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Hepatoprotective activity of *Pinus roxburghii* Sarg. wood oil against carbon tetrachloride- and ethanol-induced hepatotoxicity

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Abstract

The hepatoprotective activity of wood oil of *Pinus roxburghii* at doses of 200, 300 and 400 mg/kg body weight were studied on rat liver damage induced by CCL4 and ethanol. The substantially elevated serum enzymatic levels of SGPT, SGOT, ALP, total bilirubin, MDA and decreased level of GSH and total protein induced by hepatotoxins were significantly restored towards normalization by the wood oil at doses of 200 and 300 mg/kg. Hepatoprotective effect of oil may be due to its inhibitory effect on free radical formation as evident by recovery of GSH contents and decreased lipid peroxidation. Light microscopy of the liver tissue further confirmed the reversal of damage induced by hepatotoxins. Phytochemical analysis revealed presence of triterpenes and steroids, which have been known for their hepatoprotective activity. The results indicate that this plant possess hepatoprotective property that support the reported therapeutic use of plant in Indian system of medicine.

Introduction

The plant *Pinus roxburghii* Sarg. belonging to family pinacae are commonly known as "Chir pine" has long been known for its medicinal value (Shah et al., 2006). It is a large tree reaching 30-50 m with a trunk diameter of up to 2 m. It is found at the height of 500 to 2,500 m above sea level and grows gregariously. In India it is found in Himachal Pradesh, Kashmir and Uttaranchal.

The phytoconstituents friedelin, ceryl alcohol and β-sitostertol were isolated from bark of *P. roxburghii*. Longifolene was isolated from leaves. Xylem resin was reported to contain α-pinene, β-pinene, car-3-ene, abietic acid and isopimaric acids.

P. roxburghii has a long history of numerous traditional and ethnobotanical applications in diverse cultures (Kunwar et al., 2009). Many tribes considered it as a cure for all ailments. Plant is sweet, bitter, pungent, heating, oleaginous, intestinal antiseptic, antidyslipidemic and anti-oxidant (Puri et al., 2010) and is used in diseases of eye, ear, throat, blood and skin, bronchitis, diaphoresis, ulcer, inflammations and itching (Abbasi et al., 2010). Gum is bitter and heating, oleaginous, purgative, carminative, emmenagogue, expectorant, aphrodisiac, fattening, diuretic, anthelmintic and analgesic and is used in diseases of vegina and uterus, eye, good in dyspepsia, ulcer, diaphoresis, scabies, asthma, chronic bronchitis, ozoena, piles, diseases of liver (hepatoprotective), and spleen, gleet, ear discharge, toothache, lumbago, tuberculous glands. Resin is used as a remedy for gonorrhea and its plaster is applied to buboos and abscesses for suppuration. Wood is stimulant, diaphoretic and is used in cough, fainting and ulceration (Shah et al., 2006; Chopra et al., 2002). The literature survey reveals that some work has already been done on the plant, however most of the activities

are still without scientific backing. The present work was an attempt to evaluate hepatoprotective activity of the wood oil and to generate scientifically justified data to support the traditional use.

Materials and Methods

Animals

Wister rats of either sex weighing 150-200 g were used for the study. They were housed in polypropylene cages and fed with a standard diet and water *ad libitum*. The animals were exposed to alternating 12 hours light and dark cycles. All the experimental procedures and protocols involving animals were reviewed by the Institutional Animal Ethics Committee (Registration number: 1279/ac/09/ CPCSEA) and were in accordance with the guidelines of CPCSEA.

Collection and authentication of plant material

The wood of *Pinus roxburghii* were collected from natural source of Uttrakhand, India and authenticated by Dr. E. Roshni Nayar, National Bureau of Plant Genetic Resources, Pusa Road, New Delhi. A Voucher specimen (NHCP/ NBPGR/2010-40) was deposited at National Bureau of Plant Genetic Resources (NBPGR), New Dehi, India.

Drying and communition of plant materials

The wood of *Pinus roxburghii* were thoroughly washed and then dried under shade 25 ± 2°C for 10 days. The dried plant samples were ground well into a fine powder in a mixer grinder and sieved to give particle size 50-150 mm.

Isolation of volatile oil

The plant material was subjected to hydro distillation using Clavenger apparatus. On cooling, the essential oil was separated from the aqueous layer, dried over anhydrous sodium sulphate, and stored in an amber colored glass bottle in a cool place. The oil was emulsified by using acacia and tragacanth mixture, and administered orally at doses of 200, 300 and 400 mg/kg (Shinde et al., 1999).

Phytochemical testing

The wood oil was subjected to preliminary phytochemical screening for the presence of carbohydrates, alkaloids, amino acids, fats and fixed oils, flavonoids, glycosides, saponin, tannins, proteins, steroids and triterpenoids (Trease and Evan's, 2000). The carbohydrate was evaluated by using Molish and Fehling solution test. The alkaloids content in oil was determined by using Dragendorff's, Mayer's, Wagner's and Hager's test. Amino acid content of wood oil was determined by Hager's and ninhydrine test. The flavonoids were evaluated by Shinoda, alkaline reagent and zinc hydrochloride test. The presence of glycosides in wood oil was determined by using Borntrager's, Keller-Killiani, Legal's and Baljet's test. The presence of saponins was determined by froth formation test. The tannins content was determined by ferric chloride and gelatine test. The protein content was determined by heat and hydrolysis test. The presence of steroids and terpenoids were determined by Salkowski and sulphur powder test.

Acute toxicity study

The wood oil was administered orally in dose of 50, 100, 200, 300, 400 and 500 mg/kg to groups of mice (n = 6) and percentage mortality was noted 24 hours later.

Carbon tetra chloride-induced liver fibrosis in rats

Rats were divided into 6 groups of six animals in each group. Group I: Treated with vehicle acacia served as control. Group II: Treated with CCL_4 + Liquid paraffin served as negative control. Group III: Treated with silymarin served as standard. Group IV: Treated with oil of *P. roxburghii* (at dose 200 mg/kg) served as test. Group V: Treated with oil of *P. roxburghii* (at dose 300 mg/kg) served as test. Group VI: Treated with oil of *Pinus roxburghii* (at dose 400 mg/kg) served as test.

Group I (control) animals were administered a single daily dose of acacia (1 mL/kg body weight, p.o.). Group II received acacia before the administration of carbon tetrachloride + liquid paraffin 1:1 (1.25 mL/kg body weight, i.p.). Group III received silymarin, the known hepatoprotective compound (Sigma Chemicals Company, USA) at a dose of 100 mg/kg, p.o., before 30 min of carbon tetrachloride + liquid paraffin administration. Test groups (Groups IV-VI) were administered orally 200, 300 and 400 mg/kg body weight of wood oil respectively, in the form of acacia emulsion daily once a day before 30 minutes of carbon tetrachloride + liquid paraffin administration. Treatment duration was 3 days. On fourth day they were anaesthetized with chloral hydrate (250 mg/kg i.p) and blood was collected and kept at 37°C for one hour and then centrifuged at 2,000 rpm for 15 min. The serum was collected and estimated for SGPT, SGOT, ALP and bilirubin. After withdrawal of blood from each rat the liver was carefully removed, washed with chilled normal saline, dried by soaking well on filter paper and weighed and used for histopathological studies (Argal et al., 2010).

Biochemical estimation

The biochemical parameters like serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), serum alkaline phosphatase (SALP) and total bilirubin were assayed using assay kits (Span Diagnostic, Surat).

Histopathology

From each group small pieces of liver tissue was dissected out and fixed in 10% formalin, dehydrated in gradual ethanol (50-100%), cleared in xylene, and embedded in paraffin. Section of 5µm thick were cut in a microtome and mounted on glass slides using standards techniques (Sairam Pathology, Meerut). After staining the tissue with hematoxylin- eosin stain, the slides were viewed under a light microscope equipped for pathology. The sections were examined microscopically for the evaluation of hispathological changes including cell necrosis, fatty change, hyaline regeneration, ballooning degeneration (Vidya et al., 2007).

Ethanol-induced hepatotoxicity in rats

Rats were divided into 6 groups of six animals in each group. Group I: Treated with vehicle served as control. Group II: Treated with ethanol served as negative control. Group III: Treated with silymarin served as standard. Group IV: Treated with oil of *P. roxburghii* (200 mg/kg) served as test. Group V: Treated with oil of *Pinus roxburghii* (300 mg/kg) served as test. Group VI**:** Treated with oil of *P. roxburghii* (400 mg/kg) served as test.

On the alternate day for 7 days period of study, animals in Groups I and II were received the same amount of vehicle acacia (10 mL/kg). The rats in group II to VI were injected 25% ethanol (7.5 g/kg, i.p) 30 min after pretreatment with vehicle, silymarin and different doses of *P. roxburghii* wood oil, respectably. All animals were kept on free access to standard rat's pellets and drinking water. At the end of the study time all animals were anaesthetized using chloral hydrate (250 mg/kg i.p of 10% solution), immediately liver was removed and dipped in a normal saline (Alsaif et al., 2007).

Estimation of glutathione (GSH)

Glutathione, an anti-oxidant, protects cells from reactive oxygen species such as free radicals and peroxides. Glutathione was determined following method of Anderson, 1985 by its reaction with 5, 5′-dithiobis (2-nitrobenzoic acid) i.e. DNTB to yield a yellow chromospheres which was measured by spectrophotometrically. For 0.5 mL of processed tissue sample, 0.375 mL of sulphosalicylic acid (10%) was added and centrifuged at 12,000 x g for 15 min at 4^{o}C. The supernatant was collected and used for total glutathione analysis. 2 mL of 0.3 M potassium dihydrogen phosphate buffer (pH 8.4), 0.48 mL of distilled water and 0.02 mL of DTNB were added to 0.5 mL of supernatant. The mixture was vortexed and incubated at 37^{o}C for 10 min. The absorbance was read at 412 nm. Total glutathione concentration was expressed as micromoles per mg protein.

Lipid peroxidation assay (LPO)

Lipid peroxidation refers to the oxidative degradation of lipids. It is the process whereby free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. Certain diagnostic tests specifically malondialdehyde (MDA) are available for the quantification of the end products of lipid peroxidation. Lipid peroxidation was expressed as nanomoles per mg protein. Extent of free radical damage was quantified by estimating thiobarbituric acid reactive substances (TBARS) using 1, 1, 3, 3-tetraethoxypropane (TEP) as the standard. To 0.5 mL of tissue extract, 0.3 mL 5N HCl, 0.3 mL of thiobarbituric acid (2%) and 0.3 mL of tricholoroacetic acid (30%) were added and then heated in a water bath at 900C for 15 min. The extract was centrifuged and the absorbance of the supernatant was measured at 532 nm.

Statistical analysis

All results were expressed as mean ± SEM and data were analyzed using one-way ANOVA followed by Bonferroni'multiple comparision test. $p \leq 0.05$ was considered to be statistically significant.

Results

The wood of *P. roxburghii* was authenticated by Dr. E. Roshni Nayar, National Bureau of Plant Genetic Resources, Pusa Road, New Delhi. A Voucher specimen (NHCP/NBPGR/2010-40) was deposited at National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India.

The wood oil was obtained by hydro distillation of *P. roxburghii* wood and the % yield of light yellow color wood oil was found to be 0.5% w/v.

The phytochemical testing of wood oil of *P. roxburghii* was done in order to determine the presence of different constituents. The chemical tests showed the presence of various phytoconstituents viz. alkaloids, carbohydrate, terpenoid, steroids, fixed oil and fats in wood of *P. roxburghii* oil.

The mice treated with oral administration of 50, 100, 200, 300 and 400 mg/kg of wood oil were normal. In the study, wood oil of *P. roxburghii* was administered in 200, 300 and 400 mg/kg dosage.

The administration of CCl_4 significantly ($p<0.001$) increased the levels of serum SGPT, SGOT, ALP and total bilirubin. Treatment with silymarin and different doses (200 and 300 mg/kg, p.o.) of *P. roxburghii* wood oil reversed these parameters significantly in a dose-dependent manner, while retrograde percentage protection in marker enzyme at the dose of 400 mg/kg

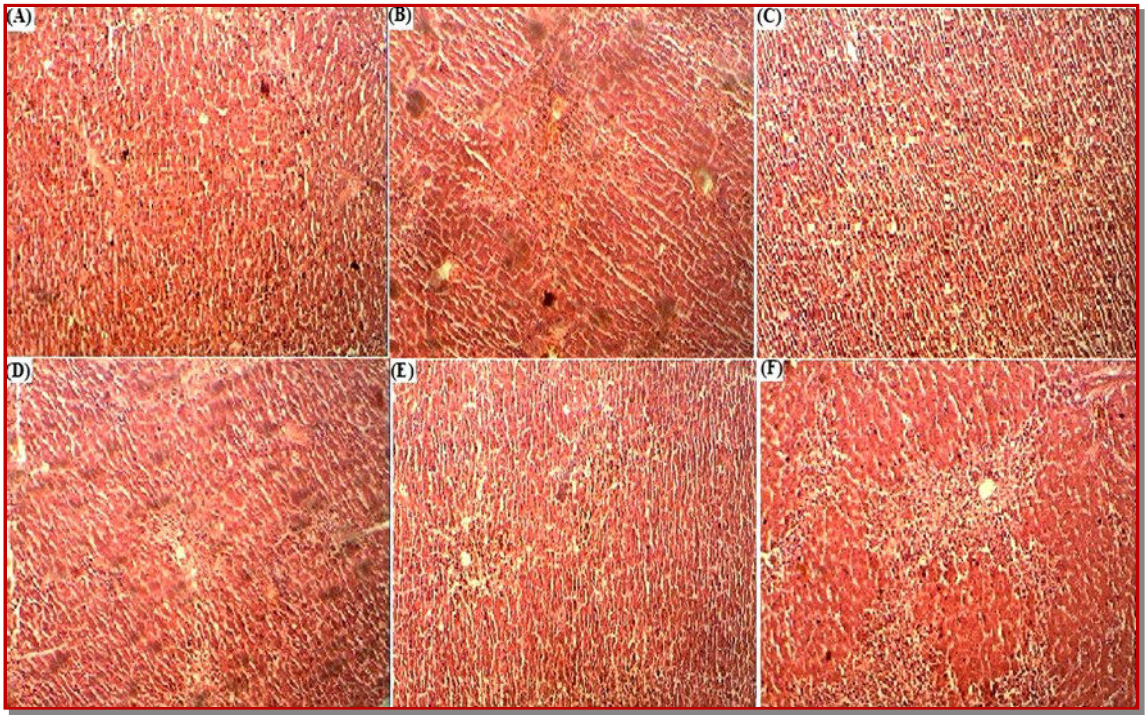

Figure 1: The photomicrographs of liver sections stained with haematoxylin and eosin (10 × 20): (A) normal rat liver, (B) CCl4 treated, (C) silymarine + CCl4 treated, (D) test group 200 mg/kg, + CCl4 treated and (E) test group 300 mg/kg + CCl4 treated (F) test group 400 mg/kg + CCl4 treated

Table I

Table I: Effect of *Pinus roxburghii* wood oil on SGPT, SGOT, ALP and total bilirubin in CCl_4 intoxicated animal

Group	Treatment	SGPT (IU/L)	SGOT (IU/L)	ALP (IU/L)	Total bilirubin (mg/dL)
I	Control	46.5 ± 0.5	33.5 ± 1.1	11.2 ± 0.3	0.3 ± 0.006
II	CCL_4	114.2 ± 0.3d	125.5 ± 0.3d	21.8 ± 0.4d	0.9 ± 0.01d
III	Silymarin + CCl_4	59.3 ± 0.3b	46.6 ± 0.5c	13.3 ± 0.2c	0.4 ± 0.003c
IV	Test 200 mg/kg + CCl_4	76.6 ± 0.5c	175.0 ± 0.3b	18.1 ± 0.3b	0.5 ± 0.007b
V	Test 300 mg/kg + CCl_4	62.5 ± 0.6c	63.5 ± 0.6c	15.4 ± 0.2a	0.3 ± 0.003c
VI	Test 400 mg/kg + CCl_4	109.6 ± 0.5	125.5 ± 0.5	2.0 ± 0.2	0.7 ± 0.01

The values were expressed as mean ± SEM. The statistical analysis was carried out by one way analysis of variance (ANOVA). $_a p<0.05$ as compared to negative control; $_b p<0.01$ as compared to negative control; $_c p<0.001$ as compared to negative control; $_d p<0.001$ as compared to control

body weight was not significant while compared to CCL_4 Group (Table I).

Histology of the liver sections of control animals showed normal hepatic cells with well-preserved cytoplasm, prominent nucleus, nucleolus and visible central veins. The liver sections of CCl4-intoxicated rats showed massive fatty changes, necrosis, degeneration and broad infiltration of the lymphocytes and the loss of cellular boundaries. The histological architecture of liver sections of the animals treated with different doses (200 and 300 mg/kg) of *P. roxburghii* wood oil showed more or less normal lobular pattern with a mild degree of fatty change, necrosis and lymphocyte infiltration almost comparable to the control and silymarin treated groups, while at the dose of 400 mg/kg improvement was negligible (Figure 1).

The effect various doses of wood oil of *P. roxburghii* were studied on GSH, MDA, Total protein in ethanol intoxicated animal. The administration of ethanol significantly increased the levels of serum MDA and decreased the level of protein and GSH. Treatment with Silymarin and different doses (200 and 300 mg/kg) of *Pinus roxburghii* wood oil reversed these parameters significantly, while retrograde percentage protection in

Table II

Effect of *Pinus roxburghii* wood oil on total protein, malondialdehyde and reduced glutathione level in ethanol- induced liver damage

Group	Treatment	Total protein (µg/mL)	MDA (µmole/mg of protein)	GSH (µmole/mg of protein)
I	Control	0.7 ± 0.1	0.7 ± 0.1	6.8 ± 1.1
II	Ethanol	0.1 ± 0.02d	1.02 ± 0.1d	3.1 ± 0.6d
III	Silymarin + ethanol	0.6 ± 0.3c	0.8 ± 0.09b	6.5 ± 2.0c
IV	Test 200 mg/kg + ethanol	0.3 ± 0.02b	0.9 ± 0.08a	4.4 ± 0.3a
V	Test 300 mg/kg + ethanol	0.3 ± 0.3b	0.8 ±0.1a	4.5 ± 0.2a
VI	Test 400 mg/kg + ethanol	0.2 ± 0.02	0.9 ± 0.1	4.0 ± 0.6

The values were expressed as mean ± SEM. The statistical analysis was carried out by one way analysis of variance (ANOVA). ap<0.05 as compared to negative control; bp<0.01 as compared to negative control; cp<0.001 as compared to negative control; dp<0.001 as compared to control

GSH, MDA and total protein at the dose of 400 mg/kg of *P. roxburghii* wood oil was not significant (Table II).

Discussion

Administration of CCl4 results in marked elevation of serum SGPT, SGOT, ALP and total bilirubin as these enzymes are liberated into systematic circulation by damaged cells. Both, prophylactic and curative, treatments with *P. roxburghii* oil (200, 300 mg/kg) reversed the increased levels of SGPT, SGOT, ALP and total bilirubin depicting a marked protective effect in a dose-dependent manner. While retrograde percentage protection in marker enzyme at the dose of 400 mg/kg body weight was not significant.

The injury and dysfunction of liver caused by CCL_4 in experimental animals simulates the human viral hepatitis. The toxic effects of CCL_4 are due to its conversion to highly reactive toxic free radical CCL_3O- by cytochrome P_{450}. The free radicals produced locally, cause autooxidation of polyenic fatty acids present within membrane phospholipids and oxidative decomposition of lipid is initiate (Vidya et al., 2007). The organic peroxides formed after reacting with oxygen leads to swelling of smooth endoplasmic reticulum and dissociation of ribosomes from rough endoplasmic reticulum. Accumulation of lipids ensues due to inability of the cells to synthesize lipoprotein from triglycerides and lipid acceptor proteins leading to the fatty liver. Further, release of products of lipid peroxidation causes damages to plasma membrane. This followed by progressive swelling of the cell death. The increase in the levels of SGPT, SGOT, ALP, total bilirubin was clear indication of cellular leakage and loss of functional integrity of the cell membrane (Ahn et al., 2007).

The comparative histopathological studies of liver from different groups further corroborated the hepatoprotective efficacy of *P. roxburghii* oil (Argal et al., 2010). Histopathological parameters indicate the structural and functional integrity of the cells and provide further support to proposed mechanism of action.

In order to probe the possible mechanism by which *P. roxburghii* oil prevents hepatic damage caused by ethanol, investigation on levels of total protein, MDA and glutathione were carried out (Alsaif et al., 2007). MDA were found to be elevated after the administration of ethanol which was significantly reversed by *P. roxburghii* oil. There was a significant rise in total protein and GSH content of liver after treatment with *P. roxburghii* oil. The effects of *P. roxburghii* oil were comparable to that of silymarin (Patel et al., 2010). Since the preliminary phytochemical analysis of the oil has shown the presence of terpenoid, and steroids compounds, which have been known for its anti-oxidant and hepatoprotective activities (Di Carlo et al., 1999).

The above results demonstrate that oil of *P. roxburghii* has significant hepatoprotective effect on acute liver injury induced by CCl4 and ethanol. It might postulate that its hepatoprotective effect may be due to its inhibitory effect on free radical formation as evident by recovery of GSH contents towards normalization and decreased lipid peroxidation. Other biochemical and histopathological parameters indicate the structural and functional integrity of the cells and provide further support to proposed mechanism of action.

Conclusion

Oil of *P. roxburghii* has hepatoprotective activity made possibly by the triterpenes and steroids via inhibitory effect on free radical formation and decreased lipid peroxidation Detailed study on the phytochemical analysis of Oil of *P. roxburghii* is in progress in the laboratory.

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