SUB-ACUTE TOXICOLOGICAL EVALUATION OF HYDROMETHANOLIC EXTRACT OF HOLARRHENA ANTIDYSENTERICA STEM BARK

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ABSTRACT

_Holarrhena antidysenterica_ (Linn.) Wall Family Apocynaceae is also known as connessi bark in English, kutaja in Sanskrit, kura or kurchi in Hindi. Its bark and seeds have been used in the treatment of dysentery and diarrhoea, anemia, epilepsy, stomach pain and cholera. In this study, toxicity profile of the plant extract on repeated administration was studied in male and female rats. The rats were treated with doses at three different levels (150, 450 and 1350 mg/kg) of the hydromethanolic extract of stem bark of the plant orally for 28 days. During the study period, the animals were observed for signs of morbidity and mortality along with behavioral changes, effect on body weight and food and water intake. After the completion of treatment, blood was collected retro-orbitally and then the animals were sacrificed after euthanasia for gross morphology and tissues were removed for histopathological studies. Study revealed that there were no adverse or toxic effects in treated animals. The extracts did not alter the structure and function of any organ or tissue as inferred from various laboratory investigations. In conclusion, _Holarrhena antidysenterica_ hydromethanolic stem bark extract was found to be safe up to 1350mg/kg dose in male and female rats.

Key words: Holarrhena antidysenterica stem bark, hydromethanolic extract, sub-acute toxicity.

INTRODUCTION

The use of herbal medicines has tremendously increased in this era. Since ages, many herbal plants have been used to treat various ailments with no scientific evidence of their safety and efficacy. Folkloric information from past generations is the basis behind using these plants which may prove hazardous if used indiscriminately. Hence it is now imperative to investigate and validate the safety of these drugs.

_Holarrhena antidysenterica_ (Linn.) Wall belonging to Family Apocynaceae is widely known as connessi bark in English, kutaja in Sanskrit and kura or kurchi in Hindi. Plant is found throughout drier or deciduous forest areas of India in the tropical Himalaya from Chenab eastwards, common in Sal forests, Aravalli hills, Bihar, Central India, South Konkan and Kerala. The active constituents isolated and identified are steroidal alkaloids conessine and holacetine (in root bark); holarrhimine, kurchine, kurchicine, conessine, norconessine, hollarine, conarrhimine, conamine, conimine, conessimine, isoconessimine, conessidine, conkurchine,
holarrhenine, lettocine and holarricine (in stem bark);antidysentericine, crystalline glucoalkaloid, kurchiline, kurchiphyllamine, kurchiphylline, holarresmine, kurchessine, holarrhidine, holonarmine, holantosine E and trimethyl conkirchine (in seeds) 1.

Bark and seeds are bitter, constipating, stomachic, astringent, powerful antidysentric, refrigerant, anthelmintic, aphrodisiac, carminative, digestive, expectorant, febrifuge, and tonic. The plant material have been found to be useful in conditions like dysentery and diarrhea, anemia, convulsions, abdominal pain and cholera infection 1. Kurchicin, an active principle of *Holarrhena antidysenterica* can cure amoebic dysentery 2. The fruit extract (50% ethanolic) showed antiprotozoal effect against *Ent. histolytica* strain STA, *Trypanosoma evansi* 3. Clinical tests with connessine on patients with intestinal and hepatic amoebiasis have been found to give results, comparable to those obtained with emetine 4.

The present study aims to see the effects of repeated oral administration of hydroalcoholic extract of *Holarrhena antidysenterica* in different doses.

**MATERIALS AND METHODS**

**Laboratory animals:**
Healthy Male Sprague Dawley (SD) rats (250-300gm, 10-11 weeks age) were housed in cages with free access to standard rat chow (diet) and water *ad libitum* and acclimatized to the surroundings for one week prior to the experiment. Animals were maintained on a light/dark cycle (12/12hr) at a constant temperature (22º±1ºC) and humidity (55±1). The experimental protocol (Protocol No. 9012 dated 26th Dec 2009) was approved by Institutional Animal Ethical Committee of Anand Pharmacy College as per the guidance of committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

**Preparation of plant extract:**
The stem bark of *Holarrhena antidysenterica* were ground to a coarse powder and stored at room temperature. 250gm of powdered plant material was extracted with 1000ml of Methanol (70%) in Soxhlet Apparatus for 5-7 hours at 65ºC. The organic extracts was concentrated by evaporation below 45ºC and then further dried at ambient temperature for 24 hr to obtain dry extract and then stored at −20 ºC. The extract of *Holarrhena antidysenterica* was labeled as MEHA.

**Grouping and drug administration:**
Subacute oral toxicity studies were conducted by following OECD guidelines- 407 5 to evaluate the safety of herbal extracts. The rats were divided into 4 groups of 10 rats (5 males and 5 females) where group I served as control group and received distilled water only. Groups II to IV received MEHA (Low dose-150 mg/kg), MEHA (Intermediate dose-450 mg/kg) and MEHA (High dose-1350 mg/kg) respectively daily for 28 days.

**Body weight, Food and water intake:**
Body weight was also recorded at every week during the entire study period. While total food intake and water intake of each group was measured on daily basis.

**Mortality, clinical signs and assessment of motor and sensory activity:**
Animals were observed with intervals for the first 4 h and afterwards every 6 h for the next 24 h. Then once daily the animals were observed for the effects on general behavior and physiology for next 27 days. Any changes in mobility, aggressiveness, sensitivity to sound and pain, as well as respiratory movements were recorded. They were also observed daily for clinical signs and mortality patterns once before dosing, immediately after dosing and up to 4 h after dosing. Any change in skin, fur, eyes, and mucous membranes, secretions, diarrhoea and effect on autonomic activity like lacrimation, salivation, piloerection, pupil size and unusual respiratory pattern were observed. Changes in gait, posture and reflexes, reaction to various stimuli, convulsions, tremors, stereotypy or bizarre behavior like self mutilation and walking backward, assessment of grip strength, motor activity assessment and occurrence of lethargy, sleep and coma were also noted.

Hematological and Biochemical parameters:
After 28 days of treatment, rats were kept for overnight fasting. Next day, blood was collected for the estimation of hematological parameters like total red blood cells (RBC), hemoglobin (Hb) content, white blood cells (WBC) and platelet count and biochemical parameters like glucose, serum creatinine, blood urea nitrogen (BUN), cholesterol, triglyceride (TG), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT). For Complete Blood Count, non-heparinized blood samples were run on fully automated hematology analyzer PCE 210. Serum glucose was estimated by GOD-POD method and all biochemical parameters were estimated using Biochemistry analyzer.

Gross pathology:
After blood collection, the rats were euthanized and liver, heart, lung and kidney were excised, rinsed in ice-cold normal saline, organs were then observed macroscopically. The external appearances of sacrificed animals and of other visceral organs like stomach, uterus, testicles, spleen etc were carefully noted.

Microscopic examination:
From each group one specimen of each organ (liver, heart, lung and kidney) from each group was examined histopathologically using H & E staining.

Statistical methods:
All the parameters were analyzed statistically using ANOVA, followed by Tukey's Multiple Comparison Test post hoc test.

RESULTS

Effect of MEHA on clinical signs and behavioral changes:
The animals fed with all the three doses of extracts were healthy. No unusual behavioral changes, disturbance in locomotors activity, any untoward clinical signs, sign of intoxication and mortality were observed. Also, color of stool, urine and eye in all the animals was normal. Signs of tremor, convulsions and reflex abnormalities were absent. No abnormal changes were seen in diarrhoea, haematuria, restlessness, ataxia, uncoordinated muscle movements during the study. The clinical indices were comparable to the control group. No changes were observed in skin, fur, eyes, mucus membrane and autonomic activity (occurrence of salivary and other secretions, lacrimation, and pupil size or unusual respiratory pattern). Gait, posture, response to handling
was also normal throughout the study. Stereotype behaviour like excessive grooming, repetitive circling, bizarre behaviour like walking backwards were not observed, thus CNS function was also normal. Sensory reactivity to stimuli of different type’s auditory, visual proprioceptive stimuli motor activity remained unaltered.

Effect of MEHA on body weight:

No significant changes were observed in the body weights of male and female rats fed with different doses of extracts as compared to control group male and female rats. (Figure 1 and 2).

![Graph](image1)

**Figure 1:** Change in weight of male rats fed with MEHA150, MEHA450 and MEHA1350 for 28 day

![Graph](image2)

**Figure 2:** Change in weight of female rats fed with MEHA150, MEHA450 and MEHA1350 for 28 days
Effect of MEHA on food and water intake:

The food and water consumption of male and female rats of control and treatment groups was similar, indicating that the MEHA extracts did not affect food and water intake. (Figure 3, 4, 5 and 6).

Figure 3: Change in food intake of male rats fed with MEHA150, MEHA450 and MEHA1350 for 28 days

Figure 4: Change in food intake of female rats fed with MEHA150, MEHA450 and MEHA1350 for 28 days
Figure 5: Change in water intake of male rats fed with MEHA150, MEHA450 and MEHA1350 for 28 days

Figure 6: Change in water intake of female rats fed with MEHA150, MEHA450 and MEHA1350 for 28 days

Data is represented as mean ± SEM. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test. P values <0.05 were considered significant.
Effect of MEHA on gross pathology:
Immediately after dissection, the gross pathology of all rats of all the groups was found to be normal with no apparent pathological abnormalities. The external appearance of heart, liver, lungs, and kidneys was similar to control group.

Effect of MEHA on hematological parameters:
After 28 days of oral administration of extracts did not have any significant variation (P<0.05) on hematological parameters when compared to control group (Table 1). Further, all values were found to be within the normal range for rats of all the groups.

Table 1: Haematological parameters of MEHA treated rats in sub acute toxicity

<table>
<thead>
<tr>
<th>Haematological parameters</th>
<th>MALE</th>
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<th>FEMALE</th>
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<tbody>
<tr>
<td></td>
<td>CONTROL</td>
<td>MEHA</td>
<td>CONTROL</td>
<td>MEHA</td>
<td>CONTROL</td>
<td>MEHA</td>
</tr>
<tr>
<td>(Normal Values)</td>
<td></td>
<td></td>
<td></td>
<td>FEMALE</td>
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<tr>
<td>RBC (x10^6/mm³) (7-10)</td>
<td>7.492±0.407</td>
<td>7.866±0.169</td>
<td>7.552±0.178</td>
<td>7.232±0.214</td>
<td>7.712±0.271</td>
<td>7.32±0.287</td>
</tr>
<tr>
<td>Hb (g/dl) (11-18)</td>
<td>12.9±0.693</td>
<td>13.86±0.384</td>
<td>14.56±0.957</td>
<td>14.23±0.274</td>
<td>14.98±2.973</td>
<td>13.02±0.662</td>
</tr>
<tr>
<td>MCV (fl) (36-58)</td>
<td>49.76±1.388</td>
<td>49.46±0.915</td>
<td>50.62±1.62</td>
<td>51.01±0.892</td>
<td>50.84±1.399</td>
<td>48.18±0.821</td>
</tr>
<tr>
<td>MCH (pg) (17.1-20.4)</td>
<td>17.24±0.623</td>
<td>17.6±0.313</td>
<td>17.9±0.524</td>
<td>17.5±0.374</td>
<td>17.7±0.317</td>
<td>17.48±0.269</td>
</tr>
<tr>
<td>MCHC (g/dl) (32.9-37.5)</td>
<td>34.9±0.366</td>
<td>35.54±0.337</td>
<td>35.44±0.204</td>
<td>36.42±0.295</td>
<td>35.9±0.557</td>
<td>34.64±0.663</td>
</tr>
<tr>
<td>WBC (x10^3/mm³) (6-17)</td>
<td>9.84±1.598</td>
<td>11.06±1.899</td>
<td>10.86±2.096</td>
<td>11.94±1.874</td>
<td>11.7±1.003</td>
<td>9.62±0.678</td>
</tr>
<tr>
<td>Lymphocytes (%) (65-85)</td>
<td>65.06±7.608</td>
<td>73.64±2.375</td>
<td>68.96±1.666</td>
<td>68.58±2.016</td>
<td>69.87±3.066</td>
<td>71.78±1.55</td>
</tr>
<tr>
<td>Monocytes (%) (0-5)</td>
<td>5.02±1.873</td>
<td>2.78±1.119</td>
<td>4.56±0.601</td>
<td>3.48±0.512</td>
<td>4.82±0.974</td>
<td>2.98±0.824</td>
</tr>
<tr>
<td>Granulocytes (%) (9-34)</td>
<td>19.42±5.751</td>
<td>13.58±1.315</td>
<td>16.48±1.225</td>
<td>16.75±1.752</td>
<td>14.63±1.205</td>
<td>14.36±0.823</td>
</tr>
<tr>
<td>PLT (x10^9/mm³) (500-1300)</td>
<td>573.6±70.77</td>
<td>634.2±22.24</td>
<td>654.2±25.44</td>
<td>657.1±22.39</td>
<td>581.6±69.85</td>
<td>659.6±21.64</td>
</tr>
</tbody>
</table>

Data is represented as mean ± SEM. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test. P values <0.05 were considered significant.

Effect of MEHA on biochemical parameters:
Results of biochemical parameters observed in the groups treated with MEHA150, MEHA450 and MEHA1350 were comparable to control groups in both sexes as shown in the Table 2 and were found to be lying within the normal range.
Table 2: Biochemical parameters of MEHA treated rats in subacute toxicity

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>MALE (Normal values)</th>
<th>FEMALE (Normal values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>MEHA (mg/kg)</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>450</td>
</tr>
<tr>
<td>RBS (mg %) (50-135)</td>
<td>130.5±18.5</td>
<td>109.6±12.59</td>
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<tr>
<td>BUN (mg %) (15-21)</td>
<td>18.59±0.468</td>
<td>18.76±2.535</td>
</tr>
<tr>
<td>Creatinine (mg%) (0.2-0.8)</td>
<td>0.764±0.041</td>
<td>0.822±0.035</td>
</tr>
<tr>
<td>Cholesterol (mg%) (40-130)</td>
<td>87.09±2.89</td>
<td>89.89±1.37</td>
</tr>
<tr>
<td>Triglycerides (mg%) (20-114)</td>
<td>69.56±2.923</td>
<td>84.5±3.846</td>
</tr>
<tr>
<td>SGPT (IU/L) (18-45)</td>
<td>42.7±3.471</td>
<td>41.16±2.552</td>
</tr>
<tr>
<td>SGOT (IU/L) (74-143)</td>
<td>62.49±9.073</td>
<td>53.35±1.289</td>
</tr>
</tbody>
</table>

Data is represented as mean ± SEM. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test. P values <0.05 were considered significant. Normal values are as per CPCSEA guidelines.

**Effect on renal function tests:**
Treatment with MEHA extracts in different doses for 28 days showed no significant changes (P<0.05) in all renal parameters in both the genders when compared to control rats.

**Effect on hepatic function tests:**
The treatment did not have any adverse effects on hepatic function as evident from the effect on SGOT and SGPT levels and the values were comparable to control group.

**Effect on serum glucose and lipid profile:**
No significant change was observed due to treatment with the drug extracts in serum glucose and lipid profile parameters like cholesterol and triglycerides.

**Effect of MEHA on histopathology of organs:**
Histopathological examinations (Figure 7) did not reveal any lesions or changes like degeneration, fibrosis or necrosis in the heart, liver, lungs and kidneys in male and female rats treated with MEHA and appeared similar to control group rats.
Figure 7: Light micrographs of tissue sections from rats of the different experimental groups (H & E stain, 100X) ((A) Normal group- heart (B) MEHA1350 group- heart (A) Normal group- lung (B) MEHA1350 group- lung (A) Normal group- liver (B) MEHA1350 group- liver (A) Normal group- kidney (B) MEHA1350 group- kidney
Liver sections of all treated groups exhibited normal cellular architecture with distinct hepatocytes, sinusoidal spaces and central vein and were devoid of any fatty infiltration. Microscopic examination of lung sections appeared normal with respect to vessels, alveoli and alveolar spaces and thus the treatments did not adversely alter the structure of lung. Signs of vascular congestion or hemorrhage, alveolar dilatation, infiltration of inflammatory cells or interstitial fibrosis were found to be absent. Slides of kidney revealed normal parenchyma without any hemorrhage or necrosis in all treated groups which was comparable to the normal group. Intact striated muscle fibers of heart were visible in all the groups. Thus, the histopathological findings were similar in both control and treated rats, which indicates that administration of MEHA extracts at all three dose levels for 28 days did not have any adverse toxicological effect on these organs.

**DISCUSSION**

The present study was done to investigate the sub-acute effects of extract of *Holarrhena antidysenterica* on rats. Toxicity studies help to determine the potential for adverse effects from chemicals (both natural and synthetic), and assesses hazard and risk to humans and animals. Globally the natural therapy by use of herbs is considered safe and hence is extensively used. Hence, it becomes necessary to assess scientifically the potential beneficial or adverse effects along with safety aspects of all medicinal plants for which various guidelines have been established. It is only after that we can claim any phytomedicine to be safe for human consumption. In preclinical toxicity studies, acute effects of single dose and sub-acute and chronic effects of repeated dose administration is established.

In this study, three fixed doses were given to the rats with 1350 mg/kg as the highest dose. There was no mortality reported during the treatment period. Generally, the reduction in body weight gain and internal organ weights is a simple and sensitive index of toxicity after exposure to potentially toxic substances. In addition, the treatment if well accepted does not affect adversely on carbohydrate, protein or fat metabolism. In our studies, the treatment with extracts in different doses did not adversely interfere with the nutritional benefits (e.g. weight gain, stability of appetite) expected of animals that are continually supplied with food and water *ad libitum* and thus did not appear to retard growth or affect food consumption and utilization.

The haematopoietic system is very sensitive to toxic compounds and serves as an important index of the physiological and pathological status for both animals and humans influenced by chemicals or any treatment. Hematological parameters reflect the status of anemia, infection, bleeding tendencies or white blood cells related disorders. No relevant changes in the haematological indices were observed due to treatment with extract. Biochemical tests are done to see the effects of phytochemicals and drugs on functions of liver and kidney and can also detect metabolic abnormalities. The SGOT and SGPT values are good indicators of liver functions whereas nephrotoxicity of the drug can be estimated by renal function markers like creatinine and BUN. Liver and kidneys are more prone to suffer the damage caused by high doses of any test compounds since they the majorly involved in metabolism and elimination for many drugs and their metabolites. No significant changes were observed in the levels of serum SGOT, SGPT, BUN and creatinine due to treatment compared to untreated group indicating that the drug preserved the structural and functional abilities of both liver and kidney. The treatment did not show any significant changes in lipid parameters and glucose levels.
The macroscopic appearance and weight of the major organs like liver, heart, kidney and lung and histopathological studies of their sections did not reveal any pathological damage, degenerative structural changes or necrosis induced by consumption of MEHA extracts at any dose levels. Since the study did not report any mortality and any adverse effects, it is suggestive that the doses were well tolerated by both male as well as female rats, and is not toxic. According to these results, the no-observed-adverse-effect level (NOAEL) for MEHA extract is 1350mg/kg body weight/day, administered orally for 28 days in rats under the conditions of this study. This may be a conclusive point in considering the suitability of MEHA for repeated administration for therapeutic use.

CONCLUSION
The subacute toxicity study did not show any treatment related mortality and adverse effects in both male and female rats at 150, 450 and 1350mg/kg p.o. dose administration of hydromethanolic extract of *Holarrhena antidysenterica*.

REFERENCES


