APPENDIX - A
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Hepatoprotective activity

*A. racemosus*, *A. precatorius* and *A. lanata* are used particularly to treat jaundice by the folklore people in the West Godavari district region of andhra pradesh. Ethanolic extract and its fractions chloroform and hexane were subjected to hepatoprotective screening after inducing acute hepatotoxicity using hepatotoxins like carbon tetrachloride and paracetamol using the following procedural details. Wistar rats (150-220 gm) purchased from mahaveer enterprises, hyderabad were used.

A.1 Experimental

A.1.1 Preparation of solutions for administration

i. Carbon tetrachloride (CCl₄): 50 % v/v solution of carbon tetrachloride was prepared in liquid paraffin. The solution was administered at the dose of 1.25 mL/kg b.wt. *i.p.*

ii. Paracetamol suspension (PC): Paracetamol powder was suspended in 0.5 % Sodium CMC and administered at the dose of 3 g/kg b. wt. *p.o.*

iii. Suspensions of test substance: All the selective extracts were suspended in 0.5 % Sodium CMC and administered at the dose levels are as follows

   i. *A. racemosus* – 200, 400 and 800 mg/kg b.wt. *p.o.*

   ii. *A. lanata* – 200, 400 and 800 mg/kg b.wt. *p.o.*

   iii. *A. precatorius* – 50, 100 and 200 mg/kg b.wt. *p.o.*

iv. Standard: Silymarin being positive control was suspended in 0.5 % Sodium CMC and administered at a dose level of 25 mg/kg b.wt. *p.o.*

A.2 Carbon tetrachloride (CCl4) induced hepatotoxicity

Ethanolic, chloroform and hexane extracts obtained from leaves of *A. racemosus*, *A. lanata* and seeds of *A. precatorius* were subjected to *in vivo* on preliminary basis against CCl₄ induced toxicity by assessing them through biochemical parameters. Each set of experiment was divided into groups consists of control, toxicant,
standard and test. Groups consisted of 6 rats each unless otherwise mentioned. The protocol followed for CCl₄ induced hepatotoxicity on preliminary basis (Kumar and Mishra, 2005) was given below

The protocol for CCl₄ induced hepatotoxicity

<table>
<thead>
<tr>
<th>Group</th>
<th>0h</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Vehicle</td>
<td>Vehicle</td>
<td>Vehicle</td>
<td>Withdrawal of blood</td>
</tr>
<tr>
<td>CCl₄</td>
<td>Vehicle</td>
<td>Vehicle + CCl₄</td>
<td>Vehicle</td>
<td></td>
</tr>
<tr>
<td>Silymarin</td>
<td>Silymarin</td>
<td>Silymarin + CCl₄</td>
<td>Silymarin</td>
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<tr>
<td>Test</td>
<td>Extract</td>
<td>Extract + CCl₄</td>
<td>Extract</td>
<td></td>
</tr>
</tbody>
</table>

Vehicle: 0.5 % Sodium CMC; Test: Extracts prepared in 0.5% Sodium CMC.

The rats of control groups received three doses of vehicle i.e. 0.5 % Sodium CMC (1mL/kg b.wt. p.o.) at 24 h interval (0 h, 24 h and 48 h). The animals in CCl₄ treated group received vehicle at 0 h, vehicle followed by CCl₄ at 24 h and only vehicle at 48 h. The animals in test group received first dose of extract at 0 h, second dose of extract at 24 h which was followed by a dose of CCl₄ and third dose of extract at 48 h. The positive control group received the first dose of silymarin (25 mg/kg b.wt. p.o.) at 0 h, second dose of silymarin at 24 h which was followed by a dose of CCl₄ and third dose of silymarin at 48 h. After 72 h blood was collected from all the groups, allowed to clot for the separation of serum. The serum was used for the estimation of biochemical parameters.

A.3 Paracetamol (PC) induced hepatotoxicity

The set of experiment was divided into groups consists of control, toxicant, standard and test. Groups consisted of 6 rats each unless otherwise mentioned. The protocol followed for paracetamol induced hepatotoxicity (Rao and Mishra, 1998) was given below

The protocol for paracetamol induced hepatotoxicity

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Vehicle</td>
<td>Vehicle</td>
<td>Vehicle</td>
<td>Vehicle</td>
<td>Withdrawal of blood</td>
</tr>
<tr>
<td>Paracetamol (PC)</td>
<td>Vehicle</td>
<td>Vehicle</td>
<td>Vehicle + PC</td>
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<tr>
<td>Silymarin</td>
<td>Silymarin</td>
<td>Silymarin</td>
<td>Silymarin + PC</td>
<td>Vehicle</td>
<td></td>
</tr>
<tr>
<td>Test</td>
<td>Extract</td>
<td>Extract</td>
<td>Extract + PC</td>
<td>Vehicle</td>
<td></td>
</tr>
</tbody>
</table>

Vehicle: 0.5 % Sodium CMC; Test: Extracts prepared in 0.5% Sodium CMC.
The rats of control group received vehicle i.e. 0.5% (CMC) carboxy methyl cellulose solution (1 mL/kg p.o.) once daily for 3 days. Rats of paracetamol group, received a single daily dose of vehicle for three days and a single dose of paracetamol (3 gm/kg p.o.) 30 min after the administration of vehicle on day 3 of experiment. Rats of test group, received a single daily dose of extract for three days and a single dose of paracetamol (3 gm/kg p.o.) 30 min after the administration of extract on day 3 of experiment. Rats of standard group (silymarin), received a single daily dose of silymarin (25 mg/kg p.o.) for three days and a single dose of paracetamol (3 gm/kg p.o.) 30 min after the administration of silymarin on day 3 of experiment. After 48 h of paracetamol administration i.e. 5th day of experiment, the blood was collected and was allowed to clot at room temperature and serum was separated. The serum was used for the estimation of biochemical parameters.

A.4 Assessment of liver function

Blood was collected from all the groups by puncturing the retro orbital plexus and was allowed to clot at room temperature and serum was separated by centrifuging at 3000 rpm for 10 min. The serum was used for estimation of biochemical parameters to determine the function of the liver.

Serum Glutamate Oxalacetate Transaminase (SGOT)/ Aspartate Aminotransferase (AST), Serum Glutamate pyruvate transaminase (SGPT)/Alanine Aminotransferase (SGPT) were estimated by a UV - Kinetic method based on the reference method of international federation of clinical chemistry. Alkaline phosphatase (ALP) was estimated method by PNPP method. Total bilirubin (TB) by jendrassik and grof method. Total cholesterol (CHL) by CHOD - PAP method. Total protein (TP) by color complexation with copper ions in an alkali solution. Allbumin was estimated by bromo cresol green method. All the estimations were carried out using standard reagents on auto analyser.
APPENDIX - B

Anti-inflammatory activity

B.1 Experimental

B.1.1 Carrageenan Suspension
Suspension of carrageenan sodium salt (Sigma-Aldrich Chemicals Ltd.), 1% was prepared by sprinkling 100 mg of carrageenan powder on 10 ml of saline (0.9%) solution and set aside to soak for 1h. A homogeneous suspension was then obtained by thorough mixing with a magnetic stirrer.

B.2 Carrageenan-Induced Rat Paw Oedema

B.2.1 Induction of Paw oedema
Sprague Dawley rats (150-200 g, purchased from Mahaveer enterprises Ltd, Hyderabad) were used. Oedema was induced by injecting, subcutaneously (s.c) into the sub plantar tissue of the left hind paw of each rat, 0.1 ml of 1% carrageenan suspension in saline. The right hind paws of the same rats received 0.1 ml of saline alone in the same manner as control. Before the induction of oedema, the thickness of the both paws of each rat between lower and upper surface was measured using an instrument consisting of a graduated micrometer combined with a constant loaded lever system (Fig. A) to magnify the small changes in paw thickness during the course of the experiment. The measurements were then taken at 1h intervals after the induction of the oedema for up to 6hs.Oedema was monitored as the percentage increase in paw thickness in the carrageena injected paw. To assess the effect of saline on the oedema produced, the percentage increase in paw thickness produced in the saline injected paw was subtracted from that of carrageenan injected left paw (Al-Haboubi and Zeitlin, 1983). The percentage increase in paw thickness was plotted against the time (h) and the maximal oedema response induced during the 6hs was determined. The total oedema response as the area under the time course curves (AUC) was also determined.
Figure A: Zeitlin’s Constant Loaded Lever (Paw thickness measuring device)

1. Place where the paws use to be kept to measure the thickness
2. Constant loaded lever
3. Graduated scale numbered between 1 – 10 and divided by 0.1 equal to 100 divisions
4. Thread to pull down the lever with right leg in order to facilitate to keep the paw in between pointer 1a and basement 1b.

B.3 Sample calculations

Paw thickness (mm)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Rat 1</th>
<th></th>
<th>Rat 2</th>
<th></th>
<th>Rat 3</th>
<th></th>
<th>Rat 4</th>
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<tbody>
<tr>
<td></td>
<td>RT</td>
<td>LT</td>
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<td>LT</td>
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</tr>
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<td>6.0</td>
<td>6.5</td>
</tr>
</tbody>
</table>

RT: Right paw; LT: Left paw
Calculations

% Increase in paw thickness = \([\frac{(Y_t - Y_o)}{Y_o}] \times 100\)

Where, \(Y_t\) = Paw thickness at time \(t\) h (after injection)
and \(Y_o\) = Paw thickness at time \(0\) h (before injection)

Accordingly, for Rat 3, the oedema in the 4th h was calculated as:
% increase in RT = \(\frac{5.8 - 3.0}{3.0} \times 100\) = 93.3%
% increase in LT = \(\frac{3.1 - 3.0}{3.0} \times 100\) = 3.3%

Therefore, % increase due to carrageenan = (93.3 - 3.3) % = 90.0%

B.4 Assessment of drug effects

For screening purposes, drugs (extract or compounds) in sodium carboxy methyl cellulose were always pre dosed to rats prior to the induction of carrageenan paw oedema. The actions of drugs were evaluated by comparing the maximal paw oedema response during 6h (monitored as % increase in paw thickness) in the drug treated groups with that produced in the drug vehicle (control) treated group. The total (area under the time course curve, AUC, calculated using Trapezoid Rule) oedema response as the area under the time course curve, produced in the drug treated groups was also compared with that from the control group.