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Full Length Research Paper

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, immuno-deficiencies, neurological disorders, and carcinogenesis. The major cellular sources responsible for the generation of damage-inducing reactive oxygen species (ROS), such as $\cdot O_2^-$, H_2O_2 and $\cdot OH$, are mitochondria and peroxi-somes. These antioxidative enzymes include cyclooxy-genase (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 ($\cdot CCl_3$). This free radical induces an adverse reaction by forming other free radicals after its administration in the early stage between intracellular uptake and trans-formation 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, $\cdot CCl_3$, are the major initial products. $\cdot CCl_3$ is converted into $\cdot CCl_3O_2$ through its reaction with molecular oxygen. Like the above-mentioned free radicals, lipid peroxidation is initiated by the interaction of this reactive free radical, $\cdot CCl_3O_2$, with polyunsaturated fatty acids (PUFA) of the membrane lipids.

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Abbreviations: GSH, Reduced glutathione; **SOD,** superoxide dismutase; **GPx,** glutathione peroxidase; **CAT,** catalase; **GST,** glutathione-S-transferase; **TBARS,** thiobarbituric acid reactive substances; **PUFA,** polyunsaturated fatty acids; **MDA,** malondialdehyde.

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 $\cdot O_2^-$ 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 hydroperoxides, 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, Hogkins disease and non-Hodgkins lymphoma (Gowdey et al., 1995; Cobb, 1990) brain tumors, bladder cancer, lung cancer and AIDS-associated Kaposi's sarcoma (Blasko 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- bot/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 homogenized in a homogenizing buffer (50 mM Tris- HCl, 1.15% KCl pH 7.4) using a Teflon homogenizer. The homogenate was centrifuged at 9,000 g for 20 min to remove debris. The supernatant was further 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 CCl_4 - treated Wistar rats.

Parameter	Group I Olive oil only	Group II CCl_4-treated	Group III CCl_4 + 20 mg/kg-day	Group IV CCl_4 + 30 mg/kg-day	Group V CCl_4 + 50 mg/kg-day
Protein (mg/100 mg tissue)	102. ± 0. 2	18.5 ± 0.3	26 ± 0.14	59 ± 0.8	64.5 ± 1.02
Reduced sulphydryl groups (nmol/g protein)	58.3 ± 4.36	10.5± 0.78[b]	15.9 ± 0.57[ab]	24.4± 1.09[ab]	31.8 ± 0.92[ab]
Glutathione reductase (µg GSSG utilized/minute/mg protein)	73.3 ± 1.987	9.2± 0.5[b]	13.0 ± 1.5[ad]	29.7± 3.42[ad]	38. ± 2.2[ad]
Glutathione peroxidase (µg GSH utilized/minute/mg protein)	67.4 ± 4.1	9.7 ± 0.54[b]	11.7 ± 0.7[ad]	29.6 ± 1.3[ab]	46.5 ± 4.3[ad]
Superoxide dismutase (units/mg protein)	34.9 ± 1.4	6.9± 1.5[b]	11.4 ± 0.1[ad]	13.3 ± 0.4[gb]	17.2 ± 1.4[db]
Catalase (nmol H_2O_2 decomposed /min/mg protein)	3510± 328	496± 68.2[b]	517. ± 43.4[ad]	1254± 120[ad]	1830± 228.3[ad]
Glutathione- S- transferase (nmol CDNB conjugated/min/mg protein)	17.2 ± 0.4	6.2 ± 0.45[b]	8.3 ± 0.15[ad]	9.2± 0.6[ad]	11.55 ± 0.6[ad]

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 CCl_4-induced group, b: $p < 0.001$ compared with normal group, d: NS compared with CCl_4-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 CCl_4 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 CCl_4 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 CCl_4 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 CCl_4 treated rats was significantly decreased. Again pretreatment with extract significantly restored the SOD levels (Tables 2).

Catalase activity

The CAT activity observed in the kidney tissue of CCl_4 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 CCl_4, CAT activity was significantly increased in a dose-dependent manner (Table 1). CAT activity in the lung of CCl_4 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 CCl_4 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 CCl_4-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 CCl_4-treated Wistar rats.

Parameters	Group I Olive oil only	Group II CCl_4-treated	Group III CCl_4 +20 mg/kg-day	Group IV CCl_4 + 30 mg/kg-day	Group V CCl_4 + 50 mg/kg-day
Protein (mg/100mg tissue)	35.2 ± 0.98	3.86 ± 0.513	6.25 ± 0.37	15.75 ± 0.63	17.84 ± 0.73
Reduced sulfhydryl groups (nmol/g protein)	34.78 ± 1	7.31 ± 0.03^{b}	8.17 ± 0.4^{db}	9.38 ± 0.4^{ab}	16.5 ± 0.84^{ab}
Glutathione reductase (µg GSSG utilized/minute/mg protein)	23.2 ± 0.95	3.37 ± 0.36^{b}	3± 0.3^{db}	5.22 ± 0.62^{ab}	11.57 ± 0.4^{ab}
Glutathione peroxidase (µg GSH utilized/minute/mg protein)	14.9 ± 1.75	2.1 ± 0.19^{b}	3.3 ± 0.2^{db}	6.1 ± 0.28^{ab}	9.1 ± 0.57^{ab}
Superoxide dismutase (units/mg protein)	28.8 ± 1.2	5.77 ± 0.76^{b}	9.2 ± 1.3^{ab}	13.7 ± 0.8^{ab}	21.1± 0.6^{ab}
Catalase activity (nm of H_2O_2 decomposed /min/mg protein)	201± 54.7	14.3 ± 1.9^{b}	28.37 ± 9$^{\$b}$	35.47 ± 6$^{\$b}$	59.30 ± 16$^{\$b}$
Glutathione- S- transferase (nmoles of CDNB conjugated/min/mg protein)	13.4± 0.1	4.563 ± 0.5^{b}	5.8 ± 0.4^{ab}	7.12 ± 1^{ab}	7.4 ± 0.3^{ab}

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 CCl_4-induced group, b: $p < 0.001$ compared with control group, d: NS compared with CCl_4-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.

Tissue	Group I Olive oil only	Group II CCl_4-treated	Group III CCl_4 +20 mg/kg-day	Group IV CCl_4 +30 mg/kg-day	Group V CCl_4 + (50 mg/kg-day
	MDA formation (nmol/g protein)				
Kidney	0.13 ± 0.06	2.1 ± 0.14	1.45 ± 0.06^{ac}	1.09 ± 0.26^{NS}	0.83 ± 0.07$^{NS;a}$
Lung	0.03 ± 0.01	2.21 ± 0.2^{ab}	0.7 ± 0.05^{ab}	0.6 ± 0.036^{ab}	0.48 ± 0.08^{ab}

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

to CCl_4. The data are shown in Table 3 ethanolic extract significantly inhibited the formation of MDA in both organs. After CCl_4 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 CCl_4 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, CCl_4. 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 CCl_4 *in vivo* are well known to be mediated through radical reactions (Recknagel et al., 1989). The CCl_3O and/or CCl_3OO radicals produced as a result of metabolic conversion of CCl_4 is reported to induce organ damage through lipid peroxidation and decreased activities of antioxidant enzymes (Aleynik et al., 1997). CCl_4 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 CCl_4 reduces the GSH (reduced form) in kidney and lung tissues (Rungby 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 CCl_4 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 CCl_4 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 CCl_4 treated group.

CCl_4 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 Reacting Substance (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 CCl_4 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 activity against CCl_4 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.

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