Original Article

Effect of Pumpkin Seed Oil on Experimentally Induced Early Liver Fibrosis in the Adult Male Albino Rat

Nesma M. Afify, Mostafa M. El-Habeby, Abeer E. El-Mehi and Manar A. Faried

Department of Anatomy and Embryology, Faculty of Medicine, Menoufia University, Menoufia, Egypt

ABSTRACT

Introduction: Liver fibrosis is a leading cause of morbidity and mortality worldwide without effective treatment. Oxidative stress and inflammation play an important role in the pathogenesis of liver fibrosis. Pumpkin Seed Oil (PSO) is known to have antioxidant, anti-inflammatory and anti-fibrotic properties.

Objectives: This work aimed to investigate the effect of PSO on the experimentally induced liver fibrosis in the adult male albino rat for the first time up to our knowledge, with reference to different underlying mechanisms.

Material and Methods: Fifty adult male albino rats were divided into Group I (n = 20): subdivided equally into plain and sham control subgroups. Group II: PSO (n = 10): PSO 4 ml/kg/day for 4 weeks. Group III: Liver fibrosis (n = 10): CCl4 1ml/kg intraperitonieally, twice a week for eight weeks. Group IV: Liver fibrosis treated with PSO (n = 10): liver fibrosis was produced as in group III. After 4 weeks of CCl4 treatment, the rats were given PSO as group II. At the end of the experiment, blood samples and liver sections were subjected to biochemical, histological (light and electron) and immunohistochemical studies.

Results: The liver fibrosis group exhibited a significant increase in the levels of liver function tests, MDA, TNFα, IL-1β and IL-6 as well as a decrease in the levels of serum albumin, SOD and CAT as in comparison to the control group. Liver sections showed vacuolated cytoplasm, pyknotic nuclei and degenerated mitochondria. Furthermore, marked fibrosis was detected by an increase in the % area of collagen deposition. Upregulation of αSMA, TGFβ1 and caspase3 immunoreactions were detected. PSO ameliorated all the examined parameters via its antioxidant, anti-inflammatory and anti-fibrotic properties.

Conclusion: There is a promising potential for PSO as a natural therapeutic agent against liver fibrosis.

Received: 27 November 2021, **Accepted:** 29 January 2022

Key Words: CCl4, liver fibrosis, oxidative stress, pumpkin seed oil.

Corresponding Author: Nesma Mahmoud Abd El Karim Afify, MSc, Department of Anatomy and Embryology, Faculty of Medicine, Menoufia University, Menoufia, Egypt, **Tel.**: +20 10 9619 0292, **E-mail:** abdelrahman.nesma@yahoo.com

ISSN: 1110-0559, Vol. 46, No. 2

INTRODUCTION

Liver fibrosis is a scarring reaction that happens almost in all chronic liver diseases[1]. Fibrotic liver diseases predispose to liver cirrhosis, the end-stage liver disease, which has been a globally growing main fitness trouble with excessive mortality and morbidity rates withinside the beyond twenty years[2]. Therefore, it is miles essential to apprehend the viable mechanisms underlying liver fibrosis for the sake of finding effective agents[3]. Liver fibrosis is characterized by the immoderate accumulation of collagen fibers and the distortion of regular hepatic architecture[4]. It is broadly diagnosed that activated hepatic stellate cells (HSCs) play an important role in the development of liver fibrosis. After they are activated in reaction to the liver damage, they come to be proliferative and get converted into myofibroblasts, and ultimately synthesize and secret ECM[5]. Additionally, activated HSCs and myofibroblasts secrete massive quantities of profibrotic cytokines that exacerbate fibrotic response inclusive of tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), endothelial growth factor and transforming growth factor-beta 1 (TGFβ1)[1].

Oxidative stress additionally has been included in the pathogenesis and progress of liver fibrosis. Reactive oxygen species (ROS) are taken into consideration as an inducement to HSCs proliferation, migration and collagen accumulation[6]. Many researches have cautioned that fibrotic development may be managed or inhibited through natural agents[7].

Pumpkin is a plant cultivated throughout the world frequently used as functional food or medicine. It has many medicinal properties as anti-diabetic, antihypertensive, hepatoprotective, antiviral, antibacterial, anti-inflammatory, antiatherogenic and anticarcinogenic[8].

Pumpkin seed oil (PSO), as a dietary antioxidant, is a wealthy supply of unsaturated fatty acids and antioxidants inclusive of vitamin E, tannins, linoleic acid, oleic acid and alkaloids[9]. Moreover, its antifibrotic and antiparasitic properties against schistosoma mansoni infection have been documented[10].

As a result, the current work was done to determine the role of PSO in the therapy of early liver fibrosis.

 DOI: 10.21608/ejh.2022.106873.1594

MATERIALS

Carbon tetrachloride was acquried from an American chemical company called Sigma Aldrich as a liquid having a molecular weight of approximately 153.82. It has a purity of ≥99.5% and a vapor pressure of 143 mmHg.

Pumpkin seed oil and corn oil were purchased from ElHawag Company for the extraction and mobilization of natural oils (Cairo, Egypt) under the supervision of the Egyptian Ministry of Health.

Animals

Fifty male albino adult rats weighing (180-200) grams have been employed. The rats have been obtained from Egypt's Theodor Bilharz Research Institute's Animal House. The animals have been saved below managed temperature and humidity situations and have been furnished with water and a stable diet. The technique was accredited with the aid of using the ethics of Menoufia University, Faculty of Medicine in accordance with global rules for the care and use of laboratory animals. After a one-week acclimatization period, Four groups of rats were divided randomly.

Group I (control group) : (n = 20). This group was equally splitted to two subgroups:

- Subgroup (Ia): no treatment was maintained.
- Subgroup (Ib): This group received corn oil one ml/kg, a CCl4 solvent, by intraperitoneal injection twice weekly for eight weeks[11].

Group II (Pumpkin Seed Oil group): (n = 10). They received PSO 4 ml/kg/day orally through a gastric tube[12] for four weeks.

Group III (Liver fibrosis group): (n = 10). They received CCl4 one ml/kg, dissolved in corn oil (1: 1), intraperitonieally, two times per a week for eight weeks[11].

Group IV (Liver fibrosis treated with PSO group): (n = 10). In this group, liver fibrosis was induced by intraperitoneal injection of CCl4 at the same dose and duration as group III. After 4 weeks of CCl4 treatment, the rats were given PSO as group II inconcomitant with CCl4 from the 5th to the 8th week.

Finally, the rats were anathesized by intraperitoneal injection of 60 mg/Kg phenobarbital followed by cervical dislocation[13]. Blood samples were taken from tail veins of the rats and the liver was dissected.

METHODS

Biochemical

Blood samples

The samples were used to measure liver function tests; alanine aminotransferase (ALT), Alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), Aspartate aminotransferase (AST), serum albumin and total bilirubin.

Liver tissue homogenate

Liver samples (10% w/v) were homogenized in ice-cold 0.1M Tris-HCl buffer (pH 7.4). Then centrifuged (3000 rpm for 10 minutes at 4°C) and the supernatant was used to determine markers of oxidative stress [Malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT)] and inflammatory cytokines [Tumor necrosis factor-alpha (TNFα), interleukin1β (IL-1β) and interleukin6 (IL-6)].

Histological study

A-Light microscopic study

For 24 hours, liver samples were fixed in (10% buffered formalin). Afterwards, the paraffin sections are prepared and stained with hematoxylin and eosin to demonstrate the histological structure and with Masson trichrome to detect collagen deposits .

Semi quantitative analysis of liver fibrosis

Using masson trichrome stained liver sections, at least five non-overlapping fields (40x) in each section were assessed for the extent of liver fibrosis and this was performed by two observer blinded to the groups under study. This was performed according to the numerical scoring system of Ishak *et al*.[14], as follows:

0= no fibrosis

- 1=fibrous expansion of some portal areas (less than three).
- 2= fibrous expansion of most portal areas (more than three).
- 3= fibrous expansion of most portal areas with occasional portal-to-portal bridging.
- 4= fibrous expansion of portal areas with marked bridging (portal to portal as well as portal to central).
- 5= marked bridging (portal to central as well as central to central) with occasional nodules formation.
- 6= cirrhosis.

B- Transmission electron microscopic examination

Using glutaraldehyde 5%, liver samples (1 mm3) were instantly fixed, and then processed and inspected with a transmission electron microscope.

Immunohistochemical study

Paraffin sections were dewaxed and rehydrated after coating the slides with polylysine. Blocking of endogenous staining was done by adding 2-4 drops of peroxide block. The process of obtaining the antigen was carried out using microwaves then, the primary antibodies were applied according to their specific dilutions [TGFβ1; a fibrogenic marker (a mouse monoclonal antibody, Midco Trade, 1:500), α-SMA; a marker for HSCs proliferation (a rabbit polyclonal antibody, Abcam, 1:100) and

caspase-3; apoptotic marker (a rabbit polyclonal antibody, Abcam, 1:400)]. Next, 1-2 drops of the goat biotinylated polyvalent secondary antibody was added. Following that, preformed streptavidin peroxidase used to incubate the sections. Finally, 1-2 drops of the prepared DAB substrate-chromogen were applied to slides until the desired brown colour was obtained.

Morphometric assessment

Sections of at least five animals/group were inspected for quantification. A Leica Microscope DML B2 / 11888111 equipped with a Leica DFC450 camera was used to randomly catch five non-overlapping slice fields. Then, using image analyzer software, morphometric analyses were performed. The different parameters including area % of TGFβ1, area % of collagen deposition (20 x) and α-SMA and caspase-3 immunoreactions (40 x) were measured and the calculated data then went through statistical analysis.

Statistical analysis

SPSS version 23 was used to analyse the data. The data were analyzed using onewayANOVA then Bonferroni test and represented in mean±standard deviation.The significance of grading of liver fibrosis was determined using the Kruskal-Wallis test. When the *p-value* was ≤ 0.05, the results were considered statistically significant[15].

RESULTS

None of the parameters investigated showed a statistically significant difference in any of the subgroups1a,1b and PSO group.

Biochemical results

Liver function tests

The liver fibrosis group showed that the levels of ALP, AST, ALT, GGT and total bilirubin rubin were significantly elevated (P <0.001), but levels of serum albumin in comparison to the control group was significantly reduced (P <0.001). However, liver fibrosis treated with PSO group showed asignificant reduction in ALP, AST, ALT, GGT and total bilirubin levels and serum albumin level was significantly elevated (P <0.001) compared to the liver fibrosis group (Table1).

Oxidative stress markers

The analysis of SOD, CAT and MDA levels indicated that the liver fibrosis group revealed a significant increment in levels of MDA than control group, while CAT and SOD levels had markedly reduced than control group (P < 0.001). Inspite of, liver fibrosis treated with PSO group revealed asignificant decline in MDA level (P <0.001) and a significant elevation in both CAT and SOD levels (P <0.001) as in comparison to the liver fibrosis group (Table2).

Inflammatory cytokines

At the end of the study, the liver fibrosis group revealed a significant increment (P <0.001) in IL-1β, IL-6 and TNF-α levels compared to the control rats. However, the liver fibrosis treated with PSO showed a significant decline in IL-1β, IL-6 and TNF-α levels (P <0.001) as in comparison to the liver fibrosis group (Table2).

Histological results

H&E

The control group's H&E stained sections revealed a normal structure of classical liver lobules. They were formed by radially located hepatocyte cords that extend from the central vein. The von Kupffer and endothelial cell-lined blood sinusoids were used to separate cell cords. Hepatocytes shape were polygonal with an eosinophilic cytoplasm and a rounded vesicular nucleus in the centre. Some hepatocytes had a double nucleus. The portal tract composed of the branches of the hepatic artery, the portal vein and the bile ducts (Figure 1 a-c). The liver fibrosis group showed markedly distorted hepatic architecture. The central vein and blood sinusoids were dilated and congested. A hemorrhage in between cells was also observed. There was degeneration of the hepatocytes in the form of cytoplasmic vacuolation and pyknotic nuclei. The changes detected in the portal triad included dilated and congested portal vein with marked perivascular inflammatory infiltration (Figure 2 a-d). The liver fibrosis treated with PSO group revealed restoration of the normal structure of liver tissues. However, some hepatocytes showed cytoplasmic vacuolation (Figure 3 a-c).

Sections of the control group stained with Masson trichrome showed fine collagen deposits around the walls of the central vein and portal tract blood vessels (Figure 4 a,b). The liver fibrosis group exhibited exaggerated collagen deposition around the central veins and portal tracts with the presence of multiple fibrous bridges connecting the portoportal, portocentral and centrocentral areas (Figure 4 c,d). The liver fibrosis treated with PSO group showed less dense collagen deposits around the walls of the central vein and portal vein (Figure 4 e,f). Statistically, there was a significant increase in the area % of collagen deposition in the liver fibrosis group compared to the control group (P <0.001) (71.40±11.67 vs 2.01±0.53). In spite of, a significant reduction was in the area % of collagen deposition in liver fibrosis treated with PSO group compared to liver fibrosis group (P <0.001) (9.49 ± 2.48 vs 71.40±11.67) (Figure 2g). According to the grading of liver fibrosis, control rat liver sections did not show fibrosis. The liver fibrosis group revealed high grades of fibrosis as compared to the controls. However, the fibrosis grade in liver fibrosis treated with PSO group was significantly diminished (P <0.001) compared to the group of liver fibrosis. (Table 3).

Electron microscopic study

In control rats, hepatocytes had euchromatic rounded nuclei with regular nuclear membrane and obvious nucleoli. Numerous mitochondria, electron-dense glycogen granules and regular rough endoplasmic

reticulum were shown in the cytoplasm. Bile canaliculi sealed by tight junction could also be clearly noticed in-between the hepatocytes (Figure 5 a,b). The liver fibrosis group showed a massive amount of collagen fiber deposition around the hepatocytes. The hepatocytes had irregular heterochromatic nucleus. The cytoplasm showed vacuolation, rarefaction, degenerated mitochondria and apparent decrease of glycogen granules. Extravasted red blood cells (RBCs) and mononuclear inflammatory cells were observed in between the hepatocytes. There were also disturbing bile canaliculi with damaging microvilli (Figure 6 a-c). liver fibrosis treated with PSO group showed the normal microstructure of hepatocytes. The hepatocytes had euchromatic nuclei with slight irregular nuclear membrane. The cytoplasm showed numerous rounded mitochondria, regular rough endoplasmic reticulumand electron-dense glycogen granules. However, some mitochondria were degenerated. Bile canaliculus sealed by tight junction could also be seen (Figure 7a,b).

Immunohistochemical results

The liver fibrosis group revealed significant up-regulation in the area% of the TGFβ1 immunoresponse in the liver fibrosis group (78.46±6.02) in comparison with the control group (1.04±0.26). Furthermore, there was a significant reduction in the area % of the TGFβ1 immunoresponse in the liver fibrosis treated with PSO group compared to group of liver fibrosis (78.46 ± 6.02) (Figure 8 a-d).

In caspase3 stained sections, the area % of caspase 3 immunoreaction was significantly elevated (P <0.001) in the liver fibrosis group (127.9±7.30) as in comparison to the control group (.70±0.11). In spite of, the area % of the caspase3 immunoreaction was significantly declined (P <0.001) in the liver fibrosis treated with PSO group (7.95±0.1.45) as in comparison to the liver fibrosis group (127.9±7.30) (Figure 8 e-h).

In sections stained with αSMA, the liver fibrosis group conceded a stastically significant increase in the area % of the αSMA immunoreactivity (P <0.001) (81.85±8.16) compared to the control group (2.52±0.46), However, the area % of the α-SMA immunoreactivity was significantly reduced (P <0.001) in the liver fibrosis treated with PSO group (11.34±2.68) in comparison with the liver fibrosis group (Figure 8 i-l).

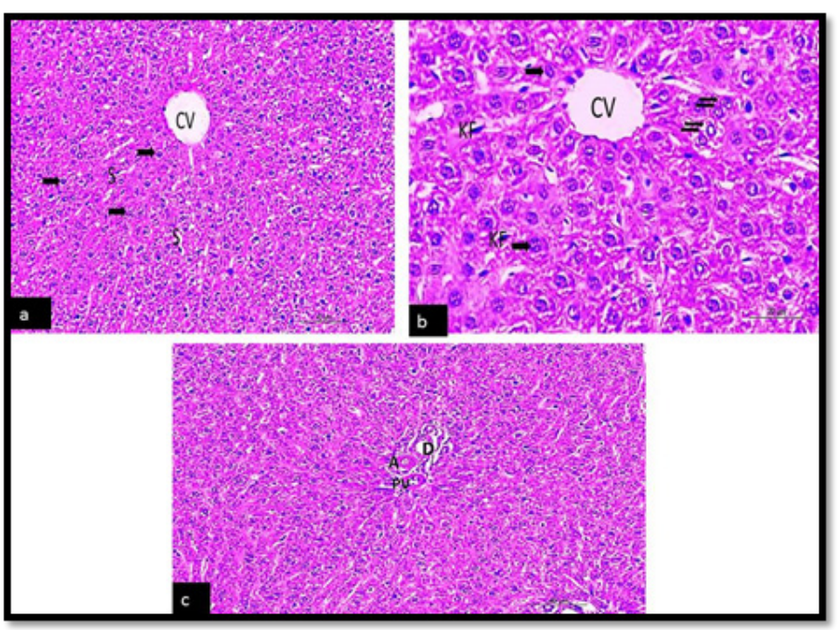

Fig. 1: H&E stain of rat liver sections of control group (a-c) showing central vein (CV), hepatocytes with vesicular nucleus in the centre (arrows) and others with double nucleui (double arrows) and blood sinusoids (S) lined with endothelial cells and von Kupffer cells (KF). The portal tract forms of hepatic artery (A), portal vein (PV) and bile duct (D). a&c (Scale bar 40 µm X200) and b (Scale bar 20 µm X400).

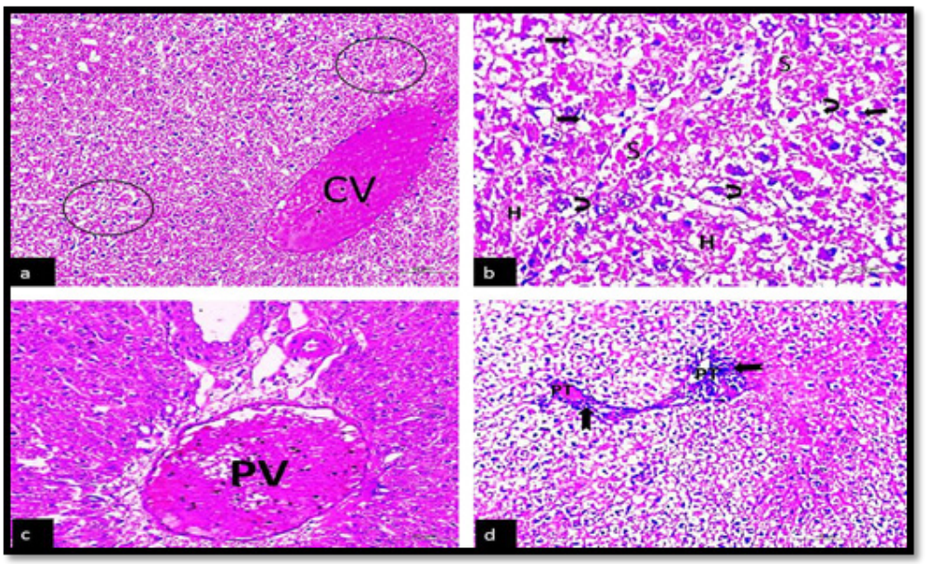

Fig. 2: H&E stain of rat liver sections of liver fibrosis group (a-d) showing dilated congested central vein (CV) surrounded by disturbed irregular hepatic cords (circles). The blood sinusoids are also dilated and congested (S) with haemorrhage (H) in between cells. There is degeneration of the hepatocytes in the form of cytoplasmic vacuolation (curved arrows) and pyknotic nuclei (arrows). The portal vein is dilated and congested with marked perivascular inflammatory infiltration (notched arrows). a,c&d (Scale bar 40 µm X200) and b (Scale bar 20 µm X400).

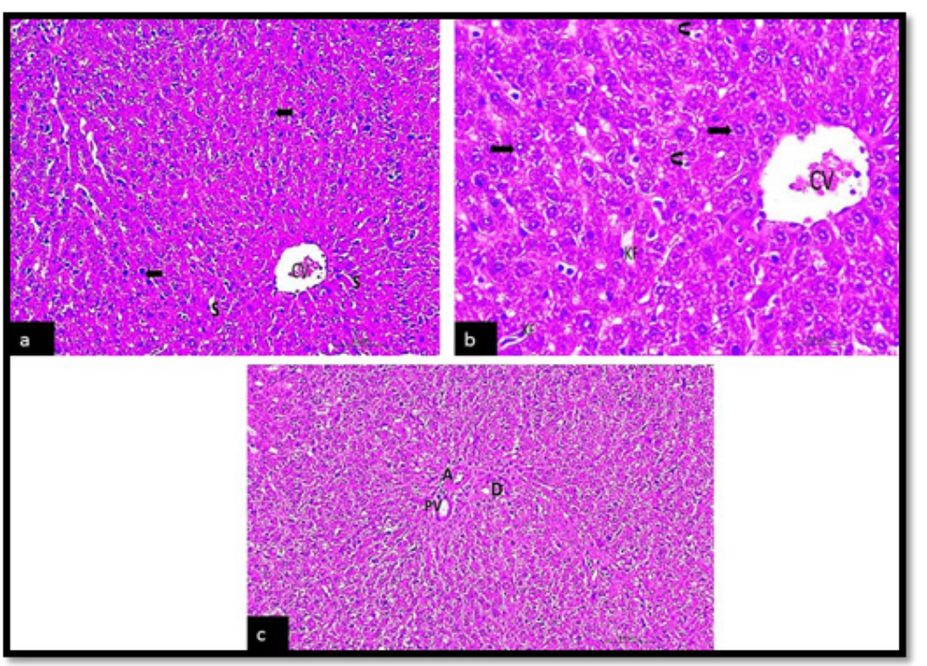

Fig. 3: H&E stain of rat liver sections of liver fibrosis+ PSO group (a-c) showing a normal structure of hepatocytes (arrows) cords surrounding the central vein (CV) and separated by blood sinusoids (S). However, some hepatocytes show cytoplasmic vacuolation (curved arrows). a&c (Scale bar 40 µm X200) and b (Scale bar 20 µm X400).

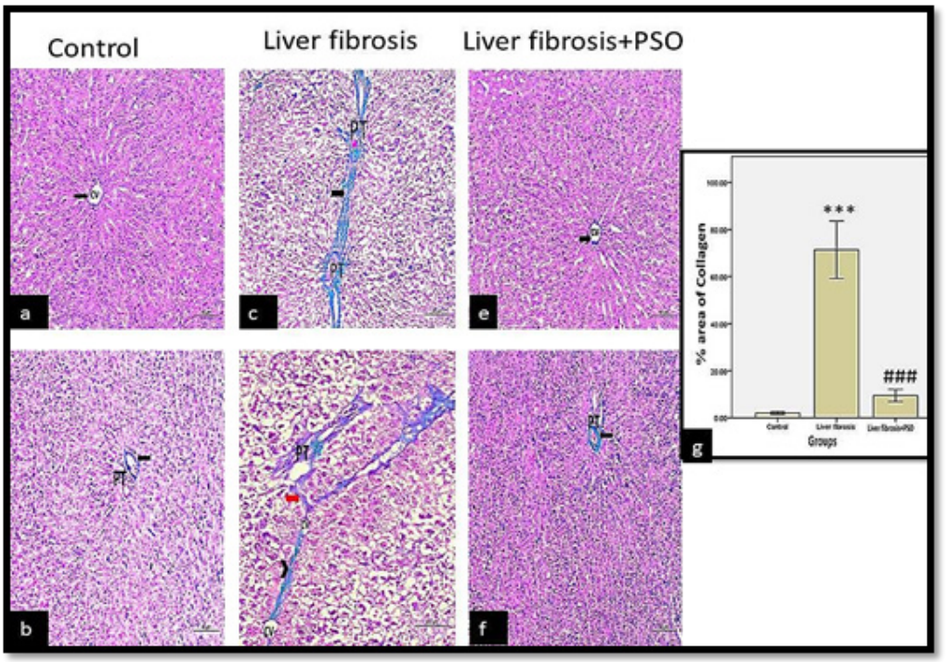

Fig. 4: Masson trichrome sections of rat liver of all groups. a&b: control group showing fine collagen deposits around the walls of the central vein and portal tract blood vessels (arrows). c&d: liver fibrosis group showing porto-portal (arrow), porto-central (red arrow) and centro-central (arrowhead) fibres. e&f: Liver fibrosis + PSO group showing less dense collagen deposits around the walls of the central and portal vein (arrows). Note: CV: central vein. PT: portal tract. (Scale bar 40µm X200). g: A histogram representing area % of collagen deposition. *** P < 0.001 liver fibrosis compared with control, ### P < 0.001 liver fibrosis + PSO compared with liver fibrosis.

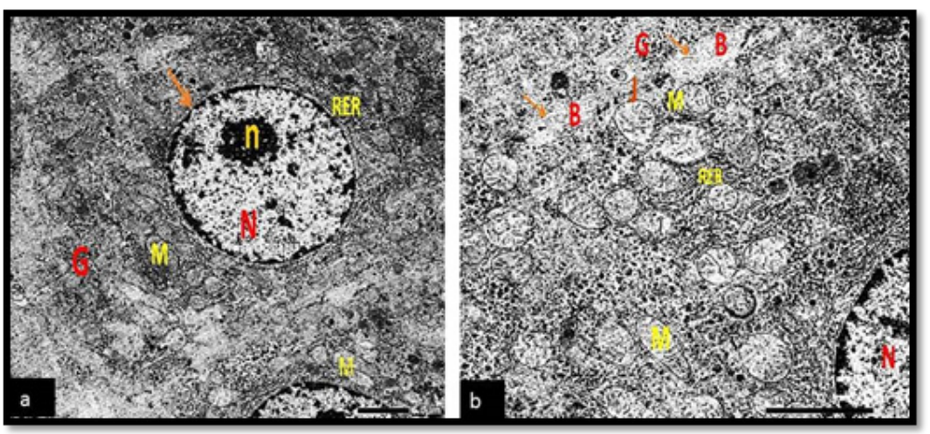

Fig. 5: electron photomicrographs of rat liver sections of control group (a&b): showing a hepatocyte has euchromatic nucleus (N) with regular nuclear membrane and obvious nucleolus (n). Numerous metochondia (M) are found in cytoplasm, regular rough endoplasmic reticulum (RER) and electron-dense glycogen granules (G). Bile canaliculi (B) sealed by a tight junction (J) can also be seen. (Scale bar 5µm X1500 for a) and (Scale bar 2 µm X3000 for b).

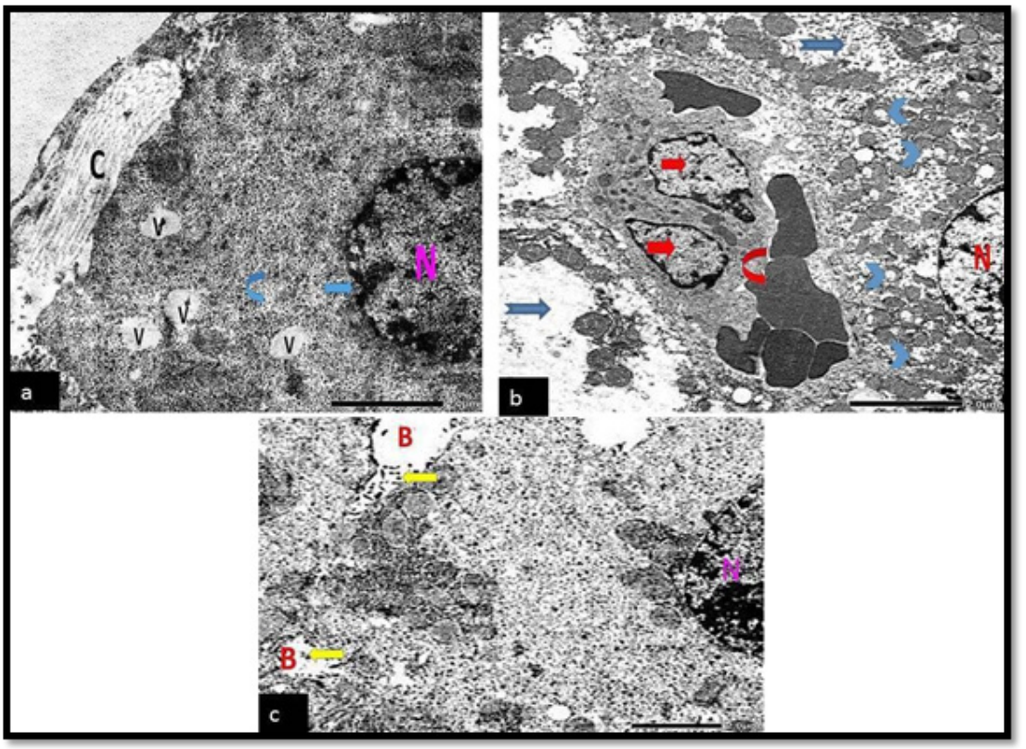

Fig. 6: electron photomicrographs of rat liver sections of Liver fibrosis group (a-c): showing a massive amount of collagen fibers (C) around the hepatocyte. The hepatocytes have heterochromatic nucleui (N) with an irregular nuclear membrane (arrow). The cytoplasm shows vacuolation (v), rarefaction (notched arrows), degenerated mitochondria (arrowheads) and apparent decrease of glycogen granules (curved arrow). Extravasted RBCs (red curved arrow) and mononuclear inflammatory cells (red arrows) can be observed in between the hepatocytes. There are also disturbing bile canaliculi (B) with damaging microvilli (yellow arrows). (Scale bar 2 µm X3000).

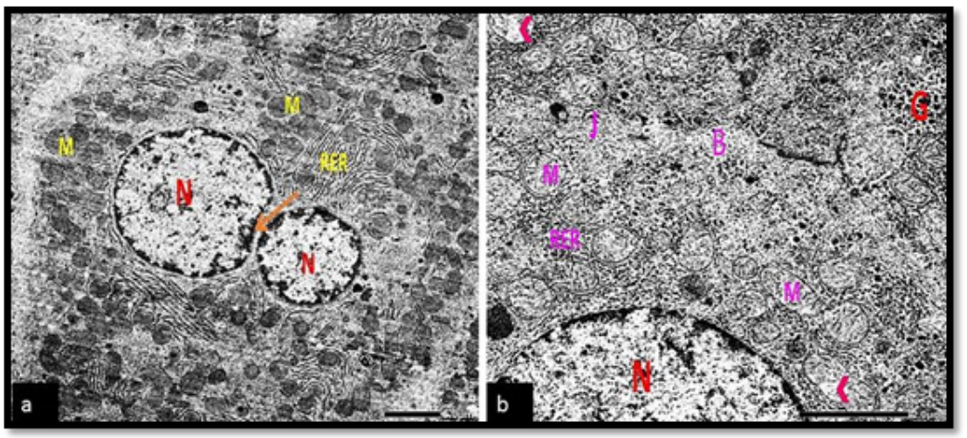

Fig. 7: electron photomicrographs of rat liver sections of Liver fibrosis + PSO group (a&b): showing a hepatocyte with double euchromatic nuclei (N) and slight irregular nuclear membrane (arrow). The cytoplasm contains normal numerous rounded mitochondria (M), regular rough endoplasmic reticulum (RER) and electron-dense glycogen granules (G). However, some mitochondria are degenerated (arrowheads). Bile canaliculus (B) sealed by a tight junction (J) can be clearly noticed. Scale bar 5µm X1500 for a) and (Scale bar 2 µm X3000 for b).

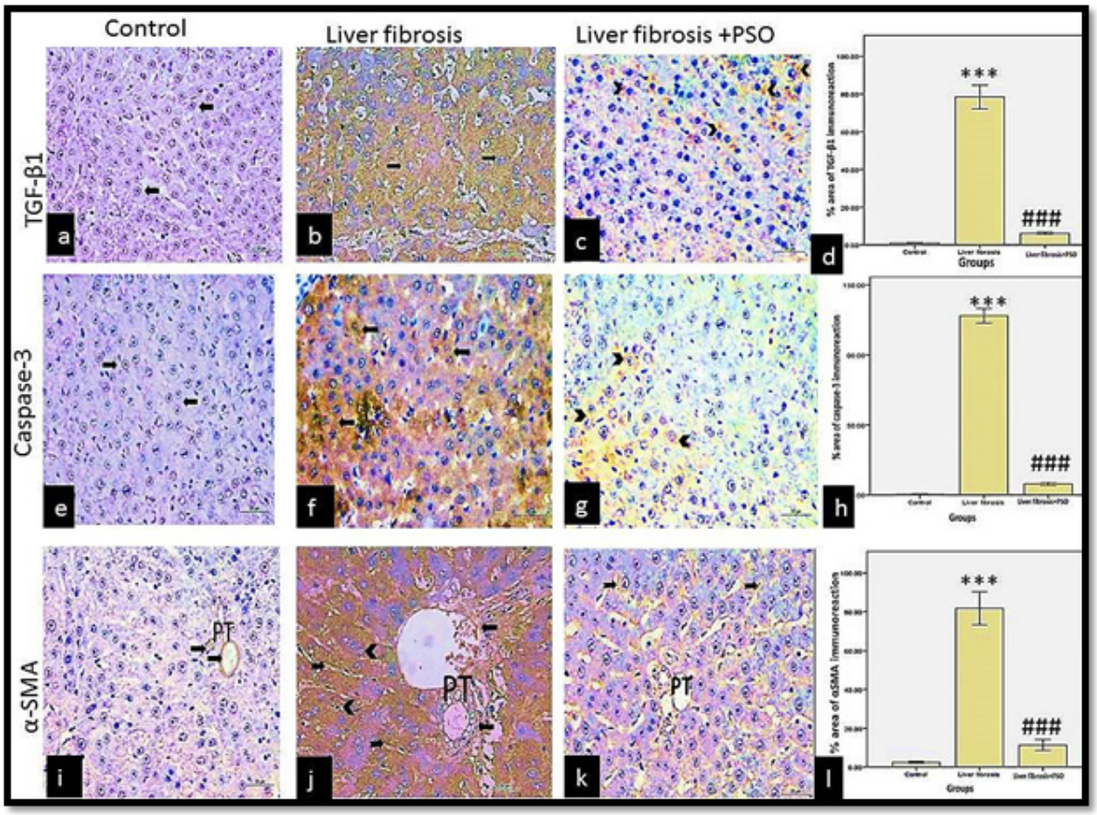

Fig. 8: micrographs of the all experimental groups showing extensive areas of positive immunoreactivity (arrows) to TGF-β1 (a-d) and Caspase-3 (e-h) in the liver fibrosis group while in the liver fibrosis + PSO group few hepatocytes exhibiting positive immunoreactivity (arrowheads). In α-SMA (i-l), the control group shows positive staining in the walls of blood vessels of the portal tract (arrows). The liver fibrosis group shows positive immunoreaction in the cytoplasm of the hepatocytes (arrowheads), around the portal area (arrows) and the walls of the blood sinusoids (notched arrows). The Liver fibrosis + PSO group shows minimal expression of α-SMA in the wall of the hepatic sinusoids (notched arrows). Note: PT: portal tract. (Scale bar 20 µm X400). *** P < 0.001 liver fibrosis compared with control, ### P < 0.001 liver fibrosis + PSO compared with liver fibrosis.

Table1: Liver function tests

Group	AST (U/L) X̄±SD	ALT (U/L) X̄±SD	ALP (U/L) X̄±SD	GGT (U/L) X̄±SD	Bilirubin (mg/dL) X̄±SD	Alb (mg/L) X̄±SD	*P value*
Control	37.18 ±3.83	52.313.18±	89.10±5.08	9.32±.53	.67±.05	42.35±.83	
Liver fibrosis	19.9±149.23	166.8117.93±	173.07±11.90	18.01±33	1.8±.093	24.15±1.35	[a] <0.001 [b] <0.001
Liver fibrosis +PSO	46.1319.92±	67.629.61±	100.72±7.54	9.76±56	.79±041	37.72±2.51	

Footnotes: Mean±SD; mean±standard deviation, a: Comparison between liver fibrosis and control, b: Comparison between liver fibrosis+PSO and liver fibrosis.

Table 2: Oxidative stress markers and inflammatory cytokines

Group	MDA (nmol/ mg protein) X̄±SD	CAT (U/mg protein) X̄±SD	SOD (U/mg protein) X̄±SD	TNF-α (pg /mg protein) X̄±SD	IL-1β (pg /mg protein) X̄±SD	IL-6 (pg /mg protein) X̄±SD	*P value*
Control	31.4.46±	38.12±4.44	164.36±5.89	50.94±3.53	86.46±3.66	59.92±2.61	
Liver fibrosis	10.51±1.16	17.18±1.17	79.21±3.36	93.16±4.06	127.09±5.64	91.18±4.02	[a] <0.001 [b] <0.001
Liver fibrosis +PSO	22 .4.98±	31.16±7.47	151.53±5.78	55.16±3.81	90.93±2.52	63.97±1.74	

Footnotes: Mean±SD; mean±standard deviation, a: Comparison between liver fibrosis and control, b: Comparison between liver fibrosis+PSO and liver fibrosis.

Table 3: Grading of liver fibrosis

Groups	Grading of liver fibrosis							
	0	1	2	3	4	5	6	*P value*
Control	10	0	0	0	0	0	0	
Liver fibrosis	0	0	0	0	4	6	0	a <0.001 b <0.001
Liver fibrosis +PSO	7	3	0	0	0	0	0	

Footnotes: Mean±SD; mean±standard deviation, a: Comparison between liver fibrosis and control, b: Comparison between liver fibrosis+PSO and liver fibrosis.

DISCUSSION

Liver fibrosis is considered a hallmark of chronic liver disease. If left untreated, the injury leads to life-threatening liver failure[16]. Herbal extracts are beneficial as alternative remedies for liver fibrosis[17].

In the present study, the male albino rat was selected because it has many biological similarities to humans[18] and a stable genetic background and a short lifespan[19]. In addition, the female liver's fibrotic reaction to carbon tetrachloride (CCl4) therapy is substantially less than that of the male liver, owing to estrogen's antifibrotic effects[20]. The protocol of CCl4 adminstration in this work was chosen as it causes liver fibrosis similar to the pattern observed in human fibrosis as reported by Varga *et al*.[21]. It was administered intraperitonieally due to good reproducibility and proper survival rates, as postulated by Scholten *et al*.[22]. Intraperitoneal injection of CCl4 at a dosage of one ml/kg twice weekly for eight weeks resulted in the development of hepatic fibrosis in concordance with Elsayed *et al*.[11], who revealed that six to eight weeks were sufficient to develop established fibrosis.

In the present work, the liver fibrosis group had a significant increase in liver enzymes (GGT, AST, ALT, and ALP) and total bilirubin, as well as a decrease in serum albumin. This was in agreement with Abdel- Sttar *et al*.[23]. Aspartate aminotransferase (AST), ALT and ALP are pronounced as sensitive indicators of hepatic damage[24] and GGT is an indicator of biliary tract injury[25]. The increase in serum enzymes can be explained by increased cell leakage and loss of liver structural integrity[26]. El-Beshbishy *et al*.[27] also attributed the increase in liver enzymes to the degradation of endogenous proteins. Increased total bilirubin and decreased serum albumin level were also considered index of hepatocyte injury and loss of its function as reported by Li *et al*.[28]. In contrast, PSO treatment attenuated elevated serum enzyme levels and caused a subsequent recovery toward normalization. It also suppresses the serum level of total bilirubin and increases the serum level of albumin. This demonstrated that PSO has the ability to restore hepatocytes, protect against membrane fragility, and reduce enzyme leakage into the circulation. This was in line with Abdel Aal[12] who stated that the protein extracted from this plant can reduce the harmful effects of protein malnutrition and, due to its antioxidant activity, can lead to an increase in liver enzyme levels.

Liver fibrosis occurs as a result of interactions between different types of cells. Fibrosis is primarily initiated by HSCs. HSCs activation can be stimulated directly by damaged and apoptotic hepatocytes through the release of ROS and other fibrotic mediators[29]. This hepatocellular damage was found in our study. Hepatocytes had irregular, heterochromatic and pyknotic nuclei. The cytoplasm showed massive deposition of lipid droplets, vacuolation, rarefaction, degenerated mitochondria, and an apparent decrease in glycogen granules. There were alterations of the bile canaliculi. Similar results were obtained by Eidi *et al*.[30] and Ahmed *et al*.[31]. The nuclear pyknosis observed in this study is evidence of apoptosis. Soliman *et al*.[32] attributed these apoptotic changes to the breakdown of chromatin into short segments as a result of endonuclease activation as a result of denosine triphosphate (ATP) depletion. This was further evidenced by a significant enhancement of the immunoreactivity of caspase 3 in the liver fibrosis group.

Alternatively, the electron and light microscopic examinations of the liver fibrosis treated with PSO group showed an improvement in the structure of the liver. Most of the hepatocytes appeared almost like those of the control group, but some hepatocytes showed cytoplasmic vacuoles, a slightly irregular nuclear membrane and some mitochondria were degenerated. These results were in line with previous reports by Elmeligy *et al*.[16]. However, Elmeligy *et al*.[16] demonstrated only routine histological study and didnot assess the ultrastrucure and different underlying mechanisms by which the PSO exerted its ameliorating effects in the model of liver fibrosis. These results demonstrated the ability of PSO to restore normal liver architecture after CCL4 poisoning. Abou Seif[33] attributed this to antiapoptotic effect of PSO. The antiapoptotic effect of PSO was also approved in this work by the significant decline in the area % of the caspase3 immunoreaction. Emam *et al*.[34] found that caspase inactivation protected hepatocytes from apoptosis, reduced cytokines that are involved in inflammatory signals, and improved fibrogenesis. Therefore, this can be used to improve liver fibrosis and develop a possible antifibrotic strategy.

ROS and oxidative stress are intermediate factors that cause fibrosis progression and HSC activation[35]. It was also reported that ROS, particularly hydroxyl radicals, have been shown to oxidize the cysteine residues' SH-

group in proteins to disulfides. It inactivates enzymes, disrupts cellular activity and disrupts many pathways[36].

In this study, oxidative stress was confirmed by a decrease in the antioxidant enzymes CAT and SOD activity and a significant increase in MDA in liver fibrosis group. This was in concordance with Ogaly *et al*.[37] and Adewale *et al*.[38] . However, treatment with PSO resulted in a substantial decrease in MDA and elevation in SOD and CAT. This was in agreement with Makni and Budawara[39]. The antioxidant effect of PSO was attributed to the properties of polyphenol, vitamin E and β-carotene content, as postulated by Rezk and Darwish[40]. They also added that these compositions block the synthesis of free radicals such as nitric oxide and prostaglandins.

Several cytokines and growth factors are essential to initiate liver fibrogenesis. TNFα and IL-6 are regarded most important hepatotoxicity mediators in liver damage. In an inflammatory reaction, TNFα is expressed by both infiltrating inflammatory cells and macrophages[41]. Kupffer cells, endothelial cells and hepatocytes in the liver release the major fibrogenic cytokine, transforming growth factor β, which has been involved in of liver fibrosis pathogenesis. It participates in hematopoietic stem cells transition to myofibroblasts by triggering the production of ECM proteins and delaying their breakdown.[42]. In the current work, the inflammatory cytokines (TNF-α, IL-1β and IL-6) were significantly elevated in the liver fibrosis group which was supported by light and electron microscopic examinations that showed severe inflammatory cell infiltration. This was in concordance with Mohamed *et al*.[43] and Gong *et al*.[44]. It was further evidenced by an overly positive immunoresponse to TGFβ1. This was consistent with Li *et al*.[45].

In contrast, there was a significant reduction in the levels of inflammatory cytokines and the immunoresponse of TGFβ1 in the liver fibrosis treated with PSO group, reflecting the anti-inflammatory effects of PSO. This was reinforced by Al-Okbia *et al*.[46] who discussed the anti-inflammatory effects of two PSO variants in an adjuvant arthritis model in rats and clarified that PSO has anti-inflammatory effects due to its inhibitory effects on TNFα and the presence of α, δ and γ tocopherol. The activity of PSO could be attributed to the promising proportions of the unsaturated fatty acids omega-6 and omega-9 that it contains, either by their individual activity or by the synergistic effect of these bioactive molecules, as indicated by Saraiva *et al*.[47]. In addition, Fortis-Barrera *et al*.[48] postulated that polyunsaturated fatty acids suppress proinflammatory cytokine production and macrophage-mediated cytotoxicity. It suppresses TNFα, which is produced by macrophages resident in liver tissue exposed to CCl4, resulting in decreased nitric oxide and IL-6 production.

Liver fibrosis was settled in the current work by the excessive deposition of collagen in the liver tissues of the liver fibrosis group as registered from the result of Masson trichrome stain, which showed multiple fibrous bridges connecting port-portal, porto-central and centro-central areas. This was further corroborated by the electron microscope results, which also showed a massive deposition of collagen fibers around the hepatocytes. These results were close to those of Emaraa *et al*.[49] and Hamza *et al*.[50]. This was explained by Hassan *et al*.[51], who reported that cytokines released by inflammatory cells modulated gene expression in hepatocytes, increasing procollagen production. Inhibitors of the enzyme that breaks down collagen were also increased. So, collagen production exceeded its breaking down resulting in liver fibrosis. α-smooth muscle actin is a dependable marker for HSCs activation into the myofibroblast cells and is taken into consideration an critical myogenic marker that performs an critical function in collagen I deposition by stimulated HSCs[52]. It was increased significantly in the liver fibrosis group, and its expression was widely distributed in the portal area, fibrous septa, and adjacent liver sinusoids. This was in agreement with Elsayed *et al*.[11], who reported a significant increase in αSMA-positive cells in rats poisoned with CCL4.

On the other hand, Liver fibrosis was also resolved after PSO administration. There was a significant decline in area % of collagen deposition. This could be due to the ability of PSO to suppress HSC activation and proliferation, as registered by a significant decline in area % of α SMA immunoresponse. Therefore, its anti-fibrotic effect might be a result of its ability to inhibit activation of HSC .

CONCLUSION

The results of this study confirmed that liver fibrosis is a reversible process if treated early. There was a promising potential of PSO as a natural therapeutic agent against liver fibrosis through its anti-oxidant, anti-inflammatory, and anti-fibrotic properties.

CONFLICT OF INTEREST

There are no conflicts of interest.

REFERENCES

1. Wu Y, Cao Y, Xu K, Zhu Y, Qiao Y, Wu Y, Chen J, Li C, Zeng R and Ge G: Dynamically remodeled hepatic extracellular matrix predicts prognosis of early-stage cirrhosis in Cell Death and Disease. (2021) 12:163-182.

2. Redman J and Sterling RK.: Non-invasive Assessment of Liver Fibrosis in Current Treatment Options Gastroenterology. (2020) 18:255–269.

3. Karsdal MA, Daniels SJ, Nielsen SH, Bager C, Rasmussen, DG, Loomba R, Surabattula R, Villesen I, Luo Y, Shevell D, Gudmann NS, Nielsen, MJ, George J, Christian R, Leeming DJ and Schuppan D: Collagen biology and non-invasive biomarkers of liver fibrosis in Liver International Journal. (2020) 40 (4): 736-750

4. Hofmann C, Djerir NEH, Danckaert A, fernandes J, Roux R, Charrueau C, Lachagès AM, charlotte F, Brocheriou I, clement K, Aron-Wisnewsky J, foufelle F, Ratziu V, Hainque B, Bonnefont-Rousselot D, Bigey P and Escriou V: Hepatic stellate cell hypertrophy is associated with metabolic liver fibrosis in Scientific Reports. (2020) 10:3850-3863.

5. Sharma S, Ghufran SM, Ghose S and Biswas S: Cytoplasmic vacuolation with endoplasmic reticulum stress directs sorafenib induced non apoptotic cell death in hepatic stellate cells in Scientific Reports. (2021) 11:3089-3105.

6. Tanwar S, Rhodes F, Srivastava A, Trembling PM and Rosenberg WM: Inflammation and fibrosis in chronic liver diseases including nonalcoholic fatty liver disease and hepatitis C in World Journal of Gastroenterology. (2020) 26(2): 109-133.

7. Trivella JP, Martin P and Andres F: Novel targeted therapies for the management of liver fibrosis in Expert Opinion on Emerging Drugs. (2020) 25(1):59-70.

8. Dotto JM and Chacha JS: The potential of pumpkin seeds as a functional food ingredient: A review in Scientific African. (2020) 10:575-590.

9. Pereira AM, Krumreochi FD, Ramos AH, Krolow ACR, Santos RB and Gularte MA: Physicochemical characterization, carotenoid content and protein digestibility of pumpkin access flours for food application in Food Science and Technology. (2020) 40:(4)1-8.

10. Beshay EVN, Rady AA, Afifi AF and Mohamed AH: Schistosomicidal, antifibrotic and antioxidant effects of Cucurbita pepo L. seed oil and praziquantel combined treatment for Schistosoma mansoni infection in a mouse model in Journal of Helminthology. (2019) 93(3): 286-294.

11. Elsayed HRH, Abd-Elmonem MM, Gabr OM and Badria FA: Saussurea lappa root extract accelerates the reversion of liver fibrosis induced by carbon tetrachloride in rats in Benha Medical Journal. (2019) 32:116–125.

12. Abdel Aal FS: The Protective Effect of Pumpkin Seed Oil on Azathioprine – Induced Hepatic Toxicity in Adult Male Albino Rats: Histological and Immunohistochemical Studyin Basic Sciences of Medicine. (2014) 3(4): 85-100.

13. Abdo Selima RR, El-Habiby MM, El-Mehi AE and Faried MA: The Protective Effect of Anise, Insulin, and their Combination in the Nephropathy of Diabetic Rat Model in Egyptian Journal of Histology (2020) 44(2) 503-519.

14. Ishak K, Baptista A, Bianchi L, Callea F, Groote JD, Gudat F, Denk H, Desmet V, Korb G, MacSween RN, Phillips MJ, Portmann PG, Poulsen H, Scheuer PJ, Schmid M and Thaler H: Histological grading and staging of chronic hepatitis in Journal of Hepatology. (1995) 22(6): 696-699.

15. Strasak A, Zaman Q, Marinell G, Pfeiffer KP and Ulmer H: The Use of Statistics in Medical Research: A Comparison of Wiener Klinische Wochenschrift and Wiener Medizinische Wochenschrift in Austrian Journal of statistics. (2007) 36(2): 141-152.

16. Maher A, Galal GM, Ahmed NS and Meghezel EM: Direct Markers of Liver Fibrosis in Sohag Medical Journal. (2020) 24 (1):86-93

17. Elmeligy MH, Farid AS and Fararh KM: Antioxidant and hepatoprotective effects of pumpkin seed oil in CCl4-intoxicated rats in Benha Veterinary Medical Journal. (2019) 36(2):77-89.

18. Aitman TJ, Critser K, Cuppen E, Dominiczak A, Fernandez-Suarez XM, Flint J, Gauguier D, Geurts AM,Gould M and Harris PC : Progress and prospects in rat genetics: a community view in Nature Genetics Journal. (2008) 40(5):516-522.

19. Aydin AF, Küçükgergin C, Ozdemirler G, Koçak N and Uysal M: The effect of carnosine treatment on prooxidant–antioxidant balance in liver, heart and brain tissues of male aged rats in Bio gerontology. (2010) 11(1):103-109.

20. Xu JW, Gong J, Chang XM, Luo JY, Dong L, Hao ZM, Jia A and Xu GP: Estrogen reduces CCL4-induced liver fibrosis in rats in World Journal Gastroenterology. (2002) 8 (5):883–887.

21. Varga J, Brenner D and Phan SH : Fibrosis research: Methods and Protocols. v volume 117, Springer Science and Business Media, Totowa, New Jersey. (2005) pp:237.

22. Scholten D, Trebicka J, Liedtke C and Weiskirchen R : The carbon tetrachloride model in mice in Laboratory Animals. (2015) 49 (1): 4–10.

23. Abdel-Sttar AR, Khalaf MM, AboYoussef AM and Abo Saif AA : Ameliorative Effect of Hesperidin on Carbon Tetrachloride Induced Liver Fibrosis in Rats in International Journal of Pharmacy and Pharmaceutical Sciences. (2017) 9(7): 45-51.

24. Motawi TK, Hamed MA, Shabana MH, Hashem RM and Abou Naser AF: Zingiber officinale acts as a nutraceutical agent against liver fibrosis in Nutrition and Metabolism. (2011) 8: 40-51.

25. Ali SA, Hamed MA, El- Rigal NS, Shabana MH and Kassem MES: Chemical constituents of Argyreia speciosa Fam. Convolvulaceae and its role against hyperglycemia in Journal of Applied Pharmaceutical Science. (2011) 1(08): 76-84.

26. El-Hawary SA, Sokkar NM, Ali ZY and Yehia MM: A profile of bioactive compounds of Rumex vesicarius L in Journal of Food Science. (2011) 76(8):1195-1202.
27. El-Beshbishy HA, Tork OM, El-Bab MF and Autif MA : Antioxidant and antiapoptotic effects of green tea polyphenols against azathioprine-induced liver injury in rats in Journal of Environmental Pathology, Toxicology and Oncology. (2010) 349-361.
28. Li R, GuoW, Fu Z, Ding G, Zou Y and Wang Z: Hepatoprotective action of Radix Paeoniae Rubra aqueous extract against CCl4induced hepatic damage in Molecules. (2011)16:8684-9331.
29. Böttcher k and Pinzani p: Pathophysiology of liver fibrosis and the methodological barriers to the development of antifibrogenic agents in Advanced Drug Delivery Reviews. (2017) 121, 3-8.
30. Eidi A, Mortazavi P, Tehrani M.E, Rohani AH and Safi S: Hepatoprotective Effects of Pantothenic Acid On Carbon Tetrachloride- Induced Toxicity In Rats in EXCLI Journal. (2012) 11:748-759.
31. Ahmed SK, Somaya A, Mohammed KG and Fikry H: Role of Bone Marrow Mesenchymal Stem Cells in the Treatment of CCL4 Induced Liver Fibrosis in Albino Rats: A Histological and Immunohistochemical Study in International Journal of Stem Cells. (2014) 7(2): 87–97.
32. Soliman MES, Kefafy MA, Mansour MA, Ali AF and Esa WA: Histological study on the possible protective effect of pentoxifylline on pancreatic acini of l-arginine-induced acute pancreatitis in adult male albino rats in Menoufia Medical Journal. (2014) 27 (4): 801.
33. AbouSeif HS: Ameliorative effect of pumpkin oil (Cucurbita pepo L.) against alcohol-induced hepatotoxicity and oxidative stress in albino rats in Beni-Suef University Journal of Basic and Applied Sciences. (2014) 3: 178-185.
34. Emam AM, Farouk SM and Abdo M: The Ameliorative Potential of Probiotics and/or Silymarin on Thioacetamide Induced Hepatotoxicity in Rats: Histological and Immunohistochemical Study in International Journal of Morphology. (2018) 36 (2):661-669.
35. Yacout GA, Elguindy NM and El Azab EF: Hepatoprotective effect of basil (Ocimum basilicum L.) on CCl4-induced liver fibrosis in rats in African Journal of Biotechnology. (2012) 11(90):15702-15711
36. Muriel P and Gordillo KR: Role of oxidative stress in liver health and disease in Oxidative Medicine and Cellular Longevity. (2016):1–2.
37. 37. Ogaly HA, Eltablawy NA and Abd-Elsalam RM: Antifibrogenic Influence of Mentha piperita L. Essential Oil against CCl4-Induced Liver Fibrosis in Rats in Oxidative Medicine and Cellular Longevity. (2018):1-10.
38. Adewale OB, Adekeye AO, Akintayo CO, Onikanni A and Saheed S : Carbon tetrachloride (CCl4)-induced hepatic damage in experimental Sprague Dawley rats: Antioxidant potential of Xylopia aethiopica in The Journal of Phytopharmacology. (2014) 3(2), 118-123.
39. Makni JT and Boudawara N: Hypolipidemic and hepatoprotective effects of flax and pumpkin seed mixture rich in Omega-3 and Omega-6 fatty acids in hypercholesterolemic rats in Food Chemical Toxicology. (2008) 46 (12): 3714-3720.
40. Rezk RG and Darwish M : Pumpkin Seed Oil Attenuates Functional and Structural Disorders in Urinogenital System of Male Albino Rats Induced by Whole Body Gamma Irradiation in Arab Journal of Nuclear Science and Applications. (2012) 45(4):210-219.
41. Latief U and Ahmad R : Herbal remedies for liver fibrosis: A review on the mode of action of fifty herbs. Journal of Traditional and Complementary in Medicine. (2018) 8:352-360.
42. Yoshida K and Matsuzaki K: Differential regulation of TGF-b/Smad signaling in hepatic stellate cells between acute and chronic liver injuries in Physiology. (2012) 3(53):1-7.
43. Mohamed NZ, Abd-Alla H, Aly HF, Mantawy M, Ibrahim N and Hassan SA: CCl4-induced hepato nephrotoxicity: protective effect of nutraceuticals on inflammatory factors and antioxidative status in rat in Journal of Applied Pharmaceutical Science. (2014) 4 (02): 87-100.
44. Gong Z, Ye H, Huo Y, Wang L, Huang Y, Huang M and Yuan X: S-allyl-cysteine attenuates carbon tetrachloride-induced liver fibrosis in rats by targeting STAT3/SMAD3 pathway in American Journal Translational Research. (2018) 10(5):1337-1346
45. Li D, Li W,Chen Y, Liu L, Ma D, Wang H, Zhan L, Zhao S and Peng Q: Anti-fibrotic role and mechanism of Periplaneta americana extracts in CCl4-induced hepatic fibrosis in rats in Acta Biochimica Biophysica Sinica. (2018) 50(5):491–498.
46. Al-Okbi SY, Mohamed SY, Kandil E, Abo-Zeid MA, Mohammed SE and Ahmed EK : Anti-inflammatory activity of two varieties of pumpkin seed oil in an adjuvant arthritis model in rats in Grasas Aceites. (2017) 68 (1):1-12.
47. Saraiva RA, Araruna MK, Oliveira RC, Menezes KDP, Leite GO, Kerntopf MR, Costa JGM, Rocha JBT, Tomé AR, Campos AR and Menezes IRA: Topical ant-inflammatory effect of Caryocar Coriaceum Wittm (Caryocaraceae) fruit pulp fixed oil on mice ear edema induced by different irritant agents in Journal of Ethnopharmacology. (2011) 136(3): 504-510

48. Fortis-Barrera, Á.; García-Macedo, R.; Almanza-Perez, J.; Blancas-Flores, G.; Zamilpa-Alvarez, A.; Flores-Sáenz, J.; Cruz, M., Román-Ramos, R. and Alarcón-Aguilar, F. (2016): Cucurbita ficifolia (Cucurbitaceae) modulates inflammatory cytokines and IFN-γ in obese mice. Canadian journal of physiology and pharmacology 95: 170-177.

49. Emaraa MW, Ahmed MI, Refaat WM and Mohammed HL: Effects of both garlic and rosemary extracts on rats liver fibrosis: A histochemical and immunohistochemical study in Zagazig University Medical Journal. (2015) 21(2):210-220.

50. Hamza AA, Mohamed MG, Lashin FM and Amin A: Dandelion prevents liver fibrosis, inflammatory response, and oxidative stress in rats in The Journal of Basic and Applied Zoology. (2020) 81(43): 1-13.

51. Hassan NF, Soliman GM, Okasha EF and Shalaby AM: Histological, immunohistochemical, and biochemical study of experimentally induced fatty liver in adult male albino rat and the possible protective role of pomegranate in Journal of Microscopy and Ultrastructure. (2018) 6: 44-55.

52. Li W, Zhu C, Li Y, Wu Q and Gao R : Mest Attenuates CCl4-Induced Liver Fibrosis in Rats by Inhibiting the Wnt/ β-Catenin SignalingPathway in Gut and Liver. (2014) 8 (3):282-291.

الملخص العربى

تأثير زيت بذور اليقطين على التليف الكبدي المبكر المستحث تجريبيا في ذكر الجرذ الابيض البالغ

نسمة عفيفي، مصطفى الحبيبي، عبير الميهي، منار فريد
قسم التشريح والأجنة، كلية الطب، جامعة المنوفية، المنوفية، مصر

الخلفية: تليف الكبد سبب رئيسي للمرض والوفيات في جميع أنحاء العالم ولا يوجد علاج فعال. يلعب الإجهاد المؤكسد والالتهاب دورًا مهمًا في التسبب في تليف الكبد. من المعروف أن زيت بذور اليقطين له خصائص مضادة للأكسدة ومضادة لالتهابات ومضادة للتليف.

الهدف من العمل: يهدف هذا العمل إلى معرفة تأثير زيت بذور اليقطين على تليف الكبد المستحث تجريبياً في ذكر الجرذ الابيض البالغ لاول مرة على حد علمنا ، مع الإشارة إلى الآليات الأساسية المختلفة.

المواد والطرق: تم تقسيم خمسين جرذًا بالغًا من ذكور الجرذان البيضاء إلى المجموعة الأولى (ن = ٢٠): مقسمة بالتساوي إلى مجموعتين.المجموعة الثانية: زيت بذور اليقطين (ن = ١٠): زيت بذور اليقطين :٤ مل / كجم / يوم لمدة ٤ أسابيع.المجموعة الثالثة: تليف الكبد (ن = ١٠): رابع كلوريد الكربون ١مل / كجم داخل الغشاءالبروتوني ، مرتين في الأسبوع لمدة ثمانية أسابيع. المجموعة الرابعة: تليف الكبد المعالج بزيت بذور اليقطين (ن = ١٠): تم تحفيز تليف الكبد كالمجموعة الثالثة.بعد ٤ أسابيع من علاج رابع كلوريد الكربون ، أعطيت الجرذان زيت بذور اليقطين كالمجموعة الثانية.في نهاية التجربة تم إخضاع عينات الدم وأقسام الكبد للدراسات الكيمياء الحيوية والنسيجية والكيمياء المناعية.

النتائج: أظهرت مجموعة تليف الكبد زيادة كبيرة في مستويات وظائف الكبد ، IL ، TNFα ، MDA-β١ و وكذلك انخفاض في مستويات الألبومين في الدم ، SOD و CAT مقارنة بالمجموعة الضابطة. أظهرت أقسام الكبد السيتوبلازم المفرغ ، النوى المتقرحة والميتوكوندريا المتحللة. علاوة على ذلك ، تم الكشف عن تليف ملحوظ بواسطة زيادة نسبة مساحة ترسيب الكولاجين. تم الكشف عن زيادة التفاعلات المناعية αSMA و TGFβ١ و caspase٣. خفف زيت بذور اليقطين جميع المعايير التي تم فحصها من خلال خصائصه المضادة للأكسدة والمضادة للالتهابات ومضادة للتليف.

الخلاصة: هناك احتمالية واعدة لبزيت بذور اليقطين كعامل علاجي طبيعي ضد تليف الكبد.