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Patchouli oil isolated from the leaves of *Pogostemon cablin* ameliorates ethanol-induced acute liver injury in rats *via* inhibition of oxidative stress and lipid accumulation

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Excessive alcohol consumption can cause serious hepatic injury which is associated with oxidative stress and fatty metabolic disturbance. Patchouli oil (PO) is a sort of food supplement with high medicinal value in hepatoprotection, but its ability against ethanol-induced liver failure has not been demonstrated. Thus, this study aimed to investigate the potential hepatoprotection of PO through an ethanol-induced hepatotoxicity rat model. Our results showed that PO pretreatment could dramatically decrease the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) in serum, paralleled by an improvement of histopathology alterations. Additionally, PO could markedly suppress the content of reactive oxygen species (ROS), tumor necrosis factor alpha (TNF- α), free fatty acid (FFA), and triglyceride (TG), while enhancing the activities of glutathione (GSH), glutathione reductase (GR), and superoxide dismutase (SOD) as well as the ratio of glutathione to oxidized glutathione (GSH/GSSG) in liver. The protective effect of PO against oxidative stress was interrelated with restraining the mRNA and protein expression of hepatic microsomal enzyme cytochrome P450 2E1 (CYP2E1). What's more, PO pretreatment could also accelerate lipometabolism *via* up-regulating expressions of adenosine monophosphate-activated protein kinase (AMPK), peroxisome proliferator-activated receptor α (PPAR- α), and carnitine palmitoyltransferase 1 (CPT-1) and down-regulating expressions of nuclear factor-kappaB (NF- κ B) p65, sterol regulatory element-binding protein 1 (SREBP-1c), fatty acid synthase (FAS), and stearoyl-CoA desaturase 1 (SCD-1). To conclude, PO showed potent effect against ethanol-induced hepatotoxicity by relieving oxidative stress and preventing lipid accumulation.

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1. Introduction

Alcoholic liver injury (ALI) refers to liver disorders caused by long-term or large consumption of alcohol, which include alcoholic fatty liver, hepatitis, hepatic fibrosis and cirrhosis.¹ With the increasing living and mental pressure, alcohol abuse prevails leading to high morbidity and even mortality rate of ALI.² Hence, it is necessary to develop an effective agent to prevent the deterioration of ALI. Actually, some progresses have

been made on the pathogenesis of ALI, but researches on specific medicine still remain stagnant.

ALI is a complex process mediated through some pathological mechanisms such as oxidative stress, lipid disorders, inflammatory response and apoptotic reaction, amongst which oxidative stress and abnormal lipid metabolism rank first for ALI occurrence.^{3,4} The interplay between oxidative stress and steatosis in ethanol-induced liver failure has been discussed by multiple researchers.^{5,6} Alcohol intake activates the activity of hepatic cytochrome P450 2E1 (CYP2E1) that accelerates the alcohol oxidation and acetaldehyde accumulation.⁷ The interaction between acetaldehyde, proteins and lipids can lead to massive reactive oxygen species (ROS) formation and severe cell damage, and cause hepatic oxidative stress.⁸ It is reported that ROS are able to induce lipid metabolic disturbance by down-regulating adenosine monophosphate-activated protein kinase (AMPK).⁹ AMPK can directly affect the expression of peroxisome proliferator-activated receptor alpha (PPAR α) and carnitine palmitoyltransferase-1 (CPT-1), protecting liver from lipidosis.¹⁰

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a gradient increase to 240 °C at a rate of 8°C min⁻¹ and maintained for 5 min. Since the inlet temperature reached 250 °C, the helium carrier gas was used to support the pressure of 12 psi on the column with a constant flow rate of 1.3 mL min⁻¹. Ion source temperature for mass spectrometer was 230 °C, operated in a splitless EI mode at 70 eV. The mass spectrum plot was applied using a full-scan monitoring mode with a mass scan range of *m/z* 35–400, and the splitting ratio was 60 : 1. The resulting peaks were identified by comparing the mass spectrums and retention index of compounds to those in the databases and values reported in the literatures.

2.3 Animals and experimental protocols

Male wistar rats (weighting 180–220 g, SCXK (YUE) 2013-0034) were purchased from Laboratory Animal Center of Guangzhou University of Chinese Medicine (Guangzhou, China). All performance accorded with the Animal Experimental Ethics Committee of Guangzhou University of Chinese Medicine (Guangzhou, China, no. 2016047). Prior to experimentation, rats were adaptively fed for 1 week with random diet and water under the controlled temperature (22 ± 2 °C) and humidity ($55 \pm 5\%$) with a 12 h light/dark cycle. Following acclimation, rats were randomly assigned into six groups (ten rats per group): normal control group (NC), ethanol group (Model), silymarin group (200 mg kg⁻¹, Sily) as the positive control, and three PO pretreatment groups (100, 200 and 400 mg kg⁻¹, respectively). Rats in Sily and PO groups were intragastrically administrated corresponding doses of drugs (10 mL kg⁻¹) once a day, while those in the NC and Model groups received an equal volume of normal saline. The doses of PO and silymarin were ascertained based on the previous investigation and our preliminary experiments.²⁸ After pretreatment for 7 consecutive days, all groups except NC group were given 65% ethanol (10 mL kg⁻¹) intragastrically²⁹ every 12 h at 6 different time points, which has successfully established severe liver injury model. At the end of the experimental period (the 11th day), all rats were injected with 1% pentobarbital sodium for anesthetization. Blood samples were collected for serum biochemistry tests and the livers were quickly dissected, weighed, frozen in liquid nitrogen, and then stored at -80 °C for further studies.

2.4 Blood biochemical studies

The blood samples were placed at room temperature for 30 min before centrifuging to obtain serum for biochemical estimations. Activities of alanine transaminase (ALT), aspartate amino transferase (AST) alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) were assayed by commercial assay kits (Nanjing, Jiancheng) following the manufacturer's protocols.

2.5 Histopathological evaluation

Part of the liver samples were separated and kept in 10% formalin for 48 h and embedded in paraffin for histopathologic examination under a light microscope (Olympus IX71, Olympus Co., Tokyo, Japan).

PO was analyzed by gas chromatography-mass spectrometry (GC-MS), on an Agilent GC 7890A/MSD5975C instrument (Agilent Technologies Co. Ltd., Santa Clara, CA, USA). Samples (2 μ L) dissolved in diethyl ether (1 mL) were injected onto the HP-5MS column (30 m \times 250 μ m \times 0.5 μ m). The temperature program was started at 100 $^{\circ}$ C. After keeping for 1 min,

Detection System (Amersham Life Science, UK). The density of each band was analyzed using Quantity One, software.

Liver samples were homogenized in cold saline (1 : 9, w/v) before being centrifuged at $10\,000 \times g$ for 10 min at 4 °C. Then the supernatant was acquired to measure glutathione (GSH), glutathione disulfide (GSSG), glutathione reductase (GR), superoxide dismutase (SOD) and triglyceride (TG) level by using commercial assay kits (Nanjing, Jiancheng) according to standard instructions.

2.7 Measurement of tumor necrosis factor (TNF- α), reactive oxygen species (ROS) and free fatty acid (FFA) levels

Liver tissue were separated from rats and the content of tumor necrosis factor (TNF- α), reactive oxygen species (ROS) and free fatty acid (FFA) were assayed using specific ELISA kits (ImmunoWay Biotech, America) respectively according to standard instructions.

2.8 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA from liver samples were isolated using TRIzol reagent and then quantified by detecting the ratio of absorbance at 260 nm to that at 280 nm, and the purity was between 1.8 and 2.0. The level of cytochrome P450 2E1 (CYP2E1), nuclear factor-kappaB (NF- κ B) p65, adenosine monophosphate-activated protein kinase (AMPK), peroxisome proliferator-activated receptor α (PPAR- α), carnitine palmitoyltransferase 1 (CPT-1), sterol regulatory element-binding protein 1 (SREBP-1c), stearoyl-CoA desaturase 1 (SCD-1), fatty acid synthase (FAS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each sample was measured using HiScript® II Q RT SuperMix (+gDNA wiper) and ChamQ™ SYBR® qPCR Master Mix Kit according to the standard protocol provided by the manufacturer (Vazyme Biotech, China). Primer sequences are listed in Table 1. PCR amplification was performed as follows: pre-denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The relative quantification of gene expression was calculated using the $2^{-\Delta\Delta C_q}$ method and GAPDH acted as an internal control.

2.9 Western blotting analyses

Liver tissue were extracted from rats and homogenized in Cell Lysis Buffer in order to obtain total proteins and the concentrations were determined by Protein Assay Kit (Nanjing, Jiancheng). Equivalent protein (50–80 μg) from each sample were separated by SDS-PAGE and transferred to a PVDF membrane. After transfection, the membranes were blocked with 5% skimmed milk under the room temperature for 1 h and then incubated with primary antibody of 1 : 500 CYP2E1, 1 : 600 NF- κB p65, 1 : 500 AMPK, 1 : 500 PPAR- α , 1 : 300 CPT-1, 1 : 500 SREBP-1c, 1 : 500 SCD-1, 1 : 1000 FAS and 1 : 1000 β -actin at 4 °C overnight. Next, each membrane was washed 10 minutes for 3 times and incubated with 1:2000 HRP-conjugated secondary antibody for 2 h. Subsequently, the bands were visualized by ECL reagent and detected by Western Blotting.

2.10 Statistical analysis

All values are exhibited as means \pm standard deviation (mean \pm SD), and a value of $P < 0.05$ was considered statistically significant. Statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by an LSD test for multiple comparisons using SPSS software 20.0.

3. Results

3.1 GC-MS analysis of PO

The total ion chromatogram of PO was shown in Fig. 1. Seven compounds with relative content above 1% were identified in patchouli oils. The retention indices and relative proportion of these eleven compounds were listed in Table 1. As exhibited, patchouli alcohol took up the largest proportion (41.993%) in PO, followed by azulene at 23.532%. Besides, α -guaiene accounted for 15.173%. The relative contents of seychellene (7.395%), α -patchoulene (4.505%), β -patchoulene (3.968%) and globulol (3.434%) were all under 10%.

3.2 Effect of PO on ALT, AST, ALP and LDH levels

Compared with NC group (all $p < 0.01$), the activities of ALT, AST, ALP and LDH in Model group were dramatically increased by 494.2% (7.23 ± 1.49 vs. 42.96 ± 6.32), 494.4% (11.29 ± 2.01 vs. 67.11 ± 10.89), 121.5% (41.04 ± 5.55 vs. 90.92 ± 13.47), and 47.4% (1163.90 ± 57.62 vs. 1716.00 ± 145.03). However, pretreatment with silymarin and PO (200 and 400 mg kg⁻¹) significantly suppressed these elevated trends (Fig. 2A–D, all $p < 0.01$). PO at 100 mg kg⁻¹ also showed a similar effect, but there were no statistical significance ($p > 0.05$) on ALP and LDH expression. Compared with Model group, PO at 400 mg kg⁻¹ exerted the best hepatic protection *via* reducing ALT, AST, ALP and LDH levels by 71.0%, 75.3%, 49.0% and 26.1%, respectively.

Table 1 Primer sequences

Targeted gene	Direction and sequence
CYP2E1	F: 5'- ATG GCG GTT CTT GGC ATC ACC -3' R: 5'- GTA GCC ATG CAG GAC CAC GAT G -3'
NF-κB p65	F: 5'- GGA ATC ATC CAG GCT GCT GAA CTG -3' R: 5'- ATC ACG CCA CTC CTC GGT CAC -3'
AMPK	F: 5'- AAC CGT TCT ATT GCC ACT CTG CTG -3' R: 5'- TGG AGG CGA GGT AGA ACT CAC TG -3'
PPAR-α	F: 5'- CTG AGG AAG CCA TTC TGC GAC ATC -3' R: 5'- GCG TCT GAC TCG GTC TTC TTG ATG -3'
CPT-1	F: 5'- TGG CTA TGG TCA AGG TCC TCT CAG -3' R: 5'- CAG CAG TAT GGC GTG GAT GGT G -3'
SREBP-1c	F: 5'- ACG CCA TCT AGC CTG CCA CTG -3' R: 5'- GGT GTT GAT GCC TGC GGT CTT C -3'
SCD-1	F: 5'- TCC TAC ACG ACC ACC ACT ACC ATC -3' R: 5'- GCC GAG CCT TGT AAG TCC TGT G -3'
FAS	F: 5'- CCG CCG AGT CCG AGT CTG TC -3' R: 5'- CCG TGA GGT TGC TGT TGT CTG TAG -3'
GAPDH	F: 5'- GTC CAT GCC ATC ACT GCC ACT C -3' R: 5'- CGC CTG CTT CAC CAC CTT CTT G -3'

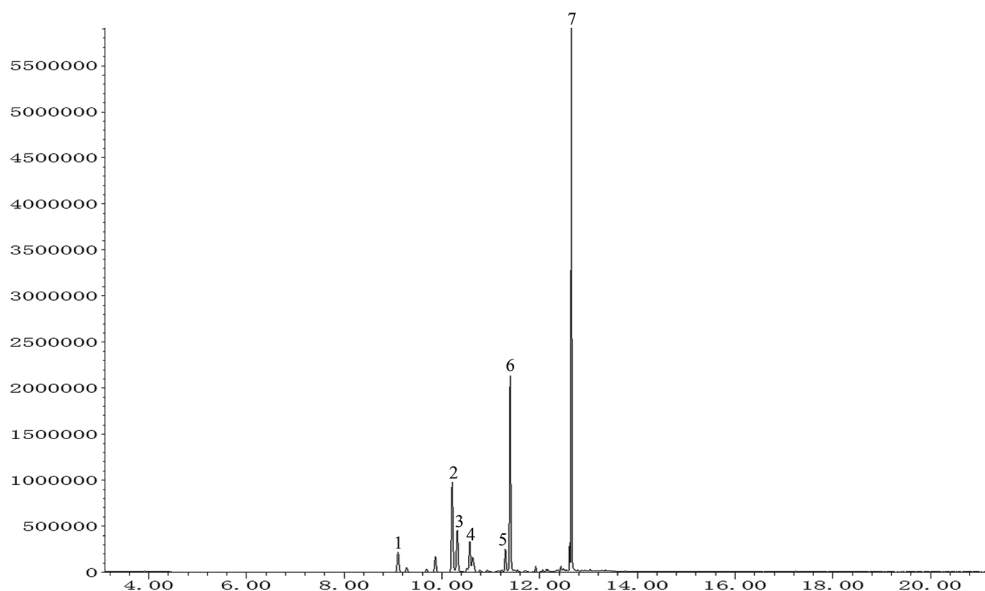


Fig. 1 GC-MS analysis of patchouli oil. Numbers indicated on peaks corresponded to those in Table 1.

3.3 Histopathological analysis on H&E staining

As shown in Fig. 3A, liver cells of normal group were in compact arrangement (Fig. 3A). Central veins ran through the middle of the lobe, surrounded by hepatic cords that radiated out in all directions. However, liver tissues in Model group appeared serious

granular denaturalization, fatty degeneration, inflammatory infiltration and hemorrhagic focus of hepatocytes (Fig. 3B). In contrast, after the administration of silymarin and PO (100, 200 and 400 mg kg⁻¹), liver histopathological lesions were distinctly repaired, as evidence by the improvement of hepatocellular steatosis and the

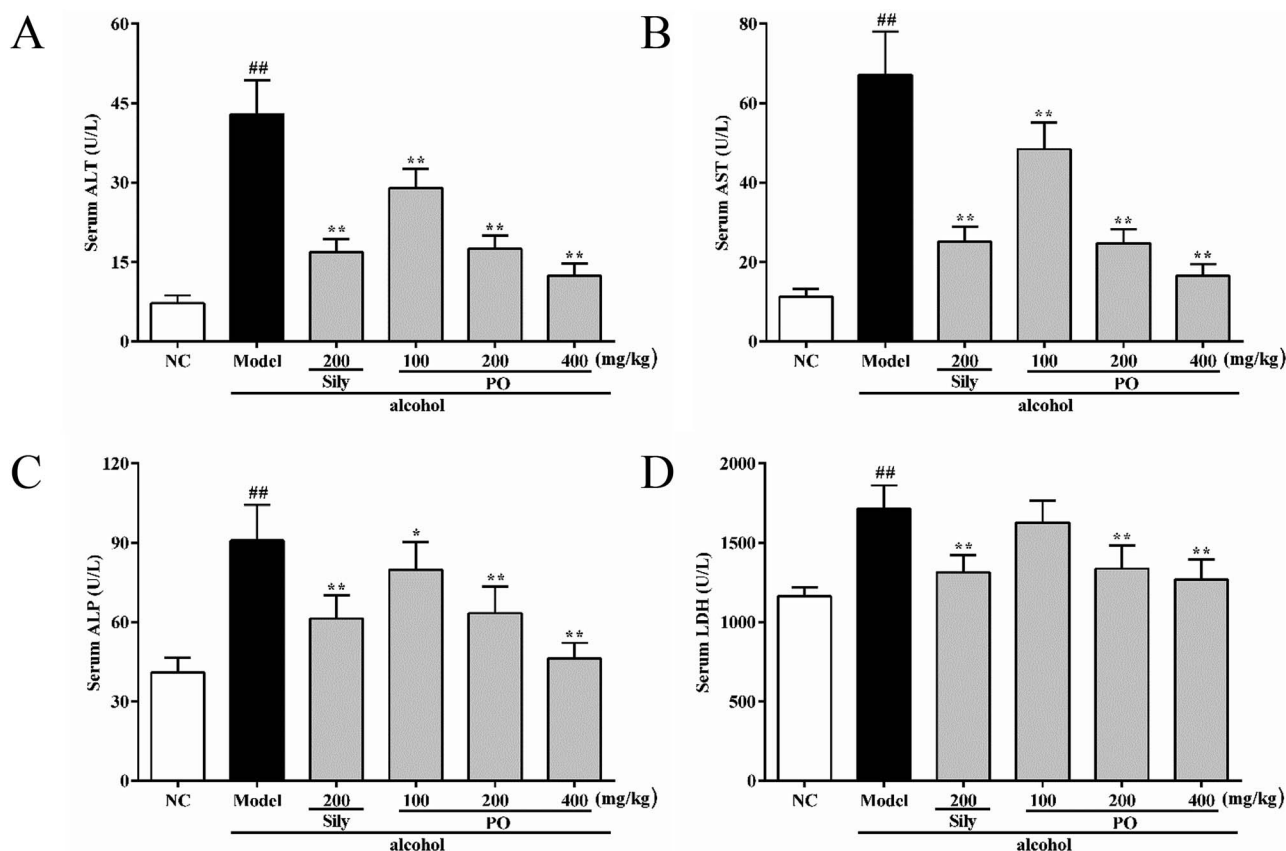


Fig. 2 Effects of PO on levels of ALT, AST, ALP and LDH in liver. (A) ALT; (B) AST; (C) ALP; (D) LDH. Values were presented as the mean \pm SD ($n = 12$). ## $p < 0.01$ vs. NC group; * $p < 0.05$, ** $p < 0.01$ vs. Model group.



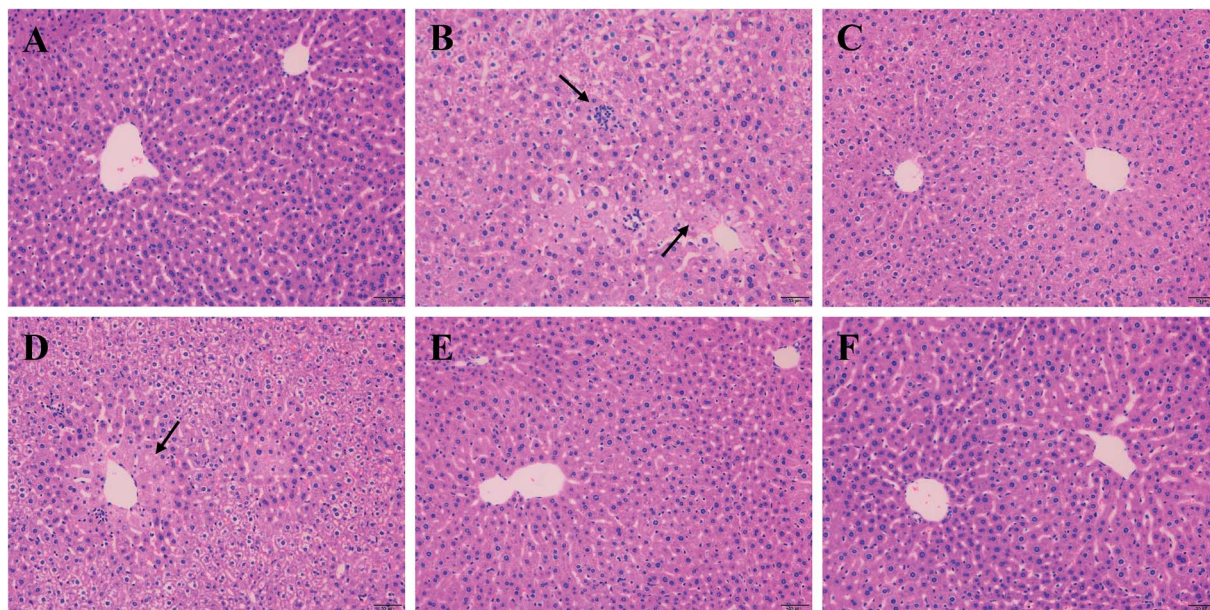


Fig. 3 Effects of PO on histological evaluation in ethanol-induced liver damage (H&E staining, magnification 200 \times). (A) NC group: visible central veins and thin sinusoids; (B) alcohol Model group: severe liver damage, inflammatory infiltration and necrosis; (C) alcohol + silymarin group; (D) alcohol + PO 100 mg kg⁻¹; (E) alcohol + PO 200 mg kg⁻¹; (F) alcohol + PO 400 mg kg⁻¹: well-formed hepatocytes and minor histopathology change. Arrows indicated both apparent focal necrosis and inflammatory infiltration.

reduction in necrosis areas (Fig. 3C–F). Moreover, restored hepatic structure could be seen in PO group at 400 mg kg⁻¹ with the fewest adipose vacuole attaching.

3.4 Effect of PO on antioxidant ability and hepatic lipidosis

As shown in Fig. 4, compared with NC group (all $p < 0.01$), alcohol administration markedly decreased the levels of GSH, GR, SOD and the ratio of GSH/GSSG by 34.3% (200.18 ± 6.79 vs. 131.45 ± 13.22), 61.9% (4.04 ± 0.56 vs. 1.54 ± 0.21), 41.1% (68.45 ± 8.85 vs. 40.32 ± 6.02), and 77.8% (2.83 ± 0.53 vs. 0.63 ± 0.09), while increased the content of TG by 560% (0.10 ± 0.02 vs. 0.66 ± 0.10). Conversely, pretreatment with PO (200 and 400 mg kg⁻¹) dramatically elevated the levels of GSH, GR, SOD and the ratio of GSH/GSSG but reduced the content of TG (all $p < 0.05$). Additionally, PO at 400 mg kg⁻¹ could almost resume the anomalous situation to normal *via* ascending GSH, GR, SOD, the ratio of GSH/GSSG by 56.0%, 480%, 86.7%, 280% and descending TG by 74%, respectively, comparing with Model group.

3.5 Effect of PO on ROS, TNF- α and FFA levels

Compared with NC group (Fig. 5, (all $p < 0.01$)), ROS, TNF- α and FFA levels significantly increased by 345.5% (55.70 ± 8.91 vs. 248.16 ± 19.50), 31.4% (208.44 ± 14.23 vs. 273.88 ± 26.79) and 132.4% (59.51 ± 9.73 vs. 138.32 ± 17.07) in Model group. PO administration (100, 200 and 400 mg kg⁻¹) reversed these trends when compared with Model group. However, it should be noticed that PO at 100 mg kg⁻¹ showed no statistical significance as compared with Model group ($p > 0.05$). In addition, PO at 400 mg kg⁻¹ decreased the elevation ($p > 0.01$) of ROS, TNF- α and FFA by 68.3%, 27.0% and 53.8%, respectively.

These data clearly manifested that PO could effectively protect the liver against ethanol-induced hepatotoxicity.

3.6 Effect of PO on mRNA expression of CYP2E1, NF- κ B p65, SREBP-1c, SCD-1, FAS, AMPK, PPAR- α , CPT-1

As shown in Fig. 6 and 7, compared with NC group, the result of RT-qPCR showed that exposure to alcohol could issue in down-regulation of AMPK, PPAR- α and CPT-1 mRNA expressions (Fig. 6, $p < 0.01$) by 72.2% (1.00 ± 0.12 vs. 0.28 ± 0.09), 58.2% (1.00 ± 0.09 vs. 0.42 ± 0.11) and 65.8% (1.00 ± 0.12 vs. 0.34 ± 0.12), respectively, while upregulating CYP2E1, NF- κ B p65, SREBP-1c, SCD-1, FAS mRNA expression (Fig. 7, $p < 0.01$) by 58.7% (1.00 ± 0.22 vs. 1.59 ± 0.20), 54.7% (1.00 ± 0.11 vs. 1.55 ± 0.20), 100% (1.00 ± 0.13 vs. 2.00 ± 0.20), 115% (1.00 ± 0.14 vs. 2.15 ± 0.22) and 212% (1.00 ± 0.24 vs. 3.12 ± 0.48). However, AMPK, PPAR- α and CPT-1 mRNA expressions in PO-administrated rats were significantly enhanced (all $p < 0.05$) in a dose-related manner while CYP2E1, NF- κ B p65, SREBP-1c, SCD-1 and FAS mRNA expressions were markedly inhibited (all $p < 0.05$). Moreover, compared to the Model group, PO at 400 mg kg⁻¹ could elevate expressions of AMPK, PPAR- α and CPT-1 by 272%, 142%, 200% and, at the same time, reduce expressions of CYP2E1, NF- κ B p65, SREBP-1c, SCD-1 and FAS by 40.4%, 51.2%, 68.9%, 68.7% and 81.2%, respectively, suggesting that pretreatment with PO prevented ethanol-induced liver steatosis.

3.7 Effects of PO on protein expression of CYP2E1, NF- κ B p65, SREBP-1c, SCD-1, FAS, AMPK, PPAR- α , CPT-1

Compared with NC group, the results revealed that the protein expressions of AMPK, PPAR- α and CPT-1 were markedly inhibited by 29.9% (1.32 ± 0.17 vs. 0.92 ± 0.10), 15.9% ($1.46 \pm$



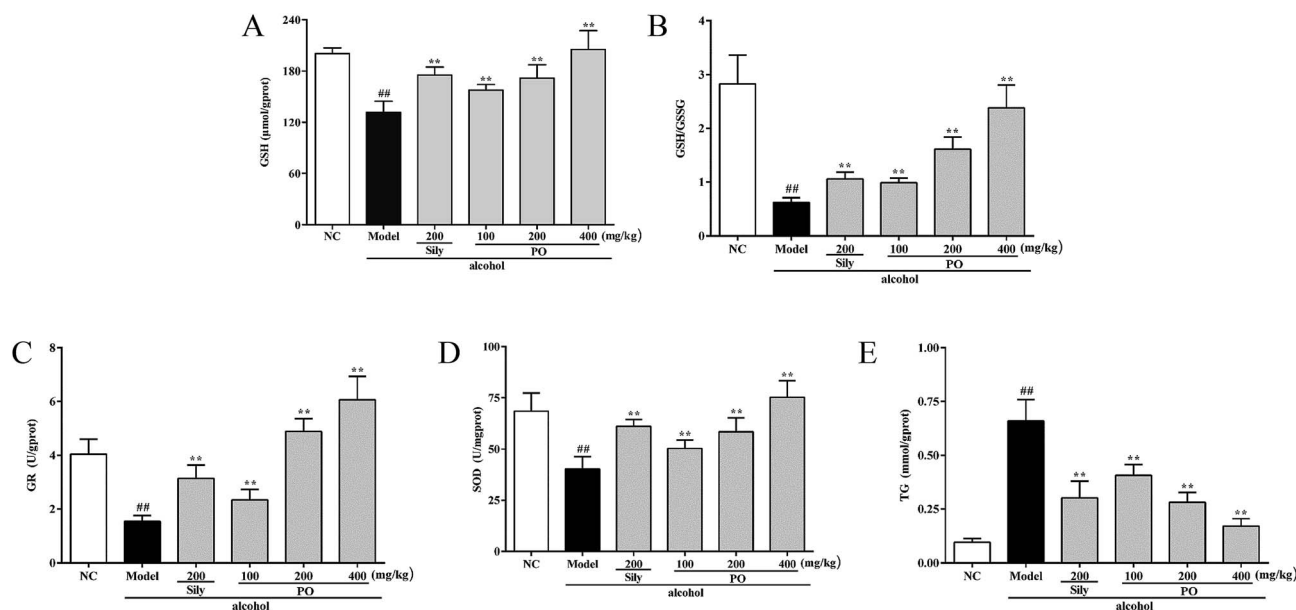


Fig. 4 Effects of PO on antioxidant ability and hepatic lipidosis levels of GSH, GSH/GSSG, GR, SOD and TG in liver. (A) GSH; (B) GSH/GSSG; (C) GR; (D) SOD; (E) TG. Values were presented as the mean \pm SD ($n = 12$). $##p < 0.01$ vs. NC group; $**p < 0.01$ vs. Model group.

0.01 vs. 1.23 ± 0.07), and 11.9% (1.26 ± 0.07 vs. 1.11 ± 0.06) while CYP2E1, NF- κ B p65, SREBP-1c, SCD-1 and FAS were noticeably enhanced by 55.7% (0.80 ± 0.21 vs. 1.25 ± 0.27), 39.8% (0.82 ± 0.03 vs. 1.15 ± 0.07), 51.4% (0.69 ± 0.04 vs. $1.04 \pm$

0.07), 34.6% (0.88 ± 0.06 vs. 1.19 ± 0.05), 22.5% (0.94 ± 0.01 vs. 1.15 ± 0.05) after repeatedly exposing to alcohol (Fig. 8 and 9, $p < 0.05$). However, PO administration (100, 200 and 400 mg kg^{-1}) were significantly increase the protein level of AMPK, PPAR-

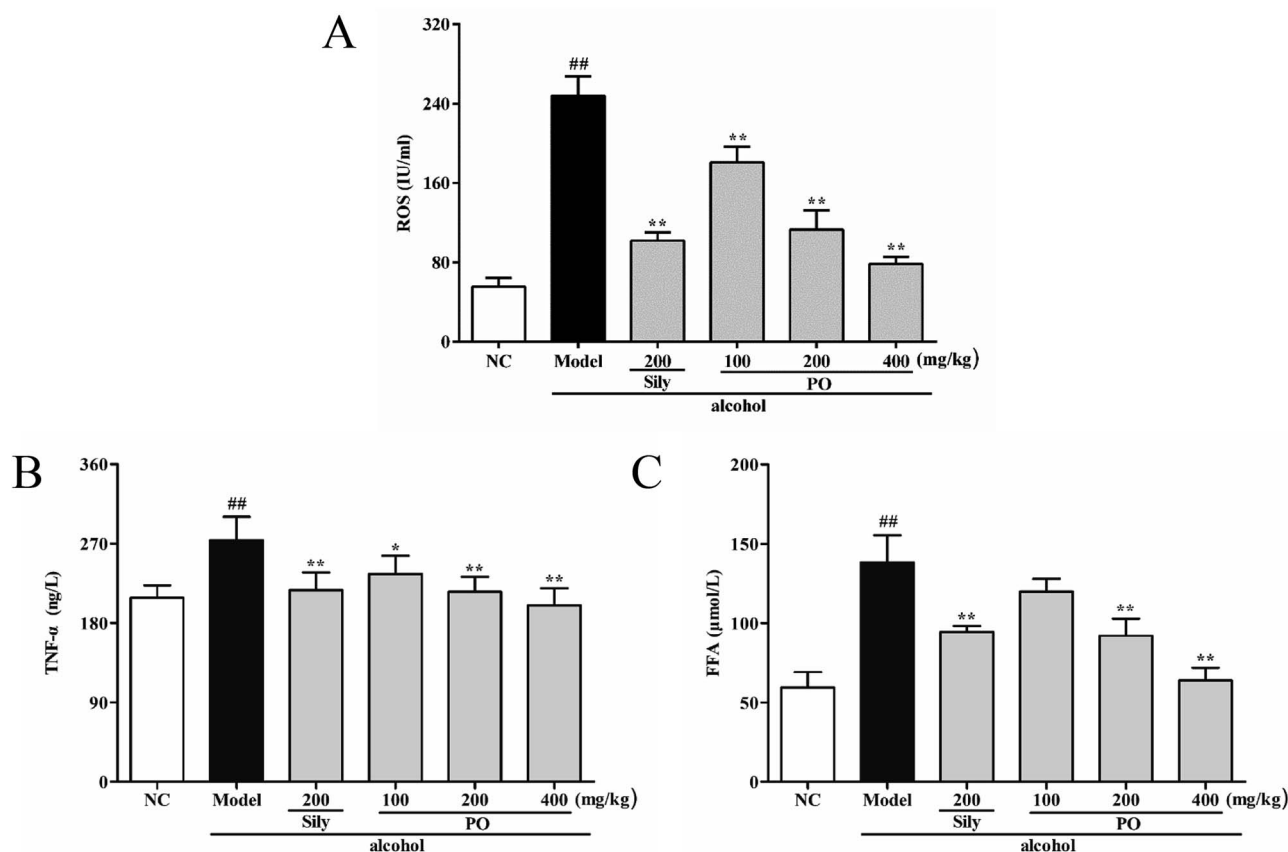


Fig. 5 Effects of PO on levels of ROS, TNF- α and FFA in liver. (A) ROS; (B) TNF- α ; (C) FFA. Values were presented as the mean \pm SD ($n = 12$). $##p < 0.01$ vs. NC group; $*$ $p < 0.05$, $**p < 0.01$ vs. Model group.



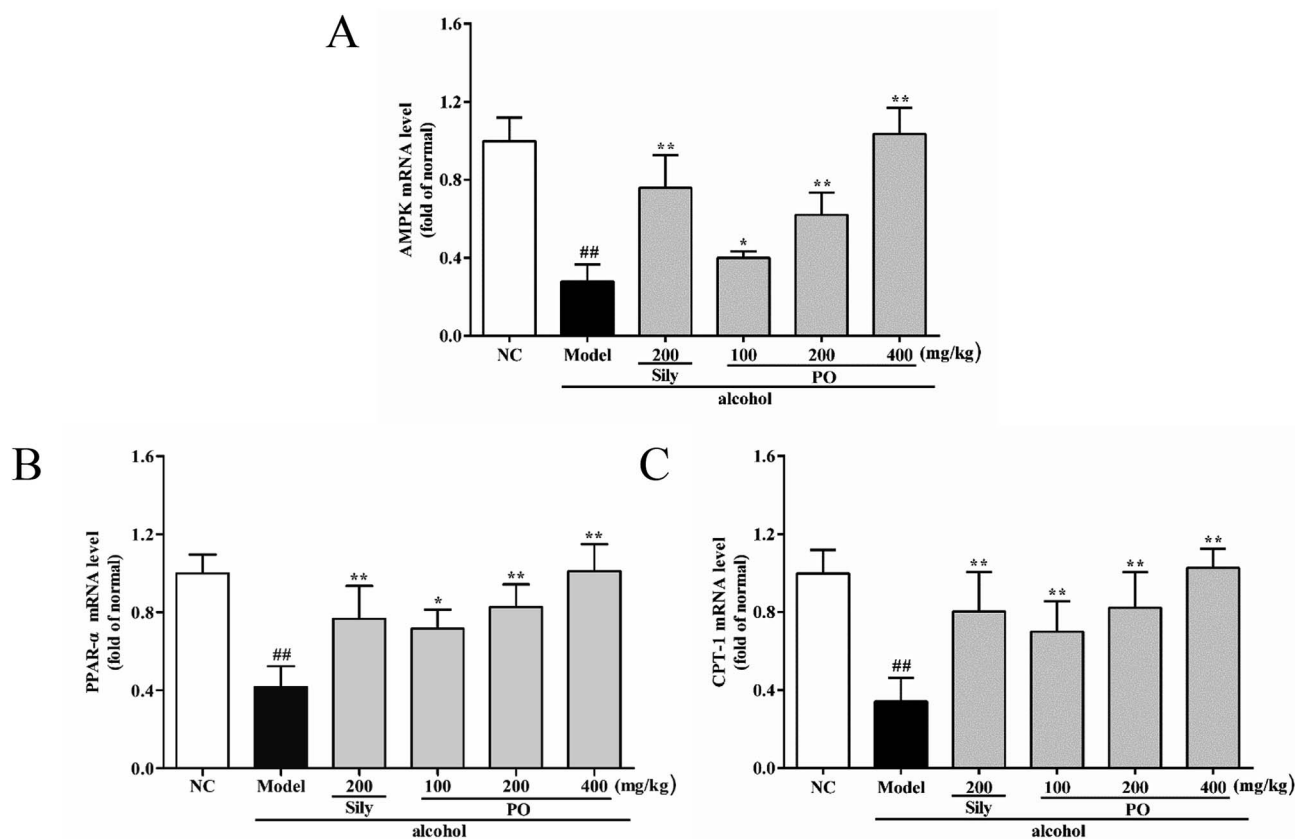


Fig. 6 Effects of PO on the transcriptional levels of (A) AMPK, (B) PPAR- α and (C) CPT-1 in liver. Values were presented as the mean \pm SD ($n = 12$). ^{##} $p < 0.01$ vs. NC group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. Model group.

α and CPT-1 (all $p < 0.05$). Meanwhile, PO pretreatment also (100, 200 and 400 mg kg⁻¹) exerted dose-dependent suppression of the protein level of CYP2E1, NF- κ B p65, SREBP-1c, SCD-1 and FAS (all $p < 0.05$). When compared to the Model group, PO

at 400 mg kg⁻¹ elevated expressions of AMPK, PPAR- α and CPT-1 by 42.9%, 28.7%, 34.0% as well as reduced expressions of CYP2E1, NF- κ B p65, SREBP-1c, SCD-1 and FAS by 47.3%, 35.3%, 32.4%, 25.3% and 27.0%, respectively.

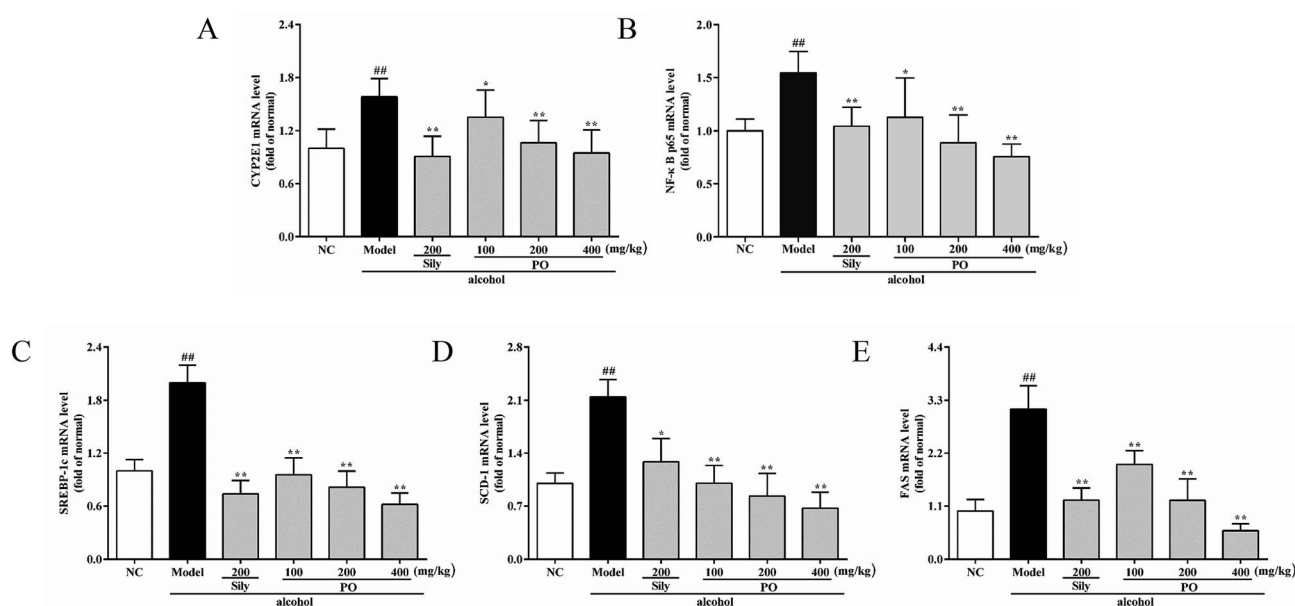


Fig. 7 Effects of PO on the transcriptional levels of (A) CYP2E1, (B) NF- κ B p65, (C) SREBP-1c, (D) SCD-1 and (E) FAS in liver. Values were presented as the mean \pm SD ($n = 12$). ^{##} $p < 0.01$ vs. NC group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. Model group.



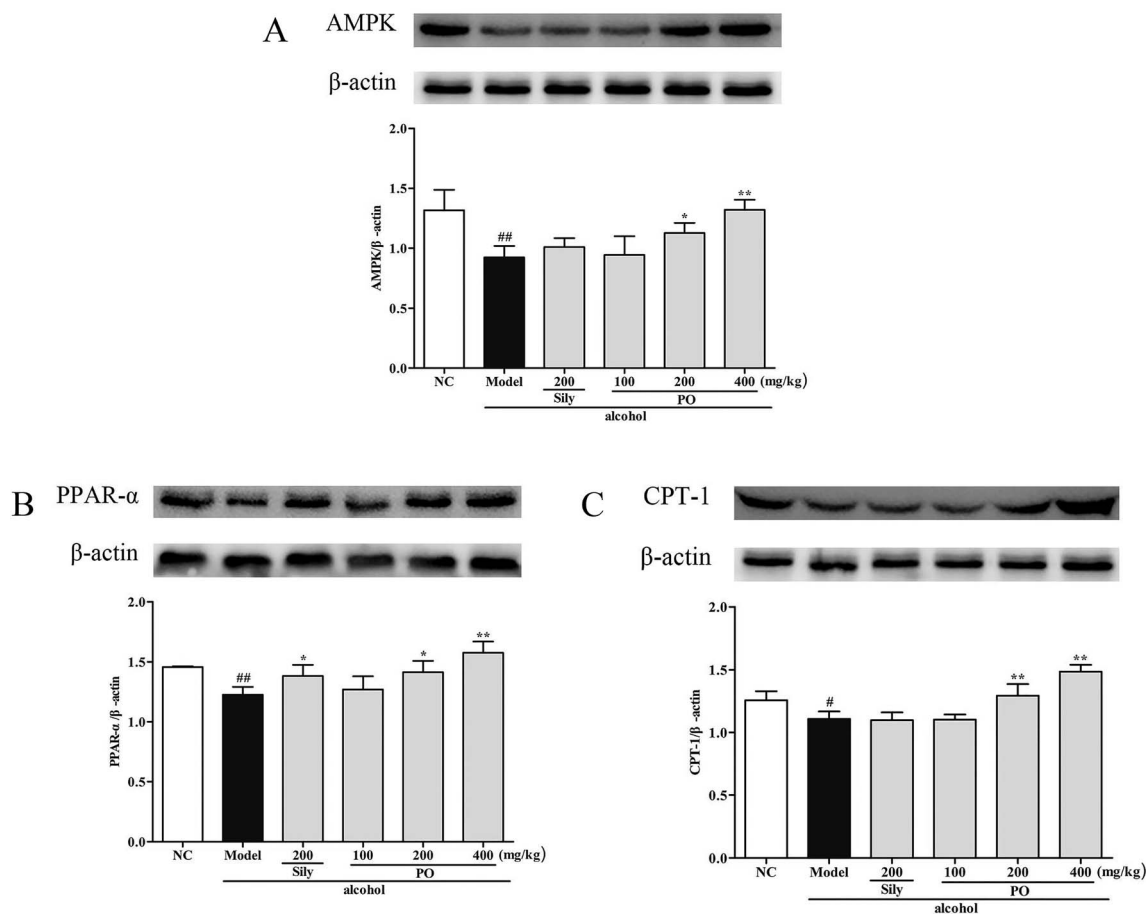


Fig. 8 Effect of PO on protein expression of (A) AMPK, (B) PPAR- α and (C) CPT-1. Values were presented as the mean \pm SD. # p < 0.05, ## p < 0.01 vs. NC group; * p < 0.05, ** p < 0.01 vs. Model group.

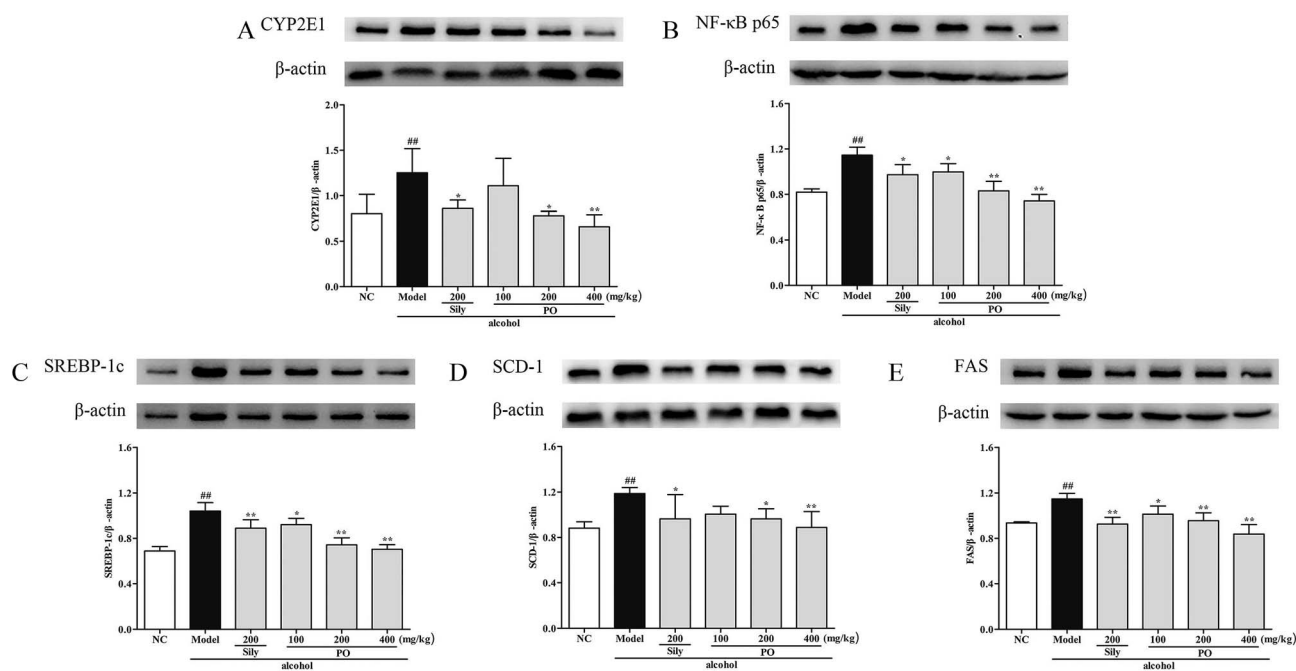


Fig. 9 Effect of PO on protein expression of (A) CYP2E1, (B) NF- κ B p65, (C) SREBP-1c, (D) SCD-1 and (E) FAS. Values were presented as the mean \pm SD. ## p < 0.01 vs. NC group; * p < 0.05, ** p < 0.01 vs. Model group.



Recent research have found that alcohol might interfere with hepatic lipid metabolism, leading to hepatic lipid accumulation and ultimate steatosis. AMPK and SREBP-1c are associated with

The interaction between oxidative stress and lipid metabolism on ethanol-induced hepatotoxicity has been investigated in this study as well. Results indicated that oxidative stress could triggered metabolic dysfunction by impacting ROS activation. Our previous study has reported that PO possesses antioxidant and anti-inflammatory ability.^{23,24} Recent pharmacological research has also proved *n*-hexane extractive from *Pogostemon patchouli* could effectively protect liver against alcoholic injury *via* modulating the extent of lipid peroxidation and augmenting the antioxidant defense system. In this experiment, the satisfactory protection of PO was first elucidated in the liver injury of rats through raising antioxidant enzyme activity, depressing

According to our GC fingerprint, PO contains abundant sesquiterpenoids including patchouli alcohol, α -guaiene, seychellene, α -patchoulene and β -patchoulene. Patchouli alcohol comprises 41.993% and serves as the major constituent of PO. It has been reported that patchouli alcohol has various pharmacological activities, including anti-oxidation by inhibiting ROS accumulation⁵⁷ and inflammation *via* suppressing NF- κ B signaling pathway.^{58,59} Based on the hepatoprotection of PO, we speculate that patchouli alcohol might act as the main active ingredient to play a protective role in liver injury. As a result, we will keep on exploring this active component of PO on ethanol-induced hepatic damage in future study.

PO possessed a potent therapeutic effect on ethanol-induced liver injury, as evidenced by relieving oxidative lesions through inhibiting ROS production, and preventing fatty degeneration through accelerating adipose metabolism. This study provided an original perspective that PO was a functional natural agent for the treatment of ethanol-induced liver damage. However, more extraneous experiments are required to further elucidate the hepatoprotective molecular mechanisms of PO and the active ingredients of PO will be explored in ethanol-induced hepatic damage in future study.

The authors declare that they have no conflicts of interest.

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