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Research Article

ANTIOXIDANT AND HEPATOPROTECTIVE PROPERTIES OF MACRO- ALGA CHAETOMORPHA LINUM AGAINST EXPERIMENTALLY INDUCED OXIDATIVE STRESS

Priyadarshini Rautray[1]*, Jyotsnarani Pradhan[2] and Luna Samanta[3]

[1]Department of Zoology, Utkal University, Bhubaneswar – 751004, India
[2]Department of Biotechnology, Utkal University, Bhubaneswar – 751004, India
[3]Department of Zoology, School of Life Sciences, Ravenshaw University, Cuttack-753003, India

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ABSTRACT

The present study aims to establish a positive correlation between the endogenous glutathione content and hepatoprotective activity, of the aqueous extract (AE) of *Chaetomorpha linum*. Presence of higher amounts of glutathione is indicative of the higher antioxidant potential of the extract. 20 female wistar strain rats were divided into four groups, with 5 animals each. Group 1 served as control, group 2 was treated with carbon tetrachloride, which is a potent and established model of hepatotoxicity. Group 3 animals were fed with 100 mg / kg body weight (aqueous extract) of *C. linum*. Group 4 animals were fed with the same dose of AE as that of group 3, followed by carbon tetrachloride treatment. Endogenous glutathione and protein contents of the macro algae were estimated. Tissue peroxidation levels and glutathione activity were assayed in the liver tissues of the experimental animals *in vivo*.

INTRODUCTION

Seaweeds are a group of macroscopic, multicellular, marine algae, with profound biological importance. They are principally grouped into three major classes depending on the colour of their thallus (i.e red- Rhodophyta, brown-Phaeophyta and green–Chlorophyta). They are rich in vitamins, minerals, trace elements, dietary fibres, pigments, proteins and amino acid, due to which their consumption has increased globally and they are widely used as neutraceutical supplements (Arunkumar *et al.*, 2010). As a natural source of antioxidant, they have gained much importance in biomedical, pharmaceutical and food industry. *C. linum* has been reported to posses highest antioxidant potential with relatively low IC_{50}, highest flavonoid content (18.177± 2.238 mg RE/g) and relatively high content of phenolics (2.895± 0.415 mg GAE/g) as compared to other species of Chaetomorpha genus (Farasat *et al.*, 2013). Due to their less adverse effects as compared to synthetic drugs, they have been studied to establish various health benefits in animal models as well as in vitro assays relating to stress related disorders. Seaweeds are well known to flourish in polluted water as well as have exceptional capacity to withstand adverse climatic conditions. These factors envisage the fact that they are provided with inbuilt defence system to combat the adversities of the surrounding environment and thrive successfully. Their inherent capacity to detoxify and resist metal pollutants is still poorly understood. The most vital non enzymatic antioxidant molecule: Reduced glutathione (GSH) are the precursor molecules for synthesis of Phytochelatins (PC). These are small sulphur-rich oligopeptides of the general structure (Glu-Cys)n-Gly, n = 2–11 and are reported to be involved in homeostasis and detoxification of metals in the cells of higher plants, eukaryotic microalgae, some fungi (Zenk, 1996) and lichens (Pawlik-Skowronska *et al.*, 2002). From experimental studies on microalgae it is known that both increases and decreases in concentrations of GSH can be associated with synthesis of PCs (Ruegsegger *et al.*, 1990; Rijstenbil and Wijnholds, 1996; Ahner *et al.*, 2002), and this appears to be true for seaweeds too. Glutathione is a multifunctional tri peptide thiol, ubiquitously found in all eukaryotes and is involved in a number of vital activities that guard the cell against toxicant insults. Its metabolism is therefore tightly regulated in the algal cell. When seaweeds are subjected to a mixture of metals, glutathione may be used to combat oxidative stress, for production of PCs, and/or to complex certain metals (e.g. Ruegsegger *et al.*, 1990; Rijstenbil and Wijnholds, 1996; Ahner *et al.*, 2002). There are extensive reviews regarding the metabolism and function of glutathione in numerous disease conditions, which is beyond the scope of the present article to enumerate (Rautray and Samanta, 2016, Townsend *et al.*, 2003). However in a nutshell the important functions of GSH are depicted in Figure1.

*Corresponding author: **Priyadarshini Rautray***
Department of Zoology, Utkal University, Bhubaneswar – 751004, India

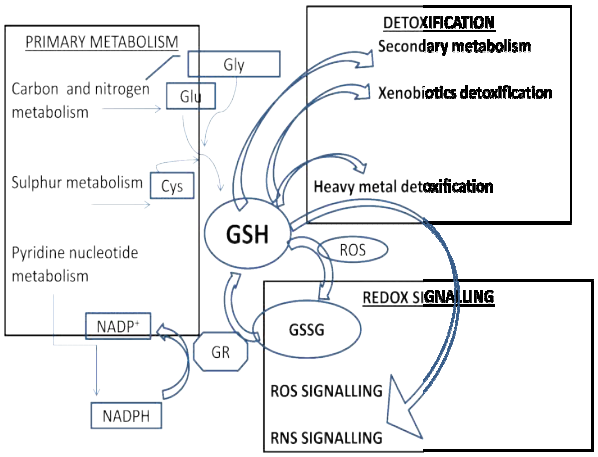

Figure 1 Schematic representation of some of the most vital functions of glutathione

Oxidative stress has been a major contributing factor for numerous health adversities. Recent advances in research are suggestive of the beneficial effects of these marine macroalga in combating many such conditions. Several in vivo and in vitro experiments have established the protective effects of these marine algae. However reports regarding the bioactive principles of these algae are scanty. A few reports indicate the presence of endogenous reduced glutathione in natural assemblages of seaweeds (Pawlik-Skowronska *et al.,* 2007). In the present study an attempt has been made to estimate the glutathione concentration of the algal extract and its probable protective mechanism elucidated using rodent model.

MATERIALS AND METHODS

Chemicals

Trichloro acetic acid (TCA), Hydrochloric acid (HCl), Di potassium hydrogen phosphate (K_2HPO_4), Pottasium di hydrogen phosphate (KH_2PO_4), Sodium dodecyl sulfate (SDS), Thiobarbituric acid (TBA), Butylated hydroxyl toluene (BHT) and Bovine serum albumin (BSA) were obtained from Sigma (St Louis, MO, USA). 5, 5′-dithiobis-2- nitrobenzoic acid (DTNB), Reduced glutathione (GSH) and Guanidine hydrochloride were procured from SISCO Research Laboratory (Mumbai, India). All other chemicals otherwise not mentioned were of analytical grade.

Collection and preparation of the algal extract

The seaweed *C. linum* (O.F.Muller) (Kützing, F. T. 1845) was harvested from the Chilika lake of Orissa (19^0 28' and 19^0 54' N latitude and 85^0 05' and 85^0 38' E longitude). The alga was rinsed carefully with freshwater thrice and then consecutively with distilled water to remove epiphytes, and shed dried. The sample was prepared as per the method described by Mansuya et al., (2010) with slight modifications. Dried finely powdered *C. linum* (10 g) was extracted with 100 ml of distilled water at 5-6° C overnight. The extracts were centrifuged (1000 g for 15 min), followed by filtration with Millipore filter (0.45 µm). The aqueous extract was lyophilised and stored at -80°C till further use.

Extract analysis

Estimation of glutathione content of the aqueous extract

The method of Moron *et al.,* (1979) was followed to determine the amount of reduced glutathione. For the estimation, algal extract (2g in 20ml DW) was treated with 5% TCA and centrifuged at 10,000 rpm for 10 minutes at 4ºC. Then supernatant was used for the estimation of GSH. To 0.1 ml of supernatant, 1.0 ml of phosphate buffer was added followed by 2.0 ml of freshly prepared DTNB (Ellman's reagent) solution. The intensity of the yellow color formed was read at 412 nm in a spectrophotometer after 10 minutes. The values are expressed as µmoles of GSH/g tissue.

Estimation of Total Protein Content of the Aqueous Extract

Protein content of the extract was carried out using biuret method according to Layne, 1957, using Bovine Serum Albumin as standard (BSA). The aqueous extract of *C. linum* was precipitated with 10% TCA and the pellet was dissolved in 0.1 M KOH, which was assayed for protein content. To 0.3 ml of sample, 0.9 ml of Biurete reagent (0.003g copper sulphate, 0.009 g sodium pottasium tartarate and 0.005g potassium iodide dissolved in 10 ml of 0.2 M sodium hydroxide) was added and kept at 37^0C for 10 minutes and thereafter absorbance was recorded at 540nm.

In vivo Experimental Design

Laboratory inbred female Wistar strain rats (120-150g) from the parental stock obtained from National Institute of Nutrition, Hyderabad, India were used for the experiment. The breeding and maintenance of the animals were approved by Institutional Animal Ethics Committee as per the guidelines of Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Rats were divided into 4 groups of 5 animals each. *Group I* served as normal control. *Group-II* received single dose of 1 ml of 20% v/v CCl_4 kg^{-1} body weight two days prior to completion of experiment (Chen *et al.,* 2006) and treated as toxin control. *Group-III* received aqueous extract of *C. linum* orally for 10 days at 100 mg kg^{-1} body weight for evaluating any adverse effect of the extract. *Group IV* received the extract as mentioned above followed by administration of 1 ml of 20% CCl_4 kg^{-1} body weight after 8 days of treatment (2 days before sacrifice).

Rats were sacrificed by decapitation. The dose of extract was decided based on the data of pilot experiment on physiological parameters which showed a significantly lowered low density lipoprotein level (LDL-cholesterol) in the serum after 10 days of treatment with 200 mg per kg body weight of the aqueous extract of *C. linum.*

Tissue processing: After rats were sacrificed, Liver tissue was excised immediately, blotted off blood and rinsed in ice cold normal saline. A 20% (w/v) homogenate was prepared in 50mM Phosphate buffer, pH 7.4 containing 0.1 % (v/v) TritonX100. The homogenate was centrifuged at 10,000 x g for 20 min at 4^0 C and the supernatant was collected for analysis. Rest of the tissues were stored for future use at -80 ^{0} C.

Lipid peroxidation in tissues samples

This was assayed according to the method of Ohkawa *et al*., (1979) by monitoring formation of thiobarbituric acid reactive substances (TBARS). Polyunsaturated lipids and other biomolecules undergo ROS-induced chain reaction to yield malondialdehyde (MDA) and other aldehydes which react with thiobarbituric acid (TBA) in an acidic medium, forming a compound with absorbance maxima at 532 nm. The assay is conducted in presence of butylated hydroxytoluene to prevent artefactual peroxidation during heating. The assay mixture contained 50 µl of appropriately diluted sample (0.5 mg protein) and 950 µl TBA-reagent and kept in boiling water bath at 95^0C for 60 mins. Thereafter the tubes were cooled followed by centrifugation at 1000g for 10 mins. Supernatants were carefully assayed for TBARS content. Concentration of TBARS in the sample was calculated from its extinction coefficient $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$(Wills, 1969) and expressed as nmol TBARS formed per mg protein.

Protein estimation of tissue samples were made according to the method of Lowry *et al., (*1951) using Bovine Serum Albumin (BSA) as standard.

Determination of Non protein – SH

These were estimated by the methods of Sedlak and Lindsay (1968) and Wudarczyk *et al*., (1996). The -SH groups can mediate reduction of 5, 5′-dithiobis-2- nitrobenzoic acid into 2-nitro-5-thiobenzoic acid, the rate of formation of which can be followed at 412 nm. Briefly, 0.2 ml of sample was treated with equal volume of 10% TCA containing 0.02 N HCl. Then contents were centrifuged at 1000 x g for 15 min to precipitate proteins. Supernatants were assayed for non protein SH. For non protein SH, distilled water 0.675 ml and 0.750 ml of 0.4 M Tris Cl (pH 8.9) was added to 0.05 ml of supernatant. To this 0.025 ml of 3.28 mM DTNB was added and incubated for 15 min and centrifuged at 1000 x g for 15 mins. A reagent blank (without sample) and a sample blank (without DTNB) were prepared in a similar manner. Absorbance was read at 412 nm in a UV-VIS spcetrophotometre. A standard curve was prepared with GSH which was linear in the concentration range of 0.1 – 0.4 mM. Final values were expressed as nmol thiol/mg tissue.

Statistics

All data were expressed as mean ± SD. For *in vivo* data one way ANOVA followed by Turkey Kramer post hoc test, was used to find out the level of significance. A difference was considered significant at $p< 0.05$ level.

RESULTS

GSH concentration of the aqueous extract of *Chaetomorpha linum* was approximately 11.76 µmol/g tissue as calculated by comparison with the standard graph of GSH. There are disparities in reports of endogenous glutathione contents of all the three major groups of alga, within and between. This disparity may be due to the differences in the site of collection as well as period of algal collection. With variation in temperature, humidity, salinity, pH and other environmental conditions, the algae tend to regulate its metabolic activities and accordingly accommodate to the environmental fluctuations. The presence of higher concentrations of endogenous glutathione may be a reason for higher anti-oxidant potential of the macroalga *C linum* in the present study.

Total protein content of the examined algal extract using biuret assay is expressed in terms of mg/g tissue dry weight of algae taking BSA as standard (standard curve equation: $y = 0.0001x + 0.0005$, $R^2 = 0.9998$).TBARS concentrations in the liver homogenates of all experimental animals are presented in Table 1. The augmented levels of TBARS in response to CCl_4 treatment were significantly declined in seaweed pretreated group to reach the level in control liver. However, treatment with seaweed extract alone didn't alter their levels. Marked decrease in NPSH was observed in the CCl_4 treated group which restored back to normal levels with the extract pretreatment (Table 1)

Table 1 Effect of aqueous extract of *Chaetomorpha linum* on liver functional markers

Parameters	Control	CCl_4	100mg extract	100mg extract+ CCl_4
Oxidative Stress Markers				
Lipid peroxidation (n mol TBARS formed mg^{-1} protein)	0.765 ± 0.307^{d}	3.272 ± 1.49^{b}	1.012 ± 0.54^{d}	$0.878\pm.1033^{d}$
Non Protein SH (n mol g^{-1} tissue)	2.20 ± 0.410^{d}	1.54 ± 0.234^{b}	2.78 ± 0.248^{d}	2.63 ± 0.330^{d}
Non Enzymatic Antioxidants				
GSH (µ mol g^{-1} tissue)	9.38 ± 1.01^{d}	7.78 ± 0.78^{b}	$11.62\pm0.328^{b,d}$	$10.93\pm0.419^{b,d}$

Data are expressed as mean ± SD of 5 animals. Data having superscripts of different letters are significantly different from each other. $^{a}p<0.05$ and $^{b}p< 0.01$ with respect to Control; $^{c}p< 0.05$ and $^{d}p<0.01$ with respect to CCl_4 treated group.

DISCUSSION

As per earlier findings, there are reports regarding presence of amines, peptides, flavonoids and terpenoids present in *C. linum* extract as evident from the FT-IR spectrum of aqueous extract of *C. linum* from Kanyakumari of southeast coast of India showed peaks at 1,020, 1,112, 1,325, 1,512, 1,535, 1,610, 1,725, 1,862, 2,924, 3,330 cm^{-1}. The vibrational bands corresponding to the bonds such as –C=C (ring), –C–O, –C–O–C and C=C (chain) by Kannan *et al.,* (2013). The results of the present study are in confirmation with the above data. The protein content of the extract was 77.50 ± 2.29 mg eq BSA/g dry weight. This indicates that the aqueous extract contains peptides and thus may be regarded as a dietary supplement in protein deficiency diseases. Presence of endogenous glutathione content in the aqueous extract is indicative of a enhanced inbuilt protective mechanism of the algae against reactive oxygen species as well as adverse climatic parameters. Reports regarding the lack of production of phytochelatins in green seaweeds (Pawlik- Skowronska *et al.,* 2007), is suggestive of lesser intracellular metal accumulation rather than inability for PC synthesis, and therefore these algal varieties can be used safely as dietary supplements .

A number of synthetic drugs and environmental toxicants can induce several cellular damages in different organs of our body by generation of reactive free radical. carbon tetrachloride is such an extensively studied toxicant. Liver is the major metabolic organ which can be survey for possible hepatotoxic

effect as well as cellular degenerative changes following carbon tetrachloride administration. Liver damage is associated with lipid peroxidation, enzyme leakage and depletion in glutathione (GSH) levels. Therefore in the present study emphasis was laid on assaying the oxidative stress markers (TBARS) in the liver tissues of the experimental animals. In liver CCl_4 is metabolized by Cytochrome P450 dependent mono-oxygenase systems followed by its conversion to the active form, trichloromethyl radical. With rise in oxidative stress, it reacts rapidly with oxygen to generate $CCl3OO^{0-}$, which initiates the peroxidation of Poly Unsaturated Fatty Acids (PUFA), causing membrane damage and binds covalently to microsomal lipids and proteins. The administration of aqueous extract, appreciably reduced the TBARS formation in the seaweed treated groups indicating a strong protective mechanism against CCl_4 hepatotoxicty. Many plants have developed an efficient antioxidant system for protection against xenobiotics and ROS. The tripeptide GSH is an important metabolite that can be utilized to detoxify exo- and endogenous toxins by the catalytic activities of some GSH-dependent enzymes (Hossain *et al.*, 2007). GSH, the major potent non enzymatic antioxidant, endogenously present in the algal extract thus helps ameliorate oxidative stress by restoring TBARS levels to normal in pretreated groups. Decrease in Non Protein SH levels is indicative of higher oxidative stress and its restoration by the AE pretreatment is suggestive of protective antioxidant capacity.

CONCLUSION

The present study provides evidence that the aqueous extract of Chaetomorpha linum, is a potent antioxidant as well as hepatoprotectant against carbon tetrachloride induced hepatotoxicity. This may be attributed partly to the endogenous glutathione content of the seaweed. Further charecterisation of the algal extract would hold promising results, in enumerating the protective effects of other potent bioactives within the algae, which are yet unexplored.

Conflict of Interest: The authors declare that there is no conflict of interest.

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