRP3 (1:2000), and β-actin (1:5000) primary antibodies at 4°C in a shaker overnight. The next day, after removing the primary antibodies and washing, the secondary antibody was added. After incubation for 1 h, enhanced chemiluminescence solution was added, and the proteins were detected.

Histological evaluation

The liver was cut into small pieces with a razor blade, rinsed with water in an embedding box overnight, and then, dehydrated with ethanol (50%, 60%, 70%, 80%, and 90%), anhydrous ethanol, and xylene. The tissue was then embedded in paraffin and cut into slices. The paraffin slices were stained with hematoxylin and eosin (H and E), and the general histological structures were observed under a microscope.

Statistical analyses

All data were presented as the mean ± standard deviation. A one-way analysis of variance followed by a *post hoc* test was performed. $P < 0.05$ was considered to be statistically significant for the statistical analysis.

RESULTS

Effect of *Lagotis brachystachys* on the liver histopathology in rats with chronic alcoholic liver disease

As shown in Figure 1, the hepatic tissue of the control rats was ruddy and delicate. The hepatic cells were normal in shape under a microscope. The nucleus was large and round and the hepatocyte cords were scattered around the nucleus. However, the surface of the hepatic tissue in ALD rats was granular. Under the microscope, the hepatic cells were scattered and enlarged and varied in size. The hepatocytes were swollen, and we observed the presence of round fat vacuoles of various sizes. In addition, there was inflammatory infiltration around the central vein. After the administration of polyene phosphatidylcholine, rats exposed to 30% and 50% ethanolic extracts of *L. brachystachys* demonstrated significantly less lipid droplet vacuoles and less inflammatory infiltration than ALD rats. The livers of rats exposed to 95% ethanolic extract of *L. brachystachys* exhibited many lipid droplet vacuoles. The hepatocyte cords were not aligned, and there was obvious hepatic sinus dilatation and inflammatory infiltration.

Effect of *Lagotis brachystachys* on the serum triglyceride, total cholesterol, alanine aminotransferase, and aspartate aminotransferase levels in rats with chronic alcoholic liver disease

As shown in Table 2, the serum levels of TG, TC, ALT, and AST significantly increased in the ALD group compared with rats in the control group. Compared with the ALD group, the ALT and AST levels in the animals treated with 30%, 50%, and 95% ethanolic extracts and with polyene phosphatidylcholine were significantly reduced. The TG levels significantly decreased in the animals treated with 30% and 50% ethanolic extracts and with polyene phosphatidylcholine.

Effect of *Lagotis brachystachys* on hepatic glutathione peroxidase and superoxide dismutase levels in rats with chronic alcoholic liver disease

As shown in Table 3, compared with those in the control group, GSH and SOD levels significantly decreased in the ALD group. Compared with the ALD group, the groups exposed to 30% and 50% ethanolic extracts of *L. brachystachys* and with polyene phosphatidylcholine experienced significantly increased GSH levels. However, only SOD levels increased after exposing the ALD rats with 50% ethanolic extract of *L. brachystachys*.

Effect of *Lagotis brachystachys* on the liver Toll-like receptor 2/myeloid differentiation factor 88/nuclear factor kappa B/NLR family pyrin domain-containing 3 signaling activity in rats with chronic alcoholic liver disease

As shown in Figure 2, rats with ALD exhibited a significant increase in the expression levels of TLR2, MyD88, NF-κB, and NLRP3 in the liver compared with the control rats. Compared with ALD rats, the

Table 1: The dose of different polar parts for oral administration

Group	Dose (converted based on crude drug dose, g/kg)	Dry extract (g)	Yield (%)	Dose (mg/kg)
Normal	-	-	-	-
Model	-	-	-	-
PPC	0.095	-	-	95.00
30% EL	0.5	218.40	4.64	23.20
30% EH	2	218.40	4.64	92.80
50% EL	0.5	164.80	3.50	17.50
50% EH	2	164.80	3.50	70.00
95% EL	0.5	43.86	0.93	4.65
95% EH	2	43.86	0.93	18.60
WL	0.5	1443.00	30.70	153.50
WH	2	1443.00	30.70	614.00

PPC: Phenylpropanoid complex; EL: Ethanol eluate, low dose; EH: Ethanol eluate, high dose; WL: Water eluate, low dose; WH: Water eluate, high dose

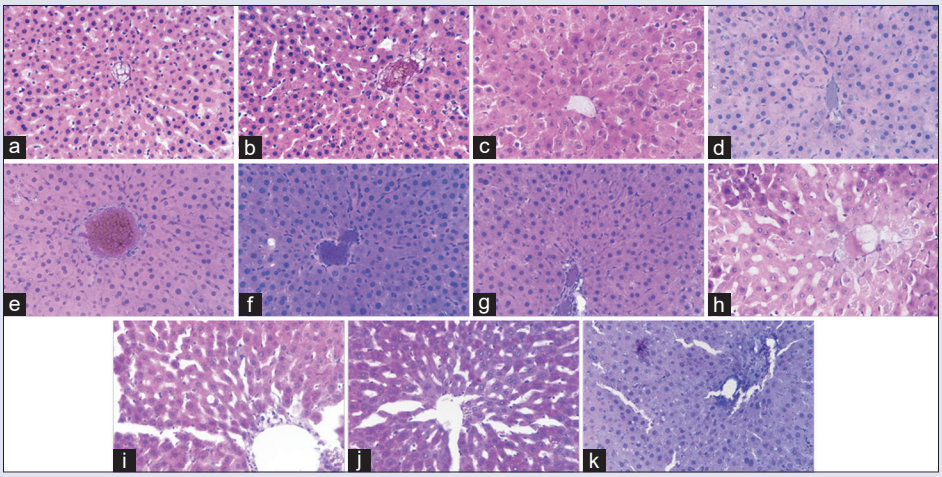

Figure 1: Effect of *Lagotis brachystachys* extracted with different polar parts on pathological morphology of liver tissue in rats with chronic alcoholic liver disease (H and E, ×400). (a) Control; (b) alcoholic liver disease; (c) PPC; (d) 30% EL; (e) 30% EH; (f) 50% EL; (g) 50% EH; (h) 95% EL; (i) 95% EH; (j) WL; and (k) WH

Table 2: Effect of *Lagotis brachystachys* extracted with different polar parts on triglyceride, total cholesterol, alanine aminotransferase and aspartate aminotransferase levels in the serum of rats with chronic alcoholic liver injury (x±s, *n*=8)

Group	Dose (g/kg)	TG (mmol/L)	TC (mmol/L)	ALT (U/L)	AST (U/L)
Normal	-	0.69±0.23	2.38±0.12	7.36±3.39	5.74±3.25
Control	-	1.60±0.53##	2.81±0.29#	14.52±3.31##	13.44±4.96##
PPC	0.095	1.02±0.42*	2.62±0.24	7.19±3.08**	7.38±2.16**
30% EL	0.5	1.06±0.17**	2.62±0.42	6.23±3.73**	7.44±2.69**
30% EH	2	0.95±0.61**	2.57±0.25	4.99±1.65**	7.31±1.25**
50% EL	0.5	0.87±0.26**	2.65±0.24	6.77±1.33**	7.50±1.19**
50% EH	2	0.58±0.14**	2.68±0.19	5.46±2.61**	6.55±1.51**
95% EL	0.5	1.32±0.44	2.71±0.25	5.97±1.73**	7.13±2.08**
95% EH	2	1.23±0.42	2.95±0.73	7.40±2.23**	7.87±1.79**
WL	0.5	1.25±0.31	3.26±0.30	13.83±4.61	11.29±5.37
WH	2	1.23±0.22	2.81±0.32	11.52±1.36	10.42±2.17

#$P < 0.05$ and ##$P < 0.01$ versus the Normal group. *$P < 0.05$ and **$P < 0.01$ versus the Control group. TC: Total cholesterol; TG: Triglyceride; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PPC: Phenylpropanoid complex; EL: Ethanol eluate, low dose; EH: Ethanol eluate, high dose; WL: Water eluate, low dose; WH: Water eluate, high dose

Table 3: Effect of *Lagotis brachystachys* extracted with different polar parts on glutathione peroxidase and superoxide dismutase content in the liver homogenate of rats with chronic alcoholic liver injury (x±s, *n*=8)

Group	Dose (g/kg)	GSH (μmol/g protein)	SOD (U/mg protein)
Normal	-	6.14±1.06	617.11±70.06
Control	-	4.48±0.27##	429.34±43.46#
PPC	0.095	6.26±0.23**	464.65±15.88
30% EL	0.5	5.74±0.32**	453.46±38.92
30% EH	2	5.79±0.53**	471.33±67.31
50% EL	0.5	6.86±1.57**	646.93±60.48**
50% EH	2	6.82±1.00**	654.17±52.40**
95% EL	0.5	5.11±1.12	475.91±25.30
95% EH	2	5.21±0.70	469.46±33.97
WL	0.5	5.24±0.78	477.19±37.34
WH	2	4.00±0.79	485.18±41.41

#$P < 0.05$ and ##$P < 0.01$ versus the Normal group. **$P < 0.01$ versus the Control group. GSH: Glutathione peroxidase; SOD: Superoxide dismutase; PPC: Phenylpropanoid complex; EL: Ethanol eluate, low dose; EH: Ethanol eluate, high dose; WL: Water eluate, low dose; WH: Water eluate, high dose

rats exposed to 30% and 50% ethanolic extracts of *L. brachystachys* significantly decreased the levels of TLR2, NF-κB, and NLRP3 in the liver. In addition, 30% ethanolic extract of *L. brachystachys* tended to decrease the levels of MyD88 in the liver, although the trend did not reach significance.

Moreover, ALD also significantly increased the serum IL-1β levels compared with the control rats. Compared with the ALD rats, 30% and 50% ethanolic extracts of *L. brachystachys* significantly decreased the serum IL-1β levels in rats [Figure 3].

DISCUSSION

The liver is a crucial organ in the human body and is involved in the glucose, protein, and fat metabolism. In addition, it detoxifies various xenobiotics into harmless chemicals that are excreted from the body. Its functional impairment has a huge impact on the body. Alcohol remains one of the key causes of liver diseases.[10] With the help of H and E staining, in this study, we confirmed that a rat model of ALD was successfully established. The following specific manifestations were observed in rat hepatocytes after exposure to alcohol: inhibition of edema, appearance of steatosis and fatty vacuoles, and unclear boundaries of the hepatic lobes. However, the administration of positive drug polyene phosphatidylcholine and *L. brachystachys* significantly improved the pathological changes in the liver tissue of rats with ALD, suggesting the hepatoprotective activity of *L. brachystachys*.

It is noteworthy that most of the alcohol is metabolized by the liver. During metabolism, many reactive oxygen species and toxic metabolites are produced. The excessive toxic metabolites lead to the lipid peroxidation of cellular unsaturated fatty acids, which increases the permeability of liver cell membranes and mitochondrial membranes. Consequently, hepatic enzymes and toxic metabolites exit the hepatic cells and enter the bloodstream. ALT and AST, two enzymes mainly located in the liver, are common indicators of liver function test in clinical practice, and their concentrations in the blood can reflect the severity of liver cell damage.[11,12] Under normal circumstances, ALT is mainly present in the cytoplasm of hepatocytes, whereas AST is mainly present in the mitochondria of the hepatocytes. ALT and AST are released into the bloodstream during alcohol exposure as a consequence of changes in membrane permeability.[13-15] In this study, we found that chronic consumption of alcohol significantly increased the levels of AST and ALT, whereas the positive control drug, polyene phosphatidylcholine, and *L. brachystachys* reversed this effect, which indicates that *L. brachystachys* attenuated the impairment of membrane permeability of hepatocytes. Another important manifestation of ALD is the disorder in the lipid metabolism. After exposure to alcohol, the metabolism of TG and TC is inhibited. In addition, due to the destruction of liver compensation, a large amount of fat stored in the body is decomposed, which causes the upregulation of TG and TC in the blood.[13] Previous studies have shown that serum TC and TG levels significantly increased after exposure to alcohol.[16,17] Consistently, this study also showed that chronic alcohol exposure induced the secretion of TC and TG in the serum, which reflected the disorder in the lipid metabolism by the liver. In contrast, the administration of polyene phosphatidylcholine and *L. brachystachys* significantly reduced the abnormal lipid levels caused due to chronic alcohol exposure.

In addition to lipid peroxidation, oxidative stress has been considered to be involved in the pathophysiology of ALD. GSH is a non-enzymatic antioxidant, which interacts with reactive oxygen species.[18] SOD is an enzyme that directly removes reactive oxygen species by catalyzing the conversion of superoxide anions into oxygen and hydrogen peroxide.[19] Consistent with previous reports,[20,21] our results showed that chronic alcohol exposure decreased the levels of GSH and the activity of SOD, indicating the impaired antioxidative functions in the liver. In contrast, we found that *L. brachystachys* increased the GSH levels and SOD activity in the liver, which indicated that *L. brachystachys* improved the antioxidant defense system in the body.

In addition to lipid peroxidation and oxidative stress, inflammation is a crucial factor in the development of ALD.[22] Inflammation, a

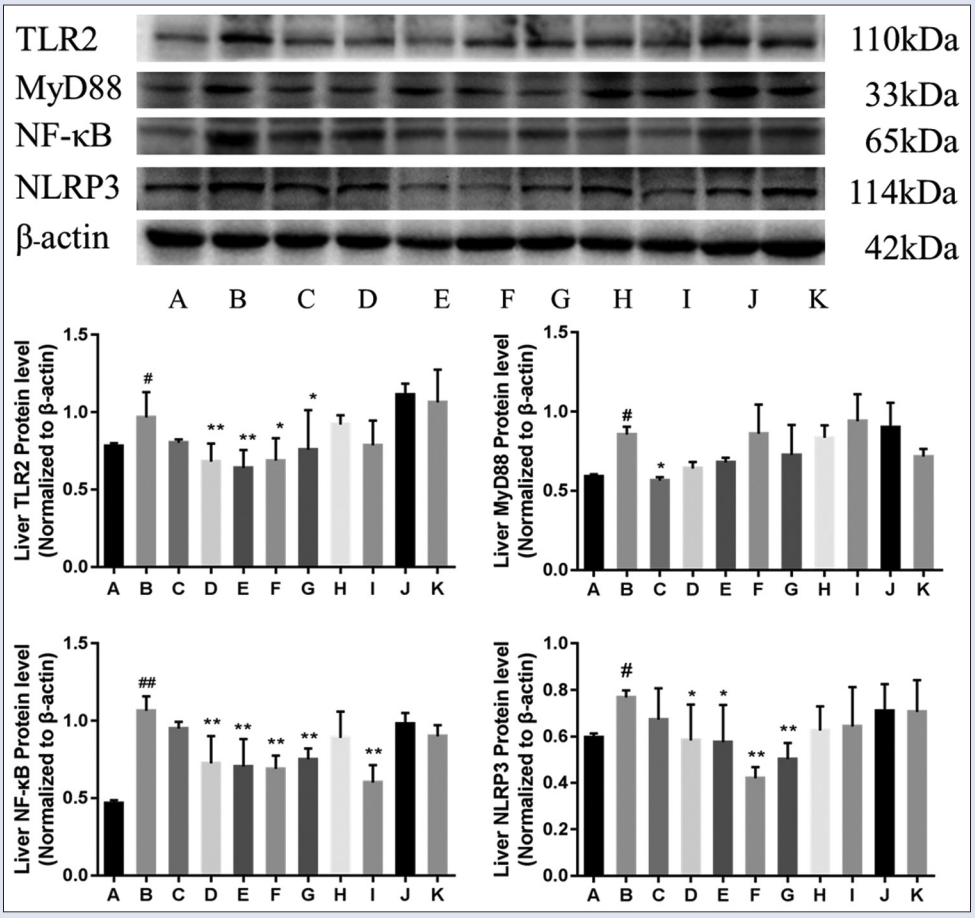

Figure 2: Effects of *Lagotis brachystachys* extracted with different polar parts on the hepatic levels of Toll-like receptor 2, myeloid differentiation factor 88, nuclear factor kappa B, and NLR family pyrin domain containing 3 in rats with chronic alcoholic liver disease. A. Normal; B. Control; C. PPC; D. 30% EL; E. 30% EH; F. 50% EL; G. 50% EH; H.95% EL; I. 95% EH; J. WL; K. WH. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus the Normal group. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the Control group

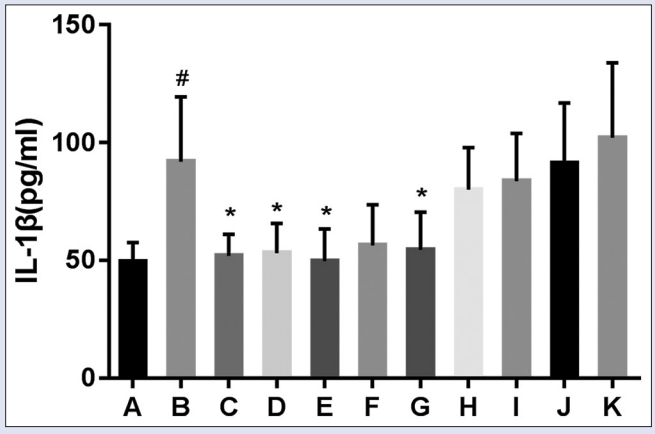

Figure 3: Effects of *Lagotis brachystachys* extracted with different polar parts on the serum interleukin-1β levels in rats with chronic alcoholic liver disease. A. Normal; B. Control; C. PPC; D. 30% EL; E.30% EH; F. 50% EL; G. 50% EH; H.95% EL; I. 95% EH; J. WL; and K. WH. $^{\#}P < 0.05$ versus the Normal group. $^{*}P < 0.05$ versus the Control group

consequence of excessive alcohol consumption, causes liver fibrosis and liver dysfunction.[23] Increasing evidence has demonstrated that many inflammatory factors, such as the TLR-related signaling pathway, mediate the pathophysiology of ALD.[24,25] A previous study demonstrated that TLR2 signaling contributed to the development of ALD through the activation of inflammasomes and IL-1 β.[26] A study conducted by Roh also revealed that MyD88, a common adaptor molecule for TLR2, participated in the development of ALD, and the study further demonstrated that the inhibition of MyD88 prevented the development of ALD via the suppression of IL-1 β production.[26] As one of the most studied pro-inflammatory cytokines, IL-1 β has been verified to be produced and processed via activation of the TLR2/MyD88/NF-κb/NLRP3 signaling pathway.[27] Similar results were observed in this study where chronic alcohol consumption increased the hepatic levels of TLR2, which activates the hepatic TLR2/MyD88/NF-κB/NLRP3 signaling, resulting in the excessive release of IL-1β and induction of ALD. We also found that treatment with *L. brachystachys* inhibited TLR2/MyD88/NF-κB/NLRP3 signaling in the liver and, in turn, decreased the production of IL-1β, thus ameliorating liver dysfunction from chronic alcohol exposure in the rats.

L. brachystachys mainly contains flavonoids, cyclic allene terpenoids, phenylpropanoid glycosides, and other components.[28,29] This study showed that, excluding 95% ethanolic and water extracts, 30% and 50% ethanolic extracts of *L. brachystachys* exerted significant hepatoprotective activity in ALD rats. This finding will be helpful in further investigations to isolate the active components.

CONCLUSION

In summary, the results of this study demonstrated that *L. brachystachys* prevented against chronic alcohol-induced hepatic injury by decreasing lipid peroxidation and oxidative stress and inhibited inflammation through the reduction of IL-1β release via inactivation of the hepatic TLR2/MyD88/NF-κB/NLRP3 signaling pathway. In addition, we also demonstrated that the main protective components were present in 30% and 50% ethanolic extracts of *L. brachystachys*. In future, we plan to isolate the active components from 30% and 50% ethanolic extracts of *L. brachystachys* and develop this herbal interventional approach for ALD.

Financial support and sponsorship

The project was supported by grants from the National Natural Science Foundation of China (No. 81660702), the Scientific Foundation of Double World-classes Subject of the Jiangxi University of TCM (No. JXSYLXK-ZHYAO032) and the Scientific Research Project of Jiangxi Provincial Health and Family Planning Commission (No. 2016A052).

Conflicts of interest

There are no conflicts of interest.

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