Hepatoprotective Efficacy of Hypnea muciformis Ethanolic Extract on CCl₄ Induced Toxicity in Rats

Giridharan Bupesh¹*, Chinnaiah Amutha³, Sakthivel Vasanth¹, Natesan Manoharan², Ramalingam Senthil Raja⁴, Raju Krishnamoorthy¹ and Periyasamy Subramanian¹

¹Department of Animal Science and Biotechnology; Bharathidasan University; Tiruchirappalli -24, India. ²Department of Marine Science; Bharathidasan University; Tiruchirappalli -24, India. ³Department of Animal Behaviour & Physiology; Madurai Kamaraj University; Madurai-21, India. ⁴Department of Virology; King Institute of Preventive medicine; Guindy,Chennai-32

ABSTRACT

The ethanolic extract of Hypnea muciformis (red algae) was tested for hepatoprotective activity against experimentally induced liver damage by Carbon tetrachloride (CCl₄) in male albino rats. The levels of serum enzymatic and biochemical parameters such as serum glutamate oxaloacetate transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), lactate dehydrogenase, 5′ nucleotidase, bilirubin, creatinine, urea, triglycerides, lipid peroxides and albumin were determined. The CCl₄ induced lesions in the liver significant increased the levels of serum marker enzymes SGPT and SGOT, bilirubin, creatinine and decreased urea. The oral treatment with ethanolic extract of H. muciformis exhibited significant hepatoprotective activity by reducing the CCL₄ caused changes in the biochemical parameters such as total protein, total bilirubin, total cholesterol, triglycerides, and urea. These parameters were restored towards the normal levels as shown by the enzymatic tests. In addition, H. muciformis significantly decreased the liver weight of CCl₄ intoxicated rats. Apparently the H. muciformis extract interfered with the free radical formation, which resulted in hepatoprotective activity. Acute toxicity studies revealed that the LD₅₀ value was more than 3 g/kg body weight. These results clearly indicated that this seaweed contained some active principles in its ethanolic extract which acted as an antidote against the hepatotoxicity induced by CCl₄.

Key words: Hypnea muciformis, Hepatoprotective activity, Carbontetrachloride

INTRODUCTION

Natural product research is increasingly turning to marine herbs as a source of natural products and is currently in preclinical and clinical evaluation showing promising biological activities in in vitro and in vivo assays (Konig and Wright 1996; Blunden 2001). The nutritional value of marine algae has long been recognized in the orient than in the western world with limited use as a dietary part (Indegaard and Ostgaard 1991; Smidtsrod and Christensen 1991). Algae are unexploited sources due to their limited distribution in natural habitats and less information on conditions for their growth and utilization. However, in recent years, algae have gained importance due to their nutritional composition and various bioactive compounds they produce to accustom to the biodiversity of marine environment. Some of the biologically significant compounds of algal origin include carrageenan, sulpholipids, and pigments such as phycocyanin (Siva Kumar et al. 2000).

*Author for correspondence: bupeshgiri@gmail.com
Seaweeds are low in fats but contain vitamins and bioactive compounds such as terpenoids and sulphated polysaccharides, which are potential natural antioxidants not found in the terrestrial plants (Lahaye and Kaffer 1997). The marine red algae are edible algae as they contain carbohydrates, proteins, vitamin A, C and B12, ash and large amount of sodium and potassium electrolytes (Boyd and Good year 1971). Hypnea muciformis is a marine red algae distributed throughout the coastal areas of Rameshwaram (Mandapam), western and eastern coasts of India, Andaman and Nicobar islands. H. muciformis contains the highest free radical scavenging antioxidant activity (Matsukawa, 1997; Yan et al. 1998). It also possesses potent antitumour and antimicrobial activity (Boyd and Bereckzy 1966). CCl4 is one of several chemicals, which cause liver injury. It has long been well documented as a hepatotoxin (Recknagel 1967; Klaassen and Plaa 1969; Harris et al. 1982). It is introduced into the water mainly as industrial wastes from its primary use in the manufacture of chlorofluorocarbons (Borzelleca et al. 1990). CCl4 causes centrilobular necrosis and fat accumulation in the liver. Studies have proved that CCl4-induced hepatotoxicity is catalyzed by the cytochrome P-450 in the endoplasmic reticulum of hepatocytes (Recknagel 1967; Slater 1984). Therefore, the present study was designed to determine the effect of the ethanolic extract of the marine red algae H. muciformis against the experimentally induced liver damage by CCl4 in albino rat model.

MATERIALS AND METHODS

Preparation of Hypnea muciformis extract
The H. muciformis used in this study was collected from the Mandampam seashore region, Tamilnadu during the month of February 2003 and the identification was confirmed with the National Facility for marine Cyanobacteria, Bharathidasan University, Tamilnadu. The fresh and healthy part of marine algae was washed in seawater and fresh water thoroughly to remove the epiphytes and other contamination. Then the sample was air-dried in the shade and coarsely powdered. The powder was soaked in alcohol for one week with occasional shaking; then the supernatant was drained. This crude solution was extracted at 45°C under reduced pressure using rotary evaporator (yield: 12–15%) and the resulting extract was then concentrated and lyophilized to a brownish residue (Anggadiredja et al. 1996).

Animals
Male Wistar albino rats (150–200 g each) were procured from the FIPPT, Chennai and were maintained under standard animal house condition (12 h light and dark cycle at 28 ± 2°C). They were fed with the balanced rodent pellet diet procured from the Poultry Research Station, Nandam, Chennai, India and dechlorinated tap water was provided ad libitum. The animals were sheltered for one week prior to the experimentation for acclimatization to laboratory temperature. The rats of uniform size were divided into four groups of six animals each for experimentation.

Experimental Protocol
The CCl4 (SRL Chemicals, India) was dissolved in gingely oil (Idhayam, India) and was introduced into the stomach of the rats by gavage oral administration through an intragastric tube. The group I (the saline vehicle control group) received only the vehicle (6.25 ml kg–1) orally. The vehicle was made of gingely oil and normal saline solution (10 ml kg–1). The group II (the CCl4 toxin treatment group) received the suitable dose of CCl4 to induce the chemical hepatitis followed 6 h later by oral saline administration. The group III was treated with the ethanolic extract of H. muciformis (200 mg kg–1, dissolved in 10 ml of 25% of Dimethyl sulfoxide (DMSO) with suitable dose of CCl4). The DMSO was purchased from SRL Chemicals, India, which was dissolved in 0.9% (v/v) saline. The ethanolic extract was administered with CCl4 to evaluate its effect on CCl4-induced hepatotoxicity. The group IV of rats were treated similarly to group III but instead of seaweed extract, they were administered with the sylimarin (50 mg kg–1, dissolved in 10 ml of 25% of DMSO vehicle), a standard drug used to compare the curative effects of H. muciformis.

Assessment of Liver Functions and Biochemical Assays
The animals were anaesthetized with ether 24 h after the last hepatotoxin treatment (CCl4) and blood (5 ml) was withdrawn from their posterior vena cava with sterile disposable syringes equipped with hypodermic needles. Serum was separated by centrifugation at 3000 x g for 15 min. The glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) were
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**Estimation of Enzymes and Biochemical Parameters**

- Alkaline phosphatase (ALP) was determined by the method of Kind and King (1971).
- Lactate dehydrogenase (LDH) was analyzed by the method of King (1965).
- Nucleotidase (5′NT) activity was analyzed by the method of Luly et al. (1972).
- Total protein was estimated by the method of Gornall et al. (1949).
- Total bilirubin was estimated by Malloy and Evelyn method (1937).
- Triglyceride was estimated by the method of Fossati and Lorenzo (1983).

**Statistical Analysis**

The mean ± SEM was calculated for each parameter. Results were statically analyzed by One way ANOVA. *P* < 0.05 indicated significant differences between the group means.

**RESULTS**

The ethanolic extract of *H. muciformis* was nontoxic when administered orally to the rats. Its LD₅₀ value was higher than 3 g/kg body weight. Administration of CCl₄ to the rats caused significant liver damage, as evidenced by the altered serum enzymatic and biochemical parameters. The treatment of the rats with the ethanolic extracts of *H. muciformis* exhibited marked protection against CCl₄ induced hepatotoxicity (Table 1, Figs. 1 and 2). The ethanolic extract of *H. muciformis* showed significant hepatoprotective activity against CCl₄ compared to the standard sylimarin.

There was a significant reduction in the liver weight (*P* < 0.001) in the group IV rats. Table 1 shows the results of *H. muciformis* treated animals compared to that of the group II CCl₄ intoxicated animals. As shown in Figure 2, the biochemical parameters such as serum bilirubin levels were also decreased significantly at a dose level of 200 mg/kg of body weight treated animals (*P* < 0.001), when compared with the CCl₄ intoxicated the group II animals which had the total bilirubin and urea 14.0 ± 0.26 and 54.0 ± 1.12 mg/dl, respectively. Figure 1 showed that in the group III, there was a significant increase in the total protein and triglyceride levels in the CCl₄ intoxicated and *H. muciformis* treated animals (*P* < 0.001), respectively. Group comparison between *H. muciformis* treated (group III) and sylimarin (group IV) treated animals showed significant variation in the biochemical parameters, viz. bilirubin, triglyceride, blood urea, GSH, ALP, SGOT and SGPT, indicating that *H. muciformis* was able to produce 87.5% activity exerted by sylimarin, the positive control in this study.

A significant increase in the serum GOT (325.21±3.41 U/L) and GPT (260.26±3.12 U/L) levels were seen in the group II CCl₄ intoxicated animals. These enzymes were reduced to near normal levels such as (178.23±1.32 U/L) and (163.0±1.64 U/L), respectively in the group III *H. muciformis* treated animals (*P* < 0.05). Similarly, the elevated ALP (348.16 ± 3.42 mU/L) and lipid peroxides (2.86 ± 0.24) enzyme levels in the group II CCl₄ intoxicated animals were also decreased to 270 ± 1.42 mU/L and 2.06 ± 0.13, respectively in the group III *H. muciformis* treated rats (Table I). The LDH (230.0 ± 1.31 U/L) and 5′NT (9.32±0.20 U/L) were also significantly decreased in the group III *H. muciformis* treated animals when compared with the group II CCl₄ intoxicated animals that showed the elevated levels of LDH (435.38±1.84 U/L) and 5′ NT (9.32±0.20 U/L), respectively (*P*<0.05). All the parameters were under normal limits in the group IV animals that acted as a positive control, which were intoxicated by the CCl₄ and treated by sylimarin.
Table 1 - Hepatoprotective effect of *H. muciformis* alcoholic extract on biochemical responses of experimental animals to CCl₄

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I control</th>
<th>Group II CCl₄</th>
<th>Group III (H. muciformis + CCl₄)</th>
<th>Group IV sylimarin + CCl₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weight (mg/g)</td>
<td>23.14 ± 0.2</td>
<td>54.26 ± 0.41</td>
<td>32.64 ± 0.82</td>
<td>28.32 ± 0.1</td>
</tr>
<tr>
<td>SGOT (U/L)</td>
<td>134.4 ± 1.49</td>
<td>325.21 ± 3.41</td>
<td>178.23 ± 1.32</td>
<td>140.36 ± 1.14</td>
</tr>
<tr>
<td>SGPT (U/L)</td>
<td>80.2 ± 6.2</td>
<td>260.26 ± 3.12</td>
<td>163.0 ± 1.64</td>
<td>126.2 ± 2.00</td>
</tr>
<tr>
<td>ALP (mU/L)</td>
<td>262.5 ± 1.40</td>
<td>348.16 ± 3.42</td>
<td>270.0 ± 1.42</td>
<td>226.63 ± 2.10</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>132.6 ± 0.52</td>
<td>435.38 ± 1.84</td>
<td>230.0 ± 1.31</td>
<td>192.20 ± 1.45</td>
</tr>
<tr>
<td>Lipid peroxides</td>
<td>1.42 ± 0.08</td>
<td>2.86 ± 0.24</td>
<td>2.06 ± 0.13</td>
<td>1.83 ± 0.09</td>
</tr>
<tr>
<td>5’nucleotidase(U/L)</td>
<td>6.24 ± 0.26</td>
<td>9.32 ± 0.20</td>
<td>5.85 ± 0.28</td>
<td>6.81 ± 0.13</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; n = 6; *p < 0.05 compared to control; †p < 0.05 compared to paracetomol

Figure 1 - Level of Bilirubin and GSH in ethonalic extract of *H.muciformis* on CCl₄ induced liver toxicity.

Figure 2 - Level of Triglyceride, Urea and Total Protein in ethonalic extract of *H.muciformis* on CCl₄ induced liver toxicity.

Each value represents the mean ± SEM of six treated rats. Values statistically significantly different from those of control group are indicated by *(One way Anova, p < 0.05) and **(p < 0.05). Treatment groups are as follow: No Treatment control (group I); CCl₄ (group II); Sylimarin + CCl₄ treatment (group III); *H. muciformis* + CCl₄ (group IV).
**DISCUSSION**

Carbon tetrachloride is commonly used as a model to study the hepatotoxicity (Chun-Kwan Wong et al. 2004). The CCl₄ is bio-transformed by the cytochrome P-450 system in the endoplasmic reticulum to produce trichloromethyl free radical (•CCl₃). Trichloromethyl free radical when combined with the cellular lipids and proteins in the presence of oxygen form trichloromethyl peroxyl radical, which may attack the lipids on the membrane of endoplasmic reticulum faster than trichloromethyl free radical. Thus, trichloromethyl peroxyl free radical leads to elicit lipid peroxidation, the destruction of Ca²⁺ homeostasis, and finally, results in cell death (Opoku et al. 2007).

In this present study, the administration of CCl₄ decreased the levels of total protein and triglycerides. These parameters were brought back to the normal levels in the group III *H. muciformis* treated animals. *H. muciformis* treatment showed a protection against the injurious effects of CCl₄ that might be due to the interference with cytochrome P-450, resulting in the hindrance of the formation of hepatotoxic free radicals. The site-specific oxidative damage in some susceptible amino acids of proteins is now regarded as the major cause of metabolic dysfunction during the pathogenesis (Uday et al. 1999). The attainment of near normalcy in protein, cholesterol, and triglycerides levels in CCl₄ intoxicated and *H. muciformis* treated rats confirmed the hepatoprotective effect of the seaweed extract. The marked elevation of bilirubin and urea level in the serum of group II CCl₄ intoxicated rats were significantly decreased in the group IV *H. muciformis* treated animals. Bilirubin is the conventional indicator of liver diseases (Girish 2004). These biochemical restorations may be due to the inhibitory effects on cytochrome P-450 or/and promotion of its glucuronidation (Cavin et al. 2001).

The health of the liver can assessed by estimating the activities of serum GOT, GPT, ALP, LDH, 5′ nucleotidase, which are enzymes originally present higher concentration in the cytoplasm. SGPT and SGOT (especially the former) are highly localized in hepatocyte cytosols (Ooi 1996). The crude ethanolic extracts of the seaweed probably acted to preserve the structural integrity of the plasma cellular membrane of the hepatocytes to protect it against the breakage by the reactive metabolites produced from exposure to CCl₄. This prevented further damage to more hepatocytes, and hence reduced further leakage of SGPT and SGOT due to cell destruction. This could explain the lower levels of these transaminases observed in the rats treated with the seaweed extract after exposure to the toxin. The tendency of other marker enzymes to return towards a near-normalcy in the *H. muciformis* treated rats was a clear manifestation of anti-hepatotoxic effect due to the presence of sulphated polysaccharides and vitamin C in the seaweed extract (Naidoo et al. 2006). The results were comparable to silymarin. Silymarin is the composite name of three flavonoids isolated from the milk thistle, *Silybum marinux*, and is used as hepatoprotectives against the experimental hepatotoxicity of various chemicals, including CCl₄ (Chhaya and Mishra 1999).

In conclusion, the ethanolic extract of seaweed provided protection from the CCl₄ induced liver damage. The protections against the liver damage by the seaweed *H. muciformis* were comparable to silymarin. Possible mechanism that might be responsible for the protection of CCl₄ induced liver damage by *H. muciformis* could involve its action as a free radical scavenger intercepting those radicals involved in CCl₄ metabolism by the microsomal enzymes. By trapping the oxygen related free radicals, the *H. muciformis* extract could hinder their interaction with the polyunsaturated fatty acids and would abolish the enhancement of lipid peroxidative processes (Upadhyay et al. 2001). It is well documented that flavonoids and glycosides are strong antioxidants (Natarajan et al. 2006). The antioxidant principles from the natural marine resources are multifaceted in their effects and provide enormous scope in correcting the imbalance through regular intake of a proper diet. Thus, from the results, it could be concluded that *H. muciformis* could a promising hepatoprotective agent and this hepatoprotective activity of *H. muciformis* might be due to its antioxidant chemicals present in it.

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