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Antioxidant Activity of Trilobatin and Its Inhibitory Effects on Oxidative Damage of Biological Macromolecules

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Abstract: Excessive levels of free radicals in the body cause oxidative damage to macromolecules by injuring DNA, proteins, and lipids, which leads to structural and functional defects and ultimately results in a number of illnesses. The antioxidation of trilobatin and its inhibitory activity of trilobatin on oxidative damage of biological macromolecules have not been thoroughly investigated. In this research, the effects of trilobatin on scavenging rates of hydroxyl l, 1,1-Diphenyl-2-Picrylhydrazyl (DPPH), and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals as well as reducing power were thus examined. Meanwhile, Cu^{2+}/ H_2O_2 and 2, 2'-Azobis (2-methylpropionamidine) dehydrochloride (AAPH) induced proteins, lipids and DNA models were used to examine the inhibitory effects of trilobatin on oxidative damage of biological macromolecules. Through the significance analysis of the repeated experiment results, it was found that trilobatin had significant scavenging effects on DPPH and ABTS free radicals, which were up to and (100.07±1.93%), respectively. However, trilobatin had a limited capacity to scavenge hydroxyl radicals and reduce power. Moreover, trilobatin had protective effects on proteins, lipids, and DNA. However, the protective effects were different in different induction systems. These findings indicated that trilobatin may function as a natural antioxidant to maintain the balance between oxidation and antioxidation in organisms. Theoretical support for the use of trilobatin in functional foods was offered by this research.

Keywords: Trilobatin, Antioxidation, Biomacromolecule, Oxidative Damage

Introduction

The generation and removal of free radicals in living organisms are in a dynamic equilibrium state (Venditti and Di Meo, 2020). Superoxide (O_2-), Hydrogen Peroxide (H_2O_2), Hydroxyl radicals (OH), and singlet Oxygen (O_2) are examples of Reactive Oxygen Species (ROS), which can be created during normal metabolic and physiological processes and are important for homeostasis and cell signaling (Borisov *et al*., 2021; Sarmiento-Salinas *et al*., 2021). With the increase of age, our tissues and organs lose their functionality gradually and our body's capacity to remove free radicals also declines (Zia *et al*., 2022). In addition, environmental pollutants, dietary factors, drugs, lifestyle, and other factors may stimulate the formation and excessive accumulation of ROS in the body, which causes oxidative damage to biological macromolecules (Rani *et al*., 2016; Sies and Jones, 2020). Oxidative damage of biological macromolecules leads to the occurrence and development of several diseases (de Bhailís *et al*. 2021; Rani *et al*., 2016; Sies and Jones, 2020). A growing body of research has linked oxidative damage to conditions like atherosclerosis, inflammation, cancer, and neurodegenerative diseases (Di Meo and Venditti, 2020; Fang *et al*., 2012; Li *et al*., 2020; Zhang *et al*., 2010). Therefore, the search for natural and effective antioxidants is crucial for preventing oxidative stress-related illnesses.

As a natural dihydrochalcone compound, trilobatin has been found in many plants such as polystachys, Strychnocotyledon, *Malus* Mill, and *Lycopersicon* esculentum Mill (De Luca *et al*., 2022; Kongkiatpaiboon *et al*., 2020; Yahyaa *et al*., 2016). In recent years, studies showed that trilobatin had hypoglycemic, anti-inflammatory, inhibition of melanin production, and

various other biological properties (Ding *et al*., 2021; Wang *et al*., 2018). Previous research preliminarily confirmed the significant antioxidant activity of trilobatin (Li *et al*., 2022). However, there are few in-depth reports on the antioxidant capacity of trilobatin and its defenses against damage to biological macromolecules. DPPH, ABTS, Hydroxyl radical scavenging rate, and reducing capacity are often used to evaluate the antioxidant activity *in vitro* (Kristiani and Kim, 2023). Therefore, the antioxidant activity of trilobatin was investigated in this study by measuring indicators such as the scavenging rates of hydroxyl, ABTS, DPPH radicals, and the reducing capacity of ferrous iron. Additionally, BSA, rat liver, and brain tissue protein were used as protein models, rat brain, liver homogenates, lecithin, and linoleic acid as lipid models and pBR322DNA was used as DNA model to investigate the inhibitory effects of trilobatin on Cu^{2+}/H_2O_2, AAPH, and Fe^{2+} induced oxidative damage of biomacromolecules.

Materials and Methods

Materials

2-Thiobarbitturic Acid (TBA), Trichloroacetic Acid (TCA), 1,1′-Diphenyl-2-Picrylhydrazyl (DPPH), 2,2′-azobis-2 - methyl-Propanimidamide (AAPH), lecithin, linoleic acid and 2,2′-azobis-3-ethylbenzthiazthiazolne-6sulfonic acid (ABTS) bought at Yuanye Biotech. Co. (Shanghai, China). Sigma-Aldrich (St Louis, MO, USA) provided the trilobatin (analytical grade, purity 98%). The analyses only employed analytical-grade reagents throughout the experiment.

DPPH Radical Scavenging Assay

The slightly modified approach was used to assess the DPPH radical scavenging activity of trilobatin (Hu *et al*., 2014). First, 1.95 mg DPPH was submerged in 25 mL methanol solution to produce 200 μM DPPH methanol solutions. Then, DPPH methanol solutions (200 μM) were mixed with the various concentrations of trilobatin solutions. The mixture reacted at room temperature without light for 30 min and a spectrophotometer (Shimadzu UV-2600, Kyoto, Japan) was used to assess their absorbance at 517 nm while utilizing Vc as a positive reference. Following the formula below, the *DPPH* radical scavenging rate of trilobatin was determined:

$$DPPH\ radical\ scavenging\ rate(\%) = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100\%$$

where, A_1 is the absorbance of the sample with *DPPH* solution, A_2 is the absorbance of the sample only (sample without *DPPH* solution), and A_0 is the absorbance of the control (*DPPH* solution without sample).

ABTS Radical Scavenging Assay

ABTS radical scavenging activity of the trilobatin was estimated according to the previously reported method with slight modification (Trujillo *et al*., 2014). First, the reaction between 7.35 mM potassium persulfate and 7 mM ABTS solution produced ABTS radical solution. Then, the combination was incubated in a dark room for a whole night. Aiming to obtain the ABTS free radical solution, the ABTS radical solution was diluted with anhydrous ethanol to obtain the absorbance of 0.7±0.02 at 734 nm measured by spectrophotometer (Shimadzu UV-2600, Kyoto, Japan). Then, 600 μL of ABTS free radical solution was combined vigorously with 120 μL of trilobatin solutions at various concentrations. After the combination had been reacting for 6 min in the darkroom, their absorbance was determined at 734 nm with the use of a spectrophotometer (Shimadzu UV-2600, Kyoto, Japan). Additionally, Vc served as a positive control. Following the formula below, the *ABTS* radical scavenging rate of trilobatin was determined:

$$ABTS\ radical\ scavenging\ rate(\%) = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100$$

where, A_1 is the absorbance of the sample with *ABTS* free radical solution, A_2 is the absorbance of the sample only (sample without *ABTS* free radical solution), and A_0 is the absorbance of the control (*ABTS* free radical solution without sample).

Hydroxyl Radical Scavenging Assay

The scavenging ability of trilobatin on hydroxyl radical was studied by the previous method (Wan *et al*., 2013). Different doses of trilobatin solutions were mixed with 200 μL of 1.8 mM $FeSO_4$, 1 mL of 1.8 mM salicylic acid-ethanol solution, and 10 μL of 0.03% H_2O_2. After 30 min of water bath at 37°C, centrifugation was performed at 3000 r/min for 20 min. With a spectrophotometer (Shimadzu UV-2600, Kyoto, Japan) set to 510 nm, the absorbance of the supernatant was measured. The hydroxyl radical scavenging capacity of trilobatin was calculated utilizing the formula:

$$OH\ radical\ scavenging\ rate(\%) = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100\%$$

where, A_1 is the absorbance of the trilobatin solutions mixed with $FeSO_4$, salicylic acid-ethanol solution, and H_2O_2, A_2 is the absorbance of the trilobatin solutions only, A_0 is the absorbance of ultra-pure water mixed with $FeSO_4$, salicylic acid-ethanol solution and H_2O_2.

Reducing Power Assay

With slight adjustments, the prior approach was used to determine trilobatin's capacity to reduce ferrous ions. (Vijayalakshmi and Ruckmani, 2016). The FRAP reagent was produced by combining 20 mM ferric chloride hexahydrate solution, 10 mM TPTZ solutions, and 300 mM sodium acetate buffer (pH3.6) (1:1: 10, v/v/v) and interacting without light for 10 min after vortex shock.

Prior to usage, the FRAP working solution was always freshly produced. After that, 60 μL trilobatin solutions in a range of concentrations were combined with 300 μL FRAP working solution, which was incubated for 30 min in a darkened environment. In order to quantify the absorbance, a spectrophotometer (Shimadzu UV-2600, Kyoto, Japan) was used at 593 nm. A standard curve was created using various concentrations of 60 μL $FeSO_4$ standard solutions (0, 5, 10, 25, 50, 100, 250 μM) mixed with 300 μL TPTZ working solution, respectively. The reducing power of trilobatin was calculated using the following formula:

$$C = A_1 - A_2 - A_0$$

where, A_1 is the absorbance of the trilobatin solutions mixed with the TPTZ working solution, A_2 is the absorbance of the trilobatin solutions only, and A_0 is the absorbance of ultra-pure water mixed with the TPTZ working solution.

Preparation of Tissue Homogenate and Protein

The rat liver homogenate and protein were prepared according to a previously reported method (Sergazy *et al*., 2021). For this study, the Shandong University Laboratory Animal Center (Shandong, China) supplied the Sprague Dawley (SD) rats for our experiments. All animals were approved by the Animal Ethics Committee of Shandong University of Technology. The relative humidity for raising SD rats was 55±10% at 25±1°C. The rats were sacrificed after being anesthesia. Then, the liver and the brain tissues were carefully separated and washed with physiological saline. After weighting, one part of the tissues was homogenized in a 0.9% (w/v) ice-cold saline solution before being centrifuged (Eppendorf 5810R, Hamburg, Germany) at 4°C (4000 r/min, 10 min). Brain homogenates or liver homogenates could be found in the supernatant. Similarly, the other part of the tissues was separated and dissolved in a solution of RIPA and PMSF solution. At 4°C (15000 r/min, 10 min), the ice bath homogenate was centrifuged. The tissue protein was then extracted from the supernatant.

Determination Protein Oxidation

Different quantities of trilobatin were incorporated into protein solutions and the mixture was incubated in water for 30 min at 37°C. Then AAPH (50 mM) or Cu^{2+}/H_2O_2 (0.1/25 mM) were added to all groups except the control group. The same volume of PBS was used instead of trilobatin in the induction group. After the vortex, the samples were placed in an AAPH induction system for 4 h or a Cu^{2+}/H_2O_2 induction system for 1.5 h at 37°C. Then sodium dodecylsulfate-palyacrylamide gel electrophoresis (SDS-PAGE) was carried out to determine the extent of protein oxidative damage (Acharya and Ghaskadbi, 2013). In order to denature the protein, the processed samples were combined with protein loading buffer and boiled in a water bath at 95°C for 10 min. After cooling, the electrophoresis apparatus was used to concentrate 20 μL of the protein sample at 80 V voltage for 30 min and then separated at 120 V voltage for 60 min. The gel was dyed with Coomassie Brillant Blue R-250 (0.1%) before scanning with Gel Doc XR System (Bio-Rad, USA). Finally, the protein band intensity was quantified using the ImageJ software.

Determination of Lipid Peroxidation

We employed lipid models made from brain and liver homogenates. Tissue homogenates were combined with varying amounts of trilobatin solutions. The mixture started an oxidation reaction with the addition of Cu^{2+}/H_2O_2 (0.1/25 mM) or AAPH (50 mM). Reactions were carried out in an open tube in a water bath that was shaking at 37°C for either 1.5 or 4 h. Additionally, Fe^{2+}-induced lipid peroxidation was investigated using linoleic acid and lecithin as lipid sources. Various concentrations of trilobatin solutions were combined with lecithin or linoleic acid solution and then $FeSO_4$ solution was added. The lecithin mixture was placed at 37°C for 40 min and the linoleic acid mixture was kept in darkness for 24 h. PBS instead of induction solution (Cu^{2+}/H_2O_2, AAPH, or $FeSO_4$ solution) was used for the control group. And trilobatin solutions were replaced by PBS in the induction group. The thiobarbituric acid method was used to determine the extent of lipid peroxidation (Sevgi *et al*., 2015). The samples were mixed with 20% TCA and 0.08% TBA. The mixture was bathed in boiling water for 30 min, instantly cooled, and then centrifuged at 6000 r/min for 10 min. The supernatant was separated and its absorbance at 532 nm was measured by spectrophotometer (Shimadzu UV-2600, Kyoto, Japan). The degree of lipid peroxidation was calculated according to the following formula:

$$Degree\ of\ lipid\ peroxidation(\%) = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100\%$$

where, A_1 is the absorbance of the sample group, A_2 is the absorbance of the induction group, and A_0 is the absorbance of the control group.

Determination of DNA Oxidative Damage

The protective role of trilobatin against DNA oxidative damage was investigated using pBR322DNA. Firstly, pBR322DNA was mixed with trilobatin solution and bathed in 37°C water for 30 min. Then pBR322DNA was oxidized using AAPH in the presence or absence of trilobatin at 37°C for 4 h. The degree of oxidative damage of DNA was detected by agar-gel electrophoresis (Tampo, 2000). Briefly, a certain amount of agarose was added into 20 mL TAE buffer to obtain 1% agarose gel. The

dissolved agarose was mixed with 2 μL ethidium bromide and poured into the electrophoresis tank. After solidification, the treated samples were added for agarose gel electrophoresis using Bio-Rad ChemiDoc XRS (120 V, 30 min). In the end, the GelDoc-XRS gel imaging system (Bio-Rad, USA) was used to acquire images, and Image J software was used to assess the grayscale of the image band.

Statistical Analysis

At least three runs of each experiment were completed. The data were analyzed using a one-way Analysis of Variance (ANOVA) and represented as the mean ± Standard Deviation (SD) using IMB SPSS statistics 2.0 software. The Least Significant Difference (LSD) test at a level of 0.05 was used to ascertain the differences between the mean values of the samples.

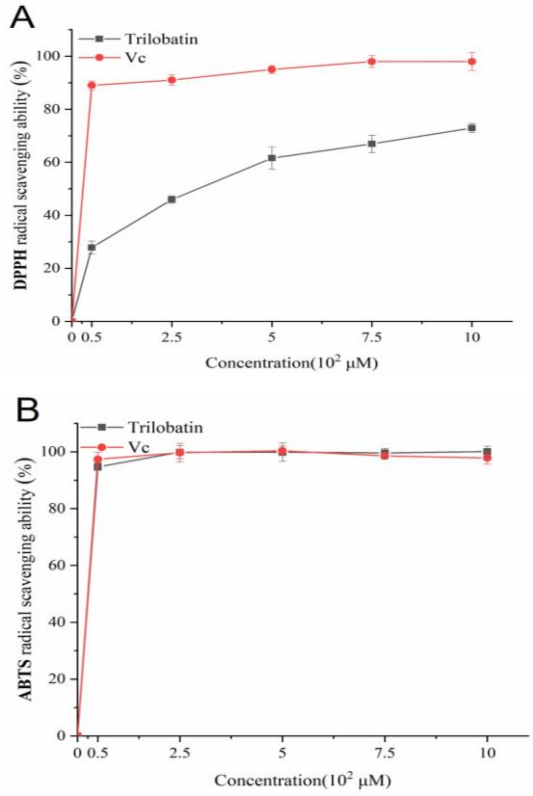
Results and Discussion

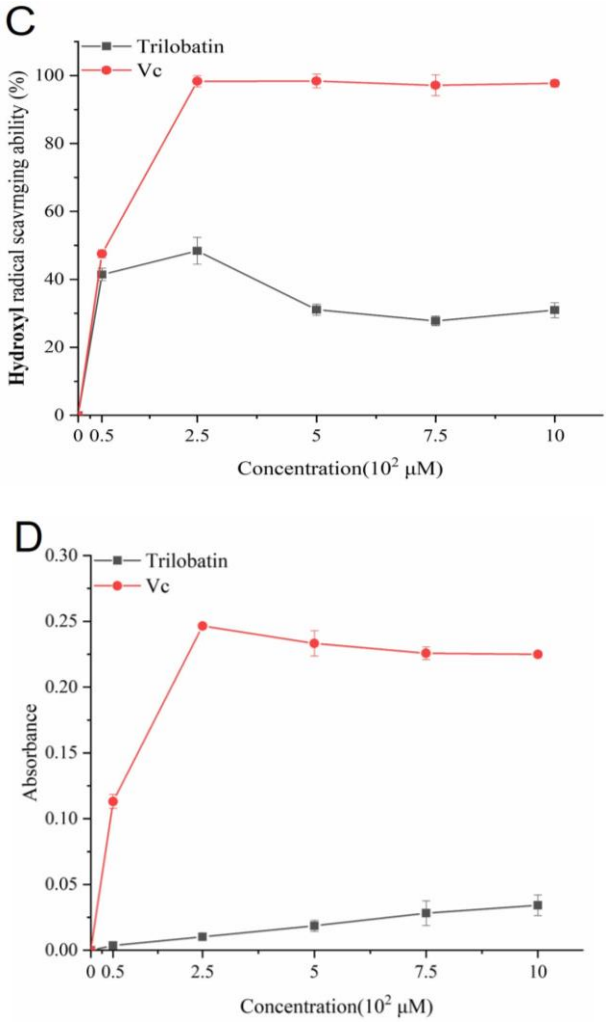
Effects of Trilobatin on Antioxidant Activities

The antioxidant properties of natural active substances are frequently assessed using DPPH, ABTS, and hydroxyl radical scavenging activities. In this research, free radical scavenging activities were determined to analyze the antioxidant potential of trilobatin *in vitro*. Figure 1A demonstrates the DPPH scavenging activity of trilobatin. According to the findings, trilobatin exhibited clear DPPH radical scavenging action dependent on concentration, the higher the concentration, the stronger the clear effect. At a concentration of 1000 μM, the DPPH scavenging ability of trilobatin was 72.97%. As presented in Fig. 1B, within the range of experimental concentration trilobatin showed the same strong ABTS free radical scavenging ability as Vc. Previous research showed that phloretin also had significant DPPH and ABTS radical scavenging activities (Lu *et al*., 2023). The similar effects of trilobatin and phloretin on DPPH and ABTS free radicals might be due to their similar structures. The scavenging ability of trilobatin on hydroxyl radical rose initially before declining as its concentration was raised and the hydroxyl radical scavenging rate peaked at 48.4% when the concentration of trilobatin was 250 μM (Fig. 1C). Reducing power is another important mean to evaluate the antioxidant property. Figure 1D, the ferric reducing powers of trilobatin were concentration dependent. These results indicate that trilobatin has a strong *in vitro* antioxidant capacity.

Effects of Trilobatin on Oxidative Damage of Protein

In this study, two induction systems were used to study the effects of trilobatin on oxidative damage of proteins including BSA, brain tissue protein, and liver tissue protein of SD rats. Figure 2A-B, SDS-PAGE findings showed that AAPH or Cu^{2+}/H_2O_2 reduced the band gray of BSA ($p<0.01$), which suggests that hydroxyl radical and alkoxy radical damaged BSA by oxidation (The more obvious the banding, the lower the degree of oxidative damage). However, trilobatin preincubation prevented oxidative damage to BSA induced by AAPH in a concentration-dependent way Fig. 2A. Moreover, Fig. 2B revealed that trilobatin pretreatment greatly decreased the oxidative damage to BSA (50-500 μM), however, the protection of trilobatin on BSA was reduced when its concentration was greater than 500 μM, indicating that the high concentration of trilobatin had pro-oxidation effect on BSA in the Cu^{2+}/H_2O_2 induction system ($p<0.01$). Previous studies also reported that high concentrations of several antioxidants, such as aloin and green tea polyphenols, had a prooxidant effect on BSA in Cu^{2+}/H_2O_2 induced system (Forester and Lambert, 2011; Naqvi *et al*., 2010). It can be seen that some antioxidant substances change their activity from antioxidant to prooxidant when their concentration or physiological conditions are changed. However, the mechanism behind this remains unexplored. As can be seen from Fig. 2C-F, AAPH or Cu^{2+}/H_2O_2 substantially raised the oxidative level of brain and liver tissue proteins compared to the control groups ($p<0.01$). The band gray levels of tissue proteins induced by AAPH or Cu^{2+}/H_2O_2 increased significantly after pretreatment with trilobatin, indicating the obvious protective effect of trilobatin on tissue proteins.





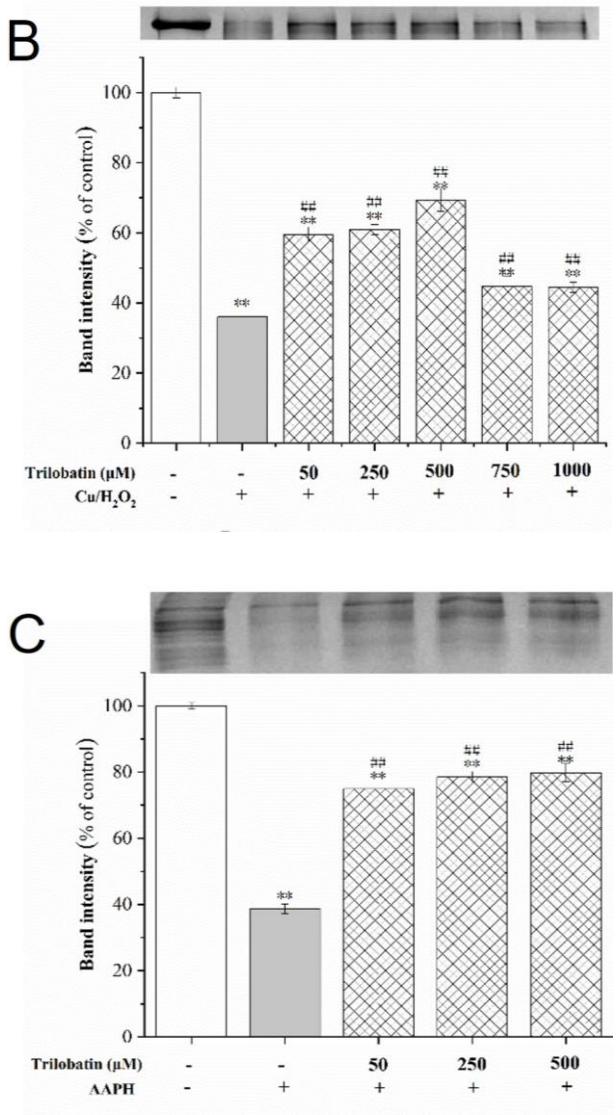
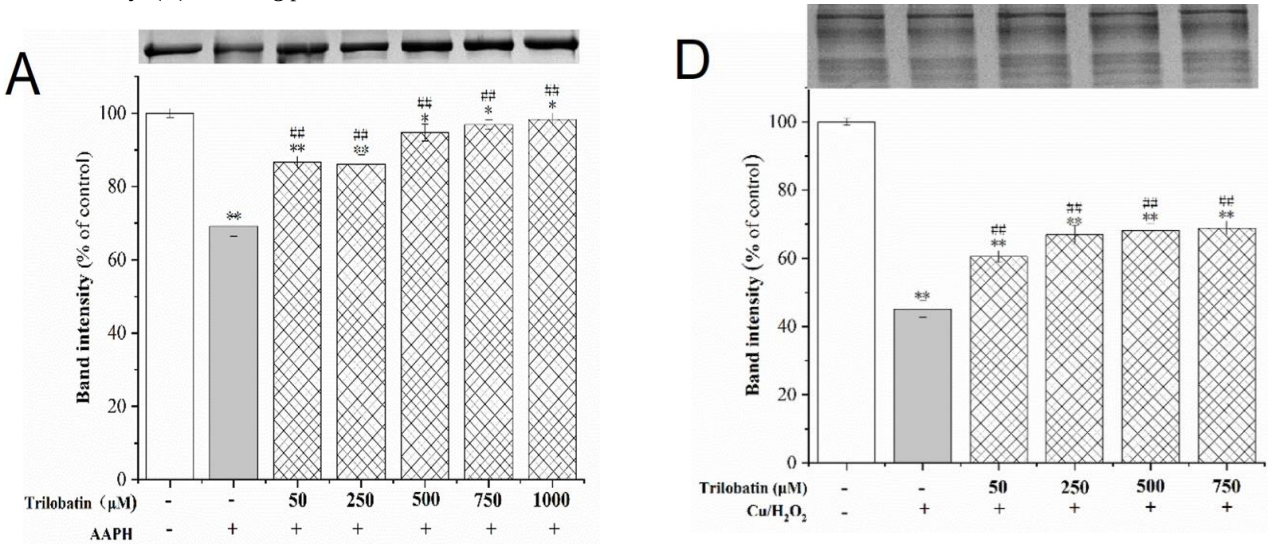


Fig. 1: Antioxidant activities of trilobatin *in vitro*; (A) DPPH free radical scavenging activity; (B) ABTS free radical scavenging activity; (C) Hydroxyl radical scavenging activity; (D) Reducing power



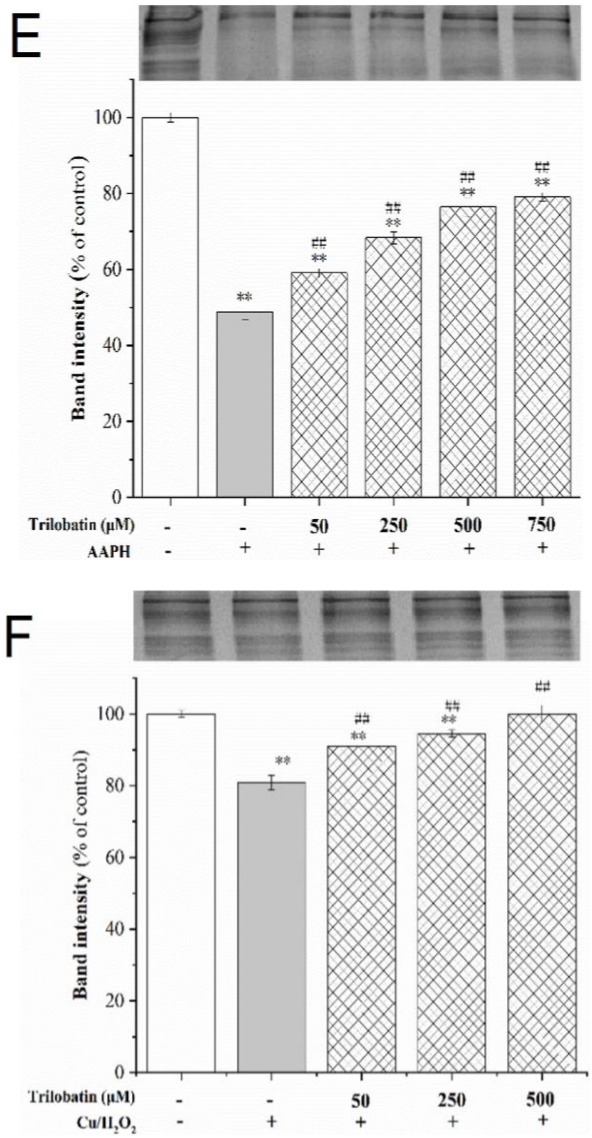
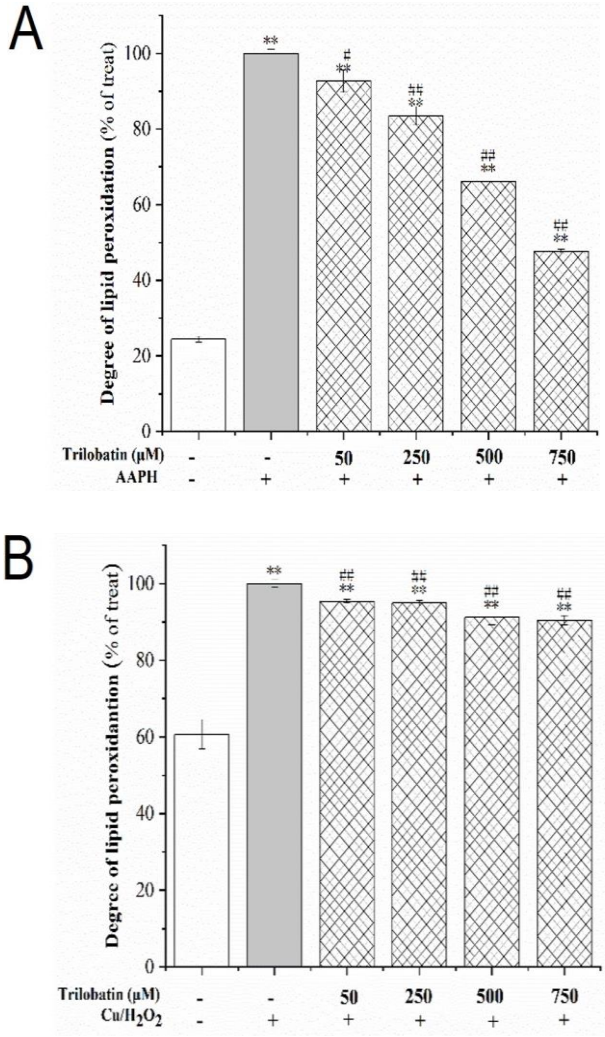
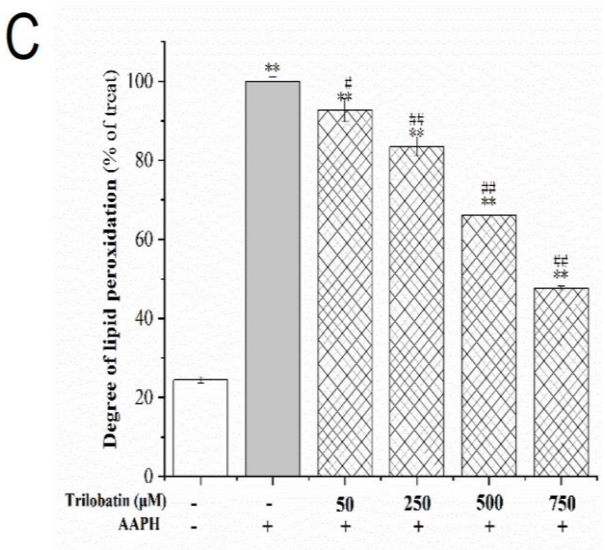


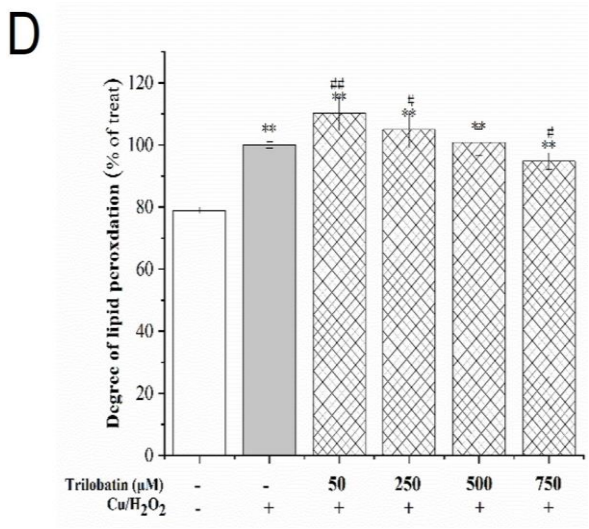
Fig. 2: Effects of trilobatin on protein oxidative damage; (A) AAPH; (B) Cu^{2+}/H_2O_2 induced BSA oxidative damage; (C) AAPH; (D) Cu^{2+}/H_2O_2 induced brain tissue protein oxidative damage; (E) AAPH; (F) Cu^{2+}/H_2O_2 induced liver tissue protein oxidative damage. (-. inexistent; +. existent. Compared with the control group, *. The difference was significant ($p<0.05$), **. Difference was extremely significant ($p<0.01$); compared with the treatment group, #. The difference was significant ($p<0.05$), ##. The difference was extremely significant ($p<0.01$). Similarly hereinafter

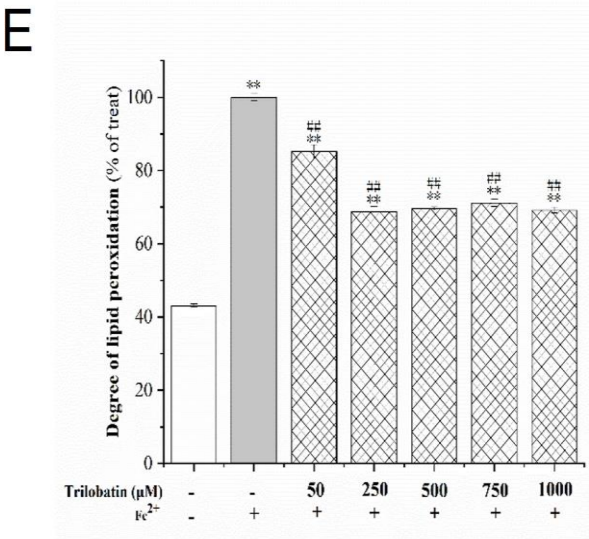
Effect of Trilobatin on Lipid Oxidative Damage

The effects of trilobatin on lipid oxidative damage were shown in Fig. 3. Figure 3A-B illustrated that the addition of AAPH or Cu^{2+}/H_2O_2 to the rat brain homogenates dramatically exacerbated the lipid peroxidation in contrast with the control group ($p<0.01$). However, the lipid peroxidation level was inhibited by the incubation of 50-750 μM trilobatin and the impact of inhibition increased with trilobatin concentration ($p<0.01$ or $p<0.05$). Figure 3C-D, trilobatin significantly reduced the lipid peroxidation of rat liver homogenates induced by AAPH or Cu^{2+}/H_2O_2 in a concentration-dependent manner ($p<0.01$ or $p<0.05$). According to the findings, trilobatin had a protective effect on lipid peroxidation in rat brains and liver homogenates. One of the important mechanisms of cell and tissue damage is lipid peroxidation and metal ions are involved in many lipid peroxidation initiation processes (Repetto *et al*., 2010). In this research, the addition of Fe^{2+} can cause obvious oxidative damage to lecithin and linoleic acid. However, preincubation of trilobatin (50-1000 μM) significantly inhibited lecithin and linoleic acid peroxidation induced by Fe^{2+} (Fig. 3E-F) ($p<0.01$). All the results showed that trilobatin effectively prevented lipid oxidation damage.









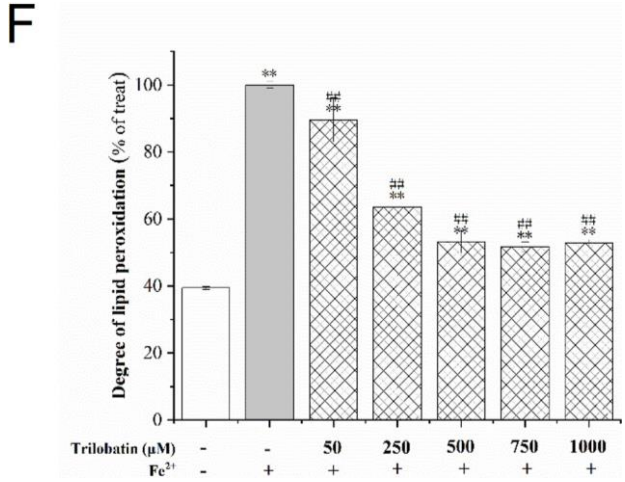


Fig. 3: Effects of trilobatin on lipid oxidative damage; (A) AAPH; (B) Cu^{2+}/H_2O_2 induced brain homogenate; (C) AAPH; (D) Cu^{2+}/H_2O_2 induced liver homogenate. Fe^{2+} induced; (E) lecithin; (F) linoleic acid liposomes peroxidation

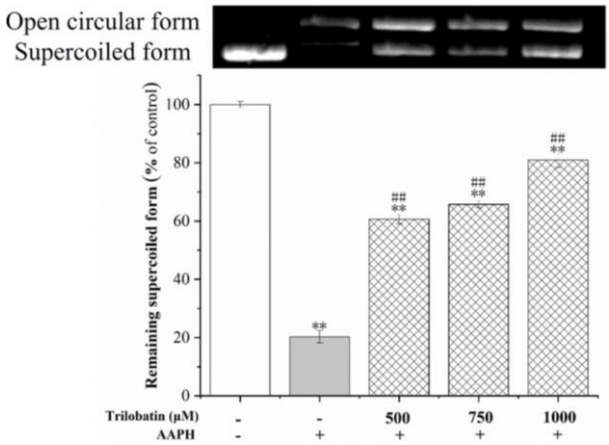


Fig. 4: Effect of trilobatin on DNA oxidative damage induced by AAPH

Effects of Trilobatin on Oxidative Damage of DNA

Excessive free radical accumulation can also lead to DNA oxidative damage in the form of DNA strand breakage, base modification, site mutation, double strand aberrant, and so on (Poetsch, 2020). Figure 4 demonstrates the effects of trilobatin on AAPH-induced pBR322DNA oxidative damage. Figure 4, most pBR422DNA structures in the control group were in the form of a superhelical structure. After treatment with AAPH, the proportion of pBR322DNA in ring-opening form increased significantly, suggesting that AAPH can cause oxidative damage to pBR322DNA by destroying its superhelix structure. The number of superhelical pBR322DNA increased significantly in a concentrate-

dependent manner after treatment with 500-1000 μM of trilobatin ($p<0.01$). Therefore, trilobatin inhibited the oxidative fracture of DNA induced by AAPH.

Conclusion

Relevant studies have shown that natural antioxidants are particularly important for clearing excessive free radicals in the human body and maintaining the equilibrium between antioxidant stress and oxidation in organisms. Our findings showed that trilobatin has significant scavenging activities on DPPH and ABTS free radicals. Trilobatin has a weak reducing power and the scavenging ability of hydroxyl radical was enhanced with the increase of the concentration of trilobatin in 0-250 μM. When the concentration of trilobatin was higher than 250 μM, the scavenging ability of hydroxyl radical began to decrease gradually. In several induction systems, trilobatin has varying protective effects on oxidative damage to biological macromolecules such as proteins, lipids, and DNA. Moreover, trilobatin displayed pro-oxidative potential at high concentrations. This research clarified the antioxidant activity and the protective effect of trilobatin on biomacromolecules. Theoretical support was given for further research and application of trilobatin in functional foods. Further research should be carried out to discuss the prooxidant effect of trilobatin thoroughly.

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Author's Contributions

Yuhan Zhai: Participate in the whole process of experimental design, experimental process, result analysis and finally write the manuscript.

Yuqing Zhang, Yaping Li and Ming He: Participated in part of the experiment.

Shuyan Yu: Participate in the part of the process of experimental design, experimental process and result analysis.

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

Shaoxuan Yu and Yuanda Song: Contributed to the guidance of experimentally designing.

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. This experiment followed the guide for the care and use of laboratory animals adopted by the Shandong University of Technology.

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