



Ameliorative effect of moringa and rosemary ethanolic extracts on thioacetamide-induced liver fibrosis in rats

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Abstract

Background/aim: Nutraceuticals have been extensively studied in recent years to find safe therapeutics of natural origin instead of relying only on pharmaceuticals. This study aimed to detect the hepato-protective role of moringa and rosemary ethanolic extracts on liver fibrosis induced by thioacetamide (TAA).

Methods: This study was conducted on 60 male albino rats divided into four groups; control, TAA-group (received 100 mg/kg thioacetamide intraperitoneal twice /week for 18 weeks), moringa-protected group (received 300 mg/kg moringa ethanolic extract orally daily with TAA), rosemary-protected group (received 200 mg/kg rosemary ethanolic extract orally daily with TAA for 18 weeks).

Results: There were significant increases in liver alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), malondialdehyde (MDA), and nitric oxide (NO) with significant decreases in reduced glutathione (GSH), superoxide dismutase (SOD) in the TAA-treated group with a high degree of fibrosis which extended to cirrhosis after 18 weeks. Meanwhile, both TAA-moringa and TAA-rosemary treated groups showed improvement in biochemical markers and fibrosis induced by TAA.

Conclusion: This study proved that both moringa and rosemary could protect the liver against TAA-induced liver fibrosis and rosemary has greater protection than moringa.

Keywords: TAA; moringa; rosemary; liver fibrosis

1. Introduction

Liver fibrosis is the result of excessive extracellular matrix (ECM) accumulation (Kisseleva and Brenner, 2011). Thioacetamide (TAA) is a potent centrilobular hepatotoxic agent which is frequently used in the experimental models of chemically induced hepatotoxicity in rodents. The most common mechanism of action of TAA for cirrhosis induction is the induction of oxidative stress (Helmy et al., 2018).

There is no FDA-approved medication for liver fibrosis (Bataller and Brenner, 2005). Many strategies have been conducted to combat liver fibrosis by inhibiting pathways or certain molecular targets that are involved in the development of liver fibrosis. Recently, substantial traditional herbs that possess low adverse effects in the treatment of chronic liver diseases have created considerable interest as protective agents for reducing liver damage (Guo et al., 2013).

Moringa oleifera leaves are a great source of flavonoids and phenolic acids. flavonoids have been shown to protect against chronic diseases associated with oxidative stress (Vergara-Jimenez et al., 2017). Moringa has been used to treat hepato-renal, cardiovascular and gastrointestinal disorders. In addition, it has efficient anti-inflammatory, antimicrobial, and anti-oxidative

properties (Kou et al., 2018).

Rosemary (*Rosmarinus officinalis*) is one of the household herbs that contains carnosic acid and several phytochemicals, including rosmarinic acid, camphor, caffeic acid, ursolic acid that act as antioxidants (Akela et al., 2018). Additionally, rosemary leaf extracts also have pharmacological activities such as anti-inflammatory, anti-obesity, and antimicrobial activity which are attributed to its bioactive ingredients (Ou et al., 2018).

Most previous studies investigated the protection of *Moringa oleifera* or rosemary on hepatotoxicity but fewer researchers are studying their protective effect on liver fibrosis. Consequently, this study aimed to investigate the protective effect of ethanolic extracts of moringa and rosemary on liver fibrosis induced by thioacetamide.

2. Materials and methods

2.1 TAA

TAA was purchased from (Sigma-Aldrich, St. Gallen, Switzerland) in the form of a white crystalline solid which was soluble in water and alcohol.

2.2 Animals

Sixty male Wistar rats weighted 120-150 g, 2 months old obtained from the research lab in Zagazig University. They were housed in cages in a well-ventilated house and allowed *ad libitum* access to food and water and kept in 12 h light-dark cycle. All experiments were approved by the Ethical Committee of Faculty of Veterinary Medicine, Benha University Egypt (BUFVTM 01/11/2019)

2.3 Preparation of plant extracts

One kilogram of each fresh moringa and rosemary leaves were air-dried in the shade for one week and samples were taken and powdered using a mill. The powder was weighed and immersed in 80% ethanol by percent 1/10 for four days at room temperature (22 °C) with gentle shaking. The contents were filtered through Whatman filter paper (No. 1), then the filtrate was dried under vacuum to evaporate alcohol to obtain a semisolid crude extract that underwent to freeze-dry method. The ethanolic extract was stored in an airtight container at 4 °C until further use (Sinha et al., 2012).

2.4. Experimental design

Sixty male rats were randomly divided into four equal groups of 15 rats per group. Group 1 (G1) was kept as control and G2 was intraperitoneally (IP) injected with 100 mg/kg TAA twice /week for 18 weeks (Hamed et al., 2011). G3 was injected with TAA as in G2 and received 300 mg/kg moringa extract orally daily for 18 weeks (Ujah et al., 2013). G4 was injected with TAA as in G2 and received 200 mg/kg rosemary extract orally daily for 18 weeks (Al-Attar and Shawush, 2014). After 6, 12, 18 weeks five rats of each group were fasted for 8 h, anesthetized by light ether then sacrificed by cervical dislocation, and immediately blood, liver samples were collected for biochemical, histopathological, and immunohistochemical investigations.

2.5. Biochemical examination

Collected blood samples were incubated for 30 min at 37 °C, centrifuged at 1500 rpm for 15 min and serum was used for estimating ALT (Biodiagnostic, Egypt, Cat. No. 264001), AST (Cat. No. 261004), and ALP (Cat.No. 241002). Some liver specimens were washed with chilled (0.15 M KCl + 10 mM Tris HCl, pH 7.4) solution, homogenized in ice-cold Tris-KCl buffer, and centrifuged for 10 min at 4 °C. The obtained supernatant was used for the estimation of MDA (Biodiagnostic, Egypt, Cat. No., MD 25 29), NO (Cat. No. NO 25 33), GSH (Cat. No. GR 25 11), and SOD (Cat. No. SD 25 21).

2.6. Histopathological examination and fibrosis scoring

Liver samples were preserved in 10% neutral buffered formalin. After proper fixation, tissue specimens were trimmed, washed in running tap water, dehydrated in different ascending grades of ethyl alcohol, cleared in xylene, embedded in paraffin, sectioned at 5 µm thickness, and stained with hematoxylin and eosin (H&E) (Bancroft et al., 1996) and Masson's trichrome (Ramos-Vara et al., 2008).

Grading and staging of liver fibrosis

The degree of liver fibrosis was scored in five sections in each group according to a scoring system described by Almpanis et al. (2016). Stages of liver fibrosis were graded into five points scale: F0 = no fibrosis; F1 = portal fibrosis without septa; F2 = portal fibrosis with a few septa; F3 = numerous septa without cirrhosis; F4 = cirrhosis.

2.7 Immunohistochemistry

Immunohistochemical examination was performed using a streptavidinbiotin peroxidase method and primary antibodies for alpha smooth muscle actin (α -SMA) (Abcam, 1:1,000; cat. no. ab5694), according to Ramos-Vara et al., (2008).

2.8 Statistical analysis

Statistical analysis of the results was expressed as the mean \pm standard error. A significant difference was used at the $P < 0.05$ probability level. One-way analysis of variance and the least significant difference test were carried out using the Statistical Package for Social science Software (Version 18, SPSS Inc., USA).

3. Results

3.1. Biochemical results

TAA treatment significantly increased ($P < 0.05$) ALT, AST, and ALP levels compared to the control group (Table 1). Meanwhile, the addition of moringa or rosemary extract with thioacetamide ameliorated the TAA effect, with significant decreases in the concentrations of ALT, AST, and ALP compared to the TAA-treated group. Moreover, the ameliorative effect of rosemary extract was higher than moringa (Table1).

TAA-treated rats that were sacrificed after 6, 12, and 18 weeks showed a significant increase in MDA, NO, and a significant decrease in GSH and SOD concentrations comparing to control rats (Table2). Meanwhile, moringa or rosemary co-treated with thioacetamide showed a significant decrease in MDA, NO, and a significant increase in GSH and SOD levels compared to the TAA-treated group. However, the ameliorative effect of moringa or rosemary extract did not restore these levels to normal concentrations (Table2).

3.2. Histopathological results

After 6 weeks

The liver of the control group showed a normal histological appearance; no excessive collagen was observed in portal areas (Fig. 1A). The liver of the TAA-treated group revealed excessive amounts of fibrous tissue proliferation in portal areas, with occasional portal-to-portal bridging, containing proliferated bile ducts and stellate cells (Fig. 1B). The liver of TAA-moringa treated rats showed moderate fibrous connective tissue proliferation in portal areas with occasional bridging from the portal area to adjacent portal areas (Fig. 1C), while liver of TAA-rosemary treated rats revealed expansion of portal areas by mild to moderate amount of fibrous tissue proliferation contains a small number of lymphocytes and macrophages-laden gold-brown pigment (Fig. 1D).

After 12 weeks

The liver of the control group showed normal histological hepatic cords, no excessive collagen in portal areas or in between hepatocytes (Fig. 2A). Meanwhile, livers of the TAA-treated group revealed disruption of the hepatic parenchyma with marked portal fibrosis, that well demonstrated by Masson's trichrome stain, with portal-to-portal bridging and formation of pseudo lobules (Fig. 2B). The liver of TAA-moringa-treated rats revealed marked multifocal portal fibrosis with fine to moderate strands of fibrous connective tissue proliferation extended in between the hepatic lobules (Fig. 2C). Additionally, the liver of TAA-rosemary treated rats revealed maintained architecture

with fine strands of fibrous connective tissue proliferation in portal areas, extending in between the hepatic cords (Fig. 2D).

After 18 weeks

The liver of the control group showed a normal histological appearance; no excessive collagen was observed in portal areas or in between hepatocytes with Masson's trichrome stain (Fig. 3A). The livers of the TAA-treated group revealed disruption of the hepatic parenchyma with marked portal fibrosis, portal-to-portal bridging tracts of fibrous connective tissue, contained proliferated bile ducts, and stellate cells. Diffusely, the normal hepatic architecture was replaced by numerous, regenerative nodules, separated, and surrounded by fibrous connective tissue proliferation indicating the characteristic picture of cirrhosis (Fig. 3B). Moreover, the architecture of the liver parenchyma in TAA-moringa treated rats was moderately disturbed with portal fibrosis and portal-portal bridging tracts resulting in more diffuse parenchymal fibrosis

(Fig. 3C). While livers of TAA-rosemary treated rats revealed diffuse normal hepatic architecture with occasional mild to moderate portal fibrosis extending in between the hepatic cords and well demonstrated with Masson's trichrome stain (Fig. 3D).

Fibrosis scoring

Histopathological grading and staging of liver fibrosis allowed the detection of several differences between control and treated groups. The fibrosis production score as illustrated in Table (3) was significantly increased ($P < 0.05$) in TAA- treated group compared with control and other treated groups, especially at 12 and 18 weeks of treatment. The moringa and rosemary-TAA treated groups significantly decreased fibrosis score in comparison with TAA treated group at 12 and 18 weeks of treatment. In addition, the ameliorative effect of rosemary extract in reducing fibrosis at the end stage of the experiment is better than that of moringa extract.

Table 1. liver function parameters

Group	ALT(U/L)			AST(U/L)			ALP(U/L)		
	6 w	12 w	18 w	6 w	12 w	18 w	6 w	12 w	18 w
Control	27.80±2.87 ^c	33.80±2.48 ^c	29.80±2.48 ^c	34.20±1.83 ^c	32.00±1.70 ^c	37.00±1.70 ^c	78.60±1.44 ^d	71.40±1.29 ^d	76.40±1.29 ^d
TAA	221.20±15.30 ^a	292.60±14.07 ^a	296.60±14.07 ^a	270.00±14.88 ^a	324.00±17.87 ^a	349.00±17.87 ^a	277.40±7.45 ^a	302.60±8.18 ^a	336.20±8.97 ^a
Moringa	102.20±2.92 ^b	87.20±2.92 ^b	79.20±2.92 ^b	85.00±2.51 ^b	76.40±2.29 ^b	71.40±2.29 ^b	256.60±6.96 ^b	228.20±6.09 ^b	195.60±5.30 ^b
Rosemary	80.40±3.50 ^b	52.20±2.78 ^c	48.20±2.78 ^c	71.80±2.82 ^b	64.60±2.48 ^b	59.60±2.48 ^{bc}	231.20±6.09 ^c	198.20±5.28 ^c	169.60±4.48 ^c

Data are presented as mean ± standard error (SE). Mean values with different superscript letters in the same column are significantly different at ($P < 0.05$).

Table 2. Liver oxidative stress and antioxidant markers

Group	MDA (nmol/g)			NO (µmol/g)			GSH (mg/g)			SOD (U/g)		
	6 w	12 w	18 w	6 w	12 w	18 w	6 w	12 w	18 w	6 w	12 w	18 w
G1	43.80±2.24 ^c	40.80±2.24 ^c	45.80±2.24 ^c	38.60±1.50 ^d	34.80±1.39 ^d	37.00±1.30 ^d	2.86±0.14 ^a	3.09±0.15 ^a	2.89±0.14 ^a	42.60±1.29 ^a	39.76±1.20 ^a	43.38±1.31 ^a
G2	225.00±18.29 ^a	255.00±18.29 ^a	305.00±18.29 ^a	89.20±2.56 ^a	96.80±2.76 ^a	103.00±3.03 ^a	0.89±0.04 ^c	0.82±0.04 ^c	0.74±0.03 ^c	23.40±1.86 ^b	19.50±1.55 ^c	16.72±1.33 ^c
G3	196.20±10.71 ^{ab}	176.20±10.71 ^b	140.20±10.71 ^b	71.80±3.41 ^b	64.60±3.04 ^b	60.20±2.94 ^b	1.43±0.07 ^b	1.61±0.08 ^b	1.75±0.08 ^b	26.00±2.07 ^b	28.60±2.27 ^b	31.78±2.53 ^b
G4	186.20±10.71 ^b	161.20±10.71 ^b	126.20±10.71 ^b	62.00±1.41 ^c	55.80±1.24 ^c	52.00±1.14 ^c	1.61±0.08 ^b	1.81±0.09 ^b	1.97±0.09 ^b	28.08±2.23 ^b	31.59±2.51 ^b	36.45±2.76 ^b

Data are presented as mean ± SE. Mean values with different superscript letters in the same column are significantly different at ($P < 0.05$).

Table 3. Liver fibrosis scoring

Group	Duration of experiment		
	6 Weeks	12 Weeks	18 Weeks
Control	0.1 ^a ±0.1	0.1 ^a ±0.1	0.1 ^a ±0.1
TAA	1.00 ^b ±0.1	3.1 ^c ±0.1	3.6 ^d ±0.1
Moringa + TAA	1.03 ^b ±0.1	1.3 ^b ±0.1	1.8 ^c ±0.1
Rosemary + TAA	0.7 ^b ±0.1	0.9 ^b ±0.1	0.7 ^b ±0.1

Data are presented as mean ± SE. Mean values with different superscript letters in the same column are significantly different at ($P < 0.05$).

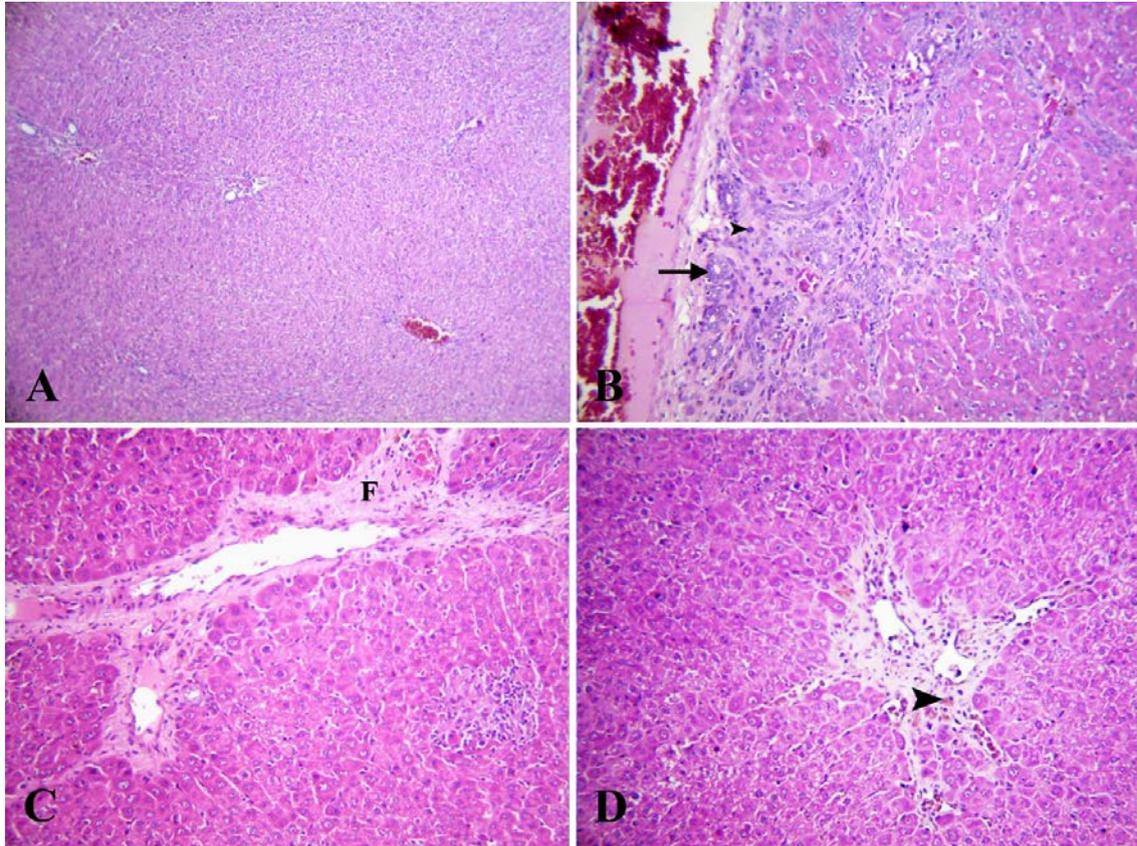


Fig. 1. Liver sections stained with H&E after 6 w. (A) Control group (X 100). (B) TAA-treated group, excessive fibrous tissue proliferation in portal areas, with portal bridging contain proliferated bile ducts (arrow) and stellate cells (arrowhead) (X 200). (C) TAA-moringa-treated group, moderate fibrosis (F) in the portal area with bridging to the adjacent portal area (X 200). (D) TAA-rosemary-treated rats, mild fibrous tissue proliferation in the portal area contain a small number of lymphocytes and macrophages-laden brown pigment (arrowhead) (X 200).

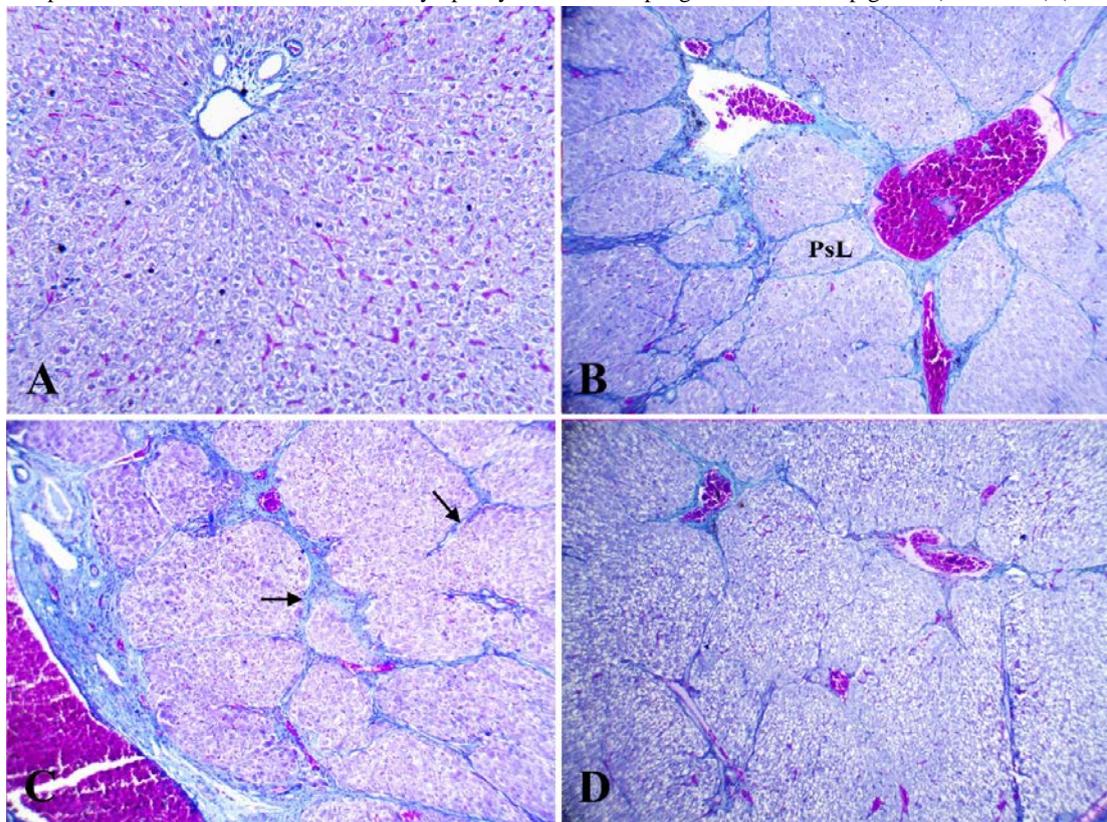


Fig. 2. Liver sections stained with Masson's trichrome staining after 12 w (x 100). A) Control group. (B) TAA-treated group, marked portal fibrosis, portal-to-portal bridging and formation of pseudo lobules (PsL). (C) TAA-moringa-treated group, marked portal fibrosis with fine to moderate strands of fibrous connective tissue (arrow) extended in between the hepatic lobules. (D) TAA-rosemary-treated rats, maintained hepatic architecture with fine strands of fibrous connective tissue in portal areas and extending in between the hepatic cords.

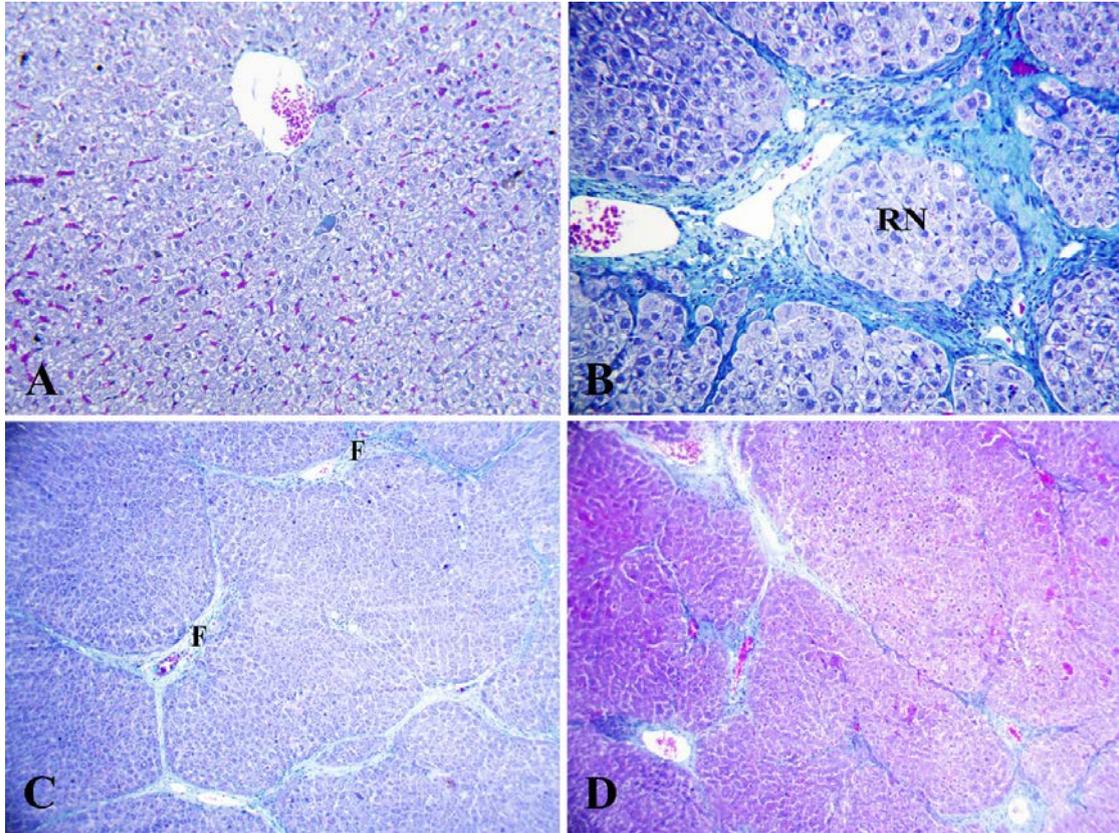


Fig. 3. Liver sections stained with Masson's trichrome staining (x 100) after 18 w. A) Control group. (B) TAA-treated group, cirrhosis characterized by replacement of normal hepatic architecture by numerous, regenerative nodules (RN), separated and surrounded by fibrous connective tissue proliferation (x 200). (C) TAA-moring-treated group, the architecture of the liver parenchyma was moderately disturbed with portal fibrosis and portal-portal bridging tracts resulting in more parenchymal fibrosis (F) (D) TAA-rosemary-treated rats, maintained hepatic architecture with mild to moderate portal fibrosis extending in between the hepatic cords.

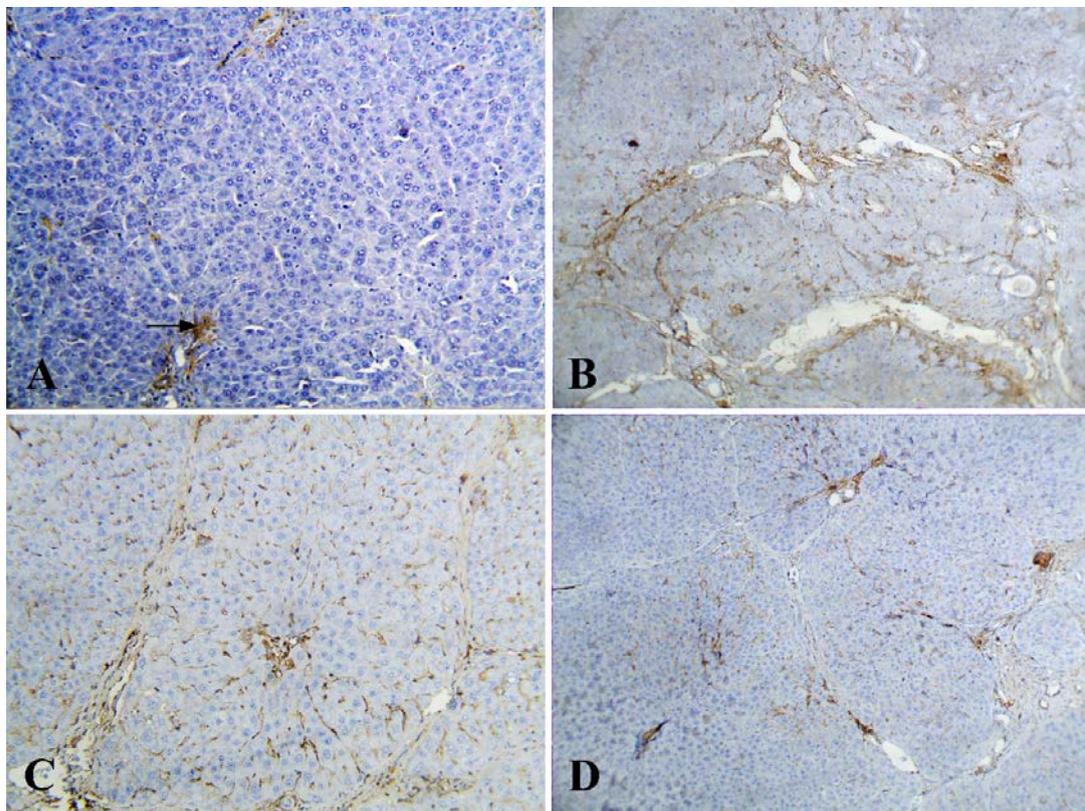


Fig.4. Immunohistochemical staining of smooth muscle actin (α -SMA) in liver sections after 18 w (100x). A) Control group, mild expression in portal areas (arrow). (B) TAA-treated group, marked increases in the expression levels of α -SMA. (C) TAA-moringa-treated group, moderate expression of α -SMA. (D) TAA-rosemary-treated group shows the lowest expression for α -SMA.

4. Discussion

Chronic liver diseases can lead to hepatic fibrosis (Lai and Afdhal, 2019). However, despite extensive research, liver fibrosis, is still a health problem that requires new therapeutic strategies. Nutraceuticals have been extensively studied in recent years to find safe therapeutics of natural origin instead of relying only on pharmaceuticals (Daliu *et al.*, 2018). Our results revealed that TAA-treated rats showed significant increase in liver enzymes (ALT, AST and ALP), oxidative stress marker (MDA, NO) with significant decrease in GSH, SOD compared to the control group. These results agree with Abdelaal *et al.*, (2019). Thioacetamide induces hepatotoxicity through binding of its metabolite TAA-S-dioxide to hepatocyte and causes damage of cellular membrane and leakage of cellular enzymes into the plasma (Hajovsky *et al.*, 2012). The administration of moringa or rosemary extracts significantly ameliorated TAA-induced functional and biochemical changes, due to high contents of flavonoids, glucosinolates, and phenolic acids in moringa leaves extract (Brunelli *et al.*, 2010), and high antioxidant activity of rosemary leaf ethanolic extract which include carnosol, carnosic acid ursolic acid, rosmarinic acid, essential oil components and caffeic acid (Akela *et al.*, 2018). Similar results were obtained (Hamdy *et al.*, 2019; Moustafa *et al.*, 2015; Said *et al.*, 2019).

Microscopically TAA-treated rats showed excessive amounts of fibrous tissue proliferation that giving the characteristic picture of cirrhosis after 18 weeks which was confirmed by marked expression of α -SMA. The pathogenesis of liver fibrosis is complex and is characterized by enhanced ECM production and altered deposition of ECM proteins (Li *et al.*, 2019). Hepatic ECM formation depending on activation of hepatic stellate cells (HSCs) that play an important role in the process of liver fibrosis (Tao *et al.*, 2015; Kisseleva *et al.*, 2011), The α -SMA is a widely accepted marker of activated HSC (Jin *et al.* 2017).

Meanwhile, liver of TAA-moringa group showed improvement in histopathological findings compared to the TAA group. These results agreed with Mousa *et al.*, (2019). Also, α -SMA showed less expression than the TAA group. The protective action of moringa ethanolic extract attributed to high phenolic compound that known as primary antioxidant which have role for inactivation of lipid free radicals and prevention decomposition of hydroperoxide into free radicals. Additionally, the combination of whole moringa crude extract has more effectiveness than single product duo to synergistic mechanism and increase their protective effect to improve hepatic damage induced by free radicals (Vergara-Jimenez *et al.*, 2017).

Rosemary ethanolic extract induced great improvement in liver fibrosis with mild to moderate fibrous tissue proliferation restricted mainly in portal areas. Diffuse normal hepatic architecture was maintained after 18 weeks of protection. Also, protein expression levels of α -SMA were decreased than TAA-moringa treated group. Our results agreed with Tousson *et al.*, (2019). The protective effect of rosemary extract against TAA hepatotoxicity attributed to its richness in carnosic acid and rosmarinic acids which have anti-inflammatory, anti-necrotizing and anti-fibrotic properties (Bahri *et al.*, 2017).

Conclusion

Both moringa and rosemary ethanolic leaf extracts have protective action against fibrosis induced by TAA and rosemary ethanolic leaf extract showed greatest protection.

Conflict of interest

The authors declare that they have no conflict of interest.

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