Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading

Hepatoprotective activity of brown alga *Padina boergesenii* against CCl_4 induced oxidative damage in Wistar rats

Rajamani Karthikeyan[1], ST Somasundaram[1], T Manivasagam[2], T Balasubramanian[1], P Anantharaman[1*]

[1]*Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai-608 502, Tamil Nadu, India*
[2]*Department of Bio-chemistry and Bio-technology, Annamalai University, Chidambaram-608 002, Tamil Nadu, India*

ARTICLE INFO

Article history:
Received 13 August 2010
Received in revised form 27 August 2010
Accepted 1 September 2010
Available online 20 September 2010

Keywords:
Hepatotoxicity
Padina boergesenii
Carbon tetrachloride
Liver fibrosis
Carotenoids

ABSTRACT

Objective: To investigate the protective effects of brown alga *Padina boergesenii*(*P. boergeseii*) against carbon tetrachloride (CCl_4) induced oxidative damage and liver fibrosis in rats. **Methods:** To assess the hepatic damage liver weight, the activities of TBARS level, glutathione, SOD, CAT and GPx in circulation and liver. **Results:** The group of rats induced with CCl_4 alone (2 mL/kg body weight), showed noticeable increase in the liver weight and TBARS level. Followed by, the level of antioxidant enzymes glutathione peroxidase, Superoxide dismutase and catalase was also significantly (P<0.01) diminished. Where as, the rats pretreated with *P. boergesenii* (150 mg/kg body weight) modulated the CCl_4 induced liver fibrosis. The level of antioxidant enzymes and lipid peroxidation products was found to be significantly (P<0.01) attenuated to near normal level, when compared with rats induced with CCl_4 alone. In order to assess the role of carotenoids in the relevant activity, total carotenoid content of the extract was analysed and found to be 0.59 mg/g fresh weight (FW). Further, the histopathogical studies provide a supportive evidence for this study to show the protective nature of *P. boergesenii*. **Conclusions:** The protective role of brown alga *P. boergesenii* extract has confirmed its potential activity through its antioxidant sparing actions against CCl_4 induced free radical damage. However, the possible mechanism of hepatoprotection is rather speculative at this stage and investigations are underway to isolate and characterize the bioactive compounds from *P. boergesenii*.

1. Introduction

Brown algae are rich sources of various bioactive compounds, including polyphenols, carotenoids and polysaccharides with different physiological effects (toxic or curative) on human health. The components of various brown algae (Phaeophyceae) species have been widely investigated and it has rich inorganic composition. More than 1 140 secondary metabolites have been reported from Phaeophyceae. The brown algae are a rich source of various natural antioxidants such as polyphenols, which play an important role in preventing lipid peroxidation[1]. In addition to potential role of polyphenols Lewin[2] have stated that, the lipids of algae comprise photosynthetic pigments-chlorophylls, carotenes and other compounds while carotenoids are powerful antioxidants. Fucoxanthin is a major carotenoid of brown algae[3], which reported to have beneficial effects on cancer as chemopreventive agent[4]. It is commonly recognized that antioxidants can able to neutralize harmful free radicals in body cells before they cause lipid and protein oxidation. Excessive production of ROS and the related oxidative stress have been proposed to play a several roles in the pathogenesis of chronic-degenerative conditions, such as neurodegenerative diseases, cancer, arteriosclerosis, malaria, rheumatoid arthritis & diabetes[5]. Hence, the development of antioxidants from natural origins has drawn more attention among the researchers against free radicals. The brown alga *Padina* is widely distributed along the Indian coast, and species of *Padina* are important source of mannitol and iodine. The brown alga *Padina boergesenii* (*P. boergesenii*) is a fan-shaped plant body, segmented, ruffled, and light brown in colour. The presence of phenolic compounds and its anticancer activity from *P. boergesenii* has proven on *in vivo* model[6]. Since, there was no report of carotenoids and

*Corresponding author: Dr. P. Anantharaman, Associate Professor, CAS-In Marine Biology, Faculty of Marine Sciences, Annamalai University, Portonovo-608 502 Tamil Nadu India.
Tel: +914144 243223
Fax: +914144 243555
E-mail: rajakarthis14@yahoo.co.in

its antioxidant activity of this particular alga *P. boergesenii*. Therefore, the aim of the present study was to investigate the ameliorative effect of *P. boergesenii* against CCl_4 induced liver fibrosis in Wistar rats. Hence, we expect to detect the higher levels of lipid peroxidation and its products in blood and liver organ. This investigation not only provides the information about the therapy or prevention of such liver disease, but also could promote an understanding of its mechanisms, especially to control the role of free radicals or oxidative stress in the pathogenesis of acute toxic liver injury.

2. Materials and methods

2.1. Chemicals

Reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione peroxidase, thiobarbituric acid (TBA), CCl_4 and diethyl ether were purchased from Sigma Aldrich. All other chemicals and solvents of analytical grade were obtained from S.D. Fine chemicals, Chennai, India.

2.2. Collection of seaweeds

In the present study the seaweed *Padina boergesenii* belonging to the class Phaeophyceae, family Dictyotaceae, collected from Vaan island, Gulf of Mannar Biosphere in the intertidal region (Lat 11° 29`N; Long 79° 46`E) during April and May 2007. The algal species were hand picked and washed thoroughly with seawater to remove all the impurities like sand particles and epiphytes. Morphologically distinct thallus of algae was placed separately in new polythene bags and were kept in an icebox containing slush ice and transported to the laboratory. Finally, the seaweeds were rinsed carefully in freshwater and shade dried then the samples were pulverized into powder (size 2 mm) using a grinder before extraction. The brown alga *P. boergesenii* were identified by their taxonomical features[7].

2.3. Preparation of seaweed extract

In general, the principle of soxhlet extraction is an extraction method by using chemical solvents[8]. The 5 g of *Padina boergesenii* grinded powder was extracted with 100 mL of diethyl ether (Non polar solvent) using a soxhlet extractor for 8 hours at 35 ℃. The extraction was repeated many times to obtain a sizable quantity of extract. The extracts were then concentrated under reduced pressure and the resultant residues were stored in a dark at 4 ℃ until further use. For the investigation of *in vivo* study diethyl ether residue were dissolved in 0.3% Carboxyl Methyl Cellulose as a vehicle.

2.4. Estimation of carotenoids

The presence of carotenoids in *P. boergesenii* extract was estimated according to the protocol prescribed by Parsons and Strickland[9].

2.5. Animal and housing

The male albino rats of the Wistar strain (4-6 weeks old), weighing 125-150 g, were obtained from Central Animal House, Faculty of Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University, India. Animal experiments were approved by the Institutional Ethical Committee Regulations. The Central animal house registration number is 160/1999/ CPCSEA. The rats were fed with pellet diet and water *ad libitum*. During experimentation they were housed in the respective polypropylene cages and allowed free access to food and water. The animals were maintained in a controlled environment under standard conditions of temperature and humidity with alternating 12 h light/dark cycles.

2.6. Experimental induction of hepatotoxicity

All rats were allowed to acclimatize for 1 week prior to experimentation. The treatment regimen for brown alga *P. boergesenii* extract was based on the preliminary *in vitro* studies carried out in our laboratory[6]. CCl_4 dose was selected according to Janbaz *et al*[10]. A total of 24 rats were used the animals were randomized and had free access to standard laboratory feed. The rats are grouped into experimental and control rats (n=6 in each group). Group I served as saline-treated negative control and was administered with saline (0.85% NaCl) alone. Group II subcutaneously injected with CCl_4 (2 mL/kg body weight) every day for 1 month. Groups III were pretreated with diethyl ether extract with dose of 150 mg/kg body weight orally by a tube, which is inserted into the mouth and down the oesophagus of the rat, where the substance is subsequently injected into the stomach by the syringe connected to the gavage tube for one month before one hour of administration of CCl_4. Group IV. Rats were orally administered with *P. boergesenii* diethyl ether extract (150 mg/kg body weight) alone for one month. At 24 h after the last treatment of extracts or pellet feed animals on the 30th day from group II and III received an i.p. injection of CCl_4 (2 mL/kg body weight).

2.7. Collection of blood

At the end of the experiment, all the animals were sacrificed to collect blood samples by cardiac puncture. Blood plasma is prepared by spinning a tube of fresh blood containing an anti-coagulant in a centrifuge until the blood cells fall to the bottom of the tube. The obtained plasma was kept at -70 ℃ before determination of the biochemical parameters for GSH, GPx, SOD and CAT.

2.8. Preparation of hemolysate

After the collection of plasma, the buffy coat was removed and the packed cells were washed with cold physiological saline. A known value of the RBC was lysed with cold hypotonic phosphate buffer, pH 7.4. The hemolysate were separated by centrifugation at 2000 g for 10 minutes at 20 ℃ to determine the activity of antioxidant enzymes SOD, CAT, GPx.

2.9. Evaluation of liver damage

Livers of all rats were excised immediately after collection of the blood. Some of the liver tissue samples were washed twice with a cold saline solution and were placed into the glass labeled bottles. The tissue samples from the group of rats were used for determination of differences in liver weight and to assess biochemical parameters as an index of lipid peroxidation. The other liver tissue specimens were used for histopathological examination.

2.10. Preparation of tissue samples

The part of the liver tissue sample was rinsed in physiological saline, and homogenized. The homogenate (25% w/v) was in ice-cold 0.25 M sucrose, then the homogenate was deproteinized with an equal volume of ice-cold (10% w/v) trichloroacetic acid. The ensuing supernatant fraction was analyzed for enzymatic and non-enzymatic biochemical assays.

2.11. Biochemical investigations

2.11.1. Vitamin E in liver

The level of vitamin E were determined by the method of reduction in Fe^{3+} to Fe^{2+} by the tocopherols with the formation of a red complex of the Fe^{2+} with α, α`-dipyridyl[11,12]. The transmissions were measured by calorimeter and it's expressed in terms of μg/mg protein.

2.11.2. Estimation of thiobarbituric acid reactive substances (TBARS)

The level of TBARS in liver were estimated in liver by measuring malondialdehyde and TBARS reactivity with thiobarbituric acid (TBA) to generate a pink coloured chromophore, which was read at 535 nm[13]. The transmissions were measured by calorimeter and it's expressed in terms of mM/100 g wet tissue.

2.11.3. Estimation of reduced glutathione

Reduced glutathione was estimated in plasma and liver by the method of Ellman[14]. The yellow colour developed was read at 412 nm. A series of standards were treated in a similar manner along with a blank containing 3.5 mL of buffer. The amount of glutathione was expressed as mg/dL plasma and mg/100 g tissue.

2.12. Quantitative analysis of enzyme activities

2.12.1. Estimation of superoxide dismutase

A superoxide dismutase (SOD) activity was determined in plasma and liver based on the method of Kakkar *et al*[15]. SOD removes the super-oxide radicals and inhibits the formation of formazan blue. The intensity of colour is inversely proportional to the activity of the enzyme. One unit of the enzyme activity is defined as the enzyme reaction, which gave 50% inhibition of NBT reduction in one minute. Thus obtained values could be expressed as specific activity in plasma as units/mL and in liver as units/mg protein.

2.12.2. Estimation of catalase

The catalase activities were determined by the method of conversion of dichromate in acetic acid to perchromic acid and then to chromic acetate in the presence of H_2O_2 when subjected to heat[16]. Thus formed chromic acetate was measured at 620 nm and the activities were expressed as μM of H_2O_2 consumed units/mL and in liver as units/mg protein.

2.12.3. Estimation of glutathione peroxidase

Glutathione peroxidase was estimated by the reaction the sample with hydrogen peroxide in the presence of GSH for a specified time period. Then the remaining GSH were measured by the method of Rotruck *et al*[17]. The activities were expressed as μg of GSH consumed units/mL and in liver as units/mg protein.

2.12.4. Histopathological examination

After animal experimentation the liver were dissected immediately and fixed in 10% neutral formalin solution for at least for 24 h and was embedded in paraffin wax (melting point 56-58 ℃). Tissue sections were made with 4 μm thickness and were stained with Mayer's hematoxylin and eosin (H&E) for histopathological examination under light microscope[18].

2.13. Statistical analysis

The results were analyzed among the experimental group of animals and statistical significance (*P<0.05, **P<0.01) between controls and treated groups were evaluated using multivariate analysis of variance (ANOVA) followed by Duncan's multiple range test.

3. Results

3.1. Effects of P. boergesenii on liver weight

The total carotenoid content of *P. boergesenii* extract was found to be 0.63 mg μ g^{-1} FW. Induction of CCl_4 (2 mL/kg, S.C.) results in acute liver injury in *Rattus novergicus*. The liver weight was increased to (5.85±0.39) g in CCl_4 induced group, where as *P. boergessenii* extract treated group (150 mg/kg, orally) showed a decrease in the liver weight to (3.57± 0.33) g.

3.2. Effects of P. boergesenii extract on vitamin-E

Since CCl_4 administration caused hepatic injury with significant decrease (P<0.01) in vitamin-E, on treatment with *P. boergesenii* extract there was a significant (P<0.01) increase in the level of vitamin-E (Figure 1).

3.3. Effects of P. boergesenii extract on TBARS & reduced GSH content

The induction of CCl_4 (2 mL/kg body weight, S.C.) was

associated with alteration of lipid peroxidation products illustrating a significant increase in TBARS content to that of control animals. On treatment with extract, the level of TBARS was significantly (P<0.01) lowered in the liver (Figure 2). Treatment with *P. boergesenii* extract was able to ameliorate CCl_4 induced hepatocellular damage as evidenced through the activity of glutathione. The level of GSH in circulation as well as in tissues was significantly decreased (P<0.01) after the rats induced with CCl_4, where as the rat treated with extract (150 mg/kg, orally) showed considerable increase in GSH level (P<0.01) as shown in Table 1. This tendency of the *P. boergesenii* extract reflects its potential antioxidant nature.

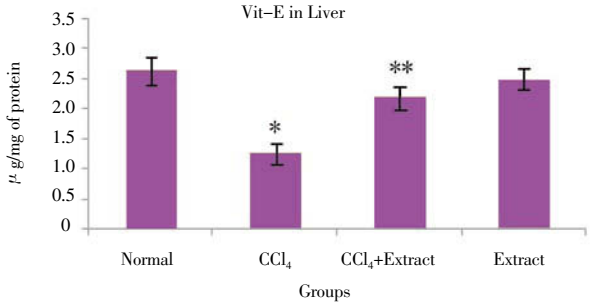

Figure 1. Activity of Vit-E in liver. Values are expressed in Mean ± S.E.M. (n=6); * significantly (P<0.01) different from control group.

Table 1
Changes in the level of reduced glutathione in plasma and tissues.

No	Groups	Plasma (mM/100 mL)	Liver (mM/100 g tissue)
1	Normal	18.66±1.43	114.37±8.71
2	CCl_4	10.05±0.23*	75.66±0.23*
3	CCl_4+Extract	16.45±1.26*	99.07±4.10*
4	Extract	22.06±1.68	118.60±4.13

Values are expressed in Mean ± SEM (n=6); *significantly (P<0.01) different from control group.

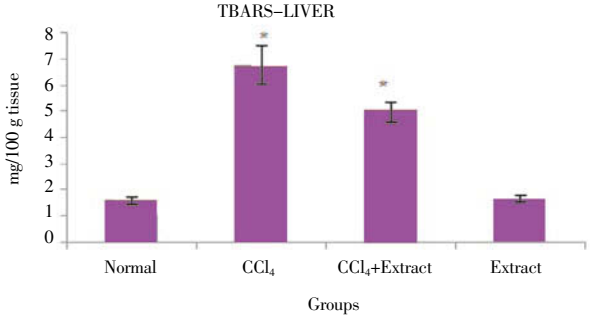

Figure 2. Level of TBARS in liver. Values are expressed in Mean ± SEM (n=6); * significantly (P<0.01) different from control group.

3.4. Changes in the activities of SOD, CAT and GPX

The activities of hepatic enzymes were altered by induction of CCl_4 (2 mL/kg body weight, S.C.) in rats, which was quantified by biochemical markers like super-oxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) in circulation as well as in liver tissue. After intoxication of CCl_4, the level of SOD, CAT and GPX was decreased both in circulation (Table 3) and liver (Table 4). Treatment with *P. boergesenii* extract (150 mg/kg, orally) was able to overcome the CCl_4 induced hepatocellular damage, which was evidenced by significant (P<0.01) increase in the activity of SOD. Since CCl_4 induction caused significant hepatic injury and inflammation, the toxicity of CCl_4 and protective effects of *P. boergesenii* extract was revealed by enzymatic assay of catalase. It showed a significant increase in catalase activity (P<0.01) both in circulation and liver as shown in Table 2 & 3. Simultaneously, the enzymatic assay of glutathione peroxidase activity was also significantly increased (P<0.01).

3.5. Histopathological changes

The tissue sections stained with H&E were examined for the extent of liver damage, inflammation, and liver cell necrosis. Extensive liver damage areas were observed in CCl_4 challenged group. Hepatocytes surrounding the central vein and perivenular regions exhibited coagulative type of necrosis against various oxidative stress conditions (Figure 3). CCl_4 (2 mL/kg body weight, S.C.) administration produced intense necrotic zones, edema, acute inflammatory infiltration, and hepatocyte degeneration characterized by cytoplasmic swelling and nuclear damage. These changes in the hepatic architecture were not observed in any of the control rats or animals treated with *P. boergesenii* extract (150 mg/kg, orally) alone. Treatment of this extract prevented CCl_4-induced morphological changes in some zones like edematous hepatocytes and showed less cytoplasmic degeneration along with less inflammatory infiltration. There was no necrosis observed in the extract treated group of animals.

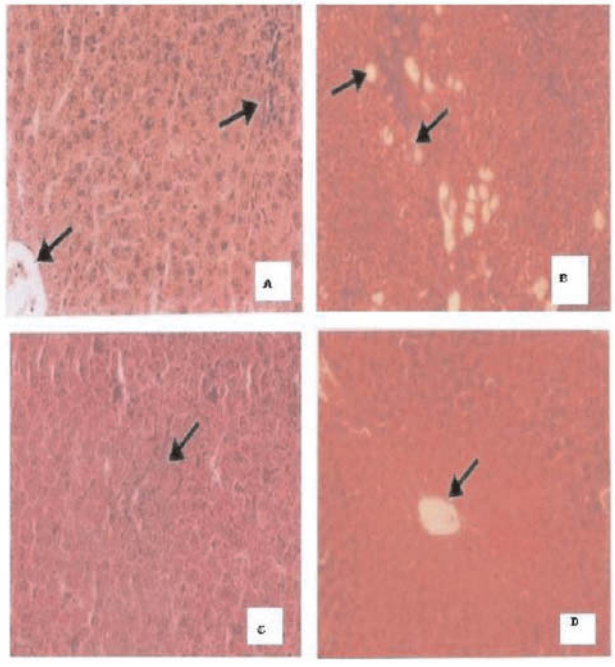

Figure 3. Histopathology of liver.
A: Shows normal central vein and portal triad, B: CCl_4 toxicity in rats (2 mL/kg body weight, S.C.,) show macro and micro vesicular changes, C: Rats pretreated with *P. boergesenii* (150 mg/kg bodyweight, orally) one hour before CCl_4 induction. D: Rats treated with extract alone (150 mg/kg bodyweight, orally) show normal histology with central vein.

Table 2
Effect of *P. boergesenii* extract on antioxidant enzymes in circulation.

No	Groups	Superoxide dismutase(units/mL)	Catalase (μ M of H_2O_2)	Glutathione peroxidase(units/mL)
1	Normal	2.17±0.17	5.86±0.56	8.48±0.36
2	CCl_4	1.48±0.11*	3.59±0.16*	5.88±0.62*
3	CCl_4+Extract	1.86±0.28*	4.90±0.34*	7.96±0.40
4	Extract	2.14±0.17	5.79±0.42	8.38±0.46

Values are expressed in Mean ± SEM (n=6); significantly different from control (*P<0.01).

Table 3
Effect of *P. boergesenii* extract on antioxidant enzymes in liver.

No	Groups	Superoxide dismutase(units/mg protein)	Catalase(units/mg protein)	Glutathione peroxidase(units/mg protein)
1	Normal	17.95±0.52	50.24±4.64	11.58±0.86
2	CCl_4	6.58±0.81**	40.99±2.39*	5.34±0.35**
3	CCl_4+Extract	9.87±1.36**	45.24±1.19*	8.38±0.46**
4	Extract	16.67±0.69	52.13±5.33	11.69±0.86

Values are expressed in Mean & SEM (n=6); significantly different from control (*P<0.05, **P<0.01).

4. Discussion

Hence, the data of this present study show that the brown alga *P. boergesenii* showed hepatoprotective activity against CCl_4 induced liver fibrosis in Wistar rats. It is well recognized that, the CCl_4 is a lipid-soluble and is thus well distributed through out the body. The biochemical mechanisms involved in the development of CCl_4 hepatotoxicity have long been investigated. It is believed that, it is due to lipid peroxidation caused by the carbon trichloromethyl radical, CCl_3 . CCl_4 is biotransformed by cytochrome P450 to the trichloromethyl-free radical that induces membrane lipid peroxidation and disturbs Ca^{2+} homeostasis to produce hepatocellular injury. Therefore, the administration of CCl_4 causes necrosis, fibrosis in liver. The CCl_4 induced hepatotoxicity lead to rapid increase in the serum enzyme levels, which have been attributed to damage in the structural integrity of cell membrane in liver and leakage of transaminases from the cytoplasm into the blood circulation[18,19]. The toxicity of CCl_4 has been well attributed in assessing changes in body weight of the rats. In addition to liver weight, we assessed the changes in body weight of the rats during the experimental periods[20]. Free radical catalyzed lipid peroxidation is thought to be one of the major processes involved in hepatotoxicity or xenobiotic hepatotoxicity. Free radicals are the essential components of energy and healthy immune function; they must be controlled to prevent cell and tissue damage. These free radicals are concerned in around sixty negative health conditions, which include heart disease, cancer, macular degeneration, cellular degradation (ageing), and numerous inflammatory diseases.

The brown alga *P. boergesenii* has proven its potential nature in two ways against such free radical damages. On the one hand, the pretreatment of rats with *P. boergesenii* extract prevented CCl_4 toxicity on lipid peroxidation, GSH and antioxidant enzyme levels in circulation as well as liver tissue. These results suggest that, the *P. boergesenii* act as an effective antioxidant or a free radical scavenger was able to stabilize membrane structures, thus, to preserve the cellular integrity and to restrain the severity of CCl_4 induced cellular injury at significant level (P<0.05, P<0.01). On the other hand, the brown alga *P. boergesenii* showed its protective role against cellular damage as evidenced by histopathological sections. Based on these results, it is reasonable to suggest that the presence of carotenoids from *P. boergesenii* extract might exert their mechanism different from its antioxidant action or free radical scavenging activity.

In conclusion, as far as the present data is concerned, this study is the first investigation to show that the presence of carotenoids from brown alga *P. boergessenii* possesses a potent antioxidant activity against CCl_4 induced acute liver damage in rats. This was revealed by changes in level of TBARS and GSH concentration in liver, in addition to the elevation of SOD, CAT and GPx activity. Whereas, *P. boergesenii* able to ameliorate acute liver damage to a high degree, as evidenced by the improvement in histopathological changes. The brown alga *P. boergesenii* extract proved its activity either through stabilization of cellular membrane or through antiperoxidase activity. The outcome of the present study reveals that, there is a strong correlation between antioxidant activity and carotenoid content. It is believed that, carotenoid has the ability to act as reducing agents, hydrogen donors, and free radical quenchers which can also act as metal chelators. Further investigations are in progress to analyze and characterize the bioactive compounds responsible for this antioxidant nature from *P. boergessenii*.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

We are grateful to Prof. Venugopal P. Menon, Director of Research, Annamalai University for purveying this opportunity to do this research work. The authors wish to thank Department of Biotechnology (DBT), India for the financial support. Finally, we would like to thank Dr. P. Subramanian for his inspiring support to carry out this work.

References

[1] Amsler CD, Fairhead VA. Defensive and sensory chemical ecology of brown algae. *Adv Bot Res* 2006; **43**:1-91.

[2] Lewin RA. Biochemical taxonomy. In: Stewart wdp.(ed.) *Algal physiology and biochemistry*. London: Blackwell Scientific Publications; 1974, p.989.

[3] Okuzumi J, Takahashi T, Yamane T, Kitao Y, Inagake M, Ohya K, et al. Inhibitory effects of fucoxanthin, a natural carotenoid, on N-ethyl-N′-nitro-N-nitrosoguanidine induced mouse duodenal carcinogenesis. *Cancer Lett* 1993; **68**: 159-68.

[4] Kim JM, Araki S, Kim D J, Park CB, Takasuka N, Baba-Toriyama H, et al. Chemopreventive effects of carotenoids and curcumins on mouse colon carcinogenesis after 1,2-dimethylhydrazine initiation. *Carcinogenesis* 1998; **19**: 81-5.

[5] Wiseman H, Halliwell B. Damage to DNA by reactive oxygen and nitrogen species: Role of inflammatory disease and progression to cancer. *Biochem J* 1996; **313**: 17-29.

[6] Rajamani Karthikeyan, Manivasagam T, Ananatharaman P, Balasubramanian T, Somasundaram ST. Chemopreventive effect of *Padina boergesenii* on ferric nitrilotriacetate (Fe-NTA) induced oxidative damage in Wistar rats, *J Appl Phycol* (2010 DOI 10.1007/s10811-010-9564-0) (in press).

[7] Allender BM, Kraft GT. The marine algae of Lord Howe Island (New South Wales): the dictyotales and cutleriales (Phaeophyta). *Brunonia* 1983; **6**: 73-130.

[8] Lim SN, Cheung PCK, Ooi VEC, Ang PO. Evaluation of antioxidative activity of extracts from brown seaweed, Sargassum siliquastrum. *J Agric Food Chem* 2002; **50**: 3862-6.

[9] Parsons TR, Strickland JD. Discussion of spectrophotometric determination of marine plant pigments with revised equations for ascertaining chlorophylls and carotenoids. *J Mar Res* 1963; **21**: 155-63.

[10] Janbaz KH, Saeedb SA, Gilani AH. Studies on the protective effects of caffeic acid and quercetin on chemical-induced hepatotoxicity in rodents. *Phytomedicine* 2004; **11**: 424-30.

[11] Emmerie A, Engel C. Calorimetric determination of alpha tocopherol (Vitamin E). *Rev Trav Chim* 1938a; 57: 1351.

[12] Emmerie A, Engel C. Calorimetric determination of tocopherol-II Absorption experiments. *Nature* 1938b; 142: 873.

[13] Nichans WG, Samuelson B. Formation of malondialdehyde from phospholipid arachidonate during microsomal lipid peroxidation. *European J Biochem* 1972; **6**:126-30.

[14] Ellman GC. Tissue sulfhydryl groups. *Arch Biochem Biophy* 1959; **82**: 70-7.

[15] Kakkar P, Das B, Viswanathan PN. A modified spectrophotometric assay of superoxide dismutase. *Indian J Biochem Biophys* 1984; **21**: 130-2.

[16] Sinha AK. Colorimetric assay of catalase. *Analytical Biochem* 1972; **47**: 389-94.

[17] Rotruck JJ, Pope AL, Gantter HE, Swanson AB. Selenium: Biochemical role as a component of glutathione peroxidase. *Science*. 1973; **179**: 588-90.

[18] Drury RAD, Wallington EA. Carleton′s Histological Technique. New York: Oxford University Press; 1980.

[19] Chenoweth MB, Hake CL. The smaller halogenated aliphatic hydrocarbons. *Annu Rev Pharmacol* 1962; **2**: 363-98.

[20] Sallie R, Tredger JM, William R. Drugs and the liver. *Biopharm Drug Dispos* 1991; **12**: 251-9.