Hepatoprotective and Antioxidant Activity of Rhizome of Podophyllum hexandrum against Carbon Tetra Chloride Induced Hepatotoxicity in Rats[*]

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Abstract

Objective   To test possible antioxidant activity of n-hexane extract of *Podophyllum hexandrum* under *in vitro* and *in vivo* conditions.

Methods   The *in vitro* antioxidant activity was evaluated by the ability of the extract to interact with the stable free radical DPPH, Superoxide (O_2^-), Hydroxyl (OH^-), Hydrogen peroxide (H_2O_2) radicals, and reducing power ability of the extract was also evaluated. Under *in vivo* conditions the extract was evaluated for its hepatoprotective activity by measuring different biochemical parameters, such as serum alanine aminotransaminase, serum aspartate aminotransaminase and serum lactate dehydrogenase and antioxidant enzymes. Antioxidant status was estimated by determining the activities of antioxidative enzymes, glutathione reductase (GR), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and superoxide dismutase (SOD), and by determining the levels of reduced glutathione (GSH) and thiobarbituric acid reactive substances (TBARS).

Results   Hexane extract of *P. hexandrum* exhibited good radical scavenging capacity in neutralization of DPPH, O_2^-, OH^-, and H_2O_2 radicals in a dose dependent manner. n-hexane extract of *Podophyllum hexandrum* at the doses of 20, 30, and 50 mg/kg-day produced hepatoprotective effect by decreasing the activity of serum marker enzymes, while it significantly increased the levels of glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GR), super oxide dismutase (SOD), and glutathione-S-transferase (GST) in a dose dependant manner. The effect of n-hexane extract was comparable to that of standard antioxidant vitamin E.

Conclusion   The extract of *Podophyllum hexandrum* possess free radical scavenging activity under *in vitro* conditions and could protect the liver tissue against CCl_4 induced oxidative stress probably by increasing antioxidant defense activities.

Key words: *Podophyllum hexandrum*; Free radicals; *in-vitro* experiments; Biochemical parameters; Antioxidant; Liver protection

INTRODUCTION

The role of free radicals in many ailments has been well established. Several biochemical reactions in our body system generate reactive oxygen species, which, if not effectively scavenged by cellular constituents, may lead to various morbid conditions[1]. Enormous researches into free radicals have confirmed that foods or plants rich in antioxidants play an essential

role in the prevention of free radical related diseases[2-3]. A wide range of antioxidants of synthetic origin such as butylated hydroxytoluene (BHT) have been proposed to be used for the treatment of various free radicals related diseases[4-5], but it has been proven that these compounds also show toxic effects like liver damage and mutagenesis[6]. Hence, search for sources of natural antioxidants is gaining much importance nowadays. The high antioxidant potential observed in many tropical plants is obviously part of their natural defense mechanism against noxious events causing oxidant damage, e.g. microbial infections. Liver is considered to be one of the most vital organs that functions as a centre of metabolism of nutrients such as carbohydrates, proteins and lipids and excretion of waste metabolites. It also handles the metabolism and excretion of drugs and other xenobiotics from the body by protecting against foreign substances via detoxifying and eliminating them. Liver cell injury caused by various toxicants including certain chemotherapeutic agents, carbon tetrachloride, thioacetamide and others, as well as chronic alcohol consumption and microbes, is well studied. Herbal drugs have gained importance and popularity in recent years because of their safety, efficacy and cost effectiveness. The association of medicinal plants with other plants in their habitat also influences their medical values in some cases. One of the important and well-documented uses of plant products is their use as hepatoprotective agents. Hence, there is an ever increasing need for safe hepatoprotective agents[7].

Podophyllum hexandrum was used by Penobscot Indians of Maine, USA for the treatment of cancer, polyps and unhealthy granulation. Its resin was used by physicians in Mississippi and Missouri and urologists in Louisiana for the treatment of venereal warts. Podophyllatoxin obtained from Podophyllun hexandrum is a natural lignin that is currently being used as a precursor to semi-synthetic anti-cancer drugs like etoposide, teniposide, and etopophos. These compounds have been used for the treatment of lung and testicular cancers as well as certain leukemias. Cytotoxic lignins derived from Podophyllatoxin are currently used in cancer chemotherapy. Besides, extracts of the plant are used in topical medications for genital warts and some skin cancers. Recent studies have also shown its effectiveness in the treatment of monocytoid leukemia, Hodgkin's disease and non-Hodgkins lymphoma, brain tumors, bladder cancer, lung cancer and AIDS associated Kaposi's sarcoma.

The objective of the present study was to evaluate the protective effects of n-hexane extract of Podophyllum hexandrum against free radical mediated damages under in vitro and in vivo conditions. The in vitro antioxidant activity was evaluated by the ability of the extract to interact with the stable free radical DPPH, Superoxide (O_2^-), Hydroxyl (OH^-), Hydrogen peroxide (H_2O_2) radicals, and reducing power ability of the extract was also evaluated.

The present research was also aimed to evaluate the efficiency of n-hexane extract of P. hexandrum in restoring metabolic disorders induced by CCl_4 related hepatotoxicity. It was previously reported that CCl_4 caused oxidative stress resulting in excessive lipid peroxidation and decrease in glutathione levels[8-9]. Acute liver injury induced by CCl_4 have been documented by measuring different biochemical parameters in liver[10]. Serum enzyme tests, hepatic tests or alterations in the chemical constituents have proved to be more sensitive indicators of damage[11]. These include disturbance in the activities of aminotransferase (AST and ALT) and lactate dehydrogenase (LDH)[12].

MATERIALS AND METHODS

Plant Material Collection and Extraction

The rhizome of Podophyllum hexandrum was collected from higher reaches of Aharbal, Shopian, J&K, India in the month of May and June 2009, 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. Then, 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 extracted with n-hexane for 48 h using a Soxhlet extractor (60-80 °C). The extract was then concentrated with the help of rotary evaporator under reduced pressure and the solid extract was stored in refrigerator for further use.

Animals

Adult male albino rats of Wistar strain weighing 200-250 g (6-7 weeks old) used throughout this study were purchased from the Indian Institute of Integrative and Medicine Jammu (IIIM). The animals had access to food 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 in accordance with the guidelines prescribed by the National Institute of Nutrition, Indian Council of Medical Research, and the study was approved by the Institutional Animal Ethical Committee of the University of Kashmir under reference number 801/03/CA/CPCSEA.

Experimental Method

DPPH Radical Scavenging Activity The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay was performed by using the method as described by Braca and his colleagues[13]. Various concentrations of plant extract (100-1000 μg/mL) were added to 1 mL of the 0.004% methanol solution of DPPH, and the mixtures were vortexed vigorously. The tubes were then incubated at room temperature for 30 min in dark, and the absorbance was taken at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. α-tocopherol and BHT were taken as known free radical scavengers. Percentage inhibition activity was calculated by using the following formula.

$$\% \text{ inhibition} = [(A_0-A_1)/A_0] \times 100 \qquad (1)$$

Where A_0 was the absorbance of the control and A_1 was absorbance in the presence of *Podophyllum hexandrum* extract/ known antioxidant.

Assessment of Hydroxyl Radical Scavenging Property Hydroxyl radical, generated from the Fe^{3+}-Ascorbate-H_2O_2 (Fenton reaction), was evaluated by degradation of deoxyribose that produced thiobarbituric acid reactive species (TBARS)[14]. The reaction mixture contained 25 mmol/L deoxyribose, 10 mmol/L ferric chloride, 100 mmol/L ascorbic acid, 2.8 mmol/L H_2O_2 in 10 mmol/L KH_2PO4 (pH 7.4) and various concentrations of *Podophyllum hexandrum* rhizome n-hexane extract. The reaction mixture was incubated at 37 °C for 1 h. Then 1 mL of 1% thiobarbituric acid and 1 mL of 3% trichloroacetic acid was added and mixture was heated at 100 °C for 20 min. TBARS was measured spectrophotometrically by taking absorbance at 532 nm. The results were expressed as percentage inhibition of deoxyribose oxidation, as determined by the following formula.

$$\text{Percentage inhibition} = [(A-B)/A] \times 100 \qquad (2)$$

Where A was the malondialdehyde produced by Fenton reaction treated alone, and B was the malondialdehyde produced in the presence of *Podophyllum hexandrum* extract/known antioxidant.

Assessment of Superoxide Anion Radical Scavenging Property Superoxide anion radical generated by the xanthine/xanthine oxidase system was spectrophotometrically determined by monitoring the product of nitroblue tetrazolium (NBT) using the method as described by Jung CH and his colleagues[15]. A reaction mixture containing 1.0 mL of 0.05 mol/L phosphate buffer (pH 7.4), 0.04 mL of 0.15% BSA, 0.04 mL of 15.0 mmol/L NBT and various concentrations of plant extract and known antioxidant was incubated at 25 °C for 10 min, and then the reaction was started by adding 0.04 mL of 1.5 U/mL Xanthine oxidase and again incubated at 25 °C for 20 min. The absorbance of the reaction mixture was measured at 560 nm. Decreased absorbance of the reaction mixture indicates increased superoxide anion radical scavenging activity.

The scavenging activity of the plant extract on superoxide anion radical was expressed as:

$$\% \text{ inhibition} = [(A_0-A_1)/A_0] \times 100 \qquad (3)$$

Where A_0 was the absorbance of the control and A_1 was absorbance in the presence of *Podophyllum hexandrum* extract/ known antioxidant.

Assessment of Hydrogen Peroxide Scavenging Activity The ability of *Podophyllum hexandrum* n-hexane extract to scavenge hydrogen peroxide was evaluated according to the method developed by Ruch RJ and his colleagues[16]. A solution of H_2O_2 (2 mmol) was prepared in phosphate buffer (pH 7.5). Plant extracts (50-300 μg/mL) were added to the hydrogen peroxide solution (0.6 mL). Absorbance of hydrogen peroxide at 230 nm was determined after 15 min against a blank solution containing phosphate buffer without hydrogen peroxide. BHT was taken as known standard. The scavenging activity of the plant extract on H_2O_2 was expressed as:

$$\% \text{ scavenged } [H_2O_2] = [(A_0-A_1)/A_0] \times 100 \qquad (4)$$

Where A_0 is the absorbance of the control and A_1 is absorbance in the presence of plant extract and known standard.

Reducing Power The reducing power of *Podophyllum hexandrum* rhizome extract was evaluated by the method developed by Oyaizu[17].

Different concentrations of the plant extract were mixed with 2.5 mL of 0.2 mol/L phosphate buffer (pH 6.6), and 2.5 mL of 1% potassium hexacyanoferrate II. The mixture was then incubated at 50 °C for 20 min, 2.5 mL of 10% TCA was added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. BHT (butylated hydroxyl toluene) was taken as the known standard. The percentage reduction of the sample as compared with BHT was calculated by using the following formula:

$$\text{Reduction (\%)} = [1- (1- A_C/A_S)] \times 100 \qquad (5)$$

Where, A_C is absorbance of standard at maximum concentration tested and A_S is absorbance of sample.

Dosage and Treatment

Rats were divided into 6 groups. Each group contains seven rats. The extract was suspended in normal saline and was used at oral doses of 20, 30, and 50 mg/kg-day. The final volume of extract at each dose was 1 mL which was fed to rats by gavage. Group I: Received olive oil vehicle only at 5 mL/kg-day; Group II: Received CCl_4 in olive oil only; Group III: Were administered with vitamin E (50 mg/kg-day); Group IV: Received 20 mg/kg-day extract orally for fifteen days, Group V: Received 30 mg/ kg-day extract orally for fifteen days; and Group VI: Received 50 mg/kg-day orally for 15 days.

On the thirteenth day, animals from groups II-VI were injected intraperitoneally with CCl_4 in olive oil vehicle at a dosage of 1 mL/kg bw. The rats were sacrificed 48 h after CCl_4 administration and the liver tissues were collected, and post mitochondrial supernatant of the liver tissue was prepared as written under preparation of PMS in section "MATERIALS AND METHODS".

Blood Collection for Estimation of AST, ALT, and LDH   Before sacrificing the experimental animals, blood was collected from retro-orbital plexus without the use of anticoagulant. The blood was allowed to stand for 10 min before being centrifuged at 5000 g for 10 min to obtain serum for analysis of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and serum lactate dehydrogenase (LDH).

Serum Alanine Aminotransaminase (ALT)   ALT was estimated by the method of Reitman and Frankel[18]. Briefly 0.5 mL of substrate (2 mmol/L α-ketoglutarate, 0.2 mol/L DL-alanine (in phosphate

buffer 0.1 mol/L pH 7.4) was incubated at 37 °C for 5 min. 0.1 mL of freshly prepared serum was added to the aliquot and again incubated at 37 °C for 30 min. At the end of incubation 0.5 mL of 2,4-dinitrophenylhydrazine was added and the aliquot was left for 30 min at room temperature. 0.5 mL of 0.4 mol/L NaOH was added and the aliquot was again left for 30 min. Absorbance was then recorded at 505 nm against water blank.

Serum Aspartate Aminotransaminase (AST)   AST was again estimated by the method of Reitman and Frankel[18]. The substrate however was 2 mmol/L α-ketoglutarate, 0.2 mol/L DL-aspartate and the rest of the procedure was identical.

Serum Lactate Dehydrogenase (LDH)   LDH was assayed by the method of King[19]. 0.1 mL of freshly prepared serum was added to 1.0 mL of buffered substrate (sodium pyruvate 37.5 mmol/L in phosphate buffer 100 mmol/L, pH 7.4), and the tubes were incubated at 37 °C for 15 min. After adding 0.2 mL of NAD^+ solution (10 mg/mL in phosphate buffer), the incubation was continued for another 15 min. The reaction was arrested by adding 0.1 mL of 2,4-dinitrophenylhydrazine (0.02% in concentrated HCl), and the tubes were incubated again at 37 °C for a further period of 15 min, after which 7.0 mL of 0.4 mol/L NaOH was added and the color developed was measured spectrophotometrically at 420 nm against a blank containing phosphate buffer only.

Preparation of Post Mitochondrial Supernatant (PMS)   Liver tissue was washed in ice-cold 1.15% KCl and was homogenized in a homogenizing buffer (50 mmol/L Tris-HCl, 1.15% KCl pH 7.4) using a teflon homogenizer. The homogenate was centrifuged at 9000 g for 20 min to remove debris. The supernatant was further centrifuged at 15 000 g for 20 min at 4 °C to get PMS, subsequently used for various biochemical assays. Protein concentration was estimated by the method of Lowry[20].

Estimation of lipid Peroxidation (PMS)   Lipid peroxidation in liver tissue homogenate was estimated by the formation of thiobarbituric acid reactive substances (TBARS) by the method of Nichans and Samuelson[21]. In brief, 0.1 mL of tissue homogenate (PMS; Tris- HCl buffer, pH 7.5) was treated with 2 mL of (1:1:1 ratio) TBA-TCA-HCl reagent (0.37% thiobarbituric acid, 0.25 mol/L HCl, and 15% TCA), placed in boiling water bath for 15 min, cooled and was centrifuged at room temperature for 10 min. The absorbance of the clear supernatant was measured against reference blank

at 535 nm.

Determination of total Sulphydryl Groups The acid soluble sulphydryl groups (non protein thiols of which more than 93% was reduced glutathione, GSH) formed a yellow colored complex with DTNB that showed the absorption maximum at 412 nm. The assay procedure followed was that of Moren[22]. 500 µL of homogenate precipitated with 100 µL of 25% TCA, was subjected to centrifugation at 300 xg for 10 min to settle the precipitate. 100 µL of the supernatant was taken in a test tube containing the 2 mL of 0.6 mmol/L DTNB and 0.9 mL of 0.2 mmol/L sodium phosphate buffer (pH 7.4). The yellow color obtained was measured at 412 nm against the reagent blank which contained 100 µL of 25% TCA in place of the supernatant. Sulphydryl content was calculated using the DTNB molar extension coefficient of 13 100.

Glutathione Peroxidase (GPx) GPx activity was assayed using the method of Sharma[23]. The assay mixture consisted of 1.49 mL of sodium phosphate buffer (0.1 mol/L pH 7.4), 0.1 mL EDTA (1 mmol/L), 0.1 mL sodium azide (1 mmol/L), 0.1 mL 1 mmol/L GSH, 0.1 mmol/L of NADPH (0.02 mmol/L), 0.01 mmol/L of 1 mmol/L H_2O_2, and 0.1 mmol/L PMS in a total volume of 2 mL. Oxidation of NADPH was recorded spectrophotometrically at 340 nm and the enzyme activity was calculated as nmoles NADPH oxidized/min/mg of protein, using extinction coefficient of 6.22×10^3 mmol·L^{-1}·cm^{-1}.

Glutathione Reductase Activity (GR) GR activity was assayed by the method of Sharma[23]. The assay mixture consisted of 1.6 mL of sodium phosphate buffer (0.1 mol/L pH 7.4), 0.1 mL EDTA (1 mmol/L), 0.1 mL (1 mmol/L) oxidized glutathione, 0.1 mmol/L of NADPH (0.02 mmol/L), 0.01 mmol/L of 1 mmol/L H_2O_2 and 0.1 mmol/L PMS in a total volume of 2 mL. The enzyme activity measured as absorbance at 340 nm was calculated as nmoles of NADPH oxidized/min/mg of protein using extinction coefficient of 6.22×10^3 mmol·L^{-1}·cm^{-1}.

Glutathione-S-transferase (GST) Activity GST activity was assayed using the method of Haque[24]. The reaction mixture consisted of 1.67 mL sodium phosphate buffer (0.1 mol/L pH 6.5), 0.2 mL of 1 mmol/L GSH, 0.025 mL of 1 mmol/L CDNB and 0.1 mL of post mitochondrial supernatant in a total volume of 2 mL. The change in absorbance was recorded at 340 nm and the enzyme activity was calculated as nmoles of CDNB conjugates formed/min/mg protein using extinction coefficient of 9.6×10^3 mmol·L^{-1}·cm^{-1}.

Super Oxide Dismutase Activity (SOD) SOD activity was estimated by Beauchamp and Fridovich[25]. The reaction mixture consisted of 0.5 mL of hepatic PMS, 1 mL of 50 mmol/L sodium carbonate, 0.4 mL of 25 µmol/L NBT, and 0.2 mL of 0.1 mmol/L EDTA. The reaction was initiated by addition of 0.4 mL of 1 mmol/L hydroxylamine-hydrochloride. The change in absorbance was recorded at 560 nm. The control was simultaneously run without tissue homogenate. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50%.

Statistical Analysis

The values are expressed as mean ± standard deviation (SD). The results were evaluated by using the SPSS (version 12.0) and Origin 6 softwares and evaluated by one-way ANOVA followed by Bonferroni *t*-test. Statistical significance was considered when value of $P<0.05$.

RESULTS

DPPH Radical Scavenging Activity

The percentage of DPPH radical scavenging activity of hexane extract of *Podophyllum hexandrum* is presented in Figure 1. The hexane extract of *Podophyllum hexandrum* exhibited dose dependent inhibition of DPPH activity, and the scavenging activities of the extract and known antioxidants increased with increasing concentration. The hexane extract of *Podophyllum hexandrum* exhibited a maximum DPPH scavenging activity of 81.10% at 1000 µg/mL whereas for α-tocopherol and BHT (standards) the scavenging activity was found to be 86.65% and 82.80 % at 1000 µg/mL. These results indicated that the extract had a noticeable effect on scavenging of DPPH free radicals.

Hydroxyl Radical Scavenging Activity

The abilities of the extract and standards catechin and α-tocopherol to inhibit hydroxyl radical-mediated deoxyribose degradation in a Fe^{3+}-ascorbic acid and H_2O_2 reaction mixture are shown in Figure 2. The effect of hexane extract of Podophyllum hexandrum and known antioxidants on hydroxyl radical was protected significantly at all concentrations. At a concentration of 300 µg/mL the extract showed the maximum inhibitory effect of about 85.26% which was comparable to that of catechin and α-tocopherol (88% and 86%). The scavenging activity was found in

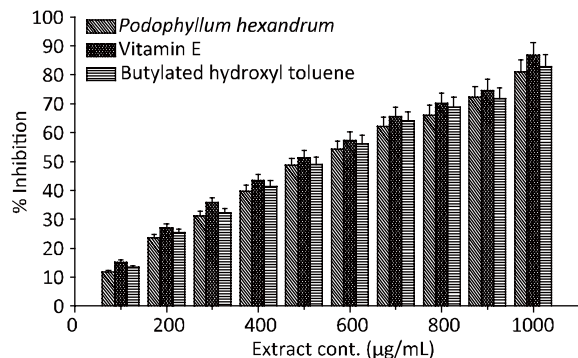the following decreasing order: catechin>α-tocopherol>hexane extract.

Figure 1. The effect of hexane extract and known antioxidants on DPPH radical scavenging activity. *Note.* The results represent mean±SD of 3 separate experiments. Absorbance at 517 nm.
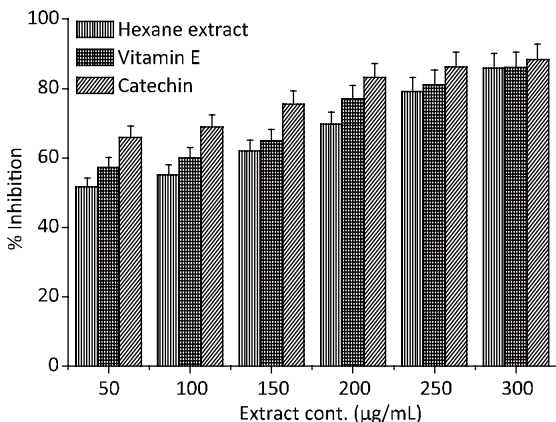

Figure 2. The effect of hexane extract and known antioxidants on hydroxyl radical scavenging activity. *Note.* The results represent mean±SD of 3 separate experiments. Results are reported as the percentage of the maximum formation of OH⁻ radical (100% deoxyribose oxidized): in absorbency, 100% is 1.516±0.007 (control). Absorbance at 532 nm.

Superoxide Radical Scavenging Activity

Percentage scavenging of superoxide anion examined at different concentrations of hexane extract of *Podophyllum hexandrum* (50, 100, 150, 200, 250, and 300 µg/mL) is depicted in Figure 3. The percentage scavenging of superoxide radical surged with the enhanced concentration of plant extract. The maximum scavenging activity of plant extract

and BHT at 1000 µg/mL was found to be 64.88% and 69.98% respectively. The results suggest that hexane extract has a potent super oxide scavenging effects which is comparable to that of BHT.
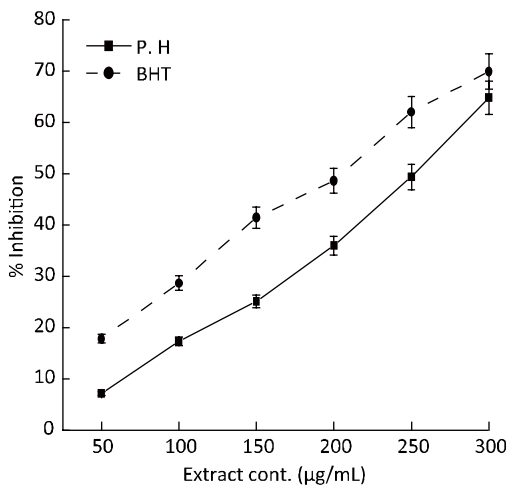

Figure 3. The effect of hexane extract and known antioxidant BHT on super oxide anion radical scavenging activity. *Note.* The results represent mean±SD of 3 separate experiments. Absorbance at 560 nm (Absorbance of control=0.883±0.23).

Hydrogen Peroxide Radical Scavenging Activity
The hydrogen peroxide scavenging activity of hexane extract of *Podophyllum hexandrum* is given in Figure 4. The extract demonstrated hydrogen peroxide decomposition activity in a concentration dependent manner. At the higher concentration (300 µg/mL) of

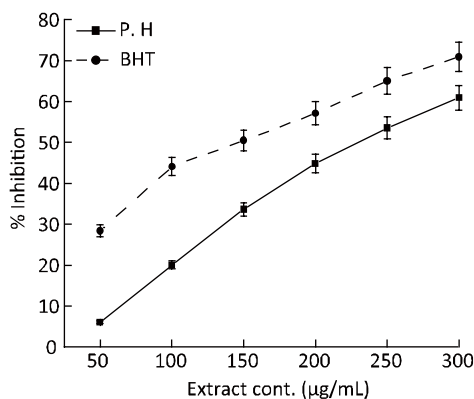

Figure 4. The effect of hexane extract and known antioxidant BHT on hydrogen peroxide radical scavenging activity. *Note.* The results represent mean±SD of 3 separate experiments. Absorbance at 230 nm (Absorbance of control=0.653±0.16).

extract and BHT the H_2O_2 scavenging activity was found as 60% and 70% respectively. These results showed that hexane extract had effective H_2O_2 scavenging activity and might also help to accelerate the conversion of H_2O_2 to H_2O.

Reducing Power Assay   As illustrated in Figure 5, Fe^{3+} was transformed to Fe^{2+} in the presence of *Podophyllum hexandrum* hexane extract and the reference compound BHT was used to measure the reductive capability. The reducing power of the extract and BHT increased with increasing in concentration. At 50 µg/mL, the absorbance of the plant extract and BHT were 0.161 and 0.214, respectively, while at the higher concentration 300 µg/mL, the absorbance of both extract and BHT increased to 0.415 and 0.675 respectively.
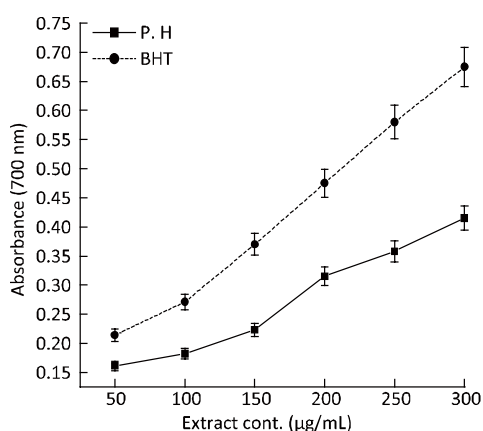

Figure 5.   The effect of hexane extract and known antioxidant BHT on reducing power method.   *Note.*   The results represent mean±SD of 3 separate experiments.

Biochemical Parameters

Effect of the Extract on Hepatic Markers   The Table 1 shows that the animals of group II, which received only CCl_4 were found to develop significant hepatic damage observed from elevated levels of AST, ALT, and LDH as compared to normal animals of group I. The treatment with *Podophyllum hexandrum* rhizome hexane extract at a dose of 20, 30, and 50 mg/kg bw-day in groups IV, V, and VI significantly deduced the CCl_4 induced elevation of liver marker enzymes in a dose dependent manner. At the higher concentration of extract (50 mg/kg bw-day) the elevated levels reduced to 70.95 U/L (AST), 61.67 U/L (ALT), and 162.40 U/L (LDH) respectively.

In Group III: (animals treated with standard vitamin E (50 mg/kg bw-day+CCl_4), there was a drastic decrease in the values of AST (72.50), ALT (63.08), and LDH (165.55) U/L. Comparison between group III and group VI showed no significant variation in these parameters indicating that hexane extract exerted the same effect as that of the vitamin E which was used as the positive control in this study.

Effect of Extract on GSH and Antioxidant Enzyme Activities   The levels of various enzymic and non enzymic antioxidants (GSH) in normal, CCl_4 controlled, *Podophyllum hexandrum* treated groups are presented in Table 2. Increased production of reactive oxygen species (ROS) due to oxidative stress plays an important role in liver diseases. CCl_4 has been reported to induce lipid peroxidation and alter the antioxidant defense system through formation of free radicals, which in turn causes damage, and degeneration of hepatic tissues.

Table 1. Effect of *Podophyllum hexandrum* Hexane Extract on Biochemical Parameters in CCl_4 Induced Hepatotoxicity in Albino Rats

Treatment	Dose	AST U/L	ALT U/L	LDH U/L
Control group (Olive oil only)	5 mL/Kg	45.38±1.96	35.21±4.15	83.18±2.44
CCl_4 treated group	1 mL/Kg	114.26±1.80	90.03±3.07	196.57±3.90
CCl_4 treated+V.E	50 mg/Kg	72.50±2.45[#,$]	63.08±2.09[#,$]	165.55±3.39[#,$]
CCl_4 treated+*P.H* extract	20 mg/Kg	95.75±2.74[#,$,@]	74.83±1.59[#,$,@]	183.50±2.07[#,$,@]
CCl_4 treated+*P.H* extract	30 mg/kg	63.21±1.39[#,$,@]	68.13±1.59[#,$,NS]	173.32±1.76[#,$,b]
CCl_4 treated+*P.H* extract	50 mg/kg	70.95±3.24[#,$,NS]	61.67±3.40[#,$,NS]	162.40±2.43[#,$,b]

Note. Each value represents the mean±SD of 6 animals. [$]$P<0.001$, as compared with normal control group; [#]$P<0.001$ as compared with CCl_4 group; [@]$P<0.001$ as compared with V.E; [NS]non significant as compared with V.E; [b]do not test as compared with V.E. The data were presented as means±SD of six parallel measures and evaluated by one way ANOVA followed by the Bonferroni *t*-test to detect inter group differences. Differences were considered to be statistically significant if $P<0.05$.

Table 2. The Treatment Effect of Hexane Extract of *Podophyllum hexandrum* on Glutathione and Antioxidant Enzymes in CCl$_4$ Challenged Rats

Parameters	Group I (Olive oil only)	Group II CCl$_4$ Treated	Group III CCl$_4$+V.E	Group IV CCl$_4$+20 mg/kg Extract	Group V CCl$_4$+30 mg/kg Extract	Group VI CCl$_4$+50 mg/kg Extract
Reduced glutathione (nm/g protein)	95.14±1.62	22.45±2.33	81.74±1.98$^{\$,\#}$	47.48±1.86$^{\$,\#,@}$	67.60±1.85$^{\$,\#,@}$	78.41±2.15$^{\$,\#,NS}$
Glutathione reductase (µg GSSG utilized/min/mg protein)	31.22±1.66	2.03±0.11	23.95±1.35$^{\$,\#}$	9.07±0.37$^{\$,\#,@}$	12.86±1.99$^{\$,\#,@}$	22.13±1.22$^{\$,\#,NS}$
Glutathione peroxidase (µg GSH utilized/min/mg protein)	33.39±1.62	2.08±0.12	29.82±2.7$^{\$,\#}$	9.4±0.30$^{\$,\#,@}$	15.09±0.30$^{\$,\#,@}$	24.98±3.52$^{\$,\#,@}$
Superoxide dismutase (units/mg protein)	32.07±1.55	13.0±0.77	26.88±1.73$^{\$,\#}$	15.95±0.57$^{\$,\#,@}$	19.80±1.31$^{\$,\#,@}$	23.74±1.41$^{\$,\#,@}$
Glutathione-S-transferase (nmoles of CDNB conjugated/min/mg protein)	15.67±0.62	3.98±0.49	10.19±0.91$^{\$,\#}$	5.32±0.35$^{\$,\#,@}$	7.42±0.31$^{\$,\#,@}$	9.13±0.42$^{\$,\#,NS}$

Note. Each value represents the mean±SD of 6 animals. $^{\$}P<0.001$, as compared with normal control group; $^{\#}P<0.001$ as compared with CCl$_4$ group; $^{@}P<0.001$ as compared with V.E; ^{NS}non significant as compared with V.E; ^{b}do not test as compared with V.E. The data were presented as means±SD of six parallel measures and evaluated by one way ANOVA followed by the Bonferroni *t*-test to detect inter group differences. Differences were considered to be statistically significant if *P*<0.05.

GSH Level

The glutathione levels were examined in this study in order to evaluate endogenous antioxidant system. Effect of hexane extract of *Podophyllum hexandrum* on GSH level for all experimental groups is shown in Table 2. CCl$_4$ treatment caused significant decrease of GSH level in liver tissue homogenates compared to the normal group. When rats were treated with CCl$_4$, GSH decreased from 95.14±1.62 mg/g protein (control group) to 22.45±2.33 mg/g protein (CCl$_4$ treated group). Pretreatment of hexane extract for 15 days at the oral doses of 20, 30, and 50 mg/kg bw-day followed by 2-day CCl$_4$ treatment enhanced the level of GSH to 47.48, 67.60, and 78.41 mg/g protein respectively. The liver of vitamin E treated animals also showed a significant increase in GSH activity as compared to CCl$_4$ treated rats.

GR, GPx, SOD, and GST Activities

The activities of GR, GPx, SOD, and GST in liver tissue of CCl$_4$-induced rats are shown in Table 2. In liver tissue, CCl$_4$ treatment caused reduction of GR (2.03±0.11), GPx (2.08±0.12), SOD (13.0±0.77 U/mg protein) and GST (3.98±0.94 U/mg protein) activities, as compared with those in the control group, showing values of 31.22, 33.39, 32.07, and 15.67 U/mg protein respectively. Enhancement of GR activity was observed in the groups treated with 20 mg/kg-day of *P. hexandrum* (9.07±0.37), 30 mg/kg-day of *P. hexandrum* (12.86±1.99) and 50 mg/kg-day of *P. hexandrum* (22.13±1.22) compared with those administered with CCl$_4$ alone. Additionally, the groups treated with *P. hexandrum* at respective doses of 20 mg/kg-day (9.40±0.57), 30 mg/kg-day (15.09±0.30), and 50 mg/kg-day (24.98±3.52) were found to cause significant increase in GPx activity, as compared with the CCl$_4$ group. Enhancement of SOD activity was observed in the groups IV, V, and VI treated with *P. hexandrum* hexane extract at the concentration of 20, 30, and 50 mg/kg-day to 15.95, 19.80, and 23.74 U/mg protein respectively as compared with that of the CCl$_4$ treated group (Table 2). Similar results were observed with GST treatment with 20, 30, and 50 mg/kg-day of *P. hexandrum* increased the activity of GST significantly in a dose dependent manner (Table 2) as compared to the CCl$_4$ treated group. In group III, continuous treatment with vitamin E a known antioxidant (50 mg/kg bw-day) increased the GR, GPx, SOD, and GST activity significantly in all experimental animals as compared to CCl$_4$ treated group (Table 2).

LPO Level

Exposures to CCl$_4$ (1 mL/kg body weight) elicited a significant increase in lipid peroxidation of liver tissue homogenate of rats as measured by the

estimation of thiobarbituric acid reactive substances (TBRAS). After 48 h CCl_4 administration the MDA level estimated was 10.74±0.84 nmol/mg protein as compared to the normal group (2.43±0.31 nmol/mg protein) (Figure 6). However, by oral administration of *Podophyllum hexandrum* extract at the concentration of 20, 30, and 50 mg/kg bw-day, the liver MDA level decreased to 7.96±0.26, 6.33±0.36, and 4.23±0.36 respectively. At meantime, the effect of vitamin E (50 mg/kg-day) on MDA levels in CCl_4 treated rats was found to be reduced to 3.8 nmol/mg protein (Figure 6).
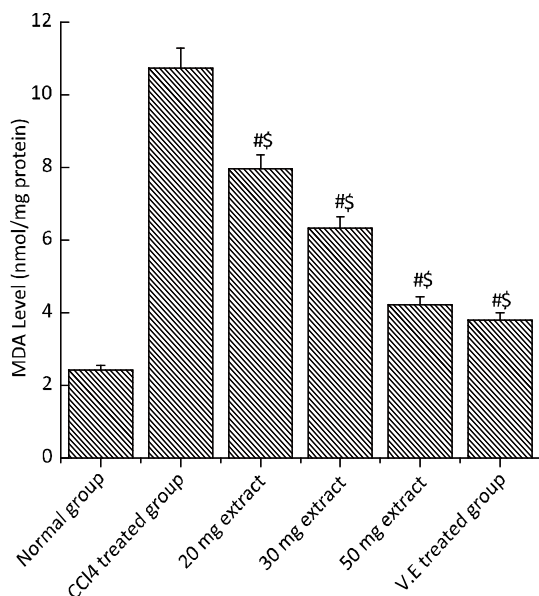

Figure 6. The effect of *Podophyllum hexandrum* hexane extract and vitamin E on liver homogenate lipid peroxidation of CCl_4 treated rats *in vivo*. *Note.* The results represent mean±SD of 6 animals in each group and evaluated by one-way ANOVA followed by the Bonferroni *t*-test. [#]$P<0.001$ when compared to normal group only, [$]$P<0.001$ when compared to CCl_4 group. Differences were considered to be statistically significant if $P<0.05$.

DISCUSSION

Natural antioxidants are closely related to their biofunctionalities, such as the reduction of DNA damage, mutagenesis, carcinogenesis and inhibition of pathogenic bacteria, which is often associated with termination of free radical propagation in biological systems[26-27]. As antioxidant capacity is widely used as a parameter for medicinal bioactive compounds. The antioxidant activity of the hexane extract of *Podophyllum hexandrum* was therefore evaluated in this study, under in vitro and in vivo conditions. Under in vitro conditions, the extract was evaluated in a series of tests like DPPH free radical scavenging, hydroxyl radical scavenging, superoxide anion scavenging, hydrogen peroxide radical scavenging and reducing power.

DPPH radical is considered to be a model of lipophilic radical. In this mode, scavenging activity is attributed to hydrogen donating ability of antioxidant[28]. DPPH is the most popular spectrophotometric methods for the determination of the antioxidant capacity of plants, foods, beverages and vegetable extracts[29]. This chromogen radical compound can directly react with antioxidants. Additionally, DPPH scavenging method has been used to evaluate the antioxidant activity of compounds due to its simple, rapid, sensitive and reproducible procedure[30]. Bleaching action of the compounds mainly depends on the number and position of the hydroxyl group present in the phytochemical[31]. With this method it is possible to determine the antiradical power of antioxidant by measuring the decrease in absorbance of DPPH at 517 nm resulting in a color change from purple to yellow. The absorbance decreased when DPPH was scavenged by an antioxidant through donation of hydrogen to form a stable DPPH molecule. In our study a dose dependent decrease in the concentration of DPPH radical scavenging was observed, and the scavenging effect of plant extract and known antioxidants decreased in the order of α-tocopherol>BHT>hexane extract.

Hydroxyl radical is a highly reactive radical formed in biological systems and is capable of damaging virtually every molecule found in living cells[32]. This radical has the capacity to induce carcinogenesis, mutagenesis and rapidly initiates lipid peroxidation[33]. In vitro study, hydroxyl radical is generated by a mixture of Fe^{3+}-H_2O_2 and ascorbic acid and is assessed by monitoring the degraded fragments of deoxyribose, through malonaldehyde (MDA) formation. If any plant extract or drug scavenges the hydroxyl radical, they may either scavenge the radical or may chelate the Fe^{2+} ion, making it unavailable for the Fenton's reaction. Plant extracts containing polyphenols are reported to quench oxygen-derived free radicals by donating a hydrogen atom or an electron to the free radical[34] or neutralize free radicals or by their chelating ability due to their high nucleophilic character of the

aromatic ring[35]. In our study the hexane extract of *Podophyllum hexandrum* was tested, the extract was found to exhibit a dose dependent hydroxyl radical scavenging activity (Figure 2).

Superoxide is biologically important as it can form singlet oxygen and hydroxyl radical[36]. Overproduction of superoxide anion radical contributes to redox imbalance and is associated with harmful physiological consequences[37]. Superoxide anions are precursor to active free radicals that have potential of reacting with biological macromolecules and thereby inducing tissue damage[38]. Also it is easily formed by radiolysis of water in the presence of oxygen and formate, which allows accurate reaction rate constants to be measured[39]. In the xanthine/xanthine oxidase system, superoxide anion derived from dissolved oxygen reduces NBT. Antioxidants are able to inhibit the blue NBT formation[40]. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. From the present study it was found that the inhibition was seen in a dose dependent manner. Ganie reported similar results[41-42] that the aqueous and ethyl acetate extracts from *Podophyllum hexandrum* showed *in vitro* antioxidant properties through the superoxide scavenger capacity method by the nitro blue tetrazolium (NBT) reduction assay.

Biological systems can produce hydrogen peroxide. Hydrogen peroxide can attack many cellular energy producing systems. For instance, it deactivates the glycolytic enzyme glyceraldehydes-3-phosphate dehydrogenase[43]. It can be formed *in vivo* by many oxidizing enzymes such as superoxide dismutase. It can cross membranes and may slowly oxidize a number of compounds. It is used in the respiratory burst of activated phagocytes[39]. In our study the H_2O_2-scavenging activity of *Podophyllum hexandrum* hexane extract and the standard BHT increased in a dose dependent manner. This ability to scavenge hydrogen peroxide could be an efficient assessment method to evaluate antioxidant property of extract of *Podophyllum hexandrum.*

Findings from different studies have showed that the electron donation capacity reflecting the reducing power of bioactive compounds is associated with antioxidant activity[44]. Antioxidants can be explained as reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one reaction species is reduced at the expense of the oxidation of the other. The presence of reductants such as antioxidant substances in the antioxidant samples causes the reduction of Fe^{3+}/ferricyanide complex the ferrous form. Therefore Fe^{2+} can be monitored by measuring the form of Perl's Prussian blue at 700 nm[45]. Our results suggest that hexane extract of *Podophyllum hexandrum* plays an important role in scavenging free radicals and the activity is increased with the increasing concentration of the plant extract. Similar results were reported by Noriham[46], who demonstrated antioxidative activity on *Pimpinella anisum* seed extracts.

In accordance with the results of the antioxidant effects of the extract of *Podophyllum hexandrum* rhizome obtained by *in vitro* assays, the *in vivo* activity of hexane extract of *Podophyllum hexandrum* was also examined.

Carbon tetrachloride (CCl_4) is a common hepatotoxin used in the experimental study of liver diseases[47-49]. Exposure to CCl_4 has been reported to induce free radical generation in tissues such as liver, heart, lung, testis, brain, and blood[50]. The first metabolite of CCl_4 trichloromethyl free radical is believed to initiate the biochemical processes leading to oxidative stress, which is the direct cause of many pathological conditions such as diabetes mellitus, cancer, hypertension, kidney and liver damages, and even death[51-52]. Liver damage caused by acute exposure to CCl_4 causes clinical symptoms such as jaundice, swollen and tender liver and elevated levels of liver enzymes in the blood[53-54]. The liver enzymes found within organs and tissues are released into the bloodstream following cellular necrosis and cell membrane permeability and are used as a diagnostic indicator of liver damage[55]. This is in consistence with the results of some previous studies[56,49] that CCl_4 treatment generates free radicals that trigger a cascade of events resulting in hepatic damage. The present study evaluated the hepatoprotective effects of *P. hexandrum* hexane extract on CCl_4 induced liver toxicity. Acute administration of CCl_4 produced a marked elevation of the serum levels of AST, ALT, and LDH in treated animals (Group II) when compared with that of the control animals (Group I). Treatment with *P. hexandrum* hexane extract at a dose of 20, 30, and 50 mg/kg-day significantly reduced the elevated levels of the enzymes in a dose dependent manner (Table 1), suggesting a protective effect of the extract against CCl_4 induced toxicity and therefore amelioration of the liver damage. Similar results were reported by Ojiako[57] that pretreatment

with the leaf extracts of *V. amygdalina* dose dependently ameliorates CCl_4 induced alterations in mouse liver enzymes.

In addition to inducing significant elevation of the levels of serum marker enzymes like ALT, AST, and LDH, this potent toxicant also caused significant decrease in GPx, GR, SOD, and GST activities, depleted the GSH content and enhanced lipid peroxidation in liver homogenate.

It has been reported that GPx, GR, SOD, and GST constitute a mutually supportive team of defense against ROS[58-59]. GSH is involved in several defense processes against oxidative damage. It protects cells against free radicals, peroxides and other toxic compounds[60]. Indeed, glutathione depletion increases the sensitivity of cells to various aggressions and also has several metabolic effects. It is widely known that a deficiency of GSH within living organisms can lead to tissue disorder and injury[61]. In our study the liver GSH level in the CCl_4 treated group was significantly decreased compared with that of the control group. However, the same parameter for CCl_4 in the hexane extract treated groups was increased compared with the CCl_4 treated group. This means that the situation is sourced by antioxidant feature of *Podophyllum hexandrum*. Recknagel[62], stated that GSH played a key role in the detoxification of the reactive toxic metabolites of CCl_4 in liver damage. GPx is partially located within the cellular membrane. It is generally believed that the protective effect of GSH against the oxidative breakdown lipids is mediated through GPx by reducing endogenously formed hydrogen peroxides of unsaturated fatty acids to hydroxyl derivates[63]. SOD is a scavenger of peroxide anion radicals, which could inhibit the initiation of lipid peroxidation by free radicals[64]. Furthermore, GPx can also terminate the chain reaction of lipid peroxidation by removing lipid hydroperoxides from the cell membrane[65]. GR is concerned with the maintenance of cellular level of GSH (especially in the reduced state) by effecting fast reduction of oxidized glutathione to reduced form. GST binds to liophilic compounds and acts as an enzyme for GSH conjugation reactions[66]. Decrease in GSH activity during CCl_4 toxicity might be due to the decreased availability of GSH resulted during the enhanced lipid peroxidation. Administration of the hexane extract of *Podophyllum hexandrum* prior to CCl_4 intoxication could not only prevent the CCl_4 induced increased lipid peroxidation, but also protect the antioxidant machineries of the liver as revealed from the enhanced levels of GPx, GR, SOD, and GST activities, increased level of GSH content.

The present study aimed to evaluate the possible antioxidant activity of the *P. hexandrum* hexane extract under *in vitro* and *in vivo* conditions. The results obtained indicate that *P. hexandrum* extract possess potent antioxidant activity, achieved by scavenging abilities observed against DPPH, hydroxyl radical, superoxide, hydrogen peroxide, and reducing power when it is compared to standard antioxidants such as catechin, BHT and α-tocopherol. The current study also demonstrates that hexane extract could reduce CCl_4-induced toxicity, particularly hepatotoxicity, by inhibiting lipid peroxidation, suppressing alanine aminotransferase (ALT), aspartate aminotransferase (AST) activities and lactate dehydrogenase activity (LDH), and increasing antioxidant enzyme activity. Therefore, hexane extract of *P. hexandrum* can be proposed to protect the liver against CCl_4-induced oxidative damage in rats, and the hepatoprotective effect might be correlated with its antioxidant and free radical scavenger effects. These *in vitro and in vivo* assays indicate that this plant extract is a significant source of natural antioxidant, which might help prevent the progress of various oxidative stresses. However, the components responsible for the antioxidative activity are currently unclear. Therefore, further studies are needed to isolate and identify the antioxidant compounds present in this plant extract, and also to clarify the mechanisms of activity of the active compounds responsible for antihepatotoxic activity.

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