Research Article

Brown Algae as A Golden Mine For Treatment of Liver Fibrosis: A Proposal Based on Experimental Animal Study

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ABSTRACT

Over the years, marine algae species offer a biological diversity in discovery-phase of new strategy for treatment of several diseases. Interest in seaweed has been on the rise, owing to the recognition of important bioactive molecules. The present study was constructed to explore the potential effectiveness of two species of brown marine algae *Cystoseira myrica* and *Padina pavonica* in regression of liver fibrosis in the experimental model. Treatment of liver fibrosis bearing rats with either fresh or dry methanolic extract of *Cystoseira myrica* and *Padina pavonica* revealed significant suppression of liver fibrosis. This finding was evidenced by the decreased urinary lamnine, and 8-Hydroxydeoxyguanosine (8-OHdG) levels, as well as hepatic malondialdehyde (MDA) content, serum hepatocyte growth factor (HGF), nuclear factor kappa-B (NF κB) and interleukin 8 (IL-8) levels, in concomitant with the increased hepatic paraxonase-1(PON-1) activity. Additionally, *Cystoseira myrica* and *Padina pavonica* extracts could downregulate the expression of Bcl2, KI-67 and transforming growth factor- β (TGF-β) in the liver of the treated rats as documented *via* immunohistochemical examination. These encouraging results provide new concepts for the development of natural therapeutic opportunities from brown marine algae in the management of liver fibrosis through their antioxidant, anti-inflammatory and antiproliferative activity.

Key words: liver fibrosis, *Cystoseria myrica*, *Padina pavonica,* oxidative stress, inflammation, proliferation.

INTRODUCTION

Seaweeds are potential renewable resource in the marine environment. They occupy an important place as a source of biomedical compounds such as carotenoids, dietary fibre, polyphenols, enzymes, protein, essential fatty acids, polysaccharides, vitamins and minerals. About 6000 species of seaweeds have been identifiedand and grouped into different classes; brown seaweeds (Phaeophytes), green seaweeds (Chlorophytes) and red seaweeds (Rhodophytes)[1]**.**

In the last decade, a great interest has been developed to isolate novel bioactive compounds from marine resources because of their numerous health beneficial effects. Hence, the preclinical pharmacological research on new marine compounds continued to be extremely active in recent history[2].

Brown seaweeds are considered as the second most abundant group of seaweeds. Most brown seaweeds contain the pigment fucoxanthin, which is responsible for the distinctive greenish-brown colour that gives them their name. Fucoxanthin occurs in great abundance in brown seaweed, while it is absent in higher plants[3]. Fucoxanthin profound physiological effects in the treatment of tumors and other cancer related problems[4]. Brown seaweeds also produce a range of active components including unique secondary metabolites such as phlorotannins which have specific biological activities that give possibilities for their economic utilization[5].

Consumption of brown marine algae is thought to ameliorate some inflammatory disorders, breast cancer and high cholesterol level[6]. The strong association between the increasing of the consumption of brown algae and human diseases prevention has been explained by their content of phytonutrients[7]. These include alkaloids (homoharringtonine), flavonoids (resveratrol, kaempferol and quercetin), coumarins (scopoletin and scopolin), and steroids (beta sitosterol).

Cystoseira is a genus of worldwide distribution with about 80% of the species. Secondary metabolites from Mediterranean species of this genus have been widely studied[8]. Very active compounds such as diterpenes and sterols have been isolated from the brown algae belonging to the genus *Cystoseira.* The isolated diterpenoids from *Cystoseira* include the linear diterpenes or the acyclic and cyclic meroditerpenoids[9,10].

Padina pavonia (L.) Gaill. is widely distributed in unpolluted environments including North Carolina to Florida in the United States, the Gulf of Mexico,

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Through out the Caribbean and tropical Atlantic and the Eastern Atlantic, Mediterranean and Adriatic Seas[11]. It is usually located on rocky substrates, various shell bottoms and coral fragments in shallow waters. Based on the data obtained by Kamenarska *et al*,[12], the active compounds responsible for the biological activity of *P. pavonia.* included twelve sterols, mainly cholesterol and fucosterol. The main fatty acids in the lipids were also identified. By gas chromatography/mass spectrometry (GC/MS) analysis of the volatile and polar fractions of this algae, forty compounds were identified. Free fatty acids, aromatic esters, benzyl alcohol and benzaldehyde were predominated. Low concentrations of terpenoids, phenols and sulfur containing compounds were also identified[12].

Liver plays a vital role in the metabolic elimination of most drugs and other foreign compounds, thus it is considered as an important target for toxicity. Liver fibrosis is the final pathway stage of most chronic liver diseases, and is the main reason for increased mortality in affected patients[13]. Liver fibrosis is triggered by chronic liver injury and develops from a series of events including apoptosis or necrosis, inflammation, tissue remodeling and repair processes. The extent of liver fibrosis displays great individual variation, even after controlling for age, gender and exogenous factors[14].

Toxins and drugs are among the basic etiopathogenetic agents of acute liver failure in Western countries[15]. Chemical toxins; acetaminophen, carbon tetrachloride, galactosamine and thioacetamide are often used as the model substances causing experimental hepatocyte injury in both *invivo* and *invitro* conditions[16,17]. Hepatotoxic agents can react with the basic cellular components and induce most types of liver lesions.

Thioacetamide (TAA) is a thiono-sulfur containing compound used as a fungicide, organic solvent, accelerator in the vulcanization of rubber, and stabilizers of motor oil[18]. A single dose of this toxin in animals can produce centrilobular necrosis with a subsequent regenerative response[19]. Chronic administration of TAA can lead to liver cirrhosis and hepatocellular carcinoma[20]. The advantages of TAA as a model hepatotoxin lies in its high specifity for the liver, regiospecifity for the previous area and a large window of time between its necrogenic effects and liver failure[21,22].

Ameliorating of liver injury induced by chemicals and drugs remains one of the major challenges in clinical aspects. Achieving effective intervention requires knowledge on the mechanisms leading to liver damage which are, unfortunately, limited by the lack of satisfactory experimental models. So, the focus of our interest was to explore the intimate mechanisms involved in liver fibrosis, and its restroation by brown marine algae *Cystoseira myrica* and *Padina pavonica* as new and promising candidtes for retrogradation of liver fibrosis.

MATERIALS AND METHODS
Materials
Collection of algae
Brown algae were collected from two different locations along the Red Sea coast. For *Cystoseira myrica*, nine kilogram was collected from the Napq protected area, South Sinai Governorate, Egypt from the mangroove area at depth about 0.5 m. While, seven kilogram of *Padina pavonica* was collected from Nuweiba coast, eastern part of Sinai Peninsula, Egypt from a coral beach at depth about 1.5 m. *Cystoseira myrica* and *Padina pavonica* were identified by Professor Muhammad Hegazi ; Professor of Marine Science, Department of Marine Science, Faculty of Science ,Suez Canal University, Ismailia, Egypt. All samples were washed thoroughly with seawater followed by tap water immediately after collection and were cleaned from any epiphytes as much as possible by using fine brush. About half of the quantity of *Cystoseira myrica* and *Padina pavonica* were frozen at about -20° C until use and the other half of the quantity of *Cystoseira myrica* and *Padina pavonica* was air-dried at room temperature in a darkened room and milled to fine powder.

Extracts preparation
The frozen quantity of *Cystoseira myrica* and *Padina pavonica* were defrost washed in tap water cut into small pieces then mixed with 80% methanol, homogenized using electrical blender and extracted three times with 80% methanol each time. Meanwhile, the resulting powder from the air-dried algae was directly extracted three times with 80% methanol each time. At each time, the extract was shaken slowly on a reciprocating shaker overnight at dark. Then, the extracts were filtered using Buchner funnel under suction and each filtrate was concentrated using rotary evaporator at 40°C till it became free of methanol.

Experimental animals
Sixty adult male albino rats of Wistar strain (2-3 month-old with body weight between 120-140 g) were obtained from the Animal House Colony of the National Research Centre, Dokki, Giza, Egypt and acclimatized in a specific area where temperature (25±1°C) and humidity (55%). Rats were controlled constantly with 12 hours light/dark cycles at National Research Centre, Animal Facility Breeding Colony. Rats were housed with *ad libitum* access to standard pellet diet and tap water. Animals were cared for according to the guidelines for animal experiments which were approved by the Ethical Committee of Medical Research at National Research Centre, Giza, Egypt.

Experimental Set-Up
After acclimatization period (one week), the animals were randomly assigned into six experimental groups (10 rats/ group). The first group was negative control group received normal saline intraperitoneally (i.p) twice weekly for 8 weeks. The groups from second to sixth were injected intraperitoneally (i.p) with thioacetamide (SIGMA, USA; dissolved in 0.9% normal saline) in a dose of 200 mg/kg b.wt[23], twice weekly for 8 weeks for induction of liver fibrosis. Then, the second group was left untreated for 8 weeks. The third and fourth groups were treated orally with *C. myrica* fresh and dry extracts respectively in a dose of 50 mg/kg b. wt. for 8 weeks. While, the fifth and sixth groups were treated orally with *P. pavonica* fresh and dry extracts respectively in a dose of 50 mg/kg b. wt. for 8 weeks[24].

After animal treatment was over, the animals were fasted overnight and 24 h urine samples were collected in sterile

containers, centrifuged for 20 min at 1800 xg by using cooling centrifugation at 4ºC and the supernatants were stored immediately at -20ºC in clean plastic eppendorf until determination of urinary laminin (LN), 8-hydroxyguanosine (8-OHdG) and creatinine levels. Afterwards, the blood samples were withdrawn from the retro-orbital venous plexus of all animals under diethyl ether anesthesia[25]. The blood samples were left to clot and the sera were separated by using cooling centrifugation at 4ºC at 1800 xg for 10 min and stored immediately at -20ºC in clean plastic eppendorf until estimation of nuclear factor kappa B (NF-κB), interleukin-8 (IL-8) and hepatic growth factor (HGF) levels. then, the whole liver of each animal was rapidly and carefully excised, thoroughly washed with isotonic saline, blotted dry and then divided into two portions. The first portion weighed and homogenized immediately in 50 mM ice cold phosphate buffer (pH 7.4) to give 2% homogenate (w/v) then, the homogenate was centrifuged at 1000 x g for 10 min in cooling centrifuge at 4ºC and the supernatent (2%) was used for the determination of malondialdehyde (MDA) content and paraoxonase1 (PON1) activity[25]. The second portion was fixed in formalin saline (10%) for immunohistochemical examination of B-cell lymphoma 2 (Bcl-2), transforming growth factor-β (TGF-β) and Ki-67 expressions.

Methods

Biochemical analyses

Urinary laminin level was determined by enzyme-linked immunosorbent assay (ELISA) technique using kit purchased from Glory Science Co., USA according to the manufacturer's instructions. While, the protocol for urinary 8-OHdG analysis was done by HPLC technique using a modified method of Kim *et al.*[27]. 8- OHdG was extracted from 1 ml urine sample. The eluent of each sample was dried under ultra- pure N_2 stream and reconstituted in 5 ml deionized water for injection in HPLC. For HPLC condition, the HPLC column was C_{18} (250 x 4.6, particle size 5 μ). The mobile phase consisted of acetonitrite/methanol/phosphate buffer (25/10/965). Phosphate buffer was prepared by dissolving 8.8 g of potassium dihydrogen phosphate in 1000 ml deionized water and pH was adjusted at 3.5. The buffer was filtered 2 times before used at a flow rate of 1 ml/min. The electrochemical detector with cell potential 600 mv was used in this system. Urinary creatinine was determined kinetically using Stanbio Direct Creatinine Liquid Color kit, USA as described by Cook[28].

Hepatic MDA content was quantifies by colorimetric method using kit purchased from Bio-diagnostic Co., Egypt, according to the method described by Ohkawa *et al.*[29].While, hepatic PON1 activity was assayed spectrophotometrically by modified method of Eckerson *et al.*[30]. Total protein content in the liver homogenate was estimated according to the method of Lowry *et al.*[31].

Serum NF-κB level was determined by ELISA technique using kit purchased from Glory Science Co., USA according to the manufacturer's instructions. While, serum IL-8 level was detected by ELISA technique using the kit purchased from Assaypro, USA according to the method described by Jeremy *et al.*[32]. Serum HGF level was assayed by ELISA technique using the kit purchased from Glory Science Co., USA according to the method described by Miyazawa *et al.*[33].

Immunohistochemistry (IHC) examination

After 24 hours of fixation in 10% formaline saline, the liver tissue samples were washed with tap water and then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for 24 hours. Sections were cut into 4μ by slidge microtome then fixed on positive slides in a 65 ºC oven for 1 hr. Slides were placed in a coplin jar filled with 200 ml of triology working solution (Cell Marque, CA-USA) which combines the three pretreatment steps: deparaffinization, rehydration and antigen unmasking. Then, the jar is securely positioned in the autoclave which was adjusted so that temperature reached 120 ºC and maintained stable for 15 min after which pressure is released. Thereafter, the coplin jar is removed to allow slides to cool for 30 min. Sections were then washed and immersed in Tris-buffer saline (TBS) to adjust the pH and these were repeated between each step of the IHC procedure. Quenching endogenous peroxidase activity was performed by immersing slides in 3% hydrogen peroxide for 10 min. Broad spectrum LAB-SA detection system (Invitrogen, USA) was used to visualize any antigen-antibody reaction in the tissue. Background staining was blocked by putting 2-3 drops of 10% goat non immune serum blocker on each slide followed by incubation in a humidity chamber for 10 min. Without washing, excess serum was drained. Then, working solution (1:500) of Bcl-2 and TGF-β primary antibodies (Biorbyt, Cambridge, UK) was prepared. In case of Ki-67, the primary antibody (Labvision, Fermont Ca, USA) was ready to use. Two-three drops of the working solution of Bcl-2 and TGF-β and the ready to use primary antibody of Ki-67 were applied. The slides were incubated in the humidity chamber overnight at 4 ºC. Henceforward, biotinylated secondary antibody was applied on each slide for 20 min followed by 20 min incubation with the streptavidin horse reddish peroxidase (HRP) enzyme conjugate. 3,3´-diaminobenzidine (DAB) chromogen was prepared and 2-3 drops were applied on each slide for 2 min. DAB was rinsed, after which counterstaining with Mayer Hematoxylin and cover slipping were performed as the final steps before slides were examined under the light microscope (Olympus Cx21 with attached digital camera)[34]. The semiquantitative estimation of Bcl-2, TGF-β and Ki-67 was based on the staining intensity.

Statistical analyses

In the present study, all results were expressed as Mean $\pm$ S.E of the mean. Data were analyzed by one way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program, version 14 followed by least significant difference (LSD) to compare significance between groups [35]. The level of significance was set at $P < 0.05$.

RESULTS

Biochemical results

Table 1: Effect of treatment of fresh and dry *Cystoseira myrica* and *Padina pavonica* extract on urinary laminin and 8-OHdG in liver fibrosis bearing rats. (Mean ±SE)

Groups	Parameters	
	Laminin (µg/mg creatinine)	8-OHdG (ng/mg creatinine)
Negative control group	0.033 ± 0.001	4.25 ± 0.21
TAA-challenged group	0.080 ± 0.002^a	16.67 ± 0.17^a
TAA + fresh *Cystocyra myrica* extract	0.068 ± 0.003^b	10.23 ± 0.71^b
TAA +dry *Cystocyra myrica* extract	0.045 ± 0.001^{bc}	8.33 ± 0.57^{bc}
TAA + fresh *Padina pavonica* extract	0.074 ± 0.003^b	11.46 ± 0.63^b
TAA + dry *Padina pavonica* extract	0.051 ± 0.001^{bd}	9.98 ± 0.71^b

a: Significant change at P< 0.05 in comparison with negative control group.
b: Significant change at P< 0.05 in comparison with the thioacetamide - challenged group.
c: Significant change at P< 0.05 in comparison with the TAA + fresh *Cystocyra myrica* extract
d: Significant change at P< 0.05 in comparison with the TAA + fresh *Padina pavonica* extract

Table 2: Effect of treatment of fresh and dry Cystoseira *myrica* and *Padina pavonica* extract on hepatic MDA level and PON-1 activity in liver fibrosis induced rats. (Mean ±SE)

Groups	Parameters	
	MDA (nM/mg protein)	PON1 (µM/mg protein)
Negative control group	2.17 ± 0.17	$6.48 \pm .30$
TAA-challenged group	5.49 ± 0.29^a	3.81 ± 0.25^a
TAA+ fresh *Cystocyra myrica* extract	3.96 ± 0.24^b	5.18 ± 0.25^b
TAA +dry *Cystocyra myrica* extract	2.88 ± 0.15^{bc}	6.00 ± 0.36^b
TAA + fresh *Padina pavonica* extract	4.41 ± 0.24^b	4.57 ± 0.33
TAA + dry *Padina pavonica* extract	3.29 ± 0.08^{bd}	5.68 ± 0.46^{bd}

a: Significant change at P< 0.05 in comparison with negative control group.
b: Significant change at P< 0.05 in comparison with the thioacetamide - challenged group.
c: Significant change at P< 0.05 in comparison with the TAA + fresh *Cystocyra myrica* extract
d: Significant change at P< 0.05 in comparison with the TAA + fresh *Padina pavonica* extract

The results in Table (1) illustrated the effect of treatment with fresh and dry *Cystoseira myrica* and *Padina pavonica* extracts on urinary laminin and 8-HdG in rats bearing liver fibrosis. In comparison with the negative control group, there was a significant increase in the urinary levels of laminin and 8-HdG1 in TAA challenged group. Treatment with either *Cystoseira myrica* or *Padina pavonica* extracts reverted this increase significantly relative to the untreated TAA-challenged group.

Data in Table (2) represented the results of hepatic malondealdehyde (MDA) content and paraxionase1(PON-1) activity in all experimental groups. It is clear from these findings that hepatic MDA content was increased significantly in TAA challenged group while PON-1 activity was decreased significantly versus the negative control group. Dry and fresh *Cystoseira myrica* and *Padina pavonica* elicited marked regression of the oxidative stress insult through decreasing hepatic MDA content and increasing PON-1 activity significantly with respect to the untreated TAA challenged group. Regarding, serum NF-κB and IL-8 levels, they showed significant increase in TAA challenged group as compared to the negative control group. Treatment with fresh and dry *Cystoseira myrica* and *Padina pavonica* extracts produced a significant decrease in these inflammatory mediators in comparison with the untreated TAA-challenged group (Table 3).

Serum hepatocyte growth factor (HGF) level showed significant increase in TAA- challenged group relative to the negative control group. On the other side, treatment with *Cystoseira myrica* and *Padina pavonica* extracts induced a significant reduction in serum HGF level when compared with the untreated TAA-challanged group (Table 4).From all the previous results we could conclude that dry *Cystoseira myrica* experienced the best ameliorative effect on regression of liver fibrosis followed by dry *Padina pavonica*.

Immunohistochemical (IHC) results
Immunohistochemical staining for liver tissue section of rats in the negative control group using antibody against BcL-2, TGF-β and Ki-67 revealed negative reaction (Fig 1a,1b,1c) respectively. However, immunohistochemical staining for liver tissue section of rat in TAA-challenged group using antibody against BcL-2, TGF-β and Ki-67 showed very sever positive reaction (Fig 2a,2b,2c) respectively. Meanwhile, photomicrograph of immunohistochemical staining of liver tissue section of rat in TAA-challenged group treated with fresh *Cystoseira myrica* extract using antibody against BcL-2, TGF-β and Ki-67 showed moderate positive reaction (Fig 3a,3b,3c) respectively. Whereas, photomicrograph of immunohistochemical staining of liver tissue section of rat in TAA-challanged group treated with dry *Cystoseira myrica* extract using antibody against Bcl-2, TGF-β and Ki-67 revealed weak positive reaction (Fig4 a,4b,4c)

Table 3: Effect of treatment of fresh and dry *Cystoseira myrica* and *Padina pavonica* extract on serum levels of NF-κB and IL-8 in liver fibrosis bearing rats. (Mean ±SE)

Groups	Parameters	
	NF-κB (ng/mL)	IL-8 (ng/mL)
Negative control group	1.10 ± 0.09	0.065 ± 0.003
TAA-challenged group	2.91 ± 0.10^a	0.149 ± 0.009^a
TAA + fresh *Cystocyra myrica* extract	1.95 ± 0.12^b	0.103 ± 0.006^b
TAA +dry *Cystocyra myrica* extract	1.48 ± 0.04^{bc}	0.073 ± 0.004^{bc}
TAA + fresh *Padina pavonica* extract	2.25 ± 0.22^b	0.117 ± 0.005^b
TAA + dry *Padina pavonica* extract	1.56 ± 0.12^{bd}	0.087 ± 0.005^{bd}

a: Significant change at P< 0.05 in comparison with negative control group.
b: Significant change at P< 0.05 in comparison with the thioacetamide - challenged group.
c: Significant change at P< 0.05 in comparison with the TAA + fresh *Cystocyra myrica* extract
d: Significant change at P< 0.05 in comparison with the TAA + fresh *Padina pavonica* extract

Table 4: Effect of treatment of fresh and dry *Cystoseira myrica and Padina pavonica* extract on serum levels of HGF in liver fibrosis bearing rats. (Mean ±SE)

Groups	Parameters
	HGF (ng/mL)
Negative control group	0.20 ± 0.006
TAA-challenged group	0.39 ± 0.037^a
TAA + fresh *Cystocyra myrica* extract	0.32 ± 0.011^b
TAA +dry *Cystocyra myrica* extract	0.23 ± 0.009^{bc}
TAA + fresh *Padina pavonica* extract	0.35 ± 0.030
TAA + dry *Padina pavonica* extract	0.26 ± 0.015^{bd}

a: Significant change at P< 0.05 in comparison with negative control group.
b: Significant change at P< 0.05 in comparison with the thioacetamide - challenged group.
c: Significant change at P< 0.05 in comparison with the TAA + fresh *Cystocyra myrica* extract
d: Significant change at P< 0.05 in comparison with the TAA + fresh *Padina pavonica* extract

respectively. While, immunohistochemical staining for liver tissue section of rats in TAA-challenged group treated with fresh *Padina pavonica* extract using antibody against Bcl-2, TGF-β and Ki-67 showed severe positive reaction (Fig 5a,5b, 5c). Finally, photomicrograph of immunohistochemical staining of liver tissue section of rats in TAA-challenged group treated with dry *Padina pavonica* extract using antibody against Bcl-2, TGF-β and Ki-67 showed mild positive reaction (Fig 6a, 6b, 6c).

DISCUSSION
Prescription drugs with side effects have become widely used in modern life and as result, liver fibrosis has become a serious health problem. The current study focused on finding new therapeutic solutions to minimize liver fibrosis. Natural products, especially plants of positive effects against liver disease are considered as alternative therapeutic approach. Seaweeds extracts probably acted to preserve the structural integrity of the plasma cellular membrane of the hepatocytes to protect it against breakage by the reactive metabolites produced from exposure to hepatotoxins.
In light of the present data, TAA elicited devastating effects on liver tissue of rats *via* inducing significant elevation in urinary laminin, 8-OHdG, hepatic MDA and serum HGF level accompanied by significant reduction in hepatic PON1activity. Furthermore, TAA administration produced significant increase in serum NFκ-B and IL-8 in

comparison with the negative control group. Immunohistochemical results showed very severe reaction in the liver of TAA-challenged rats concerning the expression of Bcl-2, TGF-β, and Ki-67. These findings confirm the hepatotoxic potential of TAA which leads to liver fibrosis. Earlier reports have been demonstrated that TAA is a typical hepatotoxin that causes centrolobular necrosis when interferes with the movement of RNA from the nucleus to the cytoplasm, leading to cell membrane injury with consequent liver fibrosis[36]. The suggested mechanism responsible for TAA induce liver fibrosis was that TAA exposure leads to hepatocytes apoptosis and hepatic stellate cells migration to the site of injury to engulf the apoptotic bodies[36]. This engulfment promotes activation of the hepatic stellate cells to hepatic myofibroblasts, which in their activated state promote deposition of extracellular matrix and scar formation in the liver. Hepatic fibrosis is a wound healing process characterized by accumulation of extracellular matrix (ECM) proteins, especially collagen types I and III, as well as an increase in other extracellular matrix constituents such as proteoglycans, fibronectin and laminin in response to liver injury[37]. These data support our results that showed a significant increase in urinary laminin level in TAA-challenged rats.
The toxicity of TAA results from its bioactivation by a mixed-function oxidase system, particularly by CYP2E1 and FAD monooxygenases[22,38]. Metabolic activation of
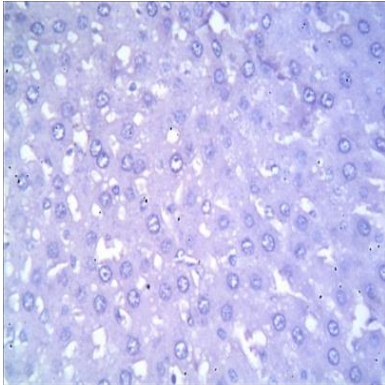
Immunohistochemical Results

Figure 1a: Photomicrograph of immuno-histochemical staining of liver tissue section of rat in the negative control group using antibody against Bcl-2 showed negative reaction
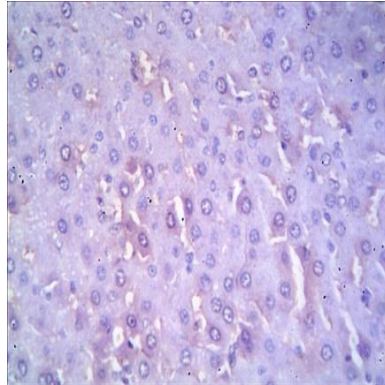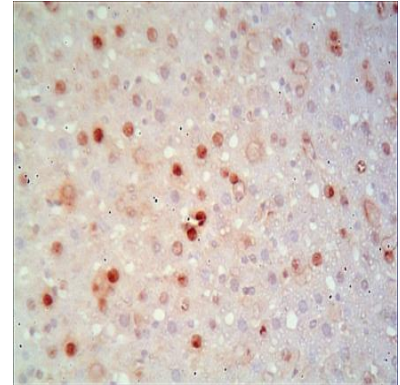
Figure 1b: Photomicrograph of immuno-histochemical staining of liver tissue section of rat in the negative control group using antibody against TGF-β showed weak positive reaction
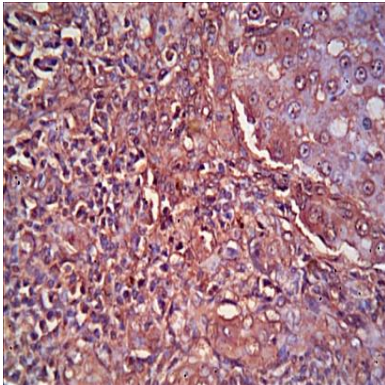
Figure 1c: Photomicrograph of immuno-histochemical staining of liver tissue section of rat in the negative control group using antibody against Ki-67 showed weak positive reaction.

Figure 2a: Photomicrograph of immuno-histochemical staining of liver tissue section of rat in TAA-challenged group using antibody against Bcl-2 showed very sever positive reaction.
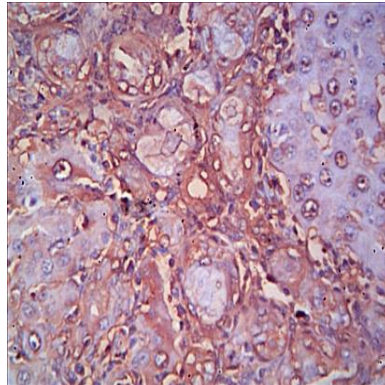
Figure 2b: Photomicrograph of immuno-histochemical staining of liver tissue section of rat in TAA-challenged group using antibody against TGF-β showed sever positive reaction.
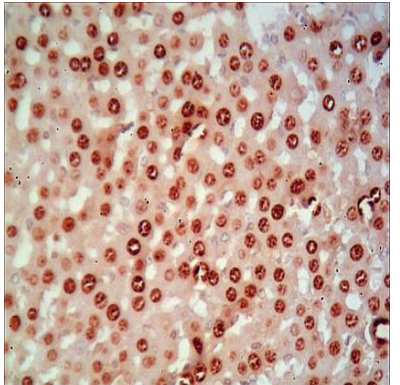
Figure 2c: Photomicrograph of immuno-histochemical staining of liver tissue section of rat in TAA-challenged group using antibody against Ki-67 showed sever positive reaction.
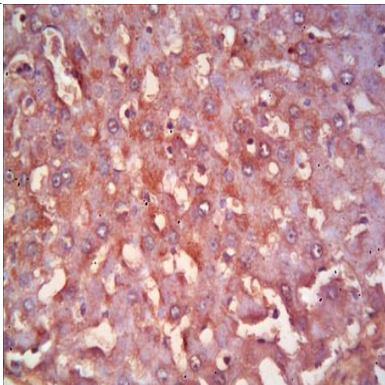
Figure 3a: Photomicrograph of immuno-histochemical staining of liver tissue section of rat in TAA-challenged group treated with fresh *Cystocyra myrica* extract using antibody against Bcl-2 showed moderate positive reaction.
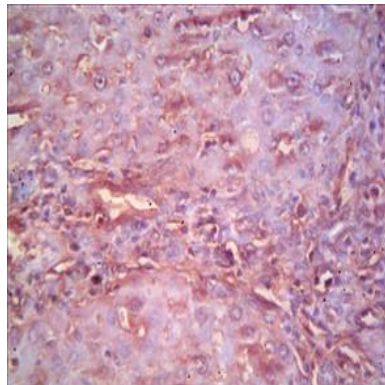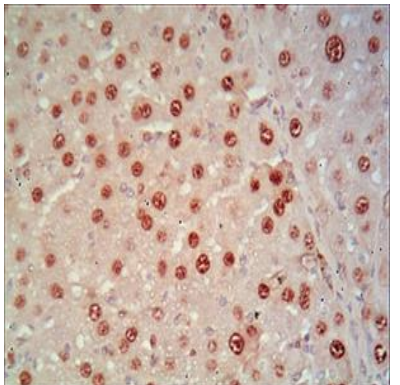
Figure 3b: Photomicrograph of immuno-histochemical staining of liver tissue section of rat in TAA-challenged group treated with fresh *Cystocyra myrica* extract using antibody against TGF-β showed moderate positive reaction.

Figure 3c: Photomicrograph of immuno-histochemical staining of liver tissue section of rat in TAA-challenged group treated with fresh *Cystocyra myrica* extract using antibody against Ki-67 showed moderate positive reaction
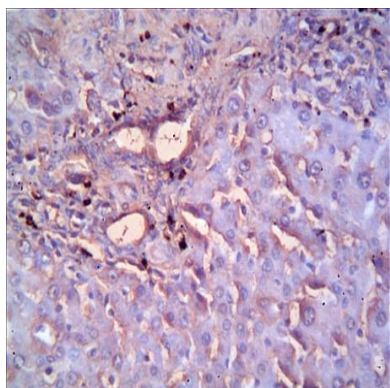
Figure 4a: Photomicrograph of immuno-histochemical staining of liver tissue sections of rat in TAA-challenged group treated with dry *Cystocyra myrica* extract using antibody against Bcl-2 showed weak positive reaction.
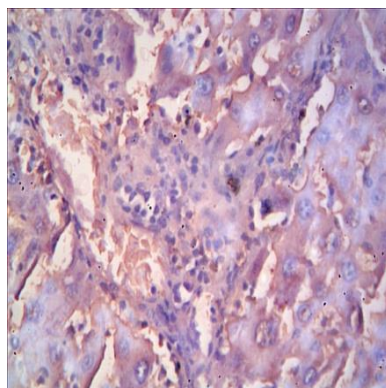

Figure 4b: Photomicrograph of immuno-histochemical staining of liver sections of rat in TAA-challenged group treated with dry *Cystocyra myrica* extract tissue using antibody against TGF-β showed mild positive reaction.
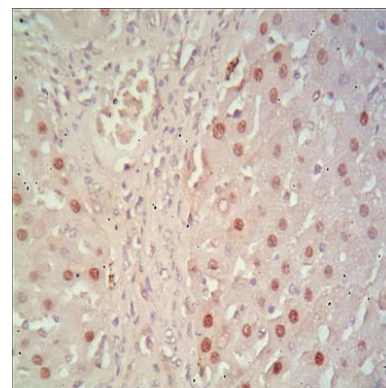

Figure 4c: Photomicrograph of immuno-histochemical staining of liver sections of rat in TAA-challenged group treated with dry *Cystocyra myrica* extract using antibody against Ki-67 showed mild positive reaction.

Figure 5a: Photomicrograph of immuno-histochemical staining of liver tissue section of rat in TAA-challenged group treated with fresh *Padina pavonica* extract using antibody against Bcl-2 showed sever positive reaction.

Figure 5b: Photomicrographof immuno-histochemical staining of liver tissue section of rat in TAA-challenged group treated with fresh *Padina pavonica* extract using antibody against TGF-β showed sever positive reaction.
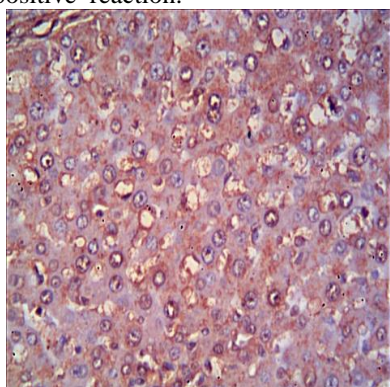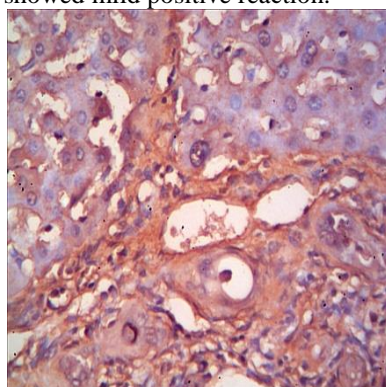

Figure 5c: Photomicrograph of immuno-histochemical staining of liver tissue section of rat in TAA-challenged group treated with fresh *Padina pavonica* extract using antibody against Ki-67 showed sever reaction.

Figure 6a: Photomicrograph of immuno-histochemical staining of liver tissue sections of rat in TAA-challenged group treated with dry *Padina pavonica* extract using antibody against Bcl-2 showed mild positive reaction.

Figure 6b: Photomicrograph of immuno-histochemical staining of liver tissue sections of rat in TAA-challenged group treated with dry *Padina pavonica* extract using antibody against TGF-β showed mild positive reaction.
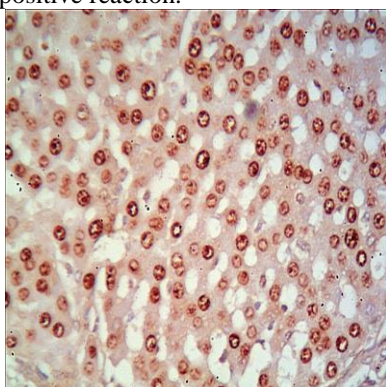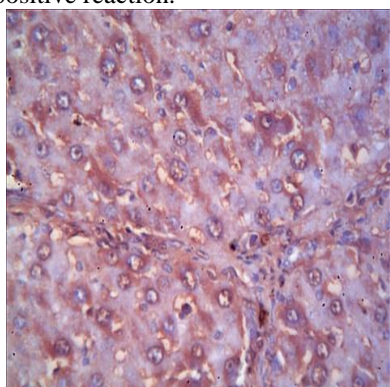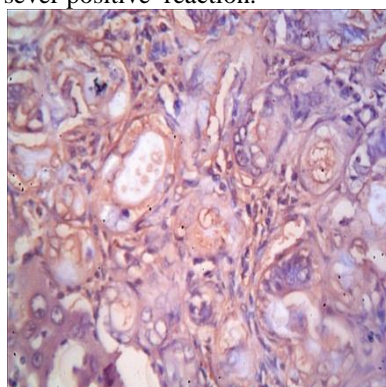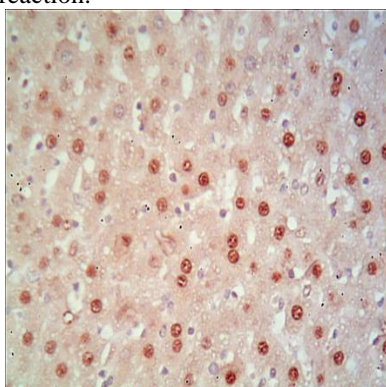

Figure 6c: Photomicrograph of immuno-histochemical staining of liver tissue sections of rat in TAA-challenged group treated with dry *Padina pavonica* extract using antibody against Ki-67 showed mild positive reaction.

TAA leads to the formation of reactive metabolites that are represented by free radicals derived from thioacetamide-S-oxide (TASO2) and by reactive oxygen species (ROS) generated as intermediates.

The development of liver fibrosis by TAA was reported to be involved multiple mechanisms. TAA induces hepatocyte damage *via* its metabolite, TASO2, which covalently binds to macromolecules of hepatocytes causing DNA damage, protein oxidation and lipid peroxidation as well as glutathione depletion and the reduction in the SH-thiol groups[14]. Salama *et al.*[39] found significant increase in urinary 8-HdG activity in TAA-treated animals which was in agreement with our results. These data strongly support our findings of the increased oxidative damage as a consequente of TAA administration which leads to hepatic MDA elevation and reduction of hepatic PON-1 activity.

Paraoxonase-1 (PON1) is an antioxidant enzyme synthesized by the liver. It protects against liver impairment and attenuates the production of the pro-inflammatory monocyte chemoattractant protein-1 (MCP-1) It also, hydrolyses lipid peroxides, and circulates in plasma bound to high-density lipoproteins (HDL)[40]. In accordance with our data it has been reported previously that serum PON1 activity is decreased in patients with liver diseases. Low PON1 levels are associated with an enhanced sensitivity to the development of liver damage. The decreased level of PON-1 in TAA -challenged group in the present study was well documented by[40].

The CXC chemokine interleukin 8 (IL- 8) is a critical chemoattractant and activator for neutrophils, basophils, and T cells and is secreted by Kupffer cells, macrophages and hepatocytes. IL- 8 secretion is complex and is regulated primarily at the transcriptional level through cooperative interactions of the transcription factors nuclear factor κβ (NF-κβ), activator protein 1 (AP -1), and IL -6 in the tissue. IL- 8 production is associated with liver injury induced by lipopolysaccharide, tumor necrosis factor (TNF), Fas, and bacterial infections. Increased plasma, hepatic and monocyte levels of IL- 8 are well documented in alcoholic liver disease (ALD) and are postulated to play a key role in the hepatic neutrophil infiltration in ALD[37]. These findings come in line with our results concerning the elevated serum level of IL- 8 as a consequent of TAA administration.

The present study revealed significant increase in hepatocyte growth factor serum level in TAA challenged group. Hepatocyte growth factor (HGF), originally identified and cloned as a potent mitogen for hepatocytes, shows mitogenic, motogenic and morphogenic activities for a wide variety of cells. Moreover, HGF plays an essential part in the development and regeneration of the liver, and shows antiapoptotic activity in hepatocytes. The receptor for HGF is a tyrosine kinase receptor encoded by c-*met*[41]. Nakagami *et al.*[42] demonstrated that the antiapoptotic action of HGF against endothelial cell death was mainly through phosphorylation of ERK on human endothelial cells. HGF has been found to be increased in liver fibrosis[43].

Immunohistochemical results of the present study revealed very severe positive reaction of Bcl-2, TGF-β and Ki-67 in the liver tissue of TAA-challenged group.

The oncogene Bcl-2 was first reported by Tsujimoto *et al.*[44]. The human BCL-2 gene is a protein consisting of 239 amino acids with a mass of 26 kDa, localized mainly in the outer mitochondrial membrane, endoplasmic reticulum and nuclear membrane. The protein encoded with the Bcl-2 protooncogene has been implicated in the prolongation of cell survival by blocking programmed cell death. In conformity with our results Frommel *et al.* [45] reported that Bcl-2 protein expression is upregulated in hepatocytes of patients with cirrhosis due to hepatitis. Chronic infection with the hepatitis virus is a predisposition for fibrosis, cirrhosis and hepatocellular carcinoma. It has been demonstrated that hepatocyte specific disruption of BCl-XL induces continuous hepatocyte apoptosis and fibrogenesis[46].

One of the characteristic features of liver disease is the hepatocytes death as a result of drug/toxicant-induced liver injury. Cytotoxic drugs and celluar stress activate the intrinsic mitochondrial apoptotic pathway so that the inhibitors of Bcl-2 induce cells necrosis[47]. *In vivo*, necrotic death is often associated with extensive damage in the tissue resulting in necroinflammatory response. Although toxicity induced by TAA was reported to cause upregulation of Bax protein and downregulation of antiapoptotic protein Bcl-2 and its translocation into the mitochondria, causing apoptosis, other studies suggested that the ROS produced from TAA biotransformation causes centrolobular necrosis[48].

The profibrogenic cytokine TGF-*β* is secreted by the immune cells gathered at site of injury to phagocytose the apoptotic bodies, further fuelling the inflammatory and fibrogenic reaction[36]. TGF-β is a central regulator in chronic liver disease contributing to all stages of disease progression from initial liver injury through inflammation and fibrosis to cirrhosis and hepatocellular carcinoma. Liver damage-induced the increasing levels of active TGF-β enhances hepatocyte destruction and mediates hepatic stellate cell and fibroblast activation. It has been suggested that TGF-β enhances damage to epithelial cells by inducing apoptosis and oxidative stress, triggers myofibroblast (MFB) activation, inhibits liver regeneration by hepatocyte apoptosis, activates regulatory T cells (TReg) and Th17 differentiation to calm down inflammatory responses, causes fibrogenesis and liver scarring in chronic disease. Hence, TGF-β is recognized as a major profibrogenic cytokine[49].

It is well documented that the expression of the human Ki-67 protein is strictly associated with cell proliferation. The fact that the Ki-67 protein is present during all active phases of the cell cycle, but its absence from resting cells, makes it an excellent marker for determining the so called growth fraction of a given cell population. Ki-67 protein expression is an absolute requirement for progression through the cell-division cycle[50].

The therapeutic potential of the selected fresh and dry brown algae against liver fibrosis was confirmed in the present study. As the treatment of TAA-challenged groups

with either *Cystoseira myrica* or *Padina pavonica* extract resulted in significant reduction in urinary lamnine and 8-OHdG levels. Moreover, hepatic MDA, serum NF- κβ, IL-8 and HGF showed significant depletion due to treatment with brown algae extracts. Meanwhile, hepatic PON-1 activity revealed significant elevation as a consequence of treatment with either *Cystoseira myrica* or *Padina pavonica* extracts.

Furthermore, in this study that both *Cystoseira myrica* or *Padina pavonica* species could downregulate Bcl-2, TGF-β and Ki-67 expression in the liver tissue of TAA-challenged rats as shown in the immunohistochemical findings. To our knowledge, this study is the first *in vivo* study investigates the effect of these algae in treatment of liver fibrosis.

Ragan and Glombitza[51] reported that the radical-scavenging activity of seaweeds is mostly related to their phenolic contents. Polyphenol-mediated scavenging of free radicals produced during fibrosis represented a good mechanism for alleviating liver fibrosis. In support with this hypothesis, polyphenols have been shown to decrease the accumulation of 4-hydroxynonenal adducts in rat livers, indicating the decreased lipid peroxidation. Moreover, oxidant-induced cell death contributes in liver fibrosis, therefore, prevention of cell death may decrease subsequent fibrosis. Polyphenols has been found to decrease the necrosis of parenchymal cells as well as minimize fibrosis[52].

In accordance with our results, Yoshie *et al.*[53] suggested that organic extracts of *Cystoseira* had significant anti-oxidant activity toward the DPPH free radical *in vitro*. Several compounds were identified as anti-oxidants, including protective enzymes, ascorbic acid, lipophilic anti-oxidants, phlorotannins and catechins. The observed antioxidant activity of *Cystoseira* could be attributed to these active compounds[53]. Moreover, the phenolic compounds present in *Cystoseira* are responsible for its antioxidant capacity, as these compounds are known to have antioxidant properties that neutralize or inactivate highly unstable and extremely reactive molecules that attack the cells.

The present results recorded a significant ameliorative effect of *Padina pavonica* against liver fibrosis**.** Brown algae represent a rich source of polysaccharides and glycosides. The antifibrotic activity of *Padina pavonica* could be attributed to these compounds, in addition to the phenolics compounds detected in *Padina pavonica* extract. Based on the published work of Kamenarska *et al.*[12], some sterols, fatty acids, aromatic esters, terpenoids, benzyl alcohol and benzaldehyde have been isolated from *Padina pavonica* which might be responsible for its biological activity.

C. myrica and *P. pavonica* have been shown anti-inflammatory effect through reducing the release of histamine, serotonin and prostaglandins. This pharmacological property is possibly ascribed to the seaweed components by which they could effectively decrease the production of the pro-inflammatory cytokins such as IL-6 and IL-8 and elevate the level of the anti-inflammatory cytokine IL-4[54,55].

C. myrica has been found to possess the ability to produce a great variety of secondary metabolites with very different skeleton types and functionalities[56]. Secondary metabolites from the genus *Cystoseira*n are polysaccharides, sterols, lipids, terpenes and many diterpenoids which have been isolated as the linear diterpenes or acyclic and cyclic meroditerpenoids[9]. These metabolites have been suggested to act synergistically to exert many pharmacological activities including anti-inflammatory activity[57].

On the other side, the antioxidant and anti-inflammatory effects of *Padina pavonica* extract is well documented in the present study which is in agreement with Karthikeyan *et al.*[55] *P. boergesenii* has proven its potential nature against free radical damages. as it has been found that the pretreatment of rats with *P. boergesenii* extract prevented CCl4-induced lipid peroxidation, GSH and antioxidant enzymes depletion in the circulation as well as in the liver tissue. These results suggested that the Padina extract acted as an effective antioxidant and/or a free radical scavenger. These properties enable *P. pavonica* to stabilize membrane structures, preserve the cellular integrity and restrain the severity of TAA -induced cellular injury. Based on these findings, it is reasonable to suggest that the presence of antioxidant compounds like carotenoids in *P. pavonica* extract might responsible for its antioxidant action or free radical scavenging activity.

In the present study, *P. pavonica* extract was able to ameliorate acute liver damage to a high degree, as evidenced by the improvement in biochemical and immnohistochemical markers. The brown alga *P. boergesenii* extract proved its nephroprotective activity either through stabilization of cellular membrane or through antiperoxidase activity[55].

The ability of *C. myrica* and *P. pavonica* extracts to downregulate Bcl-2 expression in the liver tissue of TAA-challenged group in the present work is probably ascribed to their contents from the polyphenolic compounds. Polyphenols have been found to prevent activation of Kupffer cells, thus decreasing formation of inflammatory and fibrogenic mediators. The role of Kupffer cells in fibrosis is controversial. Destruction of Kupffer cells attenuated liver fibrosis caused by CCL4 and TAA. Kupffer cells release many mediators that activate stellate cells HSCs, including TNF-α, TGF-β, HGF, PDGF, and reactive oxygen species. PDGF and TNF-α are mitogenic factors for stellate cells[58]. TNF-α production and NF-κB activation have been found to increase during cholestasis leading to fibrosis. Activation of NF-κB, probably due to oxidative stress, could lead to expression of TNF-α. In addition, the activation of NF-κB, expression of TNF-α and TGF-β were increased by cholestasis and these effects were blocked by polyphenols By scavenging oxygen radicals, polyphenols may inhibit proliferation of cholangiocytes and their production of fibrogenic and inflammatory mediators[58].

Due to the crucial role played by HSCs in liver fibrosis *via* their resistance to apoptosis, recent treatment strategies of liver disease are to inhibit their proliferation or induce their apoptosis[39]. Treating the animals with *C.myrica* and *p. pavonica* extract inhibited the necrotic effect due to TAA

administration by modifying necrosis into apoptosis, which might be through cytochrome release from mitochondria and caspase activation. This modification *in vivo* would scale down the release of the inflammatory mediators that would prevent progressive liver damage. Thus, the enhancement of damaged hepatocyte apoptosis might represent the protective mechanism by which whereby polyphenol constituents in both extracts downregulate fibrogenesis of the liver[59].

In summary, the above mentioned mechanisms might be contributed in the amelioration of liver fibrosis. Thus the selected algae extracts may provide a fundamental protective milieu for liver in coping against TAA induced liver fibrosis, by favoring the removal of oxidants and enhancing the antioxidant defense system as well as reducing the inflammatory and fibrogenic mediators.

In conclusion, the results presented here led us to conclude that supplementation of *Cystoseira myrica* or *Padina pavonica* extract could attenuate liver fibrosis by preventing the harmful cascade of events induced by TAA toxicity in rats. This was confirmed through antioxidant, anti-inflammatory, and apoptotic activity of both extracts. These findings encourage further studies on the pharmacological significance of using *Cystoseira myrica* and/or *Padina pavonica* as alternative medications for treatment of liver fibrosis.

DECLARATION OF INTEREST
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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