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Original Article

Dill extract attenuates liver fibrosis and steatohepatitis in obese rats *via* modulating TGF-β1 signaling and collagen accumulation

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Abstract

Background and purpose: Dill (*Anethum graveolens*) is an herbal plant from the Apiaceae family often used as an effective remedy for several ailments. This study aimed to investigate the potential protective effect of dill against the development of non-alcoholic fatty liver disease in obese rats.
Experimental approach: For 12 weeks, rats were fed a high-fat diet (HFD) to induce obesity. In the treatment group, the extract of dill leaves (100 mg/kg) was administered by gavage. Then, blood and liver samples were harvested for further investigations.
Findings/Results: Feeding HFD caused increased body mass index, abdominal circumference, adiposity index, weight gain, serum glucose, lipids, insulin, leptin, and insulin resistance. HFD-fed rats also showed increased hepatic triglycerides, fatty acid synthase, cytochrome P2E1, hydrogen peroxide, malondialdehyde, serum marker enzymes (AST, ALT, ALP, and GGT), and liver weight, with decreased antioxidants including superoxide dismutase, catalase, and glutathione. Besides, a significant elevation of hepatic interleukins 1β and 6, tumor necrosis factor-α, nuclear factor-kappa B, Kupfer cell markers (CD68 and CD163), fibronectin, and collagen type 1, along with an increase of transforming growth factor-β1 expression, was observed. Histological changes presented by hepatocytes, including ballooning, inflammatory cell aggregation, and deposition of collagen fibers, have also been detected. Co-administration of dill with HFD succeeded in reducing weight gain, hepatic triglyceride accumulation, oxidative reactions, inflammation, fibrosis, and liver structural injury.
Conclusion and implications: Dill extract could be approved as a promising therapeutic approach with multiple benefits for the management of obesity and associated steatohepatitis.

Keywords: Dill leaf extract; Fibrosis; Insulin resistance; Non-alcoholic fatty liver disease; Obesity; TGF-β1.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a growing health problem and a major cause of chronic liver disease worldwide (1). NAFLD refers to a variety of illnesses typically characterized by excessive accumulation of hepatic fat (steatosis) in the absence of chronic alcohol abuse and viral infection (2). Prevalence of NAFLD is about 20-30% in the general population and reaches 80-90% in the obese subjects (3). Obesity is currently considered the prime risk factor for developing NAFLD and its pathogenic switch to a more serious stage called non-alcoholic steatohepatitis (NASH) (4). Molecular mechanisms by which obesity affects the liver are complex and multifactorial; however, it is wieldy accepted that insulin resistance is a key mediator. In obese people, enlarged adipose tissue releases excess amounts of fatty acids sufficient to induce a cascade of metabolic alterations closely linked to insulin resistance (5).

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Studies have also demonstrated an association between insulin resistance and dyslipidemia, characterized by increased triglycerides (TGs) and low-density lipoprotein (LDL), with decreased high-density lipoprotein (HDL) in patients with fatty liver (6,7). This agrees with the fact that excess TGs eventually increase ectopic lipid deposition, especially in the liver, which is a hallmark of NAFLD (8). Once they occur, stored TGs provoke the generation of reactive oxygen species (ROS), ultimately leading to a pro-inflammatory milieu and progressive fibrosis (9). Normally, fibrosis is a wound healing process that preserves tissue integrity; however, sustained hepatic fibrosis becomes pathogenic, leading to evolution from steatosis to steatohepatitis, which is considered a late stage of liver disease (10). Hence, modulating hepatic fibrosis may offer therapeutic advantages for NASH.

Now, herbal remedies have drawn a lot of attention as a promising therapeutic option for a number of liver illnesses (11). *Anethum graveolens,* commonly known as dill, is a popular aromatic herb of the Apiaceae family, growing widely in the Mediterranean region, Europe, and West Asia (12). Dill has been used since ancient times for medicinal and food purposes (13). In traditional medicine, dill is applied for the management and prevention of gastrointestinal ailments (14) and breath problems (15). It has also been used as a carminative, preservative (16), antiviral (17), antifungal, and antibacterial agent (18). Moreover, dill is an abundant source of flavonoids and other phenolic compounds that possess several medicinal benefits, including anti-inflammatory, antidiabetic, antioxidant (14), and anticancer effects (13). Dill is also capable of stimulating milk flow in lactating women and curing urinary complaints (19). Besides, dill could help in lowering blood lipids (16) and in relieving cardiovascular diseases (20). Given these findings and the lack of data regarding its effect on obesity-related fatty liver, this study aimed to explore the protective potential of dill leaf extract against liver fat accumulation and progressive fibrosis in a rat model of dietary obesity.

MATERIALS AND METHODS

Chemicals

Cholesterol was purchased from Techno Pharmchem Company (New Delhi, India). Other chemicals had the highest grade of purity.

Plant collection and extract preparation

Fresh leaves of dill were obtained from the local market in Mansoura city and identified as *Anethum graveolens* (Voucher specimen No. Mans 2465) by an expert botanist from the Botany Department, Mansoura University, Egypt. Leaves were washed with clean water, air-dried for 10 days, and crushed with the help of an electric grinder. Powdered leaves (100 g) were soaked in 80% ethanol for 3 days. The extract was then filtered and concentrated using a rotary evaporator under reduced pressure at 40 °C. The yield of dry extract (8% w/w) was kept at 4 °C for further use (21).

Determination of phytochemical constituents of dill extract

The main phytochemical constituents of dill ethanolic extract were identified using standard analytical methods. The Folin-Ciocalteu assay method (22) was used to determine the total phenolic content, where a standard curve of gallic acid (GA) was used for calculating total phenolics. The results were expressed as milligrams of GA equivalent per gram of dry extract (mg GA/g dry extract). The content of flavonoids was assessed using the aluminum chloride method and a standard curve of catechin (CA). The results were expressed as milligrams of CA equivalent per gram dry extract (mg CA/g dry extract) (23). Total tannin content was determined following the procedure of vanillin-hydrochloride assay, and the attained value was expressed as milligrams of tannic acid equivalent per gram dry extract (mg TA/g dry extract) (24). Total saponin content was estimated using a sodium chloride solution for precipitation, and the yield of total saponin was determined as milligrams of saponin per gram dry extract (mg saponin/g dry extract) (25). Likewise, total alkaloids were assessed using a solution of sodium hydroxide for precipitation, and the residue of alkaloids was determined as milligrams of alkaloids per gram dry extract (mg alkaloids/g dry extract) (26).

Determination of antioxidant scavenging activity of dill extract

The antioxidant scavenging activity of dill extract was investigated based on the reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical and the decolorization of the violet DPPH• color using ascorbic acid as a standard (27). Different concentrations of the plant samples were prepared and mixed with DPPH• solution. After incubation in the dark at room temperature for 30 min, the absorbance of each sample was measured using a spectrophotometer at 517 nm. The antioxidant scavenging activity was expressed as %DPPH• inhibition and calculated using equation (1).

$$DPPH^{\bullet}\ inhibition\ (\%) = \left(\frac{(DPPH\bullet)_T}{(DPPH\bullet)_{T=0}}\right) \times 100 \quad (1)$$

where, T and T = 0 were the final and initial concentrations of DPPH•, respectively. Results were expressed as IC_{50} values, which were the concentration of antioxidants required to scavenge 50% of DPPH•.

Animals

Male Wistar rats (170 ± 10 g), obtained from the animal unit of VACSERA (Cairo, Egypt), were used in this study. Rats were kept in stainless steel cages under controlled temperature (23 ± 2 ºC) and a 12-h light/dark cycle, with free access to rodent diet and drinking water. Animals were weighed on the first and last days of the study to calculate the body weight gain. The study was carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and approved by Mansoura University Experimental Animal Ethics Committee (Ethical Code: Sci-Z-2020-14).

Experimental design and dietary protocol

Following a one-week acclimatization period, rats were randomly allocated into 5 groups (n = 6) as follows: group 1 served as control and received no treatment; group 2 was given distilled water (1 mL/kg) by gavage and served as a vehicle; group 3 was given dill extract (100 mg/kg, orally) dissolved in 1 mL of distilled water (21); group 4 was fed a high-fat diet (HFD) composed of 68% normal diet mixed with 30% animal abdominal fat and 2% cholesterol (28); and group 5 was given HFD plus dill extract in the same route and dose as the other groups. Groups 1-3 received normal diet. Dill extract dose (100 mg/kg) was selected based on Panda's study, demonstrating its effectiveness in maintaining normal glucose homeostasis, lipid metabolism, and oxidative status (21), which may afford protection against NAFLD. All treatments were continued consecutively for 12 weeks, and finally, before sacrifice, the anthropometric parameters were assessed for all rats under anesthesia by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (8 mg/kg) (29) purchased from El-Gomhouria Co. for trading chemicals and medical supplies (Mansoura, Egypt).

Anthropometric measurements

The abdominal circumference (AC) was measured on the largest region of the rat abdomen using a plastic tape with an accuracy of 0.1 cm, where the rat was positioned ventrally (30). To determine the body mass index (BMI), the body length (nose-anus length) and the body weight were used in the equation (2) (31).

$$BMI = \frac{Body\ weight\ (g)}{Body\ length\ square\ (cm^2)} \quad (2)$$

Collection of blood and tissue samples

One day later, rats were fasted overnight, anesthetized as described above, and sacrificed. Blood samples were collected, and sera were separated by centrifugation at 855 *g* for 15 min. Aliquots of sera were preserved at -20 °C for further analysis. Animals were then dissected, and the liver from each rat was removed, rinsed with ice-cold saline, and weighed. Specimens from the liver tissues were separated into two parts; the first was homogenized for various biochemical measurements, and the second was fixed in 10% neutral formaldehyde for histological investigations.

Adiposity index

Total body fat (retroperitoneal, visceral, epididymal, and subcutaneous) was collected, weighed, and divided by the body weight × 100 to calculate the adiposity index (32).

Biochemical measurements

Metabolic and hormonal parameters

Serum glucose, total lipids (TLs), total cholesterol (TC), and HDL cholesterol (HDL-C) were estimated utilizing colorimetric

assay kits (Bio-diagnostic Co., Egypt) with Cat. No. GL 1320, TL 2010, CH 1220, and CH 1230, respectively, as instructed by the manufacturer's protocol. Meanwhile, serum and hepatic TGs were estimated using commercially accessible colorimetric kits (Cat. No. TR 2030, Bio-diagnostic, Egypt). LDL cholesterol (LDL-C) was calculated by the equation (3) (33).

$$LDL-C = TC - (HDL-C) - \left(\frac{TG}{5}\right) \quad (3)$$

Also, very LDL-C (VLDL-C) was calculated using equation (4) (34).

$$VLDL-C = \frac{TGs}{5} \quad (4)$$

Serum insulin and leptin levels were estimated by the ELISA technique using kits purchased from ALPCO (Salem, NH, USA) and DRG instruments GmbH Co. (Germany) with Cat. No. 80-INSHU-E01.1 and EIA-2395, respectively. Insulin resistance (homeostasis model assessment of insulin resistance, HOMA-IR) and insulin sensitivity (quantitative insulin sensitivity check index, QUICKI) were estimated using equations (5) and (6) (35), respectively.

$$HOMA-IR = I_O \left(\mu\frac{IU}{mL}\right) \times \left(\frac{G_O \left(\frac{mg}{dL}\right)}{405}\right) \quad (5)$$

$$QUICKI = \frac{1}{\left(Log\, I_O \left(\frac{\mu IU}{mL}\right) + \log G_O \left(\frac{mg}{dL}\right)\right)} \quad (6)$$

where, I_O is the fasting serum insulin, and G_O represents the fasting glucose level.

Fatty acid synthase and nicotinamide adenine dinucleotide phosphate

Fatty acid synthase (FAS) and nicotinamide adenine dinucleotide phosphate (NADPH) were assayed in the liver using ELISA kits purchased from Cusabio (Baltimore, MD, USA) and MyBioSource (San Diego, California, USA) with Cat. No. CSB-E16440r and MBS169276, respectively.

Oxidative stress biomarkers

Liver cytochrome P450 2E1 (CYP2E1) was determined using an ELISA kit purchased from Cusabio (Cat. No. CSB-E09782r, Baltimore, MD, USA), while hydrogen peroxide (H_2O_2), malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) were estimated using colorimetric assay kits manufactured by Bio-diagnostic Co. (Egypt), with Cat. No. HP 2530, MD 2529, GR 2511, SD 2521, and CA 2517, respectively.

Liver function enzymes

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT) were determined in serum using commercial kits specific for liver function biomarkers (Bio-diagnostic, Egypt), with Cat. No. AL 1031, AS 1061, AP 1021, and GG 1041, respectively.

Inflammatory and fibrotic markers

Analysis of hepatic inflammatory cytokines, including interleukin-1β (IL-1β) and interleukin-6 (IL-6), was performed by ELISA kits from BioVision (Minneapolis, MN, USA) with Cat. No. ab214025 and K4145-100, respectively, while tumor necrosis factor-α (TNF-α) was measured using an ELISA kit obtained from ALPCO (Salem, NH, USA) with Cat. No. 30-9653. Nuclear factor-kappa β (NF-κβ) and Kupffer cell markers (kupf, CD163) were measured using ELISA kits obtained from Cusabio (Baltimore, MD, USA), with Cat. No. CSB-E13148r and CSB-E13297r, respectively. Meanwhile, the levels of fibrotic markers, including collagen type-1 (COL-1), fibronectin (FN), and transforming growth factor-β1 (TGF-β1) were determined in the liver using ELISA kits (Cusabio, Baltimore, MD, USA) with Cat. No. CSB-E08084r, CSB-E04553r, and CSB-E04727r, respectively, according to the instructions of the manufacturer's protocol.

Histopathological examination and morphometric measurements

Fixed liver specimens were dehydrated in an ascending alcohol series, cleared in xylene, and embedded in paraffin wax. Sections of 5 µm thickness were cut and stained with hematoxylin and eosin (H&E) to clarify general pathological alterations, while other sections were stained with Masson's trichrome (MTC) to demonstrate deposition of collagen fibers and the degree of liver fibrosis. The percent of areas occupied by

hepatocytes, ballooning, lipid droplets, inflammation, and deposition of collagen fibers were evaluated in each group at magnifications of ×400 (for H&E) and ×200 (for MTC) by Leica Qwin 500 image analyzer computer system (Leica image system Ltd., Cambridge, England), as described previously (36). Values were collected for each variable from 3 microscopic areas per slide and 3 slides per group.

Immunostaining and quantitative analysis of TGFβ-1

Before TGFβ-1 staining, liver sections were deparaffinized and hydrated. This was followed by adding 3% hydrogen peroxide to block endogenous peroxidase and immersing it in an antigen retrieval solution. Other non-specific binding may be inhibited using a blocking solution, phosphate-buffered solution (PBS) plus 10% normal goat serum. Next, liver sections were incubated with the primary antibody, TGF-β (Clone sc-146), diluted with PBS, then the slides were incubated with the biotinylated secondary antibody and streptavidin-peroxidase complex. Diaminobenzidine was added as a chromogen to give a brown reaction product, and Mayer's hematoxylin was used as a counterstain. TGFβ-1 expression levels were identified by the increased brown-colored staining versus normal controls (37). For evaluation of TGFβ-1 expression, the areas of positive cells with brown cytoplasmic staining in the liver of each group were measured using Leica Qwin 500 image analyzer computer system (Leica image system Ltd.; Cambridge, England), and the ratio of each stained area to the whole liver sections (area percent) was calculated at ×400 magnification (36). Microscopic images were taken 3 times from the whole stained slides, and values were collected from 3 slides per group.

Statistical analysis

Quantitative data were presented as mean ± standard error of mean (SEM) (n = 6), and analysed utilizing the GraphPad Prism 5.0 software prepared by GraphPad Software Inc.,(San Diego, CA, USA). The data were considered parametric and, therefore, the statistical comparisons were performed using one-way ANOVA followed by Tukey's post-hoc test. Values with $P < 0.05$ were considered statistically significant.

RESULTS

Assessment of phytochemical constituents

As shown in Table 1, phytochemical screening of dill extract showed a high concentration of total phenolics, total flavonoids, and tannins, varying from 74.21 mg GA/g dry extract, 32.47 mg CA/g dry extract, to 16.95 mg TA/g dry extract, respectively. Plant analysis also revealed a notable presence of saponins (42.45 mg saponins/g dry extract) and alkaloids (17.18 mg alkaloids/g dry extract).

DPPH• radical scavenging assay

As indicated in Table 1, dill extract demonstrated a potent radical scavenging action (IC_{50} = 0.11 mg/mL). It was noteworthy that the antioxidant impact was inversely correlated with the elimination of DPPH• and disappearance of violet color in the test samples, indicating that the higher the antioxidant activity, the lower the IC_{50} value (Table 1).

Table 1. Phytochemical analysis and scavenging activity of ethanolic dill extract. The scavenging activity of dill extract against DPPH• radical was expressed in the IC_{50} value.

Phytochemical constituents	Content
Phenolics (mg GA/g dry extract)	74.21
Flavonoids (mg CA/g dry extract)	32.47
Tannins (mg TA/g dry extract)	16.95
Saponins (mg saponins/g dry extract)	42.45
Alkaloids (mg alkaloids/g dry extract)	17.18
DPPH• (IC_{50} mg/mL)	0.11

Body weight gain, BMI, AC, adiposity index, and liver weight

Rats fed HFD were more likely to display obesity-related traits than control rats, as shown by a significant increase in body weight gain, BMI, AC, adiposity index, and liver weight compared to the control. The HFD + dill group revealed a significant reduction in body weight gain and other obesity-related indices compared to the HFD group. However, when normal rats were given dill extract, no significant changes were seen in the measured parameters (Table 2).

Serum glucose, insulin, leptin, insulin resistance, and insulin sensitivity

Rats fed on HFD displayed significantly increased serum levels of glucose, insulin, leptin, and insulin resistance, with decreased insulin sensitivity compared to the control group. Oral administration of dill extract to HFD-fed rats considerably reversed all these alterations; however, no detectable effects were shown when dill was given to untreated normal rats (Table 3).

Serum lipid profile

When comparing the HFD group to the control group, serum lipids (TLs, TC, TGs, LDL-C, and VLDL-C) were significantly elevated, whereas HDL-C was decreased. The administration of dill extract to the HFD-fed group resulted in a significant reduction in values of all lipid parameters except for HDL, -C, which demonstrates a significant rise compared to the non-treated group. No obvious changes were observed when dill extract was given orally to the normal untreated rats (Table 4).

Table 2. Body weight gain, BMI, AC, adiposity index, and liver weight in experimental groups. Data were presented as mean ± SEM, n = 6. The vehicle group was given distilled water (1 mL/kg) by gavage. Dill extract was administered at a dose of 100 mg/kg by gavage. $^{*}P < 0.05$ demonstrates significant difference compared with control group; $^{\#}P < 0.05$ versus HFD group.

Parameter	Control	Vehicle	Dill	HFD	HFD + dill
Body weight gain (g)	106.50 ± 4.54	106.31 ± 3.72	101.60 ± 3.88	209.30 ± 3.89*	144.11 ± 3.47$^{*\#}$
BMI (g/cm^2)	0.54 ± 0.01	0.570 ± 0.01	0.56 ± 0.013	0.75 ± 0.013*	0.66 ± 0.00$^{*\#}$
AC (cm)	14.88 ± 0.15	14.78 ± 0.27	14.87 ± 0.24	17.98 ± 0.31*	15.75 ± 0.34$^{\#}$
Adiposity index (%)	1.91 ± 0.16	1.83 ± 0.11	1.82 ± 0.08	6.56 ± 0.11*	3.05 ± 0.18$^{*\#}$
Liver weight (g)	6.08 ± 0.28	6.33 ± 0.28	6.30 ± 0.25	10.45 ± 0.32*	8.22 ± 0.47$^{*\#}$

HFD, High-fat diet; BMI, body mass index; AC, abdominal circumference.

Table 3. Serum glucose, insulin, leptin, insulin resistance, and insulin sensitivity in experimental groups. Data were expressed as mean ± SEM, n = 6. The vehicle group was given distilled water (1 mL/kg) by gavage. Dill extract was administered at a dose of 100 mg/kg by gavage. $^{*}P < 0.05$ demonstrates significant difference compared with control group; $^{\#}P < 0.05$ versus HFD group.

Parameter	Control	Vehicle	Dill	HFD	HFD + dill
Glucose (mg/dL)	90.42 ± 2.92	90.08 ± 3.98	89.5 ± 5.82	193.6 ± 10.25*	101.8 ± 3.86$^{\#}$
Insulin (µIU/mL)	58.72 ± 2.33	57.55 ± 2.30	57.53 ± 1.60	80.02 ± 5.05*	59.92 ± 2.81$^{\#}$
Leptin (ng/mL)	23.44 ± 0.51	22.62 ± 0.56	22.06 ± 0.70	38.18 ± 0.63*	28.12 ± 0.84$^{*\#}$
HOMA-IR (µIU × mg/dL)	13.15 ± 0.86	12.77 ± 0.66	12.72 ± 0.93	35.36 ± 3.78*	15.97 ± 0.84$^{\#}$
QUICKI (µIU × mg/dL)	0.26 ± 0.001	0.27 ± 0.002	0.27 ± 0.002	0.24 ± 0.003*	0.26 ± 0.002$^{\#}$

HFD, High-fat diet; HOMA-IR, insulin resistance; QUICKI, insulin sensitivity.

Table 4. Serum lipid profile in experimental groups. Data were expressed as mean ± SEM, n = 6. The vehicle group was given distilled water (1 mL/kg) by gavage. Dill extract was administered at a dose of 100 mg/kg by gavage. group. $^{*}P < 0.05$ demonstrates significant difference compared with control group; $^{\#}P < 0.05$ versus HFD group.

Parameter	Control	Vehicle	Dill	HFD	HFD + dill
TLs (mg/dL)	229.7 ± 10.93	227.3 ± 7.33	226 ± 7.22	515.3 ± 33.43*	349.2 ± 13.28$^{*\#}$
TC (mg/dL)	70.75 ± 6.51	69.32 ± 6.03	73.88 ± 4.01	250.00 ± 13.02*	118.20 ± 6.17$^{*\#}$
TGs (mg/dL)	78.23 ± 5.99	80.17 ± 6.21	78.35 ± 6.09	251.2 ± 13.85*	117.7 ± 7.83$^{*\#}$
HDL-C (mg/dL)	53.17 ± 2.96	52 ± 3.26	50.83 ± 2.83	29.17 ± 1.17*	40.50 ± 1.18$^{*\#}$
LDL-C (mg/dL)	17.19 ± 1.55	18.37 ± 2.42	16.97 ± 2.13	192.8 ± 3.59*	54.55 ± 3.93$^{*\#}$
VLDL-C (mg/dL)	18.67 ± 1.90	18.31 ± 1.81	18.43 ± 1.67	45.90 ± 3.01*	23.70 ± 2.68$^{\#}$

HFD, High-fat diet; TLs, total lipids; TC, total cholesterol; TGs, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL-C, very low-density lipoprotein cholesterol.

Hepatic TGs, FAS, and NADPH

Significant increases in the levels of hepatic TGs, FAS, and NADPH were demonstrated in HFD-fed rats compared to those of the normal diet. However, a reverse pattern of changes was exhibited following dill administration, where a marked reduction in all mentioned alterations was noticed compared to the HFD group. Indeed, after giving dill extract to the normal rats, no discernible changes were found (Fig. 1).

Hepatic oxidative stress and antioxidant biomarkers

HFD-fed rats displayed significantly elevated levels of CYP2E1, H_2O_2, and MDA, along with a significant reduction in the endogenous antioxidants (SOD, CAT, and GSH) when compared to the control group. However, when dill extract was given to the HFD group, it exhibited significant improvement in all tested parameters. No significant changes were noticed after giving dill extract to the normal rats (Table 5).

Hepatic function enzymes

Serum levels of liver enzymes, including ALT, AST, ALP, and GGT, were elevated in the HFD-fed rats compared to the control group. Indeed, the alterations significantly reduced when HFD-fed rats were given dill extract. Normal rats receiving dill extract showed no significant changes when compared to the control group (Table 6).

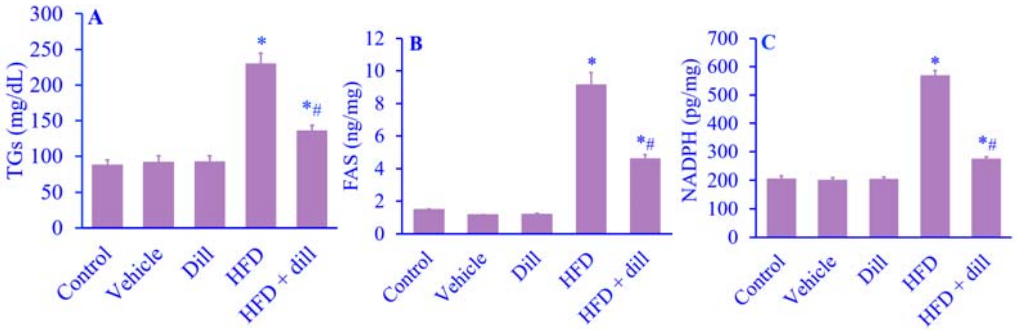

Fig. 1. Hepatic (A) TGs, (B) FAS, and (C) NADPH in experimental groups. Data were presented as mean ± SEM, n = 6. The vehicle group was given distilled water (1 mL/kg) by gavage. Dill extract was administered at a dose of 100 mg/kg by gavage. $^{*}P < 0.05$ demonstrates significant difference compared with control group; $^{\#}P < 0.05$ versus HFD group. TGs, Triglycerides; FAS, fatty acid synthase; NADPH, nicotinamide adenine dinucleotide phosphate; HFD, high-fat diet.

Table 5. Hepatic oxidative stress and antioxidant biomarkers in experimental groups. Data were expressed as mean ± SEM, n = 6. The vehicle group was given distilled water (1 mL/kg) by gavage. Dill extract was administered at a dose of 100 mg/kg by gavage. $^{*}P < 0.05$ demonstrates significant difference compared with control group; $^{\#}P < 0.05$ versus HFD group.

Parameter	Control	Vehicle	Dill	HFD	HFD + dill
CYP2E1 (ng/mg)	1.32 ± 0.12	1.30 ± 0.11	1.22 ± 0.11	7.00 ± 0.39*	3.21 ± 0.26$^{*\#}$
H_2O_2 (mM/g)	3.65 ± 0.02	3.28 ± 0.02	3.18 ± 0.07	13.56 ± 0.42*	6.67 ± 0.13$^{*\#}$
MDA (nmol/g)	831.1 ± 7.364	831 ± 3.606	816.1 ± 5.982	1143 ± 37.69*	928.4 ± 16.37$^{*\#}$
SOD (U/g)	178.6 ± 1.34	178.8 ± 1.14	186.3 ± 3.11	123.8 ± 2.46*	153.4 ± 3.34$^{*\#}$
CAT (U/g)	193.5 ± 1.08	195.1 ± 1.21	195.9 ± 2.12	114.5 ± 3.11*	172.5 ± 3.39$^{*\#}$
GSH (mg/g)	4.54 ± 0.01	4.56 ± 0.01	4.61 ± 0.02	1.49 ± 0.05*	3.43 ± 0.19$^{*\#}$

HFD, High-fat diet; CYP2E1, cytochrome P450 2E1; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GSH, glutathione.

Table 6. Liver function enzymes in the experimental groups. Data were expressed as mean ± SEM, n = 6. The vehicle group was given distilled water (1 mL/kg) by gavage. Dill extract was administered at a dose of 100 mg/kg by gavage. $^{*}P < 0.05$ demonstrates significant difference compared with control group; $^{\#}P < 0.05$ versus HFD group.

Parameter	Control	Vehicle	Dill	HFD	HFD + dill
ALT (U/L)	36.25 ± 2.02	37.42 ± 2.89	37.92 ± 3.12	72.50 ± 2.21*	47.25 ± 2.09$^{*\#}$
AST (U/L)	31.58 ± 1.15	33.83 ± 1.78	32.50 ± 1.88	120.80 ± 7.02*	46.83 ± 0.79$^{*\#}$
ALP (U/L)	207.9 ± 12.38	207.30 ± 12.43	206.10 ± 10.16	305.80 ± 3.61*	253.20 ± 2.50$^{*\#}$
GGT (U/L)	23.75 ± 2.83	24.00 ± 3.14	22.83 ± 2.99	63.67 ± 4.43*	44.83 ± 3.07$^{*\#}$

HFD, High-fat diet; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase.

Hepatic NF-κβ, inflammatory cytokines, and Kupffer cell markers

As shown in Fig. 2, HFD-fed rats exhibited prominent hepatic inflammation detected by increased levels of NF-κβ, inflammatory cytokines (TNF-α, IL-1β, and IL-6), and Kupffer cell markers (CD68 and CD163) compared to the control group. Nevertheless, oral administration of dill extract succeeded in downregulating HFD-induced inflammatory response compared to the HFD group. Meanwhile, no significant alterations were recorded when normal rats received dill extract.

Hepatic fibrotic markers

The obtained results showed significantly elevated fibrotic markers (TGF-β1, FN, and COL-1) in the liver of HFD-fed rats compared to the control group; however, all these alterations were ameliorated following administration of dill extract. Indeed, no significant changes were observed in levels of TGF-β1, FN, and COL-1 in normal rats that received dill extract (Fig. 3).

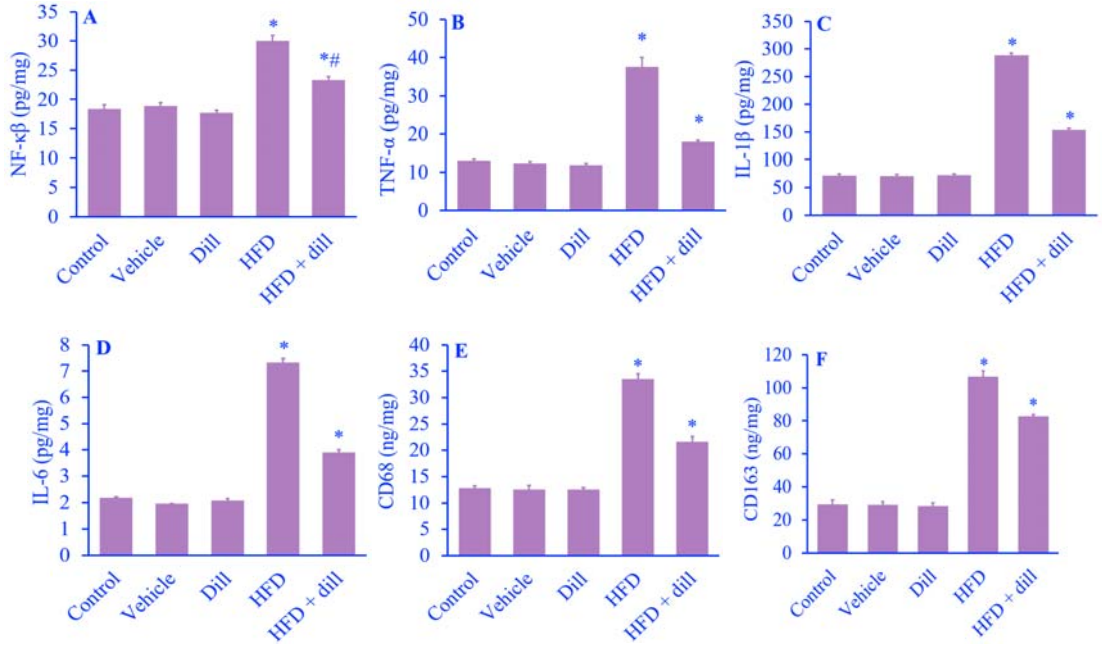

Fig. 2. Hepatic (A) NF-κβ, (B) TNF-α, (C) IL-1β, (D) IL-6, (E) CD68, and (F) CD163 in experimental groups. Data were presented as mean ± SEM, n = 6. The vehicle group was given distilled water (1 mL/kg) by gavage. Dill extract was administered at a dose of 100 mg/kg by gavage. $^{*}P < 0.05$ demonstrates significant difference compared with control group; $^{\#}P < 0.05$ versus HFD group. NF-κβ, Nuclear factor-kappa β; TNF-α, tumor necrosis factor-α; IL, interleukin; HFD, high-fat diet.

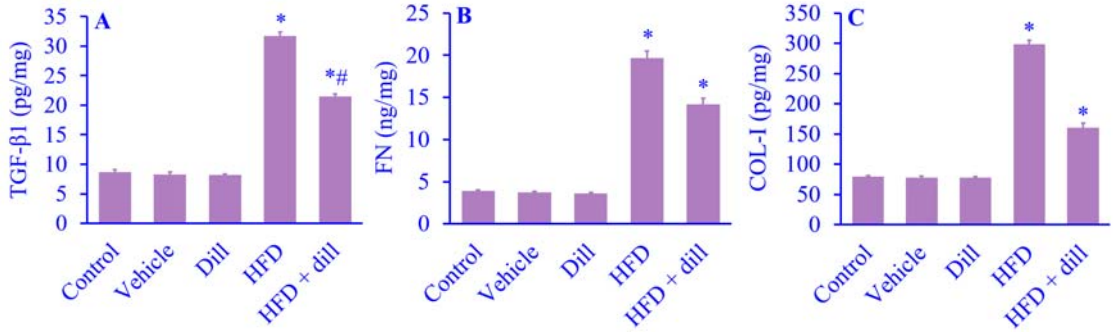

Fig. 3. Hepatic (A) TGF-β1, (B) FN, and (C) COL-1 in experimental groups. Data were presented as mean ± SEM, n = 6. The vehicle group was given distilled water (1 mL/kg) by gavage. Dill extract was administered at a dose of 100 mg/kg by gavage. $^{*}P < 0.05$ demonstrates significant difference compared with control group; $^{\#}P < 0.05$ versus HFD group. TGF-β1, Transforming growth factor-β1; FN, fibronectin; COL-1, collagen type-1; HFD, high-fat diet.

Histopathological characterization of hepatic lesions

Histopathological examination of H&E-stained liver sections from control (Fig. 4A), vehicle (Fig. 4B), and dill (Fig. 4C) groups showed normal architecture of hepatic lobules with distinct hepatocytes (H) arranged into cords radiate out from central vein (CV), and separated by blood sinusoids (BS), with several scattered Kupffer cells reside within the lumen (arrowhead). By contrast, the liver of the HFD group (Fig. 4D) showed disrupted architecture, where some hepatocytes appeared ballooned with peripheral nuclei (arrow), and others showed vacuolated cytoplasm filled with enlarged lipid droplets (LD). Dilated central veins (DCV) and blood sinusoids (DBS), with activated Kupffer cells (arrowhead) and inflammatory cells aggregation (curved arrow) were also observed. However, all these alterations exhibited a near normal appearance following dill administration to the HFD group (Fig. 4E), except for the presence of some LD with a small size.

Examination of MTC-stained sections showed small amounts of collagen fibers around the central vein (curved arrow) and portal area (arrow) in control (Fig. 5A), vehicle (Fig. 5B), and dill (Fig. 5C) groups. However, in the HFD group (Fig. 5D), extensive deposition of collagen fibers was observed throughout the whole liver section. On the other hand, these changes were markedly reduced in the HFD + dill group (Fig. 5E).

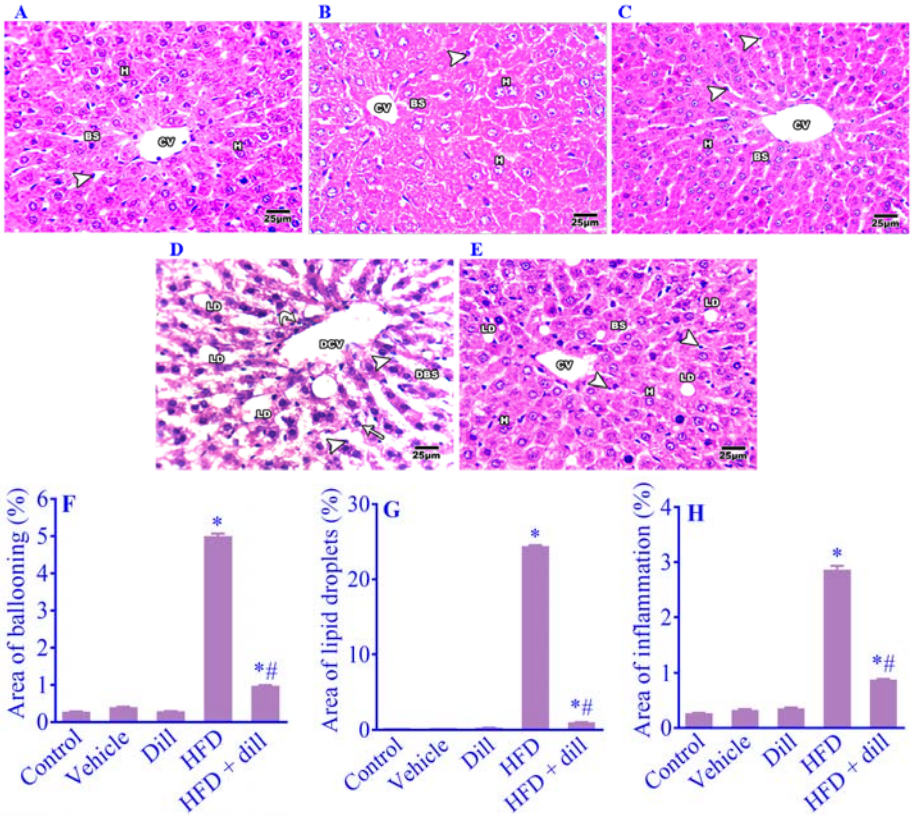

Fig. 4. Photomicrographs of liver sections in (A) control, (B) vehicle, (C) dill, (D) HFD, (E) HFD + dill groups. The vehicle group was given distilled water (1 mL/kg) by gavage. Dill extract was administered at a dose of 100 mg/kg by gavage. Tissue evaluation demonstrates normal architecture of hepatocytes, central vein, blood sinusoids, and Kupffer cells (arrowhead) in groups receiving a normal diet. HFD alone shows dilated central vein and blood sinusoids with hepatocytes ballooning (arrow), vacuolated cytoplasm filled with lipid droplets, activated Kupffer cells (arrowhead) and inflammatory cells aggregation (curved arrow). Administration of dill to HFD-fed rats showed near-normal appearance of hepatocytes, central vein, blood sinusoids, and Kupffer cells (arrowhead) with few and small lipid droplets. Histomorphometric results, including % of (F) ballooning, (G) lipid droplets, and (H) inflammation in the liver of different animal groups using image analysis. Data were expressed as mean ± SEM, n = 6. $^{*}P < 0.05$ demonstrates significant difference compared with control group; $^{\#}P < 0.05$ versus HFD group. HFD, High-fat diet; H, hepatocytes; CV, central vein; BS, blood sinusoids; LD, lipid droplets; DCV, dilated central vein.

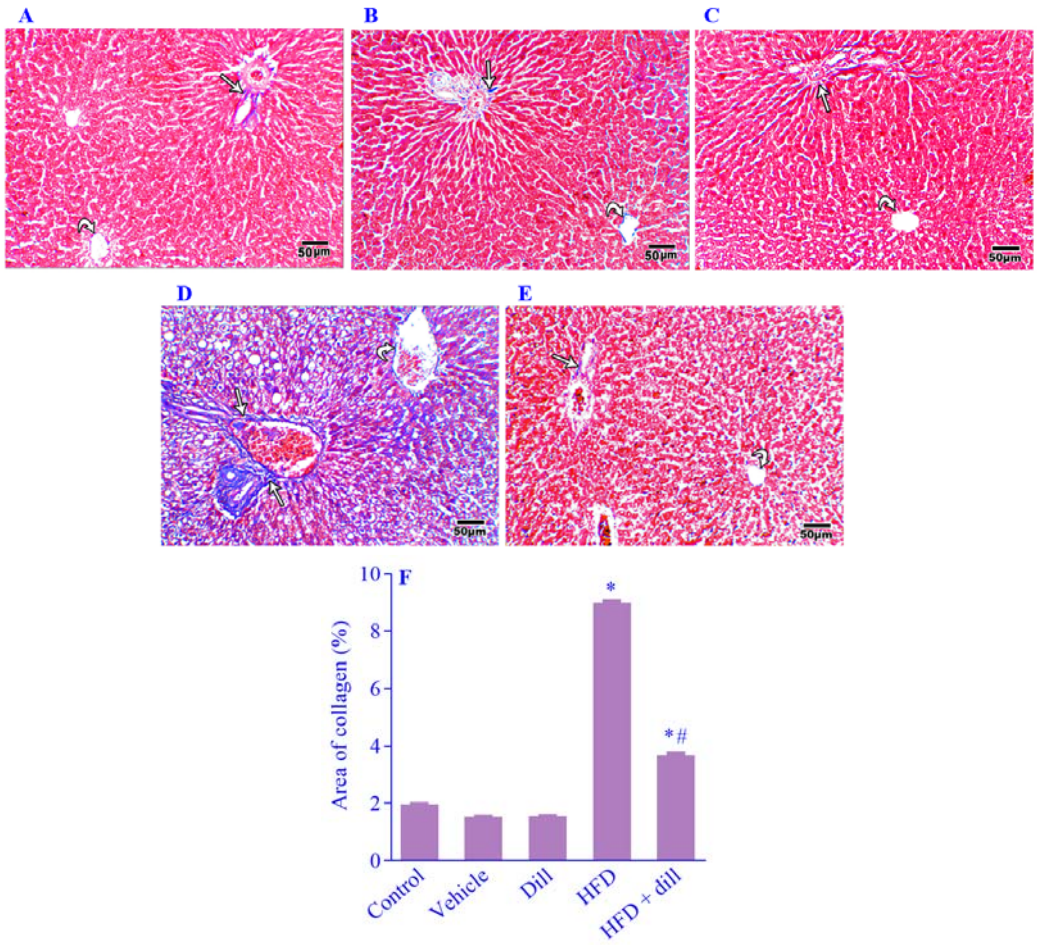

Fig. 5. Photomicrographs of Masson′s trichrome-stained liver sections in (A) control, (B) vehicle, (C) dill, (D) HFD, and (E) HFD + dill groups. The vehicle group was given distilled water (1 mL/kg) by gavage. Dill extract was administered at a dose of 100 mg/kg by gavage. Tissue evaluation illustrates normal distribution of bluish collagen fibers around the central vein (curved arrow) and portal area (arrow) in groups receiving a normal diet. HFD alone shows an increase in collagen fiber deposition around the central vein (curved arrow) and portal area (arrow), whilst administration of dill to HFD-fed rats shows a marked reduction in collagen fiber deposition around the central vein (curved arrow) and portal area (arrow). Morphometric analysis, including (F) the area percent of collagen deposition in the liver of different animal groups using image analysis. Data were expressed as mean ± SEM, n = 6. $^{*}P < 0.05$ demonstrates significant difference compared with control group; $^{\#}P < 0.05$ versus HFD group. HFD, High-fat diet.

Histomorphometric results

Morphometric analysis of hepatic lesions in the HFD group demonstrated a significant increase in the area percent of hepatocytes ballooning (Fig. 4F), LD (Fig. 4G), and inflammation (Fig. 4H), compared to the control group. However, there was significant reduction in the area percent of all assessed variables including hepatocytes ballooning (Fig. 4F), LD (Fig. 4G), and inflammation (Fig. 4H) in the HFD-fed group with dill administration (0.98%, 1.02%, and 0.88%, respectively) compared to HFD group (4.99%, 24.4%, and 2.86%, respectively). Besides, a significant elevation in the area percent of collagen deposition in the hepatic tissue of the HFD group (8.97%) was remarked compared to the control group (1.97%) (Fig. 5F). Indeed, dill administration during HFD feeding exhibited a significant reduction (3.67%) in the area percent of collagen deposition compared to the non-treated HFD-fed rats (8.97%) (Fig. 5F).

Quantitative assessment of TGF-β1 expression

Immunohistochemical staining of liver sections from control (Fig. 6A), vehicle (Fig. 6B), and dill (Fig. 6C) groups showed

non-detectable TGF-β1 expression; however, hepatic sections obtained from the HFD group (Fig. 6D) revealed strong TGF-β1 immunoexpression, indicating intense fibrogenic activity. Meanwhile, hepatic sections of the HFD + dill group (Fig. 6E) displayed weak TGF-β1 expression, suggesting anti-fibrotic activity of the used plant extract. Quantitative morphometric measurements using image analysis supported the observations, where the area percent of TGF-β1 expression was significantly increased in the HFD group (7.33%) (Fig. 6F) compared to the control animals (0.05%); however, administration of HFD group with dill extract showed significant reduction in the area percent of TGF-β1 expression (2.75%) compared to the HFD group (7.33%) (Fig. 6F).

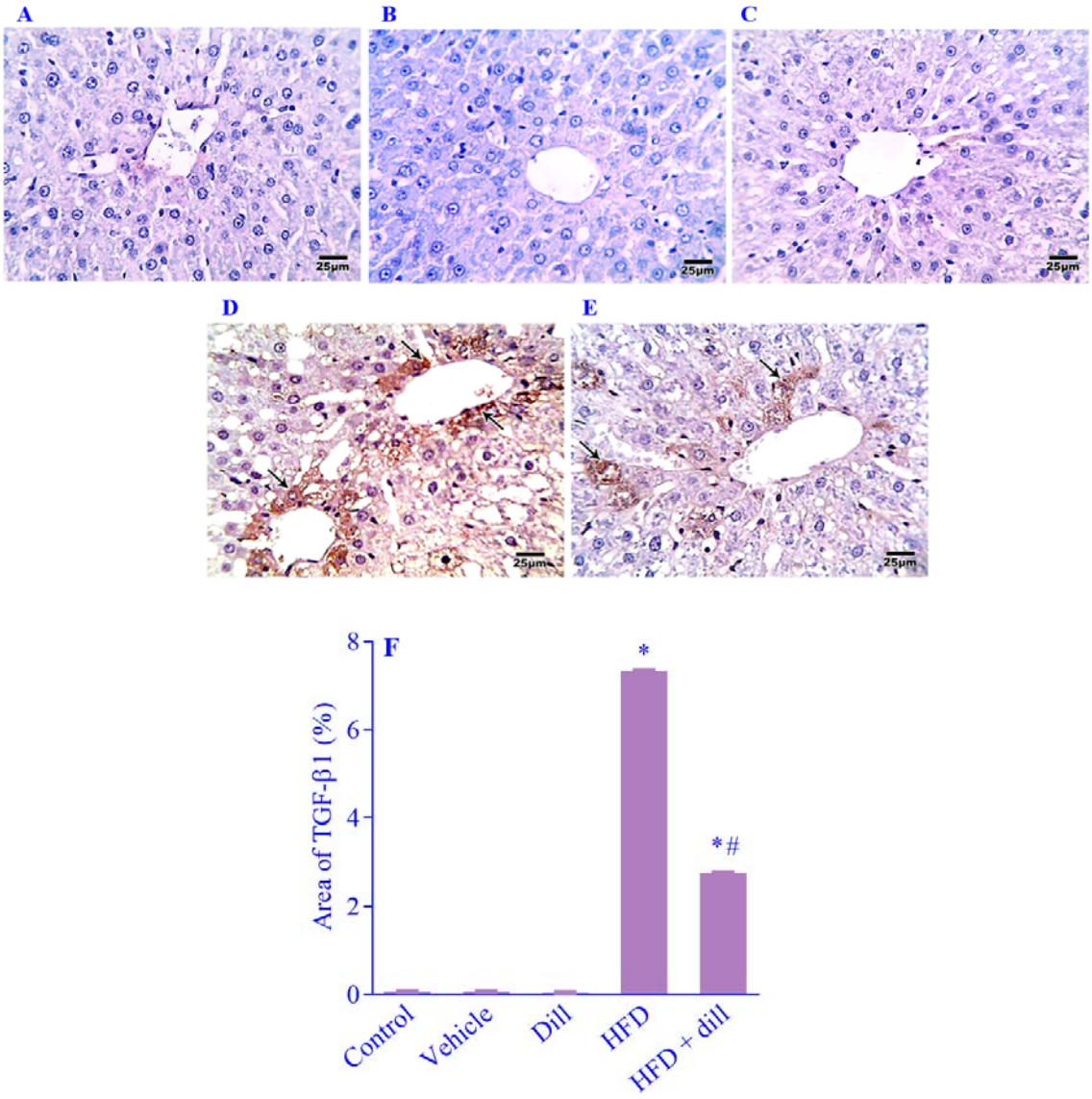

Fig. 6. Photomicrographs of TGF-β1 expression in hepatic sections of (A) control, (B) vehicle, (C) dill, (D) HFD, and (E) HFD + dill groups. The vehicle group was given distilled water (1 mL/kg) by gavage. Dill extract was administered at a dose of 100 mg/kg by gavage. Groups fed by a normal diet show negatively immunostained hepatocytes, while the HFD alone shows strong TGF-β1 immunoreactivity (arrow). Indeed, the HFD+ Dill group reveals a weak TGF-β1-stained hepatocyte (arrow). (F) The area percent of TGF-β1 expression in the liver of different animal groups using image analysis. Data were expressed as mean ± SEM, n = 6. $^{*}P < 0.05$ demonstrates significant difference compared with control group; $^{\#}P < 0.05$ versus HFD group. HFD, High-fat diet; TGF-β1, transforming growth factor-β1.

DISCUSSION

Excessive consumption of dietary fat may confer a higher risk of developing obesity and NAFLD. Although considerable studies have been conducted in this regard, there is still a critical need for more research (38). Herein, feeding rats an HFD for 12 weeks caused significantly elevated BMI, waist circumference, adiposity index, and weight gain compared to the control group. Dietary fats also contribute to various metabolic changes, including hyperglycemia, hyperinsulinemia, insulin resistance, and impaired insulin sensitivity. Meanwhile, a marked elevation of serum TGs, TC, LDL-C, and VLDL-C, with a reduction of HDL-C levels, was observed, which together may contribute to the development of obesity (39). Consumption of dill extract by HFD-fed rats was found to be effective in managing obesity, insulin resistance, hyperglycemia, and impaired lipid metabolism compared to the HFD group. The impact of dill may be derived from its abundant presence of flavonoids known for their ability to decrease weight gain (40) and maintain normal metabolic homeostasis through improving insulin sensitivity (41), suggesting a strong metabolic regulatory effect of dill extract.

Notably, obesity is generally associated with adipocyte hypertrophy accompanied by increased levels of circulating free fatty acids (FFAs) and reduced glucose uptake by muscles, resulting in a state of hyperglycemia (42). This can promote compensatory hyperinsulinemia, downregulation of insulin receptors, and systemic insulin resistance, which in turn stimulates FFAs flux into the liver and increased storage of TGs (43). This hypothesis was confirmed by results from a human study, in which insulin resistance and hyperlipidemia have been considered comorbidities primarily linked to increased risk of developing fatty liver in the obese subjects (44). An experimental study has also demonstrated that excess adipose tissue and insulin resistance are closely involved in the development of hepatic steatosis in an animal model of high-fat feeding (45). In the present study, HFD-fed rats experienced notable features of hepatic steatosis *via* significant elevation of hepatic TGs, area percent of LD, and hepatic ballooning, besides an increased liver weight, which are compatible with the earlier findings of Radhakrishnan *et al*. (46). In addition, the increased levels of liver enzymes (AST, ALT, ALP, and GGT) were observed when rats were fed on HFD compared to those with normal diet. These results agreed with data from the study of Vadivelu *et al*. (47), who demonstrated elevated levels of hepatic enzymes in the blood following chronic feeding on HFD. The underlying mechanism could be related to the fact that excess TGs in the liver may cause cellular damage and leakage of enzymes from the cytosol into the bloodstream (43). However, when dill extract was given to the HFD group, it successfully protected against increased hepatic TGs, liver weight, histological alterations, and enzyme leakage, mostly due to the ability of the plant flavonoids to inhibit precursors of TGs synthesis, such as acetyl-CoA and glycerol phosphate (48), thereby obstructing the development of hepatic steatosis.

Apart from direct fat uptake, *de novo* lipogenesis (DNL) may also contribute to hepatic steatosis (49). FAS is a major enzyme in the *de novo* synthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA, a process that utilizes NADPH for providing reducing equivalents (50). When nutrients are present in excess, FAS appears to catalyze production of TGs for storage in the liver or secretion in the circulation through VLDL particles (51). Numerous hormones are recognized to control the transcriptional level of FAS. Among these, leptin is a specific adipocyte-derived hormone that regulates appetite and energy expenditure and is known to be affected by obesity *via* disrupted leptin signaling, ultimately leading to hyperleptinemia and leptin resistance (52). This cascade consequently leads to high levels of FAS expression, thereby producing increased serum TGs and may also trigger hepatic fatty acid synthesis in response to dietary fats (53). Accordingly, the presently observed elevation of leptin secretion, FAS activity, and NADPH production could be a strong driver of lipogenesis and storage of excess TGs in the liver of obese rats, which were attenuated following dill administration. This effect can be explained by the presence of saponins as one of the plant constituents, which are important in improving leptin signaling pathways and decreasing the rate of its secretion in obese states (54). Former investigation has also reported high ability of flavonoids to reduce hepatic lipid accumulation by suppressing FAS thereby inhibiting denovo lipogenesis (55), suggesting their potential in restricting hepatic lipogenesis and availability of TGs.

Now, it is widely accepted that hepatic lipid accumulation is a key factor in creating a state of

oxidative stress that drives the development of NASH from simple steatosis (56). Evidence revealed that increased fat uptake by hepatocytes causes mitochondrial dysfunction, with an increase of non-metabolized fatty acids contributing to ROS overproduction (57). This effect is mediated in large part by promoting the activity of CYP2E1, a member of the cytochrome P450 oxidoreductase family, serving to oxidize fatty acids with subsequent production of ROS such as H_2O_2 (58). Evidently, mitochondrial CYP2E1, which is a potential source of ROS, is increased in an experimental model of NASH and in NASH patients (59,60). It has also demonstrated that decreased activity of several detoxifying enzymes may lead to increased ROS production and a high susceptibility to developing NASH (61). Similar observations have been provided by the present study through significant elevation of oxidative stress markers (NADPH oxidase, H2O2, and MDA), with accompanying depletion of endogenous antioxidants (SOD, CAT, and GSH) in the liver of HFD-fed rats compared to the control group, indicating increased oxidative stress as a part of the underlying mechanisms of NASH. Former investigations have also revealed that increased production of ROS and lipid peroxidation reactive aldehydes, such as MDA, promotes cell injury and the development of fatty liver (62). Interestingly, dill administration favorably affects HFD-induced oxidative stress, thereby neutralizing the generation of free radicals and accumulation of MDA. Results may be attributed to the presence of several phenolic constituents, primarily flavonoids, which are known to possess strong antioxidant properties and reducing power (57) as observed herein by the strong radical scavenging activity of DPPH•. Evidently, flavonoids are closely involved in cellular defense against oxidative reactions *via* the presence of attached hydroxyl groups ($OH^•$) (63). The use of flavonoids was found to decrease lipid peroxidation, increase activities of ROS detoxifying enzymes, and improve hepatic oxidative stress in rats fed an HFD (64). Moreover, flavonoids have been described for their potential to reduce the risk of NAFLD through inhibiting CYP2E1 activity and improving mitochondrial dysfunction (58). Therefore, dill can be recommended for its effectiveness in preventing cellular redox imbalance and oxidative damage.

Numerous studies have found an association between obesity and chronic inflammation, which may be explained by infiltration of macrophages into enlarged adipose tissue (65) and increased production of proinflammatory cytokines that closely contribute to the development of NASH (66). These findings have been similarly elucidated by other studies, in which excess adipose tissue with increased proinflammatory cytokines (TNF-α and IL-6) plays a crucial role in promoting inflammatory reactions often present during the development of NASH (67,68). It has also been indicated that inflammatory cytokines may develop NASH *via* multiple mediators or interactions with other pathways. In this view, TNF-α has been recognized to play a central role in the development of NASH through the release of additional cytokines, including IL-1 and IL-6 (67). Moreover, TNF-α induces systemic insulin resistance by interfering with insulin signaling pathways, which ultimately contribute to the deposition of excess TGs and liver damage (69). Another study suggested hepatic fat accumulation as a driving force in initiating sustained inflammatory response through activation of resident macrophages called Kupffer cells (70). Signaling from Kupffer cells triggers NF-κβ activation and production of a large number of cytokines contributing to hepatic inflammation. These events are further supported by the fact that activated Kupffer cells express increased surface receptors, such as CD68 and CD163, that worsen fatty liver disease in the obese populations (71). Similar outcomes have been observed by the current data *via* increased levels of NF-κβ, proinflammatory cytokines (TNF-α, IL-1β, and IL-6), and Kupffer cell markers (CD68 and CD163) in the liver of the HFD group compared to the control animals. This can be further reinforced *via* the presently observed histological alterations characterized by dilated central veins and blood sinusoids, with activated Kupffer cells and inflammatory cell aggregation, indicating provoked inflammation and progression of liver injury (72). Oral consumption of dill extract has been shown to downregulate HFD-induced inflammatory response, which may be related to the anti-inflammatory activities of the plant alkaloids (73). Administration of alkaloids to HFD-fed rodents, as a model of fatty liver, has been shown to decrease the expression of IL-6 and TNF-α, which may occur through regulation of the NF-κB pathway. Other dill constituents, mainly saponins, were identified as a potent anti-inflammatory substance. Similar evidence has been provided by using radix roots and flowers of ginseng, which are rich in saponins and can inhibit the activity of several pro-inflammatory cytokines in various

experimental models (74). Hence, dill extract could be prescribed as a natural remedy for targeting hepatic inflammation.

Rather than, inflammation has also emerged as a key factor in promoting fibrosis and evolution to end-stage liver disease (75). In obese individuals, activated Kupffer cells provoke the production of fibrotic cytokines such as TGF-β1, which in turn causes activation of hepatic stellate cells into myofibroblasts, leading to excessive production of extracellular matrix proteins, mostly COL-1. Availability of COL-1 is also stimulated by FN, which is a fibrotic protein produced in response to TGF-β1 and is often implicated in the regulation of wound healing. This cascade gives rise to an increase in scar tissue that cannot be self-repaired, thereby blocking or limiting blood flow within the liver, which promotes further tissue scarring and fibrosis (76). Similarly, the present study achieved a marked increase in hepatic expression and mean levels of TGF-β1 following HFD feeding, thus promoting increased production of FN and COL-1 compared to the control group. Histopathological examination of MTC-stained liver sections from the HFD group also showed excessive deposition of COL-1 fibers that were morphometrically detected *via* a higher percentage area, indicating progression to advanced fibrotic stages and severe liver injury. Hence, it can be suggested that reducing TGF-β1 by regular dill consumption could protect against the progression of NASH by improving effects on elevated fibrotic markers and hepatic structural injury. This effect may be attributed to the plant alkaloids, which are effective in preventing fibrosis by suppressing collagen gene transcription (77). The plant flavonoids can also alleviate hepatic fibrosis in rats by regulating the TGF-β1 signaling pathway. Thus, dill extract can be confirmed as a potent hepatoprotective therapy with a high ability to overcome hepatic fibrosis (78).

CONCLUSION

Collectively, the extract of dill leaves succeeded in protecting against obesity and associated steatohepatitis, mostly *via* targeting insulin resistance, metabolic abnormalities, and accumulation of hepatic TGs, resulting in suppressed redox imbalance, inflammation, and advanced fibrosis. Thus, dill extract can be considered a good therapeutic strategy in cases of obesity and related liver diseases. However, further investigations into the molecular mechanisms by which dill exerts its hepatoprotective effects can offer more pronounced insights.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

A. El-Wakf designed the experimental protocol; T. Obaid conducted the experiments and collected the data; R. Elmougy and T. Obaid performed the analysis and evaluated the data; A. El-Wakf and R. Elmougy wrote and revised the manuscript. All authors have read and approved the finalized article. Each author has fulfilled the authorship criteria and affirmed that this article represents honest and original work.

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