Amelioration of carbon tetrachloride induced oxidative stress in kidney and lung tissues by ethanolic rhizome extract of *Podophyllum hexandrum* in Wistar rats

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Antioxidant activity of 70% ethanolic extract of rhizome of *Podophyllum hexandrum* (Indian Mayapple) was investigated in rats by inducing kidney and lung injury with CCl$_4$. 70% ethanolic extract exhibited significant antioxidant activity in the tissues by showing increased levels of proteins, reduced glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and glutathione-S-transferase (GST) as well as decreased levels of thiobarbituric acid reactive substances (TBARS). The 70% ethanolic extract at a dose level of 20, 30 and 50 mg/kg-day, showed a dose dependent restorative activity against CCl$_4$-induced kidney and lung tissue damage. In our previous study 70% ethanolic extract of *P. hexandrum* had displayed a potent free radical scavenging activity under *in vitro* conditions. In the present study, the 70% ethanolic extract of *P. hexandrum* was effective in restoring CCl$_4$-intoxicated kidney and lung tissue enzymes towards normalcy in combating oxidative stress *in vivo*. Thus, the extract has antioxidative activity.

**Key words:** Antioxidants, carbon tetrachloride, *Podophyllum hexandrum*, GSH, catalase.

INTRODUCTION

Oxidative stress can be defined as a disturbance in the prooxidant/antioxidant balance in favour of the former. Oxidative stress may lead to a variety of diseases and degenerative processes such as aging, immunodeficiencies, neurological disorders, and carcinogenesis. The major cellular sources responsible for the generation of damage-inducing reactive oxygen species (ROS), such as ·O$_2$-, H$_2$O$_2$ and ·OH, are mitochondria and peroxisomes. These antioxidative enzymes include cyclooxygenase (COX), NADPH dehydrogenase, and oxidases such as xanthine oxidase (XOD) (Kang et al., 2003).

Carbon tetrachloride (CCl$_4$) is frequently used as a chemical inducer of experimental tissue damages. Transient tissue disorders after the administration of CCl$_4$ is believed to be induced by the trichloromethyl radical (·CCl$_3$). This free radical induces an adverse reaction by forming other free radicals after its administration in the early stage between intracellular uptake and transformation into storage types. Many biological substances such as membrane lipids, proteins, and nucleic acids are known to be injured by trichloromethyl radicals (Nomura and Yamaoka, 1999). The initial event of CCl$_4$-induced free radical generation is a carbon-halogen bond cleavage, probably through one electron reduction of CCl$_4$ aided by a particular cytochrome P-450. Chloride ion and the trichloromethyl radical, ·CCl$_3$, are the major initial products. ·CCl$_3$ is converted into ·CCl$_3$O$_2$ through its reaction with molecular oxygen. Like the above-mentioned free radicals, lipid peroxidation is initiated by the interaction of this reactive free radical, ·CCl$_3$O$_2$, with polyunsaturated fatty acids (PUFA) of the membrane lipids.
The excessive lipid peroxidation caused by these free radicals leads to a condition of oxidative stress, which results in the accumulation of malondialdehyde (MDA). MDA is one of the end products of the lipid peroxidation process and oxidative stress (Hagihara et al., 1984; Kurata et al., 1993). The MDA produced in this manner is known to cause various diseases. Enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx) form part of the defense system and are capable of eliminating ROS. SOD removes -O_2^- by converting it into H_2O_2, which is converted into molecular oxygen and water by CAT and GSH-Px (Halliwell et al., 1992). Furthermore, GSH-Px degrades hydperoxides, which originates from the oxidation of PUFA in the presence of reduced glutathione (GSH). GSH is a key component of the antioxidant defense system (Puglia and Powell, 1984). *Podophyllum hexandrum* belongs to family Berberidaceae and in Kashmir valley, it is locally known as Banwanguen. The rhizome powder is used as a laxative or to get rid of intestinal worms and also used as poultice to treat warts and tumorous growths on the skin. Recent studies have also shown its effectiveness in the treatment of monocytoid leukemia, Hodgkin disease and non-Hodgkins lymphoma (Gowdey et al., 1995; Cobb, 1990) brain tumors, bladder cancer, lung cancer and AIDS-associated Kaposi’s sarcoma (Blascko et al., 1998). Although liver is considered to be the primary target of CCl_4 toxicity, it is not the only target organ of CCl_4 and it causes free radical generation in other tissues also such as kidneys, heart, lung, testis, brain and blood (Ahmad et al., 1987; Ohta et al., 1997; Ozturk et al., 2003; Mizuguchi et al., 2006; Preethi and Kuttan, 2009). The present study was conducted with the aim of elucidating the antioxidant properties of ethanolic extract of rhizome of *P. hexandrum* against CCl_4-induced oxidative stress in kidney and lung tissues of Wistar rats.

**MATERIALS AND METHODS**

**Plant material collection and extraction**

The rhizome of *P. hexandrum* was collected from higher reaches of Aharbal, Shopian, J&K, India in the month of May and June, identified by the Centre of Plant Taxonomy, Department of Botany, University of Kashmir, and authenticated by Dr. Irshad Ahmad Nawchoo. (Department of Botany) and Akhter Hussain Malik (Curator, Centre for Plant Taxonomy, University of Kashmir). A reference specimen has been retained in the herbarium of the Department of Botany at the University of Kashmir under reference number KASH- bat/Ku/PH- 702- SAG.

The plant material (rhizome) was dried in the shade at 30 ± 2°C. The dried rhizome material was ground into a powder using mortar and pestle and passed through a sieve of 0.3 mm mesh size. The powder obtained was successively extracted in hexane, ethyl acetate, absolute ethanol, 70% ethanol using a Soxhlet extractor (60 - 80°C). The 70% ethanol extract was then concentrated with the help of rotary evaporator under reduced pressure and the solid extract was stored in refrigerator for further use. The extract was dissolved in water and given as mentioned in materials and methods. The series of extractions were done in order to extract the relatively hydrophilic components of *P. hexandrum*.

**Animals**

Adult male albino rats of Wistar strain weighing 200 - 250 g, purchased from the Indian Institute of Integrative and Medicine Jammu (IIIM), were used throughout this study. The animals were fed a pellet diet (Hindustan Lever, Ltd., Mumbai, India) and water *ad libitum*. The animals were maintained in a controlled environment under standard conditions of temperature and humidity with an alternating 12 h light and dark cycle. The animals used in the present study were maintained in accordance with the guidelines prescribed by the National Institute of Nutrition, Indian Council of Medical Research, and the study was approved by the Ethical Committee of the University of Kashmir.

**Dosage and treatment**

Rats were divided into five groups containing six rats each. The plant extract was employed at oral doses of 20, 30 and 50 mg/kg-day. The extract was suspended in normal saline such that the final volume of extract at each dose was 1 ml and was fed to rats by gavage:

- **Group I-** Served as control and received olive oil vehicle only at 5 ml/kg-day.
- **Group II-** Served as negative control and received CCl_4 in corn oil vehicle only.
- **Group III-** Animals received 20 mg/ kg-day extract orally for fifteen days.
- **Group IV-** Animals received 30 mg/ kg-day extract orally for fifteen days.
- **Group V-** Animals received 50 mg/ kg-day orally for fifteen days.

On the thirteenth day, animals from groups II - V were injected intraperitoneally with CCl_4 in olive oil vehicle at a dosage of 1 ml/kg bw. 48 h after CCl_4 administration, the rats were sacrificed, kidneys and lungs isolated, and post mitochondrial supernatant was prepared. Lung and kidney were washed in ice-cold 1.15% KCl and centrifuged at 9,000 g for 20 min to remove debris. The supernatant was centrifuged at 15,000 g for 20 min at 4°C to get Post-Mitochondrial Supernatant (PMS). This Post-Mitochondrial Supernatant (PMS) was used for the following assays: lipid peroxidation (Nichans and Samuelson, 1968), GSH (Moren et al., 1979), GR (Sharma et al., 2001), GPx (Sharma et al., 2001), GST (Haque et al., 2003), CAT (Claiborne, 1985), and SOD (Beauchamp and Fridovich, 1971); protein concentration was estimated by the method of Lowry et al. (1951).

**Statistical analysis**

The values are expressed as mean ± standard deviation (SD). The results were evaluated by using the SPSS (version 12.0) and Graphpad Prism 5 softwares and evaluated by one-way ANOVA followed by Bonferroni’s test. p < 0.05 was considered significant.

**RESULTS**

**Protein levels**

A significant decrease in protein content was noted in CCl_4 treated rat kidney tissue homogenate. Pretreatment
Table 1. Effects of 70% ethanolic extract of *Podophyllum hexandrum* on antioxidant enzymes and protein levels in kidney of CCl₄-treated Wistar rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I Olive oil only</th>
<th>Group II CCl₄-treated 20 mg/kg-day</th>
<th>Group III CCl₄ + 30 mg/kg-day</th>
<th>Group IV CCl₄ + 50 mg/kg-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (mg/100 mg tissue)</td>
<td>102. ± 0.2</td>
<td>18.5 ± 0.3</td>
<td>26 ± 0.14</td>
<td>59 ± 0.8</td>
</tr>
<tr>
<td>Reduced sulphhydryl groups (nmol/g protein)</td>
<td>58.3 ± 4.36</td>
<td>10.5 ± 0.78⁵</td>
<td>15.9 ± 0.57⁷ab</td>
<td>24.4 ± 1.09⁷ab</td>
</tr>
<tr>
<td>Glutathione reductase (µg GSSG utilized/minute/mg protein)</td>
<td>73.3 ± 1.987</td>
<td>9.2 ± 0.55²</td>
<td>13.0 ± 1.5⁸ab</td>
<td>29.7 ± 3.42⁸ab</td>
</tr>
<tr>
<td>Glutathione peroxidase (µg GSH utilized/minute/mg protein)</td>
<td>67.4 ± 4.1</td>
<td>9.7 ± 0.54⁶</td>
<td>11.7 ± 0.7⁹ab</td>
<td>29.6 ± 1.3⁹ab</td>
</tr>
<tr>
<td>Superoxide dismutase (units/mg protein)</td>
<td>34.9 ± 1.4</td>
<td>6.9 ± 1.5e</td>
<td>13.0 ± 0.1⁶⁴b</td>
<td>13.3 ± 0.4⁴⁸b</td>
</tr>
<tr>
<td>Catalase (nmol H₂O₂ decomposed /min/mg protein)</td>
<td>3510± 328</td>
<td>496± 68.2⁵e</td>
<td>517. ± 43.4⁹⁵b</td>
<td>1254± 120⁹⁵b</td>
</tr>
<tr>
<td>Glutathione- S-transferase (nmol CDNB conjugated/min/mg protein)</td>
<td>17.2 ± 0.4</td>
<td>6.2 ± 0.45⁵</td>
<td>8.3 ± 0.15⁷⁵b</td>
<td>9.2 ± 0.6⁷⁵b</td>
</tr>
</tbody>
</table>

The data are means ± SD for six animals per group and evaluated by one-way ANOVA followed by Bonferroni’s test. a: \( p < 0.001 \) compared with CCl₄-induced group, b: \( p < 0.001 \) compared with normal group, d: NS compared with CCl₄-induced group, g: NS when compared to CCl₄ + 20 mg/kg-day. 1-chloro-2, 4-dinitrobenzene (CDNB), was used as substrate for GST assay. One unit of SOD is the enzyme concentration required for inhibition of chromagen production by 50% in minute under the assay conditions.

with oral doses of *P. hexandrum* 70% ethanolic extract increased the protein levels in a dose dependent manner (Table 1). Similarly in lung tissue homogenate, the single dose CCl₄ administration decreased the protein levels significantly; however, the treatment with ethanolic extract dose dependently restored the protein levels (Table 2).

**Reduced GSH levels**

Tables 1 and 2 show the concentrations of GSH in kidney and lung homogenates. A significant (\( p < 0.001 \)) decrease in GSH concentration was observed in CCl₄ treated rats, 10.52 ± 0.78 and 7.31 ± 0.33 nmol/g protein for kidney and lung, respectively, compared with the vehicle control group (58.32 ± 4.36 and 34.78 ± 1.93 nmol/g protein, respectively). Administration of the 70% ethanolic extract of *P. hexandrum* for 15 consecutive days afforded a dose-dependent protection against GSH depletion.

**Superoxide dismutase activity**

The activity of SOD in kidney tissue homogenate was significantly decreased in CCl₄ treated animals, compared with control. However, pretreatment with 70% ethanolic extract showed significant increase in SOD activity (Table 1). Similarly, the SOD activity in lung tissue homogenate of CCl₄ treated rats was significantly decreased. Again pretreatment with extract significantly restored the SOD levels (Table 2).

**Catalase activity**

The CAT activity observed in the kidney tissue of CCl₄ treated rats was considerably lower (496 ± 68 nmol/min/mg protein) as compared to normal group (3511 ± 328 nmol/min/mg protein) (Table 1). In the groups pretreated with 70% ethanolic extract of *P. hexandrum* for 15 days prior to CCl₄, CAT activity was significantly increased in a dose-dependent manner (Table 1). CAT activity in the lung of CCl₄ treated rats was also lower than in the control group but administration of *P. hexandrum* at all tested concentrations restored its activity (Table 2).

**Glutathione-S-transferase activity**

GST activity (6.2 ± 0.45 nmol/min/mg protein) was significantly decreased in kidney tissue homogenate of CCl₄ treated rats, as compared to control rats (17.2 ± 0.4 nmol/min/mg protein), however the extract could dose dependently restore the activity towards normal (Table 1). Similar trend was observed in case of lung tissue homogenate.

**Glutathione reductase and glutathione peroxidase activities**

Significant decreases in these enzyme activities were observed in CCl₄-treated rats when compared with those in control animals. Treatment with 70% ethanolic extract of *P. hexandrum* at doses of 20, 30 and 50 mg/kg significantly restored the activities of both enzymes in both kidney and lung tissue homogenates (Table 1 and 2).

**Lipid peroxidation (PMS)**

Effects of *P. hexandrum* rhizome extracts on LPO were measured by the formation of free MDA in kidney and lung tissues of rats following exposure...
Table 2. Effects of a 70% ethanolic extract of *P. hexandrum* on antioxidant enzymes and protein levels in lung of CCl₄-treated Wistar rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I Olive oil only</th>
<th>Group II CCl₄-treated</th>
<th>Group III CCl₄ +20 mg/kg-day</th>
<th>Group IV CCl₄ +30 mg/kg-day</th>
<th>Group V CCl₄ +50 mg/kg-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (mg/100mg tissue)</td>
<td>35.2 ± 0.98</td>
<td>3.86 ± 0.513</td>
<td>6.25 ± 0.37</td>
<td>15.75 ± 0.63</td>
<td>17.84 ± 0.73</td>
</tr>
<tr>
<td>Reduced sulfhydryl groups (nmol/g protein)</td>
<td>34.78 ± 1</td>
<td>7.31 ± 0.03</td>
<td>8.17 ± 0.4</td>
<td>9.38 ± 0.4</td>
<td>16.5 ± 0.84</td>
</tr>
<tr>
<td>Glutathione reductase (µg GSSG utilized/minute/mg protein)</td>
<td>23.2 ± 0.95</td>
<td>3.37 ± 0.36</td>
<td>3 ± 0.3</td>
<td>5.22 ± 0.62</td>
<td>11.57 ± 0.4</td>
</tr>
<tr>
<td>Glutathione peroxidase (µg GSH utilized/minute/mg protein)</td>
<td>14.9 ± 1.75</td>
<td>2.1 ± 0.19</td>
<td>3.3 ± 0.2</td>
<td>6.1 ± 0.28</td>
<td>9.1 ± 0.57</td>
</tr>
<tr>
<td>Superoxide dismutase (units/mg protein)</td>
<td>28.8 ± 1.2</td>
<td>5.77 ± 0.76</td>
<td>9.2 ± 1.3</td>
<td>13.7 ± 0.8</td>
<td>21.1 ± 0.6</td>
</tr>
<tr>
<td>Catalase activity (nm of H₂O₂ decomposed /min/mg protein)</td>
<td>201 ± 54.7</td>
<td>14.3 ± 1.9</td>
<td>28.37 ± 9</td>
<td>35.47 ± 6</td>
<td>59.30 ± 16</td>
</tr>
<tr>
<td>Glutathione- S- transferase (nmoles of CDNB conjugated/min/mg protein)</td>
<td>13.4 ± 0.1</td>
<td>4.563 ± 0.5</td>
<td>5.8 ± 0.4</td>
<td>7.12 ± 1</td>
<td>7.4 ± 0.3</td>
</tr>
</tbody>
</table>

The data are means ± SD for six animals per group and evaluated by one-way ANOVA followed by Bonferroni test. a: *p* < 0.001 compared with CCl₄-induced group, b: *p* < 0.001 compared with control group, d: NS compared with CCl₄-induced group, $: did not test when compared to CCl₄ induced group.

Table 3. Effect of a 70% ethanolic extract on lipid peroxidation in kidney and lung of Wistar rats.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Group I Olive oil only</th>
<th>Group II CCl₄-treated</th>
<th>Group III CCl₄ +20 mg/kg-day</th>
<th>Group IV CCl₄ +30 mg/kg-day</th>
<th>Group V CCl₄ + (50 mg/kg-day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA formation (nmol/g protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>0.13 ± 0.06</td>
<td>2.1 ± 0.14</td>
<td>1.45 ± 0.06</td>
<td>1.09 ± 0.26</td>
<td>0.83 ± 0.07</td>
</tr>
<tr>
<td>Lung</td>
<td>0.03 ± 0.01</td>
<td>2.21 ± 0.2</td>
<td>0.7 ± 0.05</td>
<td>0.6 ± 0.03</td>
<td>0.48 ± 0.08</td>
</tr>
</tbody>
</table>

The data are mean ± SD for six animals per group.

to CCl₄. The data are shown in Table 3 ethanolic extract significantly inhibited the formation of MDA in both organs. After CCl₄ administration, MDA levels increased significantly from 0.13 ± 0.06 - 2.1 ± 0.14 nmol/mg proteins in kidney and from 0.03 ± 0.01 - 2.2 ± 0.2 nmol/mg proteins in lung. However, treatment with 20, 30, or 50 mg/kg-day 70% ethanolic extract of *P. hexandrum*, the MDA levels decreased to 1.46 ± 0.06, 1.09 ± 0.26 and 0.83 ± 0.07, respectively, in kidney tissue. Lung tissue also exhibited LPO but to a lesser extent as compared to kidney tissue as shown in Table 3. However, the test extract (70% ethanolic) in a dose-dependent manner showed inhibitory effects on lipid peroxidation.

**DISCUSSION**

The present study was conducted to evaluate the protective effect of ethanolic extract of *P. hexandrum* against CCl₄ induced oxidative stress in renal and lung tissue. Results suggest that the extract possess protective action against both renal and lung dysfunctions induced by the potent toxin, CCl₄. Data showed that the extract was effective in dose-dependent manner. Maximum protective activity of the extract was obtained when administered at the dose of 50 mg/kg-day for 15 days before toxin administration as shown in Table 1 and 2. It is well established that free radicals are implicated in a large number of diseases/ metabolic alterations. The toxic effects of CCl₄ *in vivo* are well known to be mediated through radical reactions (Recknagel et al., 1989). The CCl₃O and/or CCl₃OO radicals produced as a result of metabolic conversion of CCl₄ is reported to induce organ damage through lipid peroxidation and decreased activities of antioxidant enzymes (Aleynik et al., 1997). CCl₄ is a widely used toxicant to experimentally induce animal models of acute kidney and lung tissue damages (Ahmad et al., 1987; Ogawa et al., 1992; Ohta et al., 1997; Ozturk et al., 2003). GSH levels (reduced form) are important for maintaining the structural and functional integrity of different organs. The maintenance of cellular
GSH levels are dependent upon the activities of Glutathione Reductase (GR) and NADH (Meister and Anderson, 1983). In vivo studies carried out by other workers also indicate that CCl₄ reduces the GSH (reduced form) in kidney and lung tissues (Runge and Ernst, 1992). Glutathione Peroxidase (GPx), Glutathione Reductase (GR), Glutathione-S-Transferase (GST), Superoxide Dismutase (SOD), Catalase (CAT) constitutes a mutually supportive team of defense against ROS (Bandhopadhy et al., 1999). In the present study, it was observed that CCl₄ induced significant decrease in SOD, CAT, GSH-Px and GST activities and enhanced lipid peroxidation in kidney and lung tissues in rats. The decreased activity of SOD in lung and kidney tissues in CCl₄ treated rats may be due to the enhanced lipid peroxidation or inactivation of the antioxidative enzymes. Pretreatment with P. hexandrum was able to significantly restore the activities of SOD, CAT, GSH-Px and GST as compared to the CCl₄ treated group.

CCl₄ and its metabolites are capable of initiating a chain of lipid peroxidation reactions by abstracting hydrogen from polyunsaturated fatty acids (PUFA).

Peroxidation of lipids, particularly those containing PUFA, can dramatically change the properties of biological membranes, resulting in severe cell damage and play a significant role in pathogenesis of some diseases (Aleynik et al., 1997). Enhanced lipid peroxidation (LPO) expressed in terms of Thiobarbituric Acid Reactions (TBARS) is a measure of membrane damage as well as alteration in structure and function of cellular membranes (Halliwell et al., 1995). The level of LPO increased after the CCl₄ treatment in both kidney and lung tissues indicating membrane damage, however pretreatment with ethanolic extract of P. hexandrum decreased the LPO levels, which may be due to the free radical scavenging activity of the extract. In conclusion, present study demonstrates that ethanolic extract of P. hexandrum has potent antioxidant effect. The mode of action of ethanolic extract in affording the protective effect against CCl₄ may be due to the cell membrane stabilization, and activation of antioxidant enzymes such as SOD, CAT, GSH-Px and GST. Further study is warranted to understand the exact mode of action of the extract.

REFERENCES


