Int J Clin Exp Med 2017;10(3):5561-5577
www.ijcem.com /ISSN:1940-5901/IJCEM0042444

Original Article

Evaluation of iron-chelating activity of *Caulerpa racemosa* in iron-dextran induced iron overload in an experimental model of thalassemia

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Received July 9, 2016; Accepted January 4, 2017; Epub March 15, 2017; Published March 30, 2017

Abstract: Iron overload is induced as a result of a lot of disorders, particularly in thalassemia major, and is considered the main cause of mortality in spite of the recently-achieved advances in chelation therapy. The main aim of the present study was to investigate the *in vivo* antioxidant and Fe-chelating characteristics of both aqueous and ethanolic extracts of the marine green macroalga *Caulerpa racemosa* (Chlorophyta) to clearly assess its possible applications in Fe-chelating therapy, and to reduce iron-related complications for the Improvement of patients' lives. Forty male albino rats were randomly split into four equal groups: The first group was the control; the second one represented the iron overload group (ID); the third group was treated with the iron overload and an aqueous extract of *C. racemosa*; and the fourth group was composed of the iron overload and *C. racemosa* ethanolic extract. Rats were received six doses of iron dextran (12.5 mg/100 gm body weight (B.W.) by intraperitoneal injections (IP) and administrated *C. racemosa* (200 mg/kg B.W.) as one daily IP until the end of the experiment. The levels of iron depositions in liver, heart and brain were significantly increased in the ID treatment group compared to the control. Serum ferritin, total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC) and transferrin were also highly increased in the ID treatment group. Nevertheless, these iron profiles were significantly decreased in the ID + *C. racemosa* treatment groups (both the aqueous and ethanolic algal extracts) compared to the ID group only. Moreover, *C. racemosa* extracts distinctly down-modulated iron overload causing dramatic increases in serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK) and lactate dehydrogenase (LDH), which were significantly increased in the ID group compared to the control. This study showed that treatments with *C. racemosa* extracts effectively ameliorated the increased malondialdehyde (MDA) and nitric oxide (NO). A significant decrease in antioxidant enzyme activities such superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (Gpx) and catalase (CAT) activities were only observed in the ID group compared to the control. However, these enzymes were significantly increased in the *C. racemosa*-treated groups. No toxic effects were distinctly detected in rats treated with the applied aqueous and ethanolic *C. racemosa* extracts and this obaservation was confirmed by the histopathological studies. In conclusion, this study confirms the *in vivo* evidence that *C. racemosa* administration, especially the ethanolic extract, can highly improve the antioxidant defense systems against ID-induced hepatic, cardiac and neuro-oxidative stresses in rats. These protective characteristics of *C. racemosa* might be attributed to its remarkable antioxidant (total phenolics, total flavonoids, DPPH and ABTS radical scavenging, and reducing power) and Fe-chelation properties.

Keywords: Antioxidants, *Caulerpa racemosa* (green macroalga), iron overload, Fe-chelation, oxidative stress, thalassemia therapy

Introduction

Beta thalassemia syndromes are a group of hereditary diseases characterized by a genetic disorder causing failure of synthesis of beta-globin chains. In a homozygous state, this condition is known as thalassemia major which causes early and severe transfusion-dependent anemia, while it is identified as thalassemia minor when causes mild microcyfic anemia [1].

Iron overload constitutes the major cause of morbidity and mortality in beta thalassemia. It results from lifelong transfusion and increased iron absorption. Secondary iron over-

load causes many clinical problems such as endocrine dysfunctions, liver dysfunction, or cardiac iron deposition, which leads to life threatening high output cardiac failure [2].

Without chelating iron molecules, toxicity is an expected result of iron overload due to the remarkable increase in iron absorption from numerous blood transfusions. The presence of non-transferrin-bound iron (NTB7) causes formation of free radicals and creation of an oxidative stress, which result in damage of cell organelles and DNA [3]. Iron overload causes multiple morbidities like various endocrinopathies due to iron deposition in pituitary gland causing impaired growth and hypogonadism in thalassemia children. Furthermore, iron deposition in the pancreas causes diabetes, induces cardiomyopathy and hepatic cirrhosis as a result of iron accumulation in the heart and liver, respectively [4].

Seaweeds or marine algae are in general characterized by the presence of a wide spectrum of bioactive compounds and unconventional chemical structures with many therapeutic effects [5-8]. Some of these components are mainly functional to reduce the reactive oxygen species-related hazard effects and consequently exhibiting some medicinal benefits due to their specific antioxidant [9] and metal chelation properties [10]. The green macroalga *Caulerpa racemosa* (Forsskål) J. Agardh mainly grows in the tropical and subtropical regions worldwide [11]. It is usually eaten as a salad or cooked in some South East Asian countries [12], besides its wide utilization in folk medicine to reduce high blood pressure and to treat rheumatism [13]. Marine algae is usually rich in dietary fibers, minerals, lipids, proteins and vitamins A, B, C, and E [14-16]. Phenolic components are considered as one of their chief phytochemicals acting as antioxidants, reducing agents, metal chelators and singlet oxygen quenchers [17]. However, metal-chelating efficacy in some medicinal plants is mainly relying on some other phytochemical constituents especially flavonoids [18].

Despite the synthetic iron-chelating drugs, such as deferoxamine and deferiprone, are commonly used to prevent the increase of iron concentrations in patients' bodies, but their long-time applications cause clinically a shortage in white blood cells, mainly neutrophils [19].

Iron-chelation therapy aims to reduce the iron-related morbidity and slow down apparition of some diseases that might affect the digestive system and the endocrine. In some cases, this kind of therapy could abolish the cardiac disorders and improve human life. Consequently, iron-chelation therapy is considered an important prerequisite to reduce the expected mortality in thalassemia disease. Iron particles could be an important promoter of free-radical reactions which convert the less reactive species to more reactive ones. Therefore, living organisms have defense systems that prevent or correct such kinds of iron-induced oxidative stresses, i.e. they are characterized by strict iron sequestration and compartmentalization of iron compounds which are capable of catalyzing reactions with molecular oxygen, different enzymes and substances which can remove the harmful reactive oxygen species.

The main aim of this study was to investigate in-depth the potential effects of aqueous and ethanolic *C. racemosa* algal extracts for the treatment of iron-dextran inducing iron overload as an experimental model of thalassemia.

Materials and methods

Algal materials: collection and extraction

Caulerpa racemosa (Forsskål) J. Agardh specimens were collected in April 20th 2009 during a low tide at Al-Qusayr province (26° 07` N, 34° 13` E), Red Sea (Egypt). The materials collected were firstly washed well in the field to remove any epiphytes and then with distilled water at the lab to be completely free from any debris, salts and sand particles. They were air-dried in shade for two weeks. The dried algal specimens were ground well by a blender to 2-mm size or somewhat smaller particles. The algal materials were then stored in clean plastic bags at room temperature in a dry dark place until using. The specimens were morphologically identified following the relevant literature adopted by Aleem [20].

The aqueous and ethanolic *C. racemosa* extracts (1:10 w/v) were prepared according to the method describe by Tariq [21] with a little modification. The dried specimens were homogenized with distilled water and ethanol (95%), and then were left overnight at room temperature. After that, the algal extracts were agitated

in an orbital shaker at 120 rpm for 2 h and then were filtered through Whatmann® filter papers No. 1. This process was repeated thrice. All the three filtrates were collected and concentrated to dryness on a rotary evaporator at 40°C (Büchi R-200). The residues were stored at -20°C in liquid nitrogen until the further investigations and were dissolved in 0.9% NaCl saline solution on the application.

Determination of the algal bioactive compounds

Total phenolic content: The total phenolics (TP) of *C. racemosa* were spectrophotometrically determined using Folin-Ciocalteu reagent assay [22] with a little modification. A suitable aliquot (1 ml) of the water and ethanolic extracts, or the standard solution used for preparation of the calibration curve (Gallic acid 20-120 mg/L), was added to 25 ml volumetric flask containing 9 ml of distilled water. After that, one milliliter of Folin-Ciocalteu's phenol reagent was added to the mixture and shaken well. After 5 minutes, 10 ml of 7% Na_2CO_3 solution was added to the mixture. The solution was diluted to 25 ml with distilled water and mixed well. After incubation for 90 min at room temperature, the absorbance was determined at 750 nm using UV/VIS spectrophotometer Unicum UV-300. Phenolic contents were calculated based on the standard curve of Gallic acid (GAL). The results were expressed as mg Gallic acid (GAE) equivalent per gm dry weight of the algal extract.

Total flavonoid content: Total flavonoids (TF) were spectrophotometrically determined following the aluminum chloride method adopted by Zhishen [23] and using quercetin (QU) as a standard. One ml of the ethanolic and water algal extracts, or the standard solution (quercetin 20-120 mg/L), was mixed with 4 ml of distilled water and 0.3 ml of 5% $NaNO_2$. After 5 min, 0.3 ml of 10% $AlCl_3$ was added. At the 6^{th} min, 2 ml of 1 M NaOH were added and the total volume was completed to 10 ml with distilled water. This mixture was shaken well and the intensity of pink color was measured at 510 nm using UV/VIS spectrophotometer Unicum UV-300. The total flavonoids were expressed as mg quercetin (QU) equivalent per gm dry weight of the algal extract.

Antioxidant characterizations

DPPH radical scavenging assay: It was determined spectrophotometrically as shown previously [24]. 0.1 mM of DPPH (*2, 2-diphenyl-1-picryl hydrazyl*) in methyl alcohol was prepared and 0.5 ml of this solution was added to one ml of each algal extract at different concentrations (250, 500, 750, 1000 µg/ml). Ethanol and distilled water were used as a blank. The mixture was shaken vigorously and allowed to stand at room temperature. Butyl Hydroxytoluene (BHT, Sigma) was used as positive control and the negative control contained the entire reaction reagents except the algal extracts. The absorbance was measured at 517 nm against the blank. The lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The capacity to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging activity (inhibition %) = $(A_c\text{-}A_s/A_c) \times 100$

Where, A_c: absorbance of the control reaction; A_s: absorbance in presence of the algal extract.

ABTS radical scavenging assay: ABTS [*2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid*)] radical scavenging assay was generated by oxidation of ABTS radicals with potassium persulphate [25]. ABTS was dissolved in deionized water to 7.4 mM concentration, and potassium persulphate added to a concentration of 2.6 mM. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12-16 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using the spectrophotometer. The fresh ABTS solution was prepared for each assay. 150 µl of each ethanolic and aqueous algal extract at different concentrations (250, 500 and 750 µg/ml) were allowed to react with 2850 µl of the ABTS solution for 2 h in a dark condition. The absorbance was detected at 734 nm using the spectrophotometer. Results were expressed as in comparison with standard BHT. The highest antioxidant capacity of the sample exhibited a smaller production of free radicals.

Inhibition (%) = $(A_0\text{-}A_1/A_0) \times 100$

Where, A_0: ABTS absorbance of the control reaction; A_1: the ABTS absorbance in presence of the sample.

The reducing power: It was assayed spectrophotometrically based on the method prescribed by Oyaizu [26]. Briefly, different concentrations (100, 200 and 300 µg/ml) of each algal extract (1.0 ml) were mixed with 2.5 ml of phosphate buffer (50 mM, pH 7.0) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50°C for 20 min. Afterwards, 2.5 ml of trichloroacetic acid (10%) was added to the mixture, centrifuged at 3000 rpm for 10 min. Finally, 1.25 ml from the supernatant was mixed with 1.25 ml of distilled water and 0.25 ml $FeCl_3$ solution (0.1%). The absorbance was measured at 700 nm. The assay was carried out in triplicate and the results were expressed as mean values ± standard deviations. Increased absorbance values indicate to a higher reducing power. BHT was used as standard

Animals

Male Wistar albino rats having a weight of 180-200 g were kept in quarantine for 10 days under standard husbandry conditions (25.6°C, Relative humidity 60 ± 10%) for 12 hrs in dark and light cycle, respectively, and were supplied standard food and water *ad libitum*. This work was permitted by the Animal Auspices Committee of the National Center for Radiation Research (NCRR), Cairo, Egypt.

Acute toxicity testing

This test was performed for the ethanolic and aqueous *C. racemosa* extracts following the method described by Lorke [27]. A fixed dose level of each algal extract, starting from 50, 100, 1000, and increasing up to 2000 mg/kg body weight, was given and the symptoms of toxicity were observed for the next 48 hours.

Experimental design

Forty male albino rats weighing about 180-200 g were obtained from the Research Institute of Ophthalmology, Giza (Egypt). These animals were separately housed in well-aerated cages and fed on basal diet for 10 days as an adaptation period. Temperature and humidity were preserved at 25°C and 60%, respectively, and the animals were provided with adequate amounts of food and water. The animals were segregated into four groups, where each one is composed of ten rats. The algal extract was administered for four weeks. Group (I) was served as the normal control rats administered daily the saline solution for four weeks. Group (II) included the iron overloaded rats where they were administered received six doses (three doses per week) of 12 mg/100 gm BW of iron dextran via IP-injections and received daily saline for four weeks. Group (III) represented the iron overload rats administered by *C. racemosa* aqueous extract (200 mg/kg) IP for four weeks. Group (IV) comprised the iron overload rats administered by *C. racemosa* ethanolic extract (200 mg/kg) for four weeks.

Blood samples

Blood samples were collected by an ocular vein puncture in dry, clean and screw covered tubes. Sera were centrifuged at 2500 RPM for 15 min. The clear sera were separated and kept in a deep freeze at -20°C until using for subsequent biochemical analyses.

Liver, heart and brain tissues

At the end of the trial period, rats were euthanized. The liver, heart and brain specimens were quickly removed and perfused with cold saline to exclude the blood cells and then plotted on filter paper; then stored at -20°C. Briefly, parts of the liver tissue were weighed and crushed into small pieces, homogenized with a glass homogenizer in 9 volumes of ice-cold 0.05 mM potassium phosphate buffer (pH 7.4) to produce 10% homogenates. These homogenates were centrifuged at 5,000 RPM for 15 min at 4°C, and then the supernatant was used for the subsequent biochemical analysis. The other parts of the livers were weighed and put into glass flask, then 5 volumes of mixed acid (4 nitric acid: 1 Perchloric acid) were added and heated. The volumes of the digested specimens were adjusted to 10 ml with twofold distilled water. The obtained solutions were used to analyze iron contents.

Serum TIBC, UIBC, as well as transaminases (AST and ALT) activities were determined following the proceeding of Reitman and Frankel [28]. Moreover, iron in the liver, heart and brain, NO, Ferritin were tested using radioimmunoassay (RIA) techniques and commercial kits relying on solid phase RIA (Coat-A-Count) Diagnostic Product Corporation (DPC), Los Angles, USA.

Determination of malondialdehyde (MDA) levels

Lipid peroxidation in the liver was achieved by configuration of malondealdehyde (MDA)

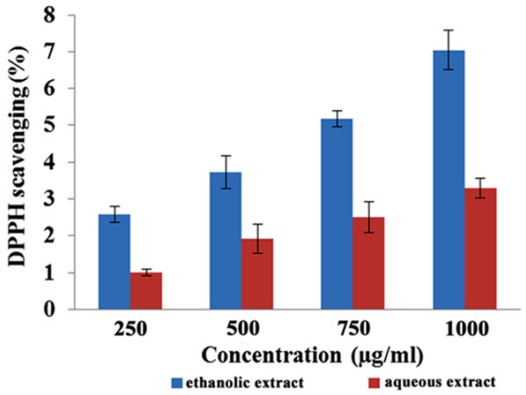

Figure 1. DPPH free radical scavenging inhibition (%) in the ethanolic and aqueous extracts of *C. racemosa* specimens included in this study. Values are expressed as mean ± standard deviation.

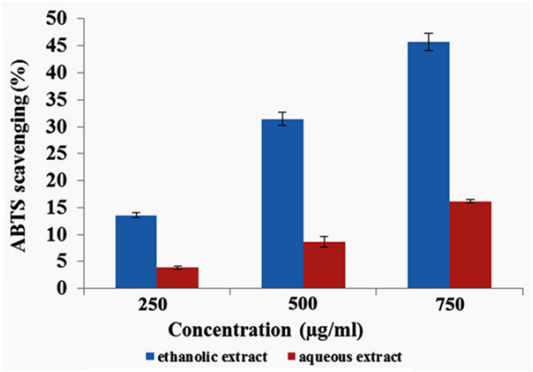

Figure 2. ABTS free radical scavenging inhibition (%) in the ethanolic and aqueous extracts of *C. racemosa* specimens included in this study. Values are expressed as mean ± standard deviation.

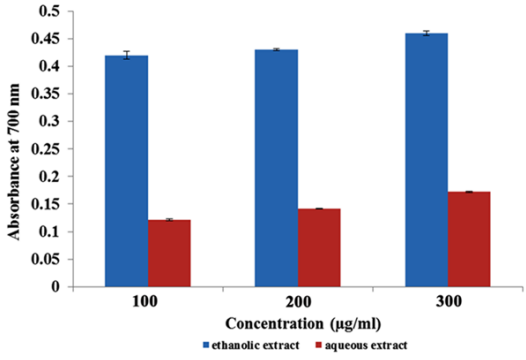

Figure 3. Reducing power of different concentrations of the ethanolic and aqueous extracts of *C. racemosa* specimens included in this study. Values are expressed as mean ± standard deviation.

and accomplished by thiobarbituric reactive (TBARS) procedure previously described by Onkawa *et al.* [29]. A reaction mixture containing homogenate (0.5 ml), about 0.5 ml of trichloroacetic acid (TCA), and 0.5 ml thiobarbituric acid (TBA) was incubated in boiling water for 15 min. The pink color of chromogen was extracted in butanol solution (2.0 ml). The admixture was centrifuged at 3000 RPM for 10 min and the absorbance of the supernatant was read at 532 nm.

Determination of glutathione (GSH and Gpx) levels in liver, heart and brain tissues

The reduced glutathione (GSH) level was determined using method previously by Ellman [30]. Homogenate (0.2 ml) was added with 25% TCA and centrifuged at 3000 RPM for 10 min. Supernatant (0.2 ml) was added with 10 mM of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in the presence of phosphate buffer (0.1 M, pH 7.4). Absorbance was read at 420 nm. As well as, the GPx activity was performed as the amount of glutathione oxidized according to the method adopted by Paglia and Valentine [31].

Determination of superoxide dismutase (SOD and GST) activities in liver, heart and brain tissues

The Cytosol fraction of the liver was used in this assay as previously described in [32]. The cytosolic fraction (0.05 ml) was added with sodium pyrophosphate buffer (0.052 M, pH 8.3, 1.2 mL), phenazine methosulphate (0.186 mM, 0.1 ml), nitroblue tetrazolium chloride (0.3 mM, 0.3 mL), and NADH (0.78 mM, 0.2 ml). The reaction was stopped after 90 sec with glacial acetic acid. The color intensity of the chromogen was extracted in butanol solution (2.0 ml) and shake strenuously. The mixture then was centrifuged at 3000 RPM for 10 min and the supernatant was measured at 560 nm.

Determination of catalase (CAT) activities in liver, heart and brain tissues

The catalase enzyme activity in tissues was assayed following the procedure of Sinha [33]. Homogenate (0.1 ml) was incubated with H_2O_2 (0.2 M, 0.5 ml) in the presence of 0.01 M phosphate buffer (pH 7.4). By adding 5% dichromate solution, the reaction was stopped. After that, samples were incubated in boiling water for 15 min. Phosphate buffer (2.0 ml) was added and shaken busily. The upper layer of the mixture was taken and the absorbance read at 570 nm.

Table 1A. Pharmacological study acute toxicity (LD) testing for the *C. racemosa* ethanolic extract

Doses (mg/kg)	The result of the first phase (mortality) n = 5
50	0/5
100	0/5
1000	1/5
Doses (mg/kg)	The result of 2nd phase (mortality) n = 5
1600	1/5
2900	2/5
5000	3/5

LD_{50} = 1300 mg/k.

Table 1B. Pharmacological study acute toxicity (LD) testing for the *C. racemosa* aqueous extract

Doses (mg/kg)	The result of the first phase (mortality) n = 5
50	0/5
100	0/5
1000	0/5
Doses (mg/kg)	The result of 2nd phase (mortality) n = 5
1600	0/5
2900	0/5
5000	1/5

LD_{50} > 2900 mg/kg.

Histopathological examination

Autopsy samples were taken from the livers, hearts and brains of rats of different groups and fixed in 10% formalin saline for 24 hrs. Washing was done with tap water, 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 degrees in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by slidge microtome. The obtained tissue sections were collected on glass slides, deparaffinized, stained by hematoxylin & eosin stain for routine examination uing the light microscopy [34].

Statistical analysis

The results were statistically analyzed using SPSS program (version 22.0), and expressed as mean and standard deviations (SE). Statistical significance ($P < 0.05$) between the groups was determined by one-way ANOVA followed by Tukey's multiple range test.

Results

In vitro analysis

Total phenolics, total flavonoids and antioxidant characterizations of C. racemosa specimens studied: Using the Folin Ciocalteu method, total phenolics of *Caulerpa racemosa* specimens studied are in general low in concentration. However, the ethanolic extract relatively exhibits a little higher concentration (0.57 ± 0.07 mg GAE/gm DW) than the aqueous one (0.13 ± 0.02 mg GAE/gm DW). The highest concentration of total flavonoids is also detected in the ethanolic extract with a value of 0.104 ± 0.005 mg QE/gm DW as compared to 0.03 ± 0.002 QE/gm DW in the water extract.

It is clearly evident from **Figures 1**-**3** that the antioxidant characterizations of the *C. racemosa* ethanolic extract including DPPH and ABTS free radical scavenging capacities, besides the reducing power, are distinctly higher than those detected in the aqueous extract. Furthermore, the scavenging capacity of each algal extract is mainly dependent on its concentration, i.e. the free radicals scavenging potentiality become higher with an increase in each algal extract concentration.

In vivo analysis

Acute toxicity testing was performed to determine LD_{50} of the extracts of tested algae and to ensure the use of safe doses in screening the therapeutic effect for iron overload. The water extracts of algae produced no mortality with increasing doses up to 1000 mg/kg BW. The LD_{50} of ethanolic extract was determined and the results are shown in (**Table 1A** and **1B**).

ID resulted in hepatic, cardiac and Neuro-iron levels of 966 ± 12.77, 272.60 ± 12.11 and 187.84 ± 8.11 µg of the Fe/dl of tissue respectively compared to control group (**Table 2**). Ethanolic and aqueous extracts of *C. rasemosa* at 200 mg/kg showed significant ($P < 0.05$) iron chelating activities compared to ID group. These algal extracts with dose 200 mg/kg reduced the iron deposition in the liver, heart and brain in iron-overloaded rats. The serum levels of ferritin, TIBC, and transferrin except

Table 2. Effects of *C. racemosa* extracts on total iron deposition (μg/dl) in liver, heart and brain in normal and iron overload in the experimental modal

Groups	Control G (I)	ID G (II)	Treatments with *C. racemosa* extracts			P
			G (III)	G (IV)	F-value	
Liver	276.48 ± 9.76[b]	966.73 ± 12.77[a]	313.45 ± 10.41[b] -208.63%	282.02 ± 7.96[b] -242.79%	45.58*	0.000
Heart	102.40 ± 6.90[b]	272.60 ± 12.11[a]	106.8 ± 6.56[b] -155.24%	100.06 ± 4.31[b] 172.44%	14.27*	0.003
Brain	91.70±4.45[c]	187.60±8.11[a]	105.24 ± 6.43[b] -78.26%	94.76 ± 7.04[b,c] -97.97%	28.89*	0.000

Data expressed as mean ± SE. Small letters: Statistically significant from control or experimental groups at $P < 0.05$ using one-way ANOVA followed by Tukey as a post-hoc test. *F value ($P < 0.05$). ID G (II): iron overload group; G (III): iron overload group treated with the aqueous extract of *C. racemosa*; G (IV): iron overload group treated with the ethanolic extract of *C. racemosa*.

Table 3. Effects of *C. racemosa* extracts on serum ferritin, transferrin and TIBC and UIBC in normal and iron overload in the experimental model

Groups	Control G (I)	ID G (II)	Treatments with *C. racemosa* extracts			P
			G (III)	G (IV)	F-value	
Ferritin (ng/ml)	3.78 ± 0.36[c]	12.20 ± 2.10[a]	5.17 ± 0.33[b] -135.98%	3.85 ± 0.36[b] -216.88%	26.76*	0.000
TIBC (μg/dl)	185.64 ± 8.16[b]	289.31 ± 10.22[a]	191.33 ± 8.76[b] -51.21%	187.60 ± 6.89[b] -54.22%	19.25*	0.000
UIBC (μg/dl)	72.60 ± 5.21[a,b]	40.08 ± 3.35[c]	68.33 ± 4.55[b] 41.34%	80.83 ± 3.75[a] 50.41%	20.24*	0.000
Transferrin (μg/dl)	3.69 ± 0.39[b]	13.80 ± 2.15[a]	4.10 ± 0.68[b] -236.59%	3.81 ± 0.35[b] 262.20%	18.36*	0.000

Data expressed as mean ± SE. Small letters: Statistically significant from control or experimental groups at $P < 0.05$ using one-way ANOVA followed by Tukey as a post-hoc test. *F value ($P < 0.05$). ID G (II): iron overload group; G (III): iron overload group treated with the aqueous extract of *C. racemosa*; G (IV): iron overload group treated with the ethanolic extract of *C. racemosa*.

UIBC were increased in all iron dextran receiver groups and the levels were significantly different from the values obtained for the control group ($P < 0.05$) (**Table 3**). Furthermore, ID ID induced significant increases in AST, ALT, ALP and LDH, CK and MDA (**Table 4**). There were also significant reductions in serum iron levels in treatment groups with the ethanolic and aqueous extracts of *C. racemosa* compared to the ID group at the end of four weeks treatment period. On the contrary, ID induced a significant decrease in NO.

There were significant decreases in antioxidant levels in the liver, heart and brain tissues in the ID group, indicating to liver damage due to iron overload in the body. Treatments with the aqueous and ethanolic extracts of *C. racemosa* produced remarkably significant increases in the antioxidant levels, and this might be attributed to the protective potential effects of these algal extracts in liver complications due to iron overload (**Tables 5-7**).

Histopathological findings

Liver: The liver of control rats showed a normal structure (**Figure 4A**), which was influenced by the administration of chronic iron dextran. Iron dextran treated rats show loss of architecture, fibrosis and fatty infiltration (**Figure 5A**). After chronic iron administration, there was heavy iron deposition in all of hepatocytes and Kupffer cells observed. Also the trabecular structure of the lobules was slightly or distinctly blurred and hepatocytes and necrotic cells were observed. Treatments with the aqueous and ethanolic *C. racemosa* extracts demonstarted minimal vacuolation, fibrosis, less disarrangement and degeneration of hepatocytes and the degree of protection was found to be lower in the water extracts (**Figures 6A** and **7A**, respectively).

Heart: Hearts from rats injected chronically with iron displayed extensive interstitial fibrosis and myocyte vacuolar degeneration with mild

Table 4. Effects of *C. racemosa* extracts on the liver function, LDH, CK, MDA & NO in iron overload experimental model

Groups	Control G (I)	ID G (II)	Treatments with *C. racemosa* extracts			P
			G (III)	G (IV)	F-value	
AST (U/l)	43.6 ± 2.72[b]	112.28 ± 6.71[a]	52.12 ± 3.97[b] -115.43%	46.69 ± 5.99[b] -140.48	26.53*	0.000
ALT (U/l)	58.30 ± 4.07[b]	131.40 ± 6.41[a]	65.12 ± 4.19[b] -101.78%	62.60 ± 6.92[b] 109.90%	31.62*	0.000
ALP (U/l)	158.20±8.12[b]	439.60±12.54[a]	168.14±3.20[b] -220.92%	150.24±4.79[b] -192.60%	64.43*	0.000
LDH (U/l)	203.01 ± 11.20[b]	528.70 ± 16.30[a]	210.20 ± 8.76[b] -151.52%	204.81 ± 6.76[b] -158.14%	34.30*	0.000
CK (U/l)	159.20 ± 7.43[c]	392.22 ± 10.02[a]	178.01 ± 6.43[b] -120.34%	166.80 ± 4.56[b,c] -135.14	27.48*	0.000
MDA(nmol/ml)	13.60 ± 1.81[b]	38.23 ± 1.51[a]	16.93 ± 1.02[b] -125.81	14.50 ± 1.27[b] -163.66%	66.51*	0.000
NO (µmol/l)	67.40 ± 4.02[b]	40.20 ± 2.90[c]	74.40 ± 2.87[a,b] +45.97%	79.94 ± 3.51[a] +49.71%	13.08*	0.000

Data expressed as mean ± SE. Small letters: Statistically significant from control or experimental groups at $P < 0.05$ using one-way ANOVA followed by Tukey as a post-hoc test. *F value ($P < 0.05$). ID G (II): iron overload group; G (III): iron overload group treated with the aqueous extract of *C. racemosa*; G (IV): iron overload group treated with the ethanolic extract of *C. racemosa*.

Table 5. Effects of *C. racemosa* extracts on antioxidants of the liver in normal and iron overload in the experimental model

Groups	Control GI	ID G (II)	Treatments with *C. racemosa* extracts			P
			G (III)	G (IV)	F-value	
GSH (µg/dl)	82.14 ± 3.39[a]	50.12 ± 2.91[b]	73.12 ± 2.76[a] +31.456%	79.85 ± 4.11[a] +37.23%	13.69*	0.000
GST (µ/g)	4.56 ± 0.47[a]	3.15 ± 0.37[b]	5.41 ± 0.23[a] +51.77%	5.14 ± 0.23[a] +38.72%	9.11*	0.005
GPx (µg/g)	473.87 ± 14.16[a]	317.60 ± 10.32[b]	441.00 ± 16.07[a] +27.98%	453.00 ± 18.22[a] +29.89%	18.76*	0.000
CAT (µ/g)	22.04 ± 2.50[a]	12.37 ± 1.79[b]	22.56 ± 2.28[a] +45.17%	24.54 ± 2.30[a] +49.59%	4.71*	0.004
SOD (U/g)	282.20 ± 14.93[a]	166.32 ± 11.56[b]	258.21 ± 8.76[a] +35.59%	279.32 ± 7.60[a] +40.46%	11.74*	0.000

Data expressed as mean ± SE. a & b: Statistically significant from control or experimental groups at $P < 0.05$ using one-way ANOVA followed by Tukey as a post-hoc test. *F value ($P < 0.05$). ID G (II): iron overload group; G (III): iron overload group treated with the aqueous extract of *C. racemosa*; G (IV): iron overload group treated with the ethanolic extract of *C. racemosa*.

inflammatory infiltrate compared to the control (**Figures 4B** and **5B**, respectively). There were vascular hemorrhage and hypertrophy observed in iron overload rats compared to control. Both algal treatments of *C. racemosa* showed protective effects on myocytes, as well as reduced fibrosis and hypertrophy of myocytes. Vascular hemorrhages were also found to be reduced in iron-overloaded rats treated with these algal extracts (**Figures 6B** and **7B**, respectively).

Brain: The hippocampus showed a nuclear pyknosis in neurons associated with focal hemorrhage, in addition to focal eosinophilic plagues detected in stratum of the ID group (**Figure 5C**), comparing to the control (**Figure 1C-E**). There was also no histopathological alteration in both cerebral cortex and hippocampus in rat models administered with the aqueous and ethanolic *C. racemosa* extracts (**Figures 6C** and **7C**).

Discussion

Marine algal extracts have already documented to largely comprise a marvelous group of antioxidants [8]. This study showed that the Egyptian marine green macroalga *Caulerpa race-*

Table 6. Effects of *C. racemosa* extracts on antioxidants of the heart in normal and iron overload in the experimental model

Groups	Control G (I)	ID G (II)	Treatments with *C. racemosa* extracts			P
			G (III)	G (IV)	F-value	
GSH (µg/dl)	85.40 ± 2.11[a]	41.84 ± 3.29[b]	79.44 ± 84.17[a] +47.33%	84.17 ± 2.92[a] +50.29	19.30*	0.000
GST (µ/g)	4.52 ± 0.22[a]	2.49 ± 0.17[c]	3.73 ± 0.11[b] +33.24%	4.24 ± 0.20[a,b] +41.27%	25.45*	0.000
GPx (µg/g)	357.40 ± 12.31[a]	186.60 ± 9.76[b]	355.50 ± 13.08[a] +47.51%	349.60 ± 14.21[a] +46.62%	24.64*	0.000
CAT (µ/g)	30.57 ± 2.82[a]	14.60 ± 1.21[b]	28.04 ± 2.22[a] +47.93	27.20 ± 2.13[a] 46.32%	5.97*	0.002
SOD (U/g)	167.44 ± 10.13[a]	135.61 ± 4.34[b]	162.60 ± 2.76[a] +16.60%	165.42 ± 4.45[a] +18.02%	11.74	0.000

Data expressed as mean ± SE. Small letters: Statistically significant from control or experimental groups at $P < 0.05$ using one-way ANOVA followed by Tukey as a post-hoc test. *F value ($P < 0.05$). ID G (II): iron overload group; G (III): iron overload group treated with the aqueous extract of *C. racemosa*; G (IV): iron overload group treated with the ethanolic extract of *C. racemosa*.

Table 7. Effects of *C. racemosa* extracts on antioxidants of brain in normal and iron overload in the experimental model

Groups	Control G (I)	ID G (II)	Treatments with *C. racemosa* extracts			P
			G (III)	G (IV)	F value	
GSH (µg/dl)	72.59 ± 4.60[a]	42.40 ± 2.77[b]	64.70 ± 3.41[a] +34.47%	71.36 ± 2.66[a] +40.58	16.68*	0.000
GST (µ/g)	3.48 ± 0.45[a]	1.70 ± 0.20[b]	3.07 ± 0.25[a] +44.63%	3.55 ± 0.3[a] +52.11%	7.70*	0.001
GPx (µg/g)	348.54 ± 17.77[a]	198.40 ± 8.43[b]	340.40 ± 10.53[a] +41.72%	342.80 ± 11.75[a] +42.12%	7.58*	0.003
CAT (µ/g)	18.60 ± 1.77[a]	7.70 ± 1.16[c]	13.68 ± 1.54[b] +43.71%	16.65 ± 1.23[a,b] 53.75%	9.91*	0.001
SOD (U/g)	230.88 ± 9.13[a]	115.84 ± 6.87[b]	219.74 ± 7.68[a] +47.28%	235.60 ± 11.34[a] +50.83%	33.78*	0.000

Data expressed as mean ± SE. Small letters: Statistically significant from control or experimental groups at $P < 0.05$ using one-way ANOVA followed by Tukey as a post-hoc test. *F value ($P < 0.05$). ID G (II): iron overload group; G (III): iron overload group treated with the aqueous extract of *C. racemosa*; G (IV): iron overload group treated with the ethanolic extract of *C. racemosa*.

mosa was characterized by the presence of total phenolics (TP) and total flavonoids (TF) acting as antioxidant components particularly in the ethanolic extract than the aqueous one. The recent contributions of [9, 35] on phytochemical properties of *C. racemosa* species from subtropical zones of Malaysia and Indonesia, respectively, support well our results in its characteristic TP and TF contents. Former study [12] during their investigation on a similar morphospecies in South East Asia reported that it had 144 ± 22 mg GAE/gm dried sample of TP content in 50% (v/v) aqueous methanolic extract, in addition to a lower reducing power and Fe-chelation ability. They also indicated that *C. racemosa* exhibited hydrophilic and hydrophobic antioxidants. This investigation demonstrated that the higher TP content in ethanolic algal extract might be attributed to the inhibition of action of polyphenol oxidases which work on oxidation of polyphenols. In a similar agreement with this observation, previous study [9] postulated that the methanolic extracts significantly contain high TP content, where the phenolic compounds are typically more polar compounds. The potent radical scavenging activity of *C. racemosa* might be due to the presence of folic acid, ascorbic acid, thiamine and vitamin A [14] and to its enzymatic and non-enzymatic antioxidants which could contribute to its medicinal benefits [9]. Accordingly, [36] pointed out that crude ethanolic extract of *C. racemosa* and its fractions were distinctly distinguished by high phenolic con-

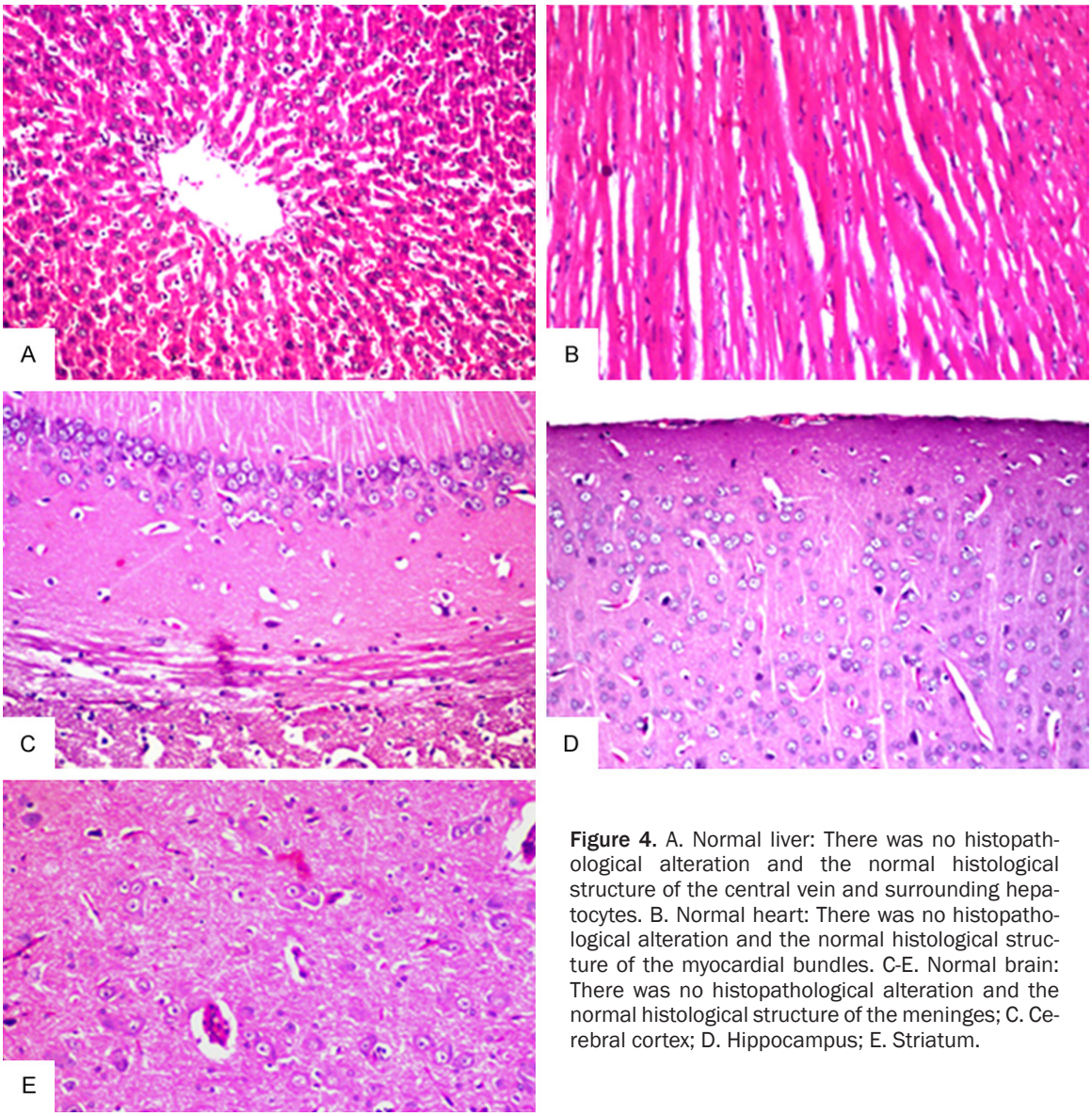

Figure 4. A. Normal liver: There was no histopathological alteration and the normal histological structure of the central vein and surrounding hepatocytes. B. Normal heart: There was no histopathological alteration and the normal histological structure of the myocardial bundles. C-E. Normal brain: There was no histopathological alteration and the normal histological structure of the meninges; C. Cerebral cortex; D. Hippocampus; E. Striatum.

tent which might be responsible for high DPPH radical scavenging activities. Concerning Fe-chelation, [37] during their deep bio-screening on cytotoxic and antioxidant potentials of some green seaweeds from India indicated to the presence of lower Fe-chelating ability in *C. racemosa* and attributed this feature to the polyphenols which could upregulate this mechanism. Moreover, [38] and Al-Shwafi & Rushdi [39] showed the natural uptake of Fe metals by *C. racemosa* specimens from the coastal waters of South Africa and Yemen, respectively.

Prevention of iron overload toxicity is the main issue of this study to overcome several complications in thalassemia disease. This necessarily entails the use of an effective chelator and regular monitoring of iron burden in the liver and heart by the use of the recent technology of MRI techniques (T2 & R2) [40]. Deferoxamine subcutaneous infusion lowers the iron burden but the compliance of patients is very poor [41]. Furthermore, there are some expected side effects due to oral chelators including hepatic toxicity, impaired liver functions and hearing loss in some patients. Neutropenia and granulocytosis recorded with DFP require following-up patients by weekly blood tests [42]. Although the combination therapy with DFO/DFX cause a distinct reduction in iron overload, but the adverse effects of both agents are still present even to a lesser extent [43].

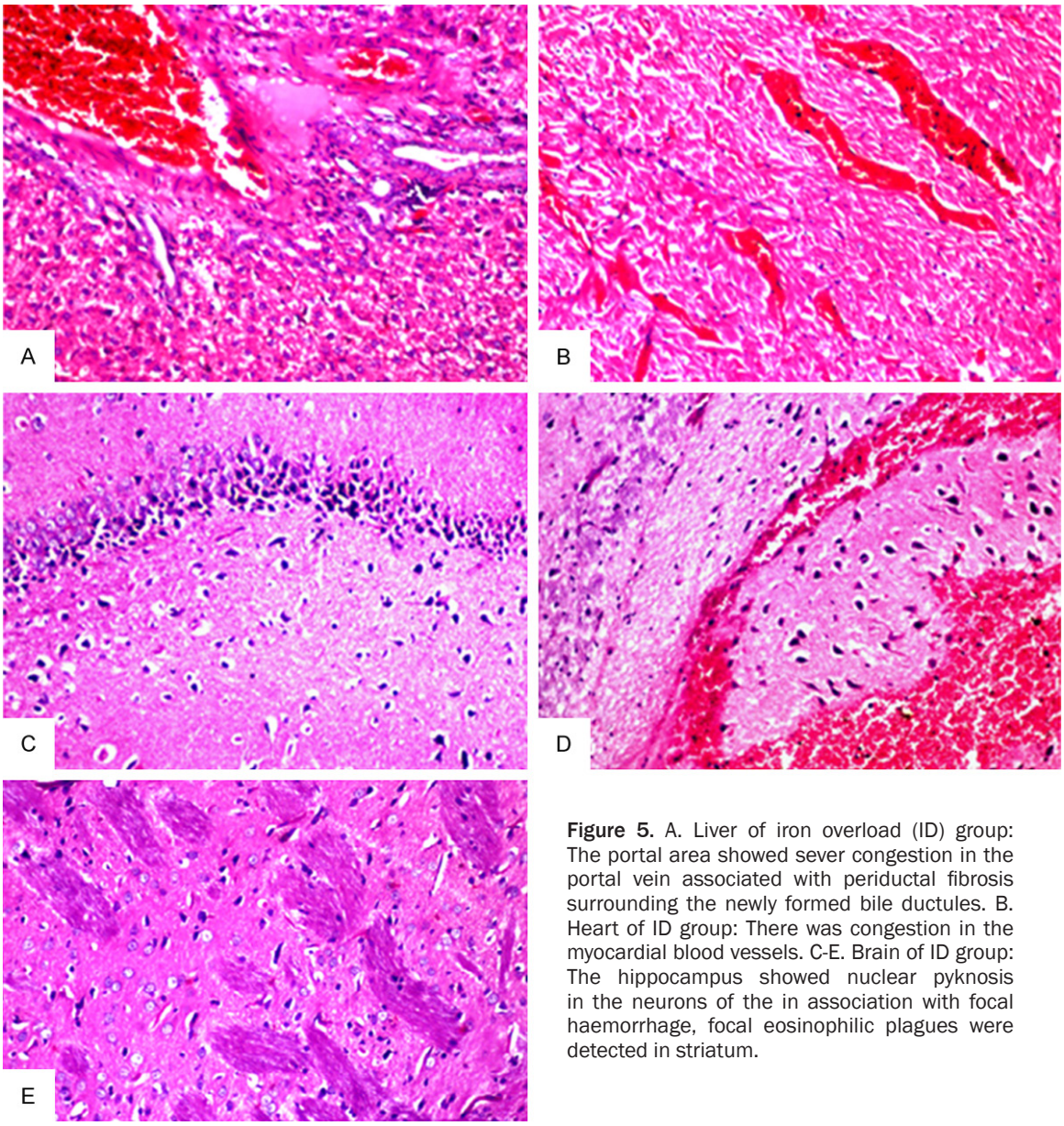

Figure 5. A. Liver of iron overload (ID) group: The portal area showed sever congestion in the portal vein associated with periductal fibrosis surrounding the newly formed bile ductules. B. Heart of ID group: There was congestion in the myocardial blood vessels. C-E. Brain of ID group: The hippocampus showed nuclear pyknosis in the neurons of the in association with focal haemorrhage, focal eosinophilic plagues were detected in striatum.

As previously study [37] investigated the methanolic extracts of some *Caulerpa* species in India, including *C. racemosa*, and indicated to their highly positive "no cytotoxic" activities which might be attributed to their excellent DPPH scavenging properties, reducing power and iron chelation characteristics. In the present study, the aqueous and ethanolic *C. racemosa* extracts with a dose 200 mg/kg reduced the iron deposition in liver, heart and brain tissues and ferritin content. It has been renowned that flavonoid compounds are mainly characterized by their antioxidant activities and the mechanism of their actions could be concluded from their ROS scavenging and/or metal-chelating process [44-46]. Phenolic compounds are also a category of antioxidant compounds which act as free radical quenching agents [45]. As inferred from the results, there were significantly decreases in the iron deposition, serum ferritin, TIBC, and transferrin levels in the ethanolic and aqueous algal extracts treated animals compared to ID group. The rate of reduction of serum iron was also more or less very near to the control group. However, the rate of Fe-reduction was found to be higher in ethanolic extract comparing to the water extract. Chelation property may give a defense

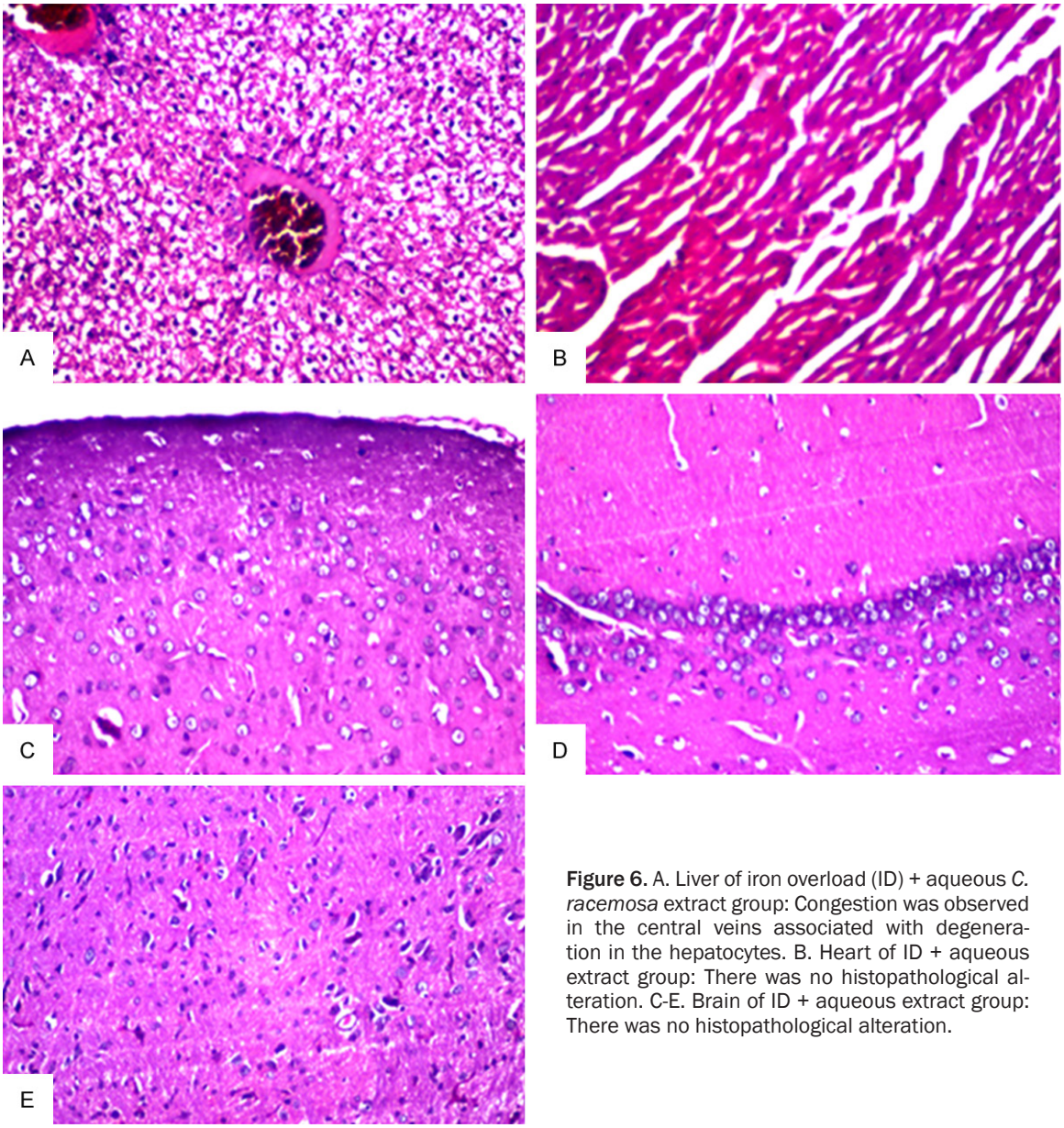

Figure 6. A. Liver of iron overload (ID) + aqueous *C. racemosa* extract group: Congestion was observed in the central veins associated with degeneration in the hepatocytes. B. Heart of ID + aqueous extract group: There was no histopathological alteration. C-E. Brain of ID + aqueous extract group: There was no histopathological alteration.

against the produced oxidative damages and iron-overload [47]. These therapeutic characteristics of the green marcoalga *C. racemosa* studied herein could be explained on the basis of its bioactive components capable of iron chelation, i.e. they could mobilize iron particles deposited in the tissues by forming soluble and steady complexes and therefore reduces iron-affined difficulties in humans and then excreted them out from the body.

In general, very few studies concerned with the mechanism of iron entering and accumulation in the brain tissues, leading to a malfunction of CNS disorders [48]. The brain blood barrier is considered one of the main factors that limits the entry of plasma iron to the brain, conversely to peripheral organs. Thus, the brain does not seem to cram iron particles when body iron stores are increased, i.e. not induce any changes in the cerebral total iron levels [49]. One finding of this study is that iron overload increased the transition of iron which release to peripheral organs, but not to the brain as evidenced by increased Ft levels in the hepatocytes and cardiac tissues but not in the brain. These results are consistent with the recent contribution of Deane [50] whom reported that suppression of

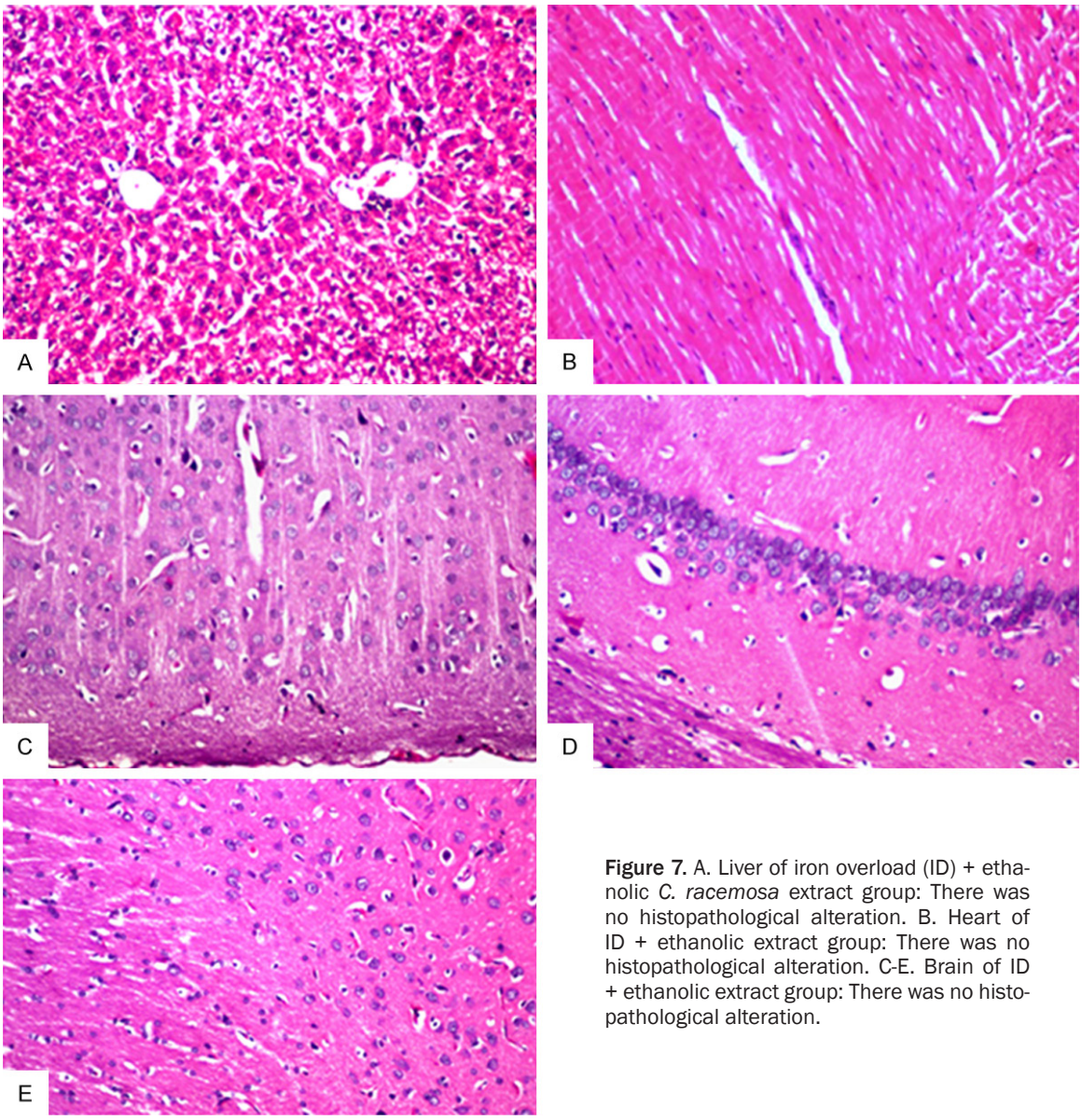

Figure 7. A. Liver of iron overload (ID) + ethanolic *C. racemosa* extract group: There was no histopathological alteration. B. Heart of ID + ethanolic extract group: There was no histopathological alteration. C-E. Brain of ID + ethanolic extract group: There was no histopathological alteration.

circulating iron by cerebral endothelial cells, as well as the recent assumption that the brain, but not systemic iron placement is important in the control of iron delivery to the cerebral cells [51]. Indeed, the amounts of free iron within cells determine the susceptibility of tissue to ischemia [52]. Moreover, the inactivation of intracellular free iron in the ischemic cerebrum by a liposoluble iron chelator was recently reported to reduce oxidative strain and infarct volume [53]. The inefficiency of iron dextran-induced iron overload to aggravate infarct magnitude indicates that patients with increased body iron stores have not an aggrandize risk for stroke. In spite of this hypothesis remains to be considered, carriers of the hemochromatosis gene were reported to be not related to stroke [54].

In the present study, the iron overload (ID) induced reduction of antioxidants in liver, heart and brain. The accumulation of excessive amounts of iron resulted in cytotoxicity and complications in these organs and caused the death. Equilibrium between ROS and enzymatic antioxidants including SOD, CAT, and GPx are decisive and could be a significant mechanism for preventing harms by oxidative stress [55]. Antioxidants, natural or artificial, other natural ROS scavengers may minimize the incidence of

free radical-mediated diseases. Antioxidants manifest to act against disease processes by raising the levels of antioxidant enzymes and diminishing toxic products such as lipid peroxidation by products [56]. Marin algae extracts have powerful antioxidant properties attributable to the multiple bioactive constituents found in diverse solvent extracts [57]. These antioxidant bioactive compounds effectively prevent liver injury from hepatotoxin-induced toxicity. Malondialdehyde (MDA) is a good indicator of the degree of lipid peroxidation [58], which relates to ID-induced tissue damage. In this study, a significant increase in the MDA level observed in the ID-intoxicated rats was reduced by the different treatments with *C. racemosa*, indicating to its ability to break the series reaction of lipid peroxidation. Based on these results, we could suggest that the therapeutic potentials of *C. racemosa* extracts are probably dependent on its antioxidant mechanism. These results concluded that *C. racemosa* extracts effectively inhibit ID-induced tissue damage due to the presence of various antioxidant bioactive compounds.

Liver function tests facilitate diagnosing of any abnormal and normal conditions of the liver. Leakage of cellular enzymes into plasma points to the hepatic tissue damage [59]. Mostly, evaluation of ALT is utilized as significant diagnostic markers to indicate liver injury due to any toxins invade the hepatocytes. Administration of *C. racemosa* extracts significantly reduced the amplitude of liver damage following a high dose of ID. There was a significant decrease of serum ALT level in rats treated with *C. racemosa* extracts as well as, AST, LDH and CK, these results correlated with other research findings [60]. This result showed that there was alleviated in the damage of parenchymal and mitochondrial sites of the liver where ALT exists. This confirmed that the integrity of the liver cells was protected from leakage as ALT enzymes were reduced in the blood stream. These results proved that *C. racemosa* extracts significantly reduced liver toxicity due to its non-poisonous nature and tissue conservative nature against various toxic metabolites. This investigation demonstrated that these algal extracts might be a new prospective prevalence of natural antioxidants which may engage in the prevention of assorted chronic degenerative diseases which are very common nowadays. Moreover, the *C. racemosa* extracts, especially the ethanolic one, could be recommended to reduce and recover iron overload in thalassemia disease.

Conclusions

This study revealed that there is a direct relationship between Fe-chelating efficiency in the experimental rats showing thalassemia disease models and the concentrations of bioactive components, including total phenolics, total flavonoids and antioxidant characteristics, in the different aqueous and ethanolic extracts of the green macroalga *Caulerpa racemosa*. The ethanolic algal extract was more effective than the aqueous one. The *in vivo* assays suggested that the antioxidant and Fe-chelating activities of *C. racemosa* specimens make it possible as a good natural source for therapy of thalassemia diseases and its accompanying damaging oxidative stress, and to maintain human health and wellness. However, more deep studies are still needed to preciously identify and characterize the *C. racemosa* bioactive components.

Acknowledgements

The authors are grateful to Dr. Adel M. Bakeer Kholoussy, Department of Pathology, Faculty of Veterinary Medicine, Cairo University for his help in the examination of the histopathological slides and for his valuable comments.

Disclosure of conflict of interest

None.

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