Penthorum chinense Pursh alleviates ethanol-induced hepatic oxidative impairment in zebrafish via AMPK / p62 / Nrf2 / mTOR pathway

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Abstract

Ethnopharmacological relevance: In China, *Penthorum chinense* Pursh (PCP) is renowned for its effectiveness in "promoting blood circulation" and "removing blood stasis". It can "relieve the liver" and its application in the field of liver protection, including viral hepatitis, alcoholic liver, liver fibrosis, has been known for hundreds of years.

Aim of the study: Oxidative stress is widely believed to exert a key role in the pathophysiology of alcoholic liver disease (ALD). Therefore, antioxidant therapy reflects a reasonable strategy for the prevention and treatment of ALD. Hence, this study aimed to elucidate the mechanism of PCP in ethanol-induced liver injury.

Methods: Treatment of liver-specific transgenic zebrafish larvae (lfabp: EGFP) at three days post-fertilization (3 dpf) with different concentrations of PCP (100, 50, 25 μg / mL) for 48 h was followed by soaking in 350 mmol / L ethanol for 32 h. Liver function and fat accumulation were identified by phenotypic indicators and biochemical kits. The related proteins and gene expression were further estimated by western blotting and quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR). Finally, high performance liquid chromatography (HPLC) was adopted to analyze the chemical composition of PCP extract.

Results: Firstly, PCP mediated alleviation of ethanol-induced steatosis and reduction of aspartate aminotransferase (AST), alanine transaminase (ALT), total cholesterol (TC) and triglyceride (TG) related indexes were evident. Dose-dependent decrease of intracellular reactive oxygen species (ROS) production, the activity of malondialdehyde (MDA) and increased the activity of glutathione (GSH), Superoxide dismutase (SOD) and catalase (CAT) in zebrafish substantiated the role of PCP in relieving oxidative stress. Furthermore, PCP induced downregulation of sequestosome 1 (p62 / SQSTM1, p62), Atg13 and Beclin 1 expression promoted autophagy. Meanwhile, PCP contributed to the hepatoprotective function by downregulating the expression of kelch-like ECH-associated protein 1 (Keap1) and upregulating the expression of nucleus factor-E2-related factor 2 (Nrf2), which activated cytoprotective related gene HO-1. Moreover, HPLC of PCP extract confirmed the presence of various polyphenols with potential antioxidant effects. Finally, PCP appeared to promote the activated protein kinase (AMPK) / p62 / Nrf2 / mTOR signaling pathways, which were related to oxidative stress and autophagy in zebrafish.

Conclusion: This study claimed that by activating the AMPK / p62 / Nrf2 / mTOR signaling pathway, PCP could attenuate ethanol-induced liver injury in zebrafish.

Highlight

1. Ethanol exposured in zebrafish caused oxidative stress and fat accumulation in the liver

2. PCP reduced ethanol-induced oxidative stress and improved autophagy

3. PCP activated AMPK / p62 / Nrf2 / mTOR signaling pathways

Introduction

Alcohol liver disease (ALD) caused by heavy ethanol consumption is worldwide problem and the resultant liver damage has been well characterized (Asrani, Devarbhavi, Eaton, & Kamath, 2019; Rehm et al., 2009). The World Health Organization (WHO) estimated that 2.3 billion people were active drinkers in 2016, about 3 million people die from ethanol each year, some of them from ALD (Mitra, De, & Chowdhury, 2020). The commonly used drug glucocorticoids have obvious side effects and are not suitable for long-term treatment, and many contraindications make its indication relatively limited (Lucey, Mathurin, & Morgan, 2009). In addition to abstinence, there is no effective clinical measures to cure ALD (You & Arteel, 2019). Thus, collective efforts are urgently needed to stem the rising tide of ALD on healthcare resources (Cholankeril et al., 2021).

Oxidative stress is widely believed to exert a key role in the pathophysiology of ALD. Therefore, antioxidant therapy reflects a reasonable strategy for the prevention and treatment of ALD (Michalak, Lach, & Cichoż-Lach, 2021). Nrf2 is a transcription factor that regulates the redox state of cells and is a promising intervention target for the prevention of ALD (N. Zhao, Guo, Xie, & Zeng, 2018). Nrf2 can mediate adaptive responses to oxidative stresses by degrading cytoplasmic Keap1-Nrf2 (Ohtsuji et al., 2008). Meanwhile, Nrf2 inhibits adipogenesis, supports β-oxidation of fatty acids and increases NADPH regeneration and purine biosynthesis (Hayes & Dinkova-Kostova, 2014). Recent study found that Nrf2 activation prevented acute ethanol-induced oxidative stress and accumulation of free fatty acids in liver (N. Zhao et al., 2018).

Autophagy is an essential survival pathway that ameliorates oxidative damage caused by ROS (Menk et al., 2018). Studies have shown that selective renewal of p62 impaired by autophagy leads to severe liver injury. In addition, there is increasing evidence of a functional association between dysfunctional autophagy and activation of the Nrf2 pathway (Komatsu et al., 2010). Furthermore, study has found that phosphorylated AMPK inhibits mTOR, thereby activating autophagy (Lu et al., 2021). Thus, the AMPK / p62 / Nrf2 / mTOR axis may play an important role in protecting ethanol-induced liver injury.

Recently researchers are increasingly turning their attention to multi-component, multi-targeted natural drugs, especially health food. *Penthorum chinense* Pursh (PCP) (Penthoraceae, Ganhuangcao in Chinese) is renowned for its effectiveness in "promoting blood circulation" and "removing blood stasis". It was firstly recorded in Jiuhuang Materia medica in Ming dynasty. Similar descriptions can be found in the National compilation of Chinese herbal medicine (A. Wang et al., 2020). It can relieve the liver and its application in the field of liver protection, including viral hepatitis, alcoholic liver, liver fibrosis, has been known for hundreds of years (A. Wang et al., 2020; X. Zhao et al., 2020). Previous studies have revealed that PCP extract confirmed the presence of various polyphenols with potential antioxidant effects *in vivo* and *in vitro* (L. He et al., 2019; Y. Sun et al., 2021; Tao et al., 2021; X. Zhao et al., 2020), respectively. And PCP have achieved good clinical effect in the treatment of liver disease (X. Sun et al., 2020; A. Wang et al.,

2020). These findings indicated that PCP has great potential to relieve oxidative stress and prevent liver injury.

Thus, the aim of this study was done to investigate the protective effect of PCP on ethanol-induced hepatic oxidative impairment in zebrafish. Further study explored the underlying mechanism involving in AMPK / p62 / Nrf2 / mTOR signaling pathways, providing a new target in identifying the molecular mechanisms of ethanol hepatic steatosis.

Materials And Methods

Chemicals and Reagents

The dried PCP, obtained from Chengdu HeHuaChi Chinese Herbal Medicine Market Co. (Sichuan, China) were extracted for three times by decoction, 1 h each time. After the solution is filtered, it is evaporated under reduced pressure to obtain a dry powder 17 % (g / g) and stored at -20°C for further use.

Oil red O dyeing solution was collected from Shanghai Solarbio Bioscience and Technology Co., Ltd (Shanghai, China). DCF-DA was purchased from Yeasen Bio-technology Co., Ltd. (Shanghai, China). Rabbit AMPK (CS-5831) and rabbit phospho-AMPK (CS-2535) antibodies were from Celling Signaling Technology (MA, USA). Rabbit mTOR (AF6308), rabbit phosphor-mTOR (AF3309), rabbit SQSTM1 / p62 (AF5384), LaminA / C and rabbit GAPDH (AF0911) were purchased from Affinity Biosciences. Rabbit Keap1 (R26935) and rabbit Nrf2 (R26935) were purchased from ZEN Bio-technology Co., Ltd. Trizol reagents were purchased from Ambion Life Technologies (Carlsbad, CA, USA). All-In-one First-Strand cDNA SynthesisMix for qPCR (One-Step-gDNA Removal) and Eva Green 2 × RT-qPCR MasterMix-Low ROX were purchased from Foregene Biotechnology Co., Ltd (Chengdu, China). PCR primer sequences were synthesized in TSINGKE Biological Technology (Chengdu, China). Other reagents used in the experiment were purchased from MultiSciences Biotech Co., Ltd.

Experimental animals and drug administration

Transgenic (lfabp: EGFP) zebrafish were obtained from China Zebrafish Resource Center (Wuhan, China), and raised at 28.5 ± 1.0°C on a 14 h light /10 h dark cycle. Transgenic (lfabp: EGFP) zebrafish larvae at 3 dpf were randomly assigned to 5 groups in a 6-well plate (30 larvae per well): larvae were maintained in filtered fish water as a control group while the ethanol treatment group was exposed to 350 mM ethanol for 32 h at 28.5°C(Howarth & Passeri, 2011). In PCP group, larvae were exposed to different concentrations PCP pre-treatment (100 μg / mL, 50 μg / mL, 25 μg / mL) for 48 h followed by 350 mM ethanol incubated in 28.5°C for 32 h (Cruz & Leite, 2013). Afterwards, larvae were collected for detection.

Assessment of liver phenotype

After treatment, zebrafish larvae were subjected to a series of pre-treatments including washed with fresh medium and subsequently anesthetized with tricaine, then fixed in CMC-Na, and adjusted to the lateral position. Then, zebrafish larvae were photographed under Leica M165Fic fluorescence microscope (Leica

Microsystems, Germany). Finally, the fluorescence integral optical density of zebrafish larvae were quantified using Image Pro Plus 6.0 software (Media Cybernetics, USA) was applied to quantify.

Assessment of tissue biochemical indicators

After treatment, zebrafish larvae were collected and broken using an ultrasonic cell disruption system at 4°C. ALT (C009-2-1), AST (C010-2-1), TG (A110-1) and TC (A111-1) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). After treatments, 30 larvae were cleaned by precooled PBS for three times, then homogenized and the supernatants were aspirated according to the instructions.

Whole-Mount Oil Red O Staining

Oil red O staining was used to determine hepatic lipid deposition. After treatment, zebrafish larvae were fixed overnight with 4 % PFA overnight. The rest of the procedure is routine(Yu & Gong, 2020). Finally, the oil red O positive staining of zebrafish livers were photographed and related parameters was measured.

Assessment of ROS accumulation

2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) was used as fluorescence probes to investigate intracellular production of ROS. After the treatments, the zebrafish were moved to a six-well plate (15 larvae per well) and treated with the DCF-DA (0.05 μM) solution. After 1 h incubation in dark, the zebrafish larvae were photographed under a Leica M165Fic fluorescence microscope and the accumulation of ROS was measured.

Assessment of oxidative stress factors

Indicators of oxidative stress and oxidation resistance were measured. After treatment, zebrafish larvae were collected and broken using an ultrasonic cell disruption system at 4°C. SOD (E-BC-K022-M), MDA (E-BC-K025-M), GSH (E-BC-K030-M), CAT (E-BC-K031-M) assay kits were obtained from Elabsciense Bio-technology Co., Ltd. (Shanghai, China). After treatments, 30 larvae were cleaned by precooled PBS for three times, 270 μL Slurry medium was added and homogenized, then the supernatants were aspirated according to the instructions.

Assessment the expression of related genes via quantitative RT-qPCR

Trizol reagent was used to extract total RNA from zebrafish larvae and dissolved it in RNase-free water at 4°C. Ct values were obtained (reaction conditions: 95°C 10 min, 95°C 15 s, 60°C 30 s (40 cycles)) and the relative gene mRNA expression was determined on ABI7500 qPCR system and calculated using the $2^{-\Delta\Delta Ct}$ method. The gene primer sequences used for RT-qPCR were listed in supplementary table 1.

Nucleus protein extraction

The extract was isolated using the Minute TM Hyston/DNA-Binding Protein Extraction Kit according to the manufacturer's protocol. In short, zebrafish larvae were digested with trypsin, then they were collected by low-speed centrifugation at 500 x g for 3 min, washed once with pre-cooled PBS, and transferred to a 1.5 mL centrifuge tube, centrifuged at 500 x g for 1 min, and the supernatant was discarded Suspend the precipitate in 100 μL cytoplasmic extraction reagent I, swirl for 15 seconds, incubate on ice for 10 min, mix 4, Centrifuge at 16000 x g for 5 min. Transfer the supernatant (the supernatant is the cytoplasmic component) to a new precooled 1.5 mL centrifuge tube and resuspend the precipitate with 0.5 mL precooled PBS. Centrifuge at 8000 x g for 3-5 min to clean the precipitate to reduce cytoplasmic protein contamination 15 seconds, incubate on ice for 1 minute and then repeat the shock for 15 seconds, incubate on ice for 1 minute for 4 times and quickly transfer the nucleus extract into the pre-cooled centrifuge tube sleeve, 16000 x g, centrifuge for 30 seconds and discard the centrifuge tube string, according to the manufacturer's instructions and heated at 95°C for 5 min.

Assessment the expression of related proteins via western blot analysis

Samples were then separated in denaturing PAGE (Novex, 10 % or 15 % bis-Tris gel) using amount of protein (50 μg) was loading per lane at 100 V for 1 h. Following electrophoresis, samples were then transferred onto a PVDF membrane (Millipore, Billerica, MA, USA) by electro-blotting at 120 V for 1 h, which were subsequently blocked in 5 % skimmed milk and probed with anti-p62, Keap1, Nrf2, p-AMPK, AMPK and p-mTOR, mTOR antibody solution (diluted 1:500). As internal reference, the membranes were detected on a Tanon 5200 automatic chemiluminescence imaging analysis system (Shanghai, China) and quantified by Image-Pro Plus (version 6.0).

Chemical composition analysis

HPLC was used to analyze the chemical composition. The analysis was performed on an Agilent 1260 HPLC ZORBAX C18 column (250 × 4.6 mm, 5 μm) and ZORBAX C18 pre-column(4.6 mm×12.5 mm, 5 μm)with UV-detector system analysis of PCP extract. Gradient elution was performed with mobile phase composed of (a) 0.1% phosphoric acid and (b) acetonitrile at the flow rate of 1 mL / min and the sample injection volume was 10 μL. Percentage of acetonitrile was 5−10 % (0−15 min), 10−45 % (15−45 min), 45−52 % (30−38 min), gradient elution was performed, the detection wavelength was set at 275 nm, and the column temperature was set at 30 ℃.

Statistical analyses

All statistics were evaluated by t-test or Mann-Whitney U test (two groups) and one-way ANOVA or Kruskal-Wallis test followed by pairwise comparisons (three or more groups) depending on whether the data were normally distributed. The statistical analyses and graphs were generated by GraphPad Prism 6.0 (GraphPad, San Diego, CA, USA). All values were expressed as means ± SD. Results were considered to be statistically significant when $p < 0.05$.

Results

PCP decreased ethanol-induced liver function injury and fat accumulation

Liver area of zebrafish (lfabp: EGFP) was reduced after ethanol treatment (Figure 1A), compared with the control group. We found that the liver fluorescence integral optical density (IOD) decreased significantly ($P < 0.001$) after ethanol treatment (Figure 1B) and caused the remarkable increasing of ALT, AST levels ($P < 0.001$) (Figure 1E, F). While PCP pretreatment strongly increased IOD in a concentration-dependent manner ($P < 0.001$), as well as decreased ALT, AST accumulation ($P < 0.001$) and significantly improved liver function. Ethanol treatment caused a significant accumulation of TG, TC concentrations ($P < 0.001$), PCP pretreatment strongly reduced TG, TC accumulation in a concentration-dependent manner ($P < 0.01$), and TG accumulation dominated (Figure 1G, H). These results were consistent with whole-mount oil red O staining (Figure 1C, D), which demonstrated that PCP reduced liver lipid deposition.

PCP reduced oxidative stress in ethanol-induced liver injury

Drinking ethanol leads to the release of large amounts of intracellular ROS, as seen in Figure 2A, a bright and strong fluorescent image was observed in the ethanol treatment group. PCP concentration-dependently decreased intracellular ROS production in zebrafish ($P < 0.001$) (Figure 2B). Compared with control group, the MDA levels of the ethanol-treated groups were significantly elevated ($P < 0.001$) (Figure 2C). Conversely, the levels of SOD, CAT and GSH markedly declined ($P < 0.001$). While PCP pretreatment reversed the process in a concentration-dependent manner (Figure 2D, E, F). However, the CAT activity was significantly increased only at 100 µg/mL PCP pretreatment ($P < 0.001$). Thus, PCP alleviated ethanol hepatosteatosis by inhibiting oxidative stress.

PCP decreased Keap1 expression and promoted Nrf2 transferring into nucleus via p62

To further explore the underlying mechanism. Western blot experiments were conducted to detect the expression of related proteins, as shown in Figure 3. In ethanol treatment group, the protein expression of p62 was upregulated ($P < 0.01$), while a decreased protein expression of p62 was observed (Figure 3A) when PCP pretreated with zebrafish in a concentration dependent manner ($P < 0.001$). And There is increasing evidence of a functional association between dysfunctional autophagy and Nrf2 pathway activation. Based on this, the Keap1 and Nrf2 expression (Figure 3B, C) were detected in our study. We found PCP could downregulate the Keap1 protein expression ($P < 0.001$) and promote Nrf2 transferring into nucleus ($P < 0.01$). These data suggested that PCP alleviated ethanol-induced liver injury via activating p62 / Keap1 / Nrf2 pathway.

PCP alleviated ethanol-induced liver injury via activating AMPK / mTOR pathway

Next, effort was made to investigate that whether PCP could activate AMPK / mTOR pathway to induce autophagy. As showed in figure 3D, E, PCP was no significant effect on total proteins expression including AMPK and mTOR, whereas it could enhance the phosphorylation of AMPK (p-AMPK) ($P < 0.01$),and decrease p-mTOR in a concentration dependent manner ($P < 0.001$), thus enhancing autophagy. In addition, a decrease of p-AMPK and an increase of p-mTOR expression was showed in

ethanol treatment group ($P < 0.001$). These data suggested that the possible mechanisms of PCP alleviated ethanol-induced liver injury were primarily through phosphorylation of AMPK / mTOR pathways.

PCP alleviated ethanol-induced liver injury via activating AMPK / p62 / Nrf2 / mTOR pathway

Subsequently, RT-qPCR for mRNA levels of AMPK, Keap1, Nrf2 and HO-1 oxidation-related, mTOR, p62, Atg13 and Beclin 1 autophagy-related genes, as shown in Figure 4. Compared with the ethanol treatment group, the mRNA expression of Keap1 was significantly decreased ($P < 0.001$), AMPK, Nrf2 (all $P < 0.001$) and its downstream target genes HO-1 ($P < 0.01$) were upregulated (Figure 4A, B, C, D) after PCP pretreatment in a concentration dependent manner. Further, the expression of p62 and mTOR increased ($P < 0.001$) (Figure 4E, F) which indicated that autophagy was inhibited and autophagy flux was blocked, while Atg13 and Beclin 1 decreased significantly ($P < 0.001$) (Figure 4G, H) in ethanol treatment group. Moreover, our data showed that 100 μg/mL PCP pretreatment significantly reversed the results ($P < 0.001$), which means that autophagy flux can be restored. These data suggested that PCP alleviated ethanol-induced liver injury via activating AMPK / p62 / Nrf2 / mTOR pathway.

Chemical characteristics of PCP extract

The extract of PCP was analyzed by HPLC to determine its main chemical constituents, which was consistent with the previous studies that PCP extract contained various polyphenols (Figure 5). Five peaks were identified as gallic acid, rutin, quercetin, luteolin, and apigenin. The contents of the five compounds were quantified using corresponding chemical standards. Specifically, the contents of gallic acid, rutin, quercetin, luteolin, and apigenin in PCP were 1.2025, 0.8244, 0.4967, 0.026, 0.0970 mg / g, respectively.

Discussion

Excessive ethanol consumption is increasing every year globally, especially among young people, affecting 10-15 per cent of the population, posing a significant medical, social and economic burden (Carvalho, Heilig, Perez, Probst, & Rehm, 2019). Apart from abstinence, there is no effective cure. Therefore, preventing the occurrence of disease may be a more reliable way to treat the disease better. PCP has been used to treat liver-related diseases with no side effects or toxicity observed in clinical use (A. Wang et al., 2020). Previous studies showed that polyphenols were the main chemical constituents of PCP, which possessed strong antioxidant activities (Hu & Wang, 2015). Our data indicated that PCP reduced ethanol-induced hepatic steatosis and oxidative stress in zebrafish and the related molecular mechanism was further discussed.

The liver is an important organ for ethanol metabolism, which can be damaged by by-products of ethanol decomposition, such as ROS. While excessive production of ROS can lead to oxidative stress, which can lead to liver damage (Yan, Nie, Luo, Chen, & He, 2021). Recently, the prevention and treatment of oxidative stress-driven liver diseases by medicinal plant extracts has attracted wide attention. PCP, acted as

traditional Miao medicine, its hepatoprotective effect on inhibiting oxidative stress *in vivo* was reported in previous literatures. It has been reported that PCP had a significant protective effect on tert-butyl hydroperoxide (t-BHP) induced hepatocyte damage, resulting in resistance to the ROS induced mitochondrial oxidative stress (A. Wang et al., 2016). Excessive ROS production induced oxidative stress, which led to the damage of protein and DNA in cells as well as the production of lipid peroxides, such as MDA. In our research, MDA level obviously increased in ethanol treatment group, whereas PCP, acting as scavengers of ROS, markedly decreased this tendency. In response to oxidative stress, the zebrafish larvae need to elevate the activities of antioxidant enzymes and activate non-enzyme antioxidant system, such as SOD, CAT and GSH. In our results, PCP exerted strong antioxidant ability, thereby attenuating the oxidative stress induced by ethanol.

In addition, recent papers reported that PCP enhanced the oxidant defense systems via the activation of Nrf2 / HO-1 pathway against chronic ethanol-induced liver injury (Cao et al., 2015). Nrf2, as important nucleus transcription factor, is retained by binding to its inhibitor and inactivating Keap1 in the cytoplasm, which serves as a Nrf2 degradation adapter. When responding to oxidative stress, Nrf2 translocates into nucleus and regulates the expression of antioxidant genes, such as HO-1, NQO-1, which become the key pathway for plant extracts and natural products to inhibit ethanol-induced oxidative stress. And our results showed the same trend.

Recently, autophagy has become a protective mechanism against ALD (Babuta & Furi, 2019). p62 is considered to be a specific autophagy marker, and the increase of p62 protein level indicates that autophagy is inhibited and autophagy flux is blocked (Pankiv et al., 2007). Previous studies have shown that disruption of early autophagy pathways leads to the accumulation of ubiquitinated proteins and an increase in ROS levels and mitochondrial dysfunction (Zhu et al., 2021). Increasing evidence suggests a functional association between dysfunctional autophagy and activation of the Nrf2 pathway, which appears to occur through the physical interaction between autophagy connector p62 and Nrf2 inhibitor Keap1, thereby binding to and competing with Keap1, promoting Nrf2 release by Keap1 and increasing Nrf2 stability and transcriptional activity of induced antioxidant gene expression (Lu et al., 2019). Our results were consistent with recent studies, indicating that PCP can effectively change the autophagy flux in ethanol-induced oxidative impairment.

In addition, AMPK is an energy sensor that is an upstream molecule of autophagy (W. S. He, Wu, Ren, Yu, & Zhao, 2021). In the former publications, Monascin regulated AMPK-mediated ethanol-induced liver injury by regulating p62 and autophagy crosstalk with the Keap1 / Nrf2 pathway (Lai, Hsu, Pan, & Lee, 2021). Similarly, in our study, an increase of p-AMPK protein expression was observed by pretreatment with PCP, and then promoting p62-mediated autophagic degradation of Keap1. The above results suggested PCP could indeed active the expression of p-AMPK to exert a vital role in the PCP alleviating oxidative stress and autophagy impairment.

Moreover, AMPK is an upstream regulator of mTOR, which can activate mTOR phosphorylation and thus mediate autophagy signaling pathway (H. Wang et al., 2019). Interactions between the p62 and mTOR

pathways have been reported, and it has been found that upregulation of p62 activates mTORC1 by directly acting on the mTOR regulatory protein Raptor, thereby inhibiting autophagy (Zhang, Bao, Cong, Fan, & Li, 2020). Combined with our experimental results, the expression of p62 and mTOR increased, blocking the autophagy flux in ethanol treatment group. While after pretreatment with PCP, phosphorylated AMPK inhibited p-mTOR, thereby activating autophagy. The above results suggested PCP could indeed active the expression of p-AMPK to inhibit p-mTOR, alleviating autophagy impairment.

Above all, the mechanism of PCP in protecting ethanol-induced hepatic oxidative impairment in zebrafish was revealed more comprehensively, and the AMPK / p62 / Nrf2 / mTOR axis may play an important role in the treatment of ethanol-induced liver injury.

Conclusion

In summary, this study claimed that by activating AMPK / p62 / Nrf2 / mTOR signaling pathway, PCP could attenuate ethanol-induced liver injury in zebrafish, which elucidated the mechanism of PCP in the treatment of ethanol-induced liver injury.

Declarations

Acknowledgements

Authors contribution

Xingtao Zhao: Writing – original draft, review & editing, Project administration. Mengting Zhou: Formal analysis. Yunxia Li and Cheng Peng: Supervision, Funding acquisition. Chaocheng Guo and Ying Deng: Visualization, Project administration. Li Liao and Linfeng He: Resources, Data curation.

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Ethics approval and consent to participate

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Competing Interest

The authors declare that there are no conflicts of interests

Abbreviations

ALD, alcohol liver disease
ALT, alanine transaminase
AMPK, activated protein kinase
AST, aspartate aminotransferase
CAT, catalase
DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate
IOD, integral optical density
GSH, glutathione
Keap1, kelch-like ECH-associated protein 1
lfabp
EGFP, Liver-specific transgenic zebrafish larvae
MDA, malondialdehyde
Nrf2, nucleus factor-E2-related factor 2
PCP, Penthorum chinense Pursh
ROS, reactive oxygen species
RT-qPCR, quantitative reverse transcriptase-polymerase chain reaction
p62 / SQSTM1, Sequestosome 1
SOD, Superoxide dismutase
SREBP-1C, sterol regulatory component binding protein 1C
TC, total cholesterol
TG, triglyceride
HPLC, High Performance Liquid Chromatography
WHO, World Health Organization
3 dpf, three days post-fertilization

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Supplementary Table 1

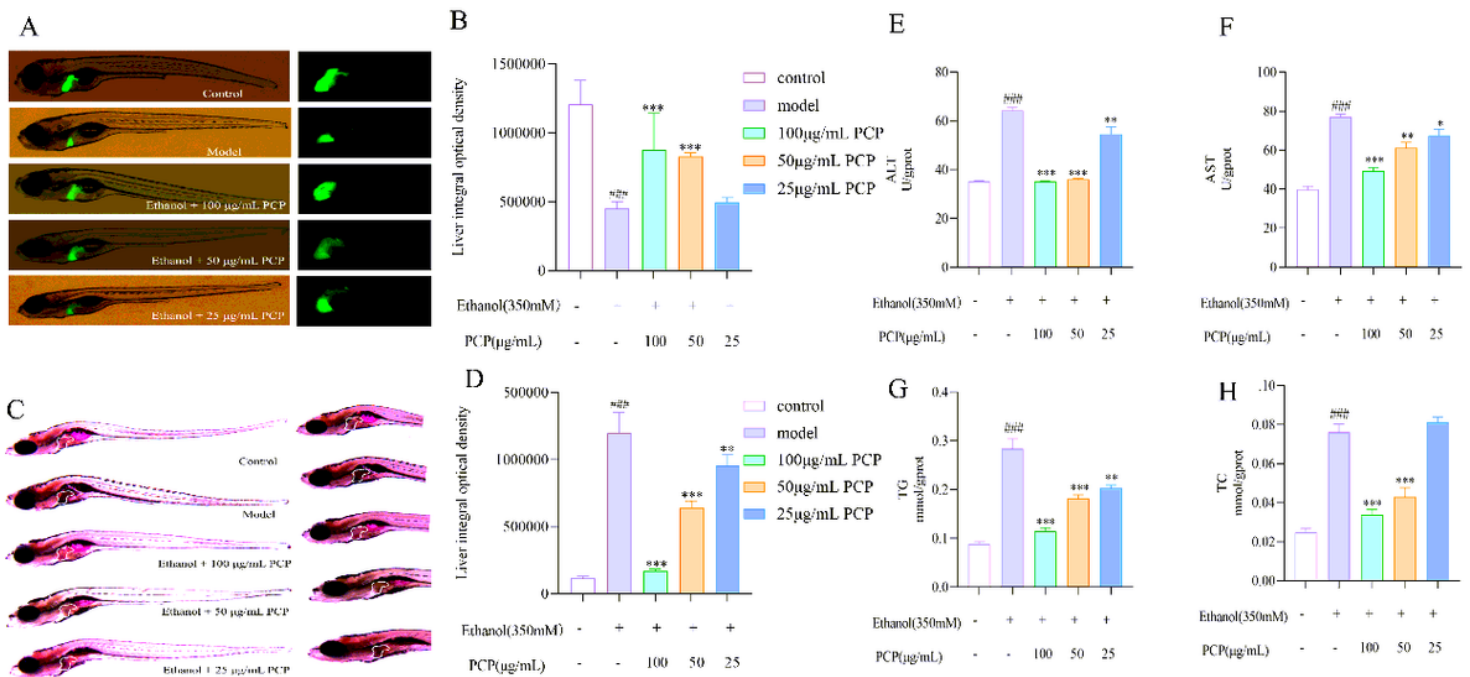Supplementary Table 1 is not available with this version.

Figures

Figure 1

Different concentrations of PCP effect on serum biochemical indicators of liver function. (A) The fluorescence intensity of the liver part; (B) The percent of IOD of the area (statistical analysis by Image Pro Plus 6.0 software). (C) the whole-mount oil red O staining of larvae zebrafish (the white a indicates the liver). (D) The percent of IOD of the area. The following four liver function markers in the tissue were assayed: (E) ALT; (F) AST; (G) TG; (H) TC. ###P < 0.001 compared with control group; ***P < 0.001, **P < 0.01, *P < 0.05 compared with ethanol treatment group
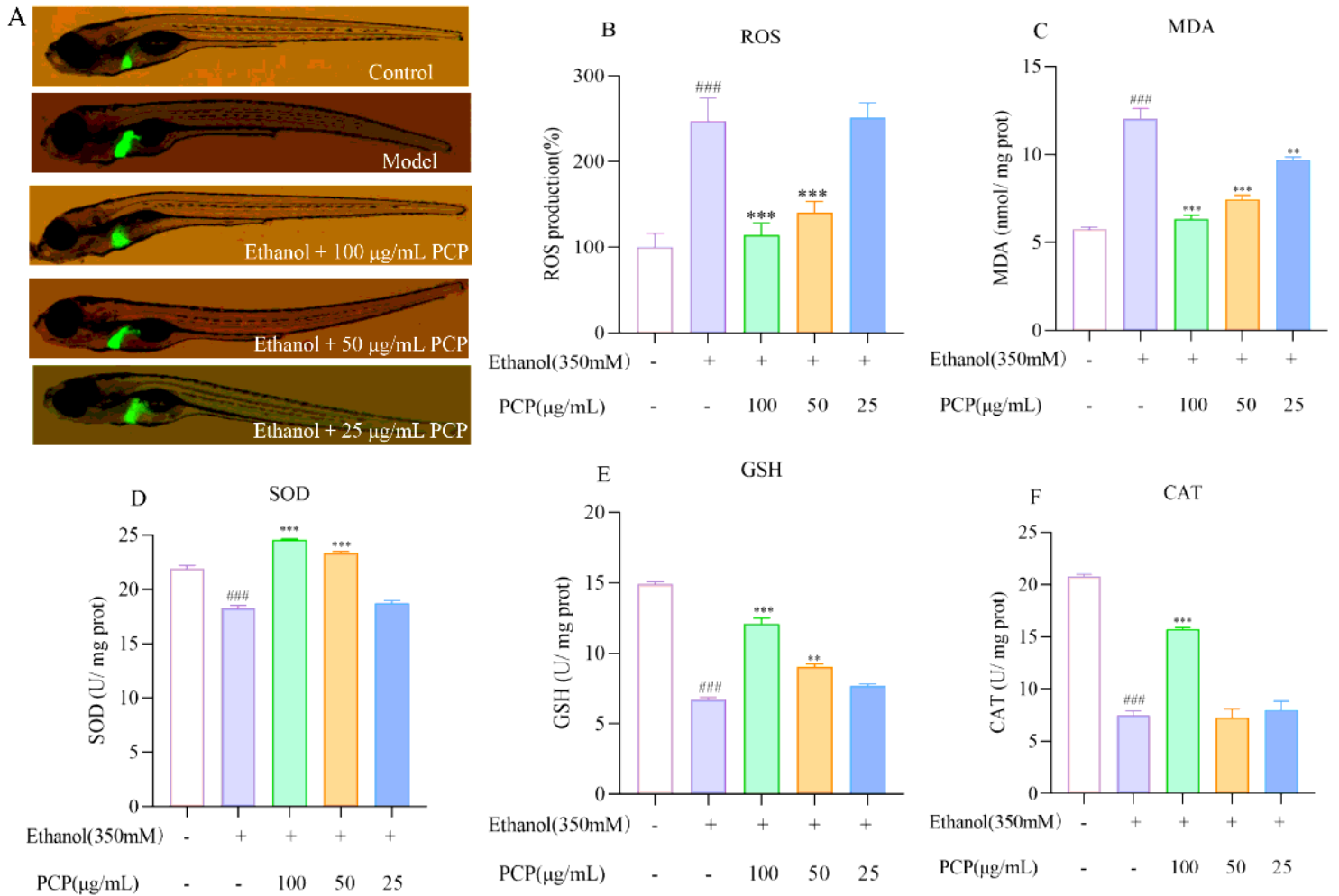
Figure 2

(A) Fluorescence micrographs of ROS in zebrafish larvae. (B) The distribution and amounts of superoxide anions were quantified according to the fluorescence intensity. The following six liver function markers in the serum were assayed: (C) MDA, (D) SOD, (E) GSH, (F) CAT. ###$P < 0.001$ compared with control group; ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ compared with ethanol treatment group.
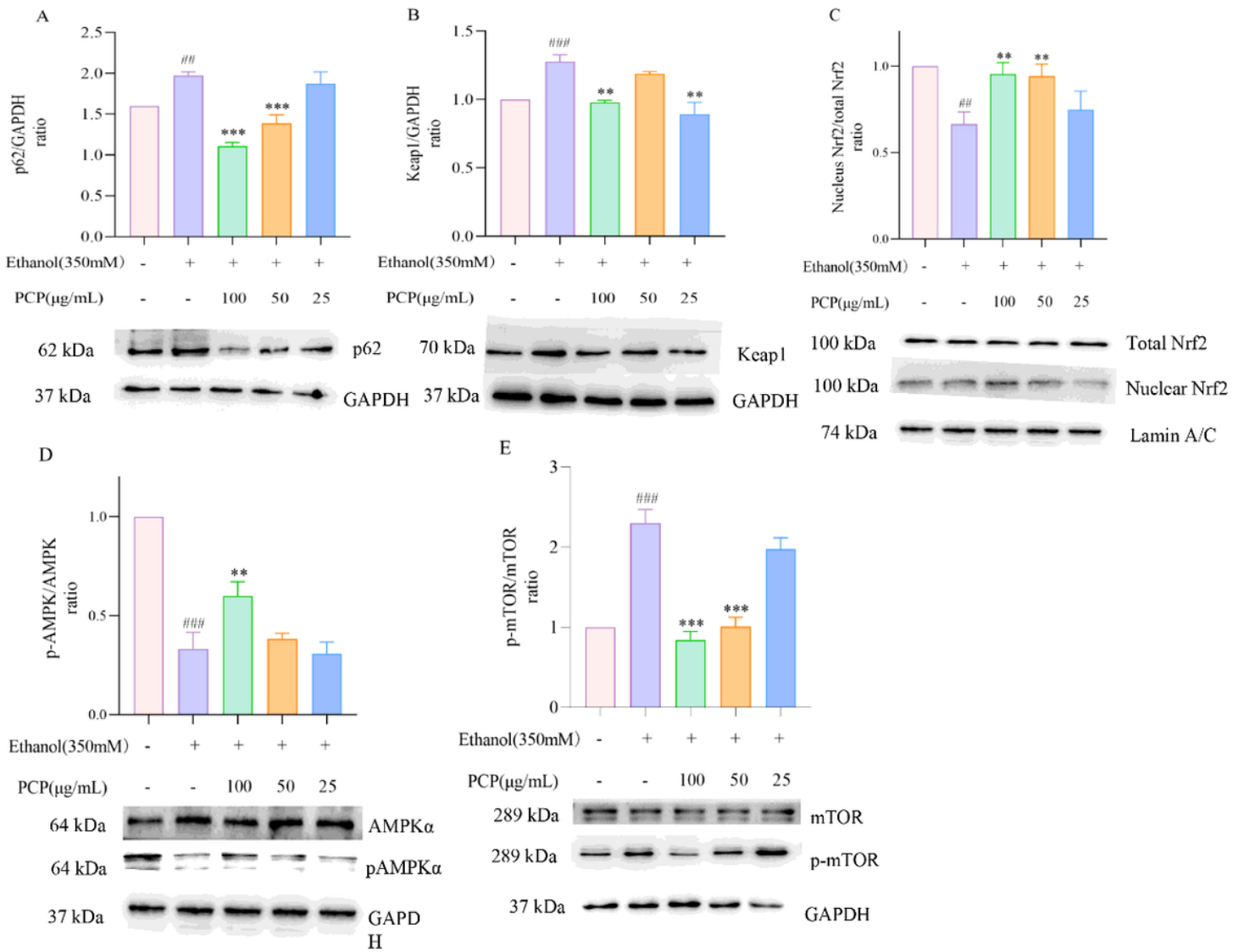
Figure 3

Effect of PCP on proteins expression profiles. Quantitation of western blot analysis of (A) p62, (B) Keap1, (C) Nrf2, (D) p-AMPK/AMPK, (E) p-mTOR/mTOR on larvae zebrafish; ###$P < 0.001$ compared with control group; ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ compared with ethanol treatment group.
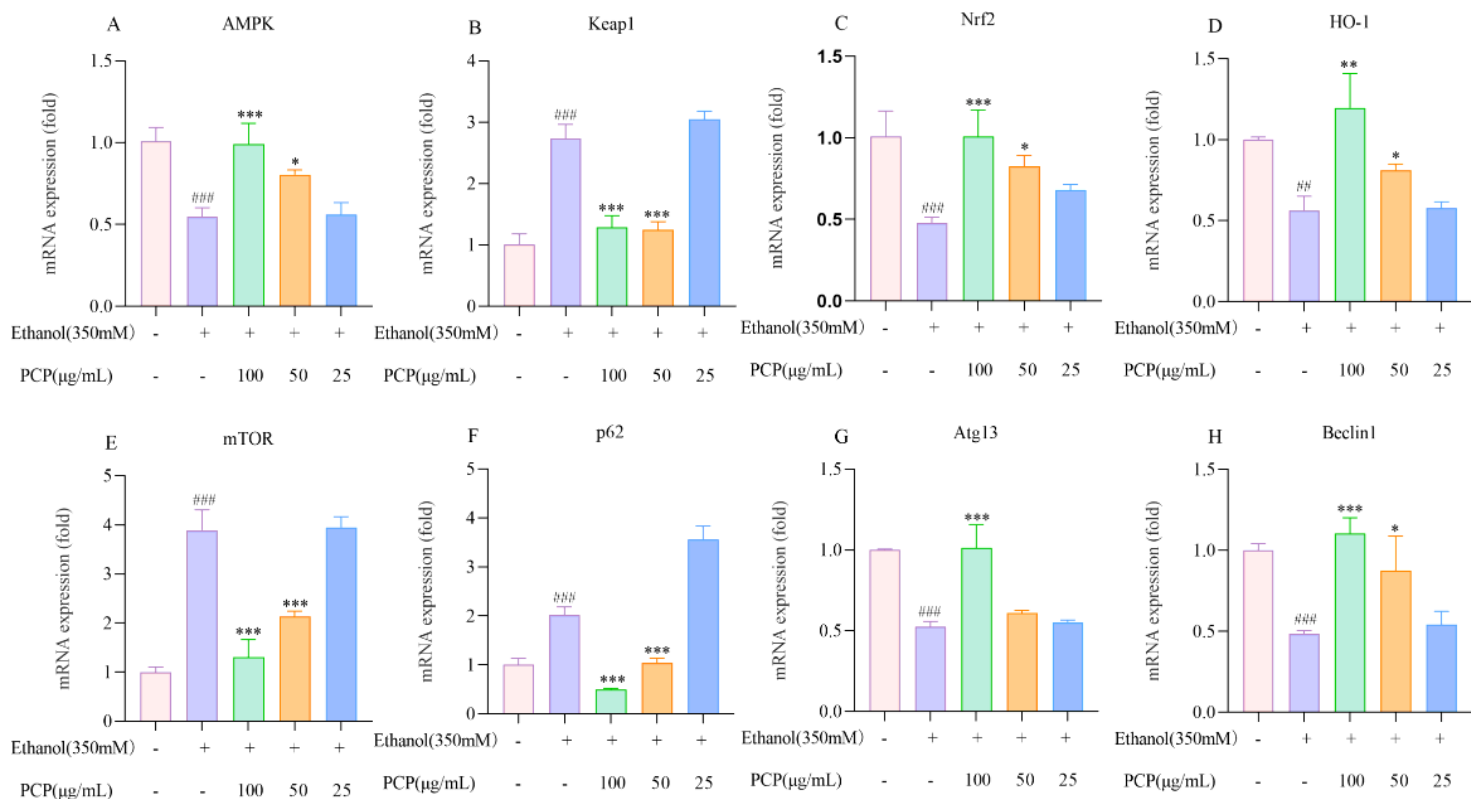
Figure 4

Effect of PCP on genes expression profiles. Quantitation of mRNA expression of (A) AMPK, (B) Keap1, (C) Nrf2, (D) HO-1, (E) mTOR, (F) p62, (G) Atg13, (H) Beclin1 on larvae zebrafish; ###$P < 0.01$ compared with control group; ***$P < 0.01$, *$P < 0.05$ compared with ethanol treatment group.
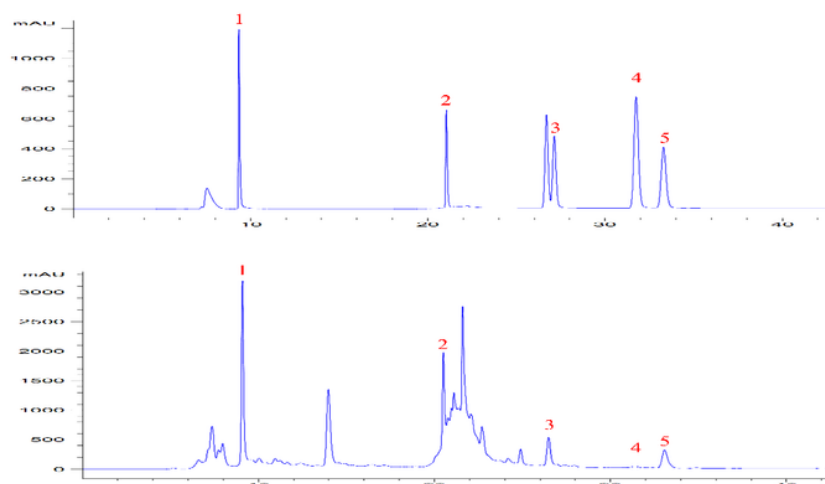

Figure 5

Representative HPLC-UV chromatograms of mixed standards and PCP extract. Gallic acid (1), lutin (2), quercetin (3), luteolin (4), and apigenin (5)

Supplementary Files

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