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Flaxseed oil attenuates experimental liver hepatitis

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ABSTRACT

D-galactosamine and lipopolysaccharide induced liver injury is a documented model of experimental liver hepatitis. Omega-3 fatty acids are considered as a potential therapeutic agents for inflammatory and autoimmune diseases through their incorporation in the cell membrane. This study aimed to investigate the role of flaxseed oil administration in two different doses in lipopolysaccharide (LPS) induced hepatitis in D-galactosamine (Dga1N) sensitized rats.Sixty healthy male albino rats were divided into five groups (control, flaxseed oil, LPS/DGalN, treated group I and treated group II). Urinary 8-hydroxyguanosine (8-OhdG) was determined by HPLC. Serum liver functions (ALT, AST and ALP) activities were determined. Liver malondialdehyde (MDA), superoxide dismutase (SOD) and reduced glutathione (GSH) activities were measured. Liver tumor necrosis factor-α (TNF- α) and Interleukin- 1α (IL- 1α) were done. Liver cyclooxygenases (COX-1, 2) were analyzed by immunohistochemistry. Results showed that LPS/DGalN administration significantly increased serum liver functions, IL-1α , TNF-α , MDA, urinary 8-OhdG concomitant with a reduction in liver GSH and SOD compared to the control group while, flaxseed oil administration attenuated these parameters . In conclusion, flaxseed oil as a rich source of omega-3 fatty acids has a powerful effect in preventing oxidative stress as well as liver inflammation.

Keywords: Hepatitis- Flaxseed oil- 8-hydroxyguanosine – Cyclooxygenases

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INTRODUCTION

D-galactosamine is a well-known chemical substance for inducing manifestation of experimental hepatitis; in addition to its properties as a transcriptional inhibitor, thus GalN is connected with an insufficiency of UDP-glucose and UDP-galactose and the loss of intracellular calcium homeostasis; these changes affect cell membranes and organelles and the synthesis of proteins and nucleic acids [1]. Liver damage induced by this chemical substance (D-GalN) is comparable to viral hepatitis in human in both function and morphology [2]. Lipopolysaccharide (LPS), the major component of gram-negative bacteria' membrane, induces liver injury when given at a high dose [3]. Administration of LPS with D-GalN induces lethal liver failure [4] through a cascade of inflammation with induction of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 and IFN-c [5].

So, it is from interest to find a natural product that has anti-inflammatory properties to limit this pathway. Omega-3 fatty acids are well-documented supplement that augment inflammation and inhibit inflammatory pass ways [6].

Flaxseed oil contains a mixture of fatty acids. It is considered the richest source of polyunsaturated fatty acids, particularly α-linolenic acid (ALA) which constitutes 57% of the total fatty acids in flax. [7].

Thus, in this study we aimed to investigate the beneficial role of flaxseed oil administration in hepatitis induced by lipopolysaccharide and D-galactosamine at sensitized rats.

MATERIALS AND METHODS

Materials

Chemicals

Lipopolysaccharide, D-galactosamine and 8-hydroxyguanosine standard (HPLC grade) were purchased from Sigma-Aldrich Company, St. Louis, MO, USA. All other chemicals were HPLC grade. Flaxseed oil was purchased from a local market.

Experimental animals

Male albino rats (Sprague Dawely Strain) weighing 180±20 g were obtained from the animal house of National Research Centre, Giza, Egypt. The animals were housed in individual suspended stainless steel cages in a controlled environment, the temperature of the animal house was fixed at about 22-25°c. Animals were maintained for one week in a controlled room (12 hour light 12 hour dark) and were fed a standard commercial diet (control diet) purchased from the Egyptian Company of Oils and Soaps. Water was available ad-libitum for acclimatization before starting the experiments. All animals received human care in compliance with guidelines of the Ethical Committee of National Research Centre (NRC), Egypt and followed the National Institutes of Health Guide Recommendations Care and Use of Laboratory Animals. The number of ethical committee permission is (13 / 096).

Induction of experimental hepatitis

LPS was dissolved in normal saline, pH was adjusted at 7.4 and intraperitonealy injected in rats (0.233 mg/kg body weight), after 2 h, D-GalN was dissolved in normal saline pH was adjusted at 7.4 and intraperitonealy injected in rats (583.3 mg/ kg body weight); blood samples were withdrawn after 22 h from D-GalN injection to check induction of hepatitis [2].

Experimental design

Sixty healthy male albino rats were divided into five groups (12 rats in each) as follows: Group I (control group): healthy rats received a vehicle. Group II (flaxseed oil group): healthy rats received flaxseed oil (1.2 ml/kg b.w./day) orally [2] for thirty days. Group III (LPS/DGalN group): healthy rats received a vehicle for 30 days before induction of hepatitis. Group IV (treated group I): healthy rats received flaxseed oil (1.2ml oil/kg b.w./day) orally for thirty days before induction of hepatitis [8]. Group V (treated group II): healthy rats received flaxseed oil (2.4 ml oil/kg b.w./day) orally for thirty days before induction of hepatitis.

At the end of the experimental period and during the injection of LPS/DGalN period, 24 h urine was collected from each animal for estimation of urinary hydroxyguanosine, then fasting blood samples were withdrawn from the retro orbital venous plexus. Liver was removed quickly from each rat and washed with ice-cold saline. A portion of the liver was homogenized in 0.1M Tris buffer for biochemical estimations. The other portion was used for immunohistochemical analysis.

Serum alanine amino- transferase (ALT) and aspartate amino- transferase (AST) activities were determined [9], serum alkaline phosphatase (ALP) was determined according to [10]. Liver malondialdehyde (MDA) was determined [11], liver superoxide dismutase (SOD) activity was measured [12] and reduced glutathione (GSH) was determined according to the method described previously [13]. Quantitative detection of liver tumor necrosis factor-α (TNF- α) and Interleukin – 1α (IL- 1α) was done by ELISA kits [14-15].

Determination of urinary 8-hydroxy 2′deoxyguanosine (8-OHdG)

8-hydroxyguanosine (8-OHdG) was estimated by HPLC system after modification of the method described previously [16] and according to Hussein et al. (2012). Briefly, 8-OHdG standard was dissolved in ultrapure water. Serial dilutions were prepared and were injected onto HPLC to draw a standard curve with different concentration.

Sample preparation
8-OHdG was extracted from 1 ml urine using Strata C18-E (55 um, 70A) column. The eluents were dried under nitrogen gas stream and were reconstituted in 5 ml ultrapure water. 20 µl from each sample were injected onto HPLC.

HPLC condition
The mobile phase consists of acetonitrile/methanol/phosphate buffer (25/10/965) v/v. Phosphate buffer was prepared by dissolving 8.8 g of potassium dihydrogen phosphate (KH_2PO_4) in 1000 ml deionized water and pH was adjusted at 3.5. The buffer was then filtered 2 times through 0.45 µm pore size sterile membrane filter before using at a flow rate of 1 ml/min through HPLC reverse phase column (250 × 4.6, particle size 5 µl) and electrochemical detector with cell potential of 600 mV. The concentration of urinary 8-OHdG was calculated from the standard curve and divided by the urinary creatinine which was estimated by kinetic method [18].

Immunohistochemistry of cyclooxygenases
Samples were embedded in 10% formalin; 5µm-thin sections were prepared on positive charged slides. The sections were deparaffinized and treated with 0.2% saponin (Thermo Fisher Scientific, Fremont Blvd, USA) at room temperature for 30 minutes. After the sections were treated with methanol containing 3% hydrogen peroxide for 15 minutes to eliminate endogenous peroxidase, the sections were reacted with 10% normal rabbit serum for 10 minutes to block nonspecific reactions. As the primary antibody, each of anti-cyclooxygenase-1 and anticyclooxygenase- 2 polyclonal antibodies (Thermo Fisher Scientific, Fremont Blvd, USA) were diluted 100 times and reacted with the sections at 4 °C for 15 hours. After the streptavidin–biotin complex method (Thermo Fisher Scientific, Fremont Blvd, USA), biotin-labeled anti-goat immunoglobulin G antibody as the secondary antibody was reacted with the sections at room temperature for 15 minutes, and the peroxidase-labeled streptavidin was reacted at room temperature for 10 minutes, followed by color development using diaminobenzidine (DAB) reagent. After counterstaining with hematoxylin, the sections were observed under a microscope.

Statistical analysis
Results in this study were represented in the form of mean ± standard error; the current data were analyzed using one-way ANOVA, version 16 of SPSS. When P value is < 0.05, it was considered a significant difference.

RESULTS

In this study, LPS/DGalN administration significantly increased liver enzymes (ALT and AST) compared to control group, while flaxseed oil administration significantly decreased these values compared to LPS/DGalN group. Moreover, administration of flaxseed oil in a dose of 2.4 ml/kg body weight significantly decreased liver functions compared to the dose of 1.2 of flaxseed oil/kg body weight (Table.1).

Data obtained from this study indicated that LPS/DGalN significantly increase hepatic MDA level and decreased hepatic GSH and SOD compared to the control group, administration of flaxseed oil in two different doses significantly increase hepatic antioxidant content compared to LPS/DGalN group (Table.2).

Chromatograms of urinary (8-OHdG) detected by HPLC indicated that LPS/DGalN administration significantly increased content of 8-OHdG as a DNA damage marker compared to the control group. Administration of flaxseed oil in two different doses significantly decreased 8-OHdG compared to LPS/DGalN group, although flaxseed oil administration in a dose of 2.4 ml/kg body weight (Treated II) significantly decreased 8-OHdG content compared to a dose of 1.2 ml /kg body weight(Treated I) (Table .2, Fig. 3).

Our data revealed that LPS/DGalN significantly increased hepatic inflammatory markers (TNF-α and IL 1 –α), while administration of flaxseed oil attenuated these levels (Fig 1, 2).

Our results were confirmed by immunohistochemical detection of both COX-1 (Fig. 4) and COX-2(Fig.5).

Table (1): liver functions in different studied groups

Parameters / Groups		ALT (U/L)	AST (U/L)	ALP (U/L)
Control	Mean±SE	9.4±0.5	33.5±0.8	77.5±2.1
Flaxseed oil	Mean±SE	9.4±0.2	31.2±0.6	82.3±1.4
LPS/DGalN	Mean±SE	55^{a}±1.1	51^{a}±0.8	286^{a}±1.9
Treated I	Mean±SE	$19.4^{a,b}$±1.1	$45.6^{a,b}$±3.2	$155^{a,b}$±4.1
Treated II	Mean±SE	$13.3^{a,b,c}$±1.3	$40.3^{a,b,c}$±0.7	$135^{a,b,c}$±1.1

Significant P value < 0.05
a=significant difference compared to control group
b=significant difference compared to LPS/DGalN
c =significant difference compared to treated II group.

Table (2): liver oxidants and antioxidants in different studied groups

Parameters / Groups		MDA (nmol/g tissue)	GSH (mg/g tissue)	SOD (U/g tissue)	8-hydroxy guanosine (pmol/ml)
Control	Mean±SE	6±0.9	7.2±0.9	265±2.2	5.8±2.1
Flaxseed oil	Mean±SE	5.6±0.5	7.3±0.2	262±2	5.7±0.1
LPS/DGalN	Mean±SE	28^{a}±0.5	5^{a}±0.4	203^{a}±1	13.3^{a}±0.6
Treated I	Mean±SE	$19.2^{a,b}$±1.3	6.5^{b}±0.1	216^{a} ±2.2	$8.7^{a,b}$±0.16
Treated II	Mean±SE	$16.7^{a,b}$±1.5	7.3^{b}±0.2	244^{b}±9	$7.7^{a,b,c}$±0.2

Data presented as mean ±SE
Significant P value < 0.05
a = significant difference compared to control group
b = significant difference compared to LPS/DGalN group.
c =significant difference compared to treated II group.

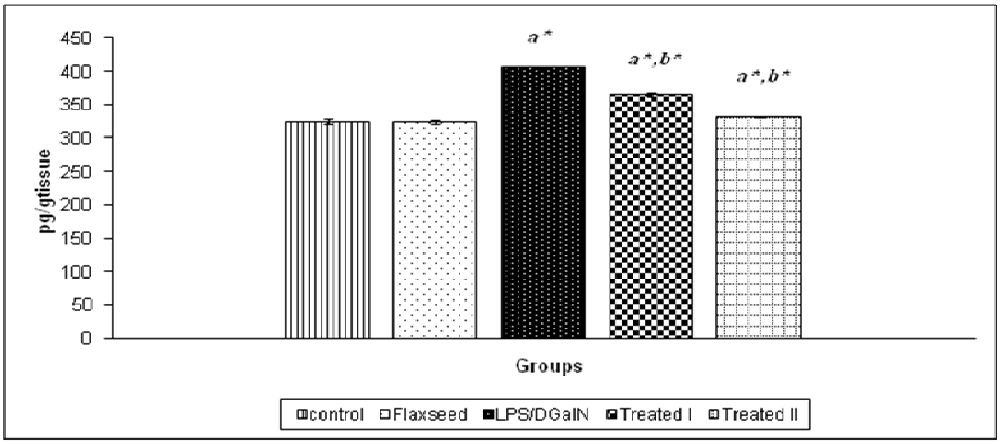

Fig. (1): Liver TNF-α levels in different studied groups
a= significant difference compared to control group, b= significant difference compared to LPS/DGalN group.

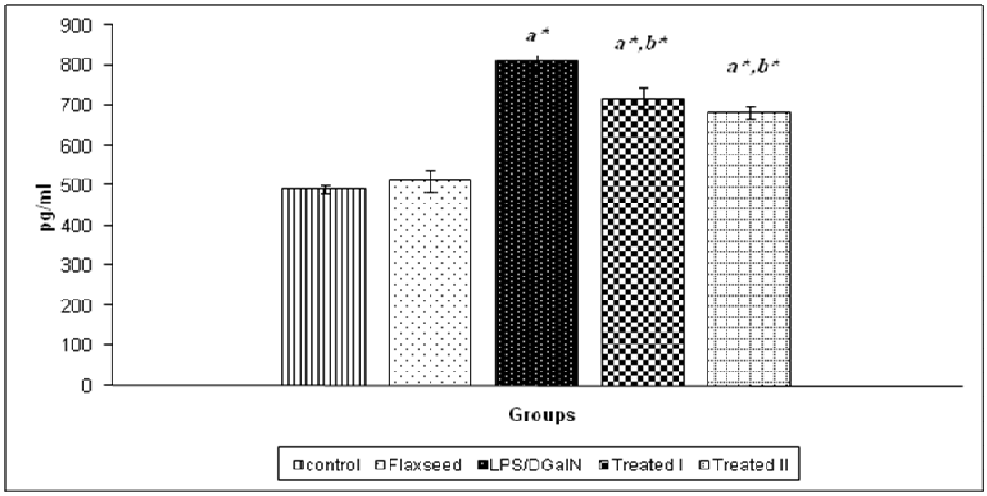

Fig. (2): Liver IL-1 α levels in different studied groups
a= significant difference compared to control group, b= significant difference compared to LPS/DGalN group.

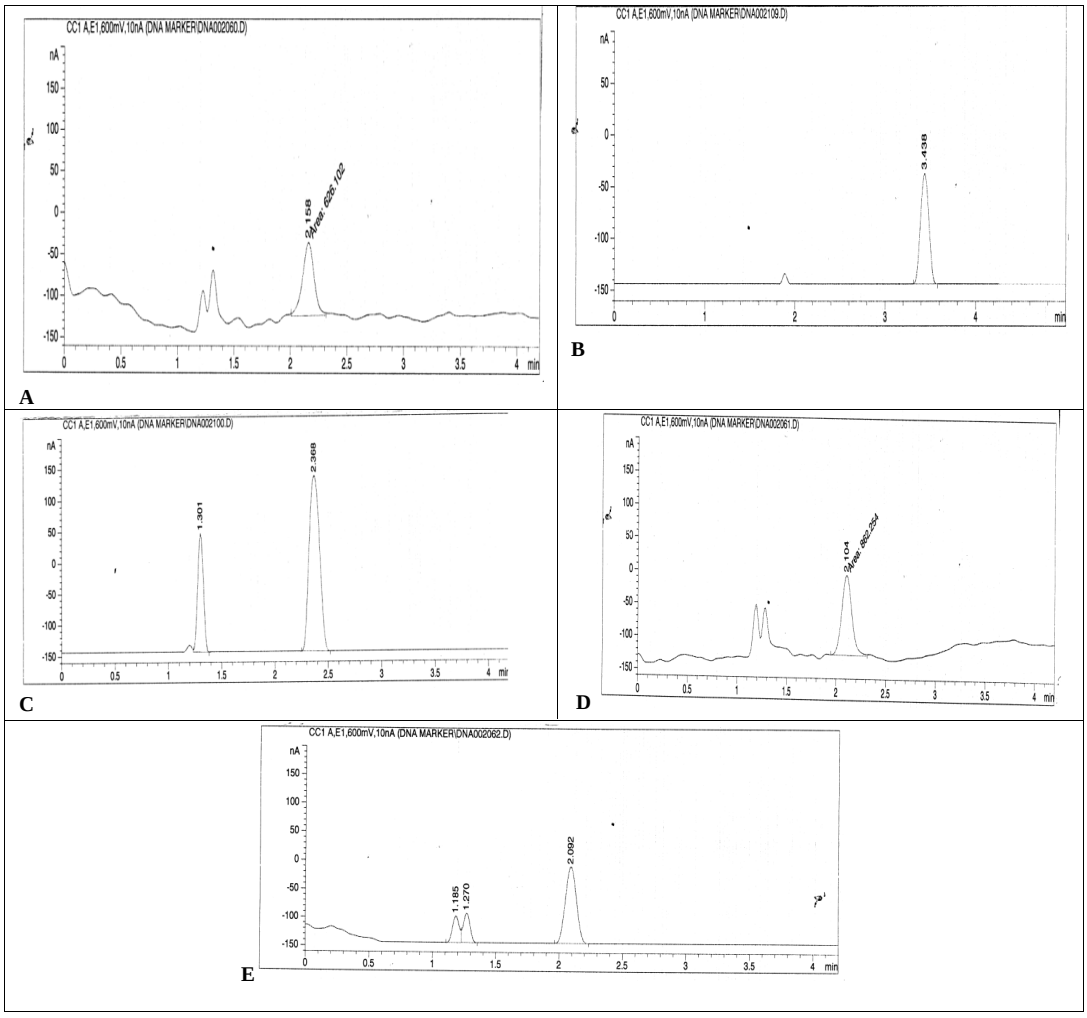

Fig.(3): HPLC chromatogram showing urinary 8-hydroxyguanosine in :A) control group; B) Flaxseed oil group; C) LPS/DGalN group; D) Treated I group(1.2 ml flaxseed oil/kg b.w./day) ; E) Treated II group (2.4 ml flaxseed oil/kgb.w./day)

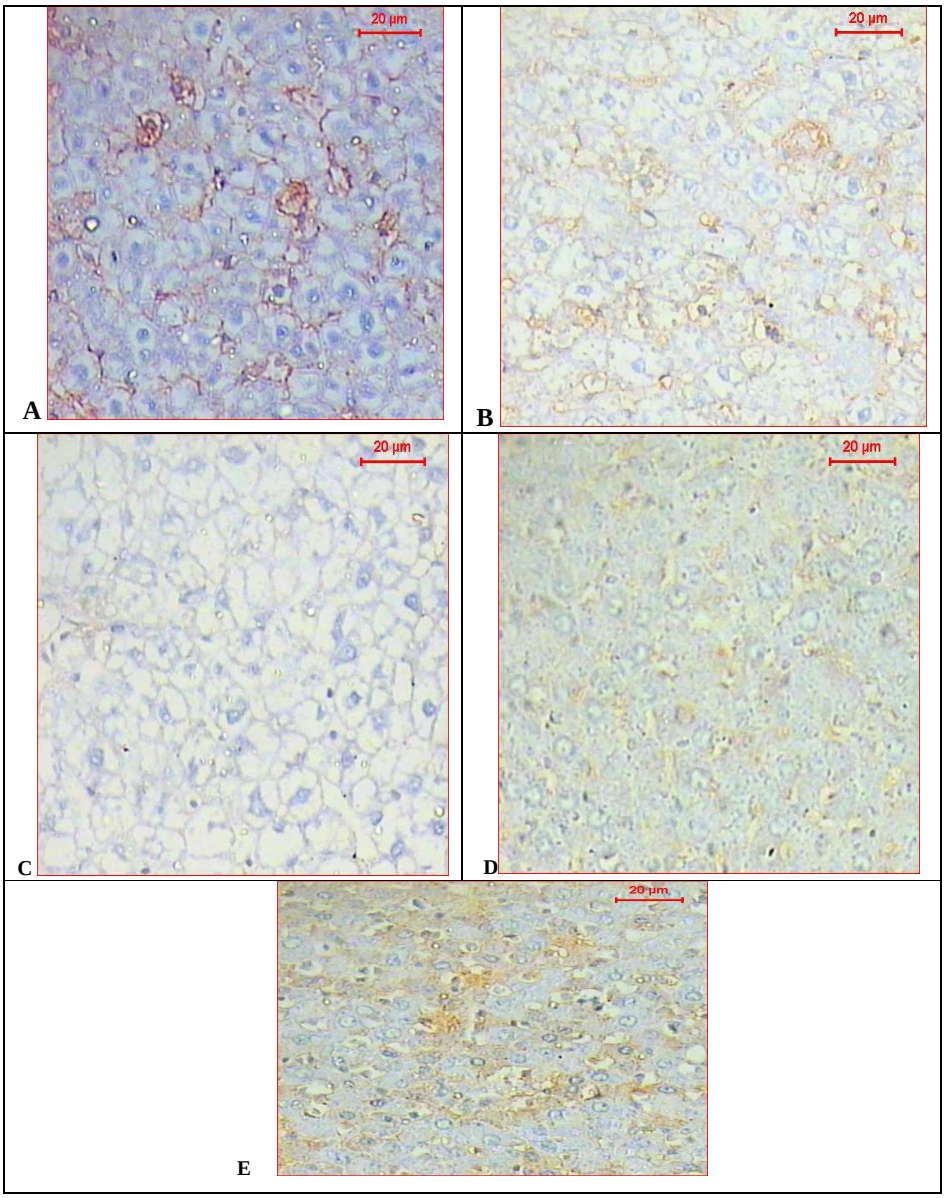

Fig.(4): A micrograph of section in liver of: A)control rat showing positive reaction of Cox-1 as indicated by brown color, B)rats administrated flaxseed oil (1.2 mg/kg/day for 30 days) showing positive reaction of Cox-1 as indicated by brown color, C)D-GalN/LPS group showing a negative reaction of Cox-1 as indicated by the absence of the brown color, D)D-GalN/LPS group treated with 1.2ml flaxseed oil showing a positive reaction of Cox-1 as indicated by brown color, E): D-GalN/LPS group treated with 2.4 ml flaxseed oil showing a positive reaction of Cox-1 as indicated by brown color.(Cox-1immunostaining, Scale bar 20 µm)

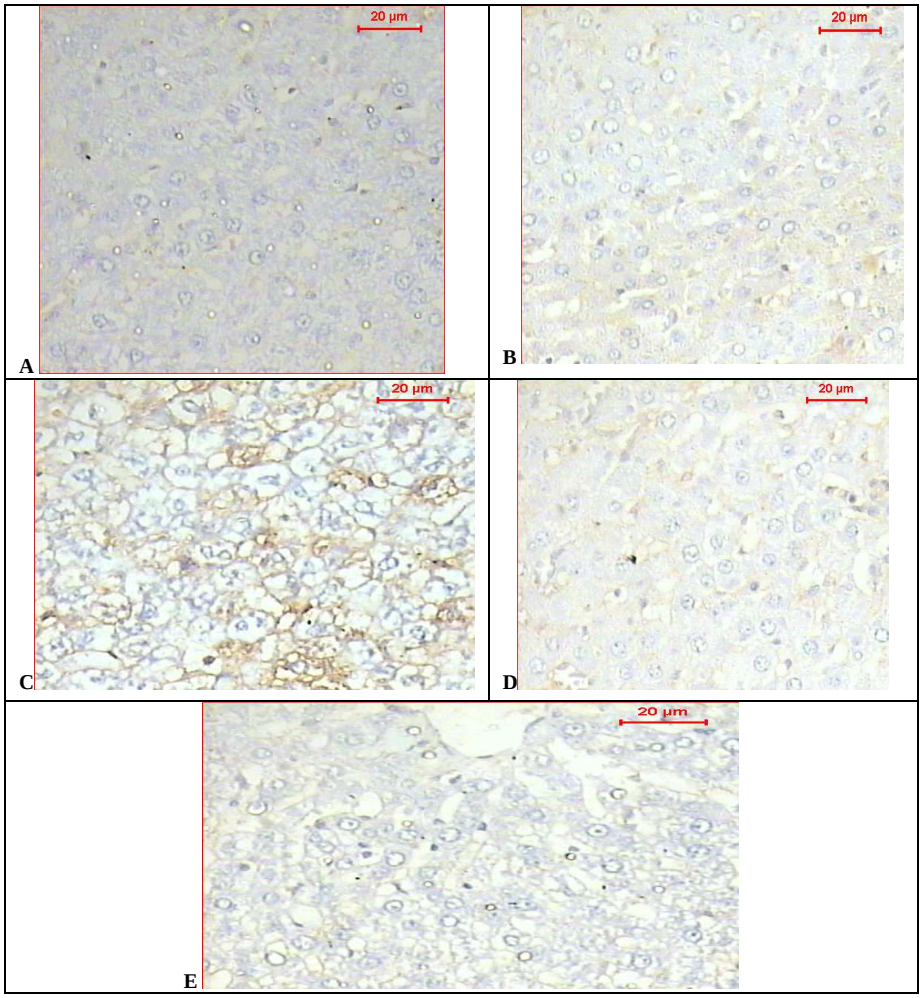

Fig.(5): A micrograph of section in liver of: A)control rat showing negative reaction of Cox-2 as indicated by the absence of the brown color, B) rats administrated Flaxseed oil (1.2 mg/kg/day for 30 days) showing negative reaction of Cox-2 as indicated by the absence brown color, C)D-GalN/LPS group showing a positive reaction of Cox-2 as indicated by the presence of the brown color, D)D-GalN/LPS group treated with 1.2ml flaxseed oil showing a little amount of brown color, positive reaction of Cox-2, E): D-GalN/LPS group treated with 2.4 ml flaxseed oil showing a negative reaction of Cox-2 as indicated by the absence of brown color.(Cox-2 immunostaining, Scale bar 20 µm)

DISCUSSION

D-GalN/LPS administration induced hepatic apoptosis and necrosis that followed by fulminate hepatitis [4]. Liver damage induced by these chemicals most like the changes that are noticed in human hepatitis so it represents an important regulation in the treatment of the disease and investigating drugs interaction [19].

The current data indicated a significant increase in serum ALT, AST and ALP levels as well as diminished liver GSH and SOD content and raised hepatic MDA content in LPS/D-GaIN group as mentioned previously [20].

Increased activities of these enzymes indicated changes in the membrane functions and permeability leading to a destruction of hepatic cells and cellular leakage indicating the powerful effect of D-GalN/LPS to induce hepatocellular toxicity as reported previously [21]; in addition to a release of pro-inflammatory cytokines which in turn lead to elevation of reactive oxygen species and oxidative stress resulting in hepatocytes damage and death [22].

D-GaIN suppresses the hepatic glucuronidation as it slashes hepatic uridyl diphosphateglucuronic acid (UDP-GA) while extrahepatic diphosphateglucuronic acid is less affected, thus the uridylate nucleotides' synthesis results in injury of the organelle is riven. When these nucleotides are depleted, it results in impairment of glycoprotein and protein synthesis which leads to a cumulative damage of the cell membrane and a change in its permeability leading to leakage of the enzymes from the cells [23].

Also D-GalN supresses the synthesis of protein and mRNA thus it causes uridine triphosphate pool' depletion [24].
A positive correlation was found between serum ALT and the 8-hydroxyguanosine content in liver showing that tissue inflammation may directly cause DNA damage.

Acute hepatitis caused by autoimmune, toxic or viral pathogenesis is distinguished by T cells and macrophages activation and an increase in cytokines production leading to liver damage and also dysfunction. Elevation of TNF-α in this study is considered one of the early stages in the liver inflammation, it takes place in liver damage in alcoholic hepatitis and steatohepatitis. TNF-α has an important role in hepatic failure induced by GalN/LPS [25] and it can cause an inflammatory cascade which induces other pro-inflammatory cytokines (IL-1, IL-6 and IFN-γ) that are responsible for inflammation and liver damage [5].

The result of increased SOD activity in treated group in this study suggested that flaxseed oil, the riche source of omega-3 fatty acids contains a free radical scavenging activity, which could exert a beneficial effect against pathological alterations caused by the presence of $O2^{-}$ and OH^{-} . The increased activity of SOD accelerates dismutation of $O2^{-}$ to hydrogen peroxide, which is removed by catalase (CAT). This action could involve mechanisms related to scavenging activity of flaxseed oil [26].

The reduction of TNF-α level in this study in the treated group may be due to a decrease in the nuclear factor kappa B (NF-k B) which is lipopolysaccharide (LPS) dependent. In addition, omega-3 fatty acids that found in flaxseed oil may be decreased TNF-α in this study through its mediators, the protectins and resolvins that have anti - inflammatory effect [27].

These observations suggest that omega-3 fatty acids may in general decrease inflammation susceptibility. Hence, the inflammatory response in liver tissue could also be decreased most probably by suppressing cytokine production and regulating Kupffer cell activation, also by suppressing IL-1 mRNA [28].

High content of unsaturated fatty acids especially omega-3 found in flaxseed oil upregulates genes involved in antioxidant enzymes expression and downregulates genes associated with reactive oxygen species production [29].
Many tissues constitutively expressed cyclooxygenase-1 (COX-1) which responsible for many physiological functions such as stomach cytoprotection, kidney vasodilatation, and production of thromboxane A2, proaggregatory, prostanoid by platelets.

Denkert et al [30] indicated that cyclooxygenase-2 (COX-2) is an inducible enzyme and is involved in prostaglandins production during inflammation.

In contrast, anti-inflammatory drugs decrease COX-2 expression [31] as was found in our experiment, thus omega-3 fatty acids in both treated groups decreased COX-2 to become more or less near the control group.

In agreement with our results, flaxseed shown to inhibit markers associated with prostate cancer; further, flaxseed oil is effective against intestinal colon cancer and tumorogenesis by downregulating the expression of COX-2 [32] and up regulating expression of COX-1 which are responsible for metabolism of arachidonic acid into prostaglandins (PGE) the main factor leading to inflammation and increasing tumor cells apoptosis.

We suggested that flaxseed oil supplementation increased cell membrane contents of omega-3 fatty acids and in contrast decreased the content of omega-6 fatty acids leading to a reduction of arachidonic acid liberation and in turn reduction of prostaglandins and pro-inflammatory cytokines and also free oxygen species , the important factors in liver inflammation and hepatitis. Increasing the supplemented dose of flaxseed oil (in treated group II) significantly increased the anti-inflammatory and anti-oxidant effect due to more reduction of arachidonic acid liberation and more reduction of pro-inflammatory markers as was found in our experiment.

CONCLUSION

We concluded that flaxseed oil confirms our idea about its powerful effect in protecting against liver injury through inhibition of inflammation as well as oxidative stress and this effect is dose dependent.

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