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ORIGINAL ARTICLE

Water-Soluble Extract of Safflower Relieved Long-Term Alcoholic Hepatic Damage through Mitigating Oxidative Stress Within Rat Model

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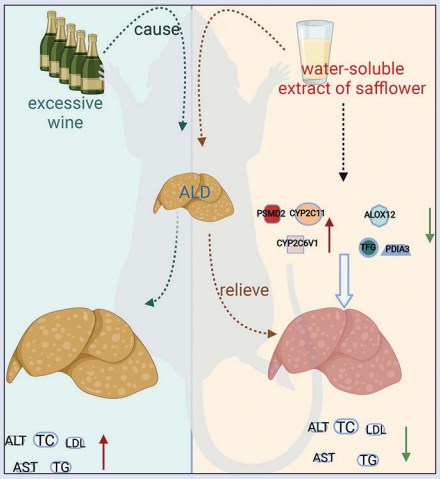
ABSTRACT

Background: The mechanism of a water-soluble extract of safflower on chronic alcoholic liver injury is still unclear. **Objective:** To study the mechanism of water extract of safflower on chronic alcoholic liver injury. **Materials and Methods:** The rats were separated in a random manner into five cohorts, namely, the normal control group (NC), alcoholic fatty liver disease model (ALD), ALD rat model treated with the water-soluble extract of safflower (30 g/kg), ALD rat model treated with the water-soluble extraction of safflower (40 g/kg), and ALD rat model with the water-soluble extract of safflower (50 g/kg). Rats in each group were anaesthetized with 2% isoflurane and euthanized by cervical dislocation after 1, 7, 14, 21, and 28 days treatment. Proteomic analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG analysis), immunohistochemistry (IHC), and western blot were employed for comparing the variations across model cohort and control cohort at the tissue level, protein level, and molecular level, respectively, and for probing potential prophylactic role/mechanisms for safflower extract on alcoholic liver injury. **Results:** KEGG analysis found that the differentially expressed genes in the alcoholic liver injury model were closely associated with alcoholism, inflammation, and serotonergic synapses. Furthermore, with the administration of water-soluble extract of safflower, these hepatic function indices and liver steatosis lesions were alleviated in both time and dose-dependent manners. Importantly, IHC analysis discovered that proteins disulfide isomerase A3 (PDIA3) and Trk-fused gene (*TFG*) were highly increased in the liver of the rats. Furthermore, the water-soluble extract of safflower alleviated liver oxidative injury and lipid peroxidation, via the upregulation of proteins such as 26S proteasome non-ATPase regulatory subunit 2 (PSMD2), cytochrome P450 2C6 (CYP2C6V1), and cytochrome P450 2C11 (CYP2C11), anddownregulation of arachidonate 12-lipoxygenase and 12S-type (ALOX12) protein expression. **Conclusion:** The water-soluble extract of safflower exhibited potential protective effects against lipid peroxidation and ethanol-driven oxidative damage within the liver via the upregulation of expression of proteins PSMD2, CYP2C6V1, and CYP2C11and downregulation of the expression of ALOX12, PDIA3, and TFG proteins.

Key words: Alcoholic fatty liver disease, lipid peroxidation, oxidative stress, safflower extraction

SUMMARY

- This study suggests that water-soluble safflower extract has the potential to protect liver against ethanol-induced oxidative injury and lipid peroxidation.



Abbreviations used: BCA: Bicinchoninic acid; SDS-PAGE: Sodium dodecylsulphate polyacrylamide gel electrophoresis; DTT: Dithiothreitol; UA: Urea; LC-MS/MS: Liquid chromatography tandem mass spectrometry; AST: Aspartate Transaminase; ALT: Alanine transaminase; LDL: Low-density lipoprotein; TG: Triglyceride; HDL: High-density lipoprotein; TC: Total cholesterol; PVDF: Polyvinylidene Fluoride; TBST: Tris-BufferedSalineTween; ECL: Enhanced chemiluminescent; LFQ: Label-free quantification.

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INTRODUCTION

Chronic excessive alcohol consumption, the main cause of the alcoholic liver disease (ALD), generates an increase in socio-economic burden and is an immense threat to public health.[1,2] Initial stages of ALD include early alcohol-related steatohepatitis and alcoholic hepatitis (AH), which ultimately progresses into alcoholic liver fibrosis, alcohol-related liver cirrhosis, and hepatocellular carcinoma.[3] Studies confirm that the excessive alcohol consumption induces hepatic oxidative stress and redox imbalances,[4] which in turn aggravates the liver microsomal cytochrome P450 (CYP) system.[5] Although, CYP enzymes are mainly

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responsible for the pharmacokinetics/pharmacogenetics of several pharmaceutical drugs, food, and toxic materials (specifically the toxic chemicals in the liver), overburdening it contributes to reactive oxygen species overproduction, being detrimental for hepatocyte organelles and DNA, thus, leading to liver dysfunction and eventually causing the liver to fail.[6] Therefore, it is important to alleviate the alcoholic oxidative damage that happens to the liver in the initial stages of ALD.[7,8]

Traditional Chinese medicine had provided effective pharmaceutical solutions for numerous diseases in the past.[9,10] Furthermore, several studies have revealed that herb formulas, natural products, and traditional Chinese herbal extracts, such as, *Pueraria lobate*, *Silybum marianum*, and aqueous extract of *Pepino* leaves have significant positive effects in reducing damage caused by ALD.[11,12] Safflower, a flower of *Carthamus tinctorius*, was reported to highlight multiple pharmacological properties, including anti-thrombotic, antioxidant, anticoagulative, protection of endothelium, antitumor antiapoptotic, and neuroprotective functions.[13-16] Therefore, considering such diverse pharmaceutical properties of safflower, we intended to study the pharmaceutical effect of safflower in rat models of ALD.

MATERIALS AND METHODS

Preparation of safflower water-soluble extract

First, a fixed quantity of safflower (Mongolian medicine, purchased from Jun Bisazza Pharmaceutical [Lot: 20181225]) was soaked in the 10X water for 30 min and then boiled (60°C for 30 min, repeated thrice) in ceramic container to obtain the water-soluble extract. Subsequently, to obtain the final preparation, all the filtrates were blended and concentrated at 65°C and –0.07 mpa pressure for 1–2 h. Approximately, 400 cc of the water-soluble extract was obtained from 2 kg of safflower.[17]

Establishment of animal model and experimental design

A total of 150 Wistar male rats (weight 200 ± 10 g) were procured through Beijing Lihua Experimental Animal Technology Co. Ltd. (ethical permission number: MDL2020-03-16-01). These were kept within climate-controlled environments (22 ± 2°C, 55 ± 5% humidity, and food/water *ad libitum*) with a 12-h light/darkcycle with 7-day acclimatization. This investigation was accepted by the animal experiment committee of Affiliated Hospital of Inner Mongolia University for the Nationalities, and rats were handled in line with the guidelines of the Animal Experiment Committee.

Randomly separated the rats within five cohorts (n = 30), namely, the normal control cohort (NC), alcoholic fatty liver disease model (ALD), ALD rat model treated with the water-soluble extract of safflower (30 g/kg), ALD rat model treated with the water-soluble extraction of safflower (40 g/kg), and ALD rat model with the water-soluble extract of safflower (50 g/kg). To establish the chronic alcoholic fatty liver model rats, the Wistar rats were fed an ethanol diet composed of 40% ethanol blended with a regular chow diet[18] (Erguotou liquor, alcohol concentration: 56°, Beijing, Niulanshan) for 4 weeks. Then, the model rats in the three cohorts were correspondingly treated with the different dosages of safflower extract by oral gavage feeding for 4 weeks. Additionally, six rats (in each cohort) were anaesthetized with 2% isoflurane and euthanized by cervical dislocation after treatment day 1, 7, 14, 21, and 28 [Figure 1]. Blood samples of the rats were collected in EDTA-filled tubes, and plasma was isolated via centrifugation at 1500 g for 10 min, 4°C. Subsequently, the livers were also removed and fixed in 10% paraformaldehyde or stored in liquid nitrogen for further studies.

Protein extraction, filter-aided proteome preparation, and desalination

Hepatic tissue samples were collected after 7 days of treatment of safflower extract and dissolved in radio-immunoprecipitation assay lysis solution containing a protease inhibitor phenylmethylsulfonyl fluoride. Centrifuged the lysates at 14,000 g, 4°C (15 min) and supernatants were passed through membrane filters (0.22 μm). Estimated the sample concentration with the BCA kit (Beyotime Biotech, shanghai, China). Proteins of the liver lysates were analyzed with SDS-PAGE. Briefly, each sample (100 μg) was boiled in 100 mmol/L DTT for 5 min, followed by the alkylation of the protein samples with 100 mmol/L iodoacetamide in UA buffer (without any light exposure) for 40 min at room temperature. Centrifuged the samples at 14,000 g, 4°C, for 15 min to obtain protein pellets. Protein sample pellets were washed twice with the dissolution buffer after which they were subjected to trypsin digestion (50 μL) at 37°C for 12 h. The tryptic peptide isolates were collected following centrifuging (14,000 g/15 min) and further washed with the dissolution buffer to obtain uncontaminated pellets. Dissolved the dried peptide mixture in 0.1% trifluoroacetic acid (TFA) and loaded it onto a 100% acetonitrile to activate desalting column and to remove the excess TFA. About 50% acetonitrile solution was used to elute peptide segments and eluted segments were subjected to centrifuging (14,000 g/15 min) for retrieving dried samples without any trace of acetonitrile.[12]

LC-MS/MS analysis

Following the column equilibrating step using 95% A solution, the tryptic peptides, dissolved within solvent A (aqueous solution of 0.1% formic acid), were inserted into an in-house manufactured reverse-phase analytical column (15-cm length, 75-μm ID) using the EASY-nLC 1200 UPLC setup. A constant flow rate of 200 nL/min was used and elution gradient conditions

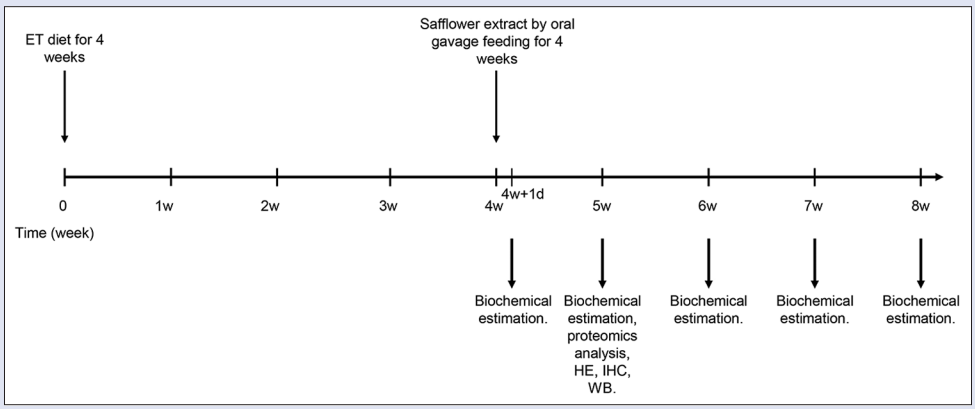

Figure 1: Experiment design. The flow-chart of animal model establishment and experimental design

were as follows: 6–25% solvent B (0.1% formic acid aqueous solution of acetonitrile [84%]) for 25 min, 25–35% for 10 min, 35–80% for 5 min, and finally 80% gradient for 5 min. Eluted peptides were analyzed in tandem mass spectrometry (MS/MS) with Orbitrap Fusion Lumos® (Thermo Scientific™, USA) connected to UPLC system. Electrospray voltage was 2.0 kV and the full scanning range was 375–1600 *m/z*. The whole peptides were analyzed with Orbitrap® in a resolution of 60,000. The MS/MS analysis of peptides was performed at 30 normalized collision energy and the fragments were detected with the Orbitrapat a resolution of 17,500. Besides, automatic gain control was at 5E4 and fixed first-mass was set at 100 *m/z*.

PROTEOMICS

The raw mass spectrometry data were analyzed with Mascot 2.5 and Proteome Discoverer 2.1 programs. The differentially expressed genes were functionally annotated with gene ontology (GO)/the Kyoto Encyclopedia of Genes and Genomes (KEGG). Also, the genes were selected according to protein function with the Uniprot database (http://www.uniprot.org). $P < 0.05$ was deemed to confer statistical significance.

Serum liver function

Anaesthetized the rat with 2% isoflurane and about 2 mL of blood was collected. Centrifuged at 2000 rpm for 10 min at 4°C. Directly transport serum within a fresh polypropylene tube. Rat serum specimens were obtained to estimate the levels of ALT (#:C009-2-1), AST (#:C010-2-1), TG (#:A110-1-1), TC (#:A111-1-1), LDL (#:A113-1-1), and HDL (#:A112-1-1). They were assayed using commercially available reagent kits following kit protocols (Nanjing Jiancheng Biology Engineering Institute, Nanjing, China). Each sample was processed twice and the experiments were repeated thrice.

Hematoxylin and eosin (HE) staining

The formalin-fixed liver tissues were washed and dehydrated. Then, treated with ethanol at different concentrations and embedded in paraffin. Finally, the tissues were sliced into 3–5 μm thickness sections and dried to remove water. After dewaxing, stained the tissue sections with HE (#C0105, Beyotime Biotech, Shanghai, China). Finally, the stained tissue sections were dehydrated and sealed within the slides in the gum. The pathological features of the tissues were visualized in an optical microscope (Leica, German).

IHC

Incubated paraffin-embedded liver sections with anti-protein disulfide-isomerase A3 antibody (PDIA3, #: ab13506, Abcam) or anti-TRK-fused gene antibody (TFG, #: ab156866, Abcam) antibodies for 4 h. Then, washed them using PBS followed by incubation using biotinylated IgG 2° antibody (#:ab99760, Abcam) for 60 min. Next,

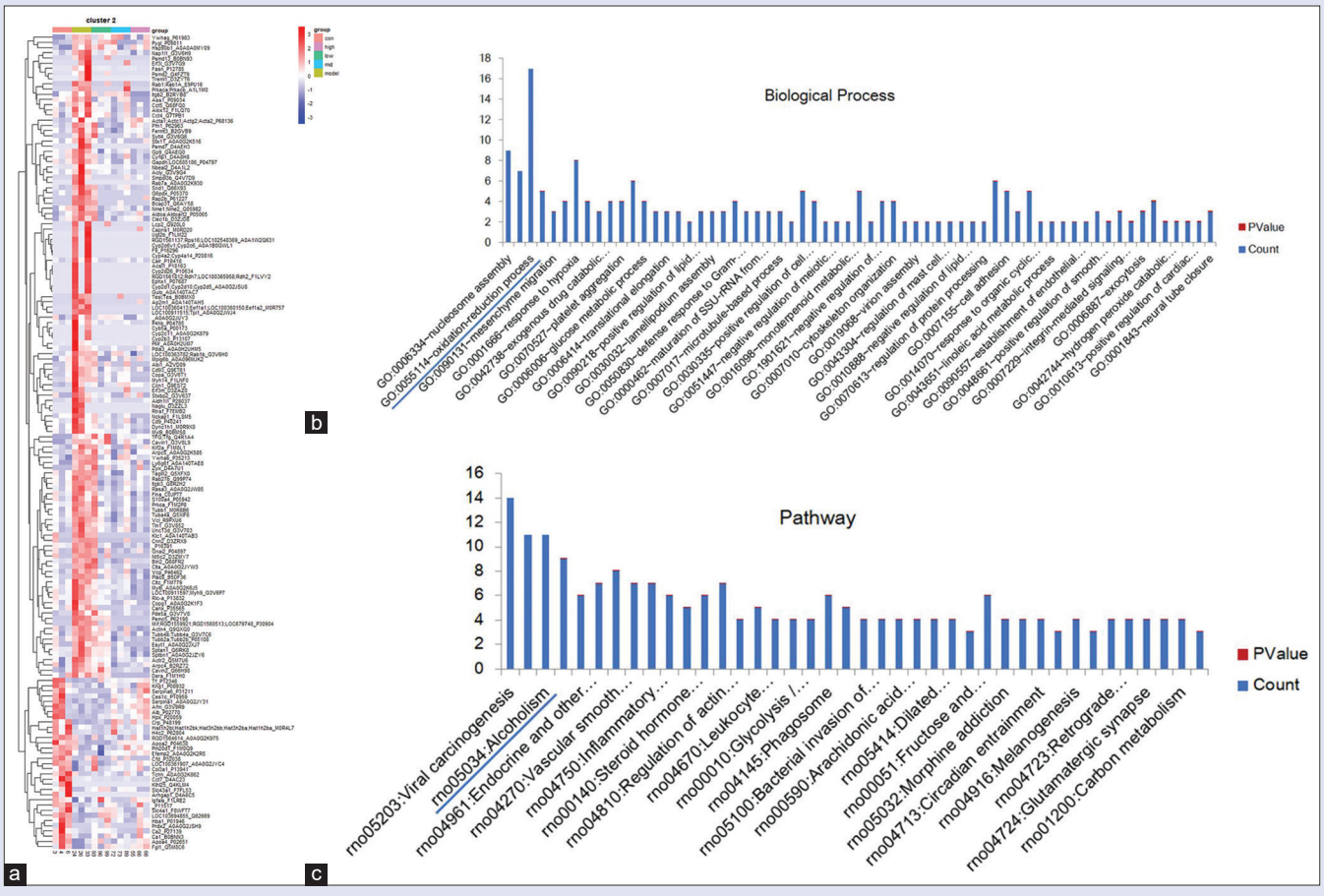

Figure 2: Proteomics analysis of the liver tissue samples (a) a heat map showing the significantly differentially expressed proteins in the control and model cohorts. Red and green denote the upregulated and the downregulated proteins, respectively. The proteins were cohorted into five broad clusters. (b) Gene ontology analysis of proteins expressed differentially among the five cohorts. The significantly associated biological processes were nucleosome assembly, oxidation–reduction process, response to hypoxia, and so on. (c) KEGG pathway enrichment revealed five prominent cohorts. It is indicated that alcoholism, inflammatory, serotonergic synapse, etc., were related closely to the response of ethanol-induced liver injury

sections were washed and incubated with 3,3'-Diaminobenzidine reagent (#: P0202, Beyotime Biotech, Shanghai, China) for 30 min, later, counterstained with hematoxylin. Washed the slides in PBS and dehydrated them for standard histological examinations. Counted the positively stained cells in >10 different high-power fields (400 × magnification) with corresponding images assessed through Image J® (NIH Bethesda, MD, USA).

Western blotting

The protein samples extracted from the liver tissues were denatured at 80–100°C. Then, 15 µl of each protein sample was isolated with SDS-PAGE and transported onto PVDF membrane (Millipore™, USA). Incubation of membranes in non-fat milk for 60 min was performed for blocking non-specific interactions. After TBST wash, membranes were placed into incubation with 1° antibodies, 26S proteasome non-ATPase regulatory subunit 2 (PSMD2, #:ab26078, Abcam), cytochrome P450 2C6 (CYP2C6V1, #:sc-53245, Santa Cruz, USA), cytochrome P450 2C11 (CYP2C11, #:ab3571, Abcam), or arachidonate 12-lipoxygenase and 12S-type (ALOX12, #:ab168384, Abcam), respectively, for 12 h. Samples were immunoblotted with the anti-mouse IgG secondary antibodies for 1 h. β-actin served control protein. Stained protein bands with chemiluminescent substrate ECL and analyzed the intensities with Image J®(NIH Bethesda, MD, USA).

Statistical analysis

Statistical analyses of the data were performed with SPSS statistic 19.0 (IBM, USA) software. Data were analyzed through one-way analysis of variance method with homogeneity of variance. A difference of $P < 0.05$ was deemed to confer statistical significance. Graphs were plotted through GraphPad Prism 6.0 software and the results are displayed as means ± standard deviation.

RESULTS

MS quantitative proteomics and functional enrichment analysis

We used a label-free MS tool to quantify and compare the protein expression profile in the liver among the control cohort, ethanol-induced ALD model cohort, and safflower-treated cohorts. The results revealed a total number of 158 proteins expressed significantly differentially ($P < 0.05$) in ALD model cohort versus control rats. Among the 158 proteins, 125 were upregulated, whereas 33 were downregulated. The list includes the mass spectrometry data, means, and LFQ intensity. Furthermore, a heat map revealed that the differentially expressed proteins were prominently clustered into five cohorts [Figure 2a]. To further understand the biological processes of these proteins, functional enrichment analysis was performed using GO and KEGG pathways. GO enrichment analysis revealed that the differentially expressed proteins were mainly involved in nucleosome assembly, oxidation–reduction process, response to hypoxia, and so on [Figure 2b]. Additionally, KEGG analysis displayed that the inflammatory and serotonergic synapses were closely linked to ethanol-induced liver injury [Figure 2c].

Safflower-attenuated ethanol-driven hepatic damage/steatosis within ALD model rats

We evaluated the level of ethanol-driven hepatic damage by analyzing the liver functional parameters, such as AST, ALT, LDL, TG, HDL, and TC. Figure 3a–f depicts a comparison with the chow diet-fed control cohort, except for HDL, the levels of TC, ALT, and LDL were markedly elevated within ALD model cohort ($P < 0.05$). Interestingly, with the treatment of water-soluble safflower extract, aminotransferase (AST and ALT) levels/lipoprotein (TC, LDL, and TG) decreased significantly in both time and dose-dependent manner. The chronic liver steatosis lesions induced by

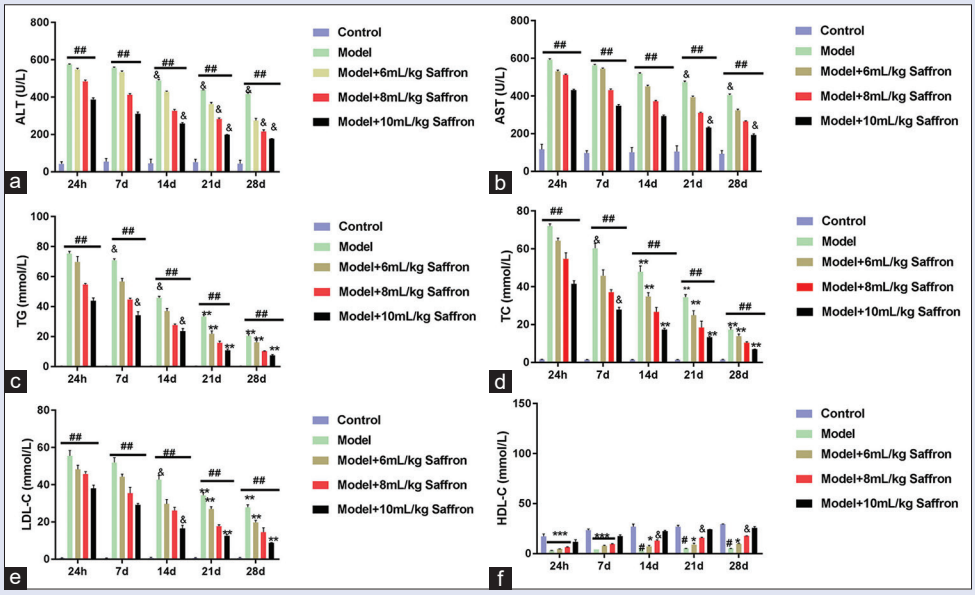

Figure 3: Biochemical estimation of liver function parameters, ALT (a), AST (b), TG (c), TC (d), LDL-C (e), and HDL-C (f). Levels of ALT, AST, TC, TG, and LDL-C were upregulated, whereas the HDL-C level was decreased in the ALD model cohort ($^{\#\#}P < 0.05$) versus the normal control cohort. AST, ALT, TC, TG, and LDL-C in safflower (50 g/kg and/or 40 g/kg) cohorts versus the ALD model cohort ($^{\&}P < 0.05$). TC, TG, and LDL-C in safflower (50 g/kg) cohort versu the ALD model cohort, safflower (30 g/kg) cohort ($^{**}P < 0.05$). HDL-C in ALD model cohort, safflower (30 g/kg, 40 g/kg) cohorts versus the normal control cohort and safflower (50 g/kg) cohort ($^{***}P < 0.05$). HDL-C in the ALD model cohort versus the other cohorts ($^{\#}P < 0.05$), and $^{*}P < 0.05$ versus the normal control cohort and safflower (40 g/kg, 50 g/kg) cohorts. HDL-C in safflower (40 g/kg) cohort versus normal control cohort, safflower (50 g/kg) cohort. The data were repeated thrice and they were presented with histogram of means, the horizontal line presented the standard deviation. Statistical analysis with one-way ANOVA method

alcohol were also reduced with decreased lipid vacuoles observed in the hepatocytes (marked by arrows in Figure 4).

Safflower-reduced ethanol-driven oxidative stress within ALD rat liver

To further analyze the proteins involved in ethanol-induced oxidative stress and fatty acid oxidation, we performed IHC and western blot analysis in the liver tissue samples. Dataset outcomes highlighted that, in comparison to the control cohort, proteins PDIA3 and TFG were highly upregulated in the ethanol-induced ALD model rats [Figure 5a]. However, the expression of the two proteins decreased significantly on treatment with water-soluble safflower extract [Figure 5b]. Furthermore, the water-soluble extraction of safflower treatment also upregulated the expression of proteins, PSMD2, CYP2C6V1, CYP2C11, and downregulated ALOX12 protein expression to relieve the liver oxidative injury and lipid peroxidation [Figure 6a and b].

DISCUSSION

In this study, with MS-based quantitative proteomics and functional enrichment analysis, the proteomic analysis revealed that a total number of 158 proteins were expressed significantly differentially. And, they were predominantly implicated within endoplasmic reticulum (ER) stress, lipid peroxidation, and sulfidation. While, in the animal study, results showed that alcohol-induced liver dysfunction and liver injury

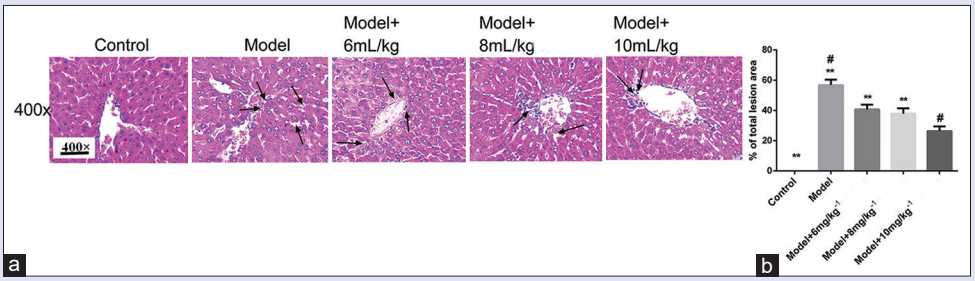

Figure 4: HE staining of rat liver tissues (a) the black arrows indicate massive steatosis and lipid droplets accumulation, scale bar = 400× (b) the quantitative data showing the % liver injury, $^{**}P < 0.05$ in model cohort, safflower (30 g/kg) cohort, safflower (40 g/kg) cohort versus the normal control cohort, $^{\#}P < 0.05$ safflower (50 g/kg) treated cohort versus the model cohort

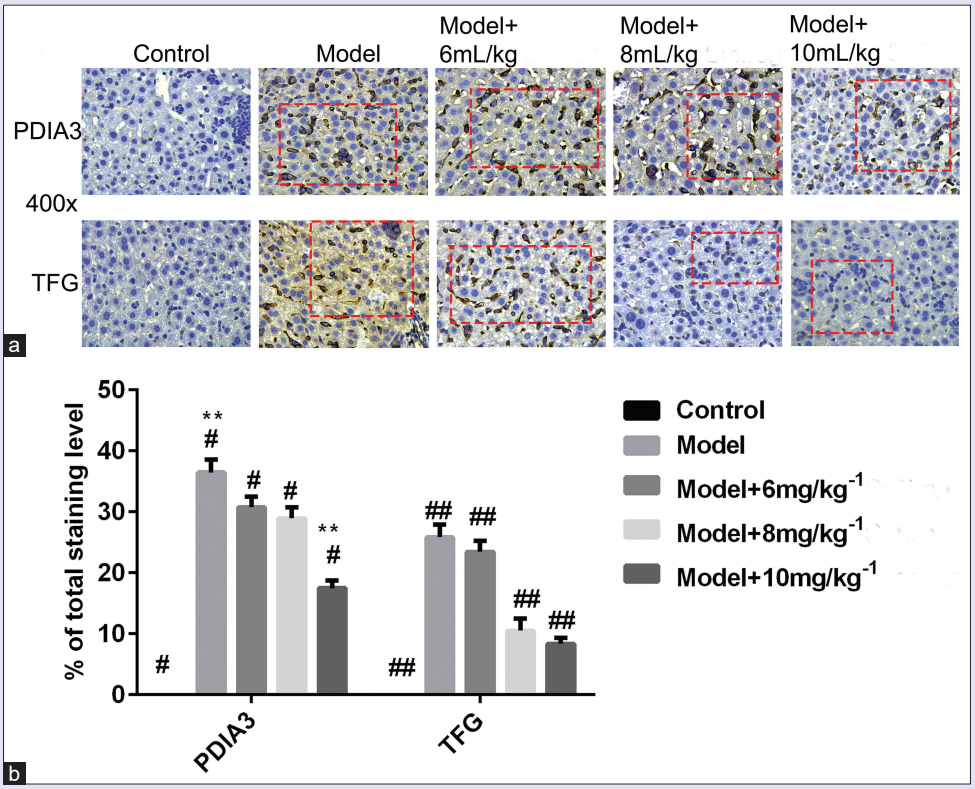

Figure 5: (a) The immunohistochemical analysis of PDIA3 and TFG expression (light black stain) in liver tissues, scale bars, 400× (b) higher levels of PDIA3 and TFG positive cytoplasmic staining in model cohort, $^{\#}P < 0.05$ PDIA3 in the model cohort, safflower (30 g/kg) cohort, safflower (40 g/kg) cohort, and safflower (50 g/kg) cohort versus the normal control cohort, $^{**}P < 0.05$ PDIA3 in the safflower (50 g/kg) cohort versus the model cohort, $^{\#\#}P < 0.05$ TFG in the model cohort, safflower (30 g/kg) cohort versus the normal control cohort, safflower (40 g/kg) cohort, and safflower (50 g/kg) cohort. The red area highlights the representative immunostaining

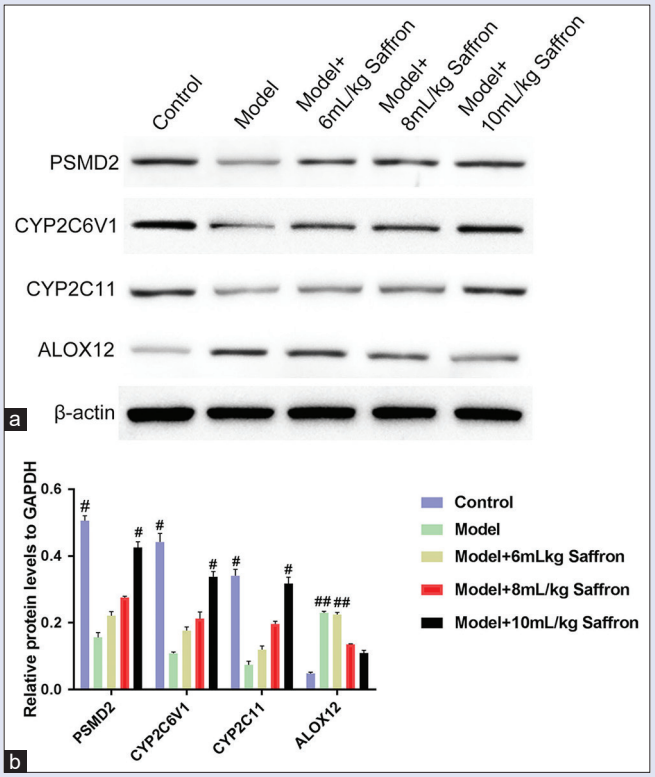

Figure 6: (a and b) Western blot analysis of proteins PSMD2, CYP2C6V1, CYP2C11, and ALOX12. Higher expression of PSMD2, CYP2C6V1, and CYP2C11 in normal control cohort, safflower (50 g/kg) cohort versus the model cohort, safflower (30 g/kg) cohort ($^{\#}P < 0.05$). The higher expression of ALOX12 in the model cohort, safflower (30 g/kg) cohort versus the normal control cohort, safflower (50 g/kg) cohort ($^{\#\#}P < 0.05$). The histogram presented the means and horizontal line depicted the standard deviation. Statistical analysis was done with one-way ANOVA method

through increasing the amount of aminotransferase (ALT and AST) and LDL lipoprotein, TG, and TC, whilst the administration of water-soluble safflower extraction reversed these biochemical indices and alleviated the chronic alcoholic liver steatosis lesion in a dose- and time-dependent way. These findings corroborated the investigation performed by Nimrouzi *et al.*[19]

This investigation probed the alcoholic liver injury rat model to study the function of the water-soluble extract of safflower that relieved chronic alcoholic liver injury as most clinical liver damage is associated with long-term alcohol consumption. Previous research done by Wang *et al.*[20] employed a drug-induced liver injury rat model (induced by acetaminophen) to study the prophylactic role of safflower extract on ethanol-driven hepatic damage. This model affected the success rate and stability of the modelling as low drug solubility was observed during modelling. Furthermore, other studies performed by Wang *et al.*[20] employed a carbon tetrachloride (CCl_4) induced liver injury model. CCl_4 is highly toxic, damaging not only the liver but also other organs. Furthermore, CCl_4 liver injury is a toxic liver injury, which is not strongly correlated with the immune regulatory response of the body. Therefore, this model is not suitable for the screening of immunomodulatory hepatoprotective drugs and the study of immune mechanisms. Furthermore, elevated levels of proteins PDIA3 and TFG expression was observed in the ER of the liver in ALD rats. Kondo *et al.*[21] reported that PDIA3 played a role of a chaperone protein and modulated the folding of novel synthesized glycoproteins in response to ER stress in hepatocellular carcinoma. Furthermore, the water-soluble safflower extract alleviated oxidative injury in the liver through upregulating proteins PSMD2, CYP2C6V1, CYP2C11, and downregulating ALOX12. Yanuo Li *et al.*[22] found hydroxysafflor yellow A (HSYA), which was extracted from safflower, had restorative effects on the liver in the hepatic fibrosis rats model. Several studies have already indicated the benefits of safflower effective components, such as in hydroxysafflor yellow A, safflower yellow, and HSYA on hepatic antioxidant enzymes, including superoxide dismutase together with cytochrome P450 (CYP2D4, CYP2C11, CYP1A2, and CYP3A1).[22,23] The current study indicated that cytochrome P450 enzymes, CYP2C6V1 and CYP2C11 might be involved in the degradation of ethanol metabolites, thus, reducing the lipid peroxidation activity. Queck *et al.*[24] reported that Alo × 12/15 deficiency could be an aggravating factor for inflammatory injury in AH. This finding was contradictory to the results obtained in our results and, therefore, further investigation is necessary to further understand the role of Alo × 12/15 in AH. However, safflower oil was shown to modulate metabolism, inflammation, and composition of gut microbiota in high fat/high sucrose diet-fed murines and defatted safflower seed extract inhibited adipogenesis.[25,26] Therefore, further studies are necessary to study the therapeutic potential of safflower extract, other than the antioxidant properties in alcohol-driven hepatic damage.

CONCLUSION

In conclusion, the present study suggests that water-soluble safflower extract has the potential to protect the liver against alcohol-driven oxidative injury and lipid peroxidation via the upregulation of expression of proteins PSMD2, CYP2C6V1, and CYP2C11and downregulation of the expression of ALOX12, PDIA3, and TFG proteins.

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Conflicts of interest

There are no conflicts of interest.

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