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Hepatoprotective Potential of Lyophilized Hydro-Alcoholic Extract of *Roylea Elegans Wall*. Against CCL_4 and PCM Induced Hepatotoxicity in Wistar Rats

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Abstract

Roylea elegans (Lamiaceae) is well known traditional plant of Garhwal and Kumaon regions of Uttarakhand, India used to treat jaundice and liver disorder. The present study was designed to evaluate its hepatoprotective effect, and to validate its traditional claims. Hepatoprotective activity of lyophilized hydro alcoholic extract of aerial parts of *Roylea elegans* (HAE) was extracted using carbon tetrachloride (CCL_4) and paracetamol (PCM) induced Hepatotoxicity at a dose level of 1 ml/kg, s.c. and 3 ml/kg, p.o, respectively. Different parameters were used for estimation of hepatic injury. Blood serum parameter as serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphate (ALP), and total billirubin (TB) was evaluated for Hepatotoxicity and tissue parameters as thio-barbituric acid reactive substances (TBARS) and reduced glutathione (GSH) levels was evaluated on oxidative stress for protective effects of plant at a different dose levels (100, 200 and 400 mg/kg). The biochemical observations were also supplemented by histopathological examination. The preliminary phytochemical sceening of HAE showed the presence of alkaloids, triterpenes and carbohydrates. The HAE extract (100, 200 and 400 mg/kg) significantly ($p<0.05$) prevented the increase levels of serum enzymes for both CCL4 and PCM induced Hepatotoxicity. Furthermore, the lyophilized HAE also exhibited antioxidant activity at above dose levels significantly ($p<0.05$) decreases the TBARS levels and increases the GSH levels against both the models, also supported by histopathological study.

Conclusion: The present investigation clearly indicates the hepatoprotective effect of *Roylea elegans Wall*. May be attributed to antioxidant potential of phytoconstituents.

Keywords: Roylea elegans; Histopathology; Hepatoprotective; Biochemical estimation; Lyophillized; Hepatotoxicity

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Introduction

Hepatotoxicity has become one of the major leading roles of morbidity and mortality all over the world. It is defined as injury to the liver that is associated with impaired liver function caused by exposure to a drug or another non-infectious agent [1]. It is a complex process that involves changes in blood flow, blood pressure, metabolic capacity and protein binding. It may be predictable or unpredictable; predictable reactions typically are dose related and occur mostly due to short exposure after some threshold for toxicity. Some chemicals as CCl_4, phosphorus, chloroform etc. are unpredictable hepatotoxins, unrelated to dose and have variable latency period [2]. Carbon tetrachloride induced Hepatotoxicity accumulates in hepatic parenchyma cells and is metabolized to CCl_3^* radicals by liver cytochrome P_{450}- dependent monooxygenases, thus rapidly damaged the hepatocytes [3]. Paracetamol (N-acetyl-par aminophenol) is a widely used analgesic drug but over dosage of paracetamol causes Hepatotoxicity and nephrotoxicity as it is detoxified in the liver in the form of glucuronide or sulphate which leads to production of reactive metabolite N-acetyl p-benzoquinonimine (NAPQI) which covalently binds to cellular macromolecules and can initiate cell damage [4]. Although, a significant advances in development of modern medicine,

Table 1: The effect of HAE on serum enzymes (SGOT, SGPT, ALP & TB) level in CCL_4-induced liver injury in rats.

Treatments	SGOT (U/L)	SGPT (U/L)	ALP (U/L)	TB (mg/dl)
Normal-control	25.26±1.62	16.92±1.14	131.08±7.96	0.26±0.03
CCl_4-control (1 ml/kg, s.c.)	181.70±15.80*	159.64±5.21*	277.69±15.03*	1.32±0.13*
Standard (silymarin, 50 mg/kg, p.o.) + CCl_4	46.05±2.13**	37.73±3.18**	149.68±9.30**	0.33±0.03**
Test extract (100 mg/kg, p.o.) + CCl_4	141.31±5.86**#	116.10±6.79**#	227.32±10.0**#	0.57 ±0.04**#
Test extract (200 mg/kg, p.o.) + CCl_4	79.80±6.61**#	62.27±4.96**#	194.62±12.25**#	0.49±0.05**#
Test extract (400 mg/kg, p.o.) + CCl_4	54.28±2.20**	44.14±3.69**	162.94±7.73**	0.44±0.03**

Each value is mean ± SEM for 6 rats in each group.
* P < 0.05 *vs.* normal control.
**P < 0.05 *vs.* CCl_4- control group.
#P< 0.05 *vs.* Standard (silymarin).

Table 2: The effect of HAE on serum enzymes (SGOT, SGPT, ALP & TB) level in PCM- induced liver Injury in rats.

Treatments	SGOT (U/L)	SGPT (U/L)	ALP (U/L)	TB (mg/dl)
Normal-control	25.03±5.71	16.92±1.14	131.08±7.96	0.26±0.03
PCM-control (3 mg/kg, p.o.)	166.71±12.67*	146.77±7.75*	245.57±14.26*	1.09±0.13*
Standard (silymarin, 50 mg/kg, p.o.) + PCM	46.44±7.27**	37.48±5.28**	144.09±12.03**	0.32±0.07**
Test extract (100 mg/kg, p.o.) + PCM	136.23±7.84**#	110.36±4.27**#	217.12±11.23**#	0.62±0.06**#
Test extract (200 mg/kg, p.o.) + PCM	77.98±7.05**#	63.34±5.75**#	184.68±8.11**#	0.50±0.06**#
Test extract (400 mg/kg, p.o.) + PCM	51.53±3.43**	40.96±5.60**	159.63±5.68**	0.47±0.04**

Each value is mean ± SEM for 6 rats in each group.
* P < 0.05 *vs.* normal control.
**P < 0.05 *vs.* PCM- control group.
#P< 0.05 *vs.* Standard (silymarin).

liver disorders remain a worldwide health problem. Numerous herbal formulations and plant extract are used to treat liver disorders against various models. Plants belonging to family lamiaceae are well reported to posses hepatoprotective potential eg. Plectranthus amboinicus, *Ocimun santum*, *Hibiscus sabdariffa*, *Rosmarinus officinalis*, *Salvia officinalis* [5,6] etc. In addition, several phytoconstituents (terpenes & alkaloids) are also reported to possess hepatoprotective properties. Terpenes, such as monoterpenes hydrocarbons (mycene, terpinolene, pinene), oxygenate monoterpenes (nerol, geraniol, linalol, thymol), sesquiterpene hydrocarbons (humulene, valencene, calarene), oxygenated sesquiterpene (trans-trans-farnesol, farnesol, farnesyl acetate, guaiol), diterpene hydrocarbons (phytol, abetine), and tetraterpene hydrocarbons (caratenoids) [7-9] and alkaloids as berberine, dictamnice, magnoflorine, xanthoplanine etc. are well known hepatoprotectives [10]. *Roylea elegans Wall.* (Lamiaceae) commonly known as titpati (Kumaon) and kauri (Garhwal) is a shrub of monotypic genus found in Western Himalayas from Kashmir to Nepal at altitudes of 600 m-1,500 m. The Aerial parts of plant are widely used against liver disorders [11] and also used in scabs and skin infections [12]. The phytochemical screening of lyophilized hydro-alcoholic extract showed the presence of alkaloids, triterpenes and carbohydrates [13]. Till date, no work has been performed on this plant as per as its pharmacological screening is concerned. Hence, the present investigation was carried to evaluate the hepatoprotective effects of lyophilized hydro-alcoholic extract of aerial parts of *Roylea elegans Wall.*

Materials and Methods

Plant material

Fresh aerial parts of *Roylea elegans* Wall. Were collected from local areas of Distt- Pithoragarh (Uttrakhand) and are authenticated by Dr. H.B. Singh, Director, Department of Raw Material And Herbarium, NISCAIR, and New Delhi (Ref. NISCAIR/RHMD/ Consult/-2010-11/1561/159). The collected aerials parts of plant was made thoroughly free from any foreign organic matter, dried under shade and powdered as per WHO guidelines.

Chemicals and reagents: CCl_4 was purchased from Merck Specialities Pvt. Ltd., Mumbai, Paracetamol was purchased from Cipla Pvt. Ltd., and Silymarin was purchased from Micro labs Pharma, Baddi and Serum Kits from CDH, India. All other chemicals and biochemical reagents were of L.R. and A.R. grade, respectively.

Preparation of HAE: The coarsely powdered aerial parts of plant (500 g) were macerated with hydro-alcohol (50 %) three times for 48 hrs each at room temperature. Solvent was removed under vacuum and the concentrated extract was lyophilized for 48 hrs, lyophilized powder was stored at minimum temperature for further use.

Preliminary phytochemical screening: The HAE was subjected for preliminary phytochemical screening for the detection of various phytoconstituents such as alkaloids, glycosides, tannins and phenolic compounds, flavonoids, steroids, saponins, proteins, amino acids, carbohydrates and triterpenoids [14,15].

FTIR spectral analysis: The FTIR spectrum of HAE was recorded on a Perkin Elmer Spectrum RXI FTIR system (FTIR-8400S, SHIMADZU, USA) by using potassium bromide sample cell.

Preparation of sample for FTIR: 1 mg of HAE was dissolved in ethanol and transfer to sample cell. The sample cell was placed in FTIR transmitting window and the spectra was recorded.

Experimental animals: Albino wistar rats (either sex) weighing 180 gm-220 gm were procured from CPCSEA approved animal house of Siddhartha Institute of Pharmacy, Dehradun, India and used throughout the experiment. The animal were housed in an air conditioned room (24 ± 4°C) with 12 h-12 h light & dark cycles; had access to standard chow diet (Ashirwad Industries, Ropar, India)

Table 3: The effect of HAE on hepatic lipid peroxidation (TBARS) level and antioxidant enzyme (GSH) activities in CCl_4-induced liver injury in mice.

Treatments	TBARS (nmol/g liver)	GSH (µmol/g liver)
Normal-control	22.405±2.904409	110.6683±7.807884
CCl_4-control (1 ml/kg, s.c.)	52.51±2.889477*	52.87667±4.800236*
Standard (silymarin, 50 mg/kg, p.o.) + CCl_4	30.245±1.809572**	89.08167±8.0331**
Test extract (100 mg/kg, p.o.) + CCl_4	48.25667±3.775779**#	59.46333±4.976311**#
Test extract (200 mg/kg, p.o.) + CCl_4	38.92167±2.821556**#	76.355±6.407598**#
Test extract (400 mg/kg, p.o.) + CCl_4	35.29333±2.24833**	82.48167±5.517113**

Each value is mean ± SEM for 6 rats in each group.
* P < 0.05 *vs.* normal control.
**P < 0.05 *vs* CCl_4- control group.
#P< 0.05 *vs* Standard (silymarin).

Table 4: The effect of HAE on hepatic lipid peroxidation (TBARS) level and antioxidant enzyme (GSH) activities in PCM-induced liver injury in mice.

Treatments	TBARS (nmol/g liver)	GSH (µmol/g liver)
Normal-control	22.405±2.904409	110.6683±7.807884
PCM-control (1 ml/kg, s.c.)	45.03±2.092711*	65.31333±4.209179*
Standard (silymarin, 50 mg/kg, p.o.) + PCM	26.55667±1.897363**	93.56167±6.533108**
Test extract (100 mg/kg, p.o.) + PCM	38.21667±2.693033**#	73.56667±5.409446**#
Test extract (200 mg/kg, p.o.) + PCM	32.35833±2.430485**#	83.40167±6.566404**#
Test extract (400 mg/kg, p.o.) + PCM	28.55167±2.209049**	87.965±7.66261**

Each value is mean ± SEM for 6 rats in each group.
* P < 0.05 *vs.* normal control.
**P < 0.05 *vs.* PCM- control group.
#P< 0.05 *vs.* Standard (silymarin).

and water *ad libitum*. The experiments were designed and conducted according to ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and the Institutional Animal Ethics Committee Guidelines (1435/PO/a/11/CPCSEA). The protocol was repeated twice to get the reproducibility of results.

Acute Toxicity

The toxicity of extract was studied as per organization for economic co-operation and development (OECD) guideline number 425. The limit test was performed initially. Swiss albino mice weighing 20 g to 25 g were used in the toxicity study. Six mice were serially administered a 2000 mg/kg dose of extract prepared in water as recommended in the guideline. After dose administration, each animal was observed after every hour for signs of toxicity and abnormality in behavior up to the 48th hour. After this, daily observations for toxicity and mortality were made up to the 14th day. The body weights of the animals were recorded every third day. On the 14th day after dosing, all the mice were sacrificed and processed for gross necropsy.

Preparation of Dose

The HAE was evaluated at three oral dose levels of 100, 200 and 400 mg/kg. The doses were prepared by accurately weighing the extract and suspending in 0.3% CMC in distilled water.

Models to Induced Hepatotoxicity

Carbon tetrachloride (CCl_4) induced hepatotoxicity

The Carbon tetrachloride (CCl_4) induced Hepatotoxicity was modified procedure of [16]. Rats were divided into six groups consisting of six animals in each group. Group I (Normal control) were administered distilled water containing 0.3% sodium carboxymethyl cellulose (CMC-Na) (1mL/kg bodyweight, p.o.) daily, for 7 days and olive oil (1 ml/kg body weight, s.c.) on days 2 and 3 Jain et al. [16]. Group II (CCl_4- control) were administered 0.3 % CMC-Na, daily for 7 days and a dose of 1 ml/kg body weight, s.c. (CCl_4: olive oil) on days 2 and 3 [17]. Group III (Standard treatement) were administered with the standard drug silymarin (50mg/kg body weight, p.o.) daily for 7 days [18] and CCl_4 on days 2 and 3, 30 min after administration of silymarin. Groups IV–VI (Test group) was administered HAE (a dose of 100, 200 and 400 mg/kg) orally for 7 days. Additionally, 30 min after administration, a dose of the CCl_4 was administerd on days 2 and 3.

Paracetamol (PCM) induced hepatotoxicity

The paracetamol (PCM) induced hepatotoxicity was modified procedure of [19]. Rats were divided into six groups consisting of six animals in each group. Group I (Normal control) were administered distilled water containing 0.3% sodium carboxymethyl cellulose (CMC-Na) (1 ml/kg body weight, p.o.) for 7 days. Group II (PCM control) were administered 0.3 % CMC daily, for 7 days and a dose of paracetamol (3 mg/kg, p.o with distilled water) on days 3 and 5 [19]. Group III (Standard treatement) were administered with the standard drug silymarin (50 mg/kg body weight, p.o) daily, for 7 days, and paracetamol on days 3 and 5, after administration of silymarin. Groups IV–VI (Test group animals) was administered HAE (100, 200 and 400 mg/kg) orally for 7 days. Additionally, 30 min after administration of extract, they received paracetamol on days 3 and 5. On day 7 for both the experimental models, animals were anaesthetized and blood was collected, allowed to clot, and serum was separated for assessment of enzyme activity. The rats were then sacrificed and the livers were carefully dissected, cleaned of excess tissue. Part of the liver tissue was immediately transferred into tris and phosphate buffer for liver tissue parameter (estimation of TBARS and reduced glutathione assay, respectively) and also in 10% formalin for histopathological investigation.

Biochemical Estimation

Serum parameters

Collected blood samples were placed at room temperature for 1h, and then centrifuged for 10 min to obtain serum. Activity of serum enzymes was measured as SGOT, SGPT, ALP and TB levels. The activities of SGOT, SGPT and ALP were expressed as U/L and for TB expressed as mg/dl.

Estimation of TBARS and reduced Glutathione level

The tissues were homogenized in 0.1 M tris buffer (pH 7.4) for estimation of thio-barbituric acid reactive substances (TBARS) and in 0.1 M phosphate buffer (pH 7.4) for estimation of reduced Glutathione (GSH) level. Lipid per-oxidation was determined by measuring the amounts of Malondialdehyde (MDA) produced primarily and levels of lipid peroxides were expressed as nmoles/g liver of Thiobarbituric acid reactive substances (TBARS). To measure the reduced glutathione (GSH) level, the tissue homogenate values were compared with a standard curve of GSH. The level of GSH was expressed as µmol/mg of live weight [20].

Histopathological studies

Different liver tissues were fixed in 10 % formalin for 24 h, embedded in paraffin, and cut into 4-5 µm thick sections using a rotary microtome. The sections were stained with Hematoxylin–

Eosin dye and observed under a microscope (IX51, Olympus, Japan) to observe histopathological changes in the liver.

Statistical analysis

Numerical results are expressed as Mean ± SEM. Data was analyzed using one way analysis of variance test. Calculations were performed using commercial software (GraphPad Software, San Diego, CA). ANOVA followed by Bonferroni post hoc test. p values < 0.05 were considered as statistically significant.

Results

Preliminary phytochemical screening

The preliminary phytochemical screening of HAE showed the presence of alkaloids, triterpenes and carbohydrates.

Acute toxicity study

In the acute toxicity study, neither death nor any observable neurobehavioral effects were observed in the limit test. No significant alterations in the necropsy after enthanasia. Hence, as stated in OECD guideline number 425, these compounds were classified as globally harmonized system (GHS) category-5 substances. Due to lack of any observable toxicity at the 2000 mg/kg dose, LD_{50} was not determined. The observed lack of toxicity of the plant is consistent with their traditional use as fodder.

Effect of Hydro-Alcoholic Extract on Serum Levels AST, ALT, ALP and TB

The serum levels of hepatic enzymes SGOT, SGPT, ALP and TB used as biochemical markers for evaluation of early Hepatotoxicity and were significantly elevated in the CCl_4-treated rats (Table 1 and Figure 1) and in paracetamol (PCM) treatment in rats (Table 2 and Figure 2). In both models, pretreatment with HAE at doses 100, 200 and 400 mg/kg/day (dose standardized and selected on the basis of acute toxicity studies) were significantly prevented the elevation of these marker enzymes compared to control group animals. The HAE at a dose of 400 mg/kg showed activity almost comparable to the group treated with silymarin, a potent hepatoprotective drug used as reference in both the models.

Effect of Hydro-Alcoholic Extract on TBARS and Reduced Glutathione (GSH) in Liver

Homogenate

There is a significant increase in TBARS level, an indicator of lipid peroxidation, was found in the livers of CCl_4-intoxicated (Table 3 and Figure 3) as well as PCM treated (Table 4 and Figure 4) rats relative to the normal group. Pretreatment with different doses of HAE (100, 200 & 400 mg/kg/day) reversed this biochemical parameter significantly towards normal level. The highest dose (400 mg/kg) was found to be comparable to the standard drug silymarin. The activities of antioxidant enzyme GSH in liver homogenate was significantly decreased in both liver injury models when compared to normal control. The HAE at a dose of 100, 200 & 400 mg/kg/day significantly increases the GSH level. The 400 mg/kg dose exerted a beneficial antioxidant effect on GSH equal to the standard drug silymarin.

Histopathological results

The histological studies showed no pathological abnormalities in the liver of normal control (Figure 5 and 6). However, CCl_4 and PCM treated rats showed several damages in the tissue. Histopathological analysis of the liver sections of toxicant administration showed centrilobular necrosis, hepatocytes ballooning and infiltration of inflammatory cells (such as macrophages and lymphocytes) into the portal tract and sinusoid in the necrotic lesion. Diffused areas of hepatitis, especially in the perivenular region which extend to the central zone were observed after the toxicant administration (Figure 7). Pretreatment with HAE at different doses (100, 200 & 400 mg/kg) reversed the hepatic lesions produced by toxicant in both models is evident from the absence of cellular necrosis and inflammatory infiltrates in the liver section of rats. Compare to the highest dose tested, which showed almost equal to the standard drug, silymarin.

Discussion and Conclusion

Liver toxicity is the well known side effect for many known drugs which requires immediate attention. Carbon tetrachloride (CCl_4), chemical hepatotoxins, is widely known for inducing Hepatotoxicity in animals. The features of CCl_4 toxicity are similar to those of acute hepatitis in humans. It interferes with triglyceride secretion and causes steatosis, fibrosis, and necrosis. It decreases the levels of plasma triglyceride-rich lipoproteins and microsomal triglyceride transfer protein (MTP) without diminishing mRNA levels [21]. CCl_4 is metabolized by various is enzymes (cytochrome P450; CYP2E1, CYP2B1 or CYP2B2, and CYP3A) present in hepatocytes leading to the formation of trichloromethyl radical (CCl3-) [22]. This radical forms a covalent bond with lipids of hepatocytes such as triacylglycerols and phospholipids with phosphatidylcholine which ultimately leads to peroxidation of membrane lipids and markedly elevate the serum levels of SGOT, SGPT, ALP and TB [23,24]. The paracetamol a commonly and widely used analgesic-antipyretic agent gets metabolized in the liver but overdosing of paracetamol leads to the formation of toxic metabolites which is metabolized by cytochrome P-450 causes centrilobular necrosis of the liver. The metabolism of PCM results in the formation of an active metabolite NAPQI (*N*-acetyl-*p*-benzo-quinone imine) along with some inactive sulfates and glucuronide conjugates. The active metabolite then reacts with the cellular membrane molecules (causing lipid peroxidation of membrane lipids), resulting in widespread hepatocytes damage and death. This further leads to marked elevations in the serum levels of SGOT, SGPT, ALP and TB due to acute hepatic necrosis [25,26]. The levels of SGOT, SGPT, ALP and TB are the most common biomarkers used to assess the liver functions during any kind of liver injury/disorder. Intoxication of both CCl_4 and PCM leads to the elevations in various biomarker enzymes, indicating considerable hepatocellular damage which further results in leakage of cellular contents into the systemic circulation [27]. The hydro-alcoholic extract dose dependently reversed the structural integrity of the hepatocellular membrane, which was evident from the reduction in serum levels of SGOT, SGPT, ALP and TB of pretreated rats in both experimental models [28-30]. Furthermore, the effect of HAE (400 mg/kg) was comparable to that of the standard drug silymarin. Consequently, it seems that HAE would certainly exert its in vivo antioxidant activity by different pathways other than the simple free radical scavenging action, such as increasing the antioxidant enzyme activities, decreasing the markers of lipid peroxidation and inhibiting the formation of advanced glycation end products [31,32]. Therefore, the effect of HAE on TBARS and GSH levels was also evaluated. There was almost a threefold increase in the TBARS level observed in liver homogenate of CCl_4 and PCM intoxicated rats. The pre-treatment with HAE almost completely controlled the levels of TBARS and GSH. The effect of HAE at highest tested dose was equivalent to the standard drug sylimarin. This indicates the ability of HAE to break the chain reaction of lipid peroxidation [33-35]. In addition, hepatoprotective

effect of plant extract was further supported by histopathological studies of the liver tissue. The histological changes, such as necrosis in hepatic lobules and inflammatory infiltration of lymphocytes and macrophages around the central vein, were simultaneously improved by the pretreatment with different doses of plant extract. Thus, reversal of both, CCl_4 and PCM induced Hepatotoxicity by the HAE could be due to its significant antioxidant potential. Furthermore, the antioxidant potential of the plant extract could be due to the presence of the triterpenoids and alkaloids.

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