EVALUATION OF ACUTE TOXICITY OF NEEM ACTIVE CONSTITUENT, NIMBOLIDE AND ITS HEPATOPROTECTIVE ACTIVITY AGAINST ACUTE DOSE OF CARBON TETRACHLORIDE TREATED ALBINO RATS

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ABSTRACT:
Objective: To investigate the neem active constituent nimbolide for the evidence of acute toxicity and its protective effects against carbon tetrachloride (CCl₄) induced liver toxicity in rats.

Materials and Methods: Group allotment in hepatoprotective activity study included vehicle, CCl₄ (1ml/kg), Silymarin (100 µg/kg/day) + CCl₄ and graded doses of nimbolide (100 and 200µg/ kg/ day) + CCl₄. On 9th day, blood was obtained for determination of biochemical parameters and liver tissue for pathological examination.

Results: There were no toxicological effects as evidenced by signs of mortality, behavior, diet consumption and tissue weights, however, some hematological parameters showed alterations in their value at higher dose level. The degree of protection was measured by using various biochemical parameters like total protein, albumin, BUN, AST, ALT and ALP levels. Nimbolide showed dose dependent hepatoprotective in nature which was further substantiated by marked decrease in incidence of hepatocellular necrosis on histopathological and transmission electron microscopic analysis.

Conclusion: This study suggests nimbolide possess hepatoprotective effect against CCl₄ induced liver damage in rats with efficiency similar to that of Silymarin standard.

INTRODUCTION: Over the years plants have emerged as veritable sources of drugs and these drugs are known to play a vital role in the management of liver diseases and there are numerous plants and polyherbal formulations claimed to have hepatoprotective activities 1,2.

Keywords: Nimbolide, Acute Toxicity, CCl₄, Hepatoprotection, Silymarin

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FIGURE 1: CHEMICAL STRUCTURE OF NIMBOLIDE (5, 7, 4’-TRIHYDROXY-3’, 5’-DIPRENYL FLAVANONE). A LIMONOID PRESENT IN THE LEAVES, SEED AND FLOWER OF THE NEEM TREE (AZADIRACHTA INDICA)

However, our previous in-vivo-in-vitro studies on nimbolide revealed changes in biochemical parameters of male reproductive functions at higher dose level; act as spermicidal and elicit depletion of antioxidant defense system in rat epididymal spermatozoa.

From the literature, the herbal hepatoprotective have less side effect or interaction as compare to synthetic medicine but in other hand scientific evidence from tests done to evaluate the safety and effectiveness of traditional these medicine products and practices is limited. Consequently, it was found worthwhile to examine the acute toxicity and hepatoprotective nature of neem bioactive phytochemical, nimbolide, in order to establish its potential use. This study deals with two different aspects.

The first part, acute toxicity including feed and water consumption, individual body weight, cage side observations, Relative organ weight (ROW) analysis, hematological analysis and the second part, is to evaluate hepatoprotective activity of nimbolide against acute dose of carbon tetrachloride (CCL4) treated albino rats; which includes serum biochemical analysis, histopathological and Electron Microscopic (EM) studies. The outcome of this study could possibly bring an additional information of nimbolide on hepatoprotective role in vivo condition that would be used in physio-pharmacological studies have therefore been designed to study the effects of nimbolide in vivo condition, by using graded concentration in treated rats.

MATERIALS AND METHODS:

Animals: Wistar albino rats weighing 206-211 g of either sex were obtained from the rat colony maintained in the department and were acclimatized for 10 days under standard housing conditions (26±2°C; 45-55% RH with 12:12 h light/dark cycle). The animals were maintained on a standard diet and water was given ad libitum and habituated to laboratory conditions for 48 h prior to the experimental protocol to minimize any non-specific stress. The animals were maintained under standard conditions in the animal house approved by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) and necessary approval from the Institutional Animal Ethics Committee (IAEC) was obtained before undertaking animal experimentation.

Chemicals and Reagents: Technical Nimbolide of purity ≥97% was obtained from SPIC Ltd., Chennai, India. Carbon tetrachloride used was of analytical grade and procured from E. Merck (India) Ltd. Mumbai; Silymarin was procured from M/s Micro Labs, Bengaluru. All other solvents and chemicals were of analytical grade and purchased from local commercial sources.

Acute Toxicity Studies: Acute oral toxicity study was performed as per OECD-404 guidelines. 10 rats/group (5 males and 5 females) were used for the study. Group 1 was control group and other groups were that of nimbolide at different doses (500, 1000, and 2000 µg /kg BW, respectively). Single dose of the nimbolide was separately administrated orally to each animal. Signs of toxicity, feed and water consumption of each animal was observed for 14 days. Individual animal body weight was recorded on day one and at the end of experiment.

Cage side observations: In acute toxicity study, the animals were observed prior to dosing. Thereafter, observations were made at every hour, for five hours and then at 24 h and then every day for 14 days. All observations were systematically recorded, with individual records being maintained for each animal. Cage side observations included the evaluation of skin and fur; eyes; respiratory effect; autonomic effects, such as salivation,
diarrhea, urination; and central nervous system effects, including tremors and convulsions, straub tail, relaxation, changes in the level of activity, gait and posture, reactivity to handling, altered strength and stereotypy.  

**Relative organ weight (ROW) analysis:** Heart, liver, brain, kidneys, lungs, thymus glands, spleen, adrenal glands, testes and uterus were mopped with filter paper, weighed and the relative weights were calculated and expressed as g/100 g b.w.

\[
\text{ROW} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rats on sacrifice day (g)}} \times 100
\]

**Hematological analysis:** At the end of study, all animals were fasted for 12 h and then under mild ether anesthesia, animals were sacrificed and blood samples were collected. Blood was collected immediately into tubes containing EDTA for analysis of hematological parameters viz. hemoglobin, total red blood cells (RBC), packed cell volume, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total white blood cells (WBC), neutrophils, lymphocytes, eosinophils, monocytes, basophiles, total platelet count using automated hematology analyzer (Sysmex KX-21, Japan).

**Hepatoprotective studies:**

**Selection of the doses for animal Studies:** Nimboide did not produce any mortality up to a dose of (2000 µg/kg, po). Hence, 1/20th (100 µg/kg, po) and 1/10th (200 µg/kg, po) of these doses were employed for further experimental pharmacological investigations.

**Hepatoprotective role of nimboide in carbon tetrachloride (CCl4) induced hepatotoxicity:** To study the CCl4 induced hepatic injury in rats, CCl4 was diluted with liquid paraffin (1:1) before intraperitoneal administration. The animals were divided into following 5 groups.

- **Group 1:** Vehicle (50% aqueous sucrose solution) for 9 days.
- **Group 2:** Vehicle + CCl4 (1 ml/kg, po) on ninth day.
- **Group 3:** Silymarin (100 µg/kg/day, po) + CCl4 (1 ml/kg, po) on ninth day.
- **Groups 4 and 5:** Nimboide (100 and 200 µg/kg/day, po) + CCl4 (1 ml/kg, po) respectively on ninth day.

To enhance the acute liver damage in animals of groups 2, 3, 4 and 5, food was withdrawn 12 h before CCl4 administration. Animals were sacrificed 24 h after administration of CCl4. Blood samples were collected by puncturing the retro-orbital plexus under light ether anesthesia and allowed to coagulate for 30 min at 37°C. Serum was separated by centrifugation at 2500 rpm at 37°C for 15 min and analyzed for various biochemical parameters. The liver was removed after sacrifice and observed for weight, volume and appearance, washed with normal and then fixed in 10% formalin for histopathological studies.

**Serum Biochemical analysis:** The hepatoprotective effect of nimboide was evaluated by the assay of liver function serum biochemical parameters according to standard methods by using test kit (Span Diagnostics Ltd.) and were analyzed at the end of the study using auto-analyzer (Erba Chem-7, Germany). Estimation of serum total protein content by modified Biuret method; serum albumin by the method given by Corcoran and Durnan; serum blood urea nitrogen (BUN) by Enzymatic Urease (Berthelot) method; alkaline phosphatase (ALP) activity by the method of Kind and King; aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities by the method of Reitman and Frankel.

**Light microscopy analysis:** Animals were sacrificed and the abdomen was cut open to remove the liver. The liver was observed for weight (LW), volume (LV) and appearance. The liver was washed with normal saline and fixed in Bouin's solution (mixture of 75 ml of saturated picric acid, 25 ml of 40% formaldehyde and 5 ml of glacial acetic acid) for 12 h, then embedded in paraffin using conventional methods and cut into 5 µm thick sections and stained using haematoxylin-eosin dye and finally mounted in di-phenyl xylene. The sections were observed under light microscope for histopathological changes.
Ultrastructural analysis: One mm³ of liver tissues were obtained from all dissected animals. The samples were fixed in a mixture of 25% glutaraldehyde / 40% formaldehyde (4:1) at pH 7.4 in room temperature for 4 h then rinsed in 0.1 M phosphate buffer and post fixed in 2.0% buffered osmic acid for 1/2 hour at 4°C.

The materials were processed to plastic blocks, ultrathin sections of 70 nm thickness were cut and picked up on copper grids. Sections were double stained by using uranyl acetate and lead citrate. The grids were examined and photographed using electron microscope (Jeol-TEM 100 C X II) at 80kV.

Statistical analysis: Data were analyzed using one way analysis of variance (ANOVA) using the Graph Pad Prism software method, followed by either Dunnet test or Turkey’s multiple comparison tests by comparing all treated groups against controls. Values represented are mean ± SEM (n=5). P ≤ 0.05 is considered to indicate a significant difference between experimental and controls.

RESULTS:

Acute toxicity study: In acute toxicity study, as shown in Tables 1 and 2, no adverse reactions or behavioral changes were observed after each administration of nimbolide (500, 1000 and 2000 µg/kgBW, respectively) during the entire period of experimentation. No significant changes in general feed and water consumption rates suggesting that this active constituent had no effect on normal growth of rats. However, there was little alteration, but not significant, in the body weight and relative weight of few organs of both either sexes (P ≤ 0.05) at higher dose level groups during experimental period.

Hematological parameters: The hematological parameters of male and female rats were shown in Tables 3 and 4 respectively. Significant difference (P ≤ 0.05) in some hematological parameters of both sexes, however, at the lower dose levels, both of either sex exhibited the variation in the some of the parameters, but the difference was insignificant against the control.

Biochemical parameters: Pretreatment with nimbolide at dose levels of 100 and 200µg/kg BW significantly controlled the change in the biochemical parameters compared to control and the effect was similar to that of standard drug as shown in Table 5.

<table>
<thead>
<tr>
<th>Group &amp; Treatment</th>
<th>Body weight (Initial)</th>
<th>Body weight (After)</th>
<th>Heart</th>
<th>Liver</th>
<th>Brain</th>
<th>Kidney</th>
<th>Lung</th>
<th>Thymus</th>
<th>Spleen</th>
<th>Adrenal</th>
<th>Testis</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (1 mL of 50% aqueous sucrose solution/rat)</td>
<td>208.3±3.76</td>
<td>212.5±2.12</td>
<td>0.342±0.26</td>
<td>2.575±1.24</td>
<td>0.670±0.50</td>
<td>0.388±0.12</td>
<td>0.785±0.28</td>
<td>0.245±0.19</td>
<td>0.278±0.10</td>
<td>0.010±0.05</td>
<td>0.576±0.12</td>
</tr>
<tr>
<td>II 500µg in 1 mL gum tragacanth / kg body weight</td>
<td>210.7±4.33</td>
<td>213.2±3.32</td>
<td>0.330±0.30</td>
<td>2.558±1.45</td>
<td>0.648±0.34</td>
<td>0.370±0.14</td>
<td>0.750±0.24</td>
<td>0.205±0.10</td>
<td>0.24±0.09</td>
<td>0.009±0.07</td>
<td>0.555±0.14</td>
</tr>
<tr>
<td>III 1000 in 1 mL gum tragacanth / kg body weight</td>
<td>207.3±4.45</td>
<td>211.1±3.16</td>
<td>0.324±0.25</td>
<td>2.534±1.57</td>
<td>0.631±0.47</td>
<td>0.356±0.17</td>
<td>0.615±0.51</td>
<td>0.199±0.22</td>
<td>0.226±0.08</td>
<td>0.073±0.09</td>
<td>0.482±0.17*</td>
</tr>
<tr>
<td>IV 2000 µg in 1 mL gum tragacanth / kg body weight</td>
<td>210.2±3.50</td>
<td>213.2±2.80</td>
<td>0.320±0.32</td>
<td>2.504±1.42</td>
<td>0.602±0.32</td>
<td>0.334±0.12</td>
<td>0.579±0.62*</td>
<td>0.193±0.15</td>
<td>0.208±0.18*</td>
<td>0.006±0.10</td>
<td>0.407±0.12*</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM (n=5) and (*) indicates significant (P ≤ 0.05) compared to control.
### TABLE 2: EFFECT OF NIMBOLIDE TREATMENT ON THE BODY WEIGHT AND OTHER ORGAN WEIGHTS (g/100g BODY WEIGHT) OF FEMALE ALBINO RATS

<table>
<thead>
<tr>
<th>Group &amp; Treatment</th>
<th>Body weight (initial)</th>
<th>Body weight (After)</th>
<th>Heart</th>
<th>Liver</th>
<th>Brain</th>
<th>Kidney</th>
<th>Lung</th>
<th>Thymus</th>
<th>Spleen</th>
<th>Adrenal</th>
<th>Uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (1 mL of 50% aqueous sucrose solution/rat)</td>
<td>210.5±1.75</td>
<td>213.1±2.36</td>
<td>0.332±0.13</td>
<td>2.728±1.09</td>
<td>0.916±0.21</td>
<td>0.376±0.18</td>
<td>0.902±0.56</td>
<td>0.228±0.35</td>
<td>0.296±0.16</td>
<td>0.0132±0.31</td>
<td>0.390±0.42</td>
</tr>
<tr>
<td>II 500µg in 1 mL gum tragacanth / kg body weight</td>
<td>209.7±1.13</td>
<td>212.2±2.06</td>
<td>0.325±0.15</td>
<td>2.636±1.32</td>
<td>0.857±0.34</td>
<td>0.354±0.12</td>
<td>0.846±0.34</td>
<td>0.216±0.40</td>
<td>0.270±0.14</td>
<td>0.0120±0.30</td>
<td>0.354±0.34</td>
</tr>
<tr>
<td>III 1000 in 1 mL gum tragacanth / kg body weight</td>
<td>210.3±2.20</td>
<td>213.7±1.05</td>
<td>0.316±0.19</td>
<td>2.589±1.43</td>
<td>0.851±0.30</td>
<td>0.338±0.27</td>
<td>0.779±0.23</td>
<td>0.198±0.52</td>
<td>0.248±0.21</td>
<td>0.0101±0.28</td>
<td>0.327±0.45*</td>
</tr>
<tr>
<td>IV 2000 µg in 1 mL gum tragacanth / kg body weight</td>
<td>208.2±2.50</td>
<td>212.4±1.30</td>
<td>0.308±0.20</td>
<td>2.475±2.07*</td>
<td>0.787±0.50*</td>
<td>0.317±0.40*</td>
<td>0.720±0.60*</td>
<td>0.172±0.55</td>
<td>0.215±0.28*</td>
<td>0.0093±0.30</td>
<td>0.304±0.54*</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM (n=5) and (+) indicates significant (P ≤ 0.05) compared to control.

### TABLE 3: EFFECT OF NIMBOLIDE TREATMENT ON HEMATOLOGICAL PARAMETERS OF MALE ALBINO RATS

<table>
<thead>
<tr>
<th>Group &amp; Treatment</th>
<th>RBC (10⁶/µl)</th>
<th>Hb (g/dl)</th>
<th>PCV (%)</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dl)</th>
<th>Platelet Count (10⁶/µl)</th>
<th>WBC (10³/µl)</th>
<th>Neutrophils (%)</th>
<th>Lymphocytes (%)</th>
<th>Eosinophils (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (1 mL of 50% aqueous sucrose solution/rat)</td>
<td>9.12±0.24</td>
<td>16.59±0.24</td>
<td>45.07±0.54</td>
<td>49.80±0.18</td>
<td>18.71±0.50</td>
<td>35.74±0.33</td>
<td>1250.0±2.30</td>
<td>12.8±1.00</td>
<td>24.70±1.12</td>
<td>82.6±0.64</td>
<td>0.40±0.58</td>
</tr>
<tr>
<td>II 500µg in 1 mL gum tragacanth / kg body weight</td>
<td>8.70±0.26</td>
<td>15.72±0.28</td>
<td>44.32±0.77</td>
<td>49.00±0.26</td>
<td>18.09±0.52</td>
<td>35.28±0.41</td>
<td>1268.1±2.84</td>
<td>11.65±1.25</td>
<td>23.32±0.80</td>
<td>80.40±0.75</td>
<td>0.30±0.50*</td>
</tr>
<tr>
<td>III 1000 in 1 mL gum tragacanth / kg body weight</td>
<td>8.35±0.17</td>
<td>15.48±0.31</td>
<td>43.83±0.82</td>
<td>50.16±0.20</td>
<td>17.80±0.48</td>
<td>34.63±0.37</td>
<td>1163.7±2.77*</td>
<td>9.97±1.17*</td>
<td>22.60±1.07</td>
<td>79.61±0.57</td>
<td>0.30±0.60*</td>
</tr>
<tr>
<td>IV 2000 µg in 1 mL gum tragacanth / kg body weight</td>
<td>8.48±0.32</td>
<td>15.25±0.34*</td>
<td>43.67±0.63</td>
<td>49.39±0.46</td>
<td>17.95±0.46</td>
<td>34.10±0.32*</td>
<td>1095.3±1.49*</td>
<td>9.89±1.19*</td>
<td>22.48±1.13*</td>
<td>77.42±0.61*</td>
<td>0.26±0.50*</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM (n=5) and (+) indicates significant (P ≤ 0.05) compared to control.

### TABLE 4: EFFECT OF NIMBOLIDE TREATMENT ON HEMATOLOGICAL PARAMETERS OF FEMALE ALBINO RATS

<table>
<thead>
<tr>
<th>Group &amp; Treatment</th>
<th>RBC (10⁶/µl)</th>
<th>Hb (g/dl)</th>
<th>PCV (%)</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dl)</th>
<th>Platelet Count (10⁶/µl)</th>
<th>WBC (10³/µl)</th>
<th>Neutrophils (%)</th>
<th>Lymphocytes (%)</th>
<th>Eosinophils (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (1 mL of 50% aqueous sucrose solution/rat)</td>
<td>8.36±0.50</td>
<td>16.30±0.34</td>
<td>42.64±0.24</td>
<td>51.76±0.33</td>
<td>19.78±0.42</td>
<td>38.00±0.24</td>
<td>1132.0±2.12</td>
<td>9.75±1.92</td>
<td>22.42±1.36</td>
<td>85.70±0.98</td>
<td>0.30±0.60</td>
</tr>
<tr>
<td>II 500µg in 1 mL gum tragacanth / kg body weight</td>
<td>8.30±0.35</td>
<td>15.92±0.30</td>
<td>42.92±0.28</td>
<td>51.60±0.41</td>
<td>19.40±0.40</td>
<td>37.86±0.31</td>
<td>1067.2±3.14</td>
<td>9.39±1.70</td>
<td>22.01±2.05</td>
<td>83.80±0.66</td>
<td>0.30±0.54</td>
</tr>
<tr>
<td>III 1000 in 1 mL gum tragacanth / kg body weight</td>
<td>8.23±0.48</td>
<td>15.23±0.48</td>
<td>41.53±0.31</td>
<td>50.84±0.32</td>
<td>19.01±0.51</td>
<td>36.88±0.43</td>
<td>1018.3±2.17</td>
<td>9.08±1.98</td>
<td>20.70±1.12</td>
<td>81.23±0.83</td>
<td>0.33±0.88*</td>
</tr>
<tr>
<td>IV 2000 µg in 1 mL gum tragacanth / kg body weight</td>
<td>7.96±0.46*</td>
<td>14.85±0.52*</td>
<td>40.30±0.35*</td>
<td>50.25±0.35</td>
<td>18.74±0.47</td>
<td>36.51±0.30</td>
<td>997.4±1.40*</td>
<td>8.60±1.45*</td>
<td>19.64±1.80*</td>
<td>79.45±0.17*</td>
<td>0.30±0.58</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM (n=5) and (+) indicates significant (P ≤ 0.05) compared to control.
TABLE 5: EFFECTS OF SILYMARIN AND NIMBOLIDE TREATMENT ON DIFFERENT SERUM BIOCHEMICAL PARAMETERS IN CCl4 INDUCED LIVER TOXICITY IN RATS (VALUES ARE EXPRESSED IN SEM OF 5 ANIMALS)

<table>
<thead>
<tr>
<th>Group &amp; Treatment</th>
<th>Total Protein</th>
<th>Albumin</th>
<th>BUN</th>
<th>ALP</th>
<th>AST</th>
<th>ALT</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>7.30 ± 0.30</td>
<td>4.45 ± 0.24</td>
<td>25.73 ± 0.63</td>
<td>393.5 ± 0.94</td>
<td>179.0 ± 1.51</td>
<td>74.4 ± 0.40</td>
</tr>
<tr>
<td>II</td>
<td>4.77 ± 0.79*</td>
<td>3.21 ± 0.49*</td>
<td>30.19 ± 1.03*</td>
<td>745.1 ± 0.57*</td>
<td>650.1 ± 1.03*</td>
<td>381.1 ± 0.44*</td>
</tr>
<tr>
<td>III</td>
<td>6.83 ± 0.41</td>
<td>4.21 ± 0.33</td>
<td>24.13 ± 0.46</td>
<td>408.2 ± 1.05</td>
<td>211.1 ± 0.76</td>
<td>56.15 ± 0.31*</td>
</tr>
<tr>
<td>IV</td>
<td>6.08 ± 0.40</td>
<td>3.68 ± 0.25</td>
<td>27.66 ± 0.49</td>
<td>522.1 ± 4.97*</td>
<td>348.5 ± 3.89*</td>
<td>83.45 ± 0.62*</td>
</tr>
<tr>
<td>V</td>
<td>6.92 ± 0.52</td>
<td>4.11 ± 0.31</td>
<td>24.75 ± 0.54</td>
<td>388.6 ± 1.84</td>
<td>191.2 ± 0.42</td>
<td>68.20 ± 0.57</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM (n=5) and (*) indicates significant (P ≤ 0.05) compared to control or CCl4.

A marked reduction (P ≤ 0.05) in the levels of total protein and albumin, sign of elevated (P ≤ 0.05) in the levels of BUN, ALP and hepato specific enzymes like AST and ALT in CCl4 induced group when compared to normal controls. Further, at higher dose level, the recovery towards normalization in these levels was similar to that of standard, however, at lower dose level, this active constituent did not have protective effects.

Histopathology: The histological changes associated with the hepatoprotective activity in nimbolide pretreated rats basically supported the estimation of the serum enzyme activities as depicted in figure 2A-E. Figure 2A of liver sections from control rats showed normal lobular architecture and normal hepatic cells with a well preserved cytoplasm and well-defined nucleus and nucleoli. In figure 2B of CCl4 treated animals, the liver pathological changes were characterized by severe hepatocellular degeneration, necrosis (arrow head) and congestion of sinusoids (arrows) along with periportal mononuclear cell infiltration due to CCl4 toxicity.

These histopathological changes were remarkably reversed in graded doses of nimbolide pretreated rats with lesser vacuolar degeneration and hepatic necrosis as shown in figure 2D and E. Nimbolide protected the liver tissue against CCl4 toxicity with mild hepatocellular degeneration, less inflammatory cell infiltration and well preserved hepatocytes were observed in most areas and the recovery from degeneration of hepatic cells of nimbolide pretreated was comparable to that of standard Silymarin as shown in figure 2C.

Ultra-structural Pathology: The results of the present ultrastructural pathology were depicted in figure 3A-J. Figure 3A and B of control liver showed hepatocytes separated by canaliculi and sinusoids. The hepatocytes exhibits normal features of nearly rounded nuclei with regular structural organization in the cytoplasm.

The condensed vacuolated cytoplasm contained degenerative changes such as atrophied mitochondria, disrupted RER and numerous lysosomes with accumulation of lipid droplets. Cell membranes were ill defined with widened intercellular spaces besides complete loss of cell-to-cell contact disturbing the morphological structure were shown in figure 3C and D.

Figure 3E and F of Silymarin treatment prevented the CCl4-induced degenerative changes to almost normal. Most of hepatocytes preserved normal appearance with prominent nucleus along with intact nuclear membrane and regular structural organization in the cytoplasm. Pretreatment with nimbolide (100 and 200 µg/kg) protected the liver tissue after CCl4 intoxication in a dose dependent
and the recovery from degeneration of hepatic cells at the ultra-structural level and was similar to that of standard drug treated animals.

In liver of 100µg/kg nimbolide pretreated revealed reduction in hepatic lesions and some hepatocytes exhibited the condensed euchromatic nuclei with intact nuclei envelope and their cytoplasmic structural organization were more or less similar to control group. Although some of the hepatic cytoplasm retained slightly dilated RER, irregular mitochondria and accumulation of lipid droplets with more lysosomes were shown in figure 3G and H. Whereas, figure 3I and J of hepatocytes structure in pretreated with 200µg/kg nimbolide restored the ultra-structure of hepatocytes almost returned to normal which correlated with the improvement of liver function panel.

FIGURE 2A-E: HISTOPATHOLOGICAL CHANGES OCCURRED IN THE LIVER AFTER CCL₄ INTOXICATION AND PREVENTION BY PRETREATMENT WITH GRADED DOSES OF NIMBOLIDE AND STANDARD Silymarin. H and E ×100. (A) CONTROL RATS; (B): CCL₄ TREATED GROUP ALONE (1ml/kg bw); (C): Silymarin Pretreated GROUP (100 µg/kg.bw) +CCL₄; (D): NimboLIDE PRETREATED GROUP (µg/kg.bw) + CCL₄ (E): NimboLIDE PRETREATED GROUP (µg/kg.bw) +CCL₄ NOTE: (C-E) SHOW DIFFERENT DEGREES OF IMPROVEMENT OF ACUTE LIVER INJURY.
FIGURE 3A-F: ULTRASTRUCTURAL CHANGES OCCURRED IN THE LIVER AFTER CCL4 INTOXICATION AND PREVENTION BY SILYMARIN DRUG. (A-B): SECTION OF CONTROL RAT LIVER ILLUSTRATING NORMAL HEPATOCYTES WITH PROMINENT NUCLEUS (N) ALONG WITH INTACT NUCLEAR MEMBRANE AND CLEAR NUCLEAR ENVELOPE (LONG HALLOW ARROW). NOTE: NORMAL FEATURES OF MITOCHONDRIA (M) AND CISTERNAE OF ENDOPHASIC RETICULUM (RER) (X 12,000 AND 8,000, RESPECTIVELY). (C-D): SECTION OF LIVER IN CCL4 TREATED GROUP ALONE (1ML/KG BW) EXHIBITING HEPATOCYTES WITH INDENTED NUCLEI OR SHOWING IRREGULAR NUCLEUS WITH LESS CHROMATIN (ARROW HEAD). THE HEPATIC CYTOPLASM CONTAINS NUMEROUS CYTOPLASMIC VACUOLES (V), DISORGANIZED OR FRAGMENTED RER AND DISRUPTED MITOCHONDRIA (ARROW). NOTE: DEGENERATED HEPATOCYTES WITH CYTOPLASMIC INCLUSION BODY (CB), DISTENDED BILE CANAICULUS (BC) WITH WIDENED INTERCELLULAR SPACES AND PRESENCE OF RED BLOOD CORPUSCLE (RC) IN BLOOD SINUSOID (S) IN THE CYTOPLASM (X 5,000 AND 8,000, RESPECTIVELY). (E-F): SECTION OF LIVER IN SELYMARIN TREATED GROUP (100 µg/kg,bw) SHOWING HEPATOCYTES WITH PROMINENT NUCLEUS (N) ALONG WITH INTACT NUCLEAR MEMBRANE AND CLEAR NUCLEAR ENVELOPE (LONG HALLOW ARROW). NOTE: NORMAL FEATURES OF MITOCHONDRIA, RER, BC WITH COMPACT INTERCELLULAR JUNCTIONAL COMPLEXES AND LYSOSOME BODIES (SMALL HAOLLOW ARROW) (X 8,000 AND 8,000, RESPECTIVELY).
FIGURE 3: ULTRASTRUCTURAL CHANGES OCCURRED IN THE LIVER AFTER CCL\textsubscript{4} INTOXICATION AND PREVENTION BY PRETREATMENT WITH GRADED DOSES OF NIMBOLIDE. (G-H): SECTION OF LIVER IN NIMBOLIDE TREATED GROUP (100 µg/kg bw) EXHIBITING ENHANCEMENT OF REGENERATION PROCESS. HEPATOCYTES SHOWING CONDENSED EUCHROMATIC NUCLEI (N) WITH INTACT NUCLEI ENVELOPES AND THEIR CYTOPLASMS CONTAIN MAJORITY OF NORMAL APPEARANCE OF MITOCHONDRIA. NOTE: HEPATOCYTES RETAINED THE FEATURES OF SLIGHTLY DILATED OR FRAGMENTED RER, FEW LYSOSOME BODIES (SMALL HALLOW ARROW), IRREGULAR MITOCHONDRIA (ARROW) AND NUMEROUS LIPIDS LIKE BODIES (L) IN THE CYTOPLASM (X 8,000 AND 8,000, RESPECTIVELY). (I-J): SECTION OF LIVER IN NIMBOLIDE TREATED GROUP (200 µg/kg bw) SHOWING NORMALIZATION OF ULTRA STRUCTURE OF HEPATOCYTES WHICH CORRELATED WITH THE IMPROVEMENT OF LIVER FUNCTION PANEL. NUCLEUS (N) WAS NEAR NORMAL IN APPEARANCE WITH INTACT NUCLEI ENVELOPES (LONG HALLOW ARROW), MAJORITY OF THE MITOCHONDRIA (M) AND WELL FORMED EXTENSIVE RER. NOTE: CYTOPLASM CONTAINS FEW LYSOSOME BODIES (SMALL HALLOW ARROW), LIPIDS LIKE BODIES (L) AND ALSO NORMAL APPEARANCE OF BC WITH INTACT INTERCELLULAR SPACES (X 8,000 AND 8,000, RESPECTIVELY).

DISCUSSION: In toxicological experiments, comparison of organ weights between control and treated groups have conventionally been used to predict toxic effect of a test material \textsuperscript{22} and no toxicity effect of the substance due to no changes in such parameters, which are often the first signs of toxicity \textsuperscript{23}. In the present study, acute toxicity test was done to establish the adverse effects of oral administration of nimboide at graded dose level and results indicate no significant changes in general, excluding little alteration in the relative weight of few organs of both either sexes at higher dose level, suggesting this active constituent had no effect on normal growth of rats. Assessment of hematological parameters are not only used to determine the extent of deleterious effect of extracts on the blood of animals, but it can also be used to explain blood relating functions of a plant extract or its products \textsuperscript{24}. In present findings, significant difference in some hematological parameters of both sexes may be indicative of direct or indirect effects of this active constituent associated with autoimmune processes \textsuperscript{25}. However, at the lower dose of 500 µg/kgbw, a increase or decrease in the some of these parameters indicates the variations may have
resulted from normal variation among animal groups or a general decrease in the values of these hematological parameters. Consequently, the parametric alterations in the higher dose treated cannot be considered as a manifestation of toxicity due to variability and physiological factors because similar differences were not observed in both gender of acute toxicity experiment. The non-toxic nature of nimbelide is evident by the absence of mortality for a period of 14 days even when maintained on limit dose indicating the active constituent could be safe up to 2000 µg/kg BW. Any compound or drug with oral LD50 estimates greater than 1000 mg/kg BW could be considered to be of low toxicity and safe. Souza- Brito reported that active principles from medicinal plants are generally found in low concentrations. In present acute toxicity study, we employed dosage values are very less concentration when compared to other studies. Though the phytochemical screening revealed many chemical constituents, which could affect the animal positively or negatively as a result of prolong usage, therefore, chronic toxicity evaluation are needed to determine the long term safety of this constituent in order to establish as medicine.

Liver is the most important key organ in the metabolism, detoxification and secretary functions in the body and it is highly affected primarily by toxic agents that why the above mentioned parameters have been found to be of great importance in the assessment of liver damage. The preventive action of liver damage by CCl4 has been widely used as an indicator of liver protective activity of drugs/ plant extracts. Studies have been demonstrated on pre-treatment with extract/ herbal formulations of various plants, at different dose levels, were found to be effective against CCl4 induced liver damage and had restored the levels of total protein, albumin and serum marker enzymes towards normalization and such effects were comparable with Silymarin standard drug.

Present study with CCl4 treated rats exhibited a marked reduction (P ≤ 0.05) in the serum levels of protein and albumin due to the hepatotoxin intoxication. Protein plays a major role in the synthesis of microsomal detoxifying enzymes to detoxify the toxicants. It is well indicated by elevated in the levels of BUN, ALP and hepato specific enzymes like AST and ALT in CCl4 induced group compared to normal controls. BUN is also a marker of liver and renal functions, which is used to diagnose acute and chronic diseases related to liver and kidney. A reduction in albumin level has been attributed to massive necrosis of the liver, deterioration of liver function and glycogen impairment of oxidative phosphorylation. The reduction is attributed to the damage produced and localized in the endoplasmic reticulum (ER) which results in the loss of P450 leading to its functional failure with a decrease in protein synthesis and accumulation of triglycerides. The rise in protein and albumin level suggests the stabilization of ER leading to protein synthesis. Preventing the induced CCl4 toxicity elevated level of BUN along with decline in the total protein and albumin levels by pretreatment with nimbelide indicating the hepatoprotective nature of the active constituent.

CCl4-induced hepatic injuries are commonly used models for the screening of hepatic drugs and the extent of hepatic damage is assessed by the level of released cytoplasmic transaminases (ALT and AST) and alkaline phosphatase (ALP) in circulation. The ability of hepatoprotective substances to reduce the harm or to preserve the mechanisms of liver function against disturbances of hepatic toxin is an indication of their protective effects. In this study, at higher dose level of nimbelide (200 µg/kg BW), the recovery towards normalization in these two levels was similar to that of standard drug, however, at lower dose level, this active constituent did not have protective effects. Significant increase in the AST, ALT and ALP levels after administration of CCl4 and reduction in these marker enzymes levels towards the normal value by pretreatment with nimbelide and Silymarin to CCl4 treated rats is an indicator of the regeneration process of the repair tissue damage caused by CCl4 liver.

Histopathological observations made after active constituent administration showed a protective effect against CCl4 liver damage, which basically supported the alterations observed in biochemical analysis. Significant increase in serum enzyme activities and the fall in protein and albumin levels caused by CCl4 have been attributed to the damaged structural integrity of the liver and hepatocellular dysfunction.
Presented regenerative effects with pretreatment with nimbolide can be considered as an expression of the functional improvement of hepatocytes and restoration of deficient functioning of marker enzymes implicating its cytoprotective role by stabilizing action at the membrane level towards normal liver cell function. EM studies revealed profound ultrastructural alterations like, disorganization of nuclear content, cytoplasmic degeneration, swelling of mitochondria and absence of cell-to-cell contact disturbing the morphological structure were more prominent in CCl₄ treated animals may be morphological evidence of liver injury or probably due to the changes in membrane structure caused by lipid peroxidation.

However, the significance of these findings lies in the fact that these changes are minimal in animals pretreated with active constituent. Furthermore, it is also possible that nimbolide may play an important role in preventing the non-alcoholic fatty liver disease (NAFLD), which has as basic mechanism of mitochondrial dysfunction and thus lead to apoptosis. This hypothesis is well supported by Tarantino et al. The most significant ultrastructural recovery with pretreated nimbolide occurred in mitochondria and RER. Mitochondria are the energy source of the cell and considered as one of the targets in CCl₄-induced subcellular damage. RER are storage of important cellular enzymes and severely dilated RER is representative of severely damaged hepatocytes. Moderate recovery of pathological effects in pretreated nimbolide against acute dose of CCl₄ treated albino rats, thus it appears that this active constituent play a key role in the reduction of hepatic injury and then preserve the structural integrity of the hepatocellular structures.

CONCLUSION: It has been suggested that A. indica is a promising hepatoprotective agent and this hepatoprotective activity of A. indica leaf extract may be due to its antioxidant and normalization of impaired membrane function activity. In present findings, nimbolide found effective in prevention of CCl₄-induced hepatic damage by preserving the structural integrity of the hepatocellular membrane as evidenced from reduction of the marker enzymes levels and thereby support the therapeutic use of this active constituent in tribal medicine for treating liver disease. Appropriate protective measures as using this constituent must be applied with hepatoprotective treatment for improving liver structure. However, the hepatoprotective mechanisms of nimbolide remain to be elucidated.

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REFERENCES:


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