

Research Article



Distinct Mechanisms Underlying the Therapeutic Potential of *Foeniculum vulgare* in Nonalcoholic Fatty Liver Disease - Experimental Study

Ahmed M. Salem¹, Hanaa H. Ahmed^{2*}, Abdelaaty A Shahat³, Mohamed R. Mohamed¹, Abdel Razik H. Farrag⁴, Safaa H. Mohamed²

¹Biochemistry Department, Faculty of Science, Ain Shams University, Cairo, Egypt. ²Hormones Department, National Research Centre, El Bohous Street, Dokki, Cairo, Egypt. ³Phytochemistry Department, National Research Centre, Dokki, Cairo, Egypt. ⁴Pathology Department, National Research Centre, Dokki, Cairo, Egypt.

*Corresponding author's E-mail: hanaaomr@yahoo.com

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ABSTRACT

The current study was undertaken to explore the underlying mechanisms for the therapeutic potential of methanolic extract of *Foeniculum vulgare* (Fennel) and its defatted fraction against nonalcoholic fatty liver disease (NAFLD) in adult female rats were classified into six groups. The first group was kept on standard rodent chow and served as healthy control group (G1). The other groups received high fat diet (HFD) for 24 weeks for NAFLD induction. These groups were assigned as NAFLD group (G2), *Foeniculum vulgare* (FCH) extract-treated groups (G3 and G4) and *Foeniculum vulgare*-defatted (FCH-defatted)-treated groups (G5 and G6). Significant increase in serum ALT activity, bilirubin (Total and direct), cholesterol, LDL, triglycerides, leptin, TNF- α , NF- κ B, Cox-2 and CD₄₀ levels has been recorded in the NAFLD group, while serum HDL and adiponectin levels were decreased significantly in the NAFLD group. On the other side, treatment of the NAFLD groups with FCH or FCH-defatted resulted in significant decrease in serum ALT activity, bilirubin (Total and direct), cholesterol, LDL and triglycerides, leptin, TNF- α , NF- κ B, Cox-2 and CD₄₀ levels showed significant decrease, while serum HDL and adiponectin levels showed significant increase. Photomicrographs of liver tissue sections of rats treated with FCH or FCH-defatted confirmed the present improvement in the studied biochemical markers. In conclusion, these results suggest that the promising therapeutic potential of *Foeniculum vulgare* extract and its defatted fraction against NAFLD could be ascribed to their hepatoprotective activity, hypolipidemic effect and anti-inflammatory property. Thus, our findings reinforce current advice recommending the consumption of natural products to modulate NAFLD.

Keywords: *Foeniculum vulgare*, *Foeniculum vulgare*-defatted, Nonalcoholic fatty liver disease, Hyperlipidemia, Inflammation, Rats.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a clinicopathologic entity increasingly recognized as a major health burden in developed countries¹. NAFLD is currently the most common form of chronic liver disease affecting both adults and children as it affects up to 20% to 30% of the population in the developed world². It includes a spectrum of liver damage ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), advanced fibrosis, and probable progression to cirrhosis³. NAFLD is believed to be a feature of metabolic syndrome because it is closely associated with visceral obesity, dyslipidaemia, insulin resistance, and type 2 diabetes mellitus (DM)⁴. Among the many causative factors of NAFLD, oxidative stress, lipid peroxidation, mitochondrial dysfunction, gut-derived lipopolysaccharide, adipocytokines and inflammation are considered the most probable etiological factors⁵.

Fennel (*Foeniculum vulgare*) is a widespread umbelliferous herb, traditionally used as food and for medicinal purposes. It is an annual, biennial or perennial aromatic herb, depending on the variety, which has been known since antiquity in Europe and Asia Minor⁶. During the last decade, fennel has been shown to have a well established role as herbal remedy⁷. The most common compounds isolated from fennel were D-limonene and β -myrcene which have been shown to improve liver function. D-limonene compound has been shown to increase the

concentration of reduced glutathione (GSH) in the liver⁸. β -myrcene compound, on the other hand, could elevate the levels of apoproteins CYP2B1 and CYP2B2, which are subtypes of the P450 enzyme system⁹. The cytochrome P450 (CYP) enzyme system consists of a superfamily of hemoproteins that catalyzes the oxidative metabolism of a wide variety of exogenous chemicals including drugs, carcinogens, toxins and endogenous compounds such as steroids, fatty acids and prostaglandins¹⁰.

Fennel extract has been found to increase the level of high density lipoprotein-cholesterol¹¹ and reduce upper gastrointestinal motility thus delaying the absorption of triacylglycerols and sugars from the small intestine¹². It has been demonstrated that, both infusions and essential oils obtained from the fruits and the aerial parts of the fennel plant, are included in the herbalist armamentarium for their relaxant, estrogenic, analgesic and anti-inflammatory properties¹³. The anti-inflammatory activity of fennel may act on both the cyclooxygenase and lipoxygenase pathways¹⁴.

The present work was designed to explore the underlying mechanisms by which *Foeniculum vulgare* aerial part methanolic extract fraction (FCH) and its defatted (FCH-defatted) could manage non-alcoholic fatty liver disease (NAFLD) in adult female rats. This goal was achieved through conducting routine biochemical analysis for liver functions, estimating the circulating levels of insulin resistance indices and evaluating serum levels of



inflammatory markers. Also, histopathological investigation of liver tissue sections was carried out to confirm the studied biochemical analyses.

MATERIALS AND METHODS

Plant materials

The fresh cultivated fennel (*Foeniculum vulgare* var. *azoricum*) was collected from the Sekem company plantation at Bilbase City, Egypt (March 2012). The plants were authenticated by Professor Ibrahim El-Garf, Department of Botany, Faculty of Science, Cairo University. Voucher samples are deposited at the Herbarium of the National Research Centre (NRC), Cairo, Egypt

Preparation of *Foeniculum vulgare* total extract (FCH)

The air-dried aerial part (4 kg) were pulverized to fine powder, extracted in a mixture of water and methanol 80% with the aid of a sonicator bath (20 min x 3 times) and then filtered. After filtration, the filtrate was evaporated under reduced pressure at 45-50°C to give a dry residue of crude aqueous methanolic extract (FCH) [11.5% from the dried aerial part]

Preparation of *Foeniculum vulgare*-defatted fraction (FCH-defatted)

The air-dried dried aerial part (2kg) were ground to fine powder and defatted with n-hexane three times; the lipid fraction was collected and dried with rotary evaporator. The residue (defatted plant material) was further extracted with 80% alcohol to give defatted alcoholic fraction.

Animals

The present study was conducted on sixty adult female albino rats of Wistar strain weighing 120 - 150g and obtained from the Animal House Colony of the National Research Centre, Cairo, Egypt. The animals were kept in well ventilated cages and maintained on standard laboratory diet and water *ad libitum* for two weeks prior to starting the experiment. All animals received adequate human care and use according to the Guidelines for Animal Experiments which were approved by the Ethical Committee of Medical Research, National Research Centre, Egypt. Non-alcoholic fatty liver disease (NAFLD) was induced in rats by using high fat diet which provided 30% of its energy from fat, 35% from carbohydrate and 35% from protein (casein) for 24 weeks. Supplements of vitamins and minerals were also included¹⁵.

Experimental set-up

The animals were classified into six groups, ten animals each. They were labeled namely (G1) Healthy control group which was fed *ad-libitum* with an isocaloric regular rat chow¹⁶, (G2) Non-alcoholic fatty liver disease (NAFLD) group which was fed *ad-libitum* with high fat diet¹⁵, (G3) NAFLD group which was treated orally with 40 mg/kg b.wt of FCH extract daily for 8 weeks, (G4) NAFLD group

which was treated orally with 20 mg/kg b.wt of FCH extract daily for 8 weeks. (G5) NAFLD group which was treated orally with 30 mg/ kg b.wt of FCH-defatted fraction daily for 8 weeks and (G6) NAFLD group which was treated orally with 15 mg/kg b.wt of FCH-defatted fraction daily for 8 weeks. The selected doses of FCH extract and FCH-defatted fraction were calculated from the chronic toxicity study for FCH extract and FCH-defatted fraction (data not shown).

After animal treatment was over, (32 weeks), the animals were subjected to one night of food deprivation and the blood samples were collected from the retroorbital plexus under diethylether anaesthesia¹⁷. The blood samples were left to clot and then centrifuged using cooling centrifuge at 1800 xg for ten minutes to obtain sera. The clear serum samples were stored at -20 °C until analysis. After blood collection, all animals were sacrificed by cervical dislocation and the liver of each rat was quickly excised, washed in isotonic saline, then cut into small pieces (0.5x0.5cm) and fixed in 10% formalin saline solution for histological examination.

Biochemical assays

Serum alanine transaminase (ALT) activity was estimated colorimetrically using kit purchased from Quimica Clinica Aplicada S.A. Co., Spain, according to the method of Reitman and Frankel¹⁸. Serum bilirubin levels (total and direct) were measured colorimetrically using kit purchased from Randox Laboratory, Crumlin, Co. Antrim, UK, according to the method of Sherlock¹⁹. Serum cholesterol (Chol) concentration was determined colorimetrically using kit purchased from Stanbio Laboratory, Boerne, Texas, USA, according to the method of Allain et al.²⁰. Serum LDL-cholesterol (LDL) concentration was assayed colorimetrically using kit purchased from Quimica Clinica Aplicada S.A. Co., Spain, according to the method of Assman et al.²¹. Serum HDL-cholesterol (HDL) concentration was measured colorimetrically using kit purchased from Stanbio Laboratory, Boerne, Texas, USA, according to the method of Lopez-Virella et al.²². Serum triglycerides (TG) level was determined colorimetrically using kit purchased from Stanbio Laboratory, Boerne, Texas, USA, according to the method of Fassati and Prencipe²³. Serum adiponectin concentration was measured by enzyme-linked immunosorbent assay (ELISA) technique using kit purchased from AssayPro, USA, according to the method of Pannacciulli et al.²⁴. Serum leptin level was measured by ELISA procedure using kit purchased from Ray Biotech Co., Georgia, USA, according to the method described by Petridou et al.²⁵. Serum TNF- α concentration was measured by ELISA procedure using kit purchased from Ray Biotech Co., Georgia, USA, according to the method of Brouckaert et al.²⁶. Serum nuclear factor kappa B p56 (NF- κ Bp56) concentration was determined by ELISA technique using kit purchased from Glory Science Co., Ltd, Veterans Blvd, Suite, USA, according to the manufacturer's instructions. Serum Cyclooxygenase-2



(Cox-2) concentration was determined by ELISA technique using kit purchased from Glory Science Co., Ltd, Veterans Blvd, Suite, USA, according to the manufacturer's instructions. Serum CD₄₀ concentration was measured by ELISA technique using kit purchased from Glory Science Co., Ltd, Veterans Blvd, Suite, USA, according to the manufacturer's instructions.

Histopathological and Histochemical examinations

After fixation of liver tissues in 10% saline buffered formalin for 24 hours, the liver tissues were dehydrated in ascending grades of ethanol, cleared in xylol and then impregnated in paraffin. Impregnated liver tissues were processed three times in pure paraffin to be embedded in blocks. Sections (5µm thick) were prepared using Leica microtome and stained with hematoxylin and eosin (H&E) for histopathological investigation²⁷. SCHARLACH Rs stain was used for histochemical identification of fatty changes. Histological variables were semiquantitated using image analysis from 0 to 4+, including macro-and microvesicular fatty changes (1⁺), the foci of necrosis (2⁺), portal and perivenular fibrosis (3⁺) and portal and perivenular fibrosis as well as the inflammatory infiltrate (4⁺). The stained slides were examined using Olympus light microscope. Photomicrographs were taken by Olympus camera fitted to the microscope at a magnification of X 300.

Statistical analysis

In the present study, all results were expressed as Mean ± S.E of the mean. Data were analyzed by one way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program, version 11 followed by

least significant difference (LSD) to compare significance between groups²⁸. Difference was considered significant when P value < 0.05. The percent of difference was calculated according to the following equation:

$$\% \text{ of difference} = \frac{\text{Treated value} - \text{Control value}}{\text{Control value}} \times 100$$

RESULTS

Biochemical Results

The effect of treatment with *Foeniculum vulgare* extract (FCH) and *Foeniculum vulgare*-defatted fraction (FCH-defatted) on serum ALT activity and bilirubin (total and direct) level in NAFLD rat model is illustrated in Table (1). A significant increase (P<0.05) in serum ALT activity (46.9 %) and bilirubin (total (195%) and direct (100%) level was recorded in NAFLD group compared with the healthy control group. By contrast, treatment of NAFLD groups with FCH extract or FCH-defatted fraction produced significant decrease (P<0.05) in serum ALT activity (-32.81%) for FCH (40 mg/kg b.wt), (-26.16%) for FCH (20 mg/kg b.wt), (-18.31%) for FCH-defatted (30 mg/kg b.wt) and (-14.8%) for FCH-defatted (15 mg/kg b.wt) compared the untreated NAFLD group. Similarly, NAFLD groups treated with FCH extract or FCH-defatted fraction showed significant decrease (P<0.05) in total and direct bilirubin levels (-57.6% and -33.3%, respectively) for FCH (40 mg/kg b.wt), (-57.6% and -23.3%, respectively) for FCH (20 mg/kg b.wt), (-49.1% and -33.33%, respectively) for FCH-defatted (30 mg/kg b.wt) and (-36.44% and -16.6%, respectively) for FCH-defatted (15 mg/kg b.wt) in comparison with the untreated NAFLD group.

Table 1: Effect of treatment with FCH extract and FCH-defatted fraction on serum ALT activity and bilirubin (total and direct) levels in NAFLD rat model.

Groups	Parameters	ALT (U/L)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)
Healthy control group (G1)		30.7 ± 2.1	0.4 ± 0.05	0.15 ± 0.01
NAFLD group (G2)		45.1 ± 1.7 ^a (46.9%)	1.18 ± 0.06 ^a (195%)	0.30 ± 0.02 ^a (100%)
NAFLD +FCH extract (40mg/kg b.wt) (G3)		30.3 ± 1.2 ^b (-32.81%)	0.5 ± 0.04 ^b (-57.6%)	0.20 ± 0.01 ^b (-33.3%)
NAFLD +FCH extract (20mg/kg b.wt) (G4)		33.3 ± 1.2 ^b (-26.16%)	0.5 ± 0.04 ^b (-57.6%)	0.23 ± 0.02 ^b (-23.3%)
NAFLD + FCH-defatted fraction (30mg/kg b.wt) (G5)		36.9 ± 1.3 (-18.31%)	0.6 ± 0.01 ^b (-49.1%)	0.20 ± 0.01 ^b (-33.33%)
NAFLD + FCH-defatted fraction (15mg/kg b.wt) (G6)		38.4 ± 2.0 ^b (-14.8%)	0.75 ± 0.02 ^b (-36.44%)	0.25 ± 0.02 ^b (-16.6%)

a: Significant change at P < 0.05 in comparison with the healthy control group; b: Significant change at P < 0.05 in comparison with NAFLD group; (%): percent difference with respect to the corresponding control value.

The Data in Table (2) show that the induction of NAFLD elicited significant elevation (P<0.05) in serum triglycerides, cholesterol and LDL levels (41.5%, 83.39% and 80.41%, respectively) in concomitant with significant decline (P<0.05) in serum HDL level (-47.84%) as compared to the healthy control group. On the other hand, treatment of NAFLD groups with FCH extract or FCH-defatted fraction resulted in significant depletion (P<0.05) in serum triglycerides, cholesterol and LDL levels (-22.9%, -32.15% and -27.98%, respectively) for FCH (40

mg/ kg b.wt), (-15.96%, -19.47% and -20.93%, respectively) for FCH (20 mg/kg b.wt), (-22.60%, -38.27% and -36.97%, respectively) for FCH-defatted (30 mg/kg b.wt) and (-17.2%, -36.56% and -29.88%, respectively) for FCH-defatted (15 mg/kg b.wt) as compared to the untreated NAFLD group. The opposite was observed regarding HDL level, where the treatment of NAFLD groups with FCH extract or FCH-defatted fraction led to a significant (P<0.05) elevation in comparison with the untreated NAFLD group. The trend of HDL elevation



behaved as follows: (49.80%) for FCH (40 mg/kg b.wt), (34.96%) for FCH (20 mg/kg b.wt), (55.87%) for FCH-defatted (30 mg/kg b.wt), (42.92%) for FCH-defatted (15 mg/kg b.wt).

The effect of treatment with FCH extract and FCH-defatted fraction on serum adiponectin and leptin levels in NAFLD rat model is presented in Table (3). In comparison with the healthy control group, significant reduction ($P < 0.05$) in serum adiponectin level (-38.39%) associated with significant elevation ($P < 0.05$) in serum leptin level (131%) were recorded in NAFLD group. In contrast, treatment of NAFLD group with FCH extract or

FCH-defatted fraction resulted in significant increase ($P < 0.05$) in serum adiponectin level (39.13%) for FCH (40mg/kg b.wt), (33.33%) for FCH (20 mg/kg b.wt), (46.08%) for FCH-defatted (30 mg/kg b.wt) and (33.62%) for FCH-defatted (15 mg/kg b.wt). Meanwhile, significant decrease ($P < 0.05$) in serum leptin level (-30.1%) was detected in NAFLD group treated with FCH (40 mg/kg b.wt), (-18.71%) in NAFLD group treated with FCH (20 mg/kg b.wt), (-38.27%) in NAFLD group treated with FCH-defatted (30 mg/kg b.wt) and (-32.03%) in NAFLD group treated with FCH-defatted (15 mg/kg b.wt) as compared to the untreated NAFLD group.

Table 2: Effect of treatment with FCH extract and FCH-defatted fraction on lipid profile of NAFLD rat model.

Groups	Parameters	Triglycerides (mg/dl)	Cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Healthy control group (G1)		55.3 ± 2.0	60.6 ± 1.5	38.5 ± 1.8	14.30 ± 0.4
NAFLD group (G2)		78.3 ± 3.0 ^a (41.5%)	111.14 ± 3.7 ^a (83, 39%)	20.08 ± 1.1 ^a (-47.84%)	25.8 ± 1.1 ^a (80.41%)
NAFLD +FCH extract (40mg/kg b.wt) (G3)		60.3 ± 2.06 ^b (-22.98%)	75.4 ± 2.9 ^b (-32.15%)	30.08 ± 1.2 ^b (49.80%)	18.58 ± 0.4 ^b (-27.98%)
NAFLD +FCH extract (20mg/kg b.wt) (G4)		65.8 ± 2.7 ^b (-15.96%)	89.5 ± 2.4 ^b (-19.47%)	27.1 ± 0.8 ^b (34.96%)	20.4 ± 0.3 ^b (-20.93%)
NAFLD + FCH-defatted fraction (30mg/kg b.wt) (G5)		60.6 ± 2.06 ^b (-22.60%)	68.6 ± 1.3 ^b (-38.27%)	31.3 ± 0.9 ^b (55.87%)	16.26 ± 0.1 ^b (-36.97%)
NAFLD + FCH-defatted fraction (15mg/kg b.wt) (G6)		64.8 ± 2.7 ^b (-17.2%)	70.5 ± 1.9 ^b (-36.56%)	28.7 ± 0.7 ^b (42.92%)	18.09 ± 0.3 ^b (-29.88%)

a: Significant change at $P < 0.05$ in comparison with the healthy control group; b: Significant change at $P < 0.05$ in comparison with NAFLD group; (%): percent difference with respect to the corresponding control value.

Table 3: Effect of treatment with FCH extract and FCH-defatted fraction on serum adiponectin and leptin levels in NAFLD rat model

Groups	Parameters	Adiponectin (ng/ml)	Leptin (pg/mL)
Healthy control group (G1)		11.2 ± 0.42	324.8 ± 3.00
NAFLD group (G2)		6.9 ± 0.2 ^a (-38.39%)	753.3 ± 3.1 ^a (131%)
NAFLD +FCH extract (40mg/kg b.wt) (G3)		9.6 ± 0.1 ^b (39.13%)	525.9 ± 2.5 ^b (-30.1%)
NAFLD +FCH extract (20mg/kg b.wt) (G4)		9.2 ± 0.2 ^b (33.33%)	612.3 ± 2.2 ^b (-18.71%)
NAFLD + FCH-defatted fraction (30mg/kg b.wt) (G5)		10.08 ± 0.2 ^b (46.08%)	465.00 ± 2.5 ^b (-38.27%)
NAFLD + FCH-defatted fraction (15mg/kg b.wt) (G6)		9.22 ± 0.3 ^b (33.62%)	512.00 ± 2.3 ^b (-32.03%)

a: Significant change at $P < 0.05$ in comparison with the healthy control group; b: Significant change at $P < 0.05$ in comparison with NAFLD group; (%): percent difference with respect to the corresponding control value.

The data depicted in Table (4) represent the effect of treatment with FCH extract and FCH-defatted fraction on serum TNF- α , NF- κ B p56, Cox-2 and CD40 levels in NAFLD rat model. The NAFLD group displayed a significant increase ($P < 0.05$) in TNF- α , NF- κ Bp56, Cox-2 and CD40 levels (66.25%, 60%, 64% and 91.4%, respectively) when compared with the healthy control group. On the other side, treatment of NAFLD group with FCH extract or FCH-defatted fraction induced significant decrease ($P < 0.05$) in serum TNF- α , NF- κ Bp56, Cox-2 and CD40 levels (-24.50%, -37.27%, -19.51% and -34.4%, respectively) for FCH (40 mg/kg b.wt), (-20.69%, -36.3%, -16.58% and -25.06%,

respectively) for FCH (20 mg/kg b.wt), (-33.75%, -42.72%, -31.41% and -30.35%, respectively) for FCH-defatted (30 mg/kg b.wt) and (-27.68%, -39.09%, -26.82% and -28.6%, respectively) for FCH-defatted (30 mg/kg b.wt) in comparison with the untreated NAFLD group.

Histopathological Results

Microscopic investigation of liver tissue sections of rats in the healthy control group showed the normal structure of the liver and the central veins (CV), hepatocytes, and blood sinusoids (Fig. 1-a). Meanwhile, histopathological examination of liver tissue sections of rats that were fed

high fat diet for 24 weeks and represented the NAFLD group showed moderate to severe macrovesicular and microvesicular fatty changes, which were diffusely distributed throughout hepatic lobule, accompanied with focal necrosis (**Fig. 1-b**). On the other hand, microscopic examination of liver tissue sections of rats in the NAFLD groups treated with FCH extracts (40 or 20 mg/kg b.wt)

for 8 weeks showed normal structure of portal area (**Fig. 1-c, and Fig. 1-d, respectively**). Similarly, histopathological investigation of liver tissue sections of rats in the NAFLD groups treated with FCH-defatted fraction (30 or 15 mg/kg b.wt) for 8 weeks showed the normal structure of the hepatic lobule (**Fig. 1- e and Fig. 1-f, respectively**).

Table 4: Effect of treatment with FCH extract and FCH-defatted fraction on serum TNF- α , NF- κ Bp56, Cox-2 and CD₄₀ levels in NAFLD rat model

Groups	Parameters	TNF- α (Pg/ml)	NF- κ Bp56 (ng/ml)	Cox-2 (U/L)	CD ₄₀ (ng/L)
Healthy control group (G1)		52.03 \pm 1.6	0.60 \pm 0.02	12.5 \pm 0.2	355.7 \pm 3.3
NAFLD group (G2)		86.5 \pm 2.1 ^a (66.25%)	1.1 \pm .05 ^a (60 %)	20.5 \pm 0.4 ^a (64.0 %)	680.9 \pm 3.4 ^a (91.4 %)
NAFLD +FCH extract (40mg/kg b.wt) (G3)		65.3 \pm 2.0 ^b (-24.50%)	0.69 \pm 0.03 ^b (-37.27 %)	16.5 \pm 0.3 ^b (-19.51 %)	446.3 \pm 2.5 ^b (-34.4 %)
NAFLD +FCH extract (20mg/kg b.wt) (G4)		68.6 \pm 2.1 ^b (-20.69%)	0.70 \pm 0.03 ^b (-36.3%)	17.1 \pm 0.32 ^b (-16.58 %)	510.2 \pm 2.7 ^b (-25.06 %)
NAFLD + FCH-defatted fraction (30mg/kg b.wt) (G5)		57.3 \pm 1.5 ^b (-33.75%)	0.63 \pm 0.02 ^b (-42.72 %)	14.06 \pm 0.3 ^b (-31.41 %)	474.2 \pm 2.4 ^b (-30.35%)
NAFLD + FCH-defatted fraction (15mg/kg b.wt) (G6)		62.4 \pm 1.3 ^b (-27.86%)	0.67 \pm 0.02 ^b (-39.09%)	15.00 \pm 0.3 ^b (-26.82%)	485.6 \pm 2.6 ^b (-28.6 %)

a: Significance change at P < 0.05 in comparison with the healthy control group; b: Significance change at P < 0.05 in comparison with NAFLD group; (%): percent difference with respect to corresponding control value

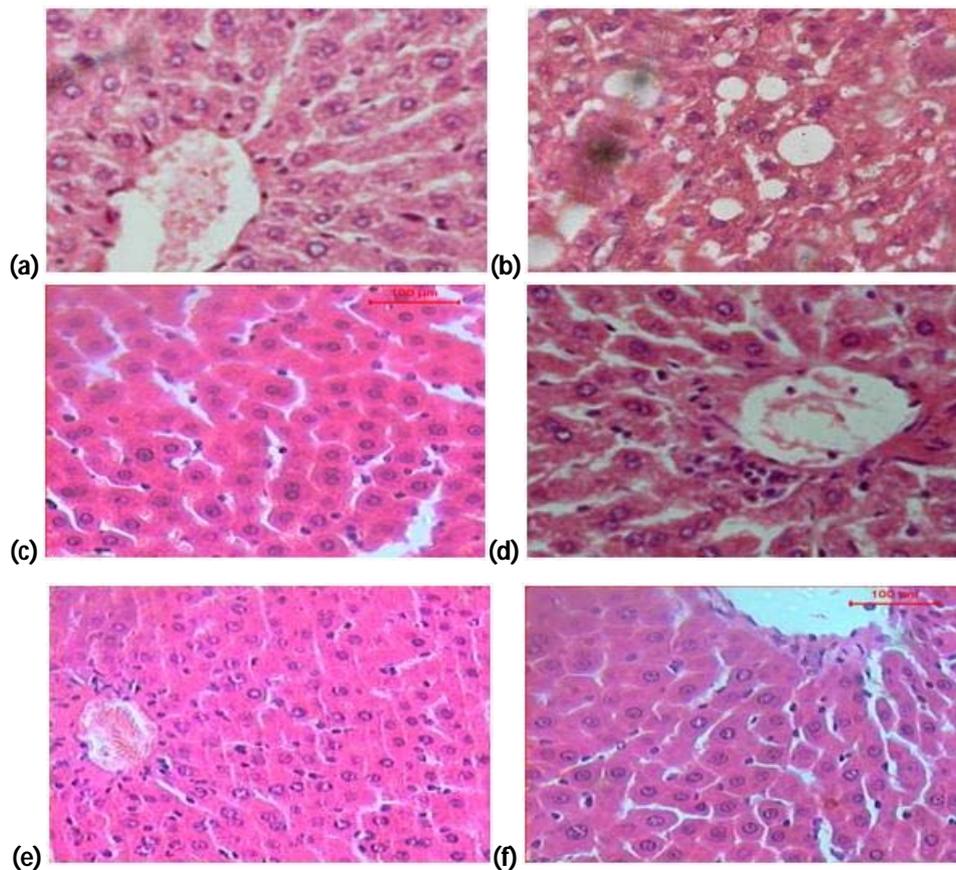


Figure 1: Photomicrograph of liver tissue sections of rats in **a**) healthy control group showing normal structure of the liver, **b**) NAFLD group showing a high degree of hepatocellular cytoplasmic vacuolation (macrovesicular and microvesicular steatosis), **c**) NAFLD group treated with 40 mg/kg b.wt of FCH extract showing normal structure of the portal area, **d**) NAFLD group treated with 20 mg/kg b.wt of FCH extract showing nearly normal structure of the portal area, **e**): rats in NAFLD group treated with 30 mg/kg b.wt of FCH- defatted

fraction showing normal structure of the hepatic lobule and **f**) NAFLD group treated with 15 mg/kg b.wt FCH- defatted fraction showing the normal structure of the hepatic lobule (**H & E X 300**).

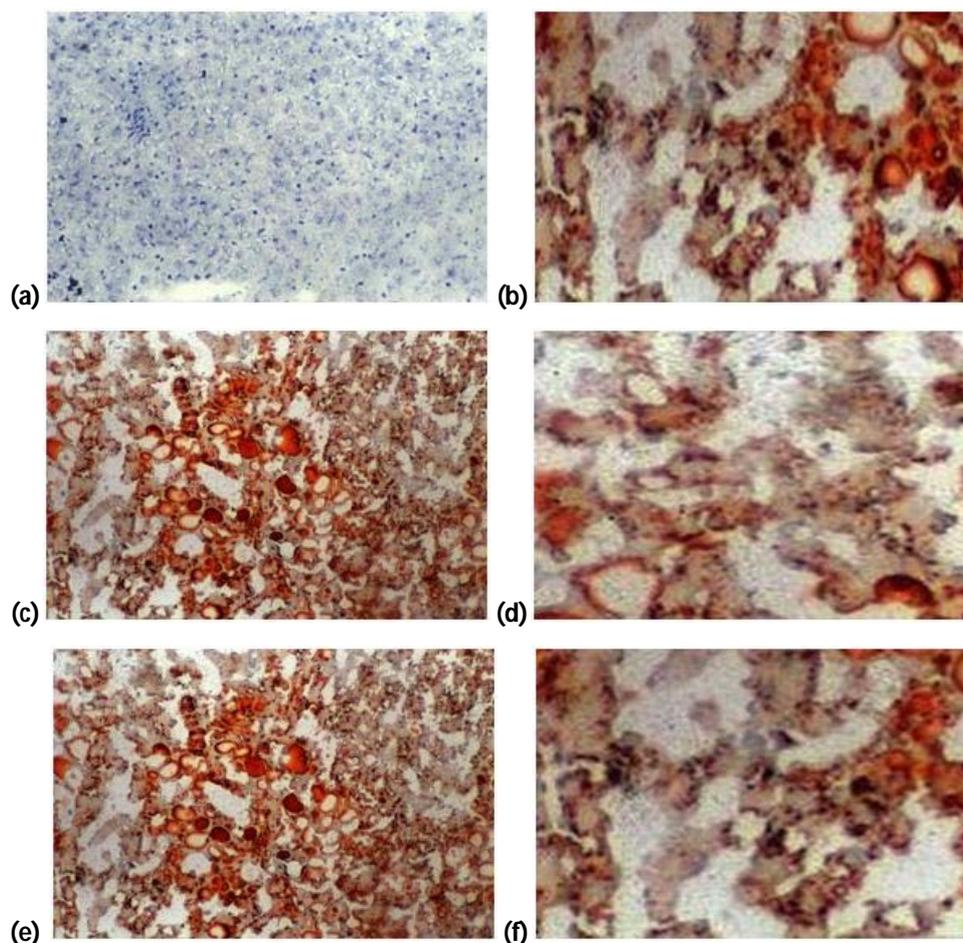


Figure 2: Photomicrograph of liver tissue sections of rats in **a**) healthy control group showing negative reaction and the hepatocytes are slightly swollen with centrally placed nuclei. No fatty changes are seen, **b**) NAFLD group showing positive reaction in the microvesicular fatty infiltration, **c**) NAFLD group treated with FCH extract (40 mg/kg b.wt) showing significant reduction in fatty deposits in liver tissues and the reaction is negative in most areas of the lobules, **d**) NAFLD group treated with FCH extract (20 mg/kg b.wt) showing negative reaction indicating marked improvement of fatty infiltration, **e**) NAFLD group treated with FCH- defatted fraction (30 mg/kg b.wt) shows significant reduction in fatty deposits in liver tissues and the reaction is negative in most areas of the lobules and **f**) NAFLD group treated with FCH-defatted fraction (15 mg/kg b.wt) showing negative reaction indicating remarkable improvement of fatty infiltration (SCHARLACH Rs x 300).

Histochemical Results

Histochemical examination of liver tissue sections obtained from rats in the healthy control group and stained with SCHARLACH Rs stain showed negative stain indicating no fatty changes (**Fig. 2-a**). However, mild macro- and microvesicular fatty changes were observed in the periportal zone of the liver obtained from rats in the NAFLD group. These changes were clearly shown in the histochemical examination of the liver tissue sections of rats in NAFLD group (**Fig. 2-b**). Meanwhile, histochemical investigation of liver tissue sections of rats in the NAFLD group treated with FCH extract (40mg/kg b.wt) showed no fatty infiltration (**Fig. 2-c**). Histochemical examination of liver sections of rats in the NAFLD group treated with FCH extract (20 mg/kg b.wt) revealed few microvesicular fatty changes (**Fig. 2-d**). Similarly, NAFLD group treated with FCH-defatted fraction (30 mg/kg b.wt) revealed no fatty infiltration in the liver as shown in the

histochemical examination of liver tissue sections of rats in this group (**Fig. 2-e**). Whereas, the histochemical investigation of liver tissue sections of rats in the NAFLD group treated with FCH-defatted fraction (15mg/kg b.wt) showed few microvesicular fatty changes (**Fig. 2-f**).

According to the results of image analysis of liver tissue sections, we classified the grades of steatosis into five stages 0, 1⁺, 2⁺, 3⁺ and 4⁺ (Table 5). Grade 0 indicates no steatosis, Grade 1⁺ indicates macro- and microvesicular fatty changes, Grade 2⁺ indicates the foci of necrosis, Grade 3⁺ indicates portal and perivenular fibrosis and Grade 4⁺ indicates portal and perivenular fibrosis as well as the inflammatory infiltrate (4⁺). Image analysis of liver tissue sections of rats in healthy control group showed 100% (10 rats) with no steatosis (Grade 0), while, rats in NAFLD group showed 70% (7 rats) with steatosis (Grade 2⁺) and 30% (3 rats) with steatosis (Grade 3⁺). Fat depositions in this group were classified as

microvesicular. Image analysis of liver tissue sections of rats in NAFLD group treated with FCH extract (40mg/kg b.wt) showed 90% (9 rats) with no steatosis (Grade 0) and 10% (1 rat) with steatosis (Grade 2⁺), whereas those treated with FCH extract (20mg/kg b.wt) showed 80% (8 rats) with no steatosis (Grade 0) and 20% (2 rats) with steatosis (Grade 2⁺). Similarly, image analysis of liver tissue sections of rats in NAFLD group treated with FCH-defatted fraction (30mg/kg b.wt) showed 90% (9 rats) with no steatosis (Grade 0) and 10% (1 rat) with steatosis (Grade 2⁺), whereas those treated with FCH-defatted fraction (15mg/kg b.wt) showed 80% (8 rats) with no steatosis (Grade 0) and 20% (2 rats) with steatosis (Grade 2⁺) (Table 5). Fat deposits in all treated groups were assigned as mixed of macro and microvesicular.

Table 5: Grades of steatosis in the different studied groups

Groups	Rats (n)	Steatosis Grades				
		0	1 ⁺	2 ⁺	3 ⁺	4 ⁺
Healthy control group (G1)	10	10	-	-	-	-
NAFLD group (G2)	10	-	-	7	3	-
NAFLD +FCH extract (40mg/kg b.wt) (G3)	10	9	-	1	-	-
NAFLD +FCH extract (20mg/kg b.wt) (G4)	10	8	-	2	-	-
NAFLD + FCH-defatted fraction (30mg/kg b.wt) (G5)	10	9	-	1	-	-
NAFLD + FCH-defatted fraction (15mg/kg b.wt) (G6)	10	8	-	2	-	-

DISCUSSION

The results of the present study indicated significant increase in serum ALT activity in NAFLD group versus the healthy control group. These findings are consistent with those of Rosa et al.²⁹. Both aminotransferases (AST and ALT) are highly concentrated in the liver and the increasing serum ALT activity is considered a consequence of hepatocyte damage in NAFLD patients³⁰. A growing body of evidence supported the possibility that insulin resistance associated with adipose tissue inflammation and hepatic microvascular dysfunction in NAFLD patients might actually contribute to the development and/or progression of ALT activity in serum².

In view of the current results, significant increase in serum total and direct bilirubin levels has been detected in NAFLD group when compared with the healthy control group. These results are in agreement with Kathirvel et al.³¹. Bilirubin is the product of hemoglobin catabolism within the reticuloendothelial system. Heme breakdown determines the formation of unconjugated bilirubin, which is then transported to the liver. In the liver, UDP-glucuronyltransferase conjugates the water-insoluble unconjugated bilirubin to glucuronic acid, and conjugated bilirubin is then excreted into the bile³². In healthy people, conjugated bilirubin is virtually absent from serum mainly because of the rapid process of bile secretion³³. But conjugated bilirubin level increases when

liver has lost at least half of its excretory capacity. Therefore, the presence of increased conjugated bilirubin is usually a sign of liver disease³⁴. Moreover, the unconjugated hyperbilirubinemia has been demonstrated in patients with non-alcoholic steatohepatitis (NASH) which may suggest the association of Gilbert's syndrome³⁵. This syndrome augments bilirubin production or decreases hepatic uptake or conjugation or both³⁴.

According to our results, significant increase in serum triglycerides, cholesterol and LDL levels, concomitant with significant decrease in serum HDL level, has been detected in the NAFLD group as compared to the healthy control group. These results coincide with those published by Adams et al.³⁶. Dyslipidemia has been found to increase oxidative stress due to increased levels of free fatty acids and triglycerides that render LDL less dense and more vulnerable to oxidation and uptake by macrophages³⁷. Cholesterol metabolism was associated with liver fat content independent of body weight, implying that the more fat the liver contains, the higher is cholesterol synthesis³⁸. Cellular cholesterol synthesis is regulated by activation of membrane bound transcription factors, designated sterol regulatory element-binding proteins (SREBPs) which are most abundant in the liver³⁹ and the excess of cellular cholesterol is esterified by the acyl CoA-cholesterol acyltransferase⁴⁰. The high level of cholesterol synthesis and the increased SREBP-2 activity have paradoxically been shown in subjects with NAFLD⁴¹.

In NAFLD disease, the ability of insulin to inhibit the production of very low density lipoproteins (VLDL) is impaired⁴². This results in hypertriglyceridemia which in turn leads to the reduction of HDL cholesterol concentration⁴³. NAFLD is strongly associated with insulin resistance which causes the inflammatory cytokine, tumor necrosis factor-alpha (TNF- α), to be over expressed in the liver. TNF- α has been found to activate cholesterol synthesis and inhibit cholesterol elimination through bile acids, which together contribute to the increased LDL-cholesterol and decreased HDL-cholesterol serum level⁴². The insufficient elimination of triglycerides, which is caused by hepatic insulin resistance⁴³ may also contribute to the development of NAFLD.

The present data revealed a significant decrease in serum adiponectin level in the NAFLD group compared with the healthy control group. Under normal condition adiponectin has been found in relatively high circulating levels but it has a decreased circulating level in patients with NAFLD and in clinical manifestations associated with insulin resistance such as metabolic syndrome and type 2 diabetes mellitus⁴⁵. In addition, plasma adiponectin levels correlated inversely with the markers of systemic oxidative stress. Many studies hypothesized that oxidative stress has an important contribution in conditions such as NAFLD and NASH due to the increased levels of free fatty acids and consequent increased levels of free radicals⁴⁶. In cultured adipocytes, under oxidative stress condition, the suppressed mRNA expression and



secretion of adiponectin were detected. Thus, the decreased serum adiponectin in NAFLD group could be attributed to the decreased gene expression level of adiponectin under this condition⁴⁷. The other suggested mechanism for decreasing serum adiponectin level is related to the increased expression of TNF- α as it has been found a decreased adiponectin mRNA expression in adipose tissue and the lower serum adiponectin levels in obese subjects. This could be explained by the increased expression of TNF- α which reduces adiponectin expression and secretion from adipocytes⁴⁸.

Serum leptin level exhibited significant increase in NAFLD group compared with the healthy control group as shown in the present study. Leptin is released into the circulation by mature adipocytes in response to changes in body fat mass and nutritional status. It has varied metabolic effects with the most significant of these being related to body weight and energy expenditure⁴⁹. In NAFLD patients, leptin levels are elevated and directly correlated with the severity of steatosis⁵⁰. The presence of hepatic steatosis, despite the presence of hyperleptinemia, is associated with the development of leptin resistance⁵¹. The increased leptin levels have been reported to be associated with the oxidative stress conditions resulting from the generation of reactive oxygen species (ROS) in accumulated fat. The elevated ROS in the adipose tissue leads to the increased adipose nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which dysregulates leptin production⁴⁷.

Significant increase in serum TNF- α , NF- κ B, Cox-2 and CD₄₀ levels has been detected in NAFLD group as compared to the healthy control group. In general, the increased oxidative stress and superoxide production decrease NO bioavailability and activates inflammatory responses of TNF- α ⁵². Higher oxidative stress status has been found in the liver of NAFLD patients which may lead to modulation of Kupffer cell function and increased serum levels of the Kupffer cell- and stellate cell-derived cytokines, such as NF- κ B⁵³, which then translocates from the cytoplasm to the nucleus to stimulate the downstream inflammatory cytokines (such as tumour necrosis factor- α (TNF- α)⁵⁴ perturbing the inflammatory cycle⁵⁵. The production of TNF- α and interleukin (IL)-1 has been found to increase the expression of Cox-2. Besides the enhanced hepatic injury and the enhanced serum ASL, ALT activities in the pristine NAFLD indicated the pristine hepatic inflammatory changes which stimulate Cox-2 expression⁵⁶. Circulating sCD₄₀ was believed to derive predominantly from platelets as a result of platelet activation. Lipid peroxidation and oxidative stress have been reported to induce platelet activation *via* TNF- α pathway (interaction of TNF- α with its receptors on platelet). Thus, oxidative stress plays a role in increasing platelet CD₄₀⁵⁷. This hypothesis has been confirmed based on the previous study indicating that (i) the up-regulation of platelet CD₄₀L expression is mediated by activation of NADPH oxidase, one of the most important cellular producers of reactive oxygen

species (ROS) and (ii) the activation of NADPH and the enhancement of oxidative stress are mediated by TNF α . TNF α upregulated platelet CD₄₀ *via* arachidonic acid-mediated oxidative stress⁵⁸.

Histopathological investigation of liver tissue sections of rats that fed high fat diet for 24 weeks (NAFLD group) showed moderate to severe macrovesicular fatty changes, which was diffusely distributed throughout the liver lobule accompanied with focal necrosis. Oxidative stress is considered to play a central role in the pathogenesis of NAFLD because the increased production of ROS is known to cause lipid peroxidation, followed by activation of the inflammatory response, and stellate cells, leading to fibrogenesis⁵⁹.

The most important histological characteristic of NAFLD is steatosis of hepatocytes. Steatosis of hepatocytes is classified into macrovesicular and microvesicular. Macrovesicular steatosis is characterized by large vacuoles occupying almost the entire cytoplasm that push the nucleus to the periphery of the cell. Microvesicular steatosis is characterized by multiple small lipid vacuoles, but the nucleus is located at the center of the cell. Typically, steatosis in NAFLD is centrilobular and macrovesicular, however, steatosis may be present throughout the lobule, and microvesicular steatosis may also be present. Steatosis in more than 5% of hepatocytes is necessary for a diagnosis of NAFLD⁶⁰. The histopathological results of the present study showed also microvesicular steatosis beside macrovesicular. Hepatic accumulation of triglycerides has been found to be associated with the development of macrovesicular steatosis of the liver⁶¹. The inhibition of mitochondrial fatty acid metabolism results in microvesicular steatosis⁶¹, and the accumulation of cytosolic triglycerides and phospholipids in the presence of initial mitochondrial damage lead to the development of a mixed type of liver steatosis over time.

The hepatoprotective activity of FCH extract and its defatted fraction has been documented in the present study by the significant inhibition of serum ALT activity and significant reduction of serum bilirubin level in treated NAFLD groups compared with the untreated group. Our results are in conformity with the previous studies Celik and Isik⁶² who reported that fennel has a hepatoprotective activity as it can protect the hepatic cells from the damaging impact of oxidative stress. The hepatoprotective potential of fennel could be attributed to its free radical scavenging property and antioxidant capacity as a result of the presence of phenolic compounds such as D-limonene which is responsible for the significant decrease in serum ALT activity⁶³. Moreover, D-limonene and β -myrcene present in fennel are active compounds with high capacity in decreasing levels of serum bilirubin³. D-limonene compound increases the concentration of reduced glutathione (GSH) in the liver⁶⁴. β -myrcene compound, on the other hand, elevates the levels of apoproteins CYP2B1 and CYP2B2,



which are subtypes of the P450 enzyme system⁹. The cytochrome P450 (CYP) enzyme system consists of superfamily of hemoproteins that catalyze the oxidative metabolism of a wide variety of exogenous chemicals including drugs, carcinogens, toxins and endogenous compounds such as steroids, fatty acids and prostaglandins¹⁰.

A lipid lowering effect afforded by FCH extract and its defatted fraction was likely attributable to the antioxidant potential of these natural products. The NAFLD groups treated with either FCH extract or its defatted fraction displayed significant reduction in serum cholesterol, triglycerides and LDL levels concomitant with significant elevation in serum HDL level with respect to the untreated NAFLD group. The antioxidant property of fennel includes total antioxidant capacity and free radical scavenging activity particularly superoxide anion radical and hydrogen peroxide radical. This property of fennel protects the liver against lipid peroxidation and free radical damaging impact which leads to the increased triglycerides, cholesterol and LDL levels⁶³. Thus, the anti lipid peroxidation capacity of fennel plays a key role in correcting lipid profile in NAFLD rats. As it has been demonstrated, free radicals diminish the cellular uptake of lipid from the blood. From another point of view, it has been reported that fennel, involved in herbal formulation, can delay upper gastrointestinal transit which promotes the reduction of fat and sugar absorption¹². These authors suggested that the constitution of fennel plays an important role in improving blood lipid profile *via* this way. The ability of FCH extract and its defatted fraction to replenish the decreased serum HDL level in the present study is similar to that of **Shahat et al.**⁶⁵ who demonstrated that fennel methanolic extract can significantly increase serum HDL level. This type of lipoprotein could stimulate the reverse cholesterol transport from the blood stream to the liver. Therefore, fennel could significantly decrease serum cholesterol, LDL and triglycerides and increase serum HDL levels. Adiponectin level is related to triglycerides level and are strong positive and independent determinants of HDL cholesterol. The current results demonstrated significant increase in serum adiponectin level upon treatment of NAFLD groups with either FCH extract or its defatted fraction compared with the untreated NAFLD group. This effect of fennel is possibly ascribed to the antioxidant activity of fennel active constituents which could decrease the signs of oxidative stress with consequent upregulation of adiponectin mRNA expression. Moreover, the ability of fennel's active ingredients to down-regulate TNF- α , which reduces adiponectin expression and secretion from adipocytes, might be another possible mechanism for enhancing serum adiponectin level⁴⁸.

Vitamin E in fennel plays an important role in health by inactivating harmful free radicals⁶⁶. Vitamin E could also inhibit NADPH oxidase activity with consequent reduction in leptin expression level⁶⁷. This explains the diminished

serum leptin level upon treatment of the NAFLD group with fennel extract or defatted fraction.

Various lines of evidences have suggested the anti-inflammatory activity of fennel. This property could be attributed to active constituents of fennel particularly D-limonene and β -myrcene. D-limonene is an effective inhibitor to macrophages which play a central role in overproduction of pro-inflammatory cytokines and inflammatory mediators such as TNF- α ⁶⁸. Meanwhile, β -myrcene has been found to directly reduce TNF- α expression⁶⁹. As mentioned above, TNF- α could upregulate platelet CD₄₀ *via* arachidonic acid-mediated oxidative stress. Thus, by suppressing the production of TNF- α , fennel could reduce serum level of CD₄₀ in the treated NAFLD group.

The suppressing effect of fennel on NF- κ B has been previously reported. This effect could be closely related to the presence of anethole compound in fennel. Anethole [1-methoxy-4-(1-propenyl) benzene] is one of the main constituents of fennel with a powerful anti-inflammatory activity⁷⁰. Anethole could repress NF- κ B-dependent gene expression induced by TNF- α . TNF- α induced NF- κ B activation through sequential interaction of the TNF receptor with TRADD, TRAF2, NIK, and IKK- β , and anethole with its antioxidant and antiinflammatory activities could phosphorylate I κ B α with consequent decrease in NF- κ B production⁷¹. In the same way, supporting evidences have suggested that in NAFLD groups treated with either FCH or its defatted.

Flavonoids in fennel have a potential role in inhibiting Cox-2 gene expression by altering NF- κ B pathway⁷². All together, the above mentioned mechanisms contribute to the reduced levels of the proinflammatory mediators (TNF- α , CD₄₀, NF- κ B and Cox-2)

Microscopic examination of liver tissue sections of rats in NAFLD groups treated with FCH extract or its defatted fraction showed significant reduction in fatty deposits in liver tissues and the reaction is negative in most areas of the lobules. Fennel is well known to have potent antioxidant activity. This activity includes total antioxidant capacity, free radical scavenging potential of superoxide anion radical and hydrogen peroxide radical thus protecting hepatic tissue property against lipid peroxidation and free radical damaging impact that lead to increased TG, cholesterol and LDL levels⁶³. Fennel rich with vitamin E, a potent fat-soluble antioxidant with a capacity to scavenge free radicals. Vitamin E could reduce aminotransferases levels and the reduction of aminotransferases has been suggested to improve hepatic histological alterations^{59,67}.

Noteworthy, the defatted fraction of fennel extract provided more pronounced effect in management of NAFLD in the present study. This could be ascribed to removing lipid fractions from the plant which increased its activity as therapeutic for NAFLD. This could be attributed to the effect of phenolic compound which

present in higher percentage in defatted extract than the total one.

The current study provides experimented evidence for the potential role of *Foeniculum vulgare* aerial part methanolic extract and or *Foeniculum vulgare*-defatted fraction in management of nonalcoholic fatty liver disease. The active constituents; flavonoids, D-limonene and β -myrcene in addition to vitamin E present in the aerial part extracts of this plant are probably responsible for this effect. These compounds proved to have hepatoprotective activity, hypolipidemic effect and anti-inflammatory property.

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