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Original Article

Unraveling the mechanisms of trans-cinnamic acid in ameliorating non-alcoholic fatty liver disease

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Abstract: Background: The prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing significantly due to high amounts of fat and fructose in the diet. Phytochemicals present in herbal plants and nutrients present in food play vital roles in the management of NAFLD. One of these is trans-cinnamic acid (TCA). We are evaluate the role of TCA in NAFLD induced by a high-fat, high-fructose diet. Methodology: Rats fed a high-fat, high-fructose (HFHF) diet for ten weeks exhibited distinct signs of NAFLD. Rats were given TCA (10 mg/kg, 20 mg/kg, and 40 mg/kg) and pioglitazone (10 mg/kg) for four weeks along with a HFHF diet. At the end, body weight, food intake, liver, lipid measurements, TNF-α, antioxidants, and histopathology were evaluated. Results: TCA significantly decreased serum glutamic-oxaloacetic transaminase and glutamic pyruvic transaminase in rats. Serum cholesterol, triglyceride, and low-density lipid levels were substantially decreased in TCA-treated rats compared to diseased controls. Superoxide dismutase, glutathione, and malondialdehyde were significantly decreased in rats treated with a high dose of TCA (40 mg/kg) compared to HFFD-fed rats. HFFD-fed rats exhibited fatty liver alterations, whereas rats treated with TCA exhibited significantly fewer morphologic changes associated with fatty liver disease. TCA at a high dose exhibited decreased TNF-α levels, thereby decreasing hepatic inflammation. Conclusion: TCA proved its role in the treatment of NAFLD by substantially reducing liver enzymes, pro-inflammatory markers (TNF-α), and lipid markers. Inclusion of TCA as a therapeutic regimen alongside diet-based treatment undoubtedly has therapeutic potential in NAFLD and related diseases.

Keywords: Trans-cinnamic acid, non-alcoholic fatty liver disease, high fat-high fructose diet, TNF-α, lipid markers

Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by an abnormal accumulation of fat in liver cells. It is frequently associated with inflammation and is not the result of heavy alcohol consumption, steatogenic medicine, or genetic abnormalities. NAFLD encompasses a number of liver conditions, including hepatic steatosis, steatohepatitis, fibrosis, cirrhosis, and liver failure. It is a multisystem disease affecting numerous extrahepatic organs and regulatory mechanisms. The global prevalence of NAFLD is estimated to be approximately 25.2%. NAFLD is classified into two types: silent liver disease, which causes hepatic damage and inflammatory reactions, and non-alcoholic steatohepatitis (NASH), which causes hepatocellular damage and inflammatory reactions with or without fibrosis [1-4]. The metabolic syndrome, which encompasses obesity, type 2 diabetes, dyslipidemia, and hypertension, is intimately linked to NAFLD. Because of the increased risk of hepatocellular carcinoma and cardiovascular disease, it is associated with a high morbidity rate. NAFLD has become a public health concern in the Asia-Pacific area, with prevalence rates ranging from 11.5% in Taiwan to 32.6% in Sri Lanka, and it is believed to arise from poor dietary trends. The "fructose hypothesis" proposes a link between a higher incidence of NAFLD and related illnesses and sweetener consumption, particularly fructose. People of various ages and races are affected by NAFLD, and pediatric NAFLD is a distinct illness with an unclear natural history. Weight loss and other lifestyle modifications are effective in treating NAFLD, while medications such as lipid-lowering, insulin-sensitizing pharmaceuticals, antioxidants, and antidiabetic and

Table 1. Animal grouping details

Group 1 (NC)	Normal diet fed rats.
Group 2 (DC)	High fat-high fructose diet rats.
Group 3 (TCA 10 mg/kg)	Received high fat-high fructose diet along with TCA 10 mg/kg.
Group 4 (TCA 20 mg/kg)	Received high fat-high fructose diet along with TCA 20 mg/kg.
Group 5 (TCA 40 mg/kg)	Received high fat-high fructose diet along with TCA 40 mg/kg.
Group 6 (pioglitazone 10 mg/kg)	STD Received high fat-high fructose diet along with pioglitazone 10 mg/kg.

hypolipidemic medications can reduce the course of symptoms. In extreme circumstances, a liver transplant may be required [5-14]. For decades, indigenous populations have been treated with many types of natural products for the management of chronic diseases such as metabolic syndrome, type 2 diabetes mellitus, gastrointestinal problems, and hyperlipidemia using traditional herbal medicines [15]. Phenolic acids and their derivatives are found in many plants, including foods, fruits, and vegetables [16-18]. Plant phenolics include monomeric cinnamic acid derivatives such as cinnamic, ferulic, p-coumaric, sinapic, and caffeic acids [19]. Cinnamic acid in plants turns into p-hydroxycinnamic (p-coumaric) acid, one of the most important lignin precursors. Apples, bananas, and raspberries are high in trans-cinnamic acid, whereas p-coumaric acid is present in apple juice, onions, carrots, red wine, chocolate, crispbread, red raspberries, and strawberries [20, 21].

Cinnamic acid is extracted from cinnamon bark or benzoin as a hydroxycinnamic acid derivative. Trans-cinnamic acid (TCA) has two isomers that have been shown to have a variety of effects. Anti-diabetic, anti-cancer, anti-aging, and anti-microbial properties are among the health benefits.

Furthermore, TCA has been shown to reduce obesity by blocking adipocyte development in 3T3-L1 cells and body weight gains in high-fat diet (HFD)-induced mice [21]. In the cinnamic acid (CA)-associated study, HepG2 cells and db/db animals were treated with CA to reduce lipid formation, and ACLY, ACC, FAS, SCD1, PPAR, and CD36 were greatly downregulated, whereas CPT1A, PGC1, and PPAR were significantly upregulated [38]. With this context in mind, the current study aimed to investigate TCA's therapeutic use in high-fat, high-fructose diet-induced non-alcoholic fatty liver disease.

TCA was selected for its multifaceted therapeutic effects in reducing hepatic lipid accumulation, which are mediated through suppression of hepatic lipogenesis and fatty acid intake as well as increased fatty acid oxidation. We studied specific pro-inflammatory marker TNF-α and histopathology in NAFLD and its proposed reversal through TCA treatment.

Materials and methods

Drugs and chemicals

Loba Chemical (Mumbai, India) supplied the fructose and cholesterol. Casein was purchased from Gujarat, India. The vegetable ghee was purchased from the local market. Pioglitazone was obtained as a free sample from the pharmaceutical industry. Diagnostic kits were used to assess the SGOT and SGPT. Total cholesterol, LDL cholesterol, and triglycerides were all tested using diagnostic kits. TNF-α was assessed using an ELISA kit.

Solubility of trans-cinnamic acid

Trans-cinnamic acid was purchased from Sigma Aldrich as a white crystal powder. It is soluble only in inorganic solvents. The trans-cinnamic acid was solubilized in PEG-400 using magnetic stirrers [14].

Animal research protocol

Adult male rats weighing 200 to 250 grams were obtained from the institute's animal house facility. The animals were housed in standard control conditions, fed a regular pellet diet, and given distilled water as needed. All experimental research was carried out in compliance with the standards established by the Committee for the Control and Supervision of Experiments on Animals (CCSEA), India. 48 rats were divided into two groups after one week of acclimatization: normal diet fed (NPD) and high-fat, high-fructose diet fed (HFFD) (**Table 1**).

Table 2. Summary of high fat-high fructose diet content

Sr No.	Ingredients	Diet (g/100 g)	Role of the ingredient
1	Normal pallet diet	37.0	-
2	Vegetable ghee	25.0	Inducing hypercholesterolemia, high-cholesterol
3	Casein	10.0	Adiposity and adipose inflammation
4	Fructose	20.0	Increased hepatic fibrosis, hepatic-inflammation
5	Cholesterol	5.0	Damage to hepatocytes and activates non-parenchymal cells
6	Vitamin and mineral mix	3.0	Nutritional supplements

NPD (normal pellet diet) consisted of 65% carbohydrates, 3% fat, 18% crude protein, 5% vitamins and minerals, and 6% crude fiber, whereas HFFD-fed rats (**Table 2**) were divided into disease control, TCA-treated, and pioglitazone-treated groups. At the end of the study, the animals were starved overnight and euthanized according to the CCSEA's suggested method. Blood samples were drawn using the retro-orbital plexus method for the analysis of biochemical parameters. The liver tissue was collected to determine organ weight, oxidative parameters, and histopathology.

Histopathology

The liver tissues were preserved for 24 hours at room temperature in a 10% formalin solution. A small piece of fixed tissue was cut, and embedded in paraffin. Hematoxylin and eosin (H and E) stain was used to stain the section, which was then examined under a light microscope (10X).

Statistical analysis

The results are presented as mean ± SEM (n = 6). The analysis of variance (ANOVA) and Dunnett's test were utilized for checking the significance levels between the TCA treatments and disease control groups for all measurements.

Results

Body weight, food intake, liver index, and platelet count

Rats in the HFFD group gained more weight than those in the TCA group. Body weight in HFFD-fed obese rats was significantly decreased by TCA treatment (**Figure 1A**). In the current investigation, we found that diseased control rats consumed substantially more food than normal control rats, and TCA-treated rats showed a non-significant and dose-dependent reduction of food intake compared to their HFHF-fed counterparts (**Figure 1D**). The formation of fat pads, most noticeably in the abdomen region, caused weight gain and an increased liver index due to the HFFD. Rats fed an HFFD had a larger liver index compared to those fed a normal diet and TCA treatment groups (20 vs. 40 mg/kg), as seen in **Figure 1B**. Platelet counts were elevated due to diminished hepatic thrombopoietin (TPO) synthesis, which is a common hematologic consequence of chronic liver illness. Platelet counts were much higher in diseased rats compared to rats treated with TCA, as seen in **Figure 1C**.

Effect of trans-cinnamic acid on liver markers

The serum values of rats were recorded at the end of a 10-week experimental period. Significant elevations in SGOT and SGPT levels were observed in the diseased control group compared to normal control group rats. TCA treatment (10, 20, and 40 mg/kg) showed significant reductions in SGOT and SGPT levels in a dose-dependent manner compared to the diseased group. However, at these levels, a significant ($P<0.05$) reduction was observed only at medium and high doses of TCA (20 and 40 mg/kg) and for pioglitazone-treated rats as compared to diseased-control rats (**Figure 2A** and **2B**).

At the end of the study, serum values were measured. As evidenced by the SGOT and SGPT data, disease-control rats had significantly higher SGOT and SGPT levels than normal control rats. In comparison to the diseased group, TCA treatment resulted in dose-dependent substantial drops in SGOT and SGPT levels (at 10, 20, and 40 mg/kg); a significant result was observed at the 40 mg/kg dose of TCA treatment. Rats treated with pioglitazone differed

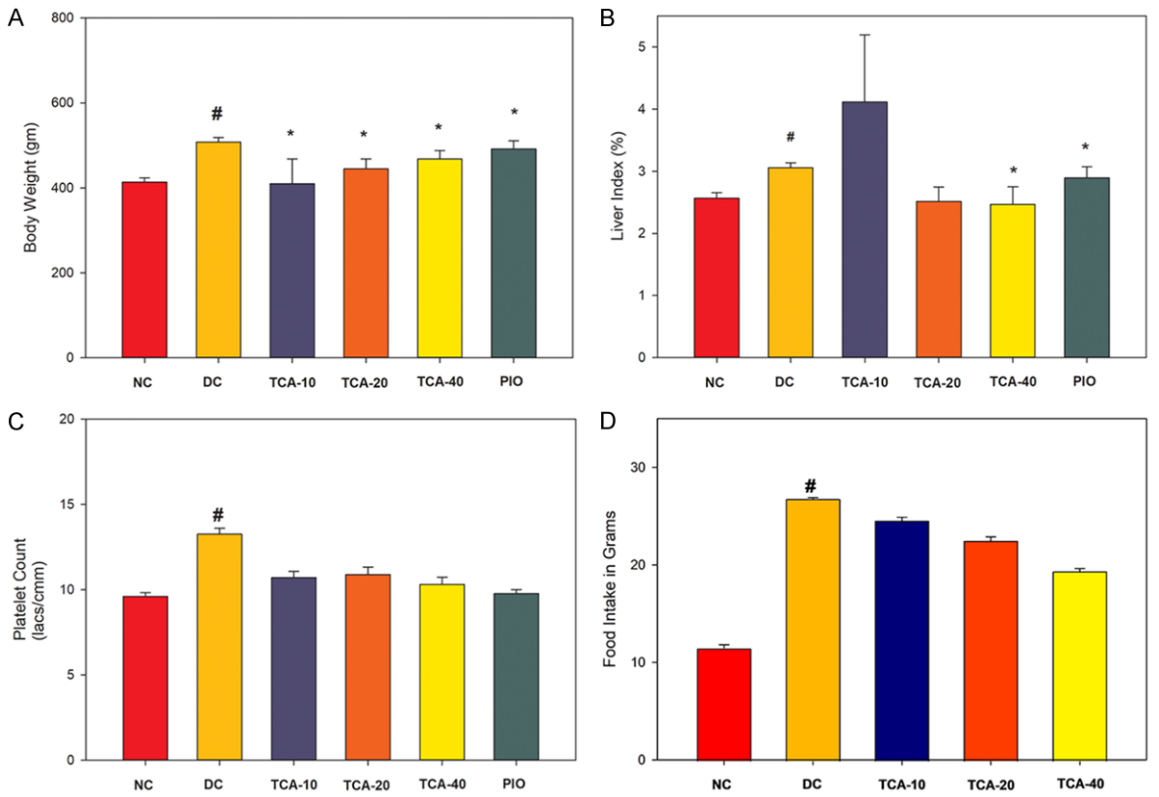

Figure 1. Effect of trans-cinnamic acid on rats' body weight change (A), liver index (liver-body weight ratio) (B), platelet count (C), and Food intake (D). NC = normal control; DC = disease control; TCA-10 = 10 mg/kg; TCA-20 = 20 mg/kg; TCA-40 = 40 mg/kg; PIO = pioglitazone (10 mg/kg). #significantly different from normal control group, P<0.05, *significantly different from disease control group, P<0.05.

from disease-control rats and showed a significant (P<0.05) reduction in SGOT and SGPT (**Figure 2A** and **2B**).

Effect of trans-cinnamic acid on lipid profile

Triglyceride (TG), cholesterol, and LDL data demonstrate that rats fed HFHF had greater TG, cholesterol and LDL level than rats fed a normal diet. However, TG, cholesterol, and LDL significantly decreased after TCA treatment. Pioglitazone-treated rats showed significantly reduced TG, cholesterol, and lower LDL levels compared to the diseased-control rats (**Figure 3A-C**).

Effect of TCA on oxidative stress markers in liver

MDA levels in liver tissue homogenate were higher in HFFD-fed rats than in normal control rats. MDA levels were decreased in TCA-treated rats when compared to diseased rats; 40 mg/kg TCA and pioglitazone-treated rats demonstrated a fall (P<0.05) in MDA levels as compared to the diseased-control rats (**Figure 4B**). Antioxidant marker SOD activity was lower (P<0.05) in the diseased-control rat liver compared to normal rats (**Figure 4A**). Rats treated with TCA (20 and 40 mg/kg) and pioglitazone had increased (P<0.05) SOD levels as compared to the diseased-control group. GSH levels were reduced significantly in HFFD-fed rats than normal rats; while TCA treated rats showed non-significant rise in GSH level (**Figure 4C**).

Effect of TCA on pro-inflammatory marker TNF-α

According to YM Yang, the hallmarks of chronic liver illness are hepatocyte loss, inflammation, and liver fibrosis. Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine that causes liver inflammation, which leads to liver fibrosis. TNF-α expression was found to be high in diseased rats in our investigation, and TCA therapy resulted in a significant reduction of TNF-α in a dose-dependent manner (**Figure 5**).

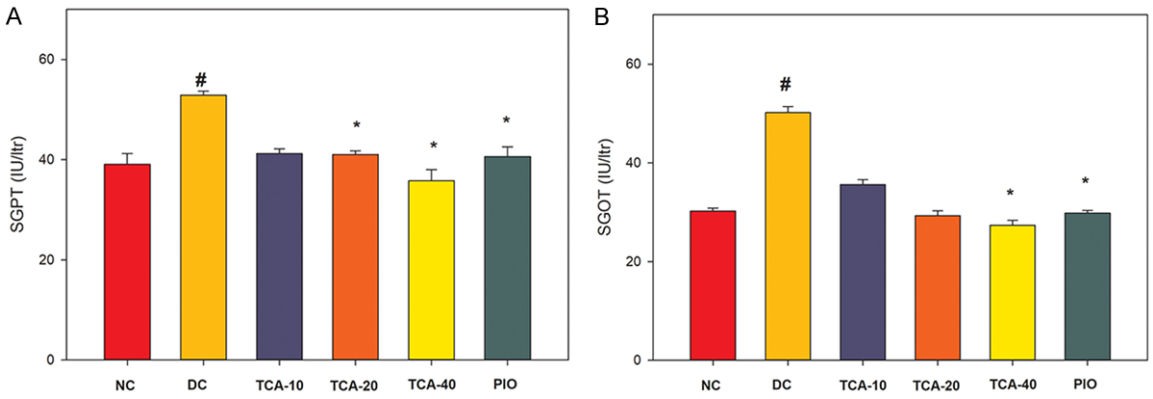

Figure 2. Effect of trans-cinnamic acid on SGPT (A) and SGOT (B) level. NC = normal control; DC = disease control; TCA-10 = 10 mg/kg; TCA-20 = 20 mg/kg; TCA-40 = 40 mg/kg; PIO = pioglitazone (10 mg/kg). #significantly different from normal control group, P<0.05, *significantly different from disease control group, P<0.05.

Histopathologic examination of liver tissue

Histopathology of the normal control group's liver tissue appeared normal (**Figure 6A**). Liver histology revealed ballooned cells, lobular inflammation, and steatosis in several regions in the HFFD-fed animals (**Figure 6B**). On the contrary, TCA supplementation significantly reduced ballooning, lobular inflammation, and low steatosis areas dose-dependently (**Figure 6C-E**). The liver histology of pioglitazone-treated rats also showed preserved normal morphology compared to diseased rats (**Figure 6F**).

Discussion

The three subgroups of phenolics - phenolic acids, flavonoids, and lignin - are a diverse and expansive class of phytochemicals that include phenol rings. Fruits such as pears, strawberries, apples, and berries generally contain polyphenols. The most common derivatives of phenolic acids are benzoic acid and cinnamic acid. Many pharmacologic actions are controlled by the phenolic moiety [22]. One scientific study revealed that the phenolic compounds found in medicinal plants, such as Ficus racemosa, had a positive impact as an anti-diabetic plant, highlighting the significance of phenolic compounds in metabolic illnesses [35-37]. The current study was the first of its kind to examine trans-cinnamic acid's effectiveness in an animal model of NAFLD.

NAFLD presents a complex challenge for developing an optimal animal model that accurately represents the course and biochemical characteristics of the illness in people. Evidence shows that dietary models are more realistic than other NAFLD animal models [23, 25]. Along with previous results of overnutrition and a sedentary lifestyle, a high-fructose diet raises the risk of developing NAFLD [25]. Portal circulation and *de novo* lipogenesis improve the distribution of fatty acids in a high-fat, high-fructose diet [26]. As a result, the liver becomes more vulnerable to subsequent events like oxidative stress and steatohepatitis, whereas steatohepatitis progression requires a lengthy period of feeding [24, 27]. The effectiveness of trans-cinnamic acid in the early stages of NAFLD was the subject of our investigation.

A high-calorie diet raises body weight and the liver index (liver/body weight ratio), per studies, as a result of the deposition of fat pads, particularly in the belly region [28]. In this study, the HFHF diet-fed groups demonstrated the same results. The current study found outcomes that were consistent with those of TCA medicine, which is known to cause a small reduction in body fat distribution. The size of the liver also increased in HFHF-fed rats, whereas it was significantly smaller in the high-dose TCA and control groups. The HFHF group consumed more food than the TCA group did. Contrarily, TCA-treated rats received more energy than untreated rats. According to Kurokawa and Ohkohchi, despite treatment showing a significant reduction in platelet count level (for a 40 mg dose level relative to DC rats), increased platelet count in blood is a common hematological complication of CLD (chronic liver disease) caused by decreased develop-

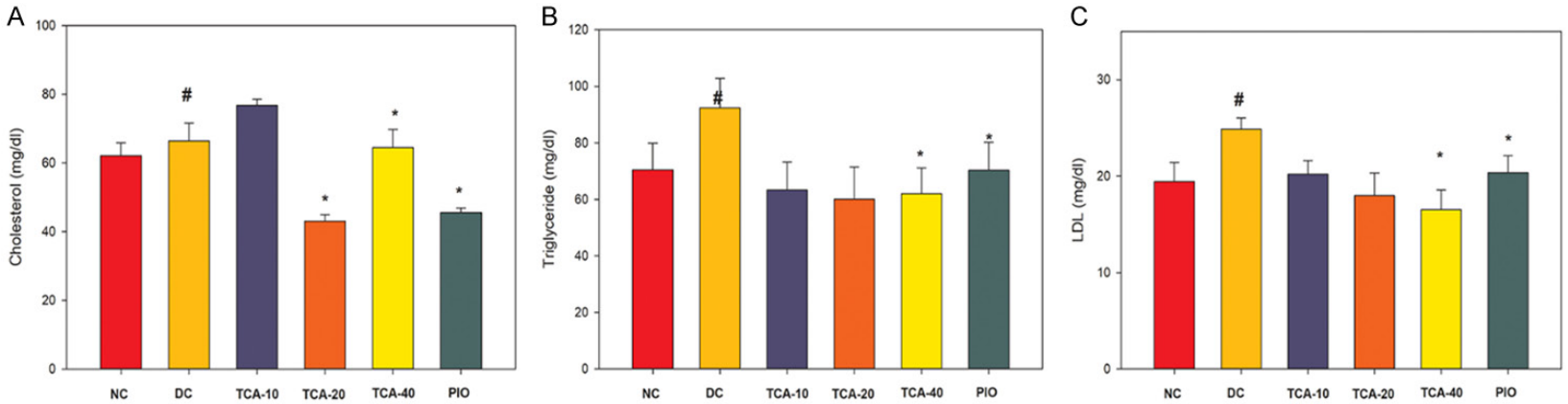

Figure 3. Effect of trans-cinnamic acid on Cholesterol (A), Triglyceride (B), LDL (C). NC = normal control; DC = disease control; TCA-10 = 10 mg/kg; TCA-20 = 20 mg/kg; TCA-40 = 40 mg/kg; PIO = pioglitazone (10 mg/kg). #significantly different from normal control group, P<0.05, *significantly different from disease control group, P<0.05.

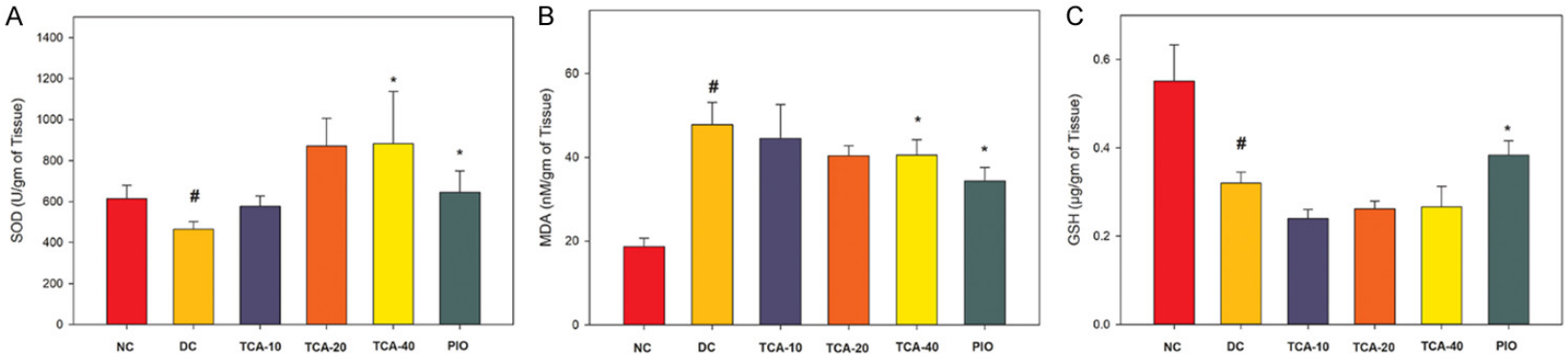

Figure 4. Effect of trans-cinnamic acid on SOD (A), MDA (B) and GSH (C) in liver. NC = normal control; DC = disease control; TCA-10 = 10 mg/kg; TCA-20 = 20 mg/kg; TCA-40 = 40 mg/kg; PIO = pioglitazone (10 mg/kg). #significantly different from normal control group, P<0.05, *significantly different from disease control group, P<0.05.

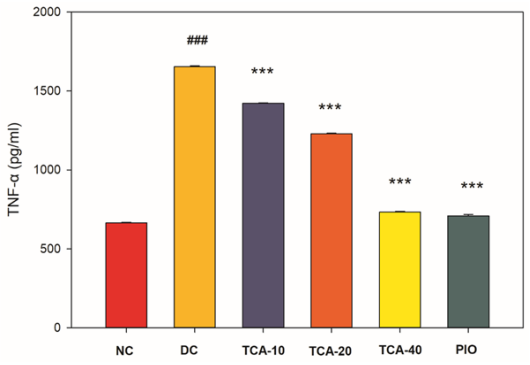

Figure 5. Effect of Trans-Cinnamic acid on serum TNF-α level. NC = normal control; DC = disease control; TCA-10 = 10 mg/kg; TCA-20 = 20 mg/kg; TCA-40 = 40 mg/kg; PIO = pioglitazone (10 mg/kg). ###significantly different from normal control group, P<0.05, ***significantly different from disease control group, P<0.05.

ment of the hormone thrombopoietin (TPO) in a weak liver and is significantly increased in diseased animals.

Hepatic function is correlated with serum ALT and AST levels, and liver injury, whether acute or chronic, results in elevated serum concentrations of both enzymes. The levels of ALT and AST were highest in the HFHF group, but TCA therapy significantly reduced each of these enzymes in the other groups. Our results are in line with an earlier *in vivo* investigation where TCA was shown to be helpful in reducing elevated ALT and AST levels in a rat model of acetaminophen-induced liver injury [29]. The results show that TCA has a significant in vivo liver protective effect, especially in NAFLD. Despite the fact that the cause of NAFLD is yet unknown, fat accumulation, particularly hepatic TG filtration, is thought to be the first step in the disease's development. This liver fat storage is caused by an imbalance of TG and FFA [30]. Fructose is a lipogenic chemical that, as was already mentioned, raises hepatic TG levels by generating DNL substrates [31]. The study found that TG, TC, HDL, and LDL values were modestly elevated in untreated NAFLD rats. In comparison to controls, TCA therapy in NAFLD rats led to significantly lower levels of TG, TC, HDL, and LDL. The TCA-treated population's serum lipid indicator levels were found to be somewhat lower than those of the control group.

Lipid dysregulation in HFHF diet-fed rats was associated with oxidative stress development in hepatocytes, which increases the development of illness. According to the progressive cycle theory, inflammation, metabolic disease, and ROS generation are all involved in the advancement of NAFLD [31]. Lipid dysregulation was linked to the activation of oxidative stress in hepatocytes in rats on an HFHF diet, which accelerates the progression of illness. The progressive cycle theory for NAFLD development assumes that inflammation, metabolic disease, and ROS generation are present [32]. ROS-induced oxidative damage results in the production of MDA as a byproduct. In the current investigation, liver tissue homogenate from rats given an HFHF diet had higher concentrations of MDA and lower concentrations of GSH, SOD, and CAT. The liver is shielded against free radicals by the enzymes GSH, CAT, and SOD, which also show when the liver is detoxifying. The disease control group had lower GSH levels, which is consistent with other studies' findings that fructose-fed rats had worse antioxidant defense activity [33]. The results of this experiment show that antioxidant therapy can reduce the symptoms of NAFLD. As free-radical terminators (antioxidants), plant phenolic compounds play a crucial role.

TCA includes phenolic compounds, according to the results of the phytochemical screening test. Rats given a high dose of TCA displayed considerably increased antioxidant defense activity when compared to the disease control group. One study found noticeably elevated antioxidant status; however, therapy with *Ficus racemosa* extract reduced high antioxidant levels and was successful in treating NAFLD [35], which supported the findings of our study.

As a key pathogenic driver of NAFLD, TNF-α has been identified. TNF-α receptor-1 (TNFR1) signaling is primarily responsible for TNF-α mediated liver injury, whereas TNFR2 mediates protective pathways [33, 34]. The etiology and development of NAFLD have been connected to the activation of pro-inflammatory cytokines like TNF-α in adipose and liver tissues. Patients with NASH reported greater serum TNF-α levels compared to those with simple steatosis, which is associated with insulin resistance. Additionally, elevated TNF-α and TNF-receptor (TNFR1) expression were linked to disease activity and fibrosis stages in the liver tissues of NASH patients [34]. In numerous diet-induced or genetic NAFLD models, TNF-α or TNFR-deficient mice displayed improved insulin

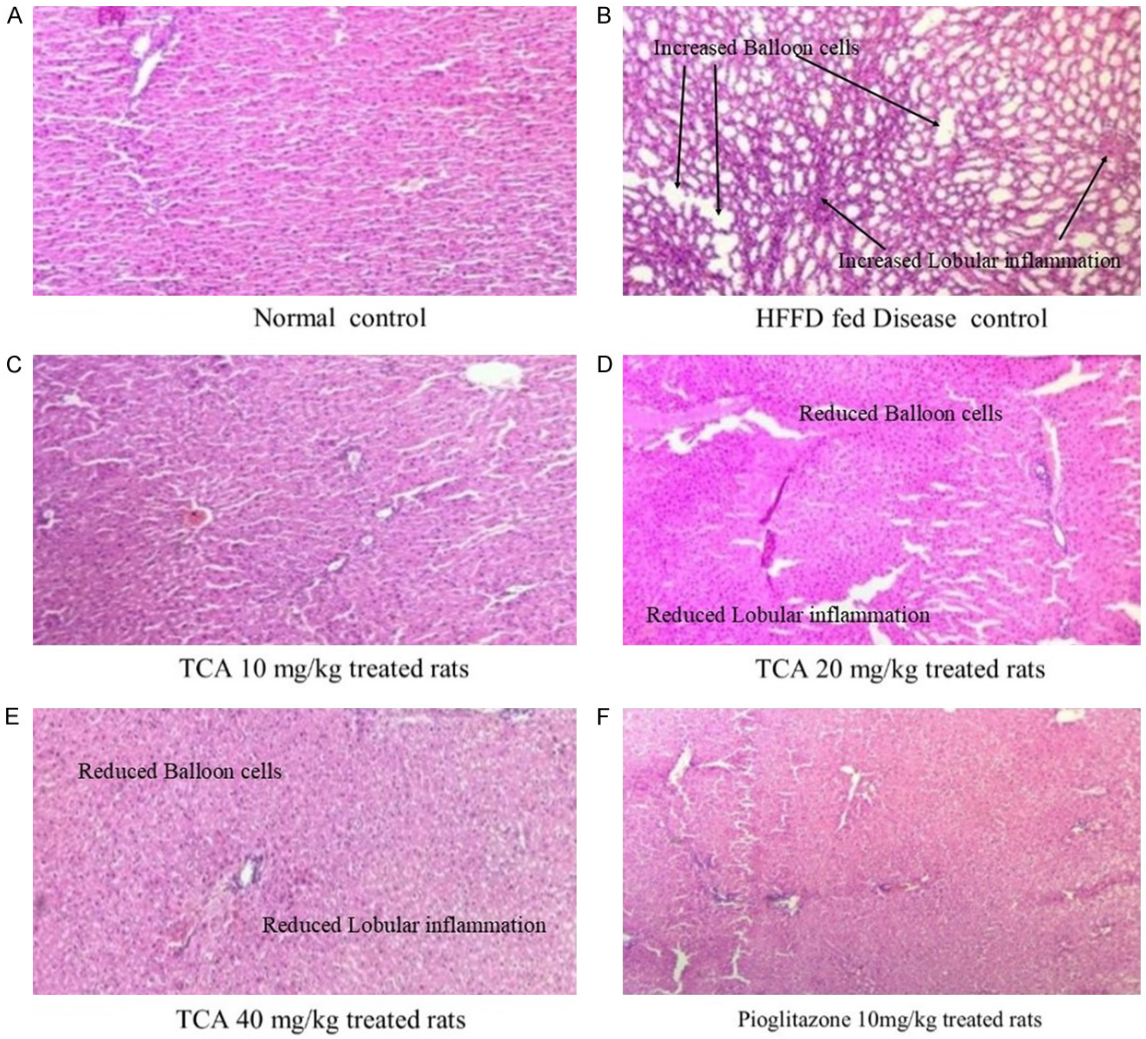

Figure 6. A-F: Histopathology of liver tissue in different groups (H/E staining of liver tissue with magnification of 10× in light microscope of Carl Zeiss).

sensitivity and less noticeable liver steatosis and fibrosis. TNF-α has a critical role in the emergence of NAFLD [28]. In our study, TNF-α expression was seen in the diseased rats, while TCA therapy significantly reduced inflammation. The histologic changes and liver inflammation may be brought on by high cholesterol and liver function indicators. Hepatic architecture improvement is considered to be a crucial indicator of NAFLD improvement [34]. Reduced serum lipid and TG levels, which were found in groups that had received large doses of TCA, explain this. According to a scientific study, the HFHF diet-fed rats' liver histology analysis revealed ballooning, inflammatory areas, and varied steatosis in various parts of the liver tissue [39]. In the present study, TCA treatment significantly reduced liver steatosis, inflammation, and ballooning.

Conclusion

TCA treatment significantly lowered body weight, fat mass, and lipid levels in obese animals, with a significant reduction in liver markers along with decreased TNF-α as a pro-inflammatory cytokine as compared to diseased-control rats. These results suggested that TCA treatment can aid in reversing NAFLD caused by a high-fat, high-fructose diet associated with obesity. TCA's ability to reduce inflammation by lowering TNF-α and improving histopathological changes in liver tissue was helpful in treating NAFLD.

Study limitation

Future studies should include expression profiling of relevant marker genes using qRT-PCR and/or western blot analysis.

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Disclosure of conflict of interest

None.

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