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Research Article

Therapeutic Effect of *Dunaliella salina* Microalgae on Thioacetamide- (TAA-) Induced Hepatic Liver Fibrosis in Rats: Role of TGF-β and MMP9

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Liver fibrosis represents a serious global health-care problem and is the outcome of many chronic liver diseases, cirrhosis, hepatitis, and toxin accumulation. The present study aimed to evaluate the antifibrotic curative effect of *Dunaliella salina* (*D. salina*) on thioacetamide- (TAA-) induced liver fibrosis in rats. Liver fibrosis was induced by TAA (200mg/kg; i.p.) twice per week for 6 weeks. *D. salina* was given orally (100 and 200 mg/kg) and silymarin was given orally (100 mg/kg) daily, for 4 weeks after TAA. Serum transaminase activities, liver inflammatory cytokines, fibrotic biomarkers, and liver histopathology were assessed. TAA significantly ($p < 0.05$) elevated serum activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and serum levels of total bilirubin, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and transforming growth factor-beta (TGF-β) with a reduction in albumin. In addition, TAA increased hepatic contents of collagen I, a-smooth muscle actin (α-SMA), and tissue inhibitors of metalloproteinase (TIMP-1), reduced matrix metalloproteinase 9 (MMP9), and finally produced marked degeneration and fibrosis of hepatocytes. Treatment with *D. salina* or silymarin improved the histological feature of hepatocytes and ameliorated the deleterious effects of TAA in a dose-dependent manner. Based on these results, it could be concluded that the use of *D. salina* could be assigned for liver fibrosis treatment via its anti-inflammatory and antifibrotic properties.

1. Introduction

Liver fibrosis is a health problem that leads to morbidity and mortality and is usually insidious [1]. Liver fibrosis results from chronic damage to the liver in conjunction with accumulation of extracellular matrix (ECM) proteins, which is a characteristic of most types of chronic liver diseases. The accumulation of ECM proteins distorts the hepatic architecture by forming a fibrous scar, and the subsequent development of nodules of regenerating hepatocytes defines cirrhosis as provoking hepatic failure or hepatocellular carcinoma [2]. Liver fibrosis is thought to be a process of hepatic parenchyma and collagen collapse, and it may happen as a result of some etiological factors such as alcohol consumption, obesity, metabolic disorders, cholestasis, steatosis, viral infection, and toxin accumulation [3].

ECM controls cellular functions and its dysregulation linked with liver disease development [4]. Matrix metalloproteinases (MMPs), endopeptidases, destroy ECM components and degrade proteins [5]. They also have an important role on non-ECM substrates, like cytokines, regulating immunity and inflammation [6]. However,

MMPs proteolytic activities are suppressed by tissue inhibitors of metalloproteinases (TIMPs) in tissues [7].

Single administration of thioacetamide (TAA), a thiosulfur compound, induced acute hepatic toxicity while hepatic cirrhosis or liver tumors developed by chronic exposure to it [8]. Lacking of safe drugs for the treatment of liver diseases is of considerable interest in finding natural products used in medicinal materials or foods as reported by the World Health Organization [9]. Medicinal plant extracts had gained increasing interest as nondrug remedy for many diseases. This may be due to their active constituents that modulate oxidative stress and inflammatory and cell damage [10]. As fibrogenesis increases, the key challenge will be translating into the development of antifibrotic drugs for patients with chronic liver disease [11].

In recent years, natural carotenoids have health benefits that attract attention, and these benefits were gained from carotenes and xanthophylls that have the ability to scavenge oxygen radicals [12]. Microalgae are the richest sources of natural carotenoids, especially strains of Chlorophyta such as *Dunaliella salina (D. salina)* which is a biflagellate unicellular green alga [13–15]. The algal cells produced three valuable products: β-carotene, glycerol, and proteins. β-carotene, an antioxidant, is used as a provitamin A food supplement and coloring agent and also used in cosmetics and a health product [16].

The aim of this study was to investigate the therapeutic effect of *D. salina* microalgae on TAA-induced hepatic liver fibrosis in rats. In addition, the role of TGF-β and MMP9 was also investigated in this study.

2. Materials and Methods

2.1. Chemicals. Thioacetamide (TAA) was purchased from Sigma-Aldrich, USA. Silymarin was obtained from MEPACO, Egypt; all other chemicals, used throughout the experiment, were of the highest analytical grade available.

2.2. Animals. Adult male albino Wistar rats weighing 150–200 gm were obtained from the animal house at the National Research Centre (Giza, Egypt) and fed a standard laboratory diet and tap water ad libitum. Experimental animals were housed in an air-conditioned room at 22–25°C with a 12 h light/dark cycle. All animals received human care, and the study protocols were carried out according to the ethical guidelines for care and use of experimental animals approved by the Ethical Committee of the National Research Centre.

2.3. Cultivation of D. salina in the Vertical Photobioreactor. Algal species *D. salina* isolated from the Nile River water and salt pond in Al-Fayoum are grown by using BG11 media for algal isolation and purification (Stanier et al., 1971). After growing *D. salina* for 10 days under lab conditions, they were then transferred to a vertical photobioreactor with a capacity of 4000 L. Reservoir (1000 L) tank associated pipe work proprietary in line pigging systems and removed all biofilms. In addition, a 10 L basket centrifuge for harvesting was connected to the system. The Alga Connect Data Acquisition System used for online measurements.

Tap water was used for the cultivation of algae in the PBR. Water was sterilized using hypochlorite after which sodium thiosulpate was added. Chlorine test was performed to insure no residual chlorine was present. Nutrient solution of BG 11 was used for growing *D. salina*. One millilitre of micronutrient solution was added to the culture medium. To insure the purity of the culture, samples were taken regularly and examined microscopically. The culture was left to grow until the biomass reached the maximum (2–2.5 gm/L). Algal biomass was harvested using a basket centrifuge at 2000 rpm, sun-dried where the temperature reached approximately 45°C, and then ground into homogeneous fine powder.

2.4. Experimental Design. After an acclimatization period of one week, male albino Wistar rats were randomly assigned to 5 groups of 8 rats per group as follows. Group 1: received water containing 0.1% Tween 80 orally for 6 weeks and served as normal control. Group 2: received TAA (200 mg/kg, i.p.) twice per week for 6 weeks and served as TAA control according to Bai et al. [17] and Afifi et al. [18]. Group 3: received silymarin (100 mg/kg, orally) [19] daily for 4 weeks after TAA. Groups 4 and 5: received *D. salina* powder (100 and 200 mg/kg, orally) [20, 21] daily for 4 weeks after TAA.

2.5. Collection of Blood Samples. At the end of the experimental period, blood samples were withdrawn from the retroorbital vein of each animal, under mild anesthesia with diethyl ether. Blood was allowed to coagulate and then centrifuged at 3000 rpm for 15 min. The obtained serum was used to estimate the activities of aspartate transaminase (AST) and alanine transaminase (ALT) and levels of total bilirubin and albumin using the Biodiagnostic kit, Egypt. Tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), transforming growth factor (TGF-β), collagen I, alpha-smooth muscle actin (α-SMA), metalloproteinase-9 (MMP9), and tissue inhibitors of metalloproteinase-1 (TIMP-1) were determined using ELIZA kits procured from SinoGeneClon Biotech Co., Ltd.

2.6. Histological Examination. At the end of the experiment, part of the liver was removed carefully and fixed in 10% formalin for 24 hours. The samples were washed under tap water, dehydrated in ascending grades of ethanol (50, 70, 80, 90, and 100%), cleared in xylene, and embedded in paraffin wax (melting point 55–60°C). Liver sections of 4 μm thickness were prepared and stained with haematoxylin and eosin. The paraffin sections were stained in Harris's haematoxylin for 5 minutes. Sections were washed in running water for blueing and then stained in 1% watery eosin for 2 minutes, washed in water, dehydrated, cleared, and mounted using Canada balsam. Other parts of the liver were stained with Masson's trichrome.

Eight microscopic fields per section were examined using a light microscope (Olympus BX50, Japan) under

high-power magnification (×400). Fibrosis staging was defined as 0, no fibrosis; 1, perisinusoidal and/or pericentral; 2, incomplete central/central bridging fibrosis; 3, portal-portal or portal-central fibrosis; and 4, cirrhosis [22].

2.7. Statistical Analysis. All the values are presented as means ± standard error of the means (SE). Data of this study were evaluated by one-way analysis of variance followed by Tukey's multiple comparisons test. Graphpad Prism software, version 5 (Inc., San Diego, USA) was used to carry out these statistical tests. The difference was considered significant when $p < 0.05$.

3. Results

3.1. D. salina Ameliorates Liver Function Changes of TAA-Induced Fibrosis. Injection of TAA, for 6 weeks, produced hepatic fibrosis evidenced by significant increases in serum AST, ALT activities, and bilirubin level and decreased albumin level as compared to normal control values. Treatment with a low dose of *D. salina* powder, for 4 weeks, reduced serum activities of ALT and AST only, while treatment with a high dose of *D. salina* powder decreased serum activity of AST and normalized serum ALT activity and bilirubin and albumin levels, as compared to the TAA group. Treatment with silymarin, for 4 weeks, decreased serum activity of AST and returned serum ALT activity and bilirubin and albumin levels to normal values, as compared to the TAA group (Table 1).

3.2. D. salina Inhibits TAA-Induced Inflammatory Biomarkers of Fibrosis. Hepatic inflammation induced, after 6 weeks, by injection of TAA was evidenced by significant increases in serum levels of TNF-α, IL-6, and TGF-β as compared to normal control values. Treatment with both doses of *D. salina* powder, for 4 weeks, reduced serum levels of TGF-β and returned TNF-α and IL-6 to their normal value, as compared to the TAA group. Treatment with silymarin, for 4 weeks, normalized IL-6 serum levels and decreased serum levels of TNF-α and TGF-β, as compared to the TAA group (Table 2).

3.3. D. salina Inhibits TAA-Induced Fibrotic Biomarkers of Fibrosis. TAA injection provoked hepatic fibrosis, after 6 weeks, and significantly elevated hepatic contents of collagen I and *a*-SMA and reduced MMP9 as compared to normal control values. In addition, treatment with both doses of *D. salina* powder, for 4 weeks, returned hepatic contents of collagen I and *a*-SMA to their normal values, while treatment with a high dose of *D. salina* powder elevated the MMP-9 hepatic content and normalised TIMP hepatic content, as compared to the TAA group. Treatment with silymarin, for 4 weeks, reduced the hepatic contents of collagen I and *a*-SMA, elevated those of MMP-9 and normalised TIMP hepatic content, as compared to the TAA group (Figure 1).

3.4. D. salina Improves Liver Tissue against TAA-Induced Fibrosis. The scoring of liver fibrosis in the TAA group, after 6 weeks, revealed that the fibrosis model was successfully established as compared to the healthy control group. However, the scoring of liver fibrosis in *D. salina* powder groups after 4 weeks treatment indicated that *D. salina* intervention was effective to treat fibrosis as compared to the TAA group (Figure 2).

The liver of normal control rat showed the normal histological structure of hepatic lobule (stage F0) (Figure 3(a)). The liver of rat from the TAA group showed ballooning degeneration of hepatocytes (small arrow), fibrosis of hepatocytes (large arrow), and mononuclear cells infiltration (arrow head) and E ×400 (stage F3) (Figure 3(b)). The liver of rat from the TAA group showed portal fibrosis (small arrow) with oval cells proliferation (large arrow) (stage F3) (Figure 3(c)). The liver of rat from the silymarin-treated group showed fine strands of fibroblasts in the portal tract (arrow) (stage F1) (Figure 3(d)). The liver of rat treated with *D. salina* (100 mg/kg) showed fatty change of hepatocytes (small arrow) and portal infiltration with inflammatory cells (large arrow) (stage F1) (Figure 3(e)). The liver of rat treated *D. salina* (200 mg/kg) showed slight hydropic degeneration of hepatocytes (small arrow) and portal infiltration with inflammatory cells (large arrow) (stage F1) (Figure 3(f)) (H&E ×400).

The liver of control rat showed no histochemical reaction for collagen fibers deposition in the portal triad (Masson's trichrome stain ×400) (F0) (Figure 4(a)). The liver of rat from the TAA group showed strong positive histochemical reaction for collagen fibers deposition in the portal triad (F3) (Figure 4(b)). The liver of rat from the TAA group showed strong positive histochemical reaction for collagen fibers deposition in the portal triad (F3) (Figure 4(c)). The liver of rat treated with silymarin showed weak positive histochemical reaction for collagen fibers deposition (F1) (Figure 4(d)). The liver of rat treated with *D. salina* (100 mg/kg) showed weak positive histochemical reaction for collagen fibers deposition (F1) (Figure 4(e)). The liver of rat treated with *D. salina* (200 mg/kg) showed no histochemical reaction for collagen fibers deposition (F0) (Figure 4(f)) (Masson's trichrome stain ×400).

4. Discussion

TAA is a hepatotoxin and used to evaluate liver fibrogenesis mechanisms [23]. One of the mechanisms underlying fibrogenesis is the liberation of reactive oxygen species (ROS), provoking oxidative stress [24]. Here, in this study, TAA produced a liver damage experimental model to mimic pathophysiological symptoms of human liver diseases via the injury of liver cell membrane, elevating activities of ALT, AST, and bilirubin level associated with a reduction in albumin compared to the control. On the contrary, treatment with *D. salina* or silymarin reduced serum activities of ALT and AST preserving hepatocyte integrity. It is worthy to note that *D. salina* also decreased the total bilirubin serum level with an increase in albumin compared to the TAA control group. Moreover, the decrease in the serum level of bilirubin after treatment with *D. salina* indicates the effectiveness in normalizing the functional state of the liver. These results come in line with those of Kumar et al. who showed that

Table 1: Effects of *D. salina* powder on serum hepatic function biomarkers.

	Normal control	TAA	Silymarin (100 mg/kg)	*D. salina* powder (100 mg/kg)	*D. salina* powder (200 mg/kg)
ALT (U/L)	43.14 ± 0.18	68.52 ± 0.62^{a}	47.95 ± 0.05^{b}	53.70 ± 0.04^{ab}	48.64 ± 0.12^{b}
% of TAA control			70	78	71
AST (U/L)	56.48 ± 0.04	89.01 ± 0.26^{a}	68.56 ± 0.37^{ab}	72.78 ± 0.15^{ab}	69.28 ± 0.03^{ab}
% of TAA control			77	82	78
Bilirubin (mg/dl)	1.44 ± 0.11	1.89 ± 0.01^{a}	1.56 ± 0.06^{b}	1.62 ± 0.06	1.55 ± 0.07^{b}
% of TAA control			83	86	82
Albumin (g/dl)	4.13 ± 0.03	3.56 ± 0.14^{a}	3.90 ± 0.05^{b}	3.59 ± 0.00^{a}	3.91 ± 0.01^{b}
% of TAA control			110	101	110

Data are presented as mean ± SE for each group ($n = 8$) and % of the TAA group. Statistical analysis was carried out by one-way analysis of variance followed by Tukey's multiple comparisons test. [a]Statistically significant from the control group. [b]Statistically significant from the TAA group at $p < 0.05$.

Table 2: Effects of *D. salina* powder on serum levels of inflammatory biomarkers.

	Normal control	TAA	Silymarin (100 mg/kg)	*D. salina* powder (100 mg/kg)	*D. salina* powder (200 mg/kg)
TNF-α (Pg/ml)	600.00 ± 8.94	808.00 ± 17.15^{a}	690.00 ± 26.83^{ab}	655.00 ± 11.18^{b}	610.00 ± 4.47^{b}
TNF-α (% of TAA control)			85	81	75
IL-6 (Pg/ml)	69.40 ± 4.73	313.80 ± 28.34^{a}	101.80 ± 0.80^{b}	71.20 ± 6.02^{b}	69.62 ± 4.74^{b}
IL-6 (% of TAA control)			32	23	22
TGF-β (ng/L)	34.33 ± 0.70	65.07 ± 0.80^{a}	40.30 ± 1.38^{ab}	47.47 ± 1.27^{ab}	39.80 ± 0.20^{ab}
TGF-β (% of TAA control)			62	73	61

Data are presented as mean ± SE for each group ($n = 8$) and % of the TAA group. Statistical analysis was carried out by one-way analysis of variance followed by Tukey's multiple comparisons test. [a]Statistically significant from the control group. [b]Statistically significant from the TAA group at $p < 0.05$.

TAA injection exhibited liver dysfunction signs evidenced by serum elevation of ALT and AST activities [25]. Moreover, Galisteo et al. found that the injection of rats with TAA produces a significant decrease in plasma albumin in relation to the control normal group [26]. Moreover, Hsu et al. revealed that *D. salina* has ameliorative effects on hepatotoxicity induced by carbon tetrachloride (CCl_4), decreasing serum activities of ALT and AST [27].

Liver fibrosis is considered as a wound-healing response and characterized by progressive inflammation, stimulating ECM deposition and formation of tissue scar. In the fibrotic liver, the principal collagen-producing cells include activated hepatic stellate cells (HSCs), portal fibroblasts, and myofibroblasts [28]. Activated myofibroblasts have an important role in forming fibrotic tissue [29]. In addition, Kupffer cells, hepatocytes, sinusoidal endothelial cells, and lymphocytes may contribute to liver fibrosis [30].

One of the first manifestations of liver fibrosis is upregulation of proinflammatory cytokine release such as TNF-α and IL-1β by Kupffer cells and stimulated HSCs. TGF-β has multiple profibrogenic effects differentiating HSCs into myofibroblasts [31]. In the present study, TAA injection, after 6 weeks, induced liver inflammation detected by a significant increase in the cytokine levels of TNF-α, IL-6, and TGF-β in serum compared to the normal control group. On the contrary, treatment with *D. salina* showed a significant decrease in TNF-α, IL-6, and TGF-β serum contents as compared to the TAA control group. In liver injury experimental models, TNF-α and IL-6 are inflammatory mediators which affect organ function homeostasis and produce hepatotoxicity. A pervious study exhibited TAA-induced acute liver injury through farnsoid X receptor AMP-activated protein kinase (FXR/AMPK) signal pathway inducing oxidative stress and inflammation in rats and mice [32]. The injection of rats with TAA produced a significant elevation in the serum level of TNF-α and IL-6 in TAA-injected rats in comparison with those in the normal control group.

The progression of fibrogenesis leads to increased synthesis of collagen [33]. The activation of quiescent HSCs and their differentiation into myofibroblast-like cells controlled by immune cells released inflammatory cytokines and some growth factors secreting ECM contents, mainly, collagen I, III, and IV, and expressing α-SMA. Collagen accumulation is a mark of fibrosis in the liver [34]. Our results exhibited that TAA induced a significant increase in hepatic collagen I and α-SMA, while treatment with *D. salina* resulted in a significant decrease in collagen I and α-SMA. In addition, during fibrogenesis, in the current work, the ECM is reworked resulting in elevations of hepatic contents of collagen I and TIMP1 coupled with a reduction in MMP-9 in the TAA group. These results explain that MMPs degrade collagen and MMPs with MMP inhibitors (TIMPs) play a key role in fibrogenesis and fibrolysis. MMP-9 is the gelatinase secreted by Kupffer cells and is implicated in the pathological process of hepatic injury [35]. Moreover, treatment with *D. salina* produced an increase in the serum concentration level of MMP-9 associated with a decrease in serum TIMP-1 compared to

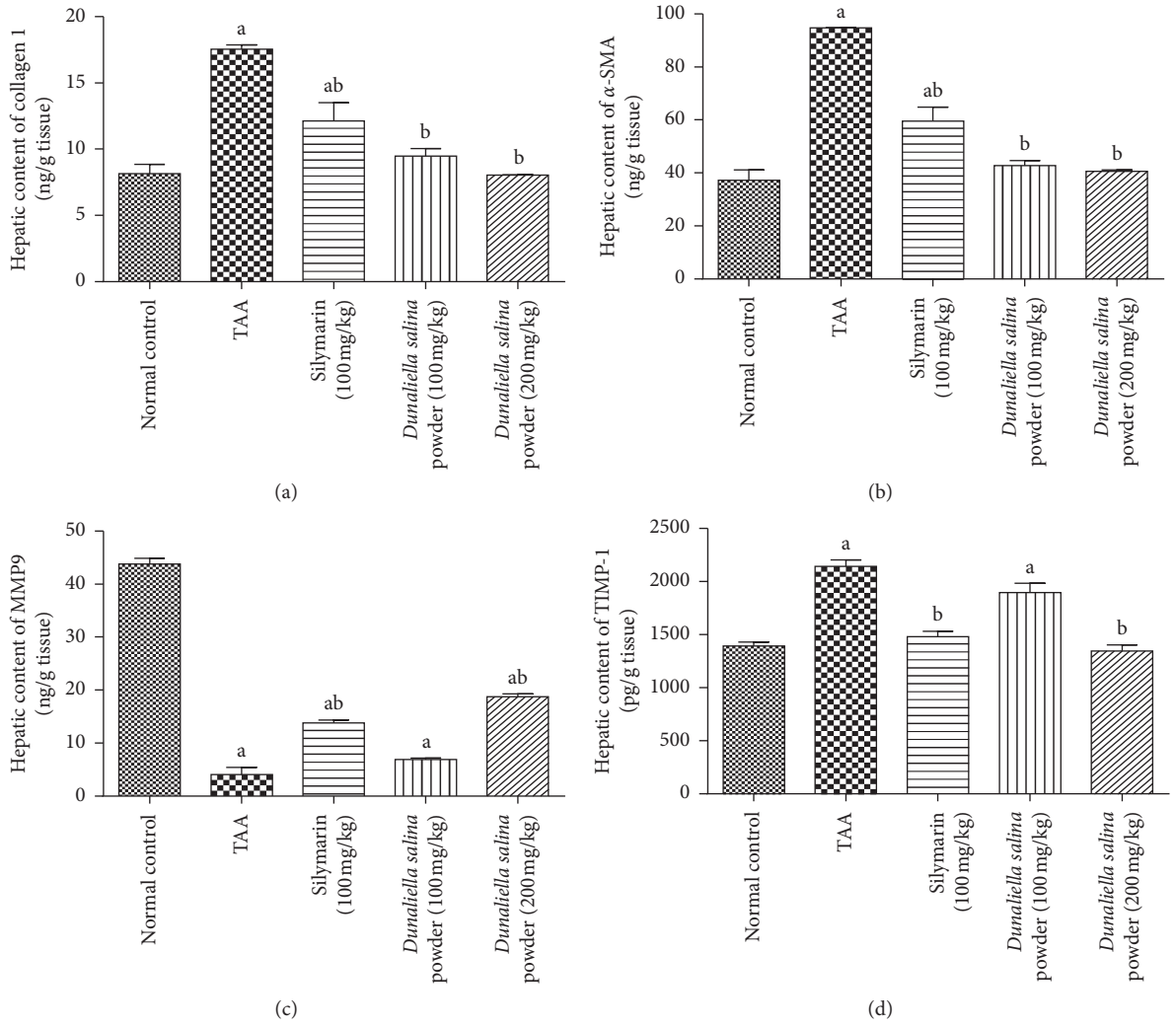


Figure 1: Effects of *D. salina* powder on hepatic contents of (a) collagen I, (b) SMA-α, (c) MMP9, and (d) TIMP-1. Data are presented as mean ± SE for each group ($n = 8$). Statistical analysis was carried out by one-way analysis of variance followed by Tukey's multiple comparisons test. [a]Statistically significant from the control group and [b]statistically significant from the TAA group at $p < 0.05$.

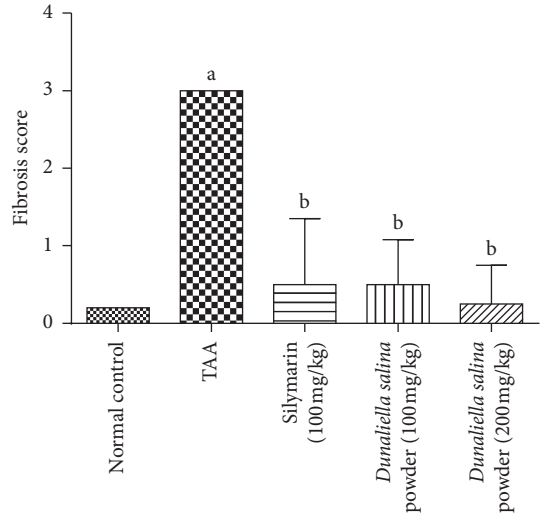


Figure 2: Effects of *D. salina* powder on fibrosis score. Data are presented as mean ± SE ($n = 8$). Statistical analysis was carried out by one-way analysis of variance followed by Tukey's multiple comparisons test. [a]Statistically significant from the control group and [b]statistically significant from the TAA group at $p < 0.05$.

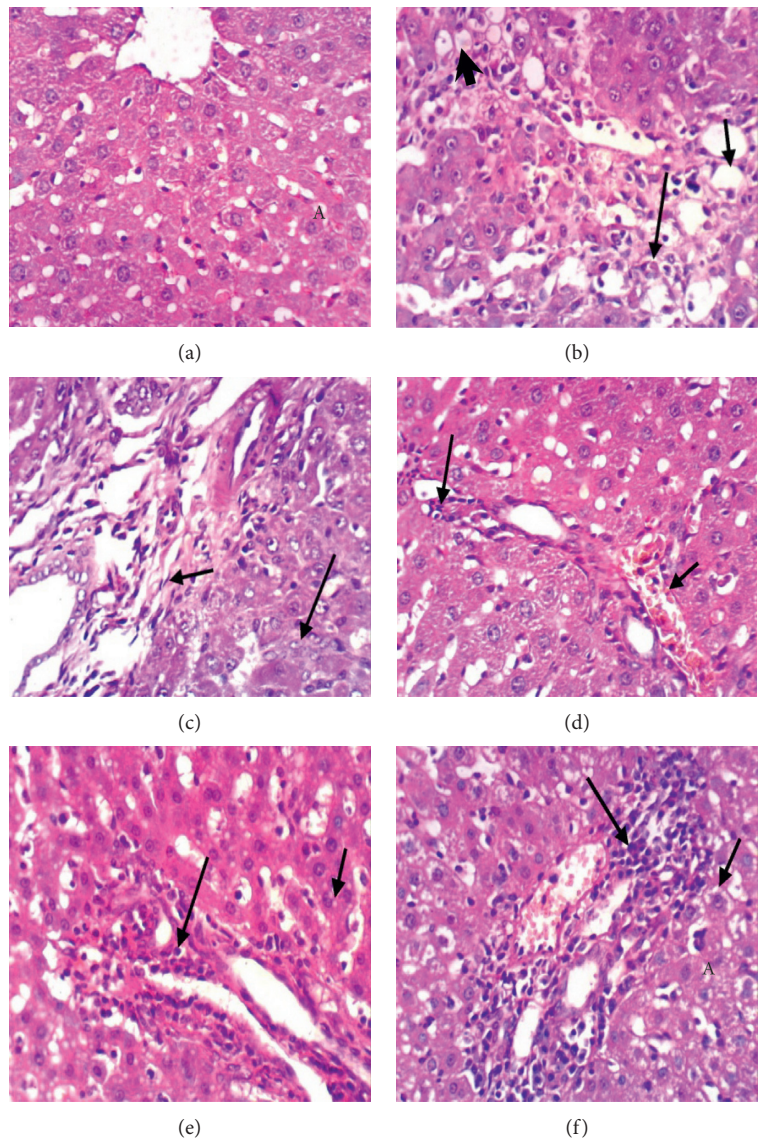


Figure 3: (a) Liver of control normal rat showing the normal histological structure of hepatic lobule (stage F0). (b) Liver of rat from the TAA group showing ballooning degeneration of hepatocytes (small arrow), fibrosis of hepatocytes (large arrow), and mononuclear cells infiltration (arrow head) (stage F3). (c) Liver of rat from TAA group showing portal fibrosis (small arrow) with oval cells proliferation (large arrow) (stage F3). (d) Liver of rat from silymarin-treated group showing fine strands of fibroblasts in portal tract (arrow) (stage F1). (e) Liver of rat from *D. salina* (100 mg/kg) showing fatty change of hepatocytes (small arrow) and portal infiltration with inflammatory cells (large arrow) (stage F1). (f) Liver of rat from *D. salina* (200 mg/kg) showing slight hydropic degeneration of hepatocytes (small arrow) and portal infiltration with inflammatory cells (large arrow) (stage F1) (H&E, ×400).

TAA-induced liver fibrosis. Furthermore, the results of our study showed the decrease in MMP-9 expression was accompanied by increased TGF-β1 expression in the TAA control group, and this result confirmed by previous hypothesis which stated that the downregulation of MMP-9 in the later stages of fibrogenesis appears to be mediated primarily by TGF-β, through a negative feedback mechanism [36] and by phosphoinositide 3-kinase (PI3K)-AKT signaling pathway [37].

Silymarin used to treat chronic liver disease in hepatofibrotic experimental animals and clinical studies [38]. However, silymarin absorption and its first-pass metabolism limited its treatment effect against severe liver damage [39], so alternative therapeutic agents from natural origin are needed as antihepatofibrotic agents. *D. salina*, a natural source of β-carotene, has a reversal role in TAA-induced liver fibrosis in rat. This study also highlights the regulation of two important pathways in fibrosis: inflammatory and fibrotic pathways. HSCs control the transport and storage of vitamin A and are named vitamin A-storing cells [40], and in the absence of these stores, a liver injury will provoke fibrosis and hepatic disease. It seems that reduced levels of β-carotene could stimulate hepatic cells damage [41]. *D. salina* contains

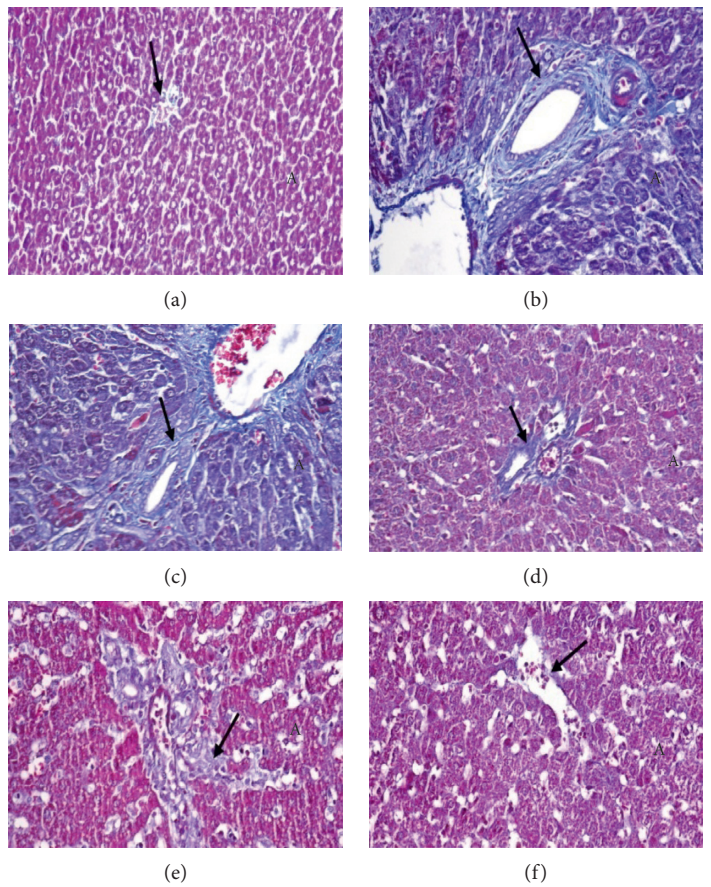


Figure 4: (a) Liver of control rat showing no histochemical reaction for collagen fibers deposition in the portal triad (F0). (b) Liver of rat from the TAA group showing strong positive histochemical reaction for collagen fibers deposition in the portal triad (F3). (c) Liver of rat from the TAA group showing strong positive histochemical reaction for collagen fibers deposition in the portal (F3). (d) Liver of rat treated with silymarin showing weak positive histochemical reaction for collagen fibers deposition (F1). (e) Liver of rat treated with *D. salina* (100 mg/kg) showing weak positive histochemical reaction for collagen fibers deposition (F1). (f) Liver of rat *D. salina* (200 mg/kg) showing no histochemical reaction for collagen fibers deposition (F0) (Masson's trichrome stain, ×400).

β-carotene and may explain its benefits to treat fibrosis in this study.

5. Conclusion

The present study demonstrates that treatment with *D. salina* markedly ameliorates hepatic inflammation and fibrosis in rats. *D. salina* has a curative effective role in maintaining liver enzymes. Antifibrotic activity of *D. salina* is mediated through hepatic activation of MMP-9 with a suppression of TIMP-1 in the liver. Notably, *D. salina* exerts an anti-inflammatory activity by decreasing serum levels of TNF-α, IL-6, and TGF-β.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Farouk El-Baz and Abeer Salama designed the study. Farouk El-Baz provided the materials. Abeer salama and Rania salama performed experimental part and biochemical analysis and drafted the manuscript. Abeer Salama analysed and interpreted the data and wrote the final draft of the article. Farouk El-Baz revised the final draft of the written article.

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