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FLAXSEED OIL AMELIORATES METHOTREXATE-INDUCED OXIDATIVE STRESS AND HEPTO-RENAL TOXICITY IN MALE RATS

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Keywords:

Methotrexate, Flaxseed oil, Liver, Kidney, Oxidative stress, Rats

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ABSTRACT: This study aimed to investigate the protective effect of flaxseed oil against hepato-renal toxicity induced by methotrexate. Rats treated with methotrexate exhibited elevations in the levels of AST, ALT, ALP, γGT, LDH, urea, creatinine, uric acid, and bilirubin. Furthermore, the levels of MDA, NO and the relative ratio of the gene expression of COX-2 and iNOS were significantly increased, accompanied by a decrease in the total protein, SOD, GPx, total thiol as well as the GSH content. Alterations in the lipid profile and hepato-renal histology were observed in rats treated with methotrexate. Also, up-regulation of α-SMA and loss of DNA bands integration were observed in methotrexate-treated rats. However, the oral treatment of flaxseed oil exhibited a protective effect against methotrexate toxicity in rats that could be attributed to its potent antioxidant, anti-inflammatory, and anti-apoptotic activities.

INTRODUCTION: Antifolates, a group of drugs imitating the structure of foliate coenzymes, have been used for the treatment of malignancies for decades [1]. Methotrexate (4-amino-10-methyl folic acid/amethopterin, MTX), a prototypical member of this group of drugs, is used commonly as a cytotoxic agent in the treatment of leukemia and other malignancies as well as in the inflammatory diseases [2]. The most common side effects of MTX are those involving gastrointestinal tract (GIT), liver, central nervous system (CNS), circulatory, and rarely respiratory [3]. MTX is used in chemotherapy regimens in which it does not discriminate between normal and malignant cells and hence promotes even normal cells to apoptosis [4].

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Flaxseed oil (FO) is approximately 53% α-linolenic acid (ALA), 17% linoleic acid (LA), 19% oleic acid, 3% stearic acid, and 5% palmitic acid, which provides an excellent n-6: n-3 fatty acid ratio of approximately 0.3:1 [5]. Consumption of flaxseed oil and flaxseed meal have become potential health benefits include anticancer, antiviral, antibacterial, anti-inflammatory, ion reduction, laxative uses and reduction of atherogenic risks [6]. The present study aimed to determine the hepato-renal toxicity of methotrexate and the possible protective effect of flaxseed oil.

MATERIALS AND METHODS:

Chemicals: Methotrexate was purchased from Shanxi PUDE Pharmaceutical Company Limited for Pharmaceutical Contract Development and Manufacturing-China. Flaxseed oil was purchased from Imtnan Health Company, Cairo, Egypt.

Animals and Experimental Design: Twenty-eight adult albino male rats weighing about 150-170 g were obtained from the animal house, Faculty of

Medicine, Alexandria University, Egypt. Rats were housed in stainless steel wire bottom cages in a room maintained at 25 °C with a 12-h light-dark cycle. Animals were fed rat pellet diet and water *ad libitum*. The experiments and the protocol were carried out according to the guidelines of the National Institutes of Health (NIH).

The experimental rats were divided as follows (7 rats each):

Group I (Control Group): Rats of this group were injected intraperitoneally with saline.

Group II (Flaxseed Oil-Treated Group): Rats of this group were ingested flaxseed oil at a dose of 1.8 ml/kg/day [7].

Group III (Methotrexate-Treated Group): Rats of this group were injected intraperitoneally with methotrexate at a dose of 3 mg/kg/week [8].

Group IV (Flaxseed Oil + Methotrexate-Treated Group): Rats of this group were ingested with flaxseed oil at a dose of 1.8 ml/kg/day and intraperitoneally with methotrexate at a dose of 3 mg/kg/week. Injection with flaxseed oil was proceeding methotrexate injection by 30 min.

Blood Collection: At the end of the experimental period (28 days), all animals of each group were anesthetized with diethyl ether and sacrificed. The blood samples were collected through an aorta section in the plain test tube. Blood samples were centrifuged at 3000 rpm for 5 min, and the serum was collected. Serum samples were left in the refrigerator at -20 °C until the measurement of the biochemical parameters.

Tissues Preparation: Liver and kidney of all experimental animals were immediately isolated, cleaned from blood adhering matters, washed in ice-cold saline and dried. Parts of the liver and kidney of each rat were sliced and immediately fixed in 10% formalin for the histological examination. One-fourth gram from each liver and kidney tissues was homogenized separately in 2 ml cold buffer (50 mM potassium phosphate pH 7.5, 1mM EDTA) per gram tissue using tissue homogenizer (Tekmar model TR-10, West Germany). The homogenate was centrifuged at 4000 rpm for 15 min. using the cooling centrifuge (Hettich model EBA 12R, Germany). Then the supernatants were stored at -80 °C for reduced glutathione (GSH) determination. The remaining liver and kidney tissues were frozen at -20 °C for the other biochemical investigations.

Biochemical Parameters: Determination of serum aspartate aminotransferase (AST; EC.2.6.1.1) alanine aminotransferase (ALT; EC.2.6.1.2), and creatinine were carried according to Murray [9] method. Alkaline phosphatase activity (ALP; EC.3.1.3.1), γ–glutamyl transferase (γ-GT; EC.2.3.2.2), and lactate dehydrogenase (LDH; E.C.1.1.1.27) were estimated according to the methods of Deutsche [10], Shaw *et al*., [11] and Friedman & Young [12], respectively. The levels of total protein (TP) [13] and albumin [14] were determined. Globulin is calculated by the equation: Globulin = Total protein-Albumin. Total bilirubin [15], urea [16], and uric acid [17] were estimated by using kits. Malondialdehyde (MDA) [18], nitric oxide level (NO) [19], antioxidant enzymes such as superoxide dismutase (SOD; EC.1.15.1.1) [20], glutathione peroxidase (GPx; EC.1.1.1.9) [21] as well as reduced glutathione (GSH; EC.1.6.4.2), [22] and the total thiol content [23] in the liver and kidney were determined. Serum total cholesterol (TC), triacylglycerides (TG), and high-density lipoprotein cholesterol (HDL-C) were analyzed according to the methods described by Tietz [24], Fossati *et al*., [25] and Grove [26], respectively. Low-density lipoprotein cholesterol (LDL-C) was estimated according to Friedewald *et al*. [27]

Determination of iNOS and COX-2: The inducible nitric oxide synthase (iNOS; E.C.1.14.13.39) and the cyclooxygenase-2 (COX-2; E.C.1.14.99.1) were determined by using a solid phase sandwich ELISA using 2 kinds of high specific antibodies.

Histological Investigation of the Liver and Kidney: The liver and kidney tissues were washed in running water overnight after fixation in 10% neutral formalin. They were dehydrated in graded ethanol (50%-100%), made transparent in xylol, and then embedded in paraffin. Sections (2-4 μm thickness) were obtained using a sliding microtome (Leica SM2000R, Germany) from the prepared paraffin blocks. These sections were stained with Hematoxylin-Eosin (H-E) [28].

Immunohistological Examination of α-Smooth Muscle Actin (*α*-SMA): Five-µm thick sections from formalin-fixed, paraffin-embedded liver and kidney tissues from all groups were cut on microscopic slides coated with 3-aminopropyl triethoxysilane for proper fixation of tissue sections of the slides and to minimize staining artifacts. After deparaffinization and subsequent blockage of the endogenous peroxidase activity by incubation in 0.3% methanolic hydrogen peroxide (10 min), the sections were then washed in phosphate buffered saline (PBS). Antigen retrieval was performed by boiling the slides twice in 10 mmol/l citrate buffer solution (pH: 6.0) for 5 min. Tissue sections were treated with normal horse serum for 10 min to avoid non-specific immunoreactivity. Duplicate liver and kidney sections were incubated overnight at 4 °C with mouse monoclonal anti-α-SMA antibody diluted 1:50. Sections were then incubated at room temperature with biotinylated goat anti-mouse antibody for 10 min followed by streptavidin-horseradish peroxidase conjugate. The reaction was visualized by the addition of diaminobenzidine substrate solution followed by counterstaining with Mayer's hematoxylin [29].

Random Amplified Polymorphic DNA (RAPD-PCR) Assay: The genomic DNA was isolated using phenol/chloroform extraction and ethanol precipitation method with minor modifications [30]. RAPD-PCR profiles from male rats DNA were generated using 2 primers **Table 1**. PCR amplification was conducted in 50 µl reaction volume containing 100 ng genomic DNA; 100 µM dNTPs; 40 nm primer; 2.5 units of Taq DNA polymerase, and 5 µl promega 10X Taq DNA polymerase buffer. The reactions were carried out in a thermocycler programmed first for denaturation of 5 min at 94 °C, followed by 45 cycles of 0.5 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C and finally, one cycle at 72 °C for 5 min. The PCR product was analyzed by electrophoresing 15 µl of the amplified mixture on an agarose gel. The Gel-Pro Analyzer (Media Cybernetics) was used to document ethidium bromide DNA gels.

TABLE 1: PRIMERS OF RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD-PCR)

Primer	Sequence
A01	5'-CAGGCCCTTC-3'
A02	5'-TGCCGAGCTG-3'

Statistical Analysis: All statistical analyses were conducted by using the Statistical Package for Windows Version 22.0 (SPSS Software, Chicago, IL). Values were compared by one-way analysis of variance (ANOVA). Post-hoc testing was performed for inter-group comparisons using the least significant difference (LSD) test, and $P \leq 0.05$ was considered statistically significant.

RESULTS:

Effect of MTX, FO and their Combination on Serum AST, ALT, ALP, γ-GT, and LDH Activities of Male Rats: The records presented in table 1 showed that the values of AST, ALT, ALP, γ-GT, and LDH were significantly ($P \leq 0.05$) increased after MTX administration compared to the control group. While the combination of FO plus MTX showed significant ($P \leq 0.05$) decrease in these enzyme levels compared to the MTX-treated group. An insignificant ($P \leq 0.05$) changes in AST, ALT, ALP, γ-GT, and LDH in the FO group whereas their levels were more or less like control.

TABLE 1: EFFECT OF MTX, FO AND THEIR COMBINATION ON SERUM AST, ALT, ALP, γ -GT, AND LDH ACTIVITIES OF MALE RATS

Parameters	Experimental groups			
	Control	FO	MTX	MTX + FO
AST (U/l)	25.61 ± 0.21 [a]	25.04 ± 0.08 [a]	40.59 ± 0.21 [b]	28.11 ± 0.09 [c]
ALT (U/l)	29.72 ± 0.71 [a]	29.09 ± 1.17 [a]	43.52 ± 2.03 [b]	31.48 ± 1.45 [c]
ALP (U/l)	340.10 ± 3.39 [a]	351.00 ± 0.20 [a]	711.38 ± 9.11 [b]	441.45 ± 0.77 [c]
γ-GT (U/l)	8.24 ± 0.31 [a]	8.29 ± 0.40 [a]	18.59 ± 0.58 [b]	12.25 ± 0.19 [c]
LDH (U/l)	2228.70 ± 14.87 [a]	2235.50 ± 12.55 [a]	5525.40 ± 122.23 [b]	2758.10 ± 316.40 [c]

Values are expressed as mean ± S.E. n=7 for each group. Mean values within a row not sharing a common superscript letter (a, b, c) were significantly different, $P \leq 0.05$.

Effect of MTX, FO and their Combination on TP, Albumin, Globulin, and TB of Male Rats: MTX-treatment caused significant ($P \leq 0.05$) decrease in the levels of TP and albumin while the values of globulin and TB were significantly ($P \leq 0.05$) increased as compared to the control group.

In contrast, the oral administration of FO with MTX revealed significant (P≤0.05) increase in the levels of TP and albumin while the levels of globulin and TB were significantly (P≤0.05) decreased concerning MTX-treated group. Administration of FO alone showed insignificantly (P≤0.05) changes in serum TP, serum albumin, globulin, and TB compared to the control group **Table 2**.

TABLE 2: EFFECT OF MTX, FO, AND THEIR COMBINATION ON TP, ALBUMIN, GLOBULIN, AND TB OF MALE RATS

Parameters	Experimental groups			
	Control	FO	MTX	MTX + FO
TP (g/dl)	6.40 ± 0.07[a]	6.36 ± 0.06[a]	5.64 ± 0.04[b]	6.27 ± 0.16[c]
Albumin (g/dl)	2.81 ± 0.09[a]	2.76 ± 0.05[a]	4.29 ± 0.09[b]	3.46 ± 0.05 [c]
Globulin (g/dl)	3.59 ± 0.08[a]	3.60 ± 0.08[a]	1.35 ± 0.08[b]	2.81 ± 0.08 [c]
TB (g/dl)	0.22 ± 0.006[a]	0.22 ± 0.006[a]	0.42 ± 0.011[a]	0.22 ± 0.006 [c]

Values are expressed as mean ± S.E. n=7 for each group. Mean values within a row not sharing a common superscript letter (a, b, c) were significantly different, P≤0.05.

Effect of MTX, FO, and their Combination on Creatinine, Urea and Uric Acid Levels of Male Rats: The values of creatinine, urea, and uric acid were significantly (P≤0.05) increased by the administration of MTX compared to the control group. On the other hand, the combination of FO plus MTX showed significant (P≤0.05) decrease in the levels of creatinine, urea, and uric acid compared to MTX-treated group **Table 3**. The oral administration of FO alone caused insignificant (P≤0.05) decrease in serum creatinine, urea, and uric acid compared to the control group.

TABLE 3: EFFECT OF MTX, FO, AND THEIR COMBINATION ON CREATININE, UREA, AND URIC ACID LEVELS OF MALE RATS

Parameters	Experimental groups			
	Control	FO	MTX	MTX + FO
Creatinine (mg/dl)	0.63 ± 0.007[a]	0.62 ± 0.009[a]	1.03 ± 0.01[b]	0.72 ± 0.008[c]
Urea (mg/dl)	36.73 ± 0.32[a]	36.09 ± 0.18[a]	45.67 ± 0.60[b]	38.08 ± 0.24[c]
Uric acid (mg/dl)	0.93 ± 0.01[a]	0.82 ± 0.02[a]	3.85 ± 0.18[b]	1.48 ± 0.06[c]

Values are expressed as mean ± S.E. n=7 for each group. Mean values within a row not sharing a common superscript letter (a, b, c) were significantly different, P≤0.05.

Effect of MTX, FO and their Combination on Liver and Kidney MDA, NO and GSH Levels of Male Rats: As shown in **Table 4**, treatment of adult male rats with MTX alone showed significant (P≤0.05) increase in liver and kidney MDA and NO levels while GSH showed significant (P≤0.05) decrease compared to the control rats. The combination of FO plus MTX showed significant (P≤0.05) decrease in the levels of MDA and NO and significant (P≤0.05) increase in GSH of liver and kidney as compared to the MTX-treated group. Oral administration of FO alone showed insignificant (P≤0.05) alterations in liver and kidney MDA and GSH. But, it showed significant (P≤0.05) decrease in kidney NO while, liver NO was similar to the control group **Table 4**.

TABLE 4: EFFECT OF MTX, FO AND THEIR COMBINATION ON LIVER AND KIDNEY MDA, NO, AND GSH LEVELS OF MALE RATS

Parameters	Experimental groups			
	Control	FO	MTX	MTX + FO
Liver				
MDA (nmol/g tissue)	3.97 ± 0.25 [a]	3.59 ± 0.28[a]	10.71 ± 0.20[b]	5.10 ± 0.59[c]
NO (µmol/g tissue)	0.46 ± 0.005 [a]	0.46 ± 0.021[a]	0.65 ± 0.007[b]	0.55 ± 0.009 [c]
GSH (µmol/g tissue)	54.49 ± 0.10[a]	53.21 ± 0.83[a]	15.64 ± 0.60[b]	49.57 ± 1.18 [c]
Kidney				
MDA (nmol/g tissue)	5.70 ± 0.20 [a]	5.28 ± 0.36[a]	16.56 ± 0.26[b]	6.51 ± 0.42 [c]
NO (µmol/g tissue)	0.36 ± 0.008[a]	0.325 ± 0.006[a]	0.741 ± 0.008[b]	0.450 ± 0.01 [c]
GSH (µmol/g tissue)	53.49 ± 0.43[a]	53.94 ± 0.50[a]	17.05 ± 0.29[b]	52.19 ± 0.28 [c]

Values are expressed as mean ± S.E. n=7 for each group. Mean values within a row not sharing a common superscript letter (a, b, c) were significantly different, P≤0.05.

Effect of MTX, FO and their Combination on Liver and Kidney Total Thiol, SOD, and GPx Levels of Male Rats: As shown in **Table 5** liver and kidney total thiol, SOD, and GPx were significantly (P≤0.05) decreased in MTX group compared to the control rats. While, the combination of FO+MTX showed significant (P≤0.05) increase in the levels of total thiol, SOD, and GPx in both liver and kidney compared to the MTX-treated group.

TABLE 5: EFFECT OF MTX, FO, AND THEIR COMBINATION ON LIVER AND KIDNEY TOTAL THOIL, SOD, AND GPX LEVELS OF MALE RATS

Parameter	Experimental Groups			
	Control	FO	MTX	MTX + FO
Liver				
Total thiol (µmol/g tissue)	4.51 ± 0.08[a]	4.66 ± 0.08[a]	1.83 ± 0.09[b]	4.10 ± 0.22[c]
SOD (U/mg protein)	4.32 ± 0.16[a]	4.43 ± 0.08[a]	2.59 ± 0.07[b]	3.33 ± 0.07[c]
GPx (mU/mg protein)	245.70 ± 4.02[a]	245.66 ± 3.43[a]	189.34 ± 1.08[b]	211.23 ± 2.12[c]
Kidney				
Total thiol (µmol/g tissue)	2.53 ± 0.08[a]	2.67 ± 0.06[a]	1.28 ± 0.09[b]	2.08 ± 0.08[c]
SOD (U/mg protein)	4.53 ± 0.16[a]	4.54 ± 0.09[a]	2.94 ± 0.05[b]	3.75 ± 0.05[c]
GPx (mU/mg protein)	193.71 ± 2.61[a]	194.50 ± 0.59[a]	116.81 ± 2.76[b]	163.61 ± 6.88[c]

Values are expressed as mean ± S.E. n=7 for each group. Mean values within a row not sharing a common superscript letter (a, b, c) were significantly different, P≤0.05.

Effect of MTX, FO and their Combination on Serum Lipid Profile of Male Rats: As presented in **Table 6**, the values of cholesterol, LDL-C, and triglycerides were significantly (P≤0.05) increased while serum HDL-C was significantly (P≤0.05) decreased after administration of MTX compared to the control group. On the other hand, the combination of FO plus MTX showed significant (P≤0.05) decrease in the levels of cholesterol, LDL-C, and triglycerides and significant (P≤0.05) increase in the level of HDL-C compared to MTX-treated group. The values of cholesterol, LDL-C, and HDL-C were insignificantly (P≤0.05) decreased by administration of FO compared to the control group.

TABLE 6: EFFECT OF MTX, FO, AND THEIR COMBINATION ON SERUM LIPID PROFILE OF MALE RATS

Parameters	Experimental groups			
	Control	FO	MTX	MTX + FO
Cholesterol (mg/dl)	123.00 ± 1.69[c]	122.00 ± 1.31[c]	143.00 ± 1.32[a]	131.00 ± 2.84[b]
Triglycerides (mg/dl)	45.45 ± 0.16[c]	47.00 ± 0.16[c]	78.33 ± 1.41[a]	54.13 ± 0.55[b]
LDL-c (mg/dl)	59.65 ± 0.54[c]	58.68 ± 1.48[c]	101.51 ± 0.58[a]	77.04 ± 0.75[b]
HDL-c (mg/dl)	54.72 ± 0.1[a]	53.81 ± 0.2[a]	25.82 ± 0.2[c]	43.13 ± 0.5[b]

Values are expressed as mean ± S.E. n=7 for each group. Mean values within a row not sharing a common superscript letter (a, b, c) were significantly different, P≤0.05.

Effect of MTX, FO and their Combination on Liver and Kidney iNOS and COX-2 of Male Rats: The data in **Table 7** showed that the values of iNOS and COX-2 in both liver and kidney were significantly (P≤0.05) increased after administration of MTX compared to the control group. The combination of FO plus MTX showed significant (P≤0.05) decrease in the values of iNOS and COX-2 in both liver and kidney compared to the MTX-treated group. The values of liver and kidney iNOS were insignificantly (P≤0.05) decreased by administration of FO while, COX-2 was insignificantly (P≤0.05) increased as compared to the control group.

TABLE 7: EFFECT OF MTX, FO, AND THEIR COMBINATION ON LIVER AND KIDNEY INOS AND COX-2 OF MALE RATS

	Experimental Groups			
Parameters	Control	FO	MTX	MTX + FO
Liver				
iNOS (nmol/g tissue)	6.80 ± 0.42[a]	6.28 ± 0.11[a]	68.60 ± 0.31[b]	20.80 ± 0.53[c]
COX-2 (nmol/g tissue)	4.30 ± 0.14[a]	4.78 ± 0.09[a]	19.09 ± 0.47[b]	10.29 ± 0.11[c]
Kidney				
iNOS (nmol/g tissue	12.58 ± 0.52[a]	10.69 ± 0.26[a]	56.09 ± 2.24[b]	24.10 ± 1.61[c]
COX-2 (nmol/g tissue	5.67 ± 0.59[a]	6.30 ± 0.15[a]	29.47 ± 0.59[b]	12.67 ± 0.19[c]

Values are expressed as mean ± S.E. n=7 for each group. Mean values within a row not sharing a common superscript letter (a, b, c) were significantly different, P≤0.05.

Effect of MTX, FO and their Combination on the Histopathological Examination of Liver and Kidney Tissues of Male Rats: Microscopic examination of control and flaxseed oil liver sections exhibited the normal histological appearance of hepatocytes, sinusoidal spaces and a central vein **Fig. 1A**, **1B** & **1C**. The MTX treatment motivated extensive necrosis and lymphocytes aggregation. The normal radial arrangements of hepatocytes from central vein were strictly distorted with pyknotic cells. Congestion and hemorrhage were disclosed throughout the hepatic parenchyma. Numerous diploid and megalo-hepatocytes with enlarged nuclei were observed. The portal areas revealed congested portal vein and round cells infiltration **Fig. 1D** & **1E**. The combination group (FO+MTX) showed a reduction in the lesions that induced by MTX alone and restored it more or less near to the normal appearance **Fig. 1F**. Meanwhile, microscopic examination of control and flaxseed oil kidney sections **Fig. 2A** & **2B** showed a normal histological pattern with normal glomerulus surrounded by the Bowman's capsule, proximal and distal convoluted tubules without any inflammatory changes. MTX-treated group **Fig. 2C** & **2D** showed degeneration of the renal tubules with disruption of the basement membranes in-between the tubules. Most of the renal tubules showed cystic luminal dilatation and their lining cells are flat. Degenerated and atrophy of glomeruli in the MTX-treated group. Treatment with FO in combination with MTX **Fig. 2E** slightly improved the kidney histology except for dilation of some proximal and distal tubules.

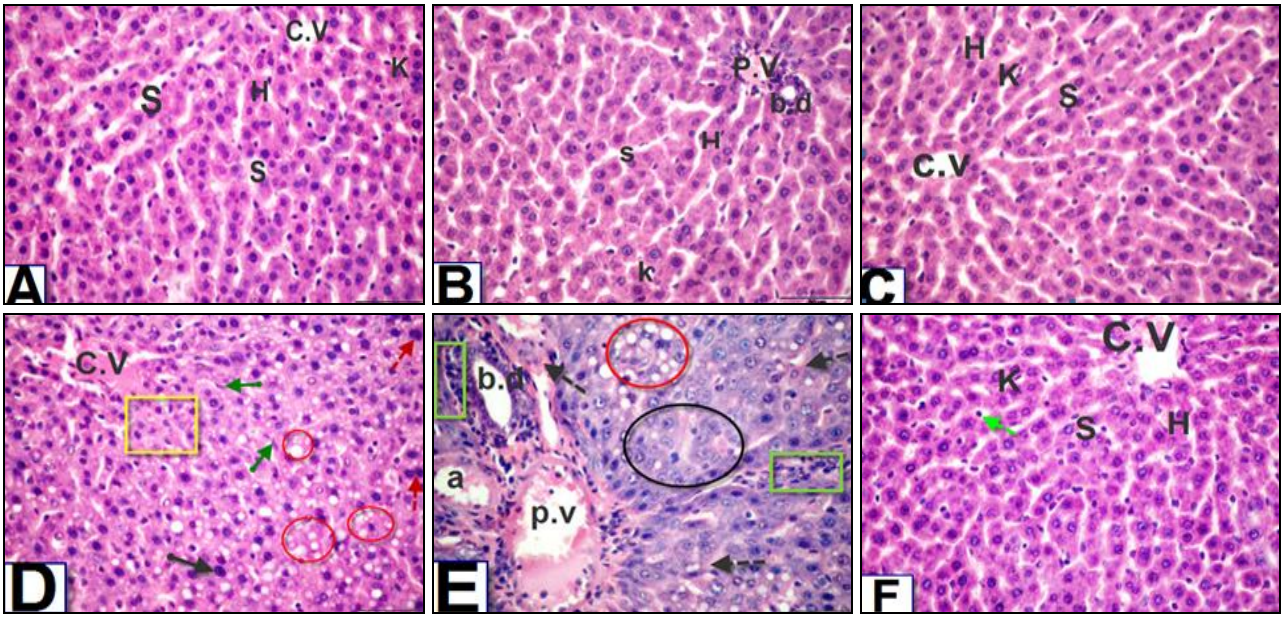

FIG. 1: PHOTOMICROGRAPHS OF LIVER CONTROL (A & B) AND FO-TREATED RAT (C) SHOWED THE NORMAL HEPATOCYTES STRUCTURE WITH NORMAL VESICULATED NUCLEI (H), CENTRAL VEIN (C.V.) BLOOD SINUSOIDS (S) WITH FEW KUPFFER CELLS (K), PORTAL VEIN (P.V.) AND BILE DUCT (B.D.). LIVER OF MTX-TREATED RATS (D & E) SHOWED A LOSS IN THE NORMAL HEPATOCYTIC ARCHITECTURE, PRESENCE OF VACUOLES (RED CIRCLE), DEGENERATION OF HEPATOCYTES (YELLOW SQUARE & BLACK CIRCLE) WITH PYKNOTIC NUCLEI, CONGESTION OF CENTRAL VEIN (C.V.), MEGALOHEPATOCYTES (BLACK ARROW), BINUCLEATED HEPATOCYTES (RED DOTTED ARROW), MORE KUPFFER CELLS (GREEN ARROW), INFLAMMATORY INFILTRATE AROUND THE PORTAL TRACT AND THE BILE DUCT AND ACTIVATION OF THE KUPFFER CELLS (GREEN SQUARE), DILATION AND CONGESTION OF PORTAL VEIN (P.V.) AND ARTERY (A) AND CONGESTION IN BLOOD SINUSOIDS (BLACK DOTTED ARROW). ON THE OTHER HAND, LIVER OF FO+MTX-TREATED RAT (F) SHOWED THAT HISTOLOGICAL ALTERATIONS WERE MARKEDLY REDUCED EXCEPT PRESENCE OF FEW PYKNOTIC NUCLEI (LIGHT GREEN DOTTED ARROW) (H & E STAIN, X 400)

Effect of MTX, FO, and their Combination on *α*-SMA Expression in Liver and Kidney Tissues: The data of immunohistochemically stained sections of control and flaxseed oil liver showed normal expression of α-SMA positively stained brown HSC **Fig. 3A** & **3B**. The immunohistochemically stained sections of liver treated with MTX **Fig. 3C** showed strong immunoreactive expression of *α*-SMA represented by brown color positively among hepatic stellate cells mainly around center veins and forming intra-acinar thick bands and in the sinusoidal lining. In

contrast, the protective group (MTX+FO) showed little brown coloration scattered around hepatic sinusoids **Fig. 3D**.

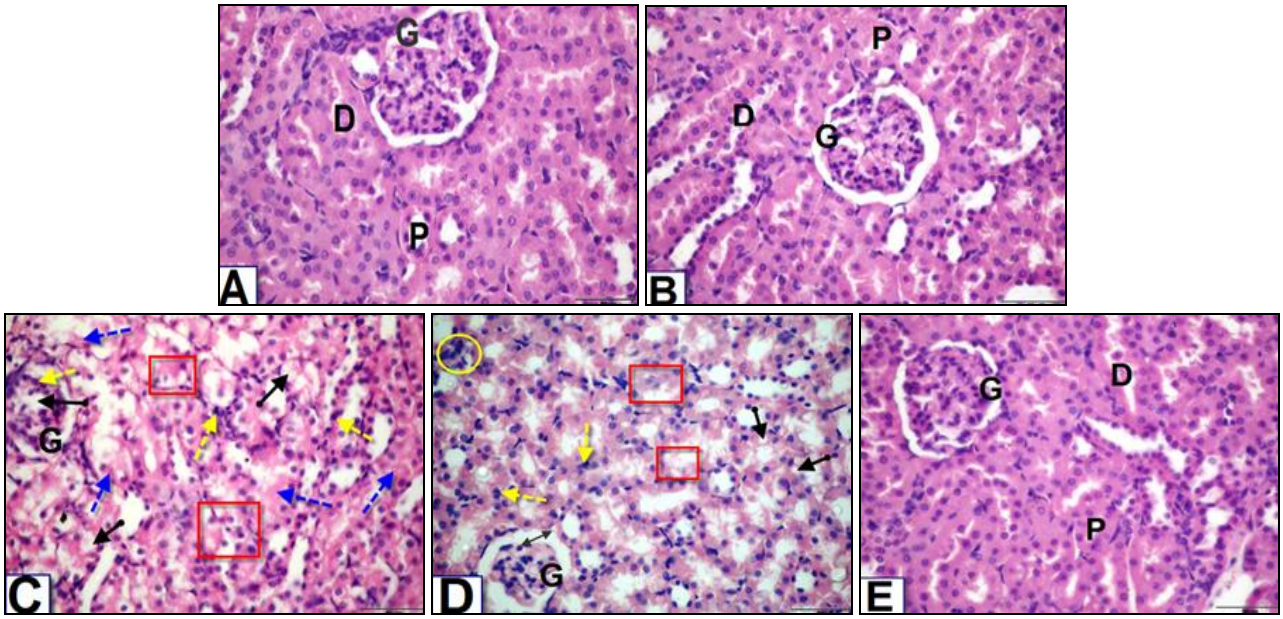

FIG. 2: PHOTOMICROGRAPHS OF T.S. KIDNEY OF CONTROL & FO-TREATED RATS (A&B) SHOWED NORMAL RENAL ARCHITECTURE, NORMAL GLOMERULAR TUFT (G) PROXIMAL CONVOLUTED TUBULE (P) AND DISTAL CONVOLUTED TUBULES (D): KIDNEY OF MTX-TREATED RATS (C & D) SHOWED ATROPHIED GLOMERULAR TUFT (G) WITH WIDE SPACE AND PRESENCE OF VACUOLES (BLACK DOUBLE HEAD ARROW & BLACK ARROW), VACUOLAR DEGENERATION IN MOST OF THE TUBULAR EPITHELIAL CELL & SEVERE DILATATION OF CORTICAL RENAL TUBULES (RED SQUARE & BLACK ARROW), EXFOLIATED CELLS, CELLULAR DEBRIS (BLUE DOTTED ARROW), PYKNOTIC NUCLEI (YELLOW DOTTED ARROW) AND FOCAL AREAS OF PERITUBULAR LYMPHOCYTIC INFILTRATION (YELLOW CIRCLE).WHILE, KIDNEY OF FO+MTX-TREATED RATS (E) SHOWED THAT HISTOLOGICAL ALTERATIONS WERE MARKEDLY REDUCED (H & E STAIN, X 400).

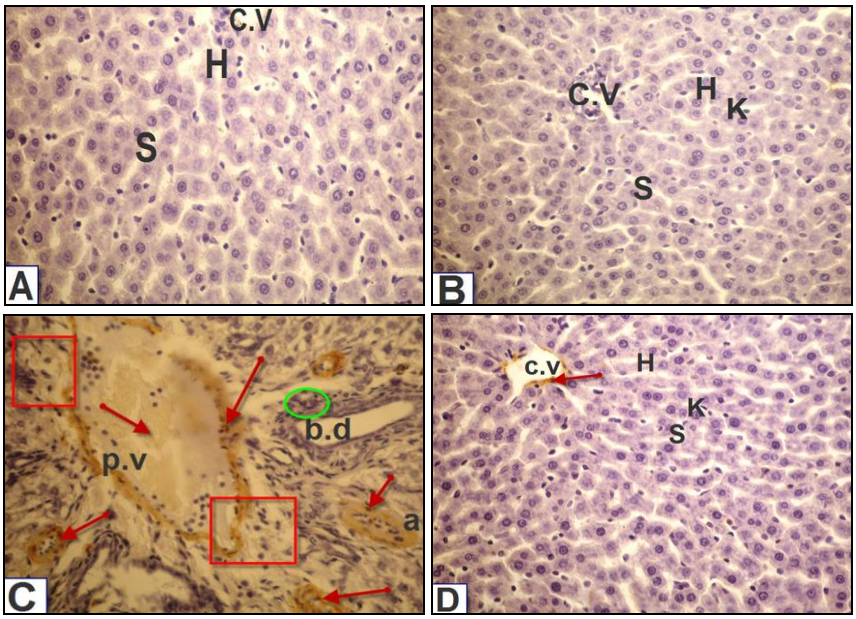

FIG. 3: PHOTOMICROGRAPHS OF T.S. LIVER OF CONTROL GROUP (A) AND FO-TREATED GROUP (B), SHOWING: NO IMMUNOREACTIVE EXPRESSION OF A- SMA, ALSO NORMAL HEPATOCYTES STRUCTURE WITH NORMAL VESICULATED NUCLEI (H), CENTRAL VEIN (C.V) AND BLOOD SINUSOIDS (S) WITH FEW KUPFFER CELLS (K). PHOTOMICROGRAPH OF T.S IN LIVER OF MTX -TREATED RAT (C) SHOWING: STRONG IMMUNOREACTIVE EXPRESSION OF A- SMA REPRESENTED BY BROWN COLOR OF A- SMA POSITIVELY AMONG HEPATIC CELLS MAINLY AROUND PORTAL VEIN, ARTERY AND IN SINUSOIDAL LINING (RED ARROW &RED SQUARE), PRESENCE OF INFLAMMATORY INFILTRATE (GREEN CIRCLE). WHILE T.S. IN LIVER OF FO+MTX-TREATED RAT (D) SHOWING: LITTLE BROWN COLORATION AROUND A CENTRAL VEIN (RED ARROW). (A- SMA IMMUNOHISTOCHEMICAL STAIN, X 400).

The immunohistochemically stained sections of control and flaxseed oil kidney showed normal expression of α-SMA positively stained brown HSC **Fig. 4A** & **4B**. The immunohistochemically stained sections of kidney treated with MTX **Fig. 4C** & **4D** showed intense staining by *α*-SMA in the tunica media of the renal blood vessels and the interstitial tissue surrounding the blood vessels. In contrast, the protective group (MTX+FO) showed little brown coloration scattered around renal blood vessels **Fig. 4E**.

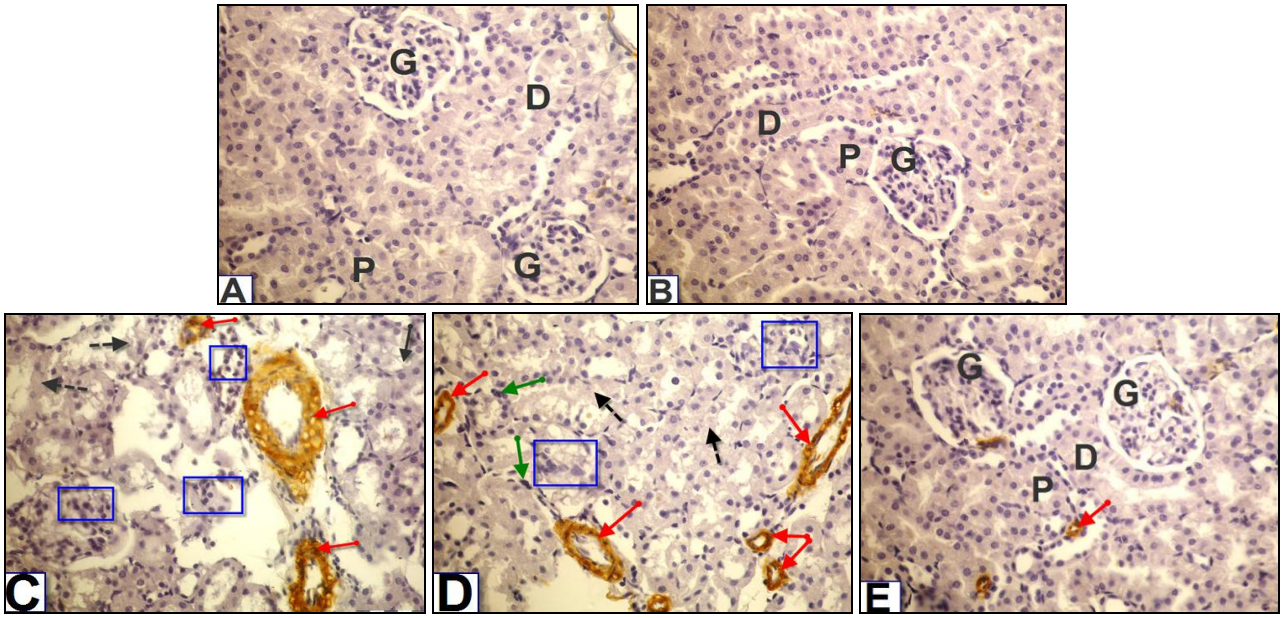

FIG. 4: PHOTOMICROGRAPHS OF CONTROL (A) AND FO-TREATED GROUPS (B) KIDNEY SHOWED NO IMMUNO-REACTIVE EXPRESSION OF A- SMA, NORMAL RENAL ARCHITECTURE, NORMAL GLOMERULAR TUFT (G) PROXIMAL CONVOLUTED TUBULE (P) AND DISTAL CONVOLUTED TUBULES (D). PHOTOMICROGRAPHS OF MTX KIDNEY (C & D) SHOWED STRONG IMMUNOREACTIVE EXPRESSION OF A- SMA REPRESENTED BY BROWN COLOR POSITIVELY AROUND RENAL TUBULES AND BLOOD VESSELS (RED ARROW), DEGENERATION OF RENAL TUBULES AND GLOMERULUS WITH PYKNOTIC NUCLEI (BLUE SQUARE & GREEN ARROW) & CELLULAR DEBRIS (BLACK DOTTED ARROW). WHILE, KIDNEY OF FO+MTX-TREATED RAT (E) SHOWED LITTLE BROWN COLORATION (RED ARROW) OF A-SMA (A-SMA IMMUNOHISTOCHEMICAL STAIN, X 400).

Effect of MTX, FO, and their Combination of RAPD-PCR DNA in Both Liver and Kidney Tissues of Male Rats: The two primers produced highly similar RAPD fingerprints for negative control and FO groups. MTX treatment caused occurrence or loss of some amplification products of different groups which indicated in both liver and kidney tissues as compared to the control group.

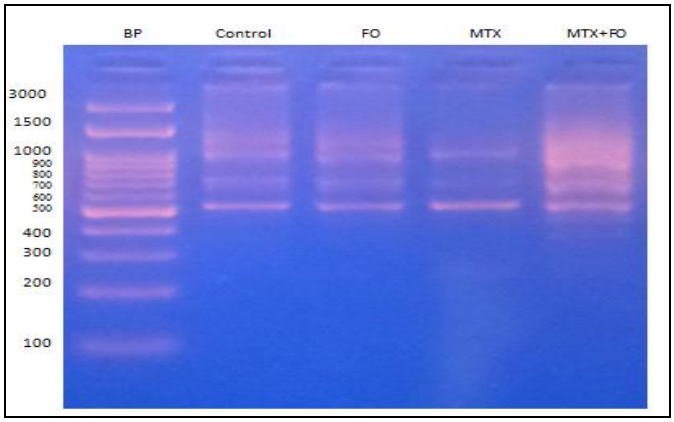

FIG. 5: PATTERN RAPD-PCR (PRIMER A01) OF HEPATIC DNA SAMPLES, LANE1CONTROL GROUP, LANE 2 FLAXSEED OIL-TREATED GROUP, LANE 3 METHOTREXATE-TREATED GROUP, LANE 4 METHOTREXATE+FLAXSEED OIL GROUP

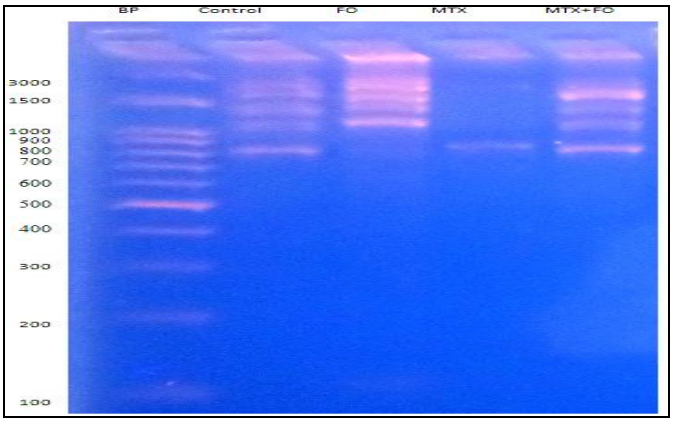

FIG. 6: PATTERN RAPD-PCR (PRIMER A01) OF KIDNEY DNA SAMPLES, LANE1CONTROL GROUP, LANE 2 FLAXSEED OIL-TREATED GROUP, LANE 3 METHOTREXATE-TREATED GROUP, LANE 4 METHOTREXATE+FLAXSEED OIL GROUP

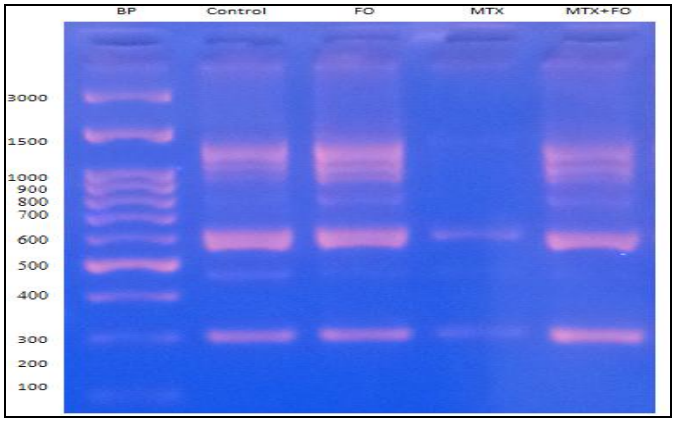

FIG. 7: PATTERN RAPD-PCR (PRIMER A02) OF HEPATIC DNA SAMPLES, LANE1CONTROL GROUP, LANE 2 FLAXSEED OIL-TREATED GROUP, LANE 3 METHOTREXATE-TREATED GROUP, LANE 4 METHOTREXATE+FLAXSEED OIL GROUP

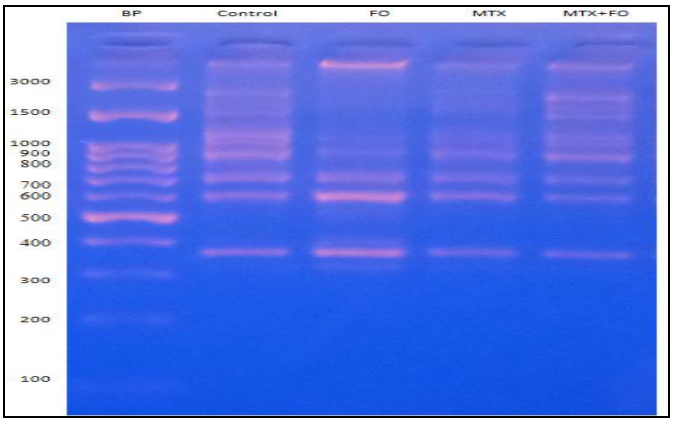

FIG. 8: PATTERN RAPD-PCR (PRIMER A02) OF KIDNEY DNA SAMPLES, LANE1CONTROL GROUP, LANE 2 FLAXSEED OIL-TREATED GROUP, LANE 3 METHOTREXATE-TREATED GROUP, LANE 4 METHOTREXATE+FLAXSEED OIL GROUP

Animals administrated MTX in combination with FO partially restore the DNA bands integration to near the control bands. FO administration alone did not alter both liver and kidney DNA bands when compared to the control one's **Fig. 5, 6, 7** & **8**. The amplified fragments of PCR products were summarized as in **Table 8**. The RAPD products were scored as present (1) or absent (0) for each primer-genotype combination. Nineteen bands were scored where sixteen were polymorphic and 3 of them were monomorphic. Jaccard's coefficient of similarity was measured and a dendrogram **Fig. 9** based on similarity coefficients generated by using the unweighted pair group method with arithmetic mean (UPGMA).

TABLE 8: RANDOM PRIMERS SHOWING POLYMORPHISM OF DNA FROM LIVER AND KIDNEY OF THE FOUR GROUPS

Primer code	Nucleotide sequence 5→ 3/span>	Total number of amplified fragments	Number of monomorphic fragments	Number of polymorphic fragments	Fragments size range (bp)
1	CAG GCC CTT C	9	2	7	540-1300
2	AAT CGG GCT G	10	1	9	300-1350
	Total	19	3	16	

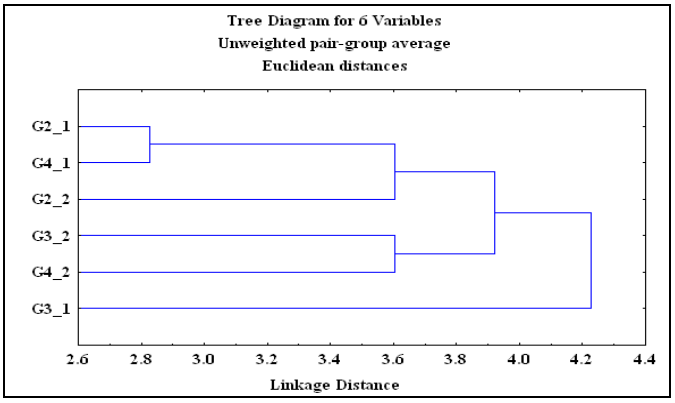

FIG. 9: DENDROGRAM OF THREE APPLIED GROUPS GENERATED BY UPGMA BASED ON 2 RAPD PRIMERS, WHERE G2 IS GROUP 2, G3 IS GROUP 3 AND G4 IS GROUP 4

DISCUSSION: The present findings suggested that methotrexate strongly disrupted hepatic function in rats as evidenced by elevated levels of ALT, AST, ALP, γGT, and LDH and reduction in serum total protein and albumin. These alterations may be due to changes in the permeability of liver cell and damage or necrosis of hepatocytes [31]. These findings have been agreed with Mukherjee e*t al*., [32] and Moghadam *et al*., [33]. Also, Patel *et al*., [34] stated that MTX at dose 0.250 mg/kg body weight given for 4 weeks induced liver cell necrosis. Increasing the total serum bilirubin levels indicated a reduction in the excretion capability of the liver as a consequence of liver injury [35].

The reduction in protein may be due to several factors like increased intestinal protein loss, protein-losing nephropathy, dietary protein deficiency as there was a decrease in feed intake and damage to liver [36]. Also, blockade of tetrahydrofolate synthesis by methotrexate which leads to the inability of cells to divide and to produce proteins [37].

While the combination of MTX and flaxseed oil significantly restored the altered liver function [38]. These results were consistent with the results of Attaia *et al*., [39] who stated that the mode of action of *ω*-3 in flaxseed oil could be intercepted pharmacologically at different levels with agents that scavenge free reactive oxygen, block their generation, or enhance endogenous antioxidant capabilities. The reported hepatoprotective action of flaxseed oil was similar to that obtained by Naqshbandi *et al*., [40] who stated that flaxseed oil ameliorated cisplatin-induced hepatoxicity and other deleterious effects due to its intrinsic biochemical antioxidant properties.

The mtx-treated group showed a significant increase in kidney markers and this matched with Asvadi *et al*., [41] who stated that pentoxifylline has a protective effect against renal toxicity after methotrexate administration. The increase in creatinine, urea and uric acid in blood during renal diseases or renal damage may be due to high activities of lipid peroxidation and increased triacylglycerol and cholesterol levels [42]. Kolli *et al*., [43] have indicated that MTX administration increases plasma BUN and creatinine levels significantly. The renal curative effect of FO was by Abdel Moneim *et al*., [44] who found a reduction in uric acid, urea, and creatinine levels during flaxseed oil treatment. Wahba and Ibrahim [45] reported that the administration of flaxseed oil produced significant decreases in the elevated BUN and UA when compared with the positive control group. El-Sayed *et al*., [46] stated that the renal failure rats fed on a diet containing 5% and 7% flaxseed get better in body weight gain% and kidney functions.

The current investigation showed that methotrexate - induced oxidative stress as documented by an increase in liver and kidney MDA levels. The lipid peroxidation mediated by oxygen-free radicals were thought to be an important cause of destruction and damage to the cell membranes and was suggested to be a contributing factor in the development of MTX-mediated tissue damage [47]. Hussein *et al*., [48] indicated that MTX causes oxidative tissue damage by increasing lipid peroxidation in the liver tissue and decreasing the level of antioxidant enzymes, which cause hepatic necrosis, inflammation, and fibrogenesis. Moreover, Ponce-Canchihuamán *et al*., [49] indicated that GPx and SOD might contribute to the explanation of the mechanisms responsible for the decrease in GSH concentration in liver and kidney. Sevgi *et al*., [50] added that MDA increased, while, GPx, SOD, GSH, and total thiol decreased in the liver tissue caused by methotrexate. As well, Asvadi *et al*., [41] showed in his study that MTX administration decreased GPX and SOD activities. Also, it was reported that the significant reduction in GSH levels promoted by MTX could lead to a reduction of effectiveness in the antioxidant enzyme defense system, sensitizing the cells to ROS [51]. Co-administration of FO ameliorated hepatic oxidative stress. The most likely explanation for this improvement in oxidative status may be due to oxygen scavenging which leads to the prevention of O-generating hydrogen peroxide [38]. The antioxidant effect of omega-3 may be due to the incorporation of long chain fatty acids into cell membranes, increasing the poly-unsaturation of the membrane [52]. Also, omega-3 has a protective role which can improve the liver and kidney total thiol concentration [53]. Galawezh [54] also reported that the antioxidant nature of flaxseed oil had been attributed to beneficial antioxidant activities of linoleic acid.

The present results indicated alterations highly in the lipid profile of methotrexate-treated group as reported by Alwachi & Alsaadi [55]. Hypercholesterolemia is an indicator of liver damage since xenobiotic intoxication obstructs with liver membrane permeability. It also may be attributed to obstruction of the liver bile ducts causing decreased or stoppage of their secretion to the duodenum. The inhibition of lipase lipoproteins may cause a triglyceride increase of [56]. Also, MTX administration caused liver toxicity, and this could be another cause for hypercholesterolemia [57]. The decrease in plasma cholesterol by administration of FO may be due to lignin, fiber and vegetable proteins present in the flaxseed which plays a role in reducing serum cholesterol in animal models. FO rich in ALA results in a higher cholesterol secretion into bile, leading to a depletion of the intra-hepatic pool of cholesterol and thus to an increase in cholesterol synthesis and turnover.

Moreover, ALA-rich diet reduces hepatic lipid accumulation both by stimulating *β*-oxidation and

suppressing fatty acid synthesis [58]. Also, Ganorkar and Jain [59] reported that ALA from flaxseed oil exerts a positive effect on blood lipids. This result matches with Aly-Aldin *et al*., [42] who found that FO treatment resulted in a significant decrease in total cholesterol, LDL, and VLDL values and a significant increase in HDL values. By our present study Morsy *et al*., [60] indicated that inflammatory mediators, including COX-2, play important roles in the pathogenesis of methotrexate toxicity. In a recent study, the effects of MTX on COX-2 activity were evaluated may be due to the increased expression of NF-κB. On the other hand, COX-2 is an inducible enzyme that governs the transformation of arachidonic acid into prostaglandins as a part of the inflammatory process. The promoter region of the COX-2 gene has two motifs comprising the binding sites for NF-κB [61]. Treatment with flaxseed oil, which is rich in PUFA have vascular benefits. The effects of ALA on the cyclooxygenase-2 (COX-2) pathway have been shown to decrease thromboxane A2 (TXA2) and increase prostacyclin-3 (PGI3) in vessels, which in turn, could augment endothelium-dependent vasodilatation [62]. Park *et al*., [63] have proposed main four antitumor actions for n-3 PUFAs one of them, modulation of COX-2 activity.

In mechanistic details, n-3 PUFAs can act as alternative substrates for COX-2 leading to a reduction in formation of protumorigenic "2-series" PGs (PGE2) in several cell types. They also bind the substrate channel of COX-2 and inhibit COX-2 activity. El-Sheikh *et al*., [61] reported that MTX also caused hepato-renal nitrosative stress, shown by an increase in NO level, confirmed by up-regulation of iNOS expression in kidney and liver. Ahmed *et al*., [64] reported that MTX-treated group whose epithelial cells exposed to oxidant stress lead to an elevation in NO release and nitrite production and a decrease in cell viability. As well, Christo *et al*., [65] stated that NO seems to worsen renal injuries because of its free radical nature, through its reaction with the superoxide radical, it probably generates the very cytotoxic peroxynitrite that could damage the tubular cells, resulting in renal failure. It was reported that flaxseed oil significantly reduced NO, and it can be suggested that the active flaxseed peptide fractions may have altered the pathway for NO synthesis in the macrophages. Polyunsaturated fatty acid and α-linolenic acid has shown that the activity of potential therapeutic agents of flaxseed oil is responsible for the inhibition of NO production [66]. Ismail *et al*., [67] and Farag *et al*., [68] also indicated that α-Linolenic acid is responsible for the inhibition of NO. The induction of the antioxidant enzyme iNOS expression is responsible for the improvement of the antioxidant and anti-inflammatory status in the hepatic tissues and could be claimed to be the hepatoprotective mechanism of FO, mainly due to the α-linolenic acid, omega-3 fatty acid and lignan constituents.

In the current study, histology outcomes confirm MTX-induced hepatotoxicity, and biochemical results are in agreement with the histological findings. Histological results revealed different changes in liver of MTX-treated rats. These results are in agreement with some investigators. Focal areas of necrosis (Hadi *et al*., [38]) and increased numbers of activated Kupffer cells (Dalaklioglu e*t al*., [69]) observed in liver tissues of MTX-administered rats. The present results also showed an apparent increase in the number of collagen fibers in the liver of MTX-treated rats.

Similarly, Al-Motabagani [70] reported that MTX caused an increase in the amount of collagen fibers particularly around blood vessels in the portal tract. Flaxseed oil treatment improved the histopathological changes induced by methotrexate in liver and kidney tissues. These observations were in the same line with Berancchia *et al*., [71] who reported that flaxseed oil was rich in lignans, flavonoids and vitamin E which protect the cells against free radical damage. So, treatment with flaxseed oil attained the normal morphological features of the liver and kidney as compared to the control group. The immunohistochemically stained sections of liver and kidney treated with MTX showed strong immunoreactive expression of α-SMA, this result was in accordance with Hussein *et al*., [48] who found increased expression of α- SMA in the liver treated with MTX and they added that liver fibrosis is a consequence of chronic hepatitis and involves the abnormal accumulation of extracellular matrix proteins, particularly collagen. Myofibroblasts, which are absent from the normal liver, are derived from two major sources: hepatic stellate cells (HSCs) and portal mesenchymal cells

in the injured liver [72]. α-SMA is a reliable marker of hepatic stellate cell activation, which precedes fibrous tissue deposition, and it can be used for identification of the earliest stages of hepatic fibrosis and for monitoring the efficacy of the therapy [73, 74]. The examination of immuno-histochemical stained tissue confirmed that flaxseed oil reduced the MTX-induced liver fibrosis.

In the present study, DNA damage induced by methotrexate was reflected by changes in RAPD profiles, the disappearance of bands and appearance of new PCR products which occurred in the profiles generated by exposed rats to methotrexate. Abdou and Hassan [75] have shown that the changes in band patterns observed in DNA "fingerprint" analyses reflect DNA alterations. Howard *et al*., [37] suggested that methotrexate binds to dihydrofolate reductase (DHFR) and completely inhibits the activity of this enzyme. The continuous inhibition of DHFR might cause an imbalance in the deoxyribonucleotide triphosphate (dNTP) pool due to the storage of thymidylate and purine nucleotides and as a consequence lead to DNA lesions. Also, Najafi *et al*., [76] recently reported that the cytotoxic effects of methotrexate had been determined in various organs and this agent inhibits the synthesis of thymidylate, serine, and methionine, leading to disruption of DNA, RNA as well as protein function and consequently cell death. Atashfaraz *et al*., [77] as well, showed that MTX caused an increase in DNA fragmentation. On the contrary, flaxseed oil in combination with methotrexate attenuated the DNA near to the normal appearance.

This may be attributed to the ability of flaxseed oil to inhibit peroxyl-radical which mediated damage of plasmid DNA and also phosphatidylcholine liposomes to normal concentrations [78]. Also, RAPD reflected the protective effect of omega-3 which is major content of flaxseed oil on DNA and suggested that omega-3 pre or post-treatment to azathioprine showed high significance in reducing the percentage of DNA fragmentation [79].

CONCLUSION: FO attenuates MTX hepato-renal toxicity with their antioxidant properties, anti-inflammatory, and anti-apoptotic activities. Based on these results, the routine clinical application of those products could be initiated after advanced further clinical studies.

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