



Ameliorative Effect of Marine Macroalgae on Carbon Tetrachloride-Induced Hepatic Fibrosis and Associated Complications in Rats

✉ Maria AZAM¹, ✉ Khan HIRA^{1,2}, ✉ Shamim A. QURESHI¹, ✉ Nasira KHATOON³, ✉ Jahan ARA⁴, ✉ Syed EHTESHAMUL-HAQUE^{5*}

¹Karachi University, Faculty of Science, Department of Biochemistry, Karachi, Pakistan

²Dow University of Health Sciences, Dow College of Pharmacy, Department of Pharmacology, Karachi, Pakistan

³Karachi University, Faculty of Science, Department of Zoology, Karachi, Pakistan

⁴Karachi University, Faculty of Science, Department of Food Science and Technology, Karachi, Pakistan

⁵Karachi University, Faculty of Science, Department of Botany, Karachi, Pakistan

ABSTRACT

Objectives: Liver fibrosis is one of the serious health concern around the globe. Persistent exposure to drugs, toxicants, and pathogens may induce liver fibrosis. Marine macroalgae are globally consumed because of nutritive and medicinal value. This study was conducted to evaluate the protective role of two seaweeds *Padina pavonia* and *Caulerpa racemosa* in carbon tetrachloride (CCl_4)-induced liver fibrosis in rats.

Materials and Methods: Animal model of hepatic fibrosis was developed by injecting 40% CCl_4 dissolved in olive oil [2 mL/kg, body weight (b.w.), i.p.] on alternate days for 30 days. Water extracts (WE) [200 mg/kg b.w., p.o.] of *P. pavonia* and *C. racemosa* were given to rats daily for 30 days. On day 31, rats were sacrificed after 12 h fasting. Serum was used for biochemical estimation. 10% neutral buffered formalin was used to preserve the liver sample for histopathological examination, while the other portion was used for the preparation of tissue homogenate to estimate antioxidant enzymes and malondialdehyde levels.

Results: WEs of both marine macro-algae significantly abrogate the elevated serum concentrations of aminotransferases (alanine aminotransferase and aspartate aminotransferases), alkaline phosphatase and lactate dehydrogenase along with a substantial ($p<0.05$) reduction in serum bilirubin levels. They also showed positive effects on oxidative stress, evident by improvement in reduced glutathione, catalase, and glutathione peroxidase activities and down regulation of lipid peroxidation level, with stabilizing the destructive cellular morphology of liver induced by repeated CCl_4 injection. Both algal extracts also improved kidney function (urea and creatinine) along with lipid metabolism (triglycerides and cholesterol).

Conclusion: WE of *C. racemosa* has shown great potential in attenuating liver fibrosis induced by CCl_4 .

Key words: *Padina pavonia*, *Caulerpa racemosa*, bilirubin, glutathione, lipid peroxidation, kidney

INTRODUCTION

The liver is the metabolic hub for the degradation, synthesis, detoxification and transformation of drugs and biomolecules. Along with all vital functions it performs, it is also prone to toxicity.¹ The liver comprises various cells, such as hepatocytes, hepatic stellate cells (HSCs), which work as a reservoir of lipid droplet (vitamin A) and Kupffer cells, usually known as resident macrophages of liver.² Viral infections, chemical/drug intoxication, or any malfunctioning of liver lead to damage of hepatocytes.³ HSCs, release extracellular matrix

(ECM) components around the injured area to prevent the further damage to the liver.⁴ Chronic exposure of the liver with toxicants resulting in continuous activation of HSCs, forming scar tissues in the liver, lead to conditions such as; fibrosis, cirrhosis, and hepatocellular carcinoma.⁵ Liver fibrosis is one of the serious health concern around the globe⁶ and it is an early stage of cirrhosis.⁷ It has been reported that the fibrotic liver contains more ECM components, which contains collagens (I, III, and IV), fibronectin, elastin, laminin, hyaluronic acid, and proteoglycans.⁸ The earlier diagnosis of liver fibrosis is

*Correspondence: sehaq@uok.edu.pk, Phone: +923012420634, ORCID-ID: orcid.org/0000-0002-8540-4068

Received: 15.03.2021, Accepted: 04.07.2021

©Turk J Pharm Sci, Published by Galenos Publishing House.

important since it is treatable and termed as reversible liver fibrosis.⁹

Carbon tetrachloride (CCl_4) has been widely used for developing animal models of hepatic fibrosis, cirrhosis, and hepatocellular carcinoma for many decades.¹⁰ The lipid-soluble nature of CCl_4 allows it to cross the lipid bilayer membrane, produces hindrance in cellular activities by promoting the lipid peroxidation, activating reactive oxygen species (ROS).¹¹ Persistent exposure of CCl_4 promotes accumulation of ECM components, which either damages the cellular structures and/or disturbs the cellular integrity.¹²

Among different marine sources, macroalgae have drawn the attention of many researchers as an interesting and unique source of bioactive natural products, besides, a great source of proteins, carbohydrates, minerals and vitamins.¹³ They have medicinal importance, including anticancer, antibacterial, anticoagulant, nephroprotective, anti-inflammatory and antioxidant activities.^{14,15} Green seaweeds are a rich source of carotenoids, phenolics, terpenoids, essential proteins, vitamins, minerals and sulfated polysaccharides (SP).¹⁶ *Ulva* and *Caulerpa* species contain higher contents of SP and rare sugars (arabinose, rhamnose and iduronic acid).¹⁷ *Caulerpa racemosa*, an edible green seaweed, has number of biological activities such as antibacterial and hypolipidemic activities against triton-induced hyperlipidemic rats.^{18,19} However, the hepatoprotective role of this seaweed against CCl_4 induced hepatic injury has not been evaluated. Brown seaweeds contain phenolic compounds and SP (alginates, fucoidans, and laminarin) and possess tremendous biological activities such as hepatoprotective, anticancer, anticoagulant, wound healing, and antiviral.¹⁴ *Padina pavonia*, has been previously reported to have anti-proliferative and pro-apoptotic activities.²⁰ Its hepatoprotective activity against azoxymethane-induced hepatotoxicity has been reported.²¹ However, its hepatoprotective activity against CCl_4 has not been explored, yet. The current study was designed to elucidate the hepatoprotective potential of *C. racemosa* and *P. pavonia* against CCl_4 -induced liver fibrosis. The study also describes the effect of these seaweeds on liver fibrosis associated complications, including renal dysfunction and lipid metabolism.

MATERIALS AND METHODS

Algal materials

Seaweeds were collected from the Karachi coast (Buleji Beach) at low tide during the month of November-April. Collected algal samples were identified by a taxonomist (Dr. Aisha Begum, Associate Professor, Department of Botany, University of Karachi). The algal material was washed using running tap water and air dried under a green shade. The dried samples were ground and stored for further use.

Water extracts

Water extract (WE) was obtained by soaking dry powder (250 g) of each seaweed in 1 L of deionized water under continuous shaking for 3 h. The filtrate obtained was lyophilized using a lyophilizer (Eyela FD-1, Japan). The dry powders were stored separately at -20°C until used according to Ismail and Tan.²²

Experimental animals

Female Wistar rats (120-200 g) acquired from Dow University of Health Sciences, Karachi. Animals were accommodated in polypropylene cages under standard laboratory conditions (23 ± 2°C and 12 h light/dark cycle). The cages were bedded with wood shaving and the rats had free access to a normal pellet diet and tap water. Rats were acclimatized for 7 days under the guidelines of the Institutional Bioethical Committee University of Karachi (IBC-KU-132/2020) before the experimental protocol.

Induction of fibrosis

The method of Iredale et al.²³ was followed to develop hepatic fibrosis in rats with slight modification. Rats received 40% CCl_4 intraperitoneally (*i.p.*) at 2 mL/kg body weight (b.w.), dissolved in olive oil, on alternative days for 30 days.

Experimental design

Effect of the WE of seaweeds in normal and liver fibrotic rat model

To evaluate the efficacy of seaweed extracts in rats, they were randomly divided into 8 groups (n= 6).

Group 1; Normal control: Rats received distilled water at 1 mL/kg b.w., daily for 30 days.

Group 2; WE of *P. pavonia*-treated rats: WE of *P. pavonia* was supplemented to rats at 200 mg/kg/mL, b.w., in distilled water, daily for 30 days.

Group 3; WE of *C. racemosa*-treated rats: WE of *C. racemosa* was supplemented to rats at 200 mg/kg /mL, b.w., in distilled water, daily for 30 days.

Group 4; Silymarin-treated rats: Rats were supplemented with silymarin [Sigma-aldrich (50 mg/kg b.w., suspended in normal saline)] daily for 30 days.²⁴

Group 5; CCl_4 control: Rats were intraperitoneally (*i.p.*) injected 40% CCl_4 (in olive oil) 2 mL/kg b.w., on alternate days for 30 days.

Group 6; WE of *P. pavonia* + CCl_4 -induced liver fibrosis: WE of *P. pavonia* was supplemented to rats at 200 mg/kg/mL, b.w., in distilled water, along with the administration of CCl_4 (*i.p.*, 2 mL/kg, b.w.), on alternate days for 30 days.

Group 7; WE of *C. racemosa* + CCl_4 -induced liver fibrosis: WE of *C. racemosa* was supplemented to rats at 200 mg/kg /mL, b.w., in distilled water daily, along with the administration of CCl_4 (*i.p.*, 2 mL/kg, b.w.), on alternate days for 30 days.

Group 8; Silymarin treatment + CCl_4 -induced liver fibrosis: Rats were supplemented with silymarin [Sigma-aldrich (50 mg/kg b.w., suspended in normal saline)] daily, along with the administration of CCl_4 (*i.p.*, 2 mL/kg, b.w.), on alternate days for 30 days.

Assessment of hepatotoxicity and associated complications

To determine the effect of seaweed on liver fibrosis and other associated complications; rats were fasted for 12 h and decapitated on the 31st day. Blood was centrifuged at 3000 rpm for 15 min to obtain serum. Liver enzymes, viz; alanine aminotransferase (ALAT) (INO-17531) aspartate

aminotransferase (ASAT) (INO-17521), lactate dehydrogenases (LDH) (INO-17653), alkaline phosphatases (ALP) (INO-17541), and other liver markers, viz; total-bilirubin (INO-17645) and direct-bilirubin (INO-17646); lipid parameters including cholesterol (INO-17501) and triglycerides (TGs) (INO-17511), renal function markers such as urea (INO-17611) & creatinine (INO-17551) and blood glucose (INO-17602) were estimated on blood chemistry analyzer (Microlab-300, Merck, France) using kits from Merck (Innoline), France as *per* manufacturer's instructions.

For histopathological studies, the liver was excised, washed with normal saline. The right lobe was preserved in 10% neutral buffered formalin. The remaining portion of liver was used for the preparation of liver tissue homogenate [Tris-HCl buffer (pH: 7.4) using Polytron (Kinematica) PT-MR 2100 homogenizer]. Tissue homogenate was used for measuring the antioxidant parameters reduced glutathione (GSH), catalase (CAT), malondialdehyde (MDA), and glutathione peroxidase (Gpx).

Assessment of hepatic reduce glutathione

Reduced GSH was estimated using the method of Moron et al.²⁵ Brief 0.1 mL homogenenatenate was mixed with 0.1 mL trichloroacetic acid (25%) and allowed to stand at room temperature for 5 min. The mixture sample was centrifuged at 3000 rpm for 10 minutes the supernatant was collected and mixed with 1.8 mL of 0.1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). Samples were allowed to incubate in dark at room temperature for 10 min and absorbance was recorded at 412 nm against the reagent blank.

Assessment of hepatic glutathione peroxidase

The activity of GPx was estimated by the method Flohé and Günzler.²⁶ Briefly 300 µL of liver homogenate was mixed with 300 µL of phosphate buffer (pH: 7.4), 200 µL of GSH (2 mM), 100 µL of sodium azide (1 mM) and 100 µL of hydrogen peroxide (1 mM). The mixture was allowed to stand for 15 min at 37°C in a water bath. 500 µL TCA (15%) was added in the mixture and centrifuged at 1500 rpm for 5 min, supernatant was mixed with 200 µL of phosphate buffer and 700 µL of DTNB (0.1 mM). The absorbance was recorded at 412 nm against the reagent blank.

Assessment of hepatic catalase

The CAT activity was evaluated by the method of Sinha.²⁷ Briefly, 100 µL of the homogenate was mixed with 1 mL of phosphate buffer (pH: 7.4) and 500 µL of hydrogen peroxide (0.2 M). The mixture was allowed to incubate 37°C for 15 min. 2 mL of dichromate solution (5%) was added to the mixture and absorbance was recorded at 570 nm against the reagent blank.

Assessment of lipid peroxidation (LPO)

MDA the end product of LPO was estimated by the method of Ohkawa et al.²⁸ Briefly, 100 µL of tissue homogenate was mixed with 100 µL of sodium dodecyl sulphate (8.1%) and incubated at room temperature for 10 min. 750 µL of 20% acetic acid and 750 µL thiobarbituric acid (0.8%) were added to the mixture and volume was adjusted up to 2 mL. The mixture was allowed to stand at 95°C for 1 hour. A 2.5 mL butanol and pyridine (1:1) solution was added to a mixture and the volume was adjusted

up to 5 mL with distilled water, and centrifuged (4000 rpm) for 10 min. The upper organic layer was collected and absorbance was recorded at 532 nm against the reagent blank.

Histological study

The liver tissue was preserved in 10% neutral buffered formalin and embedded in paraffin wax. Then, 3-4 µm thin sections were cut using a microtome. The tissues were either stained with hematoxylin and eosin or Masson's trichrome for evaluating architectural changes and evaluate the abundance of collagen in the liver tissue.²⁹ The slides were studied under the light microscope (Nikon FX-35A, Japan) and pictures were taken from an attached Nikon camera (DS Fl1). The Batts and Ludwig³⁰ scoring system was used to grade hepatic fibrosis.

Statistical analysis

The data were represented as a means ± standard deviation. Statistical analysis was executed using SPSS software (version 16). The differences between the means were subjected to One-Way ANOVA followed by Tukey's *post-hoc* test. *P* value ≤0.05 was considered a level of significance.

RESULTS

*Effect of water extracts of *P. pavonia* and *C. racemosa* on liver profile*

CCl₄ remarkably increased levels of serum aminotransferases ALAT (623.8%) and ASAT (246.3%) and ALP (392.4%), serum LDH (228.2%), total bilirubin (370%), and direct bilirubin (552.1%) levels compared to those of normal control rats. Intoxicated rats treated with WE of *P. pavonia* decreased ALAT (-62.0%), ASAT (-49.0%), ALP (-29.3%), LDH (-65.7%), total bilirubin (-70%), and direct bilirubin (-79.3%) as compared to CCl₄-treated control rats. The same pattern was observed in intoxicated rats treated with WE of *C. racemosa*, which significantly (*p*≤0.05) reduced the elevation of ALAT (-82.2%), ASAT (-46.7%), ALP (-41.3%), LDH (-25.8%), total bilirubin (-69.6%), and direct bilirubin (-69.3%) levels. Fibrotic rats concomitantly treated with silymarin showed a significant reduction in serum ALAT (-71.0%), ASAT (-41.3%), ALP (-40.7%), LDH (-45.6%), total bilirubin (-31.2%), and direct bilirubin (-43.3%). In general, WE of *C. racemosa* showed a more promising effect as compared to WE of *P. pavonia* on liver function markers in CCl₄-induced liver fibrotic rats. Further, fibrotic rats showed a significant elevation in blood glucose (25%) levels as compared with normal rats. Besides, treatment with WE, *P. pavonia* reversed the elevated blood glucose level (-51.3%) (Tables 1, 2). Overall results demonstrated that WE of both seaweeds may reciprocate the elevated level of hepatic enzymes, metabolites and also ameliorates increased blood glucose levels in response to persistent liver assault.

*Effect of water extracts of *P. pavonia* and *C. racemosa* on renal function and lipid profile*

Table 3 demonstrated that CCl₄ has significantly (*p*≤0.05) impaired the kidney function and glucose metabolism by increasing serum urea (151.8%) and creatinine (816%) levels. CCl₄ was also responsible for reducing the serum cholesterol (-75%) and TGs (-62.5%), when compared with normal rats.

Table 1. Effect of water extracts of *Padina pavonia* and *Caulerpa racemosa* on liver enzymes in normal and CCl₄-intoxicated rats

Treatments	Normal rat model				Fibrotic rat model			
	Normal control	<i>P. padina</i> (WE)	<i>C. racemosa</i> (WE)	Silymarin	CCl ₄ control	<i>P. padina</i> (WE) + CCl ₄	<i>C. racemosa</i> (WE) + CCl ₄	Silymarin + CCl ₄
ALAT (u/L)	42 ± 7.3	50.5 ± 3.25 ^a (20.2%)	44.5 ± 6.59 ^a (5.9%)	38.02 ± 0.28 ^a (-9.4%)	304.2 ± 34.06 ^{a*} (623.8%)	115.5 ± 14.45 ^{b*} (-62.0%)	54.6 ± 7.2 ^{b*} (-82.2%)	88.2 ± 5.03 ^{b*} (-71.0%)
ASAT (u/L)	123 ± 16.11	100.5 ± 15.5 ^a (-18.6%)	64 ± 8.6 ^a (-47%)	64.6 ± 5.2 ^{a*} (-47.4%)	426 ± 54.39 ^{a*} (246%)	217 ± 23.7 ^{b*} (-49.0%)	227 ± 28.34 ^{b*} (-46.7%)	140 ± 7.2 ^{b*} (-65.7%)
ALP (u/L)	79 ± 8.85	114 ± 14.5 ^a (44.3%)	72 ± 14.3.6 ^a (-8.8%)	67.6 ± 5.2 ^a (-15.1%)	389 ± 49.1 ^{a*} (392.4%)	275 ± 14.6 ^{b*} (-29.3%)	250 ± 1.5 ^{b*} (-41.3%)	230.3 ± 47.5 ^{b*} (-40.7%)
LDH (u/L)	124.6 ± 8.1	146.3 ± 19.6 ^a (17.4%)	184.0 ± 25.4 ^a (48.1%)	170.3 ± 0.57 ^a (36.9%)	409.1 ± 15.07 ^{a*} (228.25%)	162.3 ± 15.6 ^{b*} (-58.3%)	303.33 ± 24.9 ^{b*} (-25.8%)	222.3 ± 14.04 ^{b*} (-45.6%)

The data were analyzed by One-Way ANOVA followed by Tukey's post-hoc test. *p<0.05, ^aIndicates the comparison with control rats, ^bIndicates the comparison with CCl₄ control rats.

Data were expressed in means ± standard deviation (n= 6). The values in parenthesis represent percentage (%) increased or decreased as compared to their respective control. CCl₄: Carbon tetrachloride, ALAT: Alanine aminotransferase, ASAT: Aspartate aminotransferase, ALP: Alkaline phosphatases, LDH: Lactate dehydrogenases, WE: Water extract

Table 2. Effect of water extracts of *Padina pavonia* and *Caulerpa racemosa* on liver metabolite and blood glucose in normal and CCl₄-intoxicated rats

Treatments	Normal rats model				Fibrotic rats model			
	Normal control	<i>P. padina</i> (WE)	<i>C. racemosa</i> (WE)	Silymarin	CCl ₄ control	<i>P. padina</i> (WE) + CCl ₄	<i>C. racemosa</i> (WE) + CCl ₄	Silymarin + CCl ₄
Glucose (mg/dL)	116.18 ± 8.07	89.16 ± 5.7 ^a (-23.3%)	115 ± 14.30 ^a (0.94%)	98 ± 3.0 ^a (-15.59%)	146.3 ± 14.3 ^{a*} (25.8%)	71.16 ± 7.55 ^{b*} (-51.3%)	147 ± 22.4 ^b (-0.47%)	100 ± 1.5 ^{b*} (-31.64%)
Total bilirubin (mg/dL)	0.34 ± 0.04	0.21 ± 0.04 ^a (-38.2%)	0.23 ± 0.05 ^a (-32.3%)	0.6 ± 0.05 ^a (76.4%)	1.6 ± 0.12 ^{a*} (370%)	0.48 ± 0.08 ^{b*} (-70%)	0.5 ± 0.06 ^{b*} (-69.6%)	1.1 ± 0.05 ^{b*} (-31.2%)
Direct bilirubin (mg/dL)	0.23 ± 0.05	0.21 ± 0.04 ^a (-8.6%)	0.51 ± 0.07 ^a (121.7%)	0.3 ± 0.05 ^a (30.4%)	1.5 ± 0.16 ^{a*} (552%)	0.31 ± 0.04 ^{b*} (-79.3%)	0.46 ± 0.08 ^{b*} (-69.3%)	0.85 ± 0.04 ^{b*} (-43.3%)

The data were analyzed by One-Way ANOVA followed by Tukey's post-hoc test. *p<0.05, ^aIndicates the comparison with control rats, ^bIndicates the comparison with CCl₄ control rats.

Data were expressed in means ± standard deviation (n= 6). The values in parenthesis represent percentage (%) increased or decreased as compared to their respective control. CCl₄: Carbon tetrachloride, WE: Water extract

Table 3. Effect of water extract of *Padina pavonia* and *Caulerpa racemosa* on renal and lipid profile in normal and CCl₄-intoxicated rats

Treatments	Normal rats model				Fibrotic rats model			
	Normal control	<i>P. padina</i> (WE)	<i>C. racemosa</i> (WE)	Silymarin	CCl ₄ control	<i>P. padina</i> (WE) + CCl ₄	<i>C. racemosa</i> (WE) + CCl ₄	Silymarin + CCl ₄
Urea (mg/dL)	27.6 ± 6.8	23.3 ± 4.0 ^a (-15.5%)	25.6 ± 5.4 ^a (-7.2%)	22.0 ± 1.00 ^a (-20.2%)	69.5 ± 10 ^{a*} (151.8%)	28.3 ± 5.4 ^{b*} (-59.2%)	30 ± 3.07 ^{b*} (-56.8%)	39 ± 0.57 ^{b*} (-43.8%)
Creatinine (mg/dL)	0.2 ± 0.04	0.2 ± 0.05 ^a (0%)	0.43 ± 0.05 ^a (115%)	0.7 ± 0.05 ^a (250%)	1.8 ± 0.14 ^{a*} (816%)	0.2 ± 0.02 ^{b*} (-88.88%)	0.25 ± 0.039 ^{b*} (-86.1%)	0.75 ± 0.05 ^{b*} (-58.3%)
Cholesterol (mg/dL)	107 ± 9.6	79 ± 7.6 ^a (-26.1%)	137.5 ± 10.6 ^a (28.03%)	80.3 ± 0.5 ^a (-24.9%)	26.6 ± 8.6 ^{a*} (-75.1%)	64 ± 5.5 ^{b*} (140.6%)	46.4 ± 4.3 ^{b*} (74.4%)	70.3 ± 11.5 ^{b*} (164.2%)
TGs (mg/dL)	103.1 ± 16.7	84.16 ± 5.8 ^a (-18.4%)	126 ± 3.8 ^a (-22.3%)	90 ± 6.00 ^a (-12.6%)	38.6 ± 3.2 ^{a*} (-62.5%)	94 ± 6.3 ^{b*} (143.5%)	58.1 ± 6.6 ^{b*} (50.5%)	50.3 ± 3.5 ^{b*} (30.3%)

The data were analyzed by One-Way ANOVA followed by Tukey's post-hoc test. *p<0.05, ^aIndicates the comparison with control rats, ^bIndicates the comparison with CCl₄ control rats.

Data were expressed in means ± standard deviation (n= 6). The values in parenthesis represent percentage (%) increased or decreased as compared to their respective control. CCl₄: Carbon tetrachloride, WE: Water extract

Rats treated with WE of *P. pavonia* significantly ($p \leq 0.05$) reciprocated the elevation of serum urea (-59.2%) and creatinine (-88.8%) with remarkable elevation in serum lipid levels i.e. cholesterol (140.6%) and TGs (143.5%). WE of *C. racemosa* showed significant ($p \leq 0.05$) reduction in serum urea (-56.8%) and creatinine (-86.1%). It also produced elevation in serum lipid levels i.e. cholesterol (74.4%) and TGs (50.5%) against CCl_4 -induced liver fibrosis in rats. Silymarin also showed a reduction in serum urea and creatinine levels i.e. (-43.8% and -58.3%, respectively, and increased the serum cholesterol and TGs levels i.e., (164.2%, 30.3%). Conclusively, both seaweed extracts have the potential to maintain the renal and lipid metabolites, which are enhanced in response to CCl_4 administration.

*Effect of water extracts of *P. pavonia* and *C. racemosa* on liver antioxidant profile*

CCl_4 administration significantly damaged the liver tissue, which ultimately depleted the GSH (-76%) and elevated the MDA (551%) concentrations. The reduction in Gpx (-49%) and CAT (-31%) activity were also observed after administration of CCl_4 , when compared with normal rats. The CCl_4 intoxicated rats concomitantly treated with WE of *P. pavonia* showed increased hepatic antioxidant enzymes activities viz; Gpx (32.2), CAT (18.5%) along with improvement in hepatic GSH concentration (141.5%), and decreased MDA (-57.4%) respectively. The same trend was observed for WE of *C. racemosa*, which significantly ($p \leq 0.05$) combated the adverse effect of CCl_4 -induced oxidative stress indices by enhancing the activities of CAT (52.6%), Gpx (23%), and improved GSH (89.2%) concentration, whereas reduced the MDA (-55.8%) level. Silymarin-treated group showed a significant ($p \leq 0.05$) improvement in oxidative stress indices i.e. GSH (115.3%), Gpx (87.7%), CAT (23.8%), and MDA (-41.7%) (Figures 1, 2). These results validated the antioxidant capability of WE of the seaweeds, as they have the potential to attenuate the oxidative stress indices in response to CCl_4 administration.

Histological changes in liver tissue of normal and fibrotic rats

*treated with water extract of *P. pavonia* and *C. racemosa**

Normal control rats showed stabilized hepatic lobules, consisted of normal central vein and peripheral 4 to 5 portal triads along with no evidence of portal expansion or necrosis (Figures 3A, B). CCl_4 intoxicated rats showed portal fibrosis with evidence of expansion in the portal tract (grade I) with massive acidophilic bodies (Figures 3C, D). Additionally, they also showed macrophage infiltration, evidence of hepatic lobular and portal inflammation (grade II & I) along with lipid deposition evident by clear vacuoles, abundance of loosely aggregated collagen fibers also seen around the portal tract. Intoxicated rats treated with WE of *C. racemosa* did not show any presence of collagen fibers, with no evidence of lobular inflammation or piecemeal necrosis (Figure 3E, F). Whereas *P. pavonia* demonstrated mild portal expansion with no evidence of lobular inflammation or piecemeal necrosis, they also showed the deposition of collagen fibers around the portal tract in lesser intensity (Figure 3G, H). The WE of both seaweeds have the potential to stabilize the normal cellular morphology. However, further investigations must unveil the potent component(s) of seaweed, which may play a role in reciprocating the toxic effect induced by CCl_4 administration.

DISCUSSION

Liver fibrosis is one of the serious health problems around the globe and it causes apoptosis or necrosis, inflammation, tissue remodeling and repair processes.^{6,31} Excessive deposition of ECM, particularly deposition of collagen type (I & III) are major cause of liver fibrosis.³²

In this study, animal model of liver fibrosis have been developed via repeated administration of CCl_4 . Chronic exposure of CCl_4 promotes degeneration of hepatocytes, which results in excessive secretion of aminotransferases (ALAT & ASAT).³³ In the harmony of previous findings, current data demonstrated significant ($p \leq 0.05$) elevation in serum levels of transaminases (ALAT & ASAT) in CCl_4 -intoxicated model. ALP is usually distributed in microvilli of liver sinusoids and bile duct capillary.

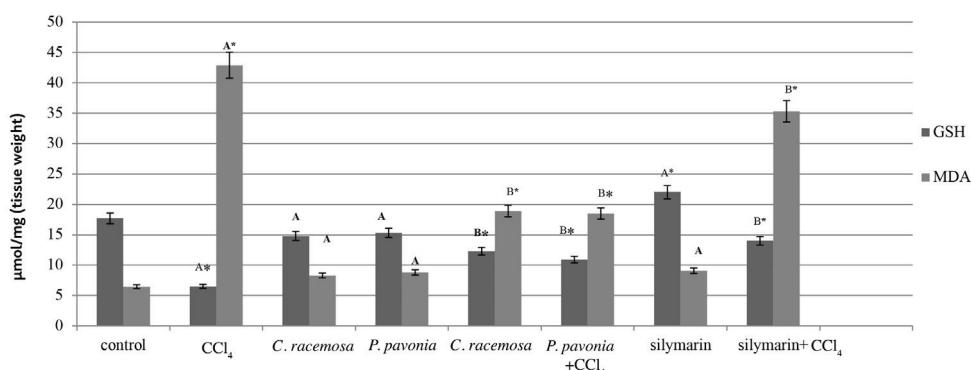


Figure 1. Effect of water extracts of *Padina pavonia* and *Caulerpa racemosa* on liver total glutathione and lipid peroxidation activities in normal and CCl_4 -intoxicated rats. The data were analyzed by One-Way ANOVA followed by Tukey's post-hoc test. Each bar expressed means \pm standard deviation (n= 6).

* $p < 0.05$, ^AIndicates the comparison with control rats while the, ^BIndicates the comparison with CCl_4 control rats, CCl_4 : Carbon tetrachloride, GSH: Glutathione, MDA: Malondialdehyde

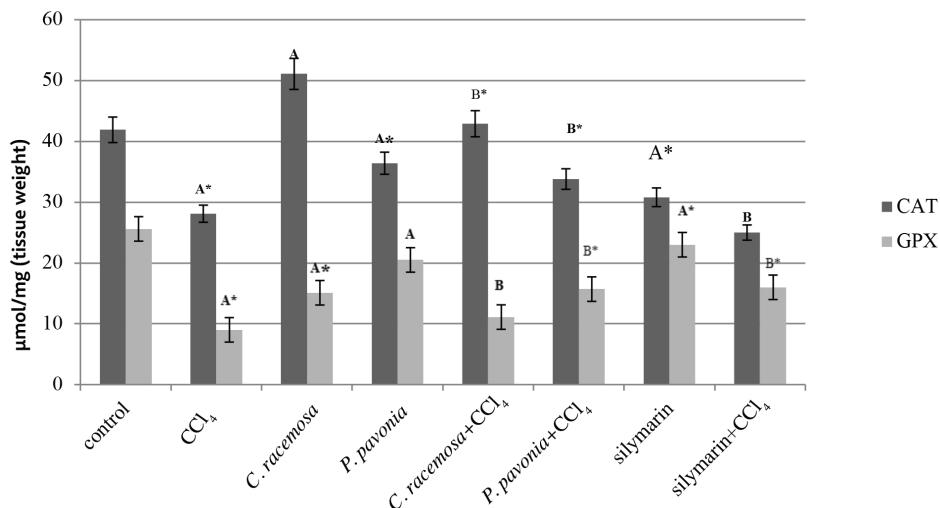


Figure 2. Effect of water extracts of *Padina pavonia* and *Caulerpa racemosa* on liver glutathione peroxidase and catalase activity in normal and CCl_4 -intoxicated rats. The data were analyzed by One-Way ANOVA followed by Tukey's post-hoc test. Each bar expressed means \pm standard deviation ($n=6$).

* $p<0.05$, ^AIndicates the comparison with control rats while the, ^BIndicates the comparison with CCl_4 control rats, CCl_4 : Carbon tetrachloride, CAT: Catalase, GPX: Glutathione peroxidase

CCl_4 administration induces hepatocyte degradation, which is responsible for exerting pressure on bile duct capillaries and promotes excessive release of ALP in serum.³⁴ Persistent liver insults by CCl_4 administration significantly ($p\leq 0.05$) elevated the serum ALP level. Moreover, the CCl_4 -intoxicated model showed a significant ($p\leq 0.05$) increase in serum LDH levels. It is usually released under hypoxic conditions, responsible for to shift metabolic cellular dependency on the anaerobic glycolytic pathway. Persistent exposure of toxicants (CCl_4) induces hepatocyte stress and promotes necrosis, which results in leakage of LDH into the serum. CCl_4 responsible for the activation of HSCs (stored retinoid) may contribute to excessive bile secretion in blood along with disturbance in redox mechanism, impaired mitochondrial integrity, and promotes apoptosis.³⁵ In the present work, CCl_4 -intoxicated model also showed a remarkable elevation in serum total bilirubin and direct bilirubin besides increasing liver enzymes. A previous study also revealed that CCl_4 induced liver fibrosis increased serum liver markers; aspartate transaminase, alanine transaminase, and total bilirubin. The current study showed that supplementation of WE of both seaweeds *C. racemosa* and *P. pavonia* caused a significant ($p\leq 0.05$) alleviation in serum levels of ALAT, ASAT, ALP, and LDH along with bilirubin in CCl_4 -induced liver fibrotic rats. Further, both seaweeds have the potential to reduce the degradation of hepatocytes and might play a role in the deactivation of activated HSC, evident by a remarkable reduction in serum aminotransferases, dehydrogenases, and phosphatase.

Among the tested seaweeds herein, *Caulerpa* species have been used around the globe due to their high nutritive value, whereas brown seaweeds have been recognized by researchers due to their medicinal importance.^{15,18} Marine macroalgae are reported to have antioxidant activity and a tendency to reciprocate the

effect induced through various toxicants.^{1,15} *P. pavonia* also demonstrated a significant effect on thioacetamide-induced hepatic fibrosis.³⁶ The hepatoprotective effect of *C. racemosa* has not been reported, yet. Repeated episodes of liver insult by CCl_4 administration caused generation of ROS, usually they are short span molecules generated by partial reduction of oxygen.³⁷ Chronic exposure of toxicants disrupts the balance between ROS and cellular antioxidant defense results in induction in oxidative stress. Further elevated levels of ROS caused depletion in antioxidants molecules and parameters i.e. GSH, Gpx, and CAT and increased LPO in terms of MDA. Nevertheless, ROS activates HSCs, which promotes the production of collagen and contributes in the progression of liver fibrosis. The present study showed a marked increase in MDA levels in CCl_4 control rats compared to normal rats. The following findings support our results that liver fibrosis promotes the generation of ROS, which ultimately caused a reduction in GSH, Gpx and CAT activities.³⁸ CCl_4 -treated rats administered with WE of both algal species significantly attenuated LPO by decreasing the formation of MDA and improved hepatic GSH levels and elevated the Gpx and CAT activities compared to CCl_4 control rats. *P. pavonia* improves the activities of superoxide dismutase and Gpx induced by azoxymethane intoxication.²¹ However, these results showed that seaweeds might affect down-regulation of ROS induced by repeated administration of CCl_4 as they showed decreased levels of MDA and improved GSH concentration and enhanced Gpx and CAT activity.

Previous findings suggested that fibrotic liver tissue showed significant morphological destruction characterized by expansion of the central vein, portal vein, and necrosis in CCl_4 treated rats.^{32,33} The current findings validate the previous findings, as repeated episodes of CCl_4 -treated rats showed expansion of the central vein, portal vein, and necrosis, whereas

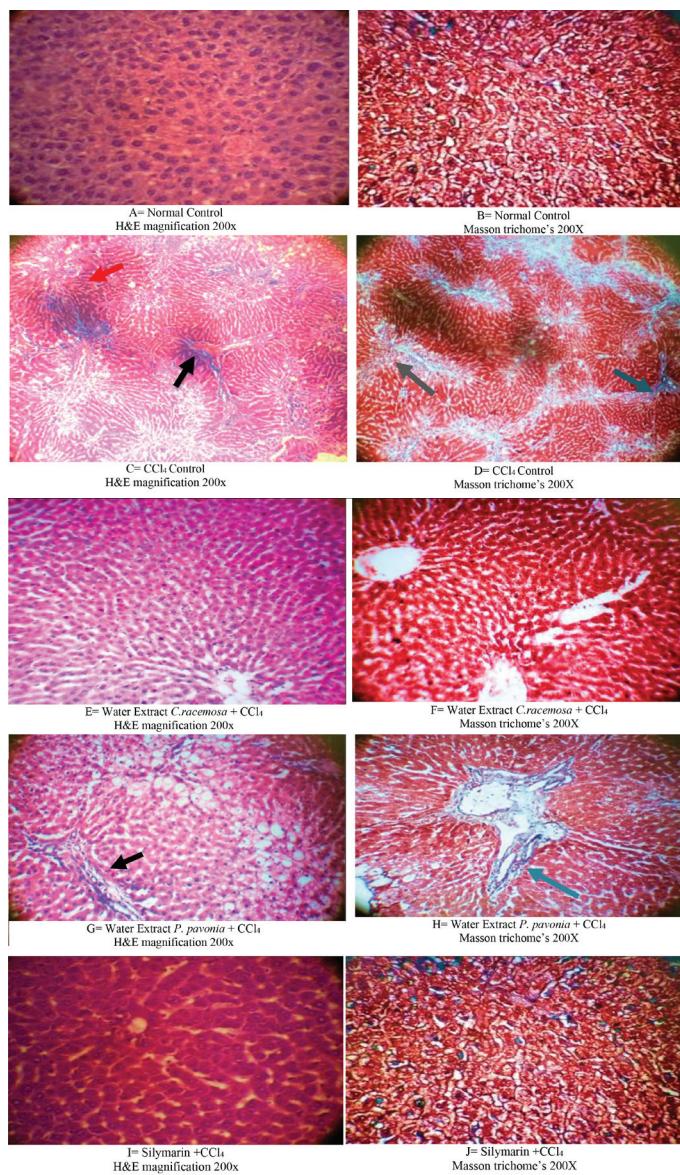


Figure 3. Light microscopy of liver tissue (200x), black arrow represent portal expansion, red arrow represent macrophages infiltration, blue arrow represent collagen deposition. Grey arrow represent necrotic cells. (A, B) Normal architecture of cellular organization along with stabilized hepatocytes, (C, D) hepatocytes damage, cellular atrophy is visible, sinusoidal cells become dilated, portal track destruction and ulcerative lesion, (E, F) stabilization of cellular morphology and attenuation of lobular destruction, foamy cells formation and lowering of intensity of highly fused cells, (G, H) stabilization of cellular morphology and attenuation of lobular destruction, foamy cells formation and lowering of intensity of highly fused cells, (I, J) stabilization of cellular morphology and lowering of lobular destruction and intensity of fused cells

CCl₄: Carbon tetrachloride, H&E: Hematoxylin and eosin

there were no changes found in normal liver tissue. In addition, intoxicated rats showed an abundance of collagen fibers, which can clearly be visualized by Masson's staining. The current study was designed to elucidate the efficacy of WEs of *C. racemosa* and *P. pavonia* against liver fibrosis. Furthermore, WEs of *C. racemosa* and *P. pavonia* showed a reciprocal effect on CCl₄ intoxication and improved the abnormal cellular

architecture of liver tissue. Both extracts showed notable reduction in the collagen fibers. Overall, results showed that these seaweeds tend to attenuate the liver fibrosis. Moreover, several reports showed that *P. pavonia* has potential to down regulate anti-apoptotic and pro-apoptotic pathways, which may relate to morphological repairs of cellular architecture.²⁰ The hepatoprotective activity of seaweeds was found to be comparable to the silymarin, in improving the cellular architecture distorted by CCl₄ intoxication.

Previous studies showed that liver-associated problems are not localized and it also affect the activity of nearby organs such as impairment in renal, lipid, and glucose metabolism.¹ Liver damage also affects renal function by increasing the concentrations of urea and creatinine. In this study, CCl₄ administration showed remarkable increases in kidney markers *i.e.*, urea and creatinine compared to normal control rats. Sohail et al.¹⁵ reported that drug induced hepatotoxicity and nephrotoxicity has been suppressed by supplementation of marine macroalgae. Another report also showed that *P. pavonia* reversed renal dysfunction induced by azoxymethane.³⁸ The current findings show that both algal species (*C. racemosa* and *P. pavonia*) extracts have the potential to attenuate the elevated levels of serum urea and creatinine. WE of both seaweeds have the potential to reciprocate the renal toxicity induced as a consequence of repeated administration of CCl₄.

The present study showed that CCl₄ administration disturbed the lipid metabolism by decreasing the cholesterol and TG levels. Ishikawa et al.³⁹ reported that CCl₄-induced liver fibrosis caused alteration in lipid metabolism evident by decreased levels of cholesterol and TGs. The distortion of hepatic parenchymal cells is responsible for impaired lipid metabolism.⁴⁰ However, the extracts of both seaweeds significantly elevated the cholesterol and TG levels toward the normal range. CCl₄ toxicity also affects the glucose metabolism, resulting in hyperglycemia in CCl₄ control rats, which was reduced in seaweed-treated groups. The ability of seaweed extracts to abrogate the elevated concentration of glucose in serum may be due to the protection of hepatocytes from toxic substances and their hypoglycemic potential.¹

CONCLUSION

The present study showed that both seaweeds (*C. racemosa* and *P. pavonia*) can reciprocate the cellular damages and associated complications produced by repeated administration of CCl₄. Both algal species showed significant positive effects on fibrotic rats. The efficacy of seaweed was compared with commonly known herbal medicine silymarin showed almost similar effect on serum enzymes and hepatic antioxidant enzymes. Generally, WE of *C. racemosa* has great potential to attenuate the liver fibrosis induced by CCl₄. Hepatoprotective activity of *C. racemosa* may be due to the presence of polysaccharides, which have been reported from other seaweeds. *C. racemosa* is an edible seaweed and its use as a diet supplement may be supportive for liver health.

ACKNOWLEDGMENTS

The help of Dr. Aisha Begum, Department of Botany, the University of Karachi for seaweed identification is acknowledged.

Ethics

Ethics Committee Approval: The approval has been received from Institutional Bioethical Committee of University of Karachi (IBC-KU-132/2020).

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: M.A., K.H., S.A.Q., N.K., J.A., S.E.H., Design: M.A., K.H., S.A.Q., N.K., J.A., S.E.H., Data Collection or Processing: M.A., K.H., S.A.Q., N.K., J.A., S.E.H., Analysis or Interpretation: M.A., K.H., S.A.Q., N.K., J.A., S.E.H., Literature Search: M.A., K.H., S.A.Q., N.K., J.A., S.E.H., Writing: M.A., K.H., S.A.Q., N.K., J.A., S.E.H.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: Financial assistance provided by the Higher Education Commission, Pakistan (Grant # nru-4505) is sincerely acknowledged.

REFERENCES

- Hira K, Sultana V, Ara J, Ehteshamul-Haque S. Protective role of *Sargassum* species in liver and kidney dysfunctions and associated disorders in rats intoxicated with carbon tetrachloride and acetaminophen. *Pak J Pharm Sci.* 2017;30:721-728.
- Blaner WS, O'Byrne SM, Wongsiriroj N, Kluwe J, D'Ambrosio DM, Jiang H, Schwabe RF, Hillman EM, Piantedosi R, Libien J. Hepatic stellate cell lipid droplets: a specialized lipid droplet for retinoid storage. *Biochim Biophys Acta.* 2009;1791:467-473.
- Laskin DL, Sunil VR, Gardner CR, Laskin JD. Macrophages and tissue injury: agents of defense or destruction? *Annu Rev Pharmacol Toxicol.* 2011;51:267-288.
- Baiocchini A, Montaldo C, Conigliaro A, Grimaldi A, Correani V, Mura F, Ciccosanti F, Rotiroti N, Brenna A, Montalbano M, D'Offizi G, Capobianchi MR, Alessandro R, Piacentini M, Schininà ME, Maras B, Del Nonno F, Tripodi M, Mancone C. Extracellular matrix molecular remodeling in human liver fibrosis evolution. *PLoS One.* 2016;11:e0151736.
- Lee UE, Friedman SL. Mechanisms of hepatic fibrogenesis. *Best Pract Res Clin Gastroenterol.* 2011;25:195-206.
- Byass P. The global burden of liver disease: a challenge for methods and for public health. *BMC Med.* 2014;12:159.
- Yoo S, Wang W, Wang Q, Fiel MI, Lee E, Hiotis SP, Zhu J. A pilot systematic genomic comparison of recurrence risks of hepatitis B virus-associated hepatocellular carcinoma with low-and high-degree liver fibrosis. *BMC Med.* 2017;15:214.
- Schiff ER, Maddrey WC, Sorrell MF. Schiff's Diseases of the Liver. (11th ed). West Sussex: John Wiley & Sons; 2011.
- Marcellin P, Gane E, Buti M, Afshar N, Sievert W, Jacobson IM, Washington MK, Germanidis G, Flaherty JF, Aguilar Schall R, Bornstein JD, Kitrinos KM, Subramanian GM, McHutchison JG, Heathcote EJ. Regression of cirrhosis during treatment with tenofovir disoproxil fumarate for chronic hepatitis B: a 5-year open-label follow-up study. *Lancet.* 2013;381:468-475.
- Dong S, Chen QL, Song YN, Sun Y, Wei B, Li XY, Hu YY, Liu P, Su SB. Mechanisms of CCl₄-induced liver fibrosis with combined transcriptomic and proteomic analysis. *J Toxicol Sci.* 2016;41:561-572.
- Halliwell B, Gutteridge JM. Free radicals in biology and medicine. (5th ed). New York, USA: Oxford University Press; 2015.
- Klaas M, Kangur T, Viil J, Mäemets-Allass K, Minajeva A, Vadi K, Antsov M, Lapidus N, Järvekülg M, Jaks V. The alterations in the extracellular matrix composition guide the repair of damaged liver tissue. *Sci Rep.* 2016;6:27398.
- Barkia I, Saari N, Manning SR. Microalgae for high-value products towards human health and nutrition. *Mar Drugs.* 2019;17:304.
- Dore CM, das C, Faustino Alves MG, Will LS, Costa TG, Sabry DA, de Souza Rêgo LA, Accardo CM, Rocha HA, Filgueira LG, Leite EL. A sulfated polysaccharide, fucans, isolated from brown algae *Sargassum vulgare* with anticoagulant, antithrombotic, antioxidant and anti-inflammatory effects. *Carbohydr Polym.* 2013;91:467-475.
- Sohail N, Hira K, Kori JA, Farhat H, Urooj F, Khan W, Sultana V, Ali MS, Ehteshamul-Haque S. Nephroprotective effect of ethanol extract and fractions of a sea lettuce, *Ulva fasciata* against cisplatin-induced kidney injury in rats. *Environ Sci Pollut Res Int.* 2020;128:9448-9461.
- Costa LS, Fidelis GP, Cordeiro SL, Oliveira RM, Sabry DA, Câmara RB, Nobre LT, Costa MS, Almeida-Lima J, Farias EH, Leite EL, Rocha HA. Biological activities of sulfated polysaccharides from tropical seaweeds. *Biomed Pharmacother.* 2010;64:21-28.
- Wang L, Wang X, Wu H, Liu R. Overview on biological activities and molecular characteristics of sulfated polysaccharides from marine green algae in recent years. *Mar Drugs.* 2014;12:4984-5020.
- Ara J, Sultana V, Qasim R, Ahmad VU. Hypolipidaemic activity of seaweed from Karachi coast. *Phytother Res.* 2002;16:479-483.
- de Gaillande C, Payri C, Remoissenet G, Zubia M. *Caulerpa* consumption, nutritional value and farming in the Indo-Pacific region. *J Appl Phycol.* 2017;29:2249-2266.
- Bernardini G, Minetti M, Polizzotto G, Biazzo M, Santucci A. Pro-apoptotic activity of French Polynesian *Padina pavonica* extract on human osteosarcoma cells. *Mar Drugs.* 2018;16:504.
- Abdella EM, Mahmoud AM, El-Derby AM. Brown seaweeds protect against azoxymethane-induced hepatic repercussions through up-regulation of peroxisome proliferator-activated receptor gamma and attenuation of oxidative stress. *Pharm Biol.* 2016;54:2496-2504.
- Ismail A Jr, Tan S. Antioxidant activity of selected commercial seaweeds. *Malays J Nutr.* 2002;8:167-177.
- Iredale JP, Benyon RC, Pickering J, McCullen M, Northrop M, Pawley S, Hovell C, Arthur MJ. Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. *J Clin Investig.* 1998;102:538-549.
- Wang L, Huang QH, Li YX, Huang YF, Xie JH, Xu LQ, Dou YX, Su ZR, Zeng HF, Chen JN. Protective effects of silymarin on triptolide-induced acute hepatotoxicity in rats. *Mol Med Rep.* 2018;17:789-800.
- Moron MS, Depierre JW, Mannervik B. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. *Biochim Biophys Acta.* 1979;582:67-78.

26. Flohé L, Günzler WA. Assays of glutathione peroxidase. *Methods Enzymol.* 1984;105:114-121.
27. Sinha AK. Colorimetric assay of catalase. *Anal Biochem.* 1972;47:389-394.
28. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem.* 1979;95:351-358.
29. Qiao JB, Fan QQ, Zhang CL, Lee J, Byun J, Xing L, Gao XD, Oh YK, Jiang HL. Hyperbranched lipid-based lipid nanoparticles for bidirectional regulation of collagen accumulation in liver fibrosis. *J Control Release.* 2020;321:629-640.
30. Batts KP, Ludwig J. Chronic hepatitis. An update on terminology and reporting. *Am J Surg Pathol.* 1995;19:1409-1417.
31. Ribeiro PS, Cortez-Pinto H, Solá S, Castro RE, Ramalho RM, Baptista A, Moura MC, Camilo ME, Rodrigues CM. Hepatocyte apoptosis, expression of death receptors, and activation of NF-κB in the liver of nonalcoholic and alcoholic steatohepatitis patients. *Am J Gastroenterol.* 2004;99:1708-1717.
32. Du WD, Zhang YE, Zhai WR, Zhou XM. Dynamic changes of type I, III and IV collagen synthesis and distribution of collagen-producing cells in carbon tetrachloride-induced rat liver fibrosis. *World J Gastroenterol.* 1999;5:397-403.
33. Yu C, Wang F, Jin C, Wu X, Chan WK, McKeehan WL. Increased carbon tetrachloride-induced liver injury and fibrosis in FGFR4-deficient mice. *Am J Pathol.* 2002;161:2003-2010.
34. Hu J, Zhang X, Gu J, Yang M, Zhang X, Zhao H, Li L. Serum alkaline phosphatase levels as a simple and useful test in screening for significant fibrosis in treatment-naïve patients with hepatitis B e-antigen negative chronic hepatitis B. *Eur J Gastroenterol Hepatol.* 2019;31:817-823.
35. Qaisiya M, Coda Zabetta CD, Bellarosa C, Tiribelli C. Bilirubin mediated oxidative stress involves antioxidant response activation via Nrf2 pathway. *Cell Signal.* 2014;26:512-520.
36. Hamza AH, Hegazi MM, Youness ER, Ahmed HH. Brown algae as a golden mine for treatment of liver fibrosis: a proposal based on experimental animal study. *Int J Curr Pharm Rev Res.* 2015;6:225-236.
37. Lambeth JD. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol.* 2004;4:181-189.
38. Mahmoud AM, El-Derby AM, Elsayed KN, Abdella EM. Brown seaweeds ameliorate renal alterations in mice treated with the carcinogen azoxymethane. *Int J Pharm Pharm Sci.* 2014;6:365-369.
39. Ishikawa M, Saito K, Yamada H, Nakatsu N, Maekawa K, Saito Y. Plasma lipid profiling of different types of hepatic fibrosis induced by carbon tetrachloride and lomustine in rats. *Lipids Health Dis.* 2016;15:74.
40. Havel RJ. Functional activities of hepatic lipoprotein receptors. *Annu Rev Physiol.* 1986;48:119-134.