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Hepatoprotective effect of *Moringa oleifera* extract on TNF-α and TGF-β expression in acetaminophen-induced liver fibrosis in rats

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Abstract

Background: It has been reported that *Moringa oleifera* (MO) has different medicinal properties. The aim of this study was to evaluate the hepatoprotective role of *Moringa oleifera* extract on acetaminophen-induced liver fibrosis in albino rats on a biochemical and histological basis. Forty male albino rats were divided into four groups: group I (control group), healthy rates; group II (acetaminophen group), rates received acetaminophen for induction of liver fibrosis; group III (treated group), liver fibrosis of rates treated with *Moringa oleifera* extract; and group IV (prophylactic group), rates treated with *Moringa oleifera* extract before and after induction of liver fibrosis. Serum liver function parameters were quantified using a spectrophotometer, while tumor necrosis factor α (TNF-α) and transformed growth factor beta (TGF- β) in liver tissue homogenate by means of enzyme-linked immunosorbent assay (ELISA), and expression of liver tissue TNF-α and TGF-genes was measured by real-time PCR after extraction and purification. Hepatic tissue was also evaluated under a microscope for histopathological changes.

Results: Our results showed a significant decrease in liver enzymes, TNF-α, and TGF-β in the treated and prophylactic groups compared to the acetaminophen group, and our biochemical data were consistent with the histopathological findings confirming the hepatoprotective effect of *Moringa oleifera* extract.

Conclusions: Biochemical parameters and histopathology results provide evidence that *Moringa oleifera* ethanolic extract has a great potential to prevent and improve liver damage due to its protective activity.

Keywords: Liver fibrosis, *Moringa oleifera*, TNF-α, TGF-β, Acetaminophen

Background

Liver is an important organ responsible for a variety of critical biochemical and physiological phenomena, such as the metabolism and detoxification of endogenous and exogenous substances, such as drugs, xenobiotics, and homeostasis [1]. Hepatotoxic agents, including drugs, alcohol, and viral infections may cause hepatic injury [2].

Acetaminophen (N-acetyl-para-aminophenol, paracetamol, APAP) is one of the most widely used analgesic and antipyretic agents that does not require a prescription. Although acetaminophen has a good safety profile at therapeutic levels, it can cause severe liver toxicity when administered in large quantities in experimental animals as well as in humans [3].

Liver disease is a global problem. The traditional medicines used to treat liver disease are sometimes inadequate and can have dire consequences. Therefore, it is necessary to search for alternative drugs to treat liver diseases to replace the currently used medicines of doubtful efficacy and safety [4]. Herbs play a role in managing various liver disorders in the absence of reliable liver protection drugs in curative medical practices. Most herbal medicines accelerate the natural healing process of the liver so that the search for an effective

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and preventive liver medicine continues because we suffer from poor treatment of liver disease [5].

In light of recent scientific developments around the world, the medicinal features of the plants have also been examined due to their potent pharmacological activity, low toxicity, and economic feasibility compared to synthetic drugs [6]. Due to its enormous medicinal potential, *Moringa oleifera* has nutritional and medicinal properties, provided that the medicinal uses, livestock feed, and nutritional value are of high economic value. Belonging to the Moringaceae family, it is a perennial tropical deciduous tree native to the southern Himalayas in northern India. *Moringa oleifera* extracts exhibit multiple nutritional or pharmacological functions including antioxidant, anticancer, anti-inflammatory, liver disease prevention, neuroprotection, hypoglycemia, and blood lipid lowering. The beneficial functions of *Moringa oleifera* are closely related to its high content of phytochemicals such as flavonoids, glucosinolates, isothiocyanates, and phenolic acids [7].

Signaling pathways of the transforming growth factor-beta family (TGF-β) play important roles in the regulation of various cellular processes, including proliferation, differentiation, migration, or cell death, which is essential for tissue and organic homeostasis. Due to the diverse and multi-orbital functions of TGF-β, the liberalization of its pathways contributes to human disease [8]. TGF-β signaling is involved in all stages of liver disease progression, from primary liver injury through inflammation and fibrosis to cirrhosis and cancer. TGF-β has cytostatic and apoptotic effects in hepatocytes, promoting liver differentiation during embryonic development and physiological liver regeneration. Thus, tracing the TGF-β signaling pathway is being explored to prevent progression of liver disease [9].

Tumor necrosis factor-α (TNFα) is a multidirectional cytokine that is generated in various immune cells including macrophages and monocytes. TNFα can lead to multiple pathways of inflammation, proliferation, and apoptosis. Although TNFα has been implicated in the pathogenesis of chronic hepatitis leading to cirrhosis, the role of TNFα in cirrhosis has not been fully defined [10].

Our aim was to investigate the hepatoprotective role of *Moringa oleifera* extract on acetaminophen-induced liver fibrosis in albino rats by evaluating a variety of biochemical and histopathological parameters.

Methods

Drugs and reagents

Acetaminophen and all chemicals were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA). *Moringa oleifera* leaves were obtained from the Egyptian Society of Moringa (inside the author's institution), Giza, Egypt.

Preparation of alcoholic extract of *Moringa oleifera*

The dried coarse powders were soaked with 1.5 L of 70% ethanol in amber-colored extraction bottle. The bottles were sealed and kept for the 7 days at room temperature with occasional shaking and stirring. The extracts were filtered through cotton followed by Whatman No.1 filter paper and were concentrated with a rotary evaporator (Bibby Sterlin Ltd, UK) under reduced pressure; extraction was repeated by using fine particles of sediment to re-soak in ethyl alcohol, and this was repeated several times till a clear supernatant was obtained and then this extract is stored in an airtight container in a refrigerator below 10 °C [11].

High-performance liquid chromatography (HPLC) assay of flavonoids and phenolic compounds in *Moringa oleifera* extract

High-performance liquid chromatography of phenols and flavonoids was performed using Agilent Technologies 1100 Series Liquid Chromatograph with automatic sampling and diode array detector. The analytical column was Eclipse XDB-C18 (150 × 4.6 μm; 5 μm) with a C18 protection column (Phenomenex, Torrance, CA). The mobile phase consists of acetonitrile (solvent A) and 2% acetic acid in water (volume/volume) (solvent B). The flow rate was maintained at 0.8 mL/min for a total run time of 70 min. The injection volume was 50 μl, and the peaks were simultaneously monitored at 280 and 320 nM for the benzoic acid and cinnamic acid derivatives respectively. All samples were filtered through an Acrodisc 0.45 μM syringe filter (Gelman's Laboratory, MI) prior to injection. Peaks were determined by matched retention times and UV-vis spectra and compared with those of the parameters [12].

Animals

Forty albino rats (180–200 g body weight) at the beginning of the experiment were obtained from the animal house of the author's institution and used in this study. Rates were individually housed in clean polypropylene cages and kept in a temperature-controlled (22 ± 2 ° C) room with 12 h light and 12 h dark cycle with free access to water and standard rat food pellets. Animals were allowed 14 days to acclimatize to laboratory conditions before the experiment. All procedures were performed in accordance with ethical guidelines and with approval by the Ethical Committee No. PBC 2019-04 and following the recommendations of the National Institutes of Health guide for the Care and Use of Laboratory Animals.

Experimental design

Forty male albino rats were divided into four groups (10 rats each): group I (control group), healthy rats were

given distilled water only; group II (acetaminophen group), induction of liver fibrosis (rats received 1 g acetaminophen/kg b.w./day orally for 10 days); group III (treated group), rats with liver fibrosis were treated with 500 mg/kg of *Moringa oleifera* extract (2 months); and group IV (prophylactic group), rats received 500 mg/kg of *Moringa oleifera* extract before (2 months) and after (2 months) induction of liver fibrosis [13].

Collection of samples

After the experimental period (4 months), the animals were fasted for 8 h; before blood samples were taken, blood was drawn from the orbital venous retrograde plexus of the eye under formalin anesthesia using capillary tubes, collected in clean tubes, allowed to clot, and then centrifuged for 10 min at 3000 rpm. The serum was separated and stored in Eppendorf at 20 °C for use in determining liver function parameters. After collecting blood samples, the mice were killed by cervical dislocation and their livers removed immediately. Moreover, rat livers were divided into 3 parts, and the first segment was kept at 80 ° C to determine the conversion of growth factor beta (TGF-β) and tumor necrosis factor alpha (TNF-α) by ELISA. The second part was used for quantitative real-time PCR analysis. The third fraction was stored in formalin phosphate-buffered 10% for further pathological examination.

Preparation of liver tissue homogenates for biochemical assays

The liver was removed and washed directly with cold phosphate saline (PBS). Further dissection was performed on an ice-cold glass plate. Liver homogenates were prepared in a Teflon-glass tissue mixer, using chilled 50 mM PBS (pH 7.4) to give 20% w/v homogeneous according to Lin et al. [14]. It was then centrifuged at 3000 rpm for 15 min separately in a cooled centrifuge. The supernatant was used to analyze biochemical variables in this study.

The following parameters were estimated:

- Liver function tests determination:
  The liver enzymes including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined according to Reitman and Frankel [15], and alkaline phosphatase (ALP) was determined as outlined by Beifield and Goldberg [16], using diagnostic kits obtained from Roche Diagnostics Ltd (Germany).
- Determination of hepatic TNF-α and TGF-β level:
  Both TNF-α and TGF-β were determined in liver homogenate using ELISA kits for rats according to Corti et al. [17] and Kim et al. [18] respectively by Avi-Bion ELISA Kit (Orgenium Laboratories, Finland)
- Quantitative real time PCR analysis:
  RNA was extracted using the RNeasy Mini kit (Qiagen) from the liver tissue of each experimental rat. RT kit (Promega) for reverse transcription was used. Real-time PCR of target gene copy numbers was performed with respect to the transcription of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using individual primers listed in Table 1. SYBR Green kit (Qiagen, Hilden, Germany) determined the levels of gene expression. MxPro qPCR (Agilent Technologies) software was used to analyze the data.

Histopathological examination

Biopsies were fixed in 10% neutral formalin, processed, and combined into paraffin blocks. For both routine H&E staining, 4-μm-thickness sections were prepared. An evaluation of changes in the portal tract, hepatocytes, and inflammatory cell reaction was performed in the four study groups [19].

Masson trichrome staining

Slides were immersed in warm Boin solution at 60 ° C and then washed in running tap water. The slides were immersed in a modified Weigert's hematoxylin to kernel and then washed in running water. Slides were dipped in anionic dyes and fuschin acid (CI 42590, Merck, Germany) for staining of the cytoplasm and erythrocytes, and then the slides were washed again with running tap water. Slides were treated with a solution of phosphomolipidic acid, and the slides were immediately immersed in a solution of methyl blue (CI 42780, Merck, Germany) for fibroblast and collagen staining. The slides were then washed in running water and finally treated with a 1% acetic acid solution. The slides were then dried into a series of ascending alcohol. Prior to observation, slides were immersed in xylene absolute and finally fitted with a sliding cap using a DPX fitting [20].

Statistical analysis

In this study, all results were expressed as mean ± standard error. One-way analysis of variance (ANOVA) followed by an LST comparison test using GraphPad

Table 1 Primer sequence of GAPDH, tumor necrosis factor alpha (TNF-α), and transforming growth factor beta (TGF-β)

Target	Sequence
GAPDH	F, 5′-ACCACAGTCCATGCCATCAC-3′ R, 5′-TCCACCACCCTGTTGCTGTA-3′
TNF-α	F, 5′-AACTCGAGTGACAAGCCCGTAG-3′ R, 5′-GTACCACCAGTTGGTTGTCTTTGA-3′
TGF-β	F, 5′-TGCGCCTGCAGAGATTCAAG-3′ R, 5′-AGGTAACGCCAGGAATTGTTGCTA-3′

Table 2 High-performance liquid chromatography assay of phenolic and flavonoids compounds in *Moringa oleifera* extract

Compound	Retention time (min)	*Moringa* leaves (μg/g)
Gallic acid	5.90	77.16
p-Hydroxybenzoic	15.22	2673.59
Catechin	18.37	2045.44
Chlorogenic acid	20.28	48.56
Caffeic acid	21.08	86.53
Ferulic acid	32.17	56.39
Sinapic acid	33.56	332.60
Rutin	36.18	599.05
p-Coumaric acid	36.95	59.19
Cinnamic acid	41.52	57.96
Quercetin	43.01	245.95

Prism (version 5.00) software was used to analyze the data. At $p < 0.05$, the difference was significant.

Results

As presented in Table 2 and Fig. 1, the most effective compound in the *Moringa oleifera* extract was phenolic compounds such as p-hydroxybenzoic acid (2673.59 μg/g), sinapic acid (332.60 μg/g), caffeic (86.53 μg/g), gallic (77.16 μg/g), p-coumaric (59.19 μg/g), ferulic (56.39 μg/g), chlorogenic (48.56 μg/g), and flavonoid compounds such as catechin (2045.44 μg/g), rutin (599.05 μg/g), quercetin (245.95 μg/g), and cinnamic acid (57.96 μg/g).

Table 3 showed a significant increase in liver enzymes (ALT, AST, ALP) in the acetaminophen and the treated groups compared to the control group. These results showed significant improvement after treatment with *Moringa oleifera* extract, and the prophylactic group showed remarkable results by returning liver enzymes to normal levels.

Table 4 showed a significant increase in TNF-α and TGF-β in the acetaminophen and treated groups compared to the control group. These results showed significant improvement after treatment with *Moringa oleifera* extract. In addition, the prophylactic group showed a significant decrease in TNF-α and TGF-B to be below normal.

Table 5 showed a significant increase in the expression of TNF-α and TGF-β genes in the acetaminophen and the treated groups compared to the control group. These results showed significant improvement after treatment with *Moringa oleifera* extract, and the prophylactic group showed significant decrease in the TNF-α and TGF-β genes to be lower than normal level.

There was a noticeable positive correlation between TNF-α and TGF-β and TNF-α and TGF-β genes as shown in Figs. 2 and 3.

Histopathological results

Examination of the liver specimens under a light microscope showed a normal liver tissue structure in the control group as shown in Fig. 4. The acetaminophen group

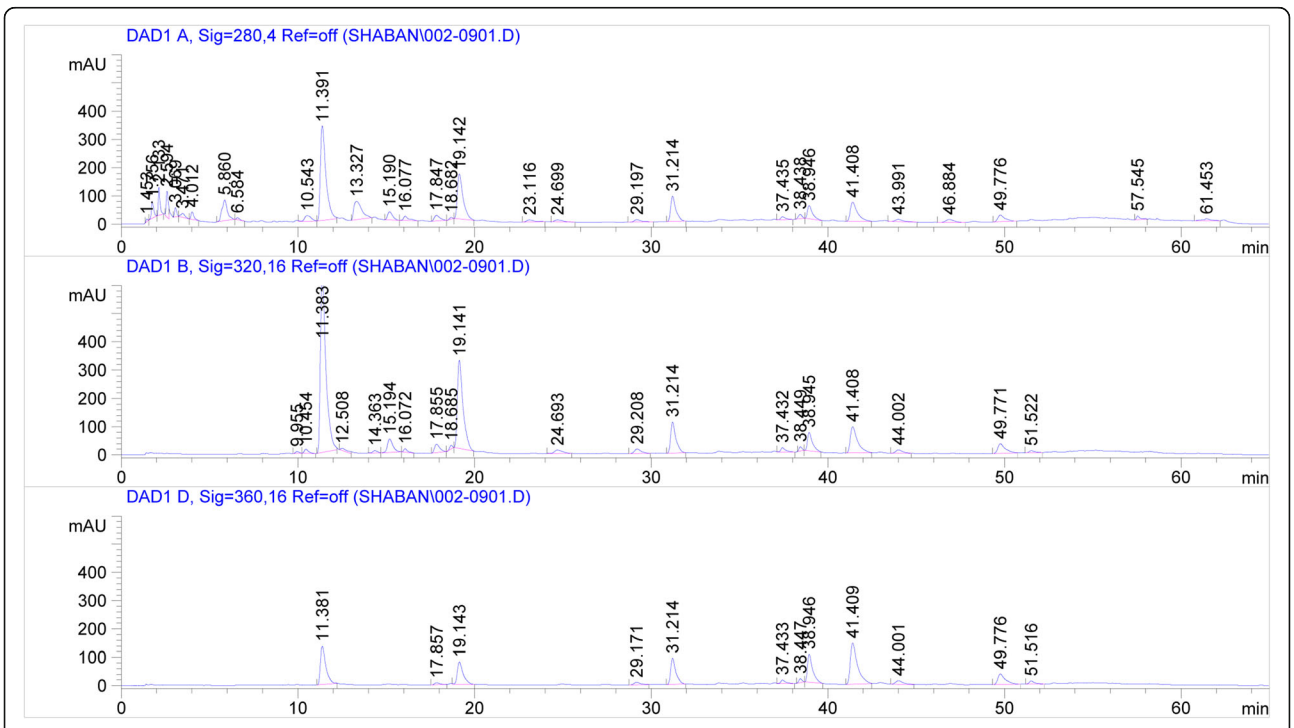

Fig. 1 High-performance liquid chromatography chromatograms of *Moringa oleifera* extract, at wavelengths 280 nm, 330 nm, and 360 nm

Table 3 Mean levels of liver function parameters in the different studied groups

Groups	Parameters		
	ALT (IU/L)	AST (IU/L)	ALP (IU/L)
Control group	51.53 ± 4.89	126.12 ± 10.94	175.59 ± 5.36
Acetaminophen group	181.99 ± 7.20[a]	580.5 ± 52.34[a]	259.34 ± 14.37[a]
Treated group	65.42 ± 2.44[a,b]	154.81 ± 8.92[a,b]	184.25 ± 5.31[a,b]
Prophylactic group	50.36 ± 1.94[b]	125.75 ± 5.18[b]	173.68 ± 8.9[b]

Values are expressed as mean ± standard error (SE)
[a]Significant difference at $p < 0.05$ compared to control group
[b]Significant difference at $p < 0.05$ compared to acetaminophen group

showed maximum cellulitis reaction with portal fibrosis bridging but with normal looking liver cells as shown in Fig. 5. The treated group showed an inflammatory mild response with minimal portal fibrosis highlighted by Masson trichrome stains as shown in Fig. 6. The prophylactic group showed insignificant inflammatory reactions compared to the previous group, and portal fibrosis was minimal and limited to the portal region as shown in Fig. 7.

Discussion

The liver is involved in many metabolic functions, and because of its central role in xenograft metabolism, it is susceptible to strange injury. Hepatotoxic drugs, such as acetaminophen, damage the liver [21].

Our data showed a significant increase in liver enzymes (ALT, AST, ALP) in the acetaminophen group compared to the control group. Increased liver enzymes activity (ALT and AST) reflects damage to liver hepatocytes and indirect impairment of liver functions due to hepatotoxicity caused by acetaminophen [22]. Our results are consistent, and the elevated serum level of ALP generally reflects hepatobiliary and hepatocellular injury. The mechanism of the ALP high levels may result from faulty liver discharges or from increased ALP generation of hepatic parenchymal or duct cells [23].

Our results showed a significant improvement after treatment with *Moringa oleifera* extract in the treated and prophylactic groups as shown in Table 3. Recovery towards normalization of the enzymes following *Moringa oleifera* pretreatment suggested that the plant extract has a role in preserving the structural integrity of the hepatocellular membrane and preventing the enzymes from leaking into circulation. Our results are consistent with generally accepted hypothesis that transaminase level return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes [24]. In addition, the significant decrease in serum levels of ALP by action of *Moringa oleifera* compared to hepatotoxic rats revealed an improvement in the effect of *Moringa oleifera* extract, which is consistent with Fakurazi et al. [25].

Table 4 Mean levels of TNF-α and TGF-β parameters in the different studied groups

Groups	Parameters	
	TNF-α ng/g	TGF-B ng/g
Control group	141.1 ± 1.17	238.8 ± 10.81
Acetaminophen group	558.8 ± 26.55[a]	968.3 ± 15.46[a]
Treated group	231.4 ± 7.18[a,b]	311.5 ± 7.17[a,b]
Prophylactic group	137.2 ± 2.34[b]	232.9 ± 9.77[b]

Values are expressed as mean ± standard error (SE)
[a]Significant difference at $p < 0.05$ compared to control group
[b]Significant difference at $p < 0.05$ compared to acetaminophen group

Table 5 Mean levels of TNF-α and TGF-β gene expression in the different studied groups

Groups	Parameters	
	TNF-α relative	TGF-B relative
Control group	1.26 ± 0.02	1.22 ± 0.02
Acetaminophen group	9.67 ± 0.47[a]	6.7 ± 0.23[a]
Treated group	2.28 ± 0.11[a,b]	1.69 ± 0.09[a,b]
Prophylactic group	1.43 ± 0.07[b]	1.19 ± 0.04[b]

Values are expressed as mean ± standard error (SE)
[a]Significant difference at $p < 0.05$ compared to control group
[b]Significant difference at $p < 0.05$ compared to acetaminophen group

This study showed a significant increase in TNF-α and TGF-β levels in both acetaminophen and treated groups compared to the control group as shown in Table 4, so this result indicates that it could induce liver inflammation in rats. The results also showed a significant improvement after treatment with *Moringa oleifera* extract and in the prophylactic group showed a significant decrease in TNF-α and TGF-β to be lower than normal levels, due to the hepatoprotective effect of *Moringa oleifera* against inflammation of the liver resulting from the reduction of the TNF-α liver content and agreed with Mahajan et al. [26].

Tumor necrosis factor alpha plays an important role in liver inflammation and fibrosis; it activates NF-κB which becomes the driving force of inflammation, activates the major fibrogenic molecule TGF-β, and

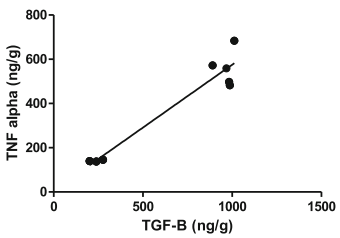

Fig. 2 Correlation between TNF-α and TGF-β (ng/g); $r = 0.9673$, $p < 0.0001$

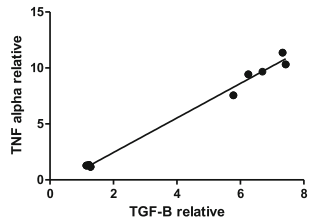

Fig. 3 Correlation between TNF-α and TGF-β relative; $r = 0.9954$, $p < 0.0001$

stimulates the survival and production of activated myofibroblasts through differentiation of hepatic stellate cells (HSCs) [27].

The gene expression of tumor necrosis factor alpha and TGF-β in the acetaminophen group was significantly higher compared to the control group as shown in Table 5. These results showed significant improvement after treatment with *Moringa oleifera* extract. The protective group also showed a significant decrease in the expressions of TNF-α and TGF-β. These results demonstrated that the induction of paracetamol was able to cause liver damage and indicated that the administration of *Moringa* leaf extract reduced TNF-α and TGF-β expression, which was shown to reduce cell death or apoptosis. The protective effect of *Moringa* leaves is due to the presence of polyphenols including quercetin (Table 2). Quercetin is a flavonoid found in *Moringa* leaf, it has a powerful antioxidant function, and it also helps cells that modify signal transmission, such as growth, reproduction, and cell death (apoptosis) [28].

Our results were consistent with Saile et al. [29] who reported that both TGF-β and TNF-α gene expression were upregulated in the chronically inflamed liver. Semyonova et al. [30] also reported that hepatic expression of inflammatory TNF-alpha occurs in many chronic and acute liver diseases, as well as after exposure to toxic chemicals to the liver, and is believed to help influence the damage and repair processes that follow these insults by regulating more mediators.

Other studies reported that TNF-α plays a major role in chronic liver injury and inflammation, in addition to improving HSC survival, hepatocyte death, and immune cell activation associated with increased liver fibrosis. Therefore, carefully targeting specific TNF-α signaling pathways must be considered as a novel therapeutic approach for liver fibrosis [31].

Our findings were agreed upon with Dooley and Dijke [32], who reported that TGF-β is a central regulator of chronic liver disease that contributes to all stages of disease progression from primary liver injury to cirrhosis and hepatocellular carcinoma through inflammation and fibrosis. Levels of active TGF-β induced by liver damage increase hepatocyte destruction and mediate activation of hepatic stellate

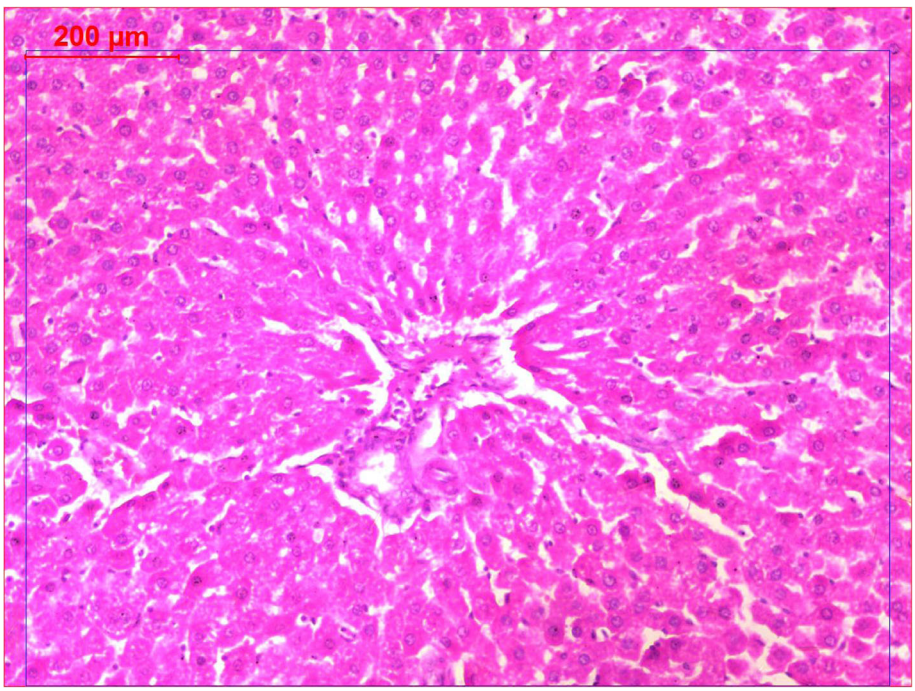

Fig. 4 Photomicrograph for the control group: normal liver tissue with preserved architecture and insignificant inflammation within normal limits (× 200)

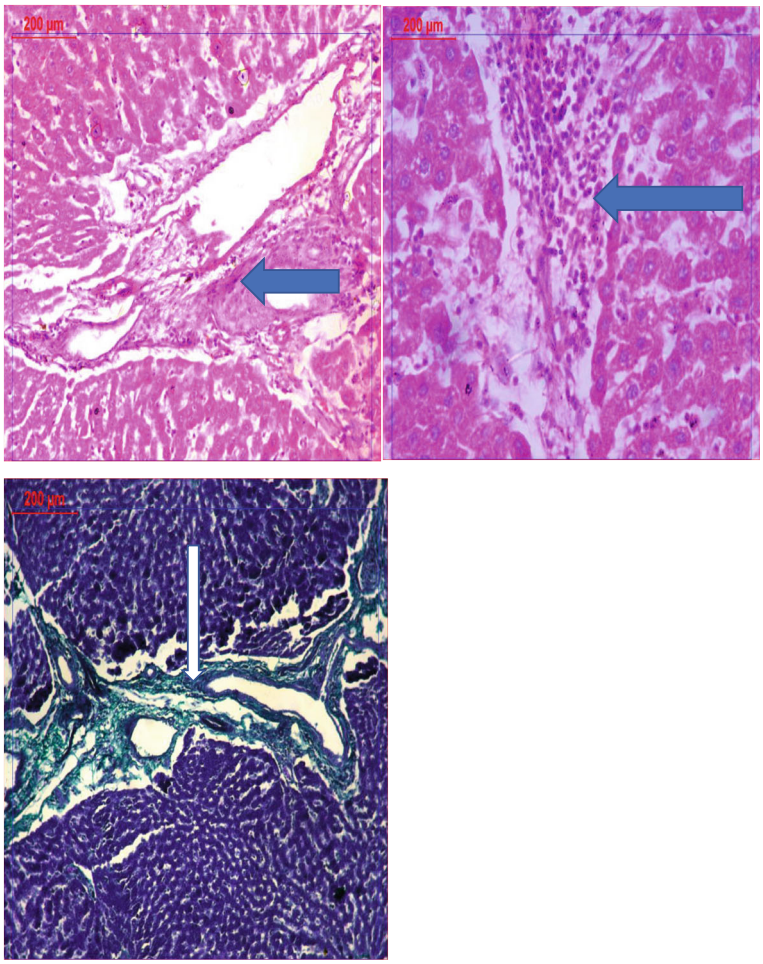

Fig. 5 Photomicrograph for the acetaminophen group: showing liver tissue with expanded portal tracts with bridging fibrosis and moderate inflammatory response (blue arrows), fibrosis demonstrated by Masson trichrome stain (white arrow) (× 200, × 400, × 100)

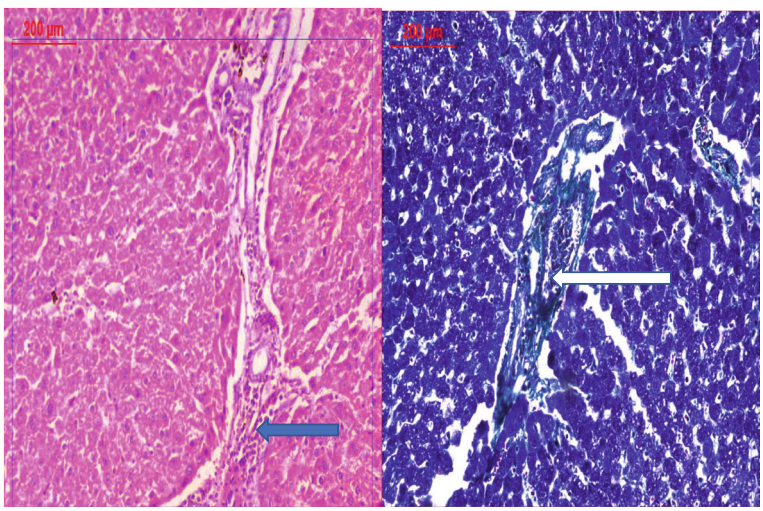

Fig. 6 Photomicrograph for the treated group: showing liver tissue with minimal portal fibrosis and mild inflammatory response (blue arrows), demonstrated by Masson trichrome stain (white arrow) (× 200, × 200)

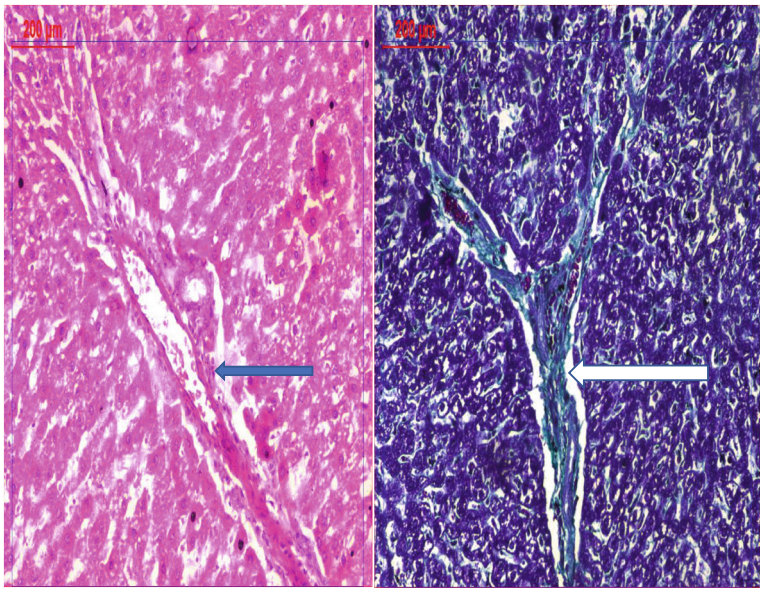

Fig. 7 Photomicrograph for the prophylactic group: showing liver tissue with minimal portal fibrosis and insignificant inflammatory response (blue arrows), demonstrated by Masson trichrome stain (white arrow) (× 200, × 200)

cells and fibroblasts resulting in a wound-healing response, including myofibroblast production and extracellular matrix deposition.

The results of the histopathological examination of this study revealed the hepatoprotective properties of the *Moringa oleifera* leaf extract, as shown in Figs. 6 and 7, where there was a minimum of pyloric fibrosis and a mild inflammatory response compared to the acetaminophen group (Fig. 5) which showed a bridging of fibrosis and a median inflammatory response. Paracetamol was used in this study to cause liver injury (Fig. 5), and hepatotoxicity has been reported [33]. Therefore, based on the results, the extract of *Moringa oleifera* leaves had some protective effect on the liver as evidenced by the decrease in infection in both the treated and prophylactic groups due in part to the presence of hepatoprotective chemical components [34]. The histopathological appearances also clearly supported the biochemical findings.

Conclusion

It was concluded that *Moringa oleifera* might be considered as an adjuvant drug in treatment of liver fibrosis and hepatotoxicity and act as hepatoprotective therapy in all liver disorders, yet more clinical studies are needed to emphasize our finding.

Limitations of the study

This study was carried out on a small sample which could yield sampling errors.

Abbreviations

ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; APAP: Acetaminophen; AST: Aspartate aminotransferase; ELISA: Enzyme-linked immunosorbent assay; HPLC: High-performance liquid chromatography; HSC: Hepatic stellate cell; MO: *Moringa oleifera*; PBS: Phosphate-buffered saline; RT-PCR: Real-time polymerase chain reaction; SE: Standard error; SPSS: Statistical Package for the Social Sciences; TGF-β: Transforming growth factor-beta; TNF-α: Tumor necrosis factor-α

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Authors' contributions

OA and HHZ put the design of this study. OA, HHZ, DMA, GAH, OGS, and AMF carried out the experimental and practical parts. OA, HHZ, and DMA contributed to the interpretation of data. All participated in the manuscript writing and revision. And finally, all authors have read and approved the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

All experimental protocols were approved by Ethics Committee of the Ahram Canadian University (ACU) with number PBC 2019-04. Consent to participate is not applicable (experimental model).

Consent for publication

Not applicable.

Competing interests

There are no competing interests.

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