SAFETY EVALUATION OF PETROLEUM ETHER, ACETONE AND HYDROALCOHOLIC EXTRACTS OF HOLOPTELEA INTEGRIFOLIA (ROXB.) PLANCH. BARK BY ORAL ADMINISTRATION IN WISTAR RATS

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ABSTRACT

Objective: There are no reports on safety information of Holoptelea integrifolia (Roxb.) Planch. bark extracts by oral administration for long duration. This study was planned to add information on the safety margin of H. integrifolia.

Methods: Acute oral toxicity study was carried out in the consideration of OECD guideline 425. Animals were observed for mortality, morbidity, behavior and toxic symptoms for 14 consecutive days. Sub-acute toxicity was planned by employing the OECD guideline 407. Dose 50, 100 and 200 mg/kg of the extracts were administered orally, daily for 28 consecutive days. Animals were observed for toxicity sign, morbidity and mortality. Body weight, relative organ weight, biochemical, hematological parameters and histopathology of vital organs like liver and kidney were evaluated.

Results: In the acute oral toxicity study, all the extracts were found to be safe at 1000 mg/kg. However, out of three extracts, petroleum ether extract of the plant showed mild damage to liver and kidney in sub-acute toxicity studies.

Conclusion: Hydroalcoholic extract of H. integrifolia is found to be safe, compared to the acetone and petroleum ether extracts for the long-term use.

Keywords: Holoptelea integrifolia, Bark, Acute toxicity, Chronic toxicity

INTRODUCTION

Most of the population in developing countries still depends on alternative systems of medicine. In India, apart from qualified Ayurvedic practitioners, many unqualified traditional healers dispense a variety of herbal preparations for common ailments. World Health Organization recommends that, the plants used for the treatment of disease need to be scientifically investigated for their toxicity [1]. Toxicity profile of the plant helps to understand the severity of toxicity for that particular herbal drug corresponding to particular dose and duration. Holoptelea integrifolia (Roxb.) Planch. is routinely used by the local traditional practitioners in the rural area of Northern Karnataka of India for the cure of various diseases viz; bronchitis, obesity [2], inflammation, gastritis, dyspepsia, vomiting, piles, wound healing, leprosy, diabetes, diarrhoea, rheumatism and skin disease [3]. H. integrifolia is from the family Ulmaceae. It is a large sized tree, found throughout India, tropical and subtropical parts of Asia and Africa [4]. The aeous leaf extract of H. integrifolia is reported to possess anti-inflammatory activity [5]. Major chemicals reported from H. integrifolia are 1,4-naphthalenedione, Holoptelene A, Holoptelene B, Friedelin, Epifriedelinol, 2 aminenaphthquinone, Hexacosanol, Octacosanol, β-amyrin and β-sitosterol [6]. Traditional Ayurvedic uses of H. integrifolia are disorder of the spleen, fever, jaundice, urinary disorder, laxative [3] and also used as an adjuvant for various therapies. H. integrifolia is scientifically studied for its anti-inflammatory activity [7], anti-diarrhoeal effect in castor oil induced diarrhea [8], adaptogenic effect of ethanolic extracts [9], anti-diabetic effect [10], anti-oxidant, wound healing property [11] and for anti-helminthic activity [12]. The plant has also been screened for anti-bacterial activity against Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa and found effective [13]. H. integrifolia parts are being frequently prescribed by the traditional Ayurvedic practitioners for various ailments as a single plant or in combination with other plant drugs. To the best of our knowledge, there are no reports on oral administration of H. integrifolia extracts for its sub-acute exposure. Therefore, there is a need to develop safety profile of H. integrifolia for its oral administration in sub-acute exposure based on hematological, biochemical and vital organ parameters. This study planned to explore the lethal dose range and effect on sub-acute administration at hematological, biochemical and vital organs to develop safety profile, which will guide for the better understanding of the least toxic dose and duration for administration.

MATERIALS AND METHODS

Experimental animals

The study was conducted in healthy, Wistar rats of male sex weighing between 150 g and 200 g and Swiss mice weighing 20 to 25 g. Animals were purchased from Shree Venkteshwara Traders, Bangalore, India. All animals were acclimatized for 7 days in the laboratory with natural light and dark cycle. Animals were fed with standard rodent pellet, tap water ad libitum. Details of the experimental procedures were reviewed and approved by Institutional Animal Ethics Committee, constituted as per CPCSEA guidelines.

Drugs and chemicals

Specific kits for serum aspartate aminotransferase, alanine transaminase, alkaline phosphatase, creatinine and urea estimation were procured from local supplier, manufactured by Erba Diagnostic Mannheim GmbH Labs, Germany. Hematoxylin and eosin Y were procured from Fisher Scientific, Hampton, NH.

Plant material

H. integrifolia (Roxb.) Planch. (RMRC-525) bark was collected from the forest areas of Gokak (Belgaum). Plant specimens were authenticated by qualified taxonomist and voucher specimen is deposited at the herbaria of Regional Medical Research Centre (ICMR), Belgaum, Karnataka, India.
Extraction and yield

The coarse powder of the shade-dried bark was subjected to successive continuous soxhlet extraction with petroleum ether, acetone and 70% ethanol. Solvents were evaporated using a rotary evaporator to prepare solvent free extracts.

Acute toxicity studies

Acute oral toxicity test of *H. integrifolia* bark extracts were conducted in Swiss mice for the limit test. The main test of up and down procedure was as per acute oral toxicity guideline 425 of Organization for Economic Co-operation and Development (OECD). Petroleum ether, acetone and hydroalcoholic extracts of *H. integrifolia* were dissolved in dimethyl sulfoxide (DMSO) and administered orally in animals. Overnight fasted with water ad libitum animals were orally administered with the dose of 2000 mg/kg and continuously observed up to 6 hrs for morbidity, mortality, other toxic signs and subsequently for 14 days. If animal showed mortality, then switched to main test to find out LD50 range. The dose for the main test was selected from default progression factor on the basis of mortality, morbidity, onset duration and severity of toxic sign. If the animal survives, then another two animals were administered with the same dose sequentially at 48 hrs intervals with the same observation as in the first animal. The high dose at which animal died and low dose at which survived was considered as LD50 range [14].

Sub-acute toxicity studies

Sub-acute toxicity studies were conducted in Wistar rats as per the OECD 407 guideline. Rats were distributed in 10 groups with 6 animals in each group. Group I received DMSO as vehicle and all the extracts were administered in the dose of 50, 100 and 200 mg/kg. Group IV received petroleum ether extract of *H. integrifolia* (HIPE), whereas Group V-VII received acetone extract (HIAC) and Group VIII-X received hydroalcoholic extract (HIHY). All the extracts and vehicle were administered orally every day for 28 consecutive days. Body weights of all animals were measured on day one before starting the study and on every 7 days interval till 28th day. Next day, overnight fasted with water ad libitum animals were anesthetized by halothane to collect the blood from retro-oralplexus in to the tubes, with or without ethylenediaminetetraacetic acid (EDTA) for hematological or biochemical assessment and subsequently animals were sacrificed to collect the organs [15].

Biochemical profile

Clotted blood tubes were subjected for centrifugation at 5000 revolutions per minute for 10 minutes to separate the serum. Sera were used to estimate various enzymes viz. serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP), creatinine, urea and protein as per the manufacturer instructions (Erba Mannheim Germany).

Hematological profile

EDTA mixed blood samples were used for red blood cells count, hemoglobin (Hb), mean corpuscular Hb (MCH), MCH concentration (MCHC), mean corpuscular volume, total white blood cell count, differential white blood cell count (neutrophils, lymphocytes, monocytes, basophils and eosinophils) and platelet count. Hematological parameters were estimated using fully automated hematological analyzer (Accurex, complete blood count 360).

Relative body weight

After blood collection, animals were sacrificed to collect the vital organs like liver, kidney, spleen, heart and lungs. The weights of the organs were measured and relative organ weight was calculated.

Relative organ body weight ratio = Weight of organ

\[ \frac{\text{Weight of organ}}{\text{Weight of animal}} \times 100 \]

Histopathology assessment

Five millimeter thick sections of liver and kidney were prepared using paraffin wax embedded tissue. They were stained with hematoxylin and eosin after processing and mounted in DPX mounting medium.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Dunnett’s post hoc test and P≤0.05 was considered as significant. Results were expressed as mean ± standard deviation.

RESULTS

Plant extract yield

The percentage yield of extracts was 0.89%, 2.26%, 7.41% in petroleum ether, acetone and hydro-alcoholic solvents, respectively.

Acute toxicity

All three extracts found toxic at limit dose 2000 mg/kg and caused mortality. In the main test, hydroalcoholic, acetone and petroleum ether extracts found safe at 1000 mg/kg and toxic at 1750 mg/kg.

Sub-acute toxicity

All extracts treated groups (50, 100 and 200 mg/kg dose at every 24 hrs interval oral administration for a period of 28 days) did not produce any signs of toxicity, mortality and morbidity.

Relative organ weight ratio

Relative organ weight ratio of kidneys, spleen and lungs did not alter significantly in any extract treated groups. However, relative weight ratio of heart were found significantly (P<0.01) altered in *H. integrifolia* acetone and petroleum ether extracts treated group at 200 mg/kg. Whereas, liver weight ratio was found significantly (P<0.01) altered in 200 mg/kg of acetone extract, 100 and 200 mg/kg of petroleum ether extract treated groups (Table 1).

Biochemical results

Biochemical profiles of serum indicated that, the elevation in urea and creatinine like liver, kidney, spleen, heart and lungs. The weights of the organs was not significant in any treated groups. However, creatinine found significantly (P<0.01) elevated in acetone and petroleum ether extracts at 200 mg/kg. SGFT found significantly (P<0.05) elevated only in 200 mg/kg of acetone extract treated group. ALP was found increased significantly (P<0.001 and P<0.01) at 200 mg/kg of acetone and petroleum ether extracts treated groups. SGOT found significantly increased (P<0.05) in acetone and petroleum ether extracts treated groups.

Table 1: Relative body organ weight ratio of *H. integrifolia* extracts treated groups

<table>
<thead>
<tr>
<th>Organ</th>
<th>Treatments</th>
<th>Vehicle</th>
<th>HIAC 50 mg/kg</th>
<th>HIAC 100 mg/kg</th>
<th>HIAC 200 mg/kg</th>
<th>HIHY 50 mg/kg</th>
<th>HIHY 100 mg/kg</th>
<th>HIHY 200 mg/kg</th>
<th>HIPE 50 mg/kg</th>
<th>HIPE 100 mg/kg</th>
<th>HIPE 200 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td>2.36±0.14</td>
<td>2.74±0.03</td>
<td>2.68±0.11</td>
<td>3.45±0.33**</td>
<td>2.78±0.10</td>
<td>2.95±0.29</td>
<td>2.95±0.33</td>
<td>2.96±0.10</td>
<td>3.26±0.12**</td>
<td>3.38±0.09**</td>
</tr>
<tr>
<td>Kidneys</td>
<td></td>
<td>0.62±0.03</td>
<td>0.75±0.03</td>
<td>0.72±0.04</td>
<td>0.75±0.07</td>
<td>0.73±0.04</td>
<td>0.73±0.08</td>
<td>0.68±0.03</td>
<td>0.77±0.04</td>
<td>0.72±0.03</td>
<td>0.73±0.03</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td>0.37±0.01</td>
<td>0.37±0.03</td>
<td>0.37±0.04</td>
<td>0.46±0.02</td>
<td>0.43±0.01</td>
<td>0.39±0.05</td>
<td>0.45±0.01</td>
<td>0.42±0.01</td>
<td>0.43±0.02</td>
<td>0.46±0.05</td>
</tr>
<tr>
<td>Lungs</td>
<td></td>
<td>0.62±0.01</td>
<td>0.71±0.02</td>
<td>0.64±0.01</td>
<td>0.64±0.05</td>
<td>0.71±0.03</td>
<td>0.61±0.02</td>
<td>0.66±0.00</td>
<td>0.66±0.06</td>
<td>0.69±0.12</td>
<td>0.61±0.03</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td>0.29±0.01</td>
<td>0.36±0.01</td>
<td>0.36±0.01</td>
<td>0.40±0.03**</td>
<td>0.35±0.01</td>
<td>0.35±0.02</td>
<td>0.37±0.03</td>
<td>0.34±0.02</td>
<td>0.38±0.02</td>
<td>0.40±0.01**</td>
</tr>
</tbody>
</table>

(N=6) *P≤0.05, **P≤0.01, HIAC: *H. integrifolia* acetone extract, HIHY: *H. integrifolia* hydroalcoholic extract, HIPE: *H. integrifolia* petroleum ether extract, H. integrifolia: Holoptelea integrifolia

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### Table 2: Biochemical parameters of *H. integrifolia* various extracts treated groups

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Vehicle</th>
<th>HIAC 50 mg/kg</th>
<th>HIAC 100 mg/kg</th>
<th>HIAC 200 mg/kg</th>
<th>HIHY 50 mg/kg</th>
<th>HIHY 100 mg/kg</th>
<th>HIHY 200 mg/kg</th>
<th>HIPE 50 mg/kg</th>
<th>HIPE 100 mg/kg</th>
<th>HIPE 200 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>49.23±12.40</td>
<td>50.02±17.08</td>
<td>54.13±13.18</td>
<td>68.35±7.97**</td>
<td>49.82±1.84</td>
<td>50.53±3.37</td>
<td>51.54±0.95</td>
<td>51.21±3.96</td>
<td>55.78±4.31</td>
<td>68.50±6.15**</td>
</tr>
<tr>
<td>SGPT</td>
<td>69.11±12.09</td>
<td>73.07±5.08</td>
<td>76.19±3.39</td>
<td>92.75±3.58*</td>
<td>70.32±2.80</td>
<td>73.07±16.22</td>
<td>80.75±12.07</td>
<td>74.46±3.47</td>
<td>89.57±7.95</td>
<td>110.20±13.63**</td>
</tr>
<tr>
<td>SGOT</td>
<td>99.94±17.79</td>
<td>102.04±1.03</td>
<td>117.67±5.45</td>
<td>123.76±6.89*</td>
<td>99.17±7.45</td>
<td>111.98±7.76</td>
<td>126.11±12.78**</td>
<td>115.16±8.62</td>
<td>126.11±10.16**</td>
<td>156.76±35.93**</td>
</tr>
<tr>
<td>Urea</td>
<td>128.35±0.32</td>
<td>127.25±1.53</td>
<td>133.64±1.39</td>
<td>139.68±3.44</td>
<td>133.07±4.91</td>
<td>137.03±9.22</td>
<td>129.96±2.12</td>
<td>133.71±5.83</td>
<td>146.21±3.42</td>
<td>146.21±3.42</td>
</tr>
<tr>
<td>Creatinine</td>
<td>1.17±0.18</td>
<td>1.22±0.06</td>
<td>1.34±0.03</td>
<td>1.54±0.22**</td>
<td>1.14±0.09</td>
<td>1.21±0.03</td>
<td>1.27±0.08</td>
<td>1.22±0.06</td>
<td>1.39±0.02</td>
<td>1.62±0.14**</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>29.88±0.71</td>
<td>32.62±0.22</td>
<td>33.35±3.94</td>
<td>33.96±2.55*</td>
<td>31.74±2.37</td>
<td>32.57±0.19</td>
<td>33.01±1.63*</td>
<td>31.74±4.45</td>
<td>32.63±0.12</td>
<td>34.34±1.99**</td>
</tr>
</tbody>
</table>

(N=6) *P≤0.05, **P≤0.01, HIAC: *H. integrifolia* acetone extract, HIHY: *H. integrifolia* hydroalcoholic extract, HIPE: *H. integrifolia* petroleum ether extract, *H. integrifolia*: Holoptelea integrifolia, SGOT: Serum glutamic oxaloacetic transaminase, SGPT: Serum glutamic-pyruvic transaminase, ALP: Alkaline phosphatase

### Table 3: Hematological parameters of *H. integrifolia* various extracts treated groups

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Vehicle</th>
<th>HIAC 50 mg/kg</th>
<th>HIAC 100 mg/kg</th>
<th>HIAC 200 mg/kg</th>
<th>HIHY 50 mg/kg</th>
<th>HIHY 100 mg/kg</th>
<th>HIHY 200 mg/kg</th>
<th>HIPE 50 mg/kg</th>
<th>HIPE 100 mg/kg</th>
<th>HIPE 200 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (10^12/l)</td>
<td>8.09±0.40</td>
<td>8.08±0.13</td>
<td>8.00±0.35</td>
<td>6.71±0.68**</td>
<td>8.01±0.09</td>
<td>7.80±0.59</td>
<td>6.79±0.82**</td>
<td>8.02±0.05</td>
<td>8.07±0.24</td>
<td>7.72±0.37</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>15.51±0.86</td>
<td>15.18±0.20</td>
<td>15.43±0.16</td>
<td>14.06±0.60***</td>
<td>15.26±0.27</td>
<td>15.2±0.35</td>
<td>15.15±0.10</td>
<td>15.3±0.10</td>
<td>14.7±0.40</td>
<td>14.61±0.33**</td>
</tr>
<tr>
<td>Platelet (10^12/l)</td>
<td>693.60±72.22</td>
<td>809.83±41.34</td>
<td>828.03±88.29**</td>
<td>845.16±87.43**</td>
<td>724.83±499.66</td>
<td>740.50±201.07</td>
<td>845.83±178.26**</td>
<td>755.66±399.03</td>
<td>824.83±134.22**</td>
<td>966.66±54.00**</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>8.46±1.42</td>
<td>10.19±0.61</td>
<td>19.00±0.53</td>
<td>19.36±1.59</td>
<td>19.1±0.39</td>
<td>19.4±2.33</td>
<td>19.9±0.31</td>
<td>18.2±0.53</td>
<td>18.8±0.24</td>
<td>19.2±0.96</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>31.78±1.02</td>
<td>32.5±0.34</td>
<td>33.11±0.90*</td>
<td>33.3±0.86*</td>
<td>32.6±0.13</td>
<td>32.5±0.31*</td>
<td>33.7±0.21**</td>
<td>32.6±0.19</td>
<td>32.67±0.61</td>
<td>33.08±0.63</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>55.41±3.49</td>
<td>57±2.53</td>
<td>58.13±2.07</td>
<td>58.9±1.93</td>
<td>57.3±2.85</td>
<td>59.3±2.29</td>
<td>65.66±4.55**</td>
<td>57.2±2.42</td>
<td>58.5±1.45</td>
<td>61.96±5.36**</td>
</tr>
</tbody>
</table>

(N=6) *P≤0.05, **P≤0.01, ***P≤0.001, HIAC: Holoptelea integrifolia acetone extract, HIHY: Holoptelea integrifolia hydroalcoholic extract, HIPE: Holoptelea integrifolia petroleum ether extract, *H. integrifolia*: Holoptelea integrifolia, RBC: Red blood count, WBC: White blood count, Hb: Hemoglobin, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, *H. integrifolia*: Holoptelea integrifolia
(P<0.05 and P<0.01) elevated in acetone and hydroalcoholic extracts at 200 mg/kg; petroleum ether extract (P<0.01) at 100 and 200 mg/kg treated groups (Table 2).

**Hematological results**

All three extracts of *H. integrifolia* did not produce any alteration in neutrophils, lymphocytes, eosinophils, basophils, monocytes and MCH, and hence not incorporated in the results table. Significant (P<0.001) decrease in hemoglobin was observed in 200 mg/kg acetone extract treated group and mean corpuscular volume was found significantly (P<0.05) elevated in 200 mg/kg of hydroalcoholic and petroleum ether extracts treated groups. However, red blood cells found decreased significantly (P<0.01) in acetone and hydroalcoholic extracts at 200 mg/kg treated groups. White blood cells found significantly (P<0.05) decreased in acetone extract 200 mg/kg and hydroalcoholic extract 50 mg/kg treated groups. Hydroalcoholic extract 100 and 200 mg/kg showed significant (P<0.001 and P<0.01) changes in white blood cell count respectively. MCHC significantly (P<0.05) elevated in 100 mg/kg of hydroalcoholic and acetone extracts and also in 200 mg/kg of acetone extract treated groups (P<0.01). Platelet count was observed significantly (P<0.01) increased in all doses of acetone extract, 200 mg/kg of...
hydroalcoholic extract and 100, 200 mg/kg of petroleum ether extract treated groups (Table 3).

**Histopathology**

Histopathology image of the liver confirmed the mild damage in petroleum ether and acetone extract treated groups. Mild tubular congestion and glomerular congestion were observed in kidneys of acetone extract treated group (Fig. 1). Inflammation, central vein congestion, portal triaditis and spotty necrosis were observed in liver of acetone and petroleum ether extract treated groups (Fig. 2).

**DISCUSSION**

Toxicity evaluation is also the major concern like efficacy of any plant based drug, because all plants on the earth are not safe for consumption. Most of the preparations of Ayurveda, Siddha and traditional practitioners are based on herbs, which they use to cure various diseases. *H. integrifolia* is one such plant, which is routinely used for various ailments like bronchitis, obesity, inflammation, gastritis, dyspepsia, vomiting, piles, wound healing, leprosy, diabetes, diarrhea, rheumatism and skin disease. World Health Organization suggested that, the plants used for the treatments of disease are needed to be scientifically investigated for their safety [1]. Therefore, this study was planned to probe into the safety profile of *H. integrifolia* extracts on acute and sub-chronic administration. The results of the acute toxicity study indicated that *H. integrifolia* showed mortality at 2000 mg/kg in all extracts treated groups and found safe at 1000 mg/kg. All three extracts of *H. integrifolia* neither show significant change in body weight nor any signs of toxicity at 50, 100 and 200 mg/kg over a period of twenty eight days of treatment. Results of the biochemical studies reflect mild liver and kidney toxicity, whereas hematology profile indicated the platelet increase which might lead to coagulopathy. Based on the resulted data, it can be concluded that hydroalcoholic extract of *H. integrifolia* is safer, compared to acetone and petroleum ether extracts on daily dose for 28 consecutive days.

**ACKNOWLEDGEMENTS**

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