Research article

Effect of procyanidins on lipid metabolism and inflammation in rats exposed to alcohol and iron

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ABSTRACT

Background: Lifestyle involving uncontrolled alcohol consumption coupled regularly with red meat and other iron sources has detrimental effects on the liver, which in the long term, results in Alcoholic Liver Disease (ALD). Procyanidin has lately garnered increasing attention and has become the focus of research owing to its antioxidant properties. This study explores the anti-inflammatory effects of procyanidins, in preventing ALD, by analyzing the biological activities of the compound on liver injury caused by excessive alcohol and iron.
Method: Male SPF Wistar rats were placed in 4 groups; the control Group A (basic diet); the model Group B (excess alcohol 8–12 mL/kg/d and iron 1000 mg/kg diet); the low dose procyanidin Group C (model group diet plus 60 mg/kg/d of procyanidin); and the high dose procyanidin Group D (model group diet plus 120 mg/kg/d of procyanidin). Serum biochemical markers for liver damage were measured spectrophotometrically. The NFκB and IκB mRNA expression levels were determined using RT-PCR; the NFκB p65 and IκB protein expression levels were assessed via western blotting, while ELISA was used to detect serum inflammatory factors.
Results: The pathological score of the model Group B, low and high dose procyanidin Groups C and D were $6.58 \pm 0.90, 4.69 \pm 0.70$ and 2.00 ± 0.73, respectively (P < 0.05). The results showed that high alcohol and iron contents in the model group led to significant damage of liver structure, increased low-density lipoproteins (LDLs), steatosis, and increased levels of inflammatory cytokines. High amounts of procyanidins led to the preservation of the liver structure, production of high-density lipoproteins, and reduction in serum inflammatory cytokines while also significantly decreasing the expression levels of NFκB p65.
Conclusion: The results prove that procyanidins have hepatoprotective potential and could be effective in reversing histopathology, possibly by alleviating inflammation and improving lipid metabolism.

1. Introduction

Jacques Masquelier was the first person who began intensive research on procyanidins in the 1940s when he investigated the pine bark brew used by Native Americans to treat Scurvy. The researcher determined that monomeric proanthocyanidins were the main components that gave the brew its healing properties, while also being safe for consumption [1, 2]. Procyanidins, molecular formula $C_{30}H_{26}O_{13}$, are polyphenols that are homo-oligomeric (epi)catechin having two B-ring hydroxyl groups and are built from (−)-epicatechin and flavan-3-ols (+)-catechin [1]. The compound can be categorized into A-type, which has an interflavan bond

and an ether linkage between the carbon-2 and the hydroxyl group of the A-ring, while B-type only has a single interflavan bond connecting the carbon-4 of the B-ring to the carbon-6 or carbon-8 of the C-ring [3].

Procyanidin, a natural health food, is commonly found in plants such as vegetables, fruits, legumes, grains, and nuts. Foods such as red wine, grapes, berries, chocolate, cranberry juice, and certain varieties of apples are known to contain large amounts of procyanidins [4, 5, 6]. Procyanidins, also known as condensed tannins, have drawn much attention for their hepatoprotective effect at the microscopic level [7] and potent activities like inhibiting levels of NO, PGE2, TNF-α, and ROS, and altering NFκB and activating IκB [8, 9]. They have been under intense

investigation by researchers as they exhibit cardioprotective, anti-oxidant, anti-cancer, anti-inflammatory, and anti-diabetic properties [6, 10, 11]. Their medicinal properties have seen their increased use in dietary supplements and alternative medicines [12].

The harmful effect of alcoholism counts for annually three million global causalities representing 5.3% of all deaths [13]. As the liver is the primary location for alcohol metabolism, it sustains the highest degree of damage [14]. Chronic alcoholism leads to alcohol-related liver disease, which is characterized by the development of hepatic steatosis, alcoholic hepatitis, fibrosis, cirrhosis, and may eventually lead to end-stage liver disease [15]. The early sign of heavy drinking is the build-up of fat in the liver, known as steatosis [15]. This simple steatosis leads to inflammation followed by fibrosis, which may eventually result in further damage as cirrhosis [16]. Alcoholic fatty liver is a reversible condition, fibrosis is variable, but cirrhosis in most cases is a sign of permanent damage [17].

The liver also plays a noteworthy role in iron homeostasis, a process that maintains plasma iron levels within a specific range [18]. High iron intake causes the activation of hepatocytes, which produce hepcidin that ensures homeostasis [19]. More than one-half of the cases with advanced ALD and one-third of the alcohol-dependent subjects exhibit high liver iron content [20], as alcohol-induced oxidative stress down-regulates hepcidin production [21, 22]. This increased iron buildup in the liver is associated with greater mortality from alcoholic cirrhosis, indicating a pathogenic role for iron in alcohol-related liver disease [22].

In the liver tissue, alcohol metabolism and high iron deposits are involved in the generation of reactive oxygen species (ROS) and oxidative stress development [23, 24], causing changes in lipid metabolism and inflammatory factors [25, 26]. Thus, drinking alcohol while eating iron-rich foods can have detrimental effects on one's health status as the two substances mediate and promote oxidative stress and hepatic fibrosis, revealing the close relation between hemochromatosis and excessive alcohol consumption [27], this means that these two substances, help in facilitating ALD development.

For determining whether procyanidin, a powerful antioxidant, can be used to rectify liver damage in patients with ALD and its effectiveness in preventing ALD, our study aimed to explore their positive effect on lipid metabolism and inflammation in rats with alcohol and iron-induced hepatic injury.

2. Materials and methods

2.1. Reagents

All reagents used in our research were of analytical purity. Grape seed procyanidin extract (GSPE), was obtained from Xi'an Tianxingjian Natural Biological Products Co., Ltd. China. At the same time, Iron(II) sulfate heptahydrate (FeSO$_4$.7H$_2$O) was purchased from Shanghai Maclean Biochemical Technology Co., Ltd. China, and ethanol was obtained from Sinopharm Chemical Reagent Beijing Co., Ltd. China.

2.2. Animal treatment and experimental design

Male SPF Wistar rats (n = 51, weighing 150–160 g) were bought from Shandong Lukang Pharmaceutical Co., Ltd. The animals (Certificate of Quality No. SCXK (Lu) 20140007) were provided humane care in compliance with international standards for the care and management of Laboratory Animals (Laboratory Animal Resources Institute, Life Science Commission, National Research Council, 1996). The research was approved by the Animal Ethics Committee of Qingdao Medical University. The animals (6 weeks old) were housed in a standard environment (25 ± 1 °C temperature, 55 ± 5% humidity, and 12/12 h natural light/dark cycle) with unlimited rodent chow and water access.

After acclimatizing the rats for three weeks, they were assigned randomly to four groups: A, Control Group (n = 11) was provided basic diet (iron content of 50 mg/kg) and normal saline; B, Model Group (n = 12) was provided with iron-rich food (1000 mg/kg) while administering 50% v/v ethanol (8 ml kg^{-1} d^{-1} for 2 weeks + 12 ml kg^{-1} d^{-1} gavage for 10 weeks); C, Low-dose Procyanidin Treatment Group (n = 14) and D, High-dose Procyanidin Treatment Group (n = 14) were given the same model group diet rich in alcohol and iron content, while also being administered procyanidin aqueous solution of 60 mg kg^{-1} d^{-1} and 120 mg kg^{-1} d^{-1}, respectively. They were kept under strict observation.

After 12 weeks under sterile conditions, the animals were anesthetized with 30 mg/kg sodium pentobarbital. For serum lipid analysis, blood samples were drawn from the abdominal aorta. Liver biopsies were taken, and the tissue samples were divided into two portions; one treated for pathological and the other for biochemical analysis. The remaining samples were frozen in liquid nitrogen and stored at -80 °C.

2.3. Liver index calculation

Formula for liver index calculation: Liver index (%) = liver mass/body mass × 100.

2.4. Preparation of liver tissue for pathological examination

Formalin-fixed liver tissues (0.9 × 0.9 × 0.5 cm) were paraffin-embedded and stained with hematoxylin-eosin (HE) to assess inflammation and steatosis, as previously described [28, 29]. Pathological observation and photography were performed using an Olympus BX60 multi-function microscope. Liver pathology scoring was done as previously described by Yin et al [30].

2.5. Preparation of liver tissue for ultrastructural observation with transmission electron microscopy (TEM)

For ultrastructural observation, as previously described [28], a portion of tissue was fixed with 2.5% glutaraldehyde for 24 hrs at 4 °C for pathological analysis (1 × 1 × 3 mm) and then washed with 0.1M cacodylate buffer, dehydrated with ethanol, fixed with 1% citric acid, dehydrated with acetone, embedded with epoxy resin (EPON812). Then the ultra-thin sections (50–70 nm) were placed on grids, stained with uranyl acetate and lead citrate, and then observed in a TEM.

2.6. Preparation of serum and liver tissue for detection of serum enzyme indices and liver metabolic indicators

Blood samples were collected into sodium citrate monovette for measuring biochemical markers such as serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transpeptidase (GGT). These markers, along with serum and liver tissue TG, TC, LDL, and HDL were measured spectrophotometrically by the automatic biochemical analyzer (AU5400, Beckman, USA) according to the kit instructions, as described previously [31].

2.7. Preparation of liver tissue for detection of NFκB and IκB expression levels

2.7.1. Detection of NFκB and IκB mRNA expression levels in liver tissue
RNA extraction, cDNA synthesis, and RT-PCR were done as previously published methods [29, 32].

2.7.1.1. Primer synthesis. Specific primers of NFκB and IκB mRNA were designed by the literature search. The gene sequences and the specificity of each primer sequence were compared by the NCBI BLAST gene pool. Shanghai Shenggong Bioengineering Co. Ltd. synthesized the primers with β-actin as the internal reference. Listed in Table 1 are the primer sequences.

Table 1. Primer sequences.

Gene	Sequence	Product length (bp)
β-actin	Upstream: CCTAGACTTCGAGCAAGAGA	140
	Downstream: GGAAGGAAGGCTGGAAGA	
NFκB p65	Upstream: TCTGTTTCCCCTCATCTTT	155
	Downstream: TGGTATCTGTGCTTCTCTC	
IκB-α	Upstream: ATGGAAGTGATTGGTCAGGTGA	184
	Downstream: AGGCAAGATGGAGAGGGGTATT	

2.7.1.2. Extraction of sample total RNA. Tissue was homogenized with liquid nitrogen, then incubated with Trizol, and allowed to stand for 5–10 min at room temperature. Again 0.2 ml chloroform was added and mixed vigorously for 15–30s. It was then allowed to stand for 3 min and centrifuged at 12,000 rpm for 15 min at 4 °C. The aqueous phase containing the RNA was transferred to a new EP tube, 0.5 ml isopropanol was added, mixed, kept in an ice bath for 10 min, and centrifuged at 4 °C, 12,000 rpm for 10 min. The aqueous phase was transferred to a fresh EP tube again, precipitated with 75% ethanol in ice, centrifuged for 5 min at 12,000 rpm at 4 °C. It was then dried and treated with DEPC to dissolve RNA, then RNA concentration was detected and stored at -80 °C. Electrophoresis of 5 μL of RNA was done on 1% agarose gel to detect RNA integrity.

2.7.1.3. Reverse transcription synthesis of cDNA. TIANScript RT instructions were followed strictly. Preparation of RT reaction was done by combining the following components in a sterile microcentrifuge tube: 1μg total RNA, 2 μL Oligo (dT)$_{15}$, 2 μL Super Pure dNTP and RNase-Free ddH$_2$O to make a volume of 14.5 μL. The mixture was heated at 70 °C for 5 min and cooled rapidly on ice for 2 min. Briefly centrifuged with the addition of the following components: 0.5 μL 5X First-Strand Buffer (including DTT), 0.5 μL RNasin and 1 μL TIANScript M-MLV; and mixed gently, with incubation at 25 °C for 10 min, 42 °C for 50 min, followed by heating at 95 °C for 5 min. The reaction system was diluted to 50 μL with RNase-Free ddH2O and stored at -20 °C.

2.7.1.4. Real-time PCR reaction. The NFκB and IκB mRNA were amplified by the target gene primer and the reference gene primer by fluorescence quantitative PCR, and the dissolution curve was analyzed at 60–95 °C. The experiment was carried out in strict accordance with the product specifications. The reaction system was prepared by mixing 10μL of SuperReal PreMix Plus, 0.6μL of upstream primer (10 μM), 0.6μL of downstream primer (10 μM), 100ng of cDNA, 0.4μL of ROX Reference Dye and RNase-Free ddH$_2$O to make a volume of 20μL. The PCR amplification procedures included initial denaturation at 95 °C for 15 min, followed by 40–50 cycles at 95 °C for 10 s, 58 °C for 30 s, and 72 °C for 30 s. The samples were heated gradually from 72 °C to 95 °C to obtain melting curves and the fusion temperature of amplicons. See Supplementary Figure 1 for amplification and dissolution curves.

Relative quantitative analysis of the data was performed using the $2^{-\Delta\Delta Ct}$ method. The formula is as follows: the ratio of the target gene of the intervention group to the target gene expression of the control group $= 2^{-\Delta\Delta Ct}$, $\triangle\triangle Ct = (Ct_1 - Ct_2) - (Ct_3 - Ct_4)$,

Ct_1: Ct value of the gene of the intervention group sample;

Ct_2: Ct value of the internal reference gene of the intervention group sample;

Ct_3: Ct value of the target gene of the control group;

Ct_4: Ct value of the internal reference gene of the control group.

2.7.2. Western blot analysis

The western blot analysis was done according to a published method [33]. Proteins were extracted from liver homogenates by SDS-polyacrylamide gel electrophoresis and then transferred onto a PVDF membrane (Millipore, Bedford, MA) for 1 h at room temperature.

The membranes were blocked with TBST containing 10% skimmed milk for 2 h at room temperature and then probed with the primary antibody against NFκB p65 (1:1000) (Santa Cruz Biotechnology, California, US), IκB (1:1000) (Santa Cruz Biotechnology, California, US), β-actin (1:5000) (Easybio, Beijing, China), and Histone H3 (1:5000) (Easybio, Beijing, China) overnight at 4 °C. After three 10-minute washes in TBST, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (1:10000) at room temperature for 60 min. The membranes were washed with TBST three times. The protein bands were then visualized with an enhanced chemiluminescence detection kit (Beyotime, Shanghai, China). The blots were analyzed by scanning densitometry with a GS-700 imaging densitometer and Quantity One software. The optical density ratio of the target proteins to their internal reference proteins was used as the relative expression of the target protein.

2.8. ELISA method for detection of serum inflammatory factors

Serum TNF-α, IL-6, IL-10, and IL-4 were detected strictly following the kit (USCN KIT Inc., Hubei, China) instructions, as previously reported [34].

2.9. Statistical analysis

Statistical analysis was performed on SPSS 17.0 software. ANOVA was used to compare multiple groups, expressed as (x̄±S), the test level α = 0.05. P < 0.05 was considered statistically significant.

3. Results

3.1. Liver index of rats in each group

There was a statistically significant difference in the liver index when comparing the high dose procyanidin Group D to the model Group B and low dose procyanidin Group C (P < 0.05) (Supplementary Table 1).

3.2. Effect of procyanidins on pathology (H&E) and ultrastructure (TEM) of rat liver tissue

H&E staining of the control Group A showed normal lobular structure, orderly hepatic cord, absence of steatosis. In the model Group B, alcohol and iron exhibited liver damage such as Mallory body, fat vacuoles, inflammatory infiltration, hepatocyte hyaline degeneration, and microvesicular steatosis. Alcohol and iron significantly increased liver pathology score in the model Group B as compared to the control Group A. In the different procyanidin Groups C and D, the liver pathology score was improved, the structure of the hepatic lobules was also damaged, but the degree of hepatocyte necrosis was significantly reduced in comparison with the model Group B. In the low dose procyanidin Group C, fat droplets were found in the cytoplasm of the hepatocytes, the hepatic cord was irregularly arranged and showed inflammatory cell infiltration. In high dose procyanidin Group D, steatosis was reduced, and hepatic histopathological changes improved significantly, hepatic cord arrangement and tissue structure were normal. High dose procyanidin improved liver

pathology score and steatosis markedly as compared to low dose pro-
cyanidin. The pathological score of the model Group B, low and high dose
procyanidin Groups C and D are 6.58 ± 0.90, 4.69 ± 0.70, and 2.00 ±
0.73, respectively (P < 0.05) (Figure 1A) (Supplementary Table 2).

Hepatic ultrastructure of the control Group A showed hepatocytes
without droplet fat, intact nuclear membrane, oval or round nuclei, clear
nucleolus, clear ridge structure, normal mitochondrial morphology and
rough endoplasmic reticulum, abundant ribosomes, capillary bile ducts,
and tight junctions were in the cell boundary when compared to the
model Group B. The model Group B showed the hepatic cells with
irregular shape and increased droplet fat, mitochondria showed defor-
mity with swelling, the nuclear membrane was fuzzy or irregular, ridge
structure was fuzzy, lysosomes increased, and the rough endoplasmic
reticulum showed disorganization, swelling, and fracture. In the low dose
procyanidin Group C, the mitochondrial lesion decreased, and the
number of mitochondria increased, the degree of rough endoplasmic
reticulum breakage and disorder was improved, and the number of ly-
sosomes and lipid droplets decreased. In high dose procyanidin Group D
as compared to the model Group B the morphology of liver cells was
normal, with the absence of fat droplets in the cytoplasm, the nuclear
membrane remained intact, the mitochondria were nearly normal, with
reduced pathological changes, while the disordered and degraded rough
ER improved (Figure 1B).

3.3. Effect of procyanidins on liver function and lipid metabolism in rats

Serum marker activity of liver functions showing levels of ALT, AST,
and GGT in the model Group B were increased significantly in compar-
ison with the control Group A (P < 0.05). The procyanidin Groups C and
D showed a dose-dependent decrease in ALT, AST, and GGT levels. Model
Group B showed an escalation in the plasma level concentrations of TG,
TC, LDL, and a decrease in HDL. Plasma lipid levels were not improved in
low dose procyanidin treatment. High dose procyanidin reduced TG, TC,
LDL, and increased HDL, suggestive of improved hepatic and blood lipid
levels (P < 0.05) (Figure 2) (Supplementary Table 3 and 4).

3.4. Effect of procyanidin on NFκB, IκB protein and mRNA expression levels in rat liver tissue

Compared to model Group B, procyanidin treated Groups C and D had
significantly reduced mRNA expression levels of NFκB (P < 0.05). The
expression level of IκB mRNA in the procyanidin Groups C and D
significantly increased (P < 0.05), as compared to the model Group B.
The western blotting results revealed, NFκB p65 protein expression level
was decreased, and IκB protein expression level was increased in pro-
cyanidin Groups C and D as compared to the model Group B (P < 0.05).
Procyanidins in a dosage-dependent manner affect protein, and their
expression levels (Figure 3) (Supplementary Table 5 and 6).

3.5. Effect of procyanidins on serum inflammatory factor levels in rats

The concentration of cytokines TNF-α, IL-6, IL-4, and IL-10 were
measured and found to be elevated in the model Group B. TNF-α and IL-4
concentrations increased in the low dose procyanidin Group C compared
to the control Group A, but no significant difference compared to the
model Group B. The concentrations of IL-6 and IL-10 in low dose Group C
significantly increased as compared to the control Group A and decreased
comparatively with the model Group B. High dose procyanidin treated
Group D showed significant improvement in cytokine levels as compared
to the model Group B, signifying the anti-inflammatory effect of pro-
cyanidins (P < 0.05) (Figure 4) (Supplementary Table 7).

4. Discussion

The outcome of the study highlights that procyanidins, members of
the procyanidin class of flavonoids, are effective in reversing the adverse
effects of high alcohol consumption and dangerous levels of iron in the
liver, which are responsible for causing ALD. These beneficial effects of
procyanidin are obvious from the following improvements: decreased
histopathological damage, suppressed serum biochemical indices,
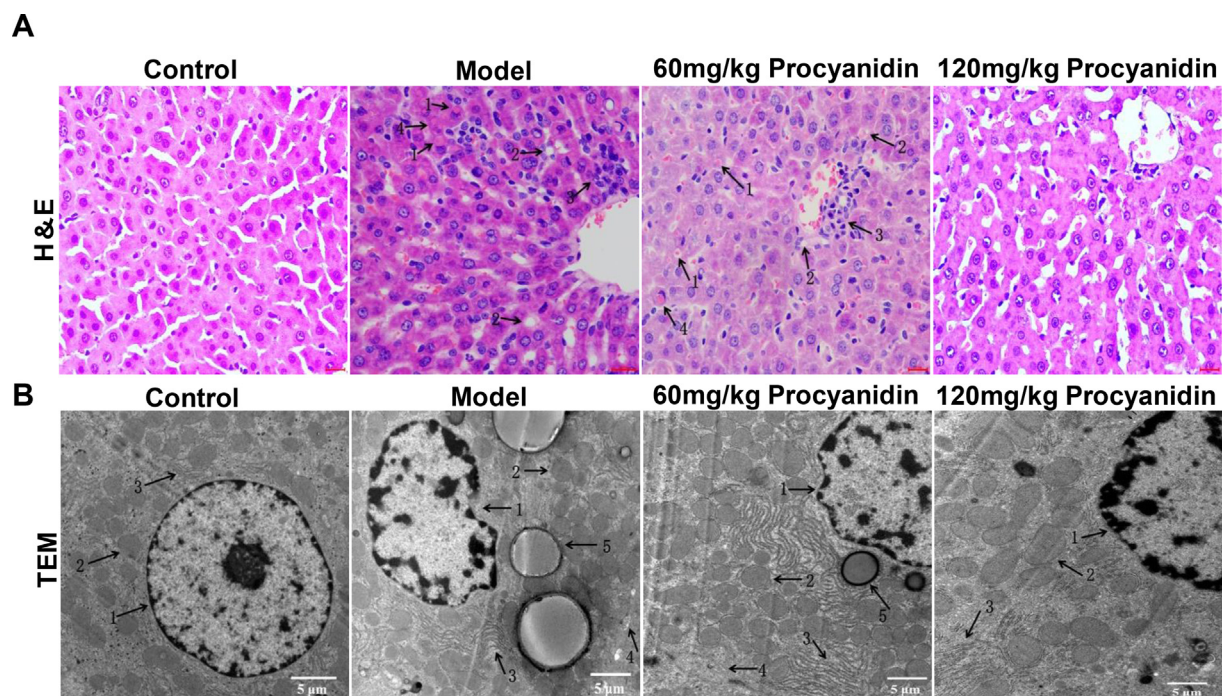improved serum lipid profiles, attenuated serum inflammatory cytokines,

Figure 1. Effect of procyanidins on pathology and ultrastructure of rat liver tissue. (A) Representative histological sections of H&E stained rat liver tissue at 12 weeks in each group. 1: Mallory body; 2: Fat Vacuoles; 3: Inflammatory Infiltration; 4: Hepatocyte Hyaline Degeneration. (B) Ultrastructure of rat liver tissue at 12 weeks in each group. 1: Nucleus; 2: Mitochondria; 3: Rough Endoplasmic Reticulum; 4: Capillary Bile Duct; 5: Lipid Droplets. Scale bar = 50 μm for A, 5 μm for B.
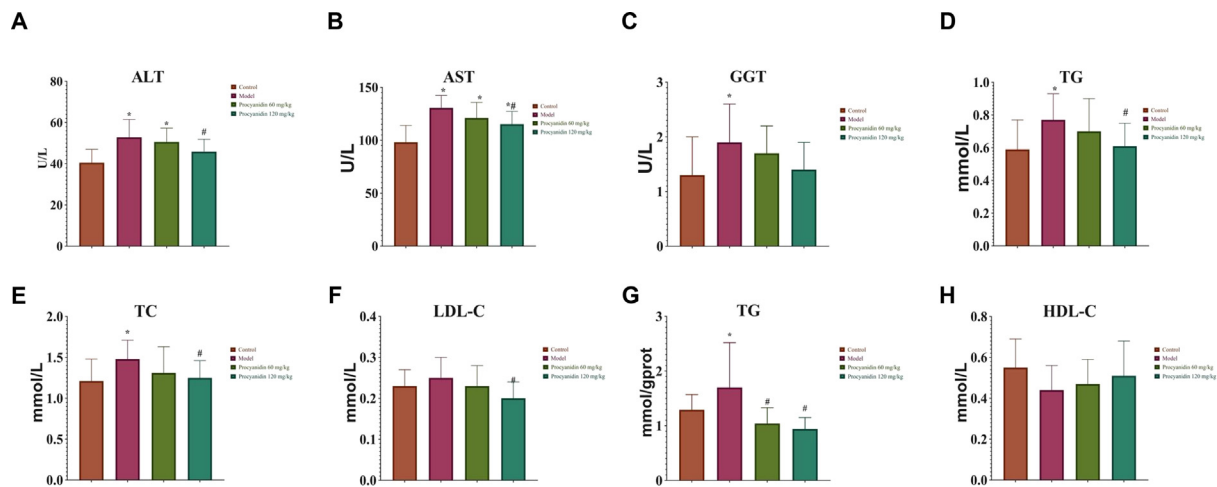
Figure 2. Effect of procyanidins on liver function and lipid metabolism in rats. The serum lipid levels of (A) ALT, (B) AST, (C) GGT showed a significant reduction with the increased dosage of procyanidins as compared to the model Group B. The lipid metabolic indicators (D) TG mmol/L, (E) TC, (F) LDL-C, (G) TG mmol/gprot showed reduction after treatment with procyanidin, with the exception of (H) HDL-C which showed an increase in high dose procyanidin Group D (*P < 0.05 vs Control; #P < 0.05 vs Model).
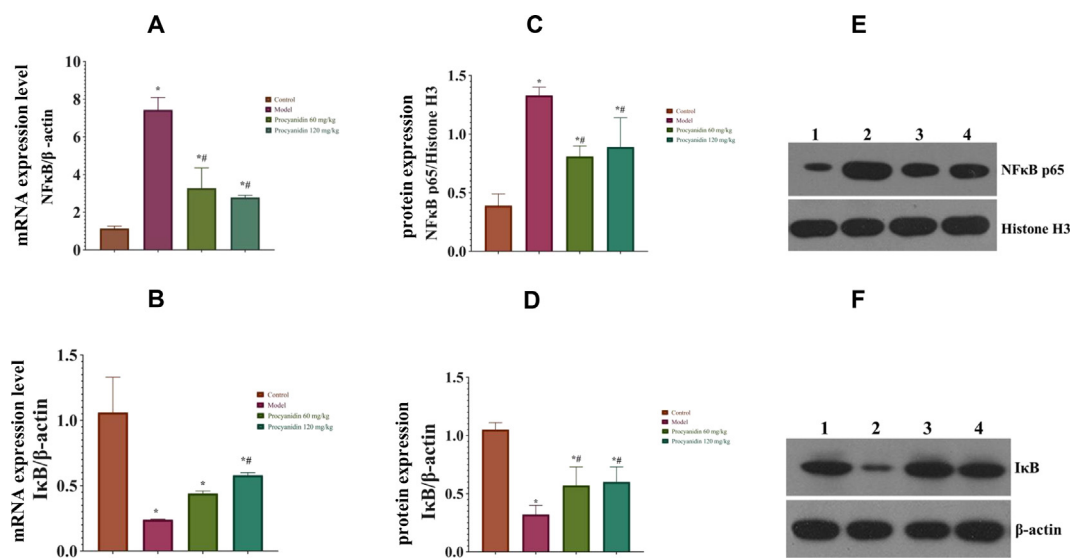

Figure 3. Effect of procyanidins on NFκB, IκB protein, and mRNA expression levels in rat liver tissue. (A) The above graphical representations show that NFκB mRNA expression is increased in the model Group B but lowered after procyanidin treatment. (B) The IκB mRNA expression levels are increased in the procyanidin treated groups. (C) NFκB protein levels increased in model Group B as compared to the procyanidin groups (D) IκB levels are increased in procyanidin treatment groups. (E) and (F) Western blot analysis was used to detect the expression levels of NFκB p65 and IκB protein in liver tissue. 1: Control Group A; 2: Model Group B; 3: Low dose procyanidin Group C; 4: High dose procyanidin Group D (*P < 0.05 vs Control; #P < 0.05 vs Model). The full, uncropped images of the western blots are presented in Supplementary Figure 2.
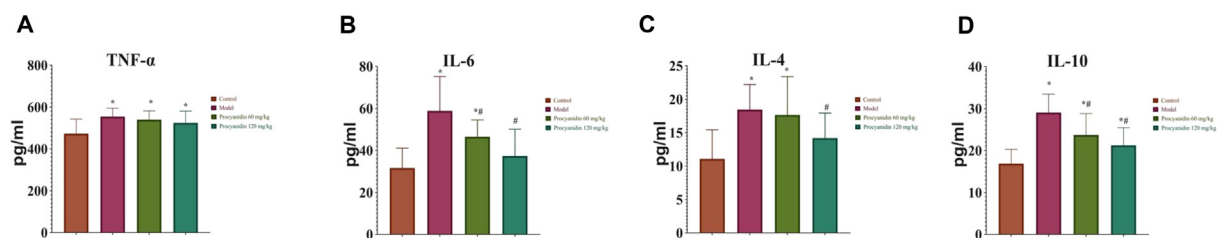

Figure 4. Effect of procyanidins on serum inflammatory factor levels in rats. (A) TNF-α, (B) IL-6, (C) IL-4, (D) IL-10 levels are reduced with the increasing dose of procyanidins as compared with the model Group B (*P < 0.05 vs Control; #P < 0.05 vs Model).

down-regulated NFκB mRNA, and NFκB p65 expressions, and up-regulated IκB mRNA expression. Procyanidin's hepatoprotective effect could be the result of its ROS scavenging nature, whose overproduction and buildup are sparked by oxidative stress, thereby alleviating hepatic injury. These findings strongly suggest that procyanidin can effectively prevent the progression of hepatic damage caused by high alcohol and iron. The result of the study correlates with previous findings on similar subjects that evaluated the effect of procyanidins on liver functions. A study by Blumberg et al. also indicated that procyanidins in cranberry juice resulted in better serum lipid profiles, glucoregulation, and improvement in the activities of serum biomarkers for inflammation control [35]. In another study, researchers confirmed the protective ability of procyanidin against hepatotoxicity in rats at the mitochondria level, owing to its antioxidant, metal chelating, and free radical scavenging properties [7].

Previous studies have shown that alcohol-iron exposure can cause histopathological changes in the liver, like hepatocyte swelling, the appearance of fat droplets, inflammatory cell infiltration, and hepatic cord derangement, reflecting the functional and morphological damage in alcohol-related liver injury [20]. Typical ALD characteristics demonstrated in Group B included the existence of Mallory bodies, fat vacuoles, inflammatory cell infiltration, hepatocyte hyaline degeneration, and microvesicular steatosis. Although there was evidence of liver damage in the procyanidin groups C and D, the degree of hepatocyte necrosis was significantly lower compared to the model Group B, as indicated by HE. However, a better recovery was observed in the high-dose procyanidin Group D, with animals exhibiting less steatosis, inflammation, and necrosis, showing that the effect of procyanidin in addressing liver damage is dose-dependent. Our analysis revealed that procyanidin intervention alleviated inflammation and fat accumulation in the liver and improved pathological injuries, the result being consistent with a previous report [36].

The high presence of iron in the liver is a major cause of oxidative stress, as research shows that it induces and increases the Fenton reaction, resulting in high production of free radicals in large amounts [37, 38]. The free radicals-antioxidant interaction is a normal process that occurs in hepatocyte cells, where the liver tries to limit the free radicals production and accumulation in the body through antioxidants [39, 40]. When the ROS production surpasses the ability of the antioxidants to neutralize them, it is referred to as oxidative stress [41]. ROS such as hydroxyl radicals, singlet oxygen, superoxide radicals, and hydrogen peroxide are produced usually by the mitochondria of inflammatory and immune cells such as the Kupffer cells and in other processes such as arachidonic acid metabolism [41]. Cells use superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) as antioxidants to defend themselves against the increased ROS in the body, they are not effective against high amounts of ROS produced when the presence of iron in the liver is high [38, 41]. The high production of ROS affects the other cell organelles, which was noted in the model Group B where the liver cells presented irregular shapes with increasing droplet fat characterized by a fuzzy or irregular nuclear membrane, swollen and deformed mitochondria, larger lysosomes, fuzzy ridge structure, with a swollen, fractured and disorganized rough endoplasmic reticulum, as indicated by TEM.

Enzyme markers, such as ALT, AST, and GGT, are widely used in animal research as indices of liver function and injury [42, 43]. The liver TG level is known to be an indicator of hepatic lipid accumulation [44]. In the current study, elevated serum ALT, AST, GGT, and liver TG levels verified the hepatic injury in rats treated with alcohol and iron. The results demonstrated that procyanidin intervention could decrease the activity levels of serum ALT, AST, GGT, and liver TG. In conclusion, procyanidin induced hepatoprotective effects in alcohol-iron induced liver injury, as indicated by liver histopathological examinations and liver function-enzyme assays.

Ioannou et al. had previously stated that chronic alcohol consumption causes increased ferritin concentration and serum transferrin saturation, and also elevated hepatic iron stores [45]. Alcohol also increases cholesterol metabolism in the liver by elevating its synthesis, absorption, and excretion while limiting the absorption of cholesterol in the intestines [31, 46]. Silva et al. found that high iron intake could cause lipid metabolism disorder in rats [47]. Alcohol and iron also cause high serum LDL concentration and proliferation of serum markers, which result in the interference of hepatocyte functions, thus making alcohol and iron, the immediate culprits of causing hepatosteatosis [48, 49]. In contrast, high levels of procyanidins are associated with the prevention of hepatosteatosis due to the downregulation of production of TG, TC, and LDL, which resulted in the preservation of liver structure and functions as found in the study.

The result of the experiment also showed that there was a marked increase in serum inflammatory cytokines TNF-α, IL-6, IL-4, and IL-10 in the model Group B. The high dose procyanidin Group D showed significant improvement in cytokine levels as compared to the model Group B signifying the anti-inflammatory effect of procyanidins [1, 8]. Research shows that ethanol-mediated microbial colonization and increase in the gut opens up the tight junctions in the intestines, facilitating the release of large amounts of endotoxins into the intestinal lumen and subsequently transported to the liver [50]. The inability of the liver to clear these high amounts of endotoxins in the blood causes them to accumulate, resulting in the activation of the immune system [51, 52]. This activation is mainly by Kupffer cells, which release large amounts of chemokines and pro-inflammatory cytokines in response to the metabolic and functional deficiencies brought about by the high presence of endotoxins [53]. Lipopolysaccharide (LPS) and other endotoxins affect the biological functions of the non-immune, immune, and parenchymal cells, and the observed inflammatory response in the experiment is usually a primary occurrence in the development of ALD.

Another observation in the experiment that can be explained using research findings is the increased level of protein expression of NFκB p65 and subsequent inhibition of IκB in the model Group B. The occurrence of this phenomenon is related to the ethanol-mediated proliferation of gram-negative bacteria in the gut, which results in the production of endotoxins. The IκB helps in the cellular reaction to inflammation and is usually phosphorylated by LPS, antigen receptors, growth factors, and cytokines through activation of the IKK complex [54]. The activity of the IκB is affected by the upregulation of NFκB p65 in the presence of high amounts of intravenous endotoxins because the latter participates in the production of cytokines, which explains why the serum inflammatory cytokines TNF-α, IL-6, IL-4, and IL-10 were observed in high amounts in the model Group B [54]. The NFκB dimer is usually kept inactive by IκB in the cytoplasm of normal cells. But when the body is invaded by pathogens, the NFκB p65 signaling pathway is activated, causing an immune response [54]. The activation of the NFκB helps in the production of antibodies that try to limit the number of endotoxins in the bloodstream. It is usually a crucial stage in ALD development as it signifies the inability of the liver to eliminate numerous endotoxins in the blood, requiring the innate immune response to help in ameliorating the conditions. In this study, high dose procyanidin Group D showed a lower NFκB activity in the liver, which is a sign of a reduction in the expression of hepatic inflammatory markers, underlining the ability of procyanidins to control liver inflammation by inhibiting the production of proinflammatory cytokines.

A study conducted by Terra et al. compared rats fed with a high-fat diet with those on a high-fat diet together with procyanidins from grape seeds. The researchers found that rats fed with only a high-fat diet had high production of C-reactive protein (CRP), while those that consumed procyanidins had lower plasma CRP levels, which is because of the downregulation of CRP mRNA expression especially in the mesenteric white adipose tissue and the liver [55]. The researchers also found out that procyanidin downregulated the expression of TNF-α and IL-6 pro-inflammatory cytokines, while increased adiponectin mRNA levels in the mesenteric white adipose tissue [55]. The study shows that procyanidin controls CRP when it is being synthesized, and in addition to the

inhibition of the expression of proinflammatory cytokines TNF-α and IL-6 and the upregulation of anti-inflammatory cytokine adiponectin the compound, can be useful in treating inflammatory illnesses [55]. The findings from this study and that from the literature underline the potential of procyanidins in lowering obesity-related adipokine dysregulation, which can be useful in managing metabolic and cardiovascular risk factors.

Researchers have also investigated the effectiveness of procyanidins in preventing oxidative stress and preserving liver integrity. In a study by Decorde et al., the researchers examined the effectiveness of polyphenolic grape seed extract (GSE) in overcoming obesity by reducing oxidative stress and addressing adipokine imbalance [56]. The study showed that procyanidins in the GSE reduced abdominal fat, insulinemia, higher plasma glucose, and leptinemia by more than 16.5% and increased the levels of adiponectin by 61% [56]. Rodríguez-Ramiro et al. determined that cocoa polyphenolic extract together with procyanidin, are effective in preventing oxidative stress caused by dietary acrylamide, by enhancing the redox status of cells and by obstructing the apoptotic pathways created by acrylamide [57]. Polyphenols such as procyanidin and epicatechin can greatly help to improve personal health.

The effectiveness of polyphenols such as procyanidins, epicatechin, phenolic acids, carotenoids, and flavonoids in preventing a myriad of illnesses such as cancers, tumors, diabetes, heart problems, liver issues, and neurogenerative disorders is due to their activities as antioxidants [56]. Antioxidants are free radical scavengers, helping to control the production of these reactive oxygen species (ROS) that are responsible for damaging small blood vessels and releasing inflammatory cytokines that result in organ damages [58]. When ROS production overwhelms the body's ability to regulate them, oxidative stress ensues a condition that adversely alters proteins, lipids, and DNA [59]. Therefore, the ability of antioxidants such as procyanidins, to control the production of ROS and reduce oxidative stress is significant in preventing diseases.

Patients with chronic ALD are usually at risk of liver failure, which can put their lives in danger when the situation is not addressed. The present study highlighted the potential of procyanidins to attenuate the debilitating effects of excess alcohol consumption and iron overload, which are the major risk factors of acquiring ALD [60, 61]. The compound can be used by individuals who are at risk of getting ALD, and in alleviating situations where prevention of liver inflammation is required to ensure the liver disease does not worsen. Procyanidins are highly abundant in plants and can be a cheaper alternative to enhancing personal health as supplements instead of relying on expensive medications. This study shows that there is great potential in using the compound to treat and prevent other ailments emanating from oxidative stress in the liver, such as diabetes and associated illnesses.

Declarations

Author contribution statement

A. Lobo: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Y. Liu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Y. Song: Performed the experiments.

S. Liu and R. Zhang: Analyzed and interpreted the data.

H. Xin and H. Liang: Contributed reagents, materials, analysis tools or data.

Competing interest statement

The authors declare no conflict of interest.

Additional information

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