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

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Objective: To establish the protective effect of seaweed Colpomenia sinuosa against carbon tetra chloride (CCl₄) 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 CCl₄ 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 CCl₄ 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 be near normal in the CCl₄ + C. sinuosa treated group and Seaweed C. sinuosa treated alone group did not change any enzyme activity. Exposure to CCl₄ resulted hydrobolic changes in epithelium and Hypercellulartity of glomerulus was seen in the CCl₄ + 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 (CCl₄) is known to be nephrotoxic as well as hepatotoxic to humans[1]. Administration of CCl₄ 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 (Phospholipids, glycolipids, 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 CCl₄ on kidney structure and function depended on the functional state of the liver. CCl₄ induces sub-lethal proximal tubular injury in the kidney and focal alteration in granular pneumocytes[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,
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 CCl4 induced nephrotoxicity[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 antiarcarcinogenic properties[28,29]. The use of seaweed as food and medicine prior to 2000 BC 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 antioxidative 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, ophthalmological 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 far 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 CCl4 induced nephrotoxicity and to investigate the possible protective role of brown algae C. sinuosa against CCl4 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 °C 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 CCl4 at the dose of 1.5 ml/kg after overnight fasting | Rats were orally administered with seaweed extract of C. sinuosa at the dose of 100 mg/kg body weight in 0.3% CMC two injection at 6 hours interval |
| Group III                 | Rats were intraperitoneally administered with CCl4 at the dose of 1.5 ml/kg after overnight fasting | Rats were orally administered with seaweed extract of C. sinuosa 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 melondialdehyde (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 °C for 60 minutes. After cooling with tap 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 nicotinamide 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 (H2O2) (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 H2O2 (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 CCl₄ 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.

CCl₄ 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 CCl₄. Treatments of rat with CCl₄ significantly increase the level of urea which could found to be normal in C. sinuosa extract.

Table 1

| Groups       | Hb (g/dl)    | Total Count x10³ cells/μl |
|--------------|-------------|---------------------------|
| Control      | 12.668±0.650| 7.166±0.338               |
| CCl₄         | 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 CCl₄ treatment ($P<0.05$) (Table 3 and Figure 1). Administration of C. sinuosa showed significantly reduction in lipid peroxidation in kidney as compared to CCl₄ 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 CCl₄. The statistical evaluation of renal SOD, CAT and GPx activities were significantly increased in the CCl₄ group ($P<0.05$) (Table 3 and Figure 2) were compared with the control group.

Table 2

| 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±2.982        | 37.000±2.280 | 53.666±5.988 |
| CCl₄        | 7.083±0.365          | 2.883±0.318    | 72.000±3.847    | 43.666±1.757        | 44.333±2.256 | 47.333±5.195 |
| Extract only| 6.950±0.501          | 2.960±0.314    | 76.666±8.981    | 50.500±5.540        | 37.000±3.162 | 48.833±4.191 |
| 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 Vs 2            | N.S          | N.S         |

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 CCl₄ 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 nephroprotective role of the extract against CCl₄ toxicity. The results of the Histopathological examination shows that the kidneys of the control and drug treated groups showed normal histological features (Figure 3). Hydroptic changes in epithelium were observed in CCl₄ treated group. Hypercellularity of glomoerulos was seen in the CCl₄ + drug group.

Table 3

| Group       | TBARS (nmol/mg protein) | Catalase (U/mg protein) | SOD (U/mg protein) | GSH (μg/mg protein) | GPx (U/mg protein) |
|-------------|-------------------------|-------------------------|-------------------|---------------------|-------------------|
| Control     | 0.841±0.035             | 34.600±2.228            | 1.278±0.277       | 143.666±21.759      | 12.25±1.417       |
| CCl₄        | 1.143±0.248             | 56.950±3.123            | 2.695±0.679       | 262.500±60.474      | 21.98±2.286       |
| Extract only| 0.661±0.125             | 33.083±2.203            | 1.491±0.261       | 163.333±16.427      | 9.71±1.430        |
| CCl₄ + Extract| 0.705±0.060           | 44.083±3.722            | 1.128±0.278       | 159.66±13.559       | 12.79±1.042       |
| Significance| 3, 4, 1 vs 2            | 3, 1 vs 2               | 4, 1, 3 vs 2      | 1, 4 vs 2           | 3, 1, 4 vs 2      |

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

Figure 1. Activity of lipid peroxidation in kidney. Values are given as mean±SD for six animals in each group ($P<0.05$).
Seaweeds contain large amounts of polysaccharides and soluble pigment capable of scavenging oxidative damage. Carotenoids, especially in fucoxanthin, which are present in seaweeds and have a diminishing risk of cardio-vascular disease, cancers, ophthalmological 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 CCl4 [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•) and trichloromethyl peroxyl radical (CCl3O2•) are reported to be involved in the pathogenesis of liver [57] and kidney damage. So CCl4 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 CCl4, 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].

CCl4, dosing with its generation of the trichloromethyl radical and the resultant lipid peroxidation would be accompanied by a decrease of antioxidants. The initial steps is reduction of CCl4 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 (’OOCCl3) [61]. Some authors have reported that production of LPO increased proportionally with the amount of fat peroxyl radical (CCl3O2•) and trichloromethyl free radical (CCl3•) [60]. 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 might be closer to control values and significantly lesser than in CCl4 administrated 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 CCl4 metabolites [63-64]. The increased activity of SOD in CCl4 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 al. [67] observed that the antioxidative defense enzymes (SOD and CAT) were found to be altered by CCl4 administration, these increased activities were detected and they were found to be normalized in the CCl4 + Betaine group. Gutierrez et al. [68] reported that antioxidant enzyme GPx level increased during the second week of treatment with HgCl2 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 (G•) can react with another G• 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 CCl4 intoxication on the cytosolic activities of reduced glutathione (GSH) increased significantly 2-3 folds from the control values.

4. Discussion

In the present study indicates the, nephroprotective effects of seaweed Colpomenia sinuosa in CCl4 induced nephrotoxic rats have been explored. Seaweeds are a lipid soluble pigment capable of scavenging 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, ophthalmological 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
In this study CCl₄ 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 CCl₄ 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 CCl₄ treated animals. The possibilities are: first, Alga might have interfered with metabolism of CCl₄ and so causing free radical generation; second, Alga might have quenched the excess free radicals generated due to CCl₄ 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 CCl₄. 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.

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