

Original Research Paper

Trilobatin Possesses Protective Potentials on H₂O₂-Induced HepG2 Cells by Suppressing Oxidative Stress: Involvement of Nrf2/Keap1 Signal Pathway

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Abstract: Trilobatin, a natural dihydrochalcone compound, possesses various biological activities. However, few investigations have been carried out on the mechanisms of trilobatin against oxidative stress. To comprehend the effects and mechanism of trilobatin on oxidative stress after hepatic injury, H₂O₂-induced HepG2 cells were used in this research. The results indicated that trilobatin enhanced the viability and improved the apoptosis morphology of HepG2 cells induced by H₂O₂. The decrease of Mitochondrial Membrane Potential (MMP) and the increase of the antioxidant-related enzymes involving Superoxide Dismutase (SOD), Glutathione Peroxidase (GSH-Px), and Catalase (CAT), as well as the reduced levels of alanine Aminotransferase (ALT) and aspartate Aminotransferase (AST), could be responsible for the protective effect of trilobatin on H₂O₂-induced HepG2 cells. Furthermore, the Nuclear erythroid-2 Related Factor 2 (Nrf2)/Kelch-like ECH Associated Protein-1 (Keap-1) pathway was activated by trilobatin in H₂O₂-induced HepG2 cells. These results indicated that trilobatin has a strong protective capacity on H₂O₂-induced HepG2 cells by inhibiting oxidative stress via Nrf2/Keap1 signaling pathway. This research implies that trilobatin might be a useful antioxidant used to prevent liver diseases caused by oxidative stress.

Keywords: Trilobatin, Oxidative Stress, HepG2 Cell, Nrf2/Keap-1

Introduction

As the largest metabolic organ of the human body, the liver plays a crucial role in several physiological functions, including metabolism, biotransformation, interpretation, and immune regulation (Zhang *et al.*, 2017). Many factors such as alcohol, drugs, and chemical pollutants lead to hepatic injury, which in turn leads to the occurrence of liver diseases. At present, liver disease has become one of the diseases seriously endangering human health and its incidence rate is increasing year by year (Asrani *et al.*, 2019). Previous research has proved that oxidative stress is closely related to liver diseases and liver cancer and plays a critical role in various stages of hepatic injury development (Asrani *et al.*, 2019; Singh *et al.*, 2019). The Nrf2/Keap1 signaling pathway is a key mechanism against oxidative stress in living organisms (Bellezza *et al.*, 2018). Studies have shown that antioxidants are particularly important for maintaining the balance between

oxidative stress and antioxidant defense (Ma *et al.*, 2022). Recently, the food industry has made extensive use of synthetic antioxidants. However, many synthetic antioxidants, like Butylated Hydroxy Anisole (BHA) and Butylated Hydroxy Toluene (BHT), can damage human liver function, induce cancer, and have other side effects (Peng *et al.*, 2021; Seedeve *et al.*, 2017). Natural antioxidants extracted from animals and plants have high safety, antioxidant abilities, no side effects, and other characteristics. Therefore, natural antioxidants have attracted wide attention from researchers in recent years. Studies have confirmed that several natural antioxidants, such as confusoside, resveratrol, and delphinine have significant antioxidant activities and can effectively induce liver injury by inhibiting oxidative stress (Zhao *et al.*, 2023; De Moraes *et al.*, 2021; Xu *et al.*, 2020). However, the existing types and quantities of natural antioxidants are not enough to meet people's needs. Therefore, more effective natural antioxidants need to be explored.

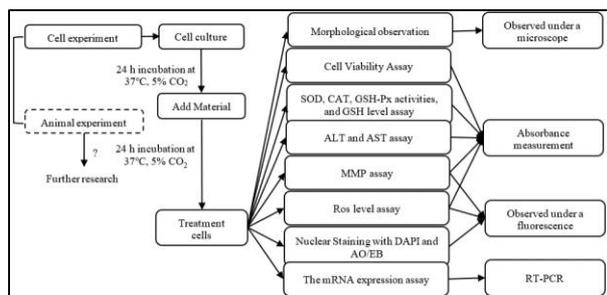


Fig. 1: Flow chart of the study. Cell: HepG2; Material: 250 μM H_2O_2 /difference concentrations of trilobatin

Because of their low energy value, high sweetness, non-participation in human metabolism, no caries and other characteristics, dihydrochalcones are often used as a low-energy sweetener for drinks, candy, and other food processing (De Luca *et al.*, 2022). As a natural dihydrochalcone compound, trilobatin is 300 times sweeter than sucrose (Sun *et al.*, 2015). In recent years, the biological activities of trilobatin have been gradually revealed. Studies showed that trilobatin possessed anti-inflammatory, anticancer, antiviral, hypoglycemic, and other biological activities (Gao *et al.*, 2018). Research preliminarily confirmed that trilobatin had significant antioxidant activity (Li *et al.*, 2022). However, trilobatin's preventive impact against oxidative stress-related liver damage has not been extensively studied. Therefore, in this study, a model of oxidative stress caused by H_2O_2 in HepG2 cells was built and the effect of trilobatin on the changes of cell viability, apoptotic morphology, antioxidant enzymes activities, ROS levels, MMP, ALT and AST contents and Nrf2/Keap1 signaling pathway were detected (Fig. 1). The research confirmed the alleviating effect of trilobatin on oxidative stress in HepG2 cells generated by H_2O_2 and provided more sufficient data for trilobatin used in oxidative stress related disease.

Materials and Methods

Materials

Dimethyl Sulfoxide (DMSO) was used to dissolve the stock solution of trilobatin (analytical grade, purity 98%), which was purchased from Sigma-Aldrich (St Louis, MO, USA), to 1 mm. The different concentrations of trilobatin solutions were diluted with Dulbecco's Modified Eagle Medium (DMEM) without Fetal Bovine Serum (FBS) or ultra-pure water. Ferrous sulfate, Bovine Albumin (BSA), and hydrogen peroxide were purchased from Sinopharm chemical reagent Co. Ltd (Shanghai, China). Dulbecco's Phosphate-Buffered Saline (PBS), DMEM, FBS, and penicillin-streptomycin were purchased from Gibco Co.

(grand island, NY, USA). 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DAPI stain Solution and AO/EB Staining Kit were obtained from BBI CO., LTD (Shanghai, China). The assay kits for 5,5',6,6'-tetrachloro-1,1'.3,3' -tetraethyl-imidaercar bocyanine iodide (JC-1), ROS, CAT, and BCA protein assay reagents were purchased from Beyootime biotechnology Co. Ltd (Shanghai, China). The assay kits for GSH, SOD, GSH-Px, AST, and ALT were purchased from Nanjing Jiancheng bioengineering institute (Nanjing, China). Trizol reagent and reverse transcription kit were purchased from Songon biotechnology Co., Ltd (Shanghai, China). All other reagents used in the experiments were of analytical grade.

Cell Culture and Treatment

The cell bank of the Chinese academy of science's institute of biochemistry and cell biology (Shanghai, China), provided the HepG2 cells. HepG2 cells were cultured in DMEM medium containing 10% FBS (v/v) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2 .

After being treated with varying doses of trilobatin (1, 10, and 50 μM) for 24 h, HepG2 cells were incubated with 250 μM H_2O_2 for a further 4 h. The same volume of DMEM was given to the control group's cells in place of trilobatin, while the cells of the positive control group were incubated with 20 mM NAC and then stimulated with H_2O_2 .

Cell Viability Assay

The MTT colorimetric test was used to evaluate the vitality of the cells (Palanisamy *et al.*, 2018). HepG2 cells were cultivated for one night in 96-well plates after being seeded at a density of 1×10^4 per well. HepG2 cells were treated experimentally and then each well received 20 μL of MTT after two PBS washes. The dish was then incubated for a further 4 h. After removing the culture media, DMSO was applied to each well to dissolve the formazone crystals that formed a purple color. Utilizing a microplate reader (Shimadzu UV-2600, Kyoto, Japan), the absorbance was measured at 490 nm.

Nuclear Staining with DAPI and AO/EB

6-well plates were seeded with 1×10^4 cells/well of HepG2 cells. After treatment, the cells were washed twice with PBS before adding the proper amount of fixative solution (methyl: Glacial acetic acid = 3:1), then the cells were maintained for 20 min at 4°C. After discarded the fixative solution, the cells were washed twice with PBS and treated with the proper amount of triton (0.5%) for 15 min to make them transparent.

Table 1: Primer sequences used for gene expression analyses

Gene	Primer sequence	
	Forward (5'-3')	Reverse (5'-3')
KEAP1	CCTTCAGCTACACCCTGGAG	AACATGGCCTGAAGACAGG
Nrf2	AGACAAACATTCAAGCCGCT	CCATCTCTTGTGTTGCTGCAG
HO-1	CAGTCTTCGCCCTGTCTAC	GCTGGTGTGTAGGGGTGAC
NQO1	AGTGCAGTGGTGTGATCTCG	GGTGGAGTCACGCCTGTAAT
SOD	GAAGGTGTGGGGAAGCATT	ACCACAAGCCAAACGACTTC
CAT	ACATGGTCTGGGACTTCTGG	CTTGGGTCGAAGGCTATCTG
GSH-Px	AACCAGTTTGGGCATCAGG	GTTACCTCGCACTTCTCG

After being cleaned with PBS solution, the cells were stained for 30 min in the dark with DAPI and AO/EB, respectively. Under a fluorescence microscope (Nikon, EclipseTS100, Japan) with a 40× magnification, the cells were observed (Li *et al.*, 2020).

Assessment of Mitochondrial Membrane Potential (MMP)

MMP of HepG2 cells was measured using the fluorescent probe JC-1 (Chang *et al.*, 2021). After treatment, HepG2 cells were twice rinsed with PBS and then JC-1 was applied to HepG2 cells at 37°C for 30 min. The green and red JC-1 fluorescence of the cells stained with JC-1 was measured using fluorescence microscopy (Nikon, EclipseTS100, Japan) and scanned of microplate fluorescence (Thermo Fisher, Shanghai, China) at 288 nm excitation, 535 and 509 nm emission, respectively.

Determination of SOD, CAT, GSH-Px Activities, and GSH Level in HepG2 Cells

Following a 24 h culture period, the cells underwent the various treatments outlined before. Following the removal of the supernatants, the cells underwent three PBS washes. The whole cell protein was then extracted by lysing the cells in a lysis buffer. After that, all the detections of GSH level, activities of SOD, CAT, and GSH-Px, as well as protein concentrations, were carried out in accordance with the kit-specified methods.

Determination of Intracellular ROS Level

The DCFH-DA probe staining was used to determine the ROS level. Briefly, HepG2 cells (1×10^4 cells/well) were planted onto 6-well plates. After the cells were treated as previously described, each well was twice cleaned with PBS and stained with 10 μM DCFH-DA solution at 37°C for 20 min. The fluorescence intensity produced by DCFH-DA in HepG2 cells was estimated with a fluorescence microplate (Thermo Fisher, Shanghai, China) quantitatively and a fluorescence microscope (Nikon, EclipseTS100, Japan) qualitatively.

Determination of AST and ALT Levels in HepG2 Cells

After being planted into a 6-well late, the cells were left to incubate for 24 h. After the cells were treated as

previously described, the supernatants were taken out and the cells underwent three PBS washes. The contents of the cells were then extracted by lysis them in a lysis buffer. Finally, the AST and ALT levels were determined in accordance with the guidelines provided by the manufacturer for AST and ALT assay kits.

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

The Trizol Kit was used to extract the HepG2 cells's total mRNA. The cells were lysed by Trizol in a 6-well plate and added to Trizol later. RT-PCR was carried out using a light cycler 96 (Roche, Switzerland) with the Sybr green RNA to CT 2-step kit (Evo M-MLV RT Mix, Accurate Biology) to assess the mRNA expression of the target gene. CT values were used for the relative quantification of mRNA. For comparative analyses, Glyceraldehyde-3 Phosphate Dehydrogenase (GAPDH) was used as the internal standard. The synthetic sequences of specific primers are listed in Table 1.

Statistical Analysis

At least three trials were conducted for each experiment. The dates were analyzed using IMB SPSS Statistics 2.0 software and the results were presented as the mean ± Standard Deviation (SD). The Least Significant Difference (LSD) test was used at a significance level of 0.05 to ascertain the differences between the sample mean values.

Results and Discussion

Effects of Trilobatin on Cell Viability Induced by H₂O₂

In this study, Fig. 2A showed that trilobatin at concentrations ranging from 1-100 μM did not appear to have any discernible effects on the viability of HepG2 cells, suggesting no toxicity of 1-100 μM trilobatin on HepG2 cells. Therefore, the concentrations of trilobatin used in this research were no more than 100 μM. In addition, MTT assay results showed that the 250 μM H₂O₂ treatment for 4 h decreased the viability of HepG2 cells significantly, which was nearly 50% lower than that of the normal group (Fig. 2B-C). Therefore, HepG2 cells were

induced by 250 μM H_2O_2 for 4 h to construct an oxidative stress model. The viability of H_2O_2 -stimulated HepG2 cells may be concentration-dependently increased by pretreatment with trilobatin for 12 h and the cell viability reached 87% after 100 μM trilobatin treatment, which was close to the effect of 20 mM NAC (Fig. 2D). These results indicated that trilobatin may be able to shield HepG2 cells from H_2O_2 -induced damage.

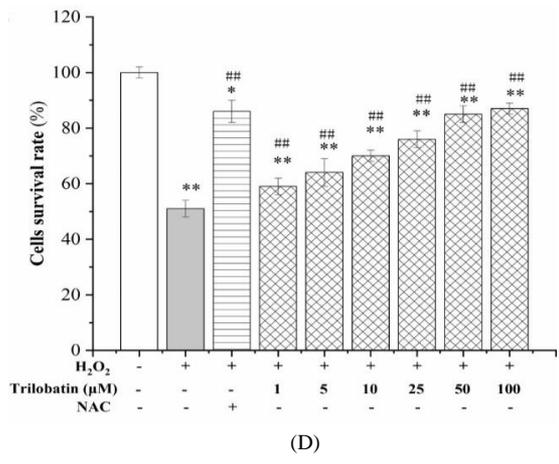
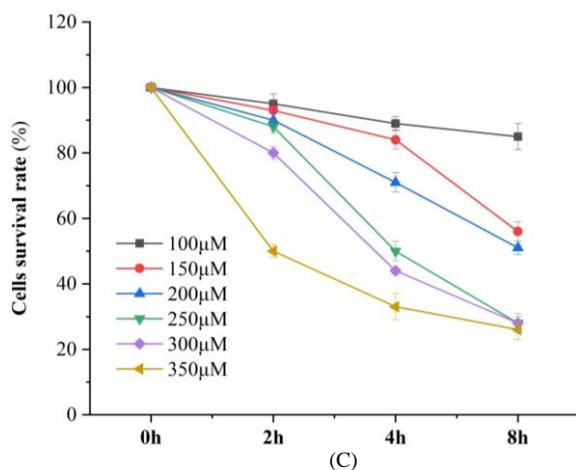
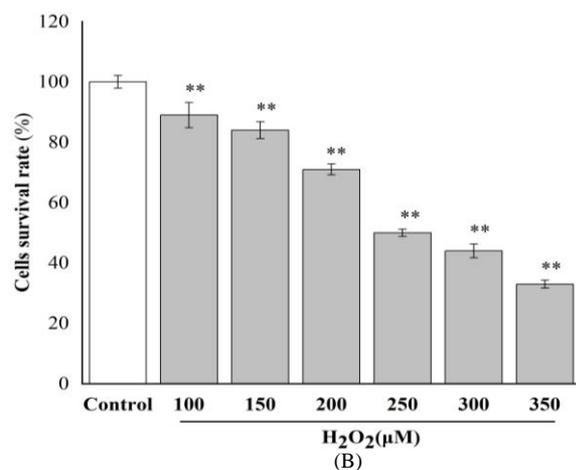
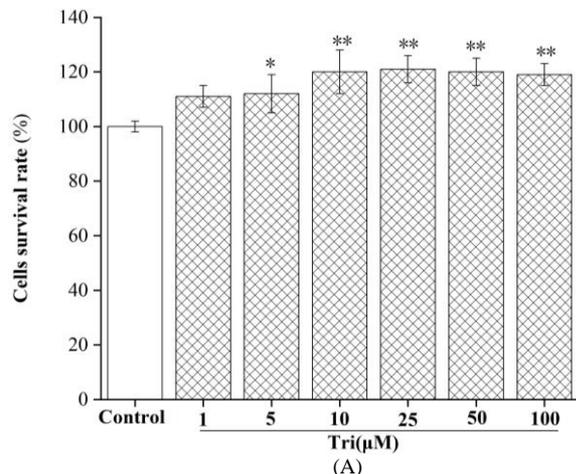


Fig. 2: (A) Effect of trilobatin on the viability of HepG2 cells. Effects of (B) H_2O_2 induction concentration and (C) H_2O_2 time on the viability of HepG2 cells; (D) Effect of trilobatin on the viability of H_2O_2 -induced HepG2 cells under different concentration

Effect of Trilobatin on Cell Morphology of HepG2 Cells Induced by H_2O_2

The primary characteristics of apoptosis include changes in morphology and biochemistry. As a result, the appearance of cells was used to assess the presence of apoptosis (Doonan and Cotter, 2008). In this study, the morphological changes of HepG2 cells are shown in Fig. 3. Whereas the cytomorphology of HepG2 cells altered following H_2O_2 treatment, the cells in the normal group had a distinct irregular fusiform outline. However, with increasing concentrations of trilobatin pretreatment, the apoptotic features (nuclear coagulation, chromatin coagulation, contraction and deformation of cells, and contour blurring) were dose-suppressed. These results suggested that trilobatin can improve the apoptosis morphology of H_2O_2 -induced HepG2 cells.

DAPI, frequently used to identify apoptosis, can penetrate the intact cell membrane and bind with double-stranded DNA, then emit strong blue fluorescence (Liu *et al.*, 2021). Normal cell membranes may be penetrated by AO, a fluorescent cationic dye that is selective for nucleic acids. When it binds with nuclear DNA, it emits a uniform green or yellow-green fluorescence. Only incomplete cells are reddened by EB (Patnaik and Padhy, 2016). Therefore, to differentiate between normal and apoptotic cells, AO is frequently double-stained with EB. To evaluate the apoptosis of HepG2 cells, DAPI and AO/EB staining were utilized. According to Fig. 4, after DAPI staining, HepG2 cells in the normal group presented uniform staining and complete nuclear morphology, whereas H_2O_2 -treated cells exhibited cell contractions and nuclear chromatin contractions. However, after pretreatment with trilobatin, the higher the concentration of trilobatin, the more uniform the nuclear staining and the more complete

the nuclear membrane of HepG2 cells. These findings suggested that the protective effect of trilobatin was achieved by reducing early apoptosis. The results of AO/EB staining showed that the green light of HepG2 cells was significantly weakened and the red light was enhanced after H₂O₂ treatment, suggesting a rise in the permeability of cell membranes. Moreover, the cells showed apoptotic features such as round, orange-red, dense nuclear chromatin. Trilobatin treatment of HepG2 cells tended to cause the green light to progressively increase and the red light to gradually become dimmer and the apoptotic characteristics of HepG2 cells were weakened, indicating that trilobatin pretreatment dramatically reduced the H₂O₂-induced apoptosis in HepG2 cells relative to the control HepG2 (Fig. 5).

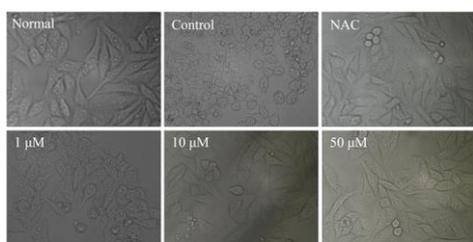


Fig. 3: Effect of trilobatin on the morphology of HepG2 cells

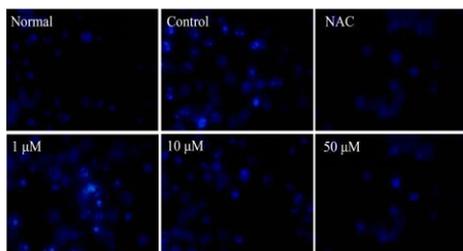


Fig. 4: DAPI staining analysis of the induction of apoptosis in HepG2 cells, visualized under fluorescence microscopy

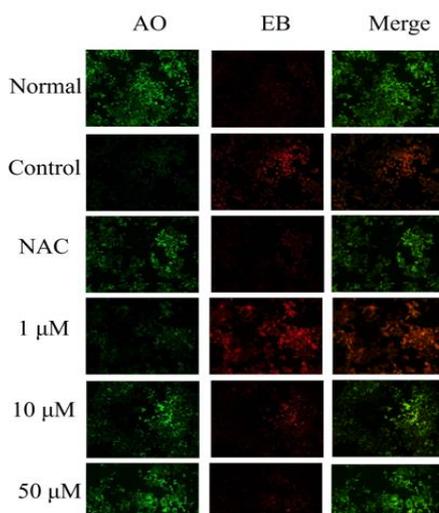


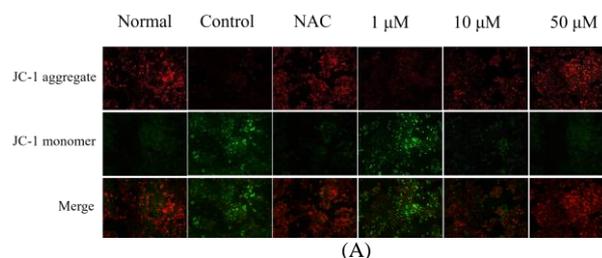
Fig. 5: AO/EB staining analysis of the induction of apoptosis in HepG2 cells, visualized under fluorescence microscopy

Effect of Trilobatin on MMP of HepG2 Cells Induced by H₂O₂

MMP reduction is an important marker for evaluating early apoptosis. The conversion of JC-1 from red to green makes it an excellent fluorescent probe that can identify any alterations in the mitochondrial membrane with speed and sensitivity (Liu *et al.*, 2020). HepG2 cells treated with trilobatin showed an increase in red fluorescence, but JC-1 emitted green fluorescence when H₂O₂ was present, as seen in Fig. 6A. Meanwhile, Fig. 6B showed a dose-dependent reduction in the intensity of green/red fluorescence in HepG2 cells following trilobatin treatment. Thus, trilobatin might effectively prevent H₂O₂-induced HepG2 cell injury by inhibiting the decline of MMP. Similarly, other research has shown that trilobatin reversed the loss of MMP in H₂O₂-induced PC12 cells (Gao *et al.*, 2018).

Effects of Trilobatin on Antioxidant Enzyme of HepG2 Cells Induced by H₂O₂

Antioxidant enzymes, including GSH-Px, CAT, and SOD, are crucial for maintaining an appropriate intracellular oxidation status (Zhang *et al.*, 2016; Yeruva *et al.*, 2021). In comparison to the normal group, the activities and expressions of antioxidant enzymes such as SOD, CAT, and GSH-Px were significantly lower in H₂O₂-induced HepG2 cells (Fig. 7). However, after the cells were incubated with trilobatin, the three enzymes' activities and expressions rose to varying degrees. Meanwhile, GSH possesses important physiological functions such as scavenging free radicals, detoxification, and the integrity of the erythrocyte cell membrane, as well as normal cell growth and development, which is a crucial component in determining the body's antioxidant capability. Figure 7 showed that the intracellular GSH level in HepG2 cells from the H₂O₂-induced group was considerably lower than in the normal group. After pretreatment with trilobatin, GSH content increased gradually compared with the H₂O₂-induced group, indicating that trilobatin's antioxidative effects on oxidative cell damage were realized. Previous research showed that phlorizin, an isomer of trilobatin, also increased the activities of antioxidant enzymes in the same oxidative damage cell model (Wang *et al.*, 2019). Moreover, it was reported that trilobatin increased the enzymatic activities of MnSOD and GSH-Px in oxidative stress PC12 cells (Gao *et al.*, 2018).



(A)

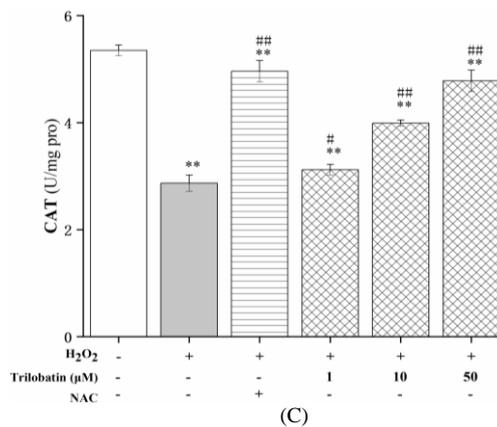
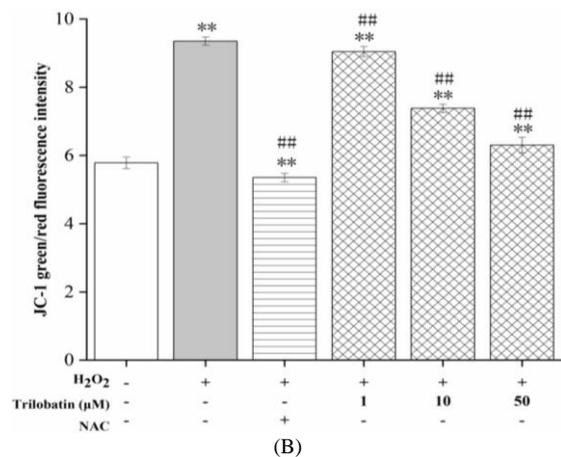
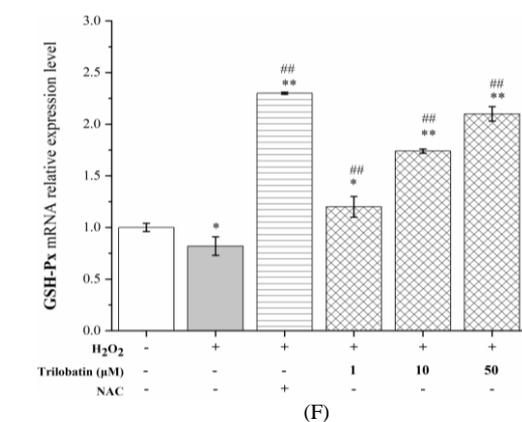
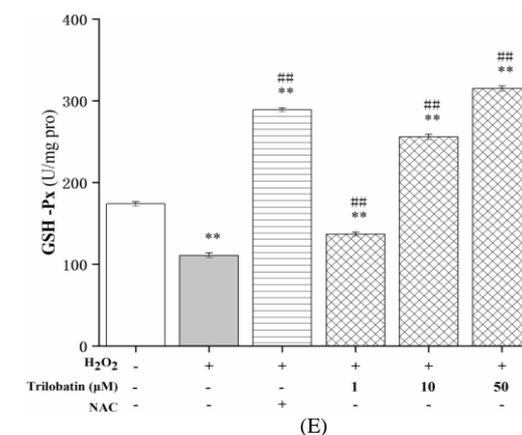
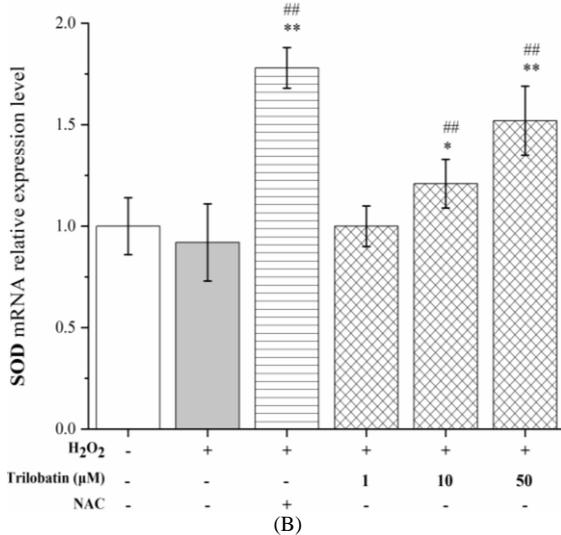
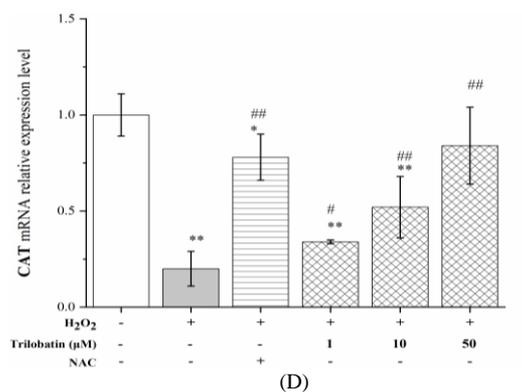
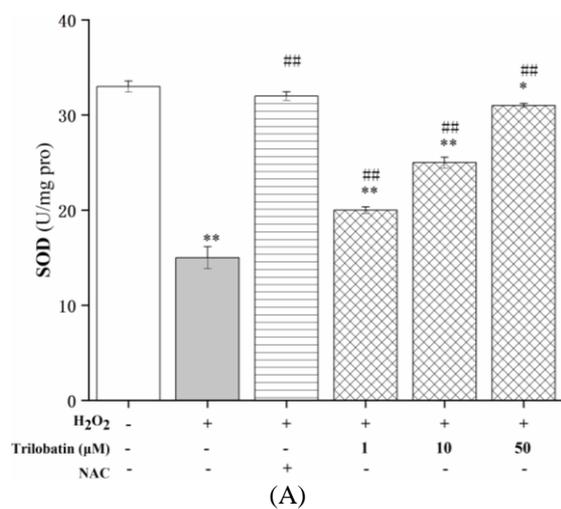


Fig. 6: Effects of trilobatin on the MMP in HepG2 cells. HepG2 cells were stained with JC-1 after being treated with trilobatin for 24 h; (A) Qualitative analysis by fluorescence microscope; (B) Quantitative analysis with a fluorescence enzyme marker



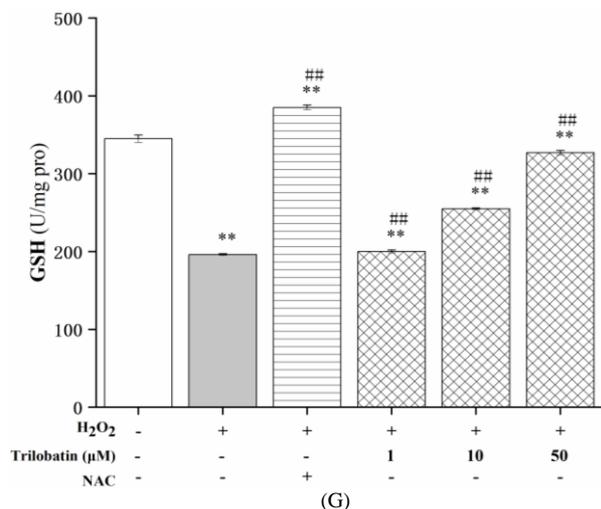


Fig. 7: Effects of trilobatin on; (A-B) SOD; (C-D) CAT; (E-F) GSH-Px and; (G) GSH in H₂O₂-induced HepG2 cells

Effect of Trilobatin on ROS Levels in HepG2 Cells

An overabundance of ROS in cells damages macromolecules oxidatively, which ultimately results in cell death (Butterfield *et al.*, 2002; Ismy *et al.*, 2022). This research utilized a DCFH-DA fluorescent probe to measure the intracellular ROS levels in HepG2 cells, was used in this study. According to Fig. 8A, compared with the normal group, the green fluorescence of HepG2 cells was greatly increased after H₂O₂ induction, which indicated that H₂O₂ treatment led to the accumulation of ROS levels in HepG2 cells. When compared to the H₂O₂-induced group, treatment with trilobatin decreased the green fluorescence intensity of HepG2 cells in a dose-dependent way (Fig. 8A-B). These results indicated that trilobatin can effectively reduce ROS content in HepG2 cells caused by H₂O₂. In mouse models with D-galactosamine/lipopolysaccharide (GalN/LPS)-induced fulminant hepatic failure (another type of hepatic damage) and LPS-stimulated Kupffer Cells (KCs) injury, trilobatin could decrease excessive cellular and mitochondrial ROS generation (Hou *et al.*, 2023). In addition, trilobatin also protected H₂O₂-induced PC12 cells by reducing ROS generation (Gao *et al.*, 2018).

Effects of Trilobatin on ALT and AST in H₂O₂-Induced Cell Culture Supernatant

The release of liver transaminases such as ALT and AST is the primary indicator of hepatocellular damage (Lee *et al.*, 2018). Figure 9 displayed the ALT and AST contents of the HepG2 cell culture supernatant. Compared with the normal group, the AST and ALT levels were increased about 2 or 3 times by H₂O₂ induction, respectively and this increase was reduced by trilobatin pretreatment. At the same time, the effect of 50 μM

trilobatin on liver transaminase level was close to that of NAC. It was reported that another dihydrochalcone compound, neohesperidin dihydrochalcone significantly decreased the serum AST and ALT activities of tetrachloride-induced acute oxidative injury mice (Hu *et al.*, 2014). The same effect of neohesperidin dihydrochalcone was also observed in paraquat-induced acute liver injury mice (Shi *et al.*, 2015).

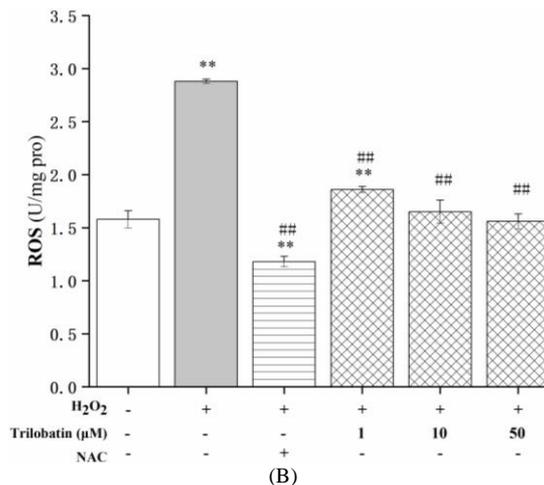
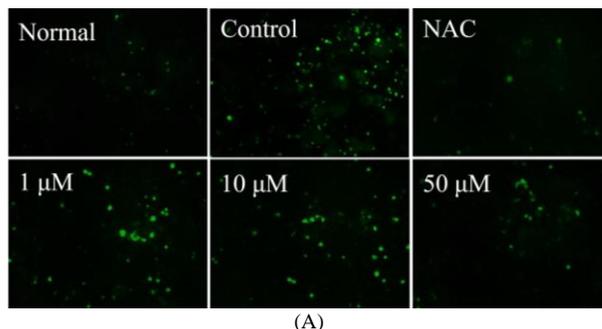
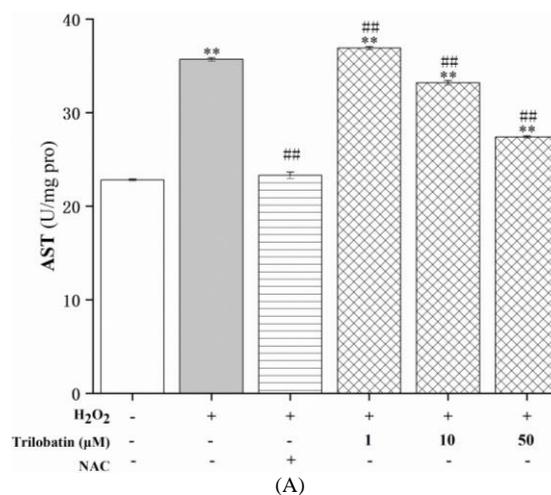


Fig. 8: Effect of trilobatin on the intracellular ROS level. (A) Qualitative analysis by fluorescence microscope; (B) Quantitative analysis with a fluorescence enzyme marker



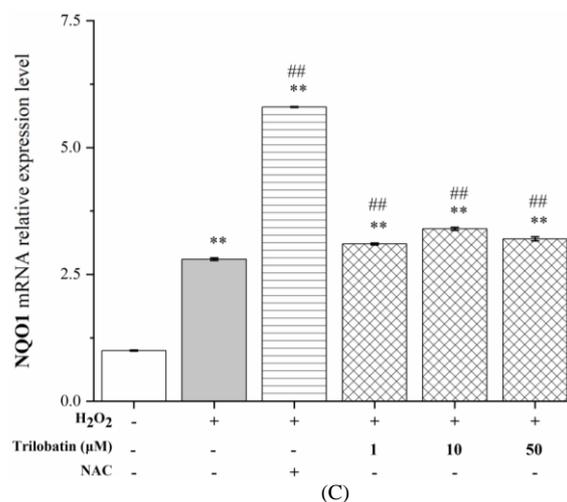
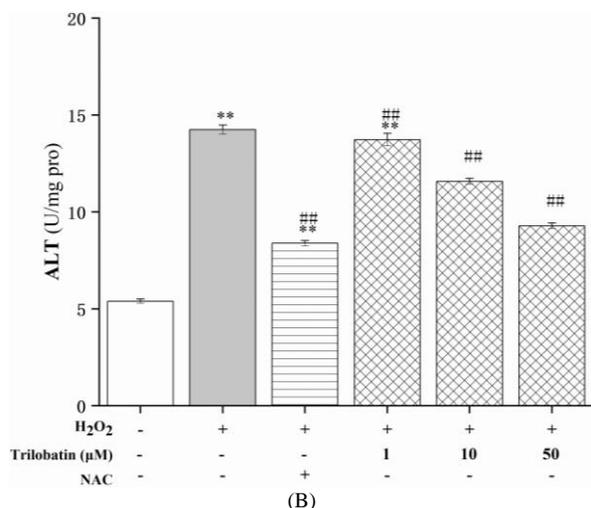


Fig. 9: Effect of trilobatin on (A) AST and (B) ALT contents in cell culture supernatant

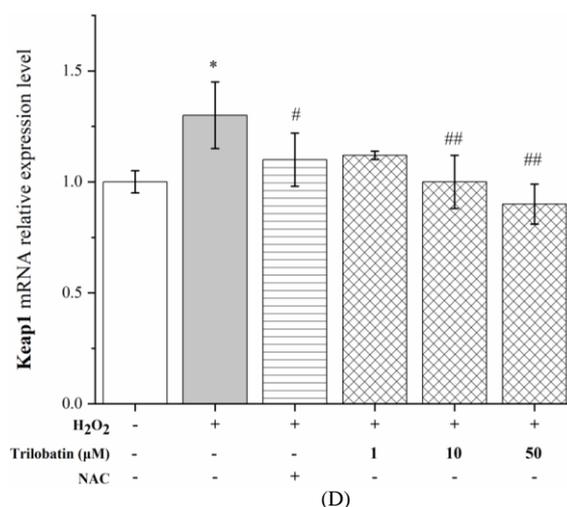
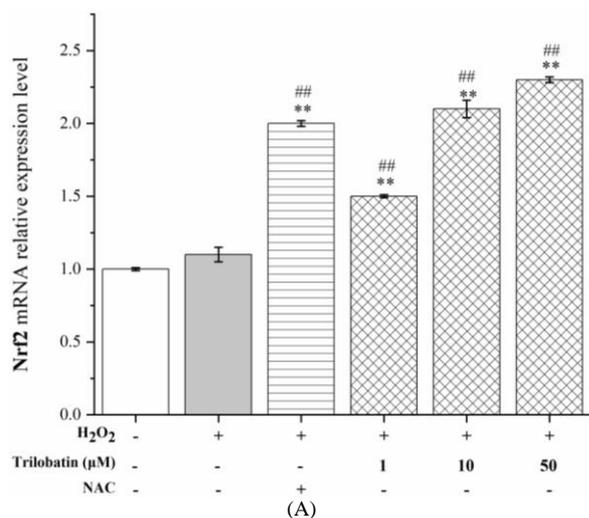
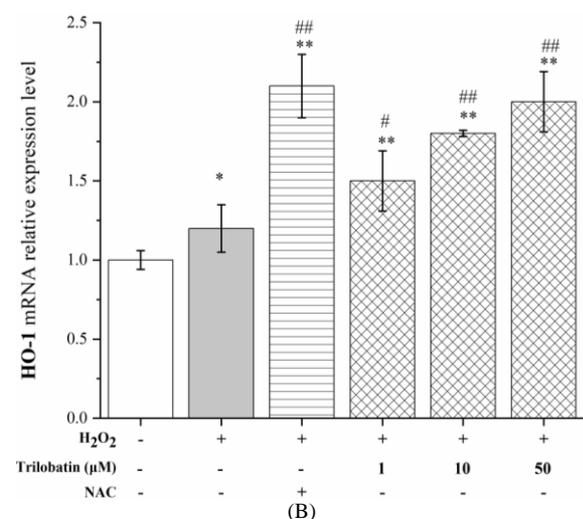


Fig. 10: Effect of trilobatin on Nrf2/Keap-1 signaling pathway of HepG2 cells. (A) Nrf2 mRNA relative expression level; (B) HO-1 mRNA relative expression level; (C) NQO1 mRNA relative expression level and (D) Keap1 mRNA relative expression level



Effects of Trilobatin on Nrf2/Keap1 Signaling Pathway in H₂O₂-Induced HepG2 Cells

The Nrf2/Keap1 signaling pathway is the most critical pathway for oxidative stress. In this research, the effect of trilobatin on the Nrf2/Keap1 signaling pathway was carried out by RT-PCR. The findings demonstrated that mRNA expression levels of Nrf2, HO-1, and NQO1 were significantly increased by trilobatin in H₂O₂-induced HepG2 cells (Fig. 10A-C). Meanwhile, the Keap1 mRNA expression level was depressed by treatment with trilobatin and NAC in H₂O₂-induced HepG2 cells (Fig. 10D). The outcomes revealed that H₂O₂ might alleviate oxidative injury of HepG2 cells by activating the Nrf2/Keap1 signaling pathway and trilobatin may via this signaling pathway,

which was consistent with the previous researches of phlorizin and raspberry on H₂O₂-induced HepG2 cells (Chen *et al.*, 2019; Wang *et al.*, 2019).

Conclusion

In conclusion, trilobatin could alleviate H₂O₂-induced oxidative damage in HepG2 cells by activating oxidative stress-related signaling pathways. The research showed that trilobatin enhanced the viability and improved the apoptosis morphology of H₂O₂-induced HepG2 cells. Trilobatin also improved the activities of antioxidant enzymes, AST and ALT. And ROS level in HepG2 cells induced by H₂O₂ was reduced effectively after trilobatin treatment. meanwhile, trilobatin activated the Nrf2/Keap1 signaling pathway to enhance antioxidant defense. Therefore, trilobatin is a potential active ingredient for the development of products used in oxidative stress-related diseases. This study provided data support for the further development of trilobatin in functional food and medicine. Further animal research needs to be carried out to explore the mechanism of trilobatin on oxidative stress in hepatic injury.

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Author's Contributions

Yuhan Zhai: Designed and conducted the experiments, analyzed the results, and wrote the manuscript.

Yuqing Zhang, Yaping Li and Ming He: Assisted in performing some of the experiments.

Shaoxuan Yu: Contributed to the study designed.

Haifang Xiao: Contributed to the study design, the result interpretation, and the manuscript preparation.

Yuanda Song: Provided guidance and supervision for the experimental designed.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the

other authors have read and approved the manuscript and that no ethical issues are involved. The research complies with ethical guidelines and standards.

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