Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

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

Document heading

Nephroprotective effects of *Colpomenia sinuosa* (Derbes & Solier) against carbon tetrachloride induced kidney injury in Wistar rats

Lekameera Ramarajan[1], Somasundaran Thirugnanasambandan Somasundaram[1], Sethupathy Subramanian[2], Vijayabaskar Pandian[3*]

[1]*Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai -608 502, Tamilnadu, India.*
[2]*Division of Biochemistry, Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalai nagar -608 002, Tamilnadu, India.*
[3]*PG Research Department of Microbiology, Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi 626 124, Tamilnadu, India*

ARTICLE INFO

Article history:
Received 25 June 2012
Received in revised from 5 July 2012
Accepted 7 Octoberr 2012
Available online 28 October 2012

Keywords:
Colpomenia sinuosa
CCl4
Kidney
Nephrotoxicity.

ABSTRACT

Objective: To establish the protective effect of seaweed *Colpomenia sinuosa* against carbon tetra chloride (CCl_4) induced oxidative stress and resultant dysfunction of rat kidney. **Methods:** Seven to eight weeks old male Wistar rats (150-220g) were exposed to CCl4 (1.5 ml/kg) injection then treated with seaweed *Colpomenia sinuosa* (100 mg/kg body weight in 0.3% CMC solution). Blood was collected at the 5th day of experimental period to estimate the Total count (TC), Hemoglobin (HB), Total protein (TP), Glucose, Albumin, Cholesterol, TGL and Urea. **Results:** The results shows significantly decreased ($P<0.01$) level of TC, the cholesterol and urea levels shows significantly increased ($P<0.05$) in CCl_4 treated groups when compared to control groups. These levels were found to be normalized by oral feeding of *C. sinuosa*. Then the rats were sacrificed and kidneys taken for enzyme analyses and histological examination. In the CCl_4 treated group significantly increased activities in TBARS, SOD, CAT, GPX, GSH ($P<0.05$) when compared to control group. These increased activities were found to near normal in the CCl_4 + *C. sinuosa* treated group and Seaweed *C. sinuosa* treated alone group did not change any enzyme activity. Exposure to CCl_4 resulted hydrobhic changes in epithelium and Hypercellularity of glomerulus was seen in the CCl_4 + drug treated group. **Conclusions:** These results suggest that the nephroprotective effect of *C. sinuosa* can be attributed to its enhancing effects on antioxidant defense system and lead to prevent the damage by exposure of CCl4 toxicity.

1. Introduction

Carbon tetra chloride (CCl_4) is known to be nephrotoxic as well as hepatotoxic to humans[1]. Administration of CCl_4 causes increased levels of lipid peroxidation[1,2] resulting from decreased activity of enzymes protecting lipid peroxidation in the kidney[3]. It inhibits the enzyme activating molecules in the tissues of vital organs such as liver, kidney etc., through covalent binding to the microsomal lipids and membrane proteins[4,5]. Oxygen radicals exert critical actions such as signal transduction, gene transcription and regulation of soluble guanylate cyclase activity in cells[6]. The consequences of oxidative stress are serious and in many cases are manifested by increased activities of enzymes involved in oxygen detoxification[7]. Reactive oxygen species (ROS) are highly reactive and react with many intracellular molecule mainly unsaturated fatty acids (Phosphoilipuids, glycolipidsd, glycerides and sterols) and transmembrane proteins with oxidizable aminoacids[8]. ROS can originate oxidation and irreversible cell damage[9]. Thus the increase of free radicals in the cells can induce lipid peroxidation with oxidative breakdown of membrane polyunsaturated fatty acids and subsequent alterations of cell membrane permeability and viscosity[10]. Stephen et al.,[11] reported that effects of CCl_4 on kidney structure and function depended on the functional state of the liver. CCl_4 induces sub-lethal proximal tubular injury in the kidney and focal alteration in granular pneumatocytes [12].

Over the years, various evidences suggest that reactive free radical species in a controlled sphere are physiologically relevant to exert a variety of biochemical reactions that regulate many of our important physiological functions including defense against microorganism, cell signaling,

*Corresponding author: Dr. P. Vijayabaskar, Assistant Professor, Post Graduate Research Department of Microbiology, Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi - 626 124, Tamil Nadu, India.
Tel: +91 9994019069
Fax: 04562 254970
E-mail: baski_bos@yahoo.co.in; heparinbaski@gmail.com

vascular control, cell generation and degeneration, control of cellular homeostasis[13-17]. Many experimental studies suggest that ROS take part in the pathogenesis of several kidney diseases for example ROS have been implicated in models of acute renal failure induced by following drugs, Gentamycin and Glycerol in animals[18-19].

There are synthetic antioxidants like butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), Propyl gallate (PG) and tertiary butyl hydroquinone (TBHQ). However, they are suspected to be responsible for complications like liver damage and carcinogenesis in laboratory animals [20-21]. In order to compensate these effects, researchers switched over to find antioxidant drugs from natural sources such as plants and vegetables. The search for powerful but non-toxic antioxidants from natural sources, especially edible or medicinal plants, is continuing for several years[22,23]. Several antioxidants agents, including ginkgo biloba, black tea extracts and vitamins (C and E) have been reported to reduce CCl_4 induced nephrotoxicity[2,24-26]. Marine algae are now being considered to be rich source of antioxidant principles[27] especially brown algae are rich in carotenoids, β-carotene and violaxanthin.

Seaweeds are the known sources of pharmacological compounds and food additives with potential health effects and are exhibiting antioxidative and anticarcinogenic properties[28,29]. The use of seaweed as food and medicine prior to 2000BC found mention in ancient Chinese medicinal literature[30]. Seaweeds also have a number of secondary metabolites that serve as chemical defence mechanisms against herbivory and fouling[31]. It is thus highly probable that algae have the potential to provide an alternative source of leads in solving many biomedical problems[32], including oxidative damage[33].

Seaweeds are rich in polyphenols, also called phlorotannins, derived from phloroglucinol units (1,3,5 – trihydroxybenzine). Phlorotannins constitute an extremely heterogeneous group of molecules providing a wide range of potential biological activity including antioxidant property[34]. Antioxidant activity of polyphenols extracted from brown and red seaweeds has already been demonstrated by in vitro assays[35]. Carotenoids are powerful antioxidants, which are present in seaweeds and have a diminishing risk of cardio-vascular disease, cancers, opthalmological diseases etc[34].

The brown seaweed *Colpomenia sinuosa* collected from Tuticorin coast (Lat. 80 45′N, long. 780 10′E) of Gulf of Mannar, Southeast coast of India were transported to the laboratory in fresh condition and identified up to species level using standard keys. As for as the present knowledge in concern that *C. sinuosa* is not much experimented for its antioxidant property. The present study was designed to observe the changes in the antioxidative defense enzymes in response to CCl_4 induced nephrotoxicity and to investigate the possible protective role of brown algae *C. sinuosa* against CCl_4 induced nephrotoxicity in rats.

2. Materials and Methods

2.1. Animals

Seven to eight weeks old male Wistar rats, weighing 150-220 g were housed in polypropylene cages, maintained in a controlled environment under standard conditions of temperature and humidity with alternating 12 h light /dark cycle. The animals were maintained on standard chow diet and water ad libitum and the study was approved by the ethical committee. After 15 days of acclimatization period they were randomly assigned in to four groups of six each.

2.2. Preparation of seaweed extracts

10 g of seaweed powder was extracted sequentially with diethyl ether in a Soxlet extractor for six hours and the extraction was repeated twice [36]. The extracts were then concentrated under reduced pressure and the resultant residues were stored in dark at 4 ℃ until further use. The diethyl ether residue dissolved in 0.3 % CMC was used in the following in vivo study to assess its antioxidant potential.

2.3. Experimental design

The animals were divided into four groups of six each.

Group of animals	First day	Second day
Group I	Untreated control rats	
Group II	Rats were intraperitoneally administered with CCl_4 at the dose of 1.5 ml/kg after overnight fasting	
Group III		Rats were orally administered with seaweed extract of C. sinousa at the dose of 100 mg/kg body weight in 0.3% CMC two injection at 6 hours interval
Group IV	Rats were intraperitoneally administered with CCl_4 at dose of 1.5 ml/kg + after overnight fasting	Rats were orally administered with seaweed extract of C. sinousa at the dose of 100 mg/kg body weight in 0.3% CMC two injection at 6 hours interval

On the 5th day of the experimental period, the animals were fasted overnight and then sacrificed by cervical dislocation. Blood was collected in heparinised tubes for the separation of plasma for biochemical estimation.

2.4. Biochemical analysis

Haemoglobin (Hb) was estimated in haemolysates by the cyanmethaemoglobin method of Jalajakumari Praveen[37].

White blood cells (WBC) total count (TC) using improved Neubauer counting chamber. Total protein content of plasma was estimated using Erba kit by Biuret method[38]. The levels were expressed as mg/g tissue. Plasma albumin was estimated using Bayer Diagnostics kit by BCG method[39]. Blood glucose was estimated using Erba kit by enzymatic glucose oxidase peroxidase (GOD-POD) method[40]. Albumin and glucose values are expressed as g/dl. The total cholesterol and urea levels in plasma were estimated by cholesterol oxidized enzymatic method using Agappe Diagnostic kit[41-42]. Cholesterol and urea volume were expressed in mg/dl.

2.5. Estimation of kidney function

Plasma creatinine and urea were estimated to asses the over all kidney function. Plasma creatinine was estimated by Jaffe's method using erzb kit[43]. Urea was estimated by Autozyme enzymatic method using urease accurex kit[42-39]. Urea and creatinine values were expressed as mg/dl.

2.6. Preparation of tissue homogenate

A known amount of kidney sample was homogenized in 0.25M sucrose and centrifuged at 10,000 r/min for 30 minutes under cold condition. Then the supernatant which was used to enzymatic and non-enzymatic antioxidants.

2.7. Assay of lipid peroxidation

The melondiialdehyde (MDA) content a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa et al.,[44]. To 0.2 ml of kidney homogenate was mixed with 0.2 ml of 8.1 % sodium dodecyl sulfate (SDS), 1.5 ml of 20 % acetic acid (pH 3.5) and 1.5 ml of 0.8 % aqueous solution of thiobarbituric acid (TBA) was added. The mixture was brought upto 4.0 ml with distilled water and heated in a boiling water bath at 95 ℃ for 60 minutes. After cooling with tab water, 1.0 ml of distilled water and 5.0 ml of n-butanol pyridine (5:1 v/v) was added and centrifuged. The organic layer was taken out and its absorbance was read at 535 nm. The level of TBARS in tissues is expressed as nmol/mg protein of tissue.

2.8. Estimation of enzymatic Antioxidants

2.8.1. Assay of superoxide dismutase (SOD)

SOD was assayed by the method of Kakkar et al.[45]. To 1 ml of kidney homogenate was taken with 1.2 ml of sodium pyrophosphate (pH 8.3, 0.052 M), 0.1 ml of Phenazine methosulfate (PMS) (186 μm) and 0.3 ml of nitroblue tetrazolium (NBT) (300 μm). The reaction was initiated by adding 0.2 ml of nicotinnamide adenine dinucleotide (NADH) (780 μM). After incubation for 90 seconds and the reaction was stopped by the addition of 1.0 ml of glacial acetic acid. The colour formed at the end of reaction was extracted in to the butanol layer and measured at 560 nm. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50 %. The SOD activity was expressed in terms of units per milligram of protein (U/mg protein).

2.8.2. Assay of catalase (CAT)

CAT was assayed by the method of Sinha[46]. To 0.1 ml of kidney homogenate was taken with 0.9 ml of phosphate buffer (0.01 M, pH 7.0) and 0.4 ml of hydrogen peroxide (H_2O_2) (0.2 M). The reduction was stopped at different time intervals by the addition of a dichromate acetic acid mixture. The rate of changes in the absorbance at 620 nm. Catalase activity was expressed in terms of units per milligram of protein tissue (U/mg protein).

2.8.3. Assay of glutathione peroxidase (GPx)

GPx was assayed by the method of Rotruck et al.[47]. To 0.2 ml kidney homogenate was taken with 0.2 ml of phosphate buffer (0.4M, pH 7.0), 0.2 ml of EDTA (0.4 mM), 0.1 ml of sodium azide (10 mM) 0.2 ml of GSH (2 mM) and 0.1 ml of H_2O_2 (0.2 mM). The reaction was stopped by the addition of 0.5 ml of 10 % TCA. The reduced glutathione hormone was allowed to react with DTNB and the developed yellow color was measured at 412 nm. The activity of glutathione peroxidase was expressed as U/mg protein

2.9. Non-Enzymatic Antioxidants

2.9.1. Estimation of reduced glutathione (GSH)

Reduced GSH was assayed by the method of Beutler and Kelly[48]. 0.2 ml of kidney tissue homogenate was taken with 0.1% of 1.8 ml EDTA solution and 3 ml of precipitate reagent were mixed thoroughly and allowed to stand for 5 minutes. To this 4.0 ml of 0.3 M disodium hydrogen phosphate solution (0.3M, pH 8.0) and 1 ml of DTNB reagent were mixed. The absorbance of this sample was read at 412 nm. The values were expressed as μg/mg protein.

2.10. Histopathology

Formalin-fixed portions of Kidney were prepared for histological studies by standard procedures from dehydration through paraffin infiltration in an automatic tissue processor. After paraffin embedding, all sections were cut at 6 μm thickness and routinely stained in hematoxylin-eosin. Selected frozen sections were made to ensure that the vacuolated appearance of the paraffin sections was due to the presence of lipid droplets[49]. Histopathological observation was recorded using Nickon Eclipse-E-200, Photomicrograph system.

2.11. Statistical analysis

Data were expressed as mean±SD, one-way analysis of variance (ANOVA) and Scheiffe multiple comparison tests were used. All tests were considered to be statistically

significant at $P<0.05$.

3. Results

In the present study CCl_4 induced a severe renal damage as represented by markedly elevated leaves of biochemical parameters, antioxidant enzymes namely SOD, CAT, GSH, GPX and lipid peroxidation. As shown in the Table 1, 2 and 3, the administration of *C. sinuosa* (100 mg/kg) brought back to this value of near control groups. However direct evidence for the antioxidant role of seaweed extract In vivo model is rare in research work.

CCl_4 treated of rats showed significantly decreased ($P<0.01$) the levels of white blood cell (WBC), total count (TC) and cholesterol in kidney as compared to control rats (Table 1 and 2). The administration of *C. sinuosa* brought this to near control rats. But the haemoglobin level, total protein, albumin, glucose and TGL leaves did not result in a significant alternation of after explore to CCl_4. Treatments of rat with CCl_4 significantly increase the level of urea which could found to be normal in *C. sinuosa* extract.

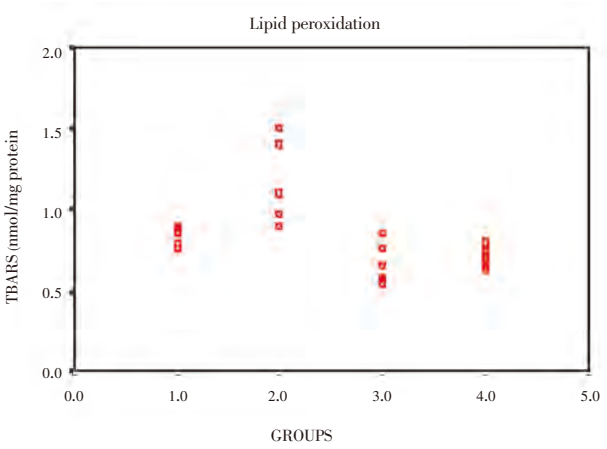
Table 1

Hematological parameters

Groups	Hb g/dl	Total Count x103 cells/ μl
Control	12.668±0.650	7.166±0.338
CCl_4	11.716±0.757	6.900±0.316
Extract only	12.583±0.702	7.583±0.371
CCl4 + Extract	12.566±0.818	7.400±0.352
Significance	N.S	2 Vs 3; 3 Vs 2

Values are given as mean±SD for six animals in each group.

The lipid peroxidation levels significantly increased as compared to normal due to the CCl_4 treatment ($P<0.05$) (Table 3 and Figure 1). Administration of *C. sinuosa* showed significantly reduction in lipid peroxidation in kidney as compared to CCl_4 induced rats. There was a significant increase in TBARS (Table 3 and Figure 1), which is an indirect measure of lipid peroxidation that suggests the possibility of enhanced free radical generation by CCl_4. The statistical evaluation of renal SOD, CAT and GPx activities were significantly increased in the CCl_4 group ($P<0.05$) (Table 3 and Figure 2) were compared with the control group.



1-Control 2-CCl_4 3-Extract only 4-CCl_4+Extract

Figure 1. Activity of lipid peroxidation in kidney.
Values are given as mean±SD for six animals in each group ($P<0.05$)

Non-enzymatic antioxidant GSH (reduced glutathione) was also increased 3-4 folds in CCl_4 treated group (Table 3 and Figure 2). There changes were nearer to control values in the case of *C. sinuosa* extract treated group. Seaweed treated group indicating the nephrotective role of the extract against CCl_4 toxicity. The results of the Histopathological examination shows that the kidneys of the control and drug treated groups showed normal histological features (Figure 3). Hydrophic changes in epithelium were observed in CCl_4 treated group. Hyper cellularity of glomoeruolous was seen in the CCl_4 + drug group.

Table 2

Baseline plasma biochemical investigation

Group	Total protein (g/dl)	Albumin(g/dl)	Glucose(mg/dl)	Cholesterol(mg/dl)	Urea(mg/dl)	TGL(mg/dl)
Control	7.183±0.263	3.050±0.225	86.666±6.282	48.666±2.582	37.000±2.280	53.666±5.988
CCl_4	7.083±0.365	2.883±0.318	72.000±3.847	43.666±1.757	44.333±2.256	47.333±5.8195
Extract only	6.950±0.501	2.966±0.314	76.666±8.981	50.500±5.540	37.000±3.162	48.833±4.1191
CCl4 + Extract	7.466±0.233	2.983±0.263	79.500±4.593	50.000±2.366	36.500±3.834	54.666±5.0761
Significance	N.S	N.S	N.S	1,4,3 Vs 2	N.S	N.S

Values are given as mean±SD for six animals in each group

Table 3

Antioxidant status in kidney

Group	TBARS(nmol/mg protein)	Catalase(U/mg protein)	SOD(U/mg protein)	GSH(μg/mg protein)	GPX(U/mg protein)
Control	0.841±0.055	34.600±2.228	1.278±0.277	143.666±21.759	12.258±1.417
CCl4	1.143±0.248	56.950±3.123	2.695±0.679	262.500±60.474	21.983±2.286
Extract only	0.661±0.125	33.083±6.203	1.491±0.261	163.333±16.427	9.716±1.430
CCl4 + Extract	0.705±0.060	44.083±3.722	1.128±0.278	159.666±13.559	12.798±1.042
Significance	3, 4, 1 vs 2	3, 1, 4 vs 2	4, 1, 3 vs 2	1, 4, 3 vs 2	3, 1, 4 vs 2

Values are given as mean±SD for six animals in each group ($P<0.05$).

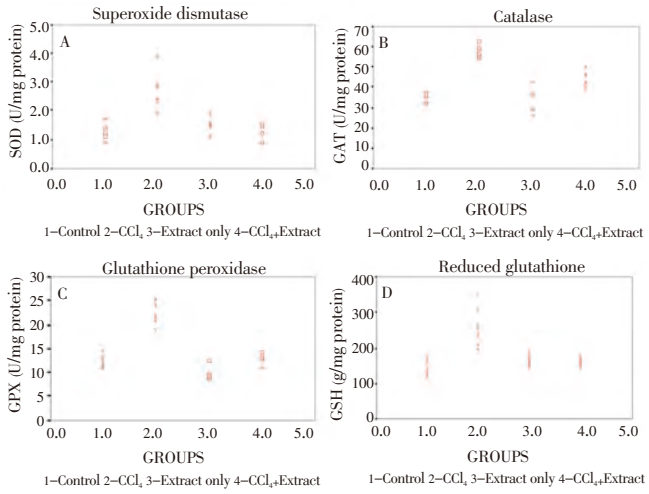


Figure 2. Activity of antioxidant status in kidney of control and experimental animals.

A: Superoxide dismutase; B: Catalase;C: Glutathione peroxidase; D: Reduced glutathione

Values are given as mean±SD for six animals in each group (P<0.05)

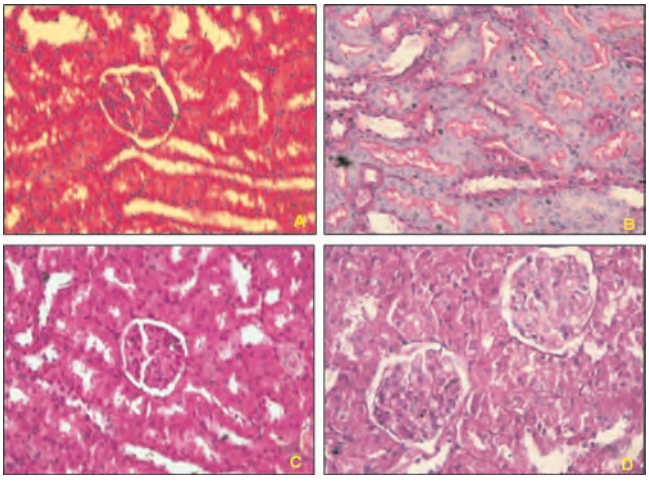


Figure 3. The histopathological examination of rat kidney tissue is depicted (H&E × 40). A: Normal kidney showing glomerulus and tubules; B: Hydropic changes seen in tubular epithelium in CCl_4 treated animals; C: Normal glomerulus and tubules seen in extract alone treated group; D: Hypercellular glomerulus in CCl_4 and drug treated group.

4. Discussion

In the present study indicates the, nephroprotective effects of seaweed *Colpomenia sinuosa* in CCl_4 induced nephrototoxic rats have been explored. Seaweeds are a lipid soluble pigment capable of scavengimg of oxidative damage. Seaweeds contain large amounts of polysaccharides and are rich in minerals. Carotenoids are powerful antioxidants, which are present in seaweeds and have a diminishing risk of cardio-vascular disease, cancers, opthalmological diseases etc [50]. Brown seaweeds are particularly rich in carotenoids especially in fucoxanthin, β-carotene and violaxanthin. It is thus highly probable that algae have the potential to provide an alternative source of leads in solving many biomedical problems[51], including oxidative damage[52-54]. Renal injuries are urea nitrogen elevation developed in balb with mice in exposure of CCl_4[55]. Nandi et al[56] also reported that urea and creatinine levels were increased after exposure to arsenic.

Carbon tetra chloride is a toxic chemical agent. It mainly causes hepatic and renal damage and its metabolites such as trichloromethyl radical ($CCl3^{\bullet}$) and trichloromethyl peroxyl radical ($CCl_3O_2^{\bullet}$) are reported to be involved in the pathogenesis of liver [57] and kidney damage. So CCl_4 induced nephrotoxic rats have been considered as a good model for evaluation of nephroprotective agents.

There was a significant increase in TBARS, which is an indirect measure of lipid peroxidation that suggests the possibility of enhanced free radical generation by CCl_4 as reported by Watson et al. [58]. During reduction of oxygen by 4 single electron steps, three intermediate superoxide, peroxide and hydroxyl radicals are formed which are responsible for oxidative damage in the cell[59].

CCl_4 dosing with its generation of the trichloromethyl radical and the resultant lipid perixidation would be accompanied by a decrease of antioxidants. The initial steps is reduction of CCl_4 by the cytochrome P450 system to the trichloromethyl free radical (CCl3)[60], which in the presence of oxygen is frequently converted into a peroxy radical ($^{\bullet}OOCCl_3$)[61]. Some authors have reported that production of LPO increased proportionally with the amount of fat accumulation and with the production of superoxide from kuffer cells, but that it was inhibited by noradrenaline[62].

In alga treated group, the enzymatic antioxidant were closer to control values and significantly lesser than in CCl_4 administration rats. These increased activities might be attributed to up-regulation in the synthesis of SOD and CAT as a self protective response against oxidative stress due to CCl_4 metabolites [63-64]. The increased activity of SOD in CCl_4 induced rats may be due to the enhanced lipid peroxidation or inactivation of the antioxidative enzymes.

Increased activity of GPx indicating an increase in the amount of organic non-organic peroxides, such as hydrogen peroxide, which are substrates for the enzyme [65]. GPx activity was significantly increased which contributes to the increased activity of GSH[66]. Feral Ozturk *et a.l*[67] observed that the antioxidative defense enzymes (SOD and CAT) were found to be altered by CCl_4 administration, these increased activities were detected and they were found to be normalized in the CCl_4 + Betaine group. Gutierrez et al.[68] reported that antioxidant enzyme GPx level increased during the second week of treatment with $HgCl_2$ rats.

It suggests that there was a decrease in synthesis or increase in utilization of GSH or both. So the possibility of decrease in the synthesis of GSH is considered and it shows that there was no need for the excess GSH which indirectly reveals the diminished free radical generation [69]. Although glutathione radical (GS●) can react with another GS● to yield GS-SG which is then reduced to GSH by the NADPH dependent glutathione reductase[70]. Paolo-di Simplico *et al.* [69] reported that the effect of CCl_4 intoxication on the cytosolic activities of reduced glutathione (GSH) increased significantly 2-3 folds from the control values.

In this study CCl_4 markedly decreased the level of P450 in the kidney because reactive oxygen or free-radical species may directly damage P450 protein, the decrease level of P450 affects cell injury by changing the arachidonate metabolizing pathway in the kidney[71]. Oral administration of *C. sinuosa* significantly reduced lipid peroxidation in kidney than CCl_4 treated group. This indicates that the alga might interfere with free radical generation. In alga treated rats, there was a significant reduction in free radical generation in comparison with CCl_4 treated animals. The possibilities are: first, Alga might have interfered with metabolism of CCl_4 and so causing free radical generation; second, Alga might have quenched the excess free radicals generated due to CCl_4 metabolites. In conclusion, The significant free radical scavenging activity of *C. sinuosa* indicated that it could be a potential source for natural antioxidant lead molecules[72,73], but also nephrotoxicity was effectively alleviated by the *C. sinuosa* pretreatment showed in these study experiments. Therefore, the brown alga *C. sinuosa* is beneficial in reducing free radical damage. The result also suggests that the lead molecules may be of polyphenolic in nature. In this study suggest that *C. sinuosa* could prevent renal damage by improving the lipid peroxidation products through the scavenging activity of free radicals induced by CCl_4. Further studies are required to elucidate the compound showing antioxidant property and the compound could evolve as an anticancer drug in near future.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgement

The authors are thankful to Prof. T. Balasubramanian, Director, Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai, Tamilnadu, India for providing all facilities.

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