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 screening 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; Lyophilized; Hepatotoxicity

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 monoxygenases, thus rapidly damaged the hepatocytes [3]. Paracetamol (N-acetyl-p-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,
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 possess hepatoprotective potential eg. Plectranthus amboinicus, Ocimum sanctum, 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 (myrcene, terpinolene, pinene), oxygenate monoterpenes (nerol, geraniol, linalol, thymol), sesquiterpene hydrocarbons (humulene, valencene, calarene), oxygenated sesquiterpene (trans-trans-farnesol, farnesol, farnesyl acetate, gaialol), 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 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 (Uttarakhand) 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 aerial parts of plant was made thoroughly free from any foreign organic matter, dried under shade and powdered as per WHO guidelines.

**Chemicals and reagents:** CCl₄ 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 to 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)

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**Table 1:** The effect of HAE on serum enzymes (SGOT, SGPT, ALP & TB) level in CCL₄-induced liver injury in rats.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>SGOT (U/L)</th>
<th>SGPT (U/L)</th>
<th>ALP (U/L)</th>
<th>TB (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal-control</td>
<td>25.26±1.62</td>
<td>16.92±1.14</td>
<td>131.08±7.96</td>
<td>0.26±0.03</td>
</tr>
<tr>
<td>CCl₄-control (1 ml/kg, s.c.)</td>
<td>181.70±15.80*</td>
<td>159.64±5.21*</td>
<td>277.69±15.03*</td>
<td>1.32±0.13*</td>
</tr>
<tr>
<td>Standard (silymarin, 50 mg/kg, p.o.) + CCl₄</td>
<td>46.05±2.13&quot;</td>
<td>37.73±3.18&quot;</td>
<td>149.68±5.30&quot;</td>
<td>0.33±0.03&quot;</td>
</tr>
<tr>
<td>Test extract (100 mg/kg, p.o.) + CCl₄</td>
<td>141.31±5.86&quot;</td>
<td>116.10±6.79&quot;</td>
<td>227.32±10.0&quot;</td>
<td>0.57±0.04&quot;</td>
</tr>
<tr>
<td>Test extract (200 mg/kg, p.o.) + CCl₄</td>
<td>79.80±6.61&quot;</td>
<td>62.27±4.96&quot;</td>
<td>194.62±12.25&quot;</td>
<td>0.49±0.05&quot;</td>
</tr>
<tr>
<td>Test extract (400 mg/kg, p.o.) + CCl₄</td>
<td>54.28±2.20&quot;</td>
<td>44.14±3.69&quot;</td>
<td>162.94±7.73&quot;</td>
<td>0.44±0.03&quot;</td>
</tr>
</tbody>
</table>

Each value is mean ± SEM for 6 rats in each group.
*P < 0.05 vs. normal control.
"P < 0.05 vs. CCl₄ 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.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>SGOT (U/L)</th>
<th>SGPT (U/L)</th>
<th>ALP (U/L)</th>
<th>TB (mg/dl)</th>
</tr>
</thead>
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<tr>
<td>Normal-control</td>
<td>25.03±5.71</td>
<td>16.92±1.14</td>
<td>131.08±7.96</td>
<td>0.26±0.03</td>
</tr>
<tr>
<td>PCM-control (3 mg/kg, p.o.)</td>
<td>166.71±12.67*</td>
<td>146.77±7.75*</td>
<td>245.57±14.26*</td>
<td>1.09±0.13*</td>
</tr>
<tr>
<td>Standard (silymarin, 50 mg/kg, p.o.) + PCM</td>
<td>46.44±7.27&quot;</td>
<td>37.48±5.28&quot;</td>
<td>149.09±12.03&quot;</td>
<td>0.32±0.07&quot;</td>
</tr>
<tr>
<td>Test extract (100 mg/kg, p.o.) + PCM</td>
<td>136.23±7.84&quot;</td>
<td>110.36±4.27&quot;</td>
<td>217.12±11.23&quot;</td>
<td>0.62±0.06&quot;</td>
</tr>
<tr>
<td>Test extract (200 mg/kg, p.o.) + PCM</td>
<td>77.98±7.05&quot;</td>
<td>63.34±5.75&quot;</td>
<td>184.68±8.11&quot;</td>
<td>0.50±0.06&quot;</td>
</tr>
<tr>
<td>Test extract (400 mg/kg, p.o.) + PCM</td>
<td>51.53±3.43&quot;</td>
<td>40.96±5.60&quot;</td>
<td>159.63±5.68&quot;</td>
<td>0.47±0.04&quot;</td>
</tr>
</tbody>
</table>

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).
Table 3: The effect of HAE on hepatic lipid peroxidation (TBARS) level and antioxidant enzyme (GSH) activities in CCl4-induced liver injury in mice.

<table>
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<tr>
<th>Treatments</th>
<th>TBARS (nM/mg liver)</th>
<th>GSH (µM/mg liver)</th>
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<tr>
<td>Normal-control</td>
<td>22.405±2.904409</td>
<td>110.8683±7.807884</td>
</tr>
<tr>
<td>CCl4-control (1 ml/kg, s.c.)</td>
<td>45.03±2.907211</td>
<td>65.3133±2.09179</td>
</tr>
<tr>
<td>Standard (silymarin, 50 mg/kg, p.o.) + CCl4</td>
<td>26.556±7.189736</td>
<td>93.5617±6.531108</td>
</tr>
<tr>
<td>Test extract (100 mg/kg, p.o.) + CCl4</td>
<td>38.216±6.2.803332</td>
<td>73.5667±5.490446</td>
</tr>
<tr>
<td>Test extract (200 mg/kg, p.o.) + CCl4</td>
<td>32.583±2.430485</td>
<td>83.4017±6.566404</td>
</tr>
<tr>
<td>Test extract (400 mg/kg, p.o.) + CCl4</td>
<td>28.551±6.2.209049</td>
<td>87.965±7.66261</td>
</tr>
</tbody>
</table>

Each value is mean ± SEM for 6 rats in each group. *P < 0.05 vs. normal control. **P < 0.05 vs. CCl4 control group.

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

<table>
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<tr>
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Each value is mean ± SEM for 6 rats in each group. *P < 0.05 vs. normal control. **P < 0.05 vs. PCM control group.

and water ad libitum. The experiments were designed and conducted according to ethical norms approved by Ministry of Social Justice 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 distilled 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 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 nmol/g liver of Thio-barbituric 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 noticeable neurobehavioral effects were observed in the limit test. No significant alterations in the necropsy after enanthasia. 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, LD₅₀ 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 were used as biochemical markers for evaluation of early Hepatotoxicity and were significantly elevated in the CCl₄-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 CCl₄-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 comparable to that of the standard drug silymarin. This indicates the ability of HAE to break the chain reaction of lipid peroxidation which requires immediate attention. Carbon tetrachloride (CCl₄), chemical hepatotoxins, is widely known for inducing Hepatotoxicity in animals. The features of CCl₄ 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]. CCl₄ is metabolized by various 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 centrifugal 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].

Discussion and Conclusion

Liver toxicity is the well known side effect for many known drugs which requires immediate attention. Carbon tetrachloride, chemical hepatotoxins, is widely known for inducing Hepatotoxicity in animals. The features of CCl₄ 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]. CCl₄ is metabolized by various 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 centrifugal 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].
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, CCl4 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.

References