

## Hepatoprotective Effects of *Tribulus terrestris* Hydro-Alcoholic Extract on Non-Alcoholic Fatty Liver-Induced Rats

Efectos Hepatoprotectores del Extracto Hidro-Alcohólico de *Tribulus terrestris* en Ratas con Inducción de Hígados Grasos no Alcólicos

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**SUMMARY:** Non-alcoholic fatty liver induces many complications to the liver tissue and also serum related parameters. Medicinal plants are the safe therapeutic strategy for the treatment of diseases. In this regards, the present study was conducted to evaluate the effect of *Tribulus terrestris* (*T. terrestris*) extract on non-alcoholic fatty liver in rats. In this experimental study, thirty male Wistar rats were divided into five groups (n=6). Animals in experimental groups were received high fructose diet (70 %) (HDF) daily alone or in combined with daily intraperitoneal injection of 500, 700 and 1000 mg/kg extract of *T. terrestris*. Control group of rats was feed with standard chow. The serum levels of biomarkers of liver and serum lipid profiles were assessed, also histopathological examination of liver tissue done. Data were analyzed using One-way ANOVA method followed by Tukey's post-hoc multiple comparison test and P<0.05 was considered statistically significant. There were significant improvements for biomarkers of liver tissue (P<0.05) and serum lipid profiles (P<0.01) in the HFD-fed rats that were treated with *T. terrestris* extract compare to HFD-fed group. In addition, accumulation of lipids in hepatocytes was significantly reduced in the HFD-fed + extract administrated groups in comparison to HFD-fed rats (P<0.01). *T. terrestris* extract has protective effects against non-alcoholic fatty liver by changing biomarkers of liver tissue, serum lipid profiles and histopathological anomalies of liver tissue, to normal range.

**KEY WORDS:** Cholesterol; NAFLD; Rat; *Tribulus Terrestris*.

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### INTRODUCTION

Non-alcoholic fatty liver is a chronic disease with high incidence of 10 % - 24 % in developing countries. Although the disease may occur without any sign, severe infection of liver with fibrosis or cirrhosis, liver failure, hepatocarcinoma, cardiovascular diseases could be seen in patients (Sherlock & Dooley, 2002). The risk factors for inducing non-alcoholic fatty liver are Insulin resistance, aging, obesity, genetic factor, misfeeding, intestinal infection and finally consumption of some drugs like methotrexate and glucocorticoids (Kotronen & Yki-Järvinen, 2008).

Two hit theory has been considered as the mechanism involving pathogenesis of non-alcoholic fatty liver disease. According to this theory, triglyceride accumulation is the first hit that is occurring by insulin resistance. The second hit is induction of oxidative stress that activates inflammation pathways, targeting mitochondrial function (Pagano *et al.*, 2002; Vanni *et al.*, 2010).

Pathological changes in non- alcoholic steatohepatitis (NASH) could be identified by micro and macro vesicular steatosis, infection and necrosis (. NASH is associated with obesity, diabetes mellitus, insulin resistance, and hyperlipidemia. Although NASH could be diagnosed by increasing alanine aminotransferase (ALT) and aspartat aminoteransferase (AST), histological evaluation of hepatic biopsies has a remarkable role in this respect Pagano *et al.*).

Adding fructose to the feeding regime of rodents induces steatohepatitis like human and introduces an available animal model to assess fatty liver. In this regard, fructose brings carbon atoms both for glycerol and acyl parts of triglycerides that plays as a suiTableinducer for De novo lipogenesis and also increases oxidant activity in the liver (Basciano *et al.*, 2005).

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Recently, consumption of medicinal plants and natural antioxidants has been attracted researchers as a strategy for treatment of fatty liver. This theory is based on the involvement of oxidative stress for development of fatty liver to steatohepatitis stage. *Tribulus terrestris* (*T. terrestris*) is a perennial creeping herb that is broadly distributed in Mediterranean and subtropical areas. It has been used since ancient times in traditional folk medicine. Studies show that *T. terrestris* contains steroids, saponins, flavonoids, alkaloids, unsaturated fatty acids, vitamins, tannins, resins, nitrate potassium, aspartic acid and glutamic acid. This plant has several medical advantages including antimicrobial, antibacterial, antioxidant and antitoxic activities used in the treatment of cardiovascular diseases, diabetes, tumors, articular pains and respiratory diseases and reproductive dysfunctions (Qureshi *et al.*, 2014).

In the case of the effect of *T. terrestris* on serum lipids, it is been shown that both *Tribulus alatus* (*T. alatus*) and *T. terrestris* extracts caused a significant decrease in the levels of glycosylated hemoglobin, total cholesterol, triglycerides and LDL-cholesterol in streptozotocin-induced diabetic rats (El-Tantawy & Hassanin, 2007). Further, administration of *T. terrestris* extract could significantly inhibit the gluconeogenesis and influence glycometabolism on normal mice and also reduces the level of triglyceride and the content of cholesterol in the plasma (Keshtmand *et al.*, 2015). In previous works we showed the efficacy of *T. terrestris* extract on kidney injury induced by cisplatin and also side effects of diabetes on testis, sperm parameters and sexual hormones (Keshtmand *et al.*, 2014; Keshtmand *et al.*, 2015; Raoofi *et al.*, 2015).

According to hypolipidemic and antioxidant effect of *T. terrestris*, this plant was chosen for examination of beneficial effects on non-alcoholic fatty liver- induced in rats and for these proposes the serum level of related parameters, and histopathological characteristic of the livers were evaluated.

## MATERIAL AND METHOD

**Plant extract.** *T. terrestris* was purchased from a traditional medicine center and identified and authenticated by a botanist. *T. terrestris* seed (300 g) was powdered and added to 80 ml of ethanol (70 %) and was left to macerate at dark condition and room temperature for 24 hours. Then, the soaked powder were extracted in 48 hours by percolation method and the obtained extract was concentrated in the vacuum and was dried in the flat surface (Keshtmand *et al.*, 2014; Raoofi *et al.*). The weight of the obtained extract was 23 g. The extract was dissolved in distilled water and was immediately administered

interaperitoneally (I.P) to rats, expressed as mg / kg of body weight and injection administered from day one for 5 weeks.

**Animals.** Thirty male Wistar rats (200-250 g) were kept at a temperature of  $22\pm 2$  °C, under controlled environmental conditions, 12-hour light-dark cycles and fed standard pellet chow and water ad libitum. All experiment procedures were conducted according to the principles for the care and use of laboratory animals in research and approved by local ethics committee of our university. All rats were weighed at the start of the experiment and before sacrificing in deep anesthesia with chloroform, the body weight and the weight of liver for each rat were measured.

**Experimental procedure.** The rats were randomly divided into five groups (n=6) and consumed for a period of 5 weeks by following descriptions of feeding ingredients; standard chow for group I (E1) and high fructose (70 %) diet for group II- V (E2- E5). The diet regime of E3- E5 groups also included 500, 700 and 1000 mg/kg extract of *T. terrestris* on it, respectively (Brunt *et al.*, 1999; Keshtmand *et al.*, 2014; Raoofi *et al.*).

**Biochemical factors evaluation.** At the end of the experiment, blood samples were taken from heart and preserved in 37 °C for 30 minutes and centrifuged (3000 g) for 15 minutes. The serum was collected and preserved in -20 °C until measuring levels of total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), Very low density lipoprotein (VLDL), triglycerides (TG), albumin (Alb) also, serum markers of hepatic damage; alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin were measured using commercially available kits (ParsAzmun, Tehran, Iran).

**Microscopic studies.** A part of each fresh liver tissue was fixed in 10 % formalin, embedded in paraffin and finally cut into 5 mm sections. From each subject, 10 sections were selected randomly and stained with hematoxylin-eosin and of them, 5 random fields were captured by Motic camera for double-blinded histological assessment, carried out by two other academic members of anatomical sciences group at Kermanshah university of Medical sciences (Khazaei *et al.*, 2012). The histopathological fatty changes were evaluated using the grading and staging system of Brunt *et al.*, that degreed from 0 to 4 as follows: 0= without steatosis, 1= <25 % steatosis, 2= approximately 26-50 % steatosis, 3= approximately 51-75 % steatosis, 4=>76 % steatosis (Brunt *et al.*).

**Statistical analysis.** The data were expressed as means  $\pm$ SE and analyzed by spss16 software (SPSS Inc, Chicago, Illinois). The data were analyzed using One Way ANOVA test followed by Tukey's post-hoc multiple comparison test.  $P < 0.05$  was considered statistically significant.

## RESULTS

The effects of *T. terrestris* extract on body and liver weights of non-alcoholic fatty liver induced rats are presented in figure 1. The mean weights of body had significantly increased in high fructose group compared to control ( $P<0.001$ ) and extract groups ( $P<0.05$ ) (Fig 1A). The mean weight of liver was increased in high fructose group ( $P<0.001$ ) but did not show any significant differences in three other ones ( $P>0.05$ ) (Fig 1B).

The effects of *T. terrestris* extract on liver histopathology of non-alcoholic fatty liver induced rats are offered in figure 2. Severe steatosis and congestion in central vein was seen in the high fructose group in compared to control one (Figs. 2A and 2B). A great number of micro and macrovesicular steatosis were seen in the E3 group (Fig. 2C), while the E4 group markedly attenuated the steatosis (Fig. 2D) and microvesicular steatosis was observed in the E5 group (Fig. 2E).

Table I. Effect of *T. terrestris* hydroalcoholic extract on the hepatic steatosis markers of rats fed high fructose diet. E1: control group, E2: High Fructose, E3: High Fructose + *T. terrestris* (500 mg/kg), E4: High Fructose + *T. terrestris* (700 mg/kg), E5: High Fructose + *T. terrestris* (1000 mg/kg).

Groups	Hepatic steatosis grading					P
	0	I	II	III	IV	
E1	6	0	0	0	0	d
E2	0	0	0	2	4	b
E3	0	1	2	3	0	ac
E4	0	4	2	0	0	d
E5	0	3	2	1	0	ac

a:  $p<0.01$ ; b:  $p<0.05$  compared with control group.  
c:  $p<0.01$ ; d:  $p<0.05$  compared with high fructose group.

The degrees of steatosis based on the microscopic observations are represented in Table I.

The effects of *T. terrestris* extract on the biochemical parameters of liver damage are represented in Table II. In comparison with control group, the serum levels of ALT, AST, ALT/ AST, ALP, bilirubin in E2 group were significantly increased but Alb decreased ( $P<0.01$ ). In E4 and E5 groups, the levels of ALT, AST, ALT/ AST, ALP, bilirubin and Alb did not show any differences with control ( $P>0.05$ ). In E3group, the levels of ALT, AST, ALT/ AST, ALP and bilirubin were increased ( $P<0.05$ ) while the levels of Alb significantly decreased ( $P<0.05$ ) (Table II).

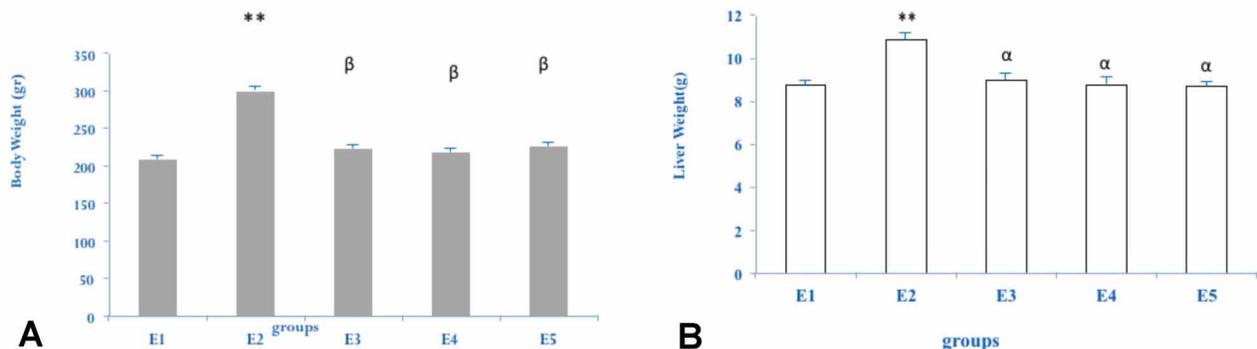


Fig. 1. The effects of *T. terrestris* extract on body weight (A) and liver weight (B) of non-alcoholic fatty liver induced rats. E1: control group, E2: High Fructose, E3: High Fructose + *T. terrestris* (500 mg/kg), E4: High Fructose + *T. terrestris* (700 mg/kg), E5: High Fructose+ *T. terrestris* (1000 mg/kg). \*\* $P<0.01$  compared with the control group. a=  $P<0.05$  compared to the High Fructose group, b =  $P<0.01$  compared with the High Fructose group. The data are presented as mean± S.E.M (Standard Error of Mean).

Table II. Effect of *T. terrestris* hydroalcoholic extract on the biochemical parameters of liver damage of high fructose diet (HFD) fed rats. The data are presented as mean± S. E. M

aGroups	ALT U/L	AST U/L	AST/ALT	ALP IU/L	Bilirubin (Mg/dl)	Alb g/dl
E1	51.23±1.2 <sup>c</sup>	50.99± 1.5 <sup>c</sup>	0.99 <sup>c</sup>	184.66±4.4 <sup>c</sup>	0.81±0.06 <sup>c</sup>	4.18±0.4 <sup>c</sup>
E2	71.02± 2.3 <sup>a</sup>	90.75± 2.7 <sup>a</sup>	1.27 <sup>a</sup>	269±4.8 <sup>a</sup>	1.12±0.05 <sup>a</sup>	2.82±0.5 <sup>a</sup>
E3	62.91± 1.3 <sup>b<sup>d</sup></sup>	70.33± 2.6 <sup>b<sup>d</sup></sup>	1.11 <sup>b<sup>d</sup></sup>	215.5±4.1 <sup>b<sup>d</sup></sup>	1.12±0.05 <sup>b<sup>d</sup></sup>	3.03±0.2 <sup>b<sup>d</sup></sup>
E4	61.13± 2.1 <sup>c</sup>	60.98± 1.9 <sup>c</sup>	0.99 <sup>c</sup>	211±5.7 <sup>c</sup>	0.83±0.04 <sup>c</sup>	3.99±0.3 <sup>c</sup>
E5	59.30± 2.1 <sup>c</sup>	58.33± 2.8 <sup>c</sup>	0.98 <sup>c</sup>	188.25±5.3 <sup>c</sup>	0.83±0.04 <sup>c</sup>	4.10±0.5 <sup>c</sup>

E1: control group, E2: High Fructose, E3: High Fructose + *T. terrestris* (500 mg/kg), E4: High Fructose + *T. terrestris* (700 mg/kg), E5: High Fructose + *T. terrestris* (1000 mg/kg). a:  $p<0.01$ ; b:  $p<0.05$  compared with control group. c:  $p<0.01$ ; d:  $p<0.05$  compared with high fructose group.

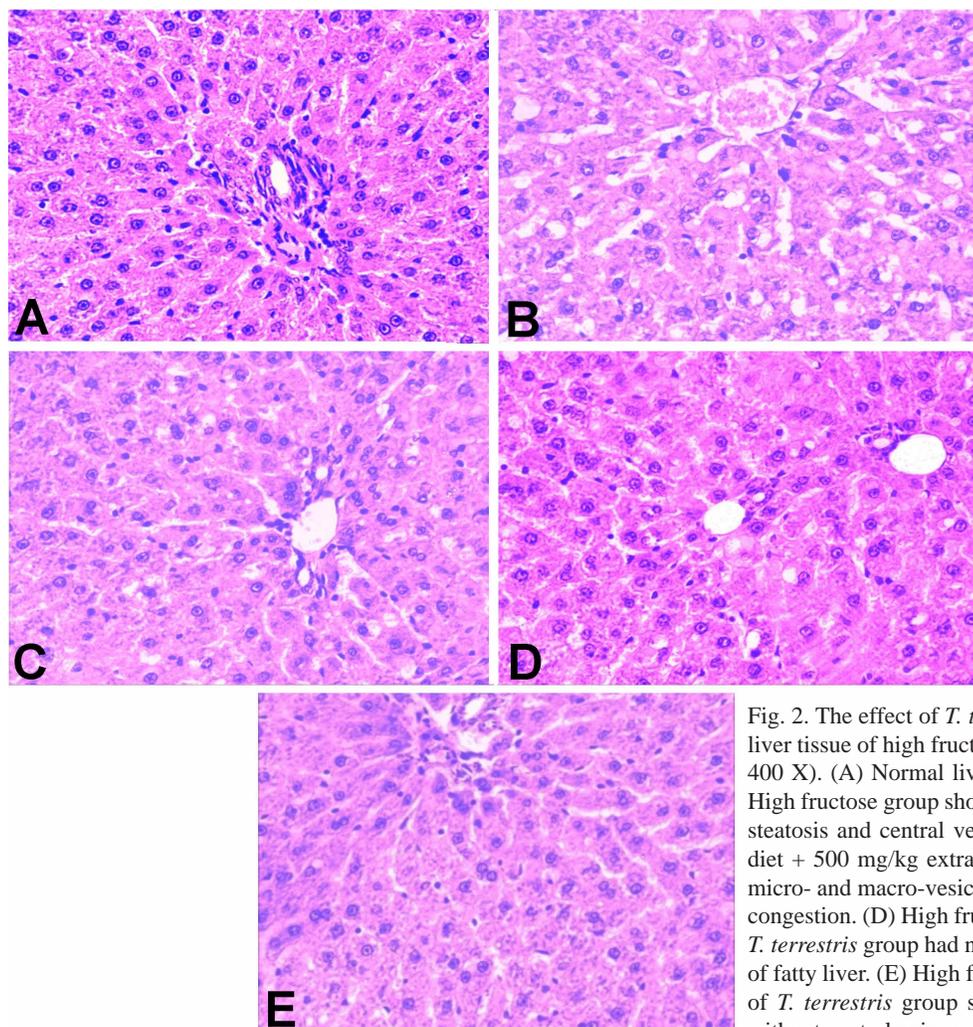


Fig. 2. The effect of *T. terrestris* hydroalcoholic extract on liver tissue of high fructose diet fed rats. (H & E staining, 400 X). (A) Normal liver histology (control group). (B) High fructose group showing micro- and macro-vesicular steatosis and central vein congestion. (C) High fructose diet + 500 mg/kg extract of *T. terrestris* group showing micro- and macro-vesicular steatosis with no central vein congestion. (D) High fructose diet + 700 mg/kg extract of *T. terrestris* group had no evidence of histological lesions of fatty liver. (E) High fructose diet + 1000 mg/kg extract of *T. terrestris* group showing microvesicular steatosis without central vein congestion.

The effects of *T. terrestris* extract on lipids profiles are shown in table III. In comparison with control group, the serum levels of TG, total cholesterol, LDL and VLDL were significantly increased in high-fructose diet (E2) ( $P < 0.01$ ) and High Fructose + *T. terrestris* (500 mg/kg) (E3) ( $P < 0.05$ ) groups while, it didn't show remarkable alterations in E4 and E5 groups ( $P < 0.01$ ). Furthermore, serum levels of

TG, total cholesterol, LDL and VLDL were significantly reduced in E3- E5 groups compared to E2 one ( $P < 0.05$ ) showed attenuation of the extract for these parameters. In contrast, the serum levels of HDL compared to control group, significantly decreased in E2 and E3 groups with  $P < 0.01$ ,  $P < 0.05$  respectively, while it didn't changed in E4 and E5 ones ( $P > 0.05$ ) (Table III).

Table III. Effect of *T. terrestris* extract on lipid levels in rats fed high-fructose diet (H.F.D).

VLDL (mg/l)	HDL (mg/l)	LDL (mg/l)	Total cholesterol mg/l	TG (mg/l)	Groups
E1	90.26±4.7 <sup>c</sup>	95.41±3.3 <sup>c</sup>	24±1.8 <sup>c</sup>	51.02±1.3 <sup>c</sup>	25.05±1.8 <sup>c</sup>
E2	211±3.7 <sup>a</sup>	201.66±2.9 <sup>a</sup>	50±2.1 <sup>a</sup>	41.18±1.8 <sup>a</sup>	73.62±2.1 <sup>a</sup>
E3	120±5.6 <sup>bd</sup>	111±2.2 <sup>bd</sup>	31.25±1.8 <sup>bd</sup>	50.12±1.6 <sup>bd</sup>	56.53±1.9 <sup>bd</sup>
E4	99±4.6 <sup>bd</sup>	99.8±2.8 <sup>bd</sup>	27.12±2 <sup>bd</sup>	49.79±1.4 <sup>bd</sup>	61.78±2.4 <sup>bd</sup>
E5	92±4.5.5 <sup>c</sup>	94.91±3.1 <sup>c</sup>	251±1.8 <sup>c</sup>	51.31±1.7 <sup>c</sup>	18.85±1.1 <sup>c</sup>

E1: control group, E2: High Fructose, E3: High Fructose + *T. terrestris* (500 mg/kg), E4: High Fructose + *T. terrestris* (700 mg/kg), E5: High Fructose+ *T. terrestris* (1000 mg/kg). Values are presented as mean ± SEM. a:  $p < 0.01$ ; b:  $p < 0.05$  compared with control group; c:  $p < 0.01$ , d:  $p < 0.05$  compared with high fructose group.

## DISCUSSION

In the present study, the protective effects of *T. terrestris* hydroalcoholic extract against non-alcoholic fatty liver in male rats were evaluated. The results showed that high fructose diet significantly increased the body and liver weight. Fructose in compared to glucose, does not induce production of insulin and secretion of leptin. Insulin and leptin play an important role in the regulation of food intake and energy homeostasis. The declination of plasma insulin and leptin increases blood lipids and weight gain (Lowette *et al.*, 2015).

In addition, fructose is metabolized by the liver into lipids directly. In summary, high fructose diet leads to stimulation of lipogenesis, hepatic insulin resistance, increased food intakes and obesity (Elliott *et al.*, 2002). Moreover, hypercholesterolemia and hypertriglyceridemia are induced by increased secretion of VLDL and decreased clearance of VLDL that justifies the increased plasma level of VLDL in the rats consumed with high fructose diet (Sleder *et al.*, 1980). Thus, the hypertriglycerolemia and hypercholesterolemia in the rats treated with high fructose diet could be explained by the direct and indirect effects of fructose on lipogenesis.

The present study also showed that high fructose consumption induced liver enlargement that should be considered as microsomal enzyme induction leading to increase storage of lipids peroxisome proliferation, and hyperfunction of the liver (Bär *et al.*, 1999). Further, the study demonstrated that *T. terrestris* extract administration decreased the body and liver weight of the rats as well as reduction in the range of steatohepatitis.

The state of liver function could be evaluated by blood assays, describing its functionality (albumin [ALB]), and its link with the biliary tract (ALP) (Agbaje *et al.*, 2009). The improvement of these two biomarkers as long as cholesterol (TC), high density lipoprotein cholesterol (HDL-C), triglycerides (TG) in *T. terrestris* groups indicated that the extract impaired hepatocellular or secretory functions of the liver in a dose dependent manner.

Two biomarkers of liver damage are AST and ALT. A mild or higher activity of AST indicates liver injury or myocardial infarction and the ratio of AST/ALT may be employed in disease diagnosis (Crook, 2006). An AST/ALT ratio greater than 1 suggests myocardial infarction while, more than 2 is indicative of alcoholic hepatitis or cirrhosis and ranges from 1.2 to 1.4, is an indication of the abnormal functioning of the liver (Su *et al.*, 2006).

In the present study, the extract relieved hepatic damage, as revealed by the results of blood chemistry analysis and histopathological assessment; the significant alterations in the indicators of liver damage (ALT, AST, ALP, HDL-C, ALB and triglycerides) and steatosis ratio.

The *T. terrestris* extract contains flavonoids which are known to have insulin-like properties and also an inhibitory effect on the lipogenase enzyme. Flavonoids improved cell viability and inhibited cellular leakage of hepatocyte AST and ALT (Su *et al.*). Further, it is been shown that flavonoids can decrease the insulin resistance and insulin requirements in adipose and muscle tissue (Wu *et al.*, 2006). Besides flavonoids, the plant contains alkaloid, glycosides, steroids and saponin that have hypolipidemic properties (Divya *et al.*, 2014).

Alkaloids reduce carbohydrate absorption and metabolism. Thus, this component of the extract could reduce the absorption of fructose and also interrupt the fructose metabolization to lipids (Gosain *et al.*, 2010). Steroids are involved in conversion of cholesterol to VLDL and exertion of cholesterol as the bile. Thus, the increased VLDL as long as decreased total serum level of cholesterol in *T. terrestris* treated rats could be explained by steroids component of the extract (Fujioka & Ui, 2001). Saponin is another component of the extract not only regulates lipid metabolism but also improves hypercholesterolemia and hypertriglyceridemia (Yan *et al.*, 1996).

Moreover, polyphenolic and flavonoids compounds of the plant having antioxidant properties are another explanation for antihepatosteatosis of the plant by blocking the second hit of the progression of the disease; oxidative stress (Kumar *et al.*, 2006).

## CONCLUSION

*T. terrestris* extract administration improves the signs of non-alcoholic fatty liver in rats. The beneficial effects of *T. terrestris* extract could be seen both in histopathological features and hepatic serum markers of the rats. At histological level, the hepatosteatosis was diminished in *T. terrestris* extract groups in a dose dependent manner.

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**ALMASI, F.; KHAZAEI, M.; CHEHREI, S. & GHANBARI, A.** Efectos hepatoprotectores del extracto hidro-alcohólico de *Tribulus terrestris* en ratas con inducción de hígados grasos no alcohólicos.

**RESUMEN:** El hígado graso no alcohólico induce muchas complicaciones al tejido hepático y también en ciertos parámetros relacionados con el suero. Las plantas medicinales son la estrategia terapéutica segura para el tratamiento de enfermedades. En este sentido, el presente estudio se realizó para evaluar el efecto del extracto de *Tribulus terrestris* (*T. terrestris*) sobre el hígado graso no alcohólico en ratas. En este estudio experimental se dividieron 30 ratas macho Wistar en cinco grupos (n = 6). Los animales de los grupos experimentales recibieron dietas altas en fructosa (70 %) (HDF) al día o en combinación con inyección intraperitoneal diaria de 500, 700 y 1000 mg / kg de extracto de *T. terrestris*. El grupo control de ratas fue alimentado con alimento estándar. Se evaluaron los niveles séricos de biomarcadores hepáticos y perfiles de lípidos séricos, así como un examen histopatológico del tejido hepático. Los datos se analizaron utilizando el método ANOVA de una vía seguido de la prueba de comparación múltiple post-hoc de Tukey y el  $P < 0,05$  se consideró estadísticamente significativo. Hubo mejoras significativas en los biomarcadores del tejido hepático ( $P < 0,05$ ) y en los perfiles de lípidos séricos ( $P < 0,01$ ) en las ratas alimentadas con HFD que se trataron con extrato de *T. terrestris* comparado con el grupo alimentado con HFD. Además, la acumulación de lípidos en los hepatocitos se redujo significativamente en los grupos alimentados con HFD + extracto, en comparación con las ratas alimentadas con HFD ( $P < 0,01$ ). El extracto de *T. terrestris* tiene efectos protectores sobre el hígado graso no alcohólico, provocando modificaciones en los marcadores biológicos del tejido hepático, los perfiles lipídicos del suero y las anomalías histopatológicas del tejido hepático, hasta un rango normal.

**PALABRAS CLAVE:** Colesterol; NAFLD; Rata; *Tribulus terrestris*.

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