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Anti-Inflammatory, Hypolipidemic And Antioxidant Activities Of Thymoquinone In Non Alcoholic Fatty Liver In Rats.

Reham E Masoud[1]*, and Amira S mohammed[2].

[1]Lecturer Of Clinincal Pharmacology, Portsaid University.
[2]Lecturer Of Clinincal Pharmacology, Suez Canal University.

ABSTRACT

Nonalcoholic steatohepatitis (NAFLD) is a progressive hapatic disease leading to liver cirrhosis and failure. The present study aimed to investigate the possible protective and curable effect of thymoquinone (TQ) on liver functions, lipid profile, blood sugar and blood pressure in NAFLD. Rats were divided into four study groups: normal study group received normal drinking water and fructose drinking water group F20 which drank 20% fructose in drinking water, low dose TQ group (10 mg/kg) and high dose TQ group (20 mg/kg) for 12 weeks. Fructose caused hepatic damage in the form of significant increase in liver index, liver enzymes compared to normal group. There was also significant increase in blood sugar, serum cholesterol, triglyceride, with significant decrease in HDL. There was significant increase in body weight and blood pressure. Additionally, increased oxidative stress assessed by MDA associated with significant decrease in antioxidant activity assessed by glutathione perioxidase. TNF-α as a marker of inflammation was significantly increased in F20 group as compared to normal control group. Treatment with TQ significantly improved all measured parameters. High dose was more effective, with statistically nonsignificant difference versus normal control. Therefore, we concluded a hepatoprotective effect of TQ against fatty liver and metabolic syndrome.
Keywords: Thymoquinon, non alcoholic steatohepatitis, TNF-α, MDA, glutathione perioxidase.

Corresponding author

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INTRODUCTION

Bad dietary habits and overconsumption of sugar lead to obesity, which affects not only adults but also children. Obesity and fatty liver are among the serious health problems all over the world [1]. Fatty liver not only caused by consuming alcohol but there are many other causes as increasing body weight, type 2 diabetes mellitus I, hyperlipidemia, and hypertension, and all the causes other than alcohol are called nonalcoholic fatty liver disease (NAFLD) [1]. Although patients with NAFLD can lead a normal life, it can cause morbidity and mortality [2].

The prevalence of NAFLD is about 75–100 % in obese individuals and type-II diabetes [3]. The pathogenesis of NAFLD consists of accumulating triglyceride in hepatocytes, then developing oxidative stress and progression to steatohepatitis [4]. There are increased oxidative stress in liver cells, increasing lipid peroxidation, and toxic substances accumulation as malondialdehyde (MDA) and excess iron [5].

Drinking soft drinks leads to increase in fructose consumption therefore increasing energy intake from 3.9% to 9.2% [6]. Fructose increases hepatic lipogenesis and stimulates production of triglyceride [7]. Hence, increasing consumption of fructose mostly causes formation of macrovesicular and microvesicularsteatosis [8]. Consumption of drinking water containing 20% of fructose for eight weeks in rats caused increasing body weight and increasing body mass index[9].

Fatty liver may progress to steatohepatitis , cirrhosis and leads to advanced liver disease [10]. Several agents were proven to be of value in treatment of NAFLD for short time, but no agent was beneficial for longer period [11]. The first line management of fatty liver is lifestyle modification. Some agents like Omega 3 polyunsaturated fatty acids and statins may be beneficial. Bariatric surgery may be considered in morbid obesity [12].

No specific therapy is available for treating or preveting NAFLD till now. Therefore, complementary and alternative medicine is required *Nigella sativa* has been used extensively because of its biological properities and therapeutic potential as diuretic, antihypertensive, antidiabetic, anticancer and immunomodulatory, analgesic, antimicrobial, anthelmintics, analgesics and anti-inflammatory, spasmolytic, bronchodilator, gastroprotective, hepatoprotective, renal protective, and antioxidant properties [13].

Nigella Thymoquinone (TQ) is main component of the volatile oil from the seeds of Nigella sativa and it has been proven to have antioxidant effect in carbon tetrachloride-induced hepatotoxicity [14].Thus, in the present study, we used a model of fructose induced diabetes mellitus to help us understanding the pathogenesis of NAFLD induced by fructose and effect of TQ on prevention and control of this condition.

MATERIALS AND METHODS

Experimental animals

Male wistar rats (200-220 g) were used. . They were purchased from the Egyptian Organization for Biological Products and Vaccines (Egypt).Animals were kept and housed in polypropylene cages and kept in the standard laboratory environmental conditions; with free access to food and water *ad libitum*. Animals were left to acclimatize to laboratory conditions before starting the study. The care and handling of the animals were approved by the Animal Care and Use Committee at the Suez Canal University and were in accordance with the National Institutes of Health guide for the care and use of laboratory animals (Maryland, USA).

All drugs and reagents were purchased from Sigma chemical co. Egypt.

Animals were kept and housed in cages and kept in the standard laboratory environmental conditions; with free access to food and water *ad libitum*. Animals were left to acclimatize to laboratory conditions before starting the study. All parts of the study were done during the light period (08:00–16:00 h). All drugs and reagents were purchased from Sigma chemical co. Egypt.

Animals and Diets

Twelve male Wistar rats (200–250 grams) were included in the study . The animals were housed in cages under control conditions of temperature (20–22°C) and lighting (12 light/12 h dark). The rats were randomly divided into four groups, the control group (C) and fructose-drinking water group (F20) , fructose (F20) + small dose TQ and fructose(F)+ large dose TQ. There were six rats in each experimental group. Each group was fed with normal rat chow diet; however, they differ in water intake. Control group (C) was administered tap water while other groups were administered 20% fructose in the drinking water. The food and water intake was given *ad libitum* for 12 weeks.

Doses of TQ:

Small dose TQ (10 mg/kg for 12 weeks, by gavage) [15]
Large dose TQ (20 mg/kg for 12 weeks, by gavage) [16]

Preparation of Fructose-Drinking Water

The preparation of 20% fructose in the drinking water was similar to a past study [17]. The fructose used was D-fructose >99%.

Physiological parameters:

Body weight, blood pressure, fasting plasma glucose and fasting lipid profile, were assessed at the start of the study as baseline data and at the end of study.

Liver Enzymes

Blood samples were taken at baseline and at end of the experiment from tail vein in. Serum ALT and AST were determined enzymatically using commercially available kits

Liver index was calculated according to the following formula liver weight/body weight × 100.

Assessment of inflammatory marker

TNF-α levels in liver homogenate were measured by using ELISA (quantikine R&D system USA) kit according to the manufacturer's instructions.

Determination of MDA and GSH-Px

One small section (200 mg) of liver was removed from the rats and weighed. Subsequently, saline was added according to the tissue weight: Saline volume=1:9 (w/v). then homogenization was done at 4°C by a DY89-I electric homogenate (Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China), the homogenates were then centrifuged at 1,100xg for 15 min at room temperature., MDA content and GSH-Px (glutathione perioxidase) levels were determined by using commercially available kits, according to manufacturer's protocol.[18-19]

Histology of Liver

The liver tissues were immediately fixed in 10% formalin for three days. These tissue samples were processed using an automatic-tissue processing machine and followed by embedding in paraffin wax. Thin sections (5 μm) were obtained and stained with hematoxylin and eosin (H&E) and PAS stain. Photomicrographs of the liver sections were captured.

Statistical Analysis

All data were analysed using Statistical Package Social Sciences (SPSS) version 20 software and were presented in mean ± SEM subjected to one-way ANOVA followed by Turkey post hoc test. Value of $P < 0.05$ was taken as significant.

RESULTS

The physiological parameters measured at the beginning of the study showed nonsignificant differences between all study groups (p value >0.05), but at the end of the study; body weight, systolic blood pressure, and fasting blood sugar of fructose drinking water group(F20 group) were significantly higher than normal control group (p value<0.05) which indicates that fructose caused obesity, diabetes mellitus and hypertension. Treatment with low dose of thymoquinone caused significant reduction of all these parameters compared to F20 group, but fasting blood sugar was still significantly higher than normal, whereas high dose of thymoquinone caused significant lower level of all these measures compared to F 20 group and nonsignificant difference compared to normal indicating better control (Table 1).

Table 1: Physiological parameters of study groups at the end of the study

	FBS	Body weight	SBP
Normal control	77±8.1	290±20	100±12
F20	220±10*	350±30*	140±10*
F20+TQ (LD)	150±12*#	300±10#	100±5#
F20+TQ(HD)	96±5.5#	305±11#	90±8#

Data are the mean ± SD ($n = 6$). TQ (LD): TQ (10 mg/kg/day), TQ (HD): large dose TQ (20 mg/kg/day),F20: 20 % fructose, SBP: systolic blood pressure.

* Significantly different from normal control at $P < 0.05$ using ANOVA followed by Tukey post hoc test

\# Significantly different from F 20 group at $P < 0.05$ using ANOVA followed by Tukey post hoc test

Liver enzymes measured at the beginning of the study showed nonsignificant differences between all study groups (p value >0.05), but at the end of the study; AST and ALT level of (F20 group) were significantly higher than normal control group (p value<0.05) and significant increase of liver index which indicate increase weight of liver due to fat accumulation. Treatment with low dose of thymoquinone caused significant reduction of all these parameters compared to F20 group, but liver enzymes were still significantly higher than normal, whereas high dose of thymoquinone caused significant lower level of all these measures compared to F 20 group and nonsignificant difference compared to normal indicating better control (Table 2)

Table 2: liver parameters of study groups measured at the end of the study

	Liver index	ALT (U/l)	AST(U/l)
Normal control	0.04 ± 0.001	18.7 ± 1.4	11.4 ± 0.5
F20	0.07 ± 0.001	75.8 ± 17.5*	39.8± 4.5*
F20+TQ (LD)	0.04 ± 0.001	45.1 ± 5.6*#	26.7 ± 3.1*#
F20+TQ (HD)	0.04 ± 0.001	20.2 ± 0.5#	15.3± 1.1#

Data are the mean ± SD ($n = 6$). TQ (LD): TQ (10 mg/kg/day), TQ (HD): large dose TQ (20 mg/kg/day),F20: 20 % fructose

* Significantly different from normal control at $P < 0.05$ using ANOVA followed by Tukey post hoc test

\#Significantly different from F 20 group at $P < 0.05$ using ANOVA followed by Tukey post hoc test

Assessment of lipid profile at the beginning of the study showed nonsignificant differences between all study groups (p value >0.05), but at the end of the study; cholesterol, LDL and triglycerides level (F20 group) were significantly higher than normal control group (p value<0.05) and HDL level was significantly lower compared to normal. Treatment with low dose of thymoquinone caused significant reduction of

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cholesterol, LDL and triglycerides and increased HDL compared to F20 group, but high dose of thymoquinone caused more significant difference versus F 20 group and nonsignificant difference compared to normal group (table 3).

Table 3: lipid profile of study groups at the end of the study

	Cholesterol	TG	HDL	LDL
normal	120.1 ± 1.5	45.6 ± 2.5	49.1 ± 1.9	70.1 ± 3.4
F 20	250.5 ± 10.1*	90.0 ± 10.1*	36± 0.5*	180.8 ± 15.5*
F20+ TQ (LD)	178.4 ± 10.8*#	64.2 ± 13*#	40.6 ± 2.2*#	120.3 ±
F20 + TQ (HD)	130.5 ± 10*	60.5 ± 8.8*#	49.2 ± 0.9*	100 ± 11*#

Data are the mean ± SD (n = 6). TQ (LD): TQ (10 mg/kg/day), TQ (HD): large dose TQ (20 mg/kg/day),F20: 20 % fructose.

* Significantly different from normal control at $P < 0.05$ using ANOVA followed by Tukey post hoc test

#Significantly different from F 20 group at $P < 0.05$ using ANOVA followed by Tukey post hoc test

Assessment of antioxidant activity of the liver showed that in (F20 group) MDA and TNFα level was significantly higher than normal control group (p value<0.05) and glutathione perioxidase level was significantly lower compared to normal. Treatment with low dose of thymoquinone caused significant reduction of MDA and TNF α and increased glutathione level compared to F20 group, but high dose of thymoquinone caused more significant difference versus F 20 group and nonsignificant difference compared to normal group (fig.1).

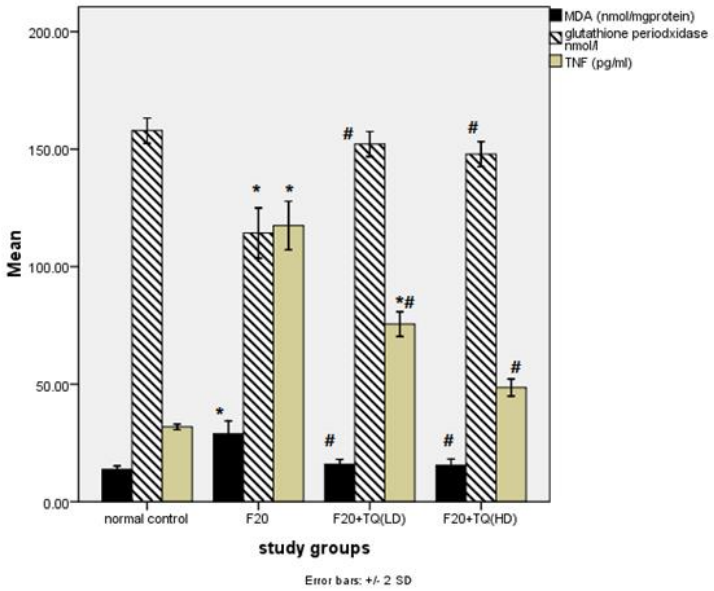

Fig 1: MDA, glutathione perioxidase level and TNFα level of study groups. Data are the mean ± SD (n = 6). TQ (LD): TQ (10 mg/kg/day), TQ (HD): large dose TQ (20 mg/kg/day), F20: 20 % fructose.

* Significantly different from normal control at $P < 0.05$ using ANOVA followed by Tukey post hoc test

Significantly different from F 20 group at $P < 0.05$ using ANOVA followed by Tukey post hoc test

Histopathological analysis of study groups (fig 2-4) showed that in F20 group, there were inflammatory infiltration, vacuolation of cells indicate fat accumualation in the cell, distortion of liver architecture. Treated groups showed improvement of all these finding.

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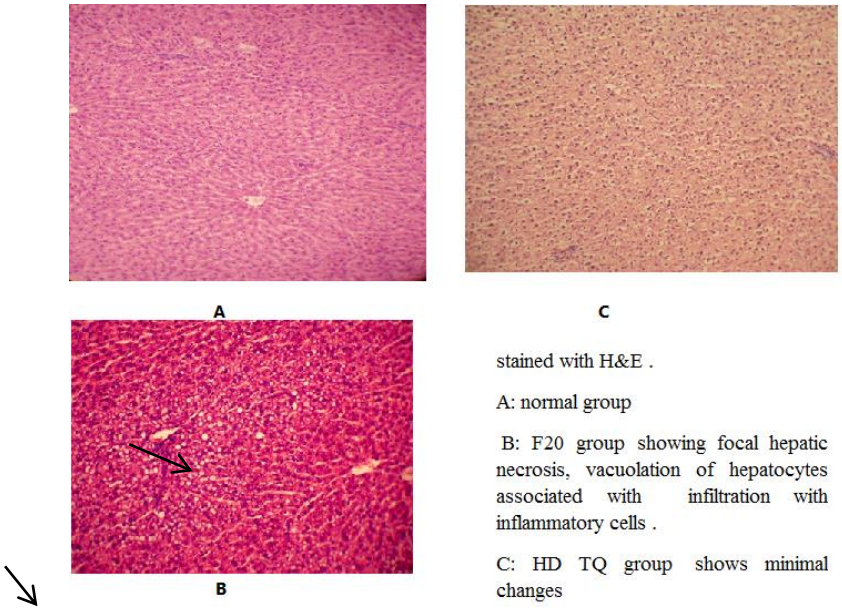


Fig 2: Photomicrographs of liver sections of study groups stained with H&E.×100

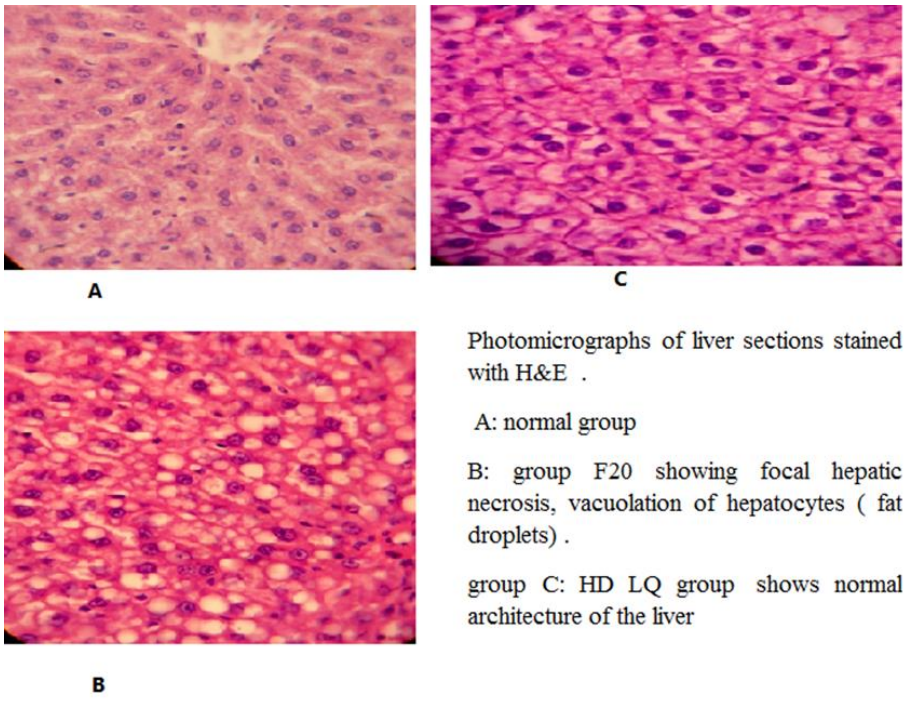
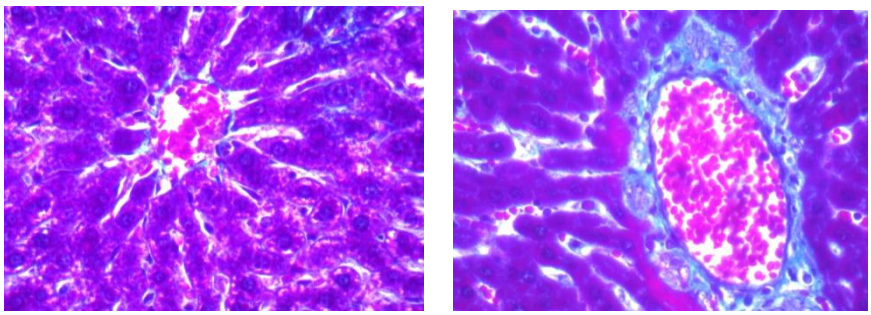


Fig 3: Photomicrographs of liver sections stained with H&E × 400



A normal group **C HD TQ group**

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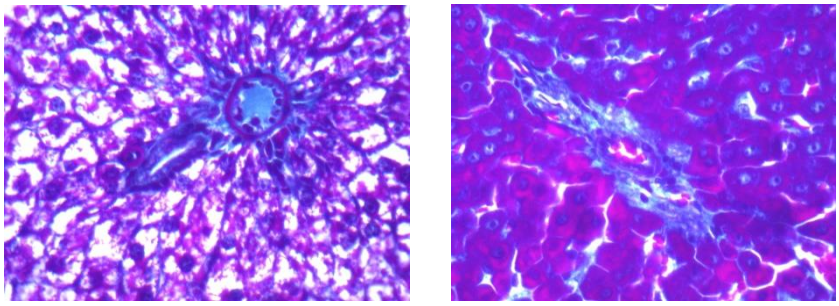

B1 F20 group shows vacolation **B2 F20 group shows fibrosis**

Fig 4: Photomicrographs of liver sections stained with PAS × 40

DISSCUSION

The leading cause of chronic hapatic malfunction is NAFLD , and it is usually related to bad lifestyle habits as increased consumption of fat and sugar which lead to obesity, type 2 diabetes mellitus, insulin resistance, and metabolic syndrome. These factors contribute to progression of fatty liver and eventually cirrhosis[20].

Fatty liver is a serious problem because there is no effective treatment is present. However, it is strongly recommended to reduce body weight, consume healthy diet and regular exercise which may help in prevention of progression of the disease but modification of life style alone is not very much effective [21].

We need method to reverse or delay the progression of fatty liver and cirrohsis. Our target should be reversal of pathological conditions which lead to the disease as insulin resistance, dyslipidemia, steatosis, fibrosis, and inflammation[22]. The present study aimed to investigate the possible protective effects of TQ on fatty liver , and study the underlying mechanism using two different doses (small and large), fatty liver was induced by high fructose in rats.

The TQ is the main component of oil of nigella sative, its concentration in seeds is between 0.12 and 0.25 % [23]. In the essential oil, which is about 0.41–0.44 % of the total seed, the TQ concentration is between 28–57 % [24]. The concentrations in our study were 20 mg/kg body weight for TQ-high and 10 mg/kg for TQ-low dose. Thus, the concentrations used are likely well tolerated[25].

Models of NAFLD should show human characteristics of hepatic pathology and pathophysiology. Therefore ,there should be liver steatosis, hepatic inflammation,liver fibrosis. Rats should show metabolic syndrome features as increased body weight and hyperipidemia [26]. After eight weeks of consuming fructose 20%, there was deposition of fat vacuoles in the hepatocyte cytoplasm[17]. The weight of the liver was increased , besides inflammatory cells infliltration and fibrosis after high fat high carbohydrate consumption for eight weeks in male Wistar rat [27]Another study proved that consuming drinking water with 20% of fructose for 8 weeks increased body weight and obesity, hyperlipidemia, hypertension, and hyperglycemia [28]. This model of metabolic syndrome induced by dietary modification is similar to humans. Thus, in the present study, we used a model of fructose induced metabolic syndrome to investigate the protective effect of TQ in NAFLD pathogenesis.

All of the data observed after 12 weeks confirmed that this the model caused development of characterisitcs of metabolic sundrome and development of fatty liver. TQ treatment decreased all the changes that occurred in the group with control group with fructose in drinking water with more obvious effect in high dose. To confirm the protective effect of TQ on the liver , liver enzymes were evaluated. We found that ALT and AST significantly increased in F20 group as compared to normal control group. High and low dose TQ significantly decrease ALT and AST as compared to F20 group , but high dose was more effective than low dose. In a study by Ahmad and Beg [29] , they proved that TQ at a dose of 80 mg/kg for 30 days caused decreased hepatic enzyme activities and blood sugar.

In our study ,fructose caused significant increase in the FBG,body weight and blood pressure, it caused increase in lipid profile. Impairment in the glucose metabolism and insulin resistance are ususally present in fatty liver individaulss. Decreased insulin sensitivity in the liver increases fatty acids transfer to the liver, decreases fatty acid oxidation, and increases lipogenesis[30]. Our study found that both groups of TQ significantly improved all paramters meaured in the F20 group. Low TQ treated group showed comparable but lesser effective effects than HD TQ rats which showed non signifiacnt difference compared to normal group. Pari and Sankaranarayanan [31] stated that oral TQ for 45 decreased fasting blood sugar in rats with streptozotocin induced diabetic.

Oxidative stress and inflammation are responsible for the progression of fatty liver. TQ has antioxidant properities therefore may be beneficial for prevention of NAFLD. Oxidative stress is a cause in many diseases [32]. We found that fructose caused significant increase in the MDA level and decrease in the antioxidant capacity manifested by decreased glutathione perioxidase level and these effects were ameliorated significantly in TQ treated groups with more effect in the high dose.

We also found that fructose caused significant increase in hepatic TNF-α level, which was ameliorated with TQ treated groups with more effect in high dose. Oxidative stress causes oxidation of free fatty acids, increases TNF-α. TNF-α is the classic cytokine suspected to cause fibrosis and lead to fatty liver [33]. Therefore reduction of TNF-α production may decrease inflammation and fatty liver.

The present study showed that fructose causedinflammatory infiltration, vacuolation of cells indicate fat accumualation in the cell, distortion of liver architecture, TQ ameliorated the pathological changes in the liver caused by fructose.

TQ high dose was more effective in decreasing all NAFLD features, and there was nonsignificant difference between TQ high dose and normal control.

We concluded that TQ can be used to improve metabolic syndrome, oxidative stress and inflammation in the fatty liverand prevent further liver damage and fibrosis , daily consumption of TQ should be ensouraged to prevent metabolic syndrome and progression of progression to fatty liver.

There is no conflict of interest or funding agency for this work

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