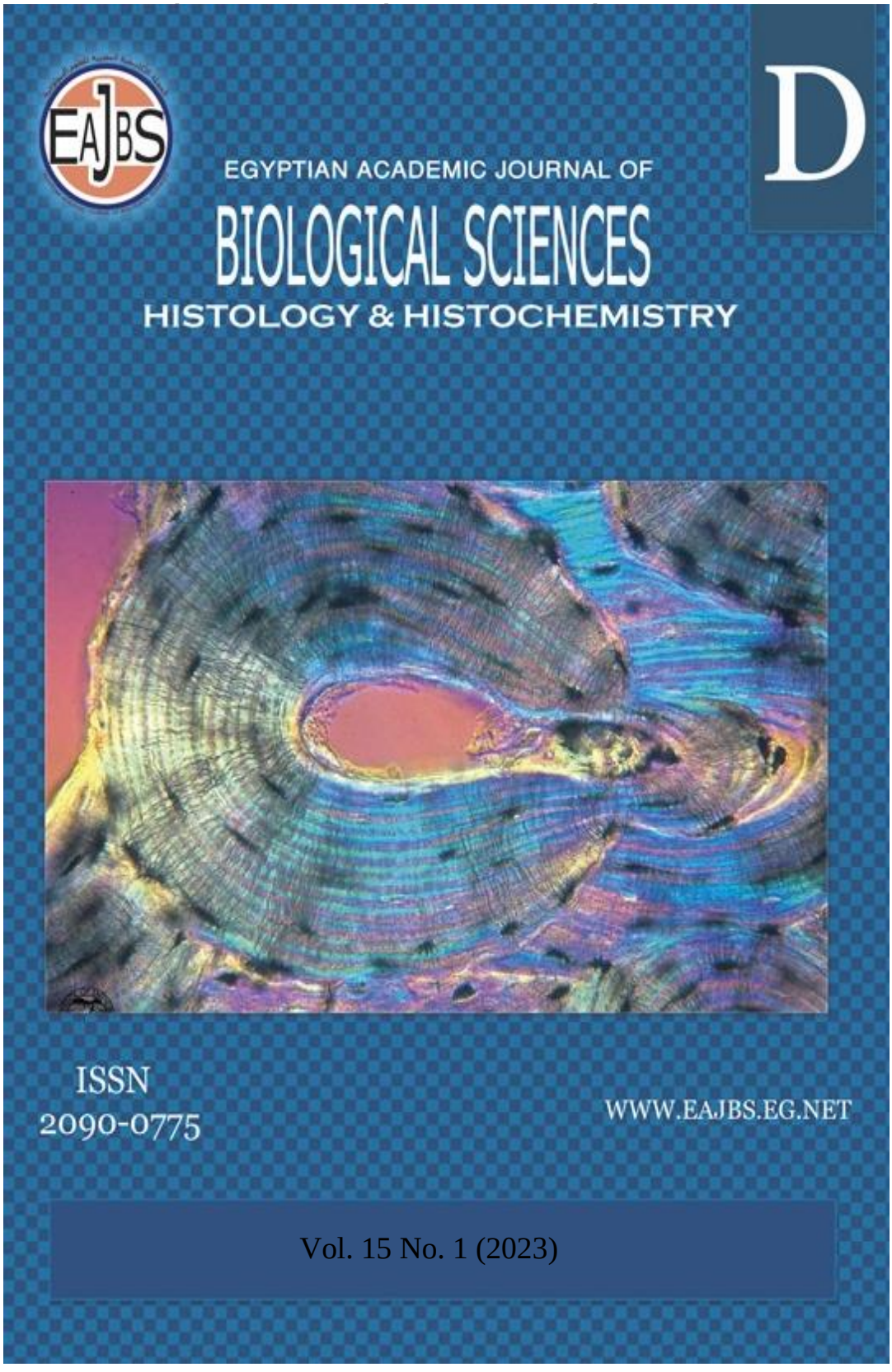

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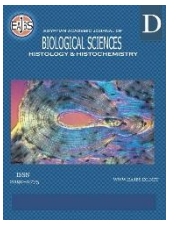

Effect of Flaxseed on Experimentally Induced Non-Alcoholic Fatty Liver Disease in Albino Rats

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ABSTRACT

Background: Nonalcoholic fatty liver disease is now known to be the most frequent chronic liver disease worldwide. **Aim of work:** Evaluate the progress of fibrosis in experimentally induced NAFLD in albino rats and the potential protective effect of the flaxseed using biochemical, histopathological and immunohistochemical parameters. **Material and methods:** We used twenty-four rats and divided them into 4 groups; Negative control group, rats were fed a basal diet only; Positive control group, rats were fed a basal diet plus ground flaxseed at a dose of 8mg/kg body weight daily by gastric tube for 12 weeks; High fat diet group, rats received high fat diet (20 mg butter/100 gm diet); High fat diet plus flaxseed group, rats received high fat diet plus ground flaxseed at a dose of 8mg/kg body weight daily by gastric tube. They were sacrificed after 12 weeks. Assessment of serum level of liver enzymes (ALT and AST), fasting blood glucose levels and lipid profile assessment [total cholesterol (TC) and triglycerides (TG)] was done. Oxidative stress was assessed in the liver. Fresh frozen sections were used for oil red O staining. Paraffin sections were prepared from liver specimens and stained with H&E, Sirius red and immunohistochemical staining for α-SMA. **Results**: Flaxseed suppressed excessive weight gain in rats that received HFD. Flaxseed decreased the fasting blood glucose level, serum level of ALT and AST enzymes, serum cholesterol and triglyceride levels and increased GSH levels as compared to HFD animals. Flaxseed attenuated the hepatic steatosis, inflammation, fibrosis and α-SMA immunohistochemical expression.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) represents a batch of correlated chronic liver disorders which include elevated fat deposition within hepatocytes (simple steatosis), portal and lobular inflammation and fibrosis (nonalcoholic steatohepatitis, NASH), that may progress to cirrhosis or hepatocellular cancer (McPherson *et al.*, 2014). Changes of lifestyle and diet have accelerated the frequency of obesity and metabolic syndrome (MS), resulting in higher incidence of NAFLD (Koplay *et al.*, 2015). Epidemic proportions of the disease have been reached because of rapid growth of the number of affected patients. It is now thought to be the hepatic manifestation of the metabolic syndrome and a separate determinant of cardiovascular morbidity and mortality (Cauchy *et al.*, 2014).

Raising the possibility of cardiovascular diseases, chronic kidney disease and type 2 diabetes mellitus because of NAFLD, has been reached recently (Byrne and Targher, 2015). The synchronization of hepatic steatosis and insulin resistance could cause more serious metabolic sequalae (Smith and Adams, 2011).

In NAFLD patients, pharmacological drugs targeted the basic MS elements (e.g., obesity, diabetes, hypertension, and dyslipidemia) as well as liver dysfunction itself. Numerous clinical research has studied multiple drugs and supplements, but researchers have not yet picked out a fully safe and potent medication that can be approved for NAFLD treatment (Baran and Akyüz, 2014).

The effective management of NAFLD is being targeted by herbal medicine, because of their least side effects and their numerous modes of action in controlling lipid metabolism (Thounaojam *et al.*, 2012). One of the richest sources of lignans is Flaxseed (Bhathena and Velasquez, 2002). The effective antioxidant impact of flaxseed is due to its active constituent (lignans - secoisolariciresinol diglucoside - SDG) which is responsible for diminished release of reactive oxygen species in addition to the inhibition of the DNA's divisions and lipid peroxidation (Cerovic *et al.*, 2013).

This study was planned to assess the progress of fibrosis in experimentally induced NAFLD in albino rats and the potential protective effect of the flaxseed using biochemical, histopathological and immunohistochemical parameters.

MATERIALS AND METHODS

Animals Used:

Twenty-four adult male Spraque-Dawley rats, weighing 200-250 gm aging 6 months were obtained from College of Veterinary Medicine, Mansoura University, Egypt. Under controlled condition of temperature (23ºC±3) and relative humidity, the rats were housed in stainless steel mesh cages with soft wood chips for bedding. The animals were allowed free access to standard commercial diet and tap water ad libitum with 12:12-hours light/dark cycle for two weeks before the experiment for acclimatization and to ensure normal growth and behaviour. All rats were maintained in the animal house under specific pathogen-free conditions.

All the experiments were carried out according to the rules and regulations lay down by the committee of animals' experimentation of Mansoura University ,Egypt, meeting the NH Guide for animal use. The date of IRB acceptance was on 29/12/2016.

Materials Used:

- Flaxseeds: Flaxseeds were bought from a local herbalist, and identified by department of pharmacognosy, Faculty of Pharmacy, Mansoura University, Egypt. The seeds were crushed, dissolved in carboxymethyle cellulose (0.5%) and given orally using gastric tube at a dose of 8 mg/kg body weight daily (Saad *et al.*, 2014).
- 0.5% carboxymethyle cellulose: 100 ml distilled water and 0.5 gm carboxymethyle cellulose powder.

Experimental Design:

After two weeks of accommodation, the rats were randomly divided into four groups with six rats in each one:

Group I: Negative control (NC) group, rats were fed a basal diet only and were sacrificed after 12 weeks.

Group II: Positive control (PC) group, rats were fed a basal diet plus ground flaxseed (8mg/kg) body weight daily intra-gastric for 12 weeks (Saad *et al.*, 2014).

Group III: High fat diet (HFD) group, rats received high fat diet for 12 weeks (20 mg butter/100 gm diet) (Qamar *et al.*, 2016).

Group IV: High fat diet plus flaxseed (HFD + Flax) group, rats received high fat diet plus ground flaxseed (8mg/kg) body weight daily by gastric tube for 12 weeks.

The absolute body weights of all rats were recorded at the beginning of the experiment and every 4 weeks.

Samples Collection:

At the exact times, the animals were weighted then they were anaesthetized with diethyl ether (after 8 hours fasting); Blood samples were collected from the left ventricles and put in polyethylene tubes for assessment of fasting blood glucose levels, liver enzymes, reduced glutathione (GSH), serum cholesterol and triglycerides. Via median abdominal incisions, livers were rapidly removed, washed with cold normal saline, dissected and divided; fresh part was processed for oil red O staining. Another portion was homogenized for quantitative analysis of GSH in liver homogenate. Immediately, the remaining parts were fixed in 10% buffered formalin and processed for paraffin sectioning for light microscopic examination.

Evaluation Methods:

I. **Biochemical Studies:**

A. **Assessment of Fasting Blood Glucose (FBG):** Precision Xtra Plus test strips and an optium Xceed device (Abbott Diabetes Care, Ltd., Maidenhead, UK) were used to measure the levels of fasting blood glucose (Vashist *et al.,* 2011).

B. **Assessment of Liver Functions, Serum Lipids and Reduced Glutathione (GSH):**

Blood samples were centrifuged for 10 min at 5000 g at 4°C to obtain the serum samples. Assessment of the serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) were done using clinical test kits (Elitech, UK) spectrophotometrically as a routine biochemical analysis (Jia *et al.,* 2013). Assessment of the serum levels of triglycerides and total cholesterol were done using clinical test kits (Elitech, UK) by standard procedures depending on enzymatic and colorimetric approaches, spectrophotometry, following manufacturer`s recommendations (Freitas *et al.,* 2013). Determination of reduced glutathione was done by using the appropriate kits (Biodiagnostic kits, Giza, Egypt). Homogenization of portions of livers (10% w/v) were done in ice-cold 0.1 M Tris-HCl buffer (pH 7.4). Centrifugation of the homogenate was done at 3000 rpm for 10 min at 4°C. Reduced glutathione was detected in the resultant supernatant of liver homogenate as previously described by Jia *et al.,* (2013).

II. Histopathological Assessment:

Haematoxylin and Eosin stain (Bancroft and Layton, 2013): to assess the general changes of hepatic architecture, Oil red O stain (Mehlem *et al.,* 2013): to demonstrate fat content in the frozen section; fat appears red while nuclei appear blue, Picro-Sirius red stain (Chun and Inoue, 2014): to detect collagen deposition in liver and determine the degree of fibrosis; collagen appears red on a pale-yellow background and immunohistochemical stain for α-smooth muscle actin (α-SMA) (Yoshiji *et al.,* 2001): to detect the expression of α-SMA and assessment of the activated hepatic stellate cells (aHSCs); α-smooth muscle actin positive cells appear brown colour in cytoplasm.

III. Image Analysis:

Quantitative assessment of steatosis, fibrosis and activated HSCs was done for all groups by image analysis of the oil red O, Sirius red and anti α-SMA stained sections, respectively. Liver sections were examined and photographed using a light microscope (Olympus, Japan) at a magnification of 100 for Sirius red and anti α-SMA stained sections and magnification of 400 for oil red O-stained sections, captured by digital camera (Olympus LC20). The RBG image was converted to a 8-bit grayscale image using NIH Image J (version 1.48, Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA) software. The picrosirius red and oil red stained area percentage was measured. Several readings were obtained from the different slides of all groups and at least

six random fields were measured in each slide.

Statistical Analysis:

Data were analyzed using the computer program SPSS (Statistical package for social science) version 22. Descriptive statistics were calculated in the form of mean ± Standard deviation. Analysis of variance (ANOVA) was used to test the significance of difference to compare between more than two groups of numerical (parametric) data followed by post-hoc Tukey for multiple comparisons. Microsoft Excel for windows (Microsoft Inc., USA) was used to perform all graphic representations of the data.

RESULTS

Assessment of Body Weight (Table 1):

No significant difference ($P > 0.05$) was detected between the groups regarding the body weight as detected by ANOVA test at the beginning of the experiment. Till the end of the experiment, no significant difference ($P > 0.05$) was detected between negative control (NC) and positive control (PC) groups. At the 4th week, there was significant increase in body weight of high fat diet (HFD) group when compared with (NC) group ($P < 0.05$) which becomes highly significant from the 8th week until the end of the experiment ($P < 0.001$). High fat diet plus flaxseed (HFD + Flax) group revealed no significant difference in body weight when compared with (NC) group in the first 8 weeks ($P > 0.05$), but significant increase was noticed at 12th week ($P < 0.05$). Additionally, significant decrease in body weight of (HFD + Flax) group when compared to (HFD) group was detected in the first 8 weeks ($P < 0.05$) and becomes highly significant at 12th week ($P < 0.001$).

Table 1: Body weight (gm) of the rats in the different groups.

	NC	PC	HFD	HFD + Flax	ANOVA
At beginning	206.67 ± 5.32	207.17 ± 5.78	208 ± 7.56	207.33 ± 6.65	0.99
4th week	233.33 ± 9.31	232.33 ± 8.31	254.67± 5.05	239.17 ± 7.47	0.000
P1		1	0.001	0.82	
P2				0.03	
8th week	260.83 ± 9.67	260 ± 7.59	289.83 ± 9.79	267.17 ± 11.27	0.000
P1		1	0.000	0.84	
P2				0.002	
12th week	285.33 ± 9.61	278.33 ± 6.22	332 ± 9.49	305.50 ± 6.72	0.000
P1		0.69	0.000	0.003	
P2				0.000	

The data is presented as mean ± standard deviation. ♣ P: Probability ♣ P1: significance relative to (NC) group ♣ P2: significance relative to (HFD) group.

Biochemical Assessment:

1. **Assessment of Serum Level of ALT and AST (Table 2).**

No significant difference ($P > 0.05$) was detected in ALT and AST levels between (NC) and (PC) groups. A highly significant increase ($P < 0.001$) was detected in ALT and AST levels in (HFD) group when compared to (NC) group. Additionally, a highly significant ($P < 0.001$) increase was recorded in ALT and AST levels in (HFD + Flax) group when compared to (NC) group and a highly significant ($P < 0.001$) decrease when compared with (HFD) group.

Table 2: Serum ALT and AST levels (U/L) in different groups**.**

	NC	PC	HFD	HFD + Flax	ANOVA
ALT	25.83 ± 4.45	16.71 ± 4.83	64.65 ± 8.66	46.83 ± 8.31	0.000
P1		0.13	0.000	0.000	
P2				0.000	
AST	134.67 ± 3.38	132.17 ± 4.92	364.17 ± 47.30	234.5 ± 53.32	0.000
P1		1	0.000	0.000	
P2				0.000	

The data is presented as mean ± standard deviation. ♣ P: Probability ♣ P1: significance relative to (NC) group ♣ P2: significance relative to (HFD) group.

2. Assessment of Serum Level of Triglycerides (Table 3):

The serum levels of triglycerides for (NC) and (PC) groups were 84.17 ± 7.49 mg/dl and 83.83 ± 6.65 mg/dl respectively. This represented no significant difference in triglycerides level between both groups (P > 0.05). The serum level of triglycerides for (HFD) group was 182.17±12.43 mg/dl. A highly significant increase was detected in triglycerides level when compared to (NC) group (P < 0.001). The serum level of triglycerides for (HFD + Flax) group was 147.33 ± 9.75 mg/dl. This represented a highly significant increase in triglycerides level in comparison to (NC) group (P < 0.001) and a highly significant decrease when compared with (HFD) group (P < 0.001).

3. Assessment of Serum Level of Total Cholesterol (Table 3):

The serum levels of total cholesterol for (NC) and (PC) groups were 59.67 ± 3.98 mg/dl and 56.50 ± 5.43 mg/dl respectively. This represented no significant difference in total cholesterol level between both groups (P > 0.05). The serum level of total cholesterol for (HFD) group was 117.50 ± 11.01 mg/dl. There was a highly significant (P < 0.001) increase in total cholesterol level when compared to (NC) group. The serum level of total cholesterol for (HFD + Flax) group was 78 ± 4 mg/dl. This represented a highly significant increase in total cholesterol level in comparison with (NC) group (P < 0.001) and a highly significant decrease in comparison with (HFD) group (P < 0.001).

Table 3: Serum triglycerides (TG) level (mg/dl) and serum total cholesterol (TC) level (mg/dl) in different groups.

	NC	PC	HFD	HFD + Flax
TG	84.17 ± 7.49	83.83 ± 6.65	182.17 ± 12.43	147.33 ± 9.75
P1		1	0.000	0.000
P2				0.000
TC	59.67 ± 3.98	56.50 ± 5.43	117.50 ± 11.01	78 ± 4
P1		0.96	0.000	0.000
P2				0.000

The data is presented as mean ± standard deviation. ♣ P: Probability ♣ P1: significance relative to (NC) group ♣ P2: significance relative to (HFD) group.

4. Assessment of serum level of oxidative stress marker (Table 4):

The serum levels of GSH for (NC) and (PC) groups were 1.46 ± 0.06 mmol / L and 1.41 ± 0.11 mmol / L respectively. This represented no significant difference in GSH level between both groups (P > 0.05). The serum level of GSH for (HFD) group was 0.50 ± 0.12 mmol / L. There was a highly significant (P < 0.001) decrease in GSH level in comparison with (NC) group. The serum level of GSH for (HFD + Flax) group was 1.13 ± 0.11 mmol / L. This represented a highly significant (P < 0.001) decrease in GSH level in comparison with (NC) group and a highly significant (P < 0.001) increase in comparison with (HFD) group.

5. Assessment of GSH level in liver homogenate (Table 4):

The hepatic levels of GSH for (NC) and (PC) groups were 1.84 ± 0.06 mmol / gm tissue and 1.83 ± 0.08 mmol/ gm tissue respectively. This represented no significant difference in GSH level between both groups (P > 0.05). The hepatic level of GSH for (HFD) group was 0.78 ± 0.14 mmol/ gm tissue. There was a highly significant (P < 0.001) decrease in GSH level when compared to (NC) group. The hepatic level of GSH for (HFD + Flax) group was 1.51 ± 0.05 mmol/ gm tissue. This represented a highly significant (P < 0.001) decrease in GSH level in comparison with (NC) group and a highly significant (P < 0.001) increase in comparison with (HFD) group.

Table 4: Serum GSH level (mmol/L) and hepatic GSH level (mmol/ gm) in different groups.

	NC	PC	HFD	HFD + Flax
Serum GSH	1.46 ± 0.06	1.41 ± 0.11	0.50 ± 0.12	1.13 ± 0.11
P1		0.96	0.000	0.000
P2				0.000
Hepatic GSH	1.84 ± 0.06	1.83 ± 0.08	0.78 ± 0.14	1.51 ± 0.05
P1		1	0.000	0.000
P2				0.000

The data is presented as mean ± standard deviation. ♣ P: Probability ♣ P1: significance relative to (NC) group ♣ P2: significance relative to (HFD) group.

6. Assessment of Fasting Blood Glucose Level (Table 5):

The fasting blood glucose levels for (NC) and (PC) groups were 99.67 ± 3.98 mg/dl and 100.33 ± 8.98 mg/dl respectively. This represented no significant difference in fasting blood glucose level between both groups (P > 0.05). The fasting blood glucose level for (HFD) group was 238 ± 30.97 mg/dl. There was a highly significant (P < 0.001) increase in fasting blood glucose level in comparison with (NC) group. The fasting blood glucose level for (HFD + Flax) group was 171.33 ± 11.78 mg/dl. There was a highly significant (P < 0.001) increase in fasting blood glucose level when compared with (NC) group and a highly significant (P < 0.001) decrease in comparison with (HFD) group.

Table 5: Fasting blood glucose (FBG) level (mg/dl) in different groups.

	NC	PC	HFD	HFD + Flax
FBG	99.67 ± 3.98	100.33 ± 8.98	238 ± 30.97	171.33 ± 11.78
P1		1	0.000	0.000
P2				0.000

The data is presented as mean ± standard deviation. ♣ P: Probability ♣ P1: significance relative to (NC) group ♣ P2: significance relative to (HFD) group.

Histopathological and Immunohistochemical Assessment:

A- Haematoxylin and Eosin-Stained Sections:

Light microscopic examination of liver sections of rats of both negative and positive control groups revealed the normal hepatic architecture of the classical hepatic lobules with plates of hepatocytes radiating from the central vein. The hepatocytes possessed central rounded nuclei with acidophilic cytoplasm. Some hepatocytes were binucleated. The hepatocytes plates were separated by hepatic sinusoids lined with flat endothelium. Kupffer cells with elongated nuclei were observed among hepatocytes plates. The portal tracts lay at the corners of lobules and showed the portal triad formed of a small branch from the hepatic artery, a branch from the

portal vein and a bile ductule (Fig. 1 a & b).

The histopathological investigation of liver sections of HFD group showed distorted hepatic architecture with variable amounts of lipid droplets accumulating in hepatocytes (micro-vesicular &macro-vesicular steatosis). Some hepatocytes nuclei were pushed to one side by large lipid droplets filling most of the cytoplasm. There was inflammatory cell infiltration in between hepatocytes and also surrounding the central vein. The cytoplasm of some hepatocytes appeared vacuolated (hydropic degeneration) (Fig. 2). Administration of flaxseed simultaneously with HFD showed less distorted hepatic architecture on investigation of liver sections of rats of this group. Minimal hepatic steatosis was observed. It appeared mainly micro-vesicular and was concentrated around central vein. Minimal inflammatory cell infiltration was noticed around central vein and in between hepatocytes (Fig. 2).

Fig. 1 a

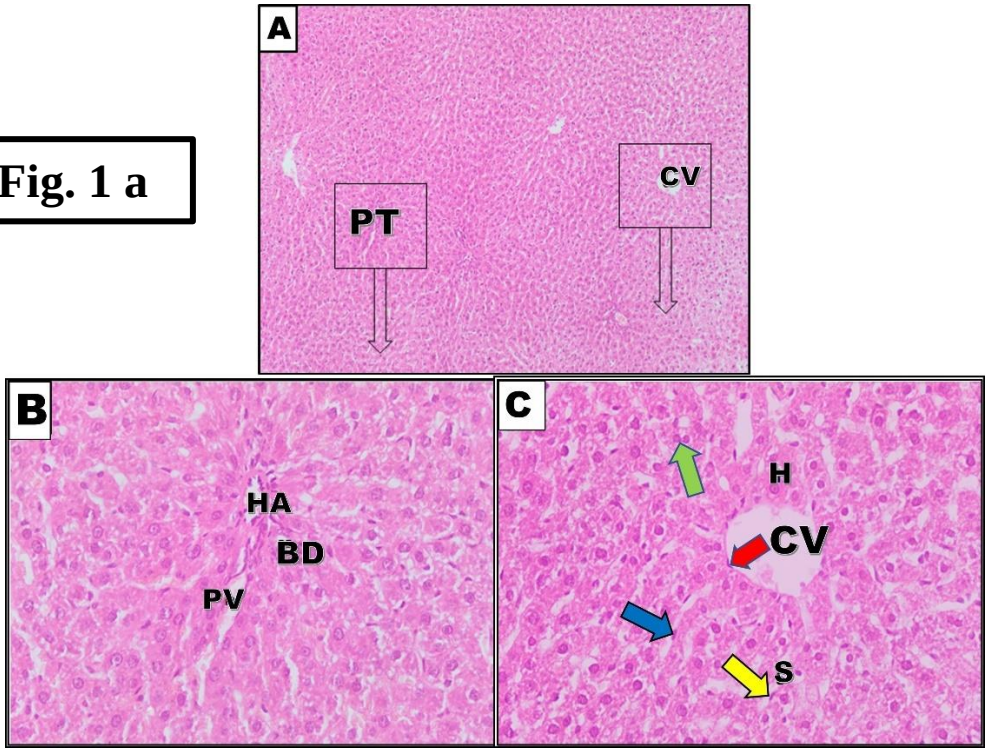

Fig. 1 b

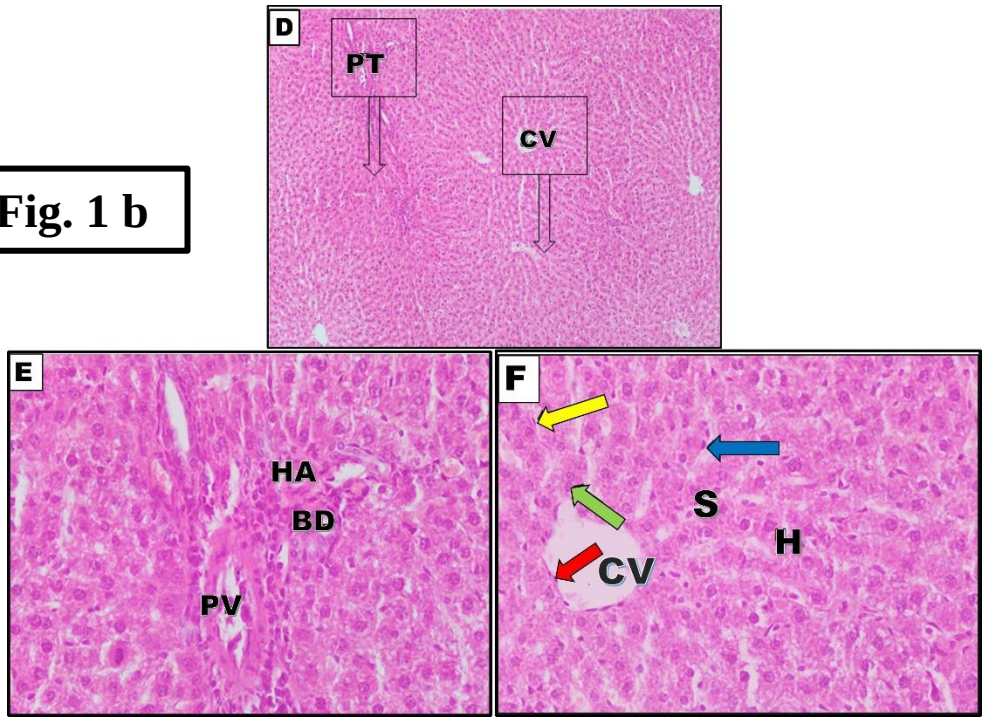

Fig. 1 a & b: Photomicrographs of rat liver sections of the control groups (negative control (1a) and positive control (1b)), respectively, showing the normal histological structure of the liver. A and D: Hepatic lobules with anastomosing plates of polyhedral hepatocytes arranged extending from the central vein (CV) with intervening hepatic sinusoids. Portal tract (triad) (PT) lies at the periphery (H&E; x 100). B and E: Photomicrographs of higher magnification of the left selected areas in figures A & D, respectively, showing portal tract with a portal venule (PV), hepatic arteriole (HA) and bile ductule (BD). (H&E; x 400). C and F: Photomicrographs of higher magnification of the right selected areas in figures A&D, respectively, showing polyhedral hepatocytes (H) with acidophilic cytoplasm and rounded nuclei forming anastomosing plates extending from central vein (CV) which is lined by flat endothelium (red arrows) and separated by blood sinusoids (S) lined with flat endothelium (yellow arrows). Kupffer cells (blue arrows) are seen among hepatocytes plates. Binucleated hepatocytes (green arrows) could be seen. (H&E; x 400).

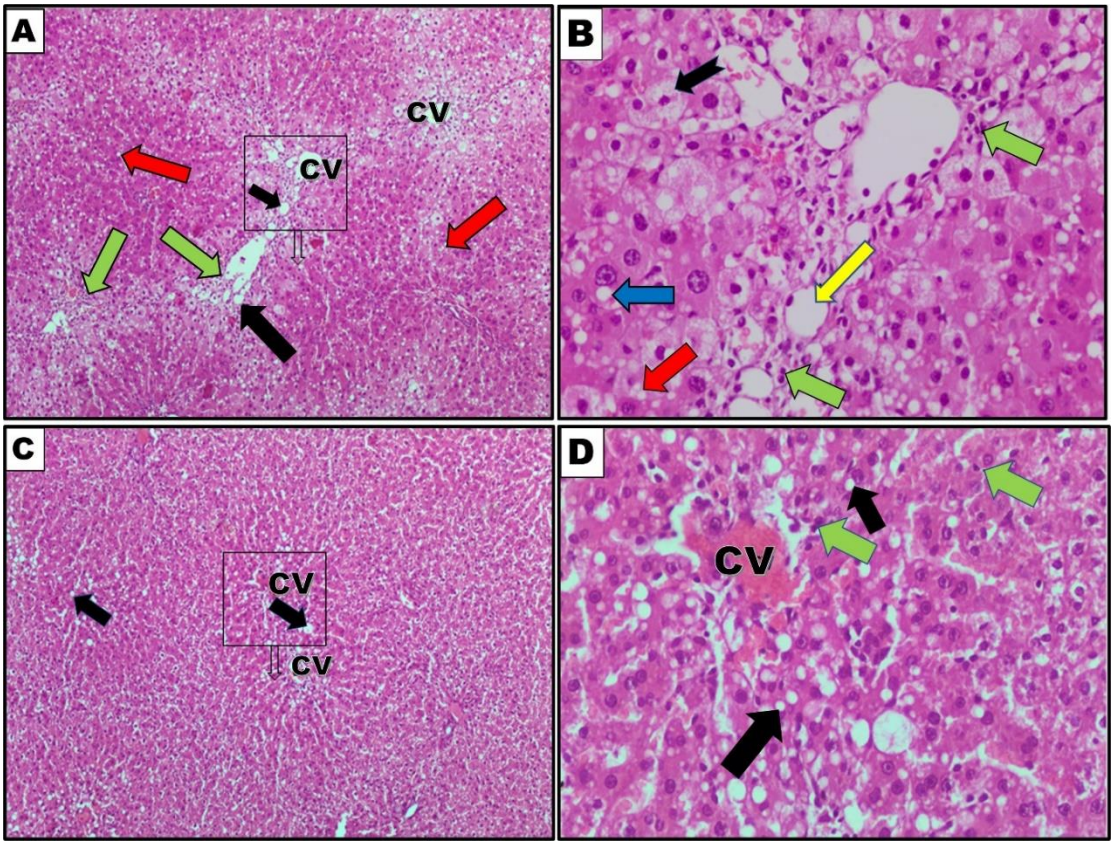

Fig. 2: A: a photomicrograph of rat liver sections of HFD group showing micro-vesicular (red arrows); macro-vesicular (black arrows) steatosis and inflammatory cell infiltrate (green arrows). Central vein (CV) is seen **(**H&E; x 100). B: a photomicrograph of a higher magnification of the previous section showing hepatocytes loaded with variable amounts of lipid droplets. Hepatocytes show numerous small lipid droplets (red arrow), single large lipid droplet (blue arrow) or very large lipid droplet pushing the nucleus eccentrically (yellow arrow). Some hepatocytes show vacuolated cytoplasm "hydropic degeneration" (forked arrow). Inflammatory cells infiltrate around the central vein and in between hepatocytes are detected (green arrow). C**:** a photomicrograph of rat liver sections of (HFD + Flax) group showing less distorted hepatic architecture than HFD group. Some hepatocytes show steatosis (arrows). Central vein (CV) (H&E; x 100). D**:** a photomicrograph of a higher magnification of the previous section showing minimal steatosis mainly micro-vesicular (arrows) around central vein (CV). Minimal inflammatory cell infiltration around central vein or in between hepatocytes can be seen (green arrow). (H&E; x 400).

B- Oil red O-stained Sections:

Oil red O-stained sections were evaluated and assessed in (Fig. 3) and (Table 6). Investigation of frozen liver sections of rats of both negative and positive control groups revealed oil red-stained small lipid droplets scattered in some hepatocytes. By image analysis, the area occupied with lipid droplets was 2.06 ± 0.35% and 1.74 ± 0.45% respectively. No significant difference ($P > 0.05$) was detected between (NC) and (PC) groups.

Frozen liver sections of rats of HFD group showed increase in size and amount of oil red stained lipid droplets. By image analysis, the area occupied with lipid droplets was 26.35 ± 2.17%. A highly significant ($P < 0.001$) increase in fat accumulation in comparison to the (NC) group.

Frozen liver sections of rats received flaxseed simultaneously with HFD revealed decrease in the size and amount of oil red stained lipid droplets. By image analysis, the area occupied with lipid droplets was 9.02 ± 1.73%. A highly significant ($P < 0.001$) increase was detected in fat accumulation in comparison to (NC) group and a highly significant ($P < 0.001$) decrease in comparison to (HFD) group.

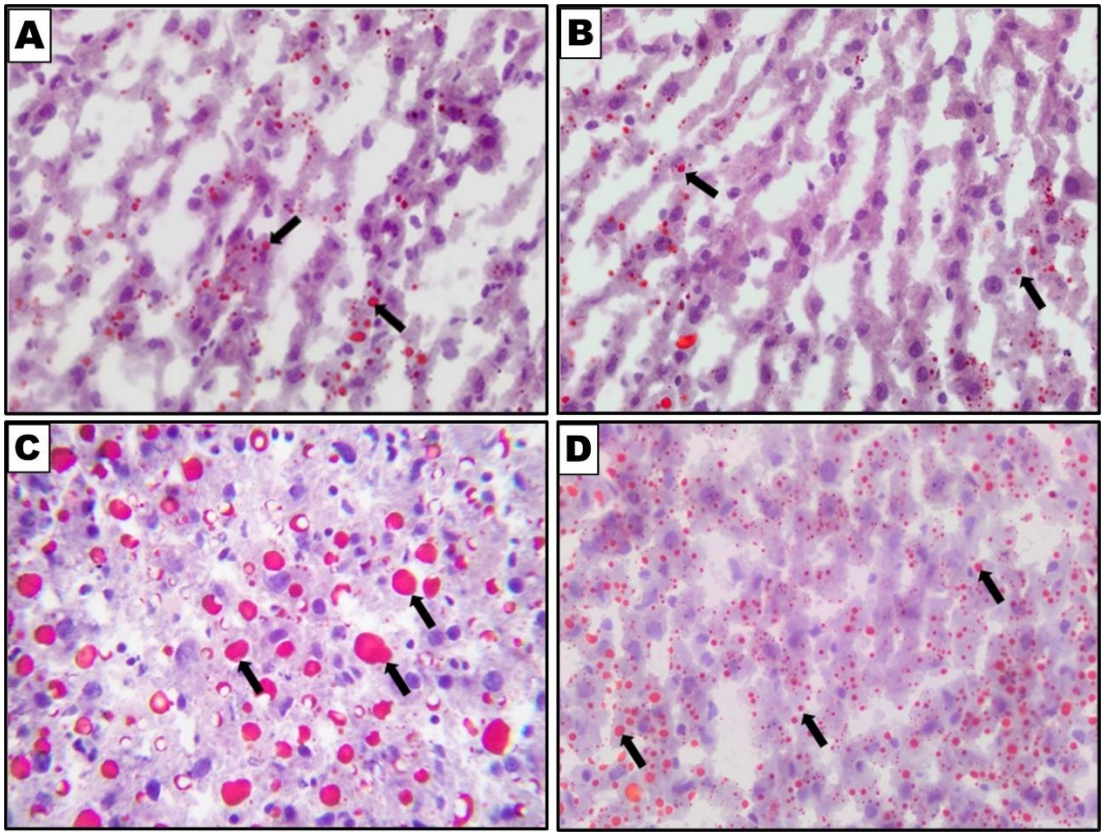

Fig. 3: Photomicrographs of frozen sections of liver from rats of the different groups. **A**: negative control and **B**: positive control, showing small lipid droplets scattered in some hepatocytes (arrows). **C**: HFD group showing increase in size and amount of lipid droplets (arrows). **D:** HFD with flaxseed showing variable sized lipid droplets scattered in the section (arrows). **(Oil Red O; x 400).**

C- Sirius Red Stained Sections:

Sirius red stained sections were evaluated and assessed in (Table 6). Investigation of rat liver sections of both negative and positive control groups revealed normal distribution of connective tissue in the form of fine collagen fibres surround the portal areas and the central veins (Figure 4). Morphometrically, the area percentage of fibrous tissue was 0.91 ± 0.06 % and 0.76 ± 0.09 % respectively. No significant difference ($P > 0.05$) was detected between (NC) and (PC) groups.

Liver sections of rats of HFD group showed noticeable increase in amount of collagen fibres surrounding the portal areas and the central veins with formation of thick well developed fibrous septa between them (Figure 5A). By image analysis, the area percentage of fibrous tissue was 15.20 ± 1.15 %. A highly significant ($P < 0.001$) increase in collagen deposition in comparison to the (NC) group.

Liver sections of rats received flaxseed simultaneously with HFD revealed an increase in the amount of collagen fibres deposited around the central vein and the portal tract with few thin fibrous septa observed extending from them (Figure 5B). By image analysis, the area percentage of fibrous tissue was 1.79 ± 0.31%. Significant increase ($p< 0.05$) was detected in collagen deposition in comparison to the (NC) group and a highly significant decrease when compared with (HFD) group ($P < 0.001$).

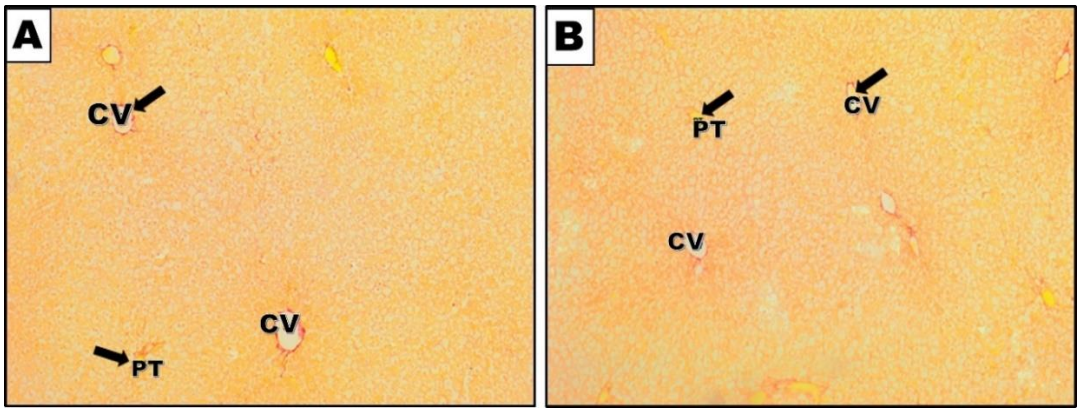

Fig. 4: Photomicrographs of rat liver sections of the control groups; A: negative control, B: positive control, showing few collagen fibers (arrows) around central vein (CV) and portal tract (PT). **(Sirius red; x100).**

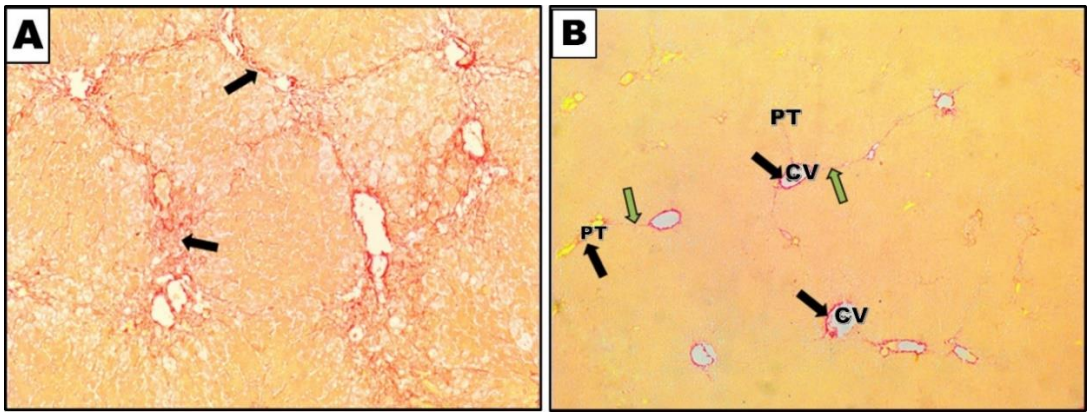

Fig. 5: A: a photomicrograph of a liver section from a rat received HFD showing manifest increase in the amount of collagen fibers with thick well developed septa forming bridges between portal areas and central veins (black arrows). **B:** A photomicrograph of a liver section from a rat received flaxseed simultaneously with HFD showing increased collagen deposition (black arrows) around central vein (CV) and portal tract (PT) with few thin fibrous septa extending from portal tract and central vein (green arrows). **(Sirius red; x100)**

D- Anti α-SMA Stained Sections:

Anti α-SMA stained sections were evaluated and assessed in (Table 6). Investigation of liver sections of rats of both negative and positive control groups revealed positive α-SMA reaction within the portal tracts in the muscle layers of both portal veins and hepatic arteries (Fig. 6). By image analysis, the area occupied by anti α-SMA was 0.39 ± 0.13 % and 0.27 ± 0.12 % respectively. No significant difference was detected in Anti α-SMA positively stained area between (NC) and (PC) groups ($P > 0.05$).

Liver sections of rats of HFD group revealed positive αSMA reaction around central veins, portal areas and in the fibrous tissue septa extending from them (Fig. 7A and B). Morphometrically, the area occupied by anti α-SMA was 11.09 ± 1.51%. A highly significant ($P < 0.001$) increase was detected in Anti α-SMA positively stained area in comparison to the (NC) group.

Liver sections of rats received flaxseed simultaneously with HFD revealed apparent decrease in anti α-SMA positively stained area in all liver sections as compared with the animals of HFD group (Fig. 7C and D). By image analysis, the area occupied by anti α-SMA was 1.69 ± 1.21%. There was significant increase in anti α-SMA positively stained area when compared with the (NC) group ($p< 0.05$) and a highly significant ($P < 0.001$) decrease when compared with (HFD) group.

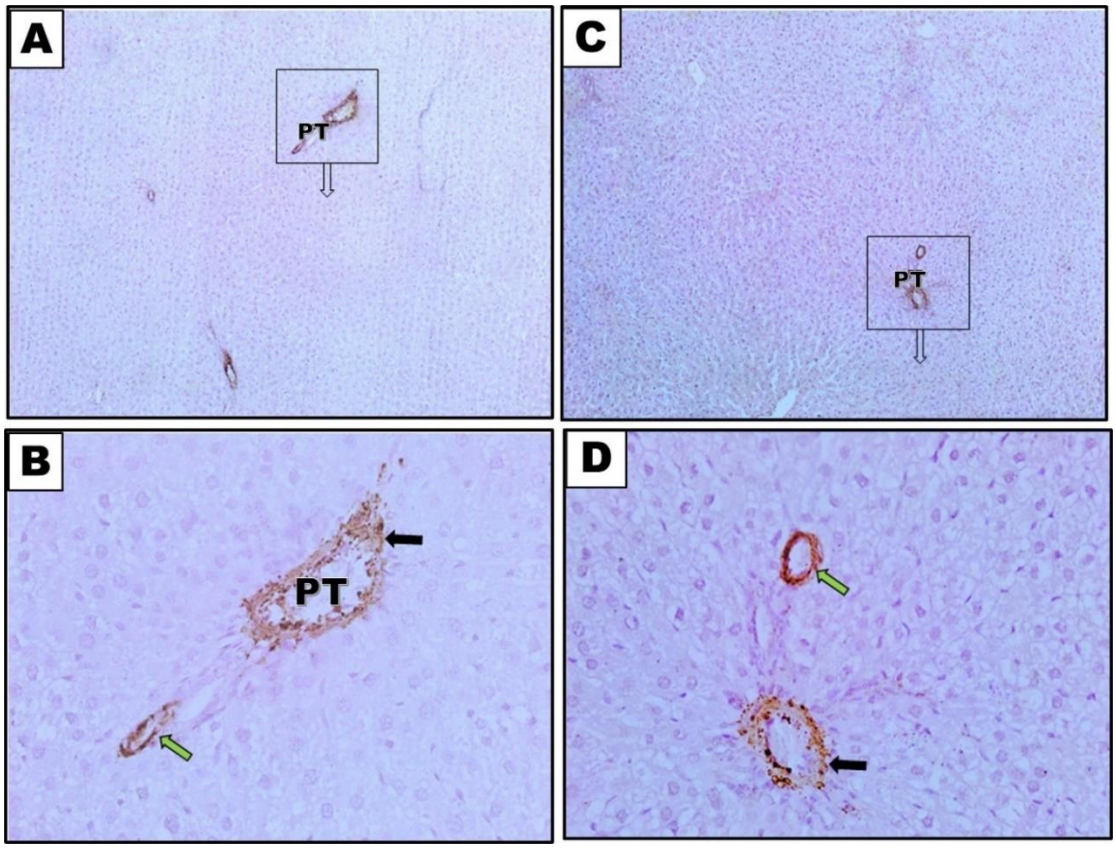

Fig. 6: Photomicrographs of liver sections stained for α-SMA from rats of the control groups; A and B: negative control, C and D: positive control, showing positive stain (brown) in portal tract (PT). B and D: Photomicrographs of a higher magnification of the selected areas in figures A & C, respectively, showing positive α-SMA immune stain in the wall of portal vein (black arrow) and hepatic artery (green arrow). **A** and **C: x100. B** and **D: x400.**

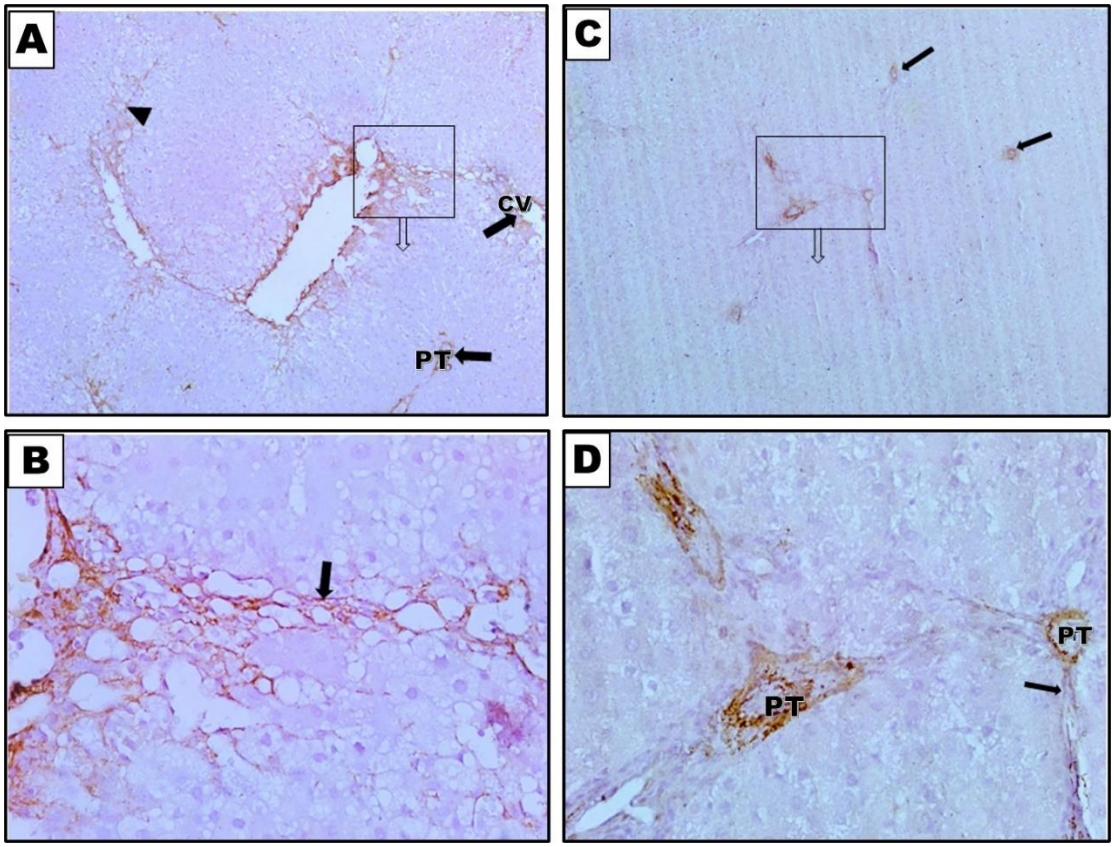

Fig. 7: A: a photomicrograph of a liver section stained for α-SMA from a rat received HFD showing strong positive stain (brown) in portal tract (PT), central vein (CV) and fibrous septa (arrowhead). **B:** a photomicrograph of a higher magnification of the previous section showing strong positive α-SMA immune stain (brown) in fibrous tissue septa (arrow). **C**: a photomicrograph of a liver section from a rat received flaxseed simultaneously with HFD stained for α-SMA showing positive stain (brown) in portal tract and central vein (black arrows). **D**: a photomicrograph of a higher magnification of the previous section showing positive stain (brown) in portal tract and thin fibrous septa (arrow). **A and C: x100. B and D: x400.**

Table 6: Area percentage of oil red O, area percentage of Sirius red and area percentage of anti α-SMA positive reaction in different groups**.**

	NC	PC	HFD	HFD + Flax
Oil red O area %	2.06 ± 0.35	1.74 ± 0.45	26.35 ± 2.17	9.02 ± 1.73
P1		1	0.000	0.000
P2				0.000
Sirius red area %	0.91 ± 0.06	0.76 ± 0.09	15.20 ± 1.15	1.79 ± 0.31
P1		0.99	0.000	0.004
P2				0.000
anti α-SMA area %	0.39 ± 0.13	0.27 ± 0.12	11.09 ± 1.51	1.69 ± 1.21
P1		1	0.000	0.007
P2				0.000

The data is presented as mean ± standard deviation. ♣ P: Probability ♣ P1: significance relative to (NC) group ♣ P2: significance relative to (HFD) group.

DISCUSSION

Non-alcoholic fatty liver disease is currently considered the most prevalent chronic liver disease worldwide (Dassanayake, 2018). NAFLD incorporates a range of diseases starting from simple steatosis to NASH, which in its most severe form causes liver fibrosis, cirrhosis, and hepatocellular carcinoma (Koplay *et al.*,

2015). There is a complex relationship between diabetes and NAFLD since both share similar risk factors. Each condition may accelerate the progress of the other (Williams *et al.,* 2012). Currently, there is no treatment that is effective for all NAFLD patients. Studies aiming to recognize adequate treatment for NAFLD, and NASH have not yet found a common protocol that treats this enlarging problem successfully (Milic *et al.,* 2015). The use of the naturally occurring components and plant medicine for the purpose of NAFLD prevention and/or treatment has become increasingly popular (Song *et al.,* 2014). Flaxseed is an underused crop but acquired significance in the last few decades in that it has a special nutrient profile, especially omega-3 fatty acid, lignans, and fibres (Goyal *et al.,* 2014), all of which play a role and have positive effects in disease prevention (Udenigwe *et al.,* 2009). Need for flaxseed has been increased as consumer became aware of the strong relationship between diet and health (Eyres, 2015). In the current study, we aimed to evaluate liver fibrosis in NAFLD and to examine the potential protective effect of the flaxseed on it. The high-fat diet rat model closely resembles the pathophysiology found in human NAFLD (Neuschwander-Tetri *et al.,* 2010). Administration of high fat diet for 12 weeks resulted in progressive increase in body weight compared with negative control group congruent with Fukumitsu *et al.* (2008), Lin *et al.* (2016) and Sun *et al.* (2016). Weight gain can be attributed to excess calorie intake which promotes positive energy balance leading to increased visceral fat deposition with abdominal obesity and increased body weight (Amin and Nagy, 2009). In ketogenic diet, a state of physiological ketosis is attained by creating a state of carbohydrate starvation via consuming minimal carbohydrates. The insulin requirement decreases as carbohydrate substrate is minimized. This leads to resolution of IR and reduction in insulin secretion, with a simultaneous reduction in glucagon production (the islet de-stress hypothesis). Caloric and nutrient intake is preserved through fats and protein. Fats are favourably consumed to produce energy for the body and are burnt up (Kalra *et al.,* 2018). The rise of the body weight of rats fed flaxseed simultaneously with HFD is less than that of rats of HFD group. This is in harmonious with Fukumitsu *et al.* (2008) and Sun *et al.* (2016). Suppression of excessive weight gain by flaxseed administration may be attributed to the role of flaxseed dietary fibres. These fibres suppress postprandial appetite, food intake and lipaemia which decrease fat accumulation and suppress excessive increase in body weight (Kristensen *et al.,* 2012; Marpalle *et al.,* 2014). The gel formed by the absorption of water by soluble fibres delayed digestion and emptying of stomach (Ibrügger *et al.,* 2012). Insoluble fibres do not absorb water and form gel as soluble fibres. It adds bulk and helps to speed up the transit of food through stomach and intestine (Mishra, 2016). Besides, evidence recommend that the plenty of polyunsaturated fatty acids in the diet can act as a regulator for the body fat deposition (Wu *et al.,* 2010). Regarding the liver enzymes, rats of HFD group showed marked elevation in the serum levels of liver enzymes (ALT and AST) in comparison to the negative control group. These results were consistent with Adams and Feldstein (2011), Zhang *et al.* (2012) and Han *et al.* (2015). ALT is present in the hepatocellular cytosol, whereas AST is located mostly within the mitochondria (Vernon *et al.,* 2011). Significantly increased serum ALT activity usually suggests its leakage from injured hepatocytes and refers to liver cell damage. ALT is a more specific than AST as indicator of hepatic inflammation as AST levels may also increase in diseases of other organs (Safwat *et al.,* 2009). The elevated liver enzymes can be attributed to the oxidative stress and the direct hepatotoxic effect caused by free fatty acids (Mota *et al.,* 2016). Administration of flaxseed decrease liver enzymes in rats of HFD group. As

flaxseed contains lignans which is one of the phenolic compounds, it may counterbalance the free radicals and peroxides thus showing its hepatoprotective effectiveness (Fukumitsu *et al.,* 2008; Han *et al.,* 2015; Shakir and Madhusudhan, 2007). The rats of HFD group showed hyperlipidemia. This is indicated by marked elevations in total serum cholesterol and triglycerides, in rats of HFD group as compared with the negative control group. These findings came in accordance with Yao *et al.* (2013), Sun *et al.* (2016) and Gou *et al.* (2016). Low adiponectin may be a cause of obesity and adipose tissue-specific insulin resistance (Di Chiara *et al.,* 2012). Experimental studies have revealed that replenishment of hypoadiponectinemia can restore insulin sensitivity and reverse the accompanying weight gain, hyperglycaemia and fatty liver (Cheng *et al.,* 2014). In the current study, flaxseed administration simultaneously with HFD led to significant decrease in total cholesterol and triglycerides. This is congruent with Yang *et al.* (2009); Saad *et al.* (2014); Sun *et al.* (2016) and Kaur *et al.* (2017). Han *et al.* (2015) found that flaxseed oil intervention elevates peroxisome proliferator-activated receptor α (PPARα) and reduce sterol regulatory element binding protein 1c (SREBP-1c), respectively. PPAR α and SREBP-1c are principal adjustors of hepatic lipogenic genes (Castrillo and Tontonoz, 2004). The anti-obesity effects of flaxseed lignan Secoisolariciresinol diglucoside (SDG) result from the suppression of genes concerned in fatty acid and triacyl glyceride (TAG) formation via the regulatory activity of SREBP-1c. Sterol regulatory element binding proteins are chief transcription components that adjust the hepatic metabolism of fatty acid and cholesterol. SREBP-1c, particularly, plays a crucial function in the dietary control of nearly all lipogenic genes of the liver (Fukumitsu *et al.,* 2008). Correction of dyslipidaemia also may be attributed to the hypercholesteraemic effect of flaxseed soluble fibres. Soluble fibres absorb water and form a gel which helps lowering LDL blood cholesterol by interfering with the absorption of dietary cholesterol (Mishra, 2016). The gel surrounds cholesterol suppressing its absorption and facilitating its excretion (Ibrügger *et al.,* 2012). Also, bacteria in the colon ferment the fibres to compound acetate, propionate and butyrate, which inhibit cholesterol synthesis (Mishra, 2016).

In the present study, rats of HFD groups exhibited a significant increase in fasting blood glucose levels in congruent with Frantz *et al.* (2014) and Sun *et al.* (2016). Hyperglycemia in NAFLD can be attributed to hepatic lipid aggregation of di-acyl glycerols and protein kinase C activity, suppressing the insulin signalling pathway and encouraging IR. Also, there is impaired post prandial glycogen production. In addition, stimulation of glucose production by the liver occurs as a result of increased fluxes of hepatic long-chain fatty acyl CoA, thereby raising the hazard of glucose intolerance and diabetes (Samuel and Shulman, 2016).

It was noticed that administration of flaxseed in rats of HFD lower the increased level of fasting blood glucose. This is in congruent with Fukumitsu *et al.* (2008) and Sun *et al.* (2016). One probable explanation for lowering this glycemia is the presence of SDG, through its antioxidant activity which decreases incidence of diabetes (Brant *et al.,* 2012). The antioxidant activity of SDG metabolites successively prevents the development of type 1 and 2 diabetes (Kaur *et al.,* 2017). Another cause of this reduction is that flaxseeds inhibit glucosidase and pancreatic α-amylase causing reduction in hydrolysis of the starch and finally decreasing glucose levels (Bhat *et al.* 2011; Sudha *et al.* 2011). In the current study, rats of HFD groups revealed significant reduction in the levels of reduced glutathione (GSH) when compared with negative control group. These findings were consistent

with Zhu *et al.* (2017). The decrease in GSH level may be due to its increased consumption by the hepatocytes to neutralize the increased formation of lipids peroxides (Amini and Yazdanparast, 2009). However, the significant reduction in the levels of reduced glutathione in HFD group was improved by the administration of flaxseed in accordance with Yang *et al.* (2009). Hemmings *et al.* (2004) reported that protection of the liver is via the ability of flax lignan to activate liver Gamma-glutamyl transferase (GGT) in the resting state and keep this activation in response to injury, which by turn increase reduced glutathione level. Flax lignans act as safeguards against destruction to DNA and liposomes and have an antioxidant capacity as segregators of hydroxyl radicals, and as estrogenic compounds with their structural similarity to 17-β-oestradiol (Hu *et al.,* 2007). Recent studies reveal the power of SDG to hunt hydroxyl free radicals and displayed that it is a powerful antioxidant (Singh *et al.* 2011). Kaur *et al.* (2017) stated that the use of different doses of flaxseed extract retrieved oxidative stress through raising the level of antioxidant enzymes.

Rats receiving HFD exhibited the features of fatty liver showing the accumulation of lipid droplets in the form of micro and macro vesicular steatosis and inflammation through histopathological examination in comparison to the negative control group. This is in match with Zhang *et al.* (2012), who referred this to the upregulated expression of SREBP-1c mRNA. It causes activation of the transcription of lipogenic genes whose promoter regions contain sterol regulatory elements, like fatty acid synthase (FAS) (Raghow *et al.,* 2008). Fatty acid synthase is an important enzyme that regulates the rate of fatty acid synthesis. Overaccumulation of hepatic fatty acids and triglycerides may be the result of increased the expression of FAS (Yan *et al.,* 2007). Fatty liver may progress to steatohepatitis as a result of a disparity between proinflammatory and anti-inflammatory cytokines, enhancing reactive oxygen species production and intrahepatic lipid peroxidation (Tamura and Kawamori, 2006).

Hepatic steatosis is accompanied by peripheral insulin resistance. Besides, hyperglycaemia and hyperinsulinemia caused by insulin resistance aggravate hepatic steatosis through de novo lipogenesis (Smith and Adams, 2011).

In this work, a significant increase in the area percentage of fat stained with Oil red O was observed following the administration of HFD in agreement with Zaitone and Essawy (2012) and Asgharpour *et al.* (2016). The hepatic steatosis was significantly reduced in rats that received flaxseed simultaneously with HFD in agreement with Sun *et al.* (2016). They explained the protecting action of SDG by its role in AMPK activation. AMPK is a detector of the condition of the cell energy which controls cellular and whole-body energy balance (Grahame Hardie, 2014). Dysregulation of AMPK has been crucial in the pathogenesis of hepatic steatosis, insulin resistance, and metabolic syndrome-associated diseases (Ruderman *et al.,* 2013). Viollet *et al.*, 2009 stated that triggering of AMPK in the liver augments fatty acid oxidation and concurrently inhibits hepatic lipogenesis, cholesterol synthesis, and glucose production, resulting in the enhancement of hepatic steatosis and insulin resistance. According to Saad *et al.* (2014), they reported positive effects of flaxseed in the improvement of lipid disorders related to NAFLD in rabbits, but it was not efficient in reducing hepatic steatosis. The area percentage of the fibrous tissue stained with Sirius red stain was significantly increased in the HFD group in agreement with Asgharpour *et al.* (2016). The appearance and progress of liver fibrosis is critical in all long-lasting liver diseases (Marra *et al.,* 2014). Activation of hepatic stellate cells promotes the generation of fibrogenic myofibroblasts

(Mederacke *et al.,* 2013). The initiation of fibrogenesis may result from changed pattern of circulating adipokines, oxidative stress and the hormonal profile associated with the metabolic syndrome (Rombouts and Marra, 2010).

Liver sections of rats of HFD group stained with Anti α-SMA revealed positive reaction around central veins, portal areas and in the fibrous tissue septa extending from them, in agreement with Asgharpour *et al.* (2016). Long term hepatic injury leads to alterations in hepatic architecture and progressive fibrosis. Hepatic stellate cells when activated acquire myogenic features including expression of alpha smooth muscle actin (Mehta *et al.,* 2014). According to Weiskirchen *et al.,* 2018, both activated HSCs and transdifferentiated myofibroblasts (MFBs) are positive for α-smooth muscle actin. At the cellular level, many factors activate quiescent hepatic stellate cells including soluble mediators (chemokines and cytokines) released by liver resident macrophages (Kupffer cells), infiltrating leukocytes, and other cell types including damaged hepatocytes. Administration of flaxseed in HFD groups led to significant decrease in the amount of collagen fibres deposited around central vein and portal tract and also the area percentage of anti α-SMA stained area. This beneficial effect of flaxseed can be explained by PUFA mediated modulation of cell proliferation and anti-inflammatory effects which may promote wound healing (Otranto *et al.,* 2010). Flaxseed replaces trans and saturated fatty acids of blood with unsaturated ones (Villeneuve *et al.,* 2013). PUFA play an important role in the prophylaxis and the treatment of dyslipidemia, cardiovascular diseases and metabolic syndrome (Marik and Varon, 2009). They have anti-inflammatory, vasodilatory, and antithrombotic effects. They reduce insulin resistance and decrease cytokine production (Shearer *et al.,* 2012). The anti-inflammatory effect is exerted by suppressing interleukin 1b, tumour necrosis factor-α and interleukin-6 (Simopoulos, 2008). Resolvins, neuroprotectins and protectins are endogenous metabolic products of eicosapentaenoic acid and docosahexaenoic acid. The resolvins act as powerful anti-inflammatory mediators. Specifically, they chunk the actions of prostanoids, and clear the site of inflammation from breakdown products of the inflammatory process, so restricting the extent of inflammation (Simpolous, 2011).

In Conclusion, there is a very strong link between NAFLD and diabetes. Each condition promotes the development of the other. Flaxseed induced favorable effects on both conditions. Administration of flaxseed improved body weight, relieved oxidative stress, and decreased the serum levels of liver enzymes, triglycerides, total cholesterol and fasting blood glucose. Flaxseed improved the liver histopathology and prevented progression of NASH to hepatic fibrosis. Further studies are needed to ensure the benefits of flaxseed on NAFLD regarding the mechanisms, duration, doses especially in human.

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