Chapter 8

Invivo studies

8.1 ACUTE TOXICITY STUDY IN RATS WITH METHANOL ELUATE OF C. macrophylla ROOTS ETHANOL (50%) EXTRACT (ME-CMRH) - (OECD GUIDELINES 423)

Two groups, each of three female Wistar albino rats of average body weight 200 g, were treated with ME-CMRH by oral route at a dose of 2000 mg/kg body weight. The test item was formulated in the vehicle (distilled water) at a concentration of 2000 mg/ml and administered at the dose volume of 10 ml/kg.

The animals were observed daily during the acclimatization period and the mortality/viability and clinical signs were recorded. All animals were observed for clinical signs during the first 30 minutes and at approximately 1, 2, 3 and 4 hours after administration on test day 0 and once daily during test days 1-14. Mortality/viability was recorded twice daily during days 1-14. Body weights were recorded on test day 0 (prior to test dose administration), test days 7 and 14. All animals were examined macroscopically.

8.1.1 TREATMENT

The animals received a single dose of the test dose by oral administration at 2000 mg/kg body weight, after being fasted for approximately 18.0 hours, but with free access to water. Food was provided again at approximately 3.0 hours after dosing. The administration volume was 10 ml/kg body weight. The animals were dosed using 18 G oral stainless steel feeding tubes.

8.2 GLUCOSE UPTAKE BY RAT HEMI DIAPHRAGM

8.2.1 ISOLATION OF DIAPHRAGM

The rats were fasted overnight and killed by cervical dislocation. The diaphragms were dissected out quickly with minimal trauma and divided into two
equal halves. Two diaphragms from the same rat were not used for the same set of experiments.

![Isolated rat hemi-diaphragm](Image)

**Fig. 8.1** Isolated rat hemi-diaphragm

8.2.2 EXPERIMENTAL DESIGN

Five sets of six graduated test tubes each, were grouped as follows:

- **Group 1**: 2 ml Tyrode solution with 2 g % glucose (Normal control);
- **Group 2**: 2 ml Tyrode solution with 2 g % glucose + insulin (Novo Nordisk) 0.62 ml of 0.4 U/ml solution (insulin-treated group)
- **Group 3**: 2 ml of Tyrode solution with 2 g % glucose + extract (500 µg/ml)
- **Group 4**: 2 ml of Tyrode solution with 2 g % glucose + extract (250, µg/ml)

The volumes of all solutions in graduated test tubes were made up to 4 ml with distilled water. The hemi-diaphragms were placed in test tubes and incubated for 30 min at 37°C, bubbled with oxygen and shaken continuously. Glucose uptake per g of tissue was calculated as the difference between the initial and final glucose content in the incubated medium (Ghosh et al., 2004).
8.2.3 STATISTICAL EVALUATION

The data were expressed as mean ± SEM. Statistical comparisons were performed by one-way ANOVA followed by Dunnett's multiple comparison tests using Graph Pad Prism version 5.0.

8.2.4 CHEMICALS AND KITS

Streptozotocin was procured from Sigma Aldrich, St. Louis, USA. Citrate buffer was prepared in water for injection and used. CMC was purchased from Himedia, Mumbai. All biochemical kits were procured from ERBA diagnostics, India.

8.3 STUDIES ON MALE Wistar albino RATS

8.3.1 GROUPING AND DRUG TREATMENT

Adult healthy male Wistar albino rats with body mass of approximately 200–225 g were used. The animals were conditioned to room temperature and at natural photoperiods for 1 week before study. A commercial balanced diet and tap water ad libitum were provided. The animals (36) were initially divided into two groups, the first group (6) received saline solution intraperitonially (i.p.) and it was kept as control (Group I). The second group (30 rats) was injected with a single intravenous dose of streptozotocin (STZ) at 50 mg/kg of body weight, dissolved in 0.01 M ice-cold citrate buffer, pH 4.5, immediately before use. Nicotinamide dissolved in sterile normal saline was injected intraperitonially at a dose of 150 mg/kg body weight to the second group of (30 rats). This was performed fifteen minutes prior to streptozotocin injection. Around 20% of the animals died during the induction of diabetes (Pellegrino Masiello et al., 1998).

Three days later blood glucose levels were determined in this group in whole blood samples collected from the tip of the tail to confirm the diabetic condition. Diabetic animals (24) were further divided into four groups of 6 rats each.

Group I: normal rats treated with water orally once a day for 4 weeks.

Group II: diabetic rats treated with water orally once a day for 4 weeks.
**In vivo studies**

**Group III:** diabetic rats treated with Gliclazide (5 mg/kg b.wt.) orally once a day for 4 weeks.

**Group IV:** diabetic rats treated with test extract ME-CMRH (250 mg/kg b.wt.) orally once a day for 4 weeks.

**Group V:** diabetic rats treated with test extract ME-CMRH (500 mg/kg b.wt.) orally once a day for 4 weeks.

Body weights were monitored and recorded weekly during the entire experiment.

8.3.2 BLOOD AND ORGAN COLLECTION

At the end of the experiment, rats were fasted overnight and anesthetized with sodium pentothal (intraperitonially) and 2 ml of blood was withdrawn through the retro-orbital plexus using a glass capillary (microhaematocrit capillaries) and collected into fresh centrifuge tubes. The animals were sacrificed and organs were separated and subjected for biochemical and histopathological studies.

8.3.3 PREPARATION OF HEMOLYSATE

The collected blood was centrifuged for 10 minutes at 3000 rpm. The serum separated was taken in fresh serum bottles, capped and stored in refrigerator at 2-4 °C for biochemical studies. The serum thus obtained was used for glucose, glycosylated haemoglobin (HbA1c)(Trivelli, Lia. et al., 1971), (Bijukumar Gopalakrishna Pillai et al., 2003), (Bhagyajyothi M Bhat et al., 2012) creatinine kinase (CK) (Tietz, 2006), (Jade I K et al., 2009) and lactate dehydrogenase (LDH) (Vizir O O, 1977), (Chang A Y, Rothorck D, 1977) estimation using commercial kits (ERBA diagnostics).

The percentage change in blood glucose levels between the zero day of treatment and twenty eighth day in all the six study groups were determined.

Percentage change =

\[
\frac{\text{Blood glucose of individual animal on day zero} - \text{Blood glucose of same animal on day 28}}{\text{Avg. blood glucose on day zero}} \times 100
\]
The percentage change between diabetic control and normal group as well as all the four treated groups were also determined.

Percentage change =

\[
\frac{\text{Average blood glucose on day 28 on diabetic control group} \ - \ \text{Blood glucose on individual animal on day 28}}{\text{Average blood glucose on day 28 on diabetic control group}} \times 100
\]

### 8.4 HISTOPATHOLOGY STUDIES ON PANCREAS

Slides were prepared from the tissues of pancreas of the necropsied test animals and examined for the evaluation of changes.

### RESULTS

#### 8.5 ACUTE TOXICITY STUDIES

All animals survived in the acute toxicity studies until the end of the experimental period. None of the animals dosed at 2000 mg/kg body weight showed evident toxicity throughout the experimental period. The animals which survived throughout the experiment increased their body weight by day fourteen as compared to day zero. No abnormalities were detected in any of the animals by visual observation. So necropsy was not performed.

Based on the results, the median lethal dose of ME-CMRH after single oral dose administration to female rat was decided as 2000 mg/kg body weight (LD₅₀ cut off value). Thus it was classified as category 4. (OECD 423)

On the basis of the above results, it was decided to perform animal studies with 250 mg/kg and 500 mg/kg dose of ME-CMRH eluate.
8.6 GLUCOSE UPTAKE STUDY IN RAT HEMIDIAPHRAGM OF TEST EXTRACT (ME-CMRH)

Table 8.1 Data of glucose uptake study in rat hemidiaphragm

<table>
<thead>
<tr>
<th>Test</th>
<th>Glucose uptake (mg/dl/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3.28±1.00</td>
</tr>
<tr>
<td>Insulin</td>
<td>17.43±2.93***</td>
</tr>
<tr>
<td>High dose (500µg/ml)</td>
<td>11.14±1.94*</td>
</tr>
<tr>
<td>Low dose (250µg/ml)</td>
<td>5.791±0.58</td>
</tr>
</tbody>
</table>

Each value represents mean ± SEM, n = 6. * p<0.05, ***p<0.001 compared to normal control (Dunnett's multiple comparison test)

Fig. 8.2 Schematic representation of rat hemidiaphragm glucose uptake

8.7 INFERENCE

The estimation of glucose content in isolated rat hemidiaphragm was employed for the study of peripheral glucose uptake. The effects of the extract on glucose uptake are shown in table 8.1.
8.8 BIOCHEMICAL FINDINGS

Table 8.2 Effect of test extracts (ME-CMRH) on blood glucose levels during treatment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Blood glucose level in mg/dl</th>
<th>% Change between day 0 and day 28</th>
<th>% Change between diabetic control and treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 day</td>
<td>7th day</td>
<td>14th day</td>
</tr>
<tr>
<td>I</td>
<td>Normal control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>96.00</td>
<td>±4.82</td>
<td>101.33</td>
</tr>
<tr>
<td>II</td>
<td>STZ treated (Diabetic control)</td>
<td>268.07</td>
<td>±7.76</td>
<td>316.30</td>
</tr>
<tr>
<td>III</td>
<td>STZ + ME-CMRH (250 mg/kg)</td>
<td>291.63</td>
<td>±9.16</td>
<td>314.23</td>
</tr>
<tr>
<td>IV</td>
<td>STZ + ME-CMRH (500 mg/kg)</td>
<td>296.33</td>
<td>±16.45</td>
<td>268.83</td>
</tr>
<tr>
<td>V</td>
<td>STZ + Glimepiride (25 mg/kg)</td>
<td>289.67</td>
<td>±10.93</td>
<td>242.50</td>
</tr>
</tbody>
</table>

n=6

Values are expressed as mean ± SEM for six animals in each group aP<0.05, bP<0.01 and cP<0.001 between normal and diabetic group values. dP<0.05, eP<0.01 and fP<0.001 between diabetic control and treated groups.

Out of the six diabetic control animals, one died on the twenty fifth day of studies.
### Table 8.3: Effect of test extracts (ME-CMRH) on STZ induced changes on the body weight, HbA1c, CK and LDH

<table>
<thead>
<tr>
<th>Group(s)</th>
<th>Treatment</th>
<th>Body weight (g)</th>
<th>HbA1c (%)</th>
<th>CK (IU/L)</th>
<th>LDH (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>236.46±6.12</td>
<td>4.22±0.38</td>
<td>201.33±9.95</td>
<td>91.50±6.47</td>
</tr>
<tr>
<td>II</td>
<td>STZ treated control</td>
<td>147.07±8.35c</td>
<td>8.19±0.66c</td>
<td>286.40±14.74b</td>
<td>163.67±8.82c</td>
</tr>
<tr>
<td>III</td>
<td>STZ + ME CMRH (250 mg/kg)</td>
<td>181.50±8.72</td>
<td>6.91±0.80</td>
<td>257.83±17.55</td>
<td>125.67±6.89</td>
</tr>
<tr>
<td>IV</td>
<td>STZ + ME CMRH (500 mg/kg)</td>
<td>203.47±9.47</td>
<td>5.79±0.42d</td>
<td>237.33±17.51l</td>
<td>115.67±11.40e</td>
</tr>
<tr>
<td>V</td>
<td>STZ + Gliclazide (25 mg/kg)</td>
<td>238.00±8.63</td>
<td>5.56±0.29e</td>
<td>266.00±11.03d</td>
<td>134.67±8.80d</td>
</tr>
</tbody>
</table>

n=6

Values are expressed as mean ± SD for six animals in each group, a,d - *, b,e - **, c,f - ***

aP<0.05, bP<0.01 and cP<0.001 between normal and diabetic group values. dP<0.05, eP<0.01 and fP<0.001 between diabetic control and treated groups.

The above results show a significant decrease in body weight in STZ-induced diabetic rats when compared to controls. However, diabetic rats treated with test extract showed augmented body weight when compared with STZ only treated rats. The blood glucose increased in STZ-diabetic rats as compared to normal rats. However, treatment of STZ-diabetic rats with the test extract ME-CMRH significantly reduced the hyperglycaemia when compared with STZ only treated rats. Rats lost their weight after STZ treatment, which was reversed by the treatment of test extracts and Gliclazide. HbA1c levels were higher in the STZ-induced diabetic rats compared to the control rats. The supplementation of ME-CMRH decreased the HbA1c level of the STZ induced diabetic rats. Antidiabetic activity of the test extract ME-CMRH at 500 mg/kg body wt. dose was comparable with the
standard drug gliclazide in restoring the levels of blood glucose, body weight and HbA\textsubscript{1C} towards normal levels.

8.9 GRAPHICAL REPRESENTATION OF RESULTS

**Fig 8.3:** Effect of test extracts (ME-CMRH) on blood glucose levels during treatment.

**Fig 8.4:** Effect of test extracts (ME-CMRH) on body weight levels during treatment.
**Fig 8.5:** Effect of test extracts (ME-CMRH) on CK levels during treatment.

**Fig 8.6** Effect of test extracts (ME-CMRH) on %HbA1c levels during treatment.
**In vivo studies**

**Fig 8.7:** Effect of test extracts (ME-CMRH) on LDH levels during treatment.

**Fig. 8.8:** Percentage change of blood glucose from day day 0 to day 28

8.10 HISTOPATHOLOGY REPORT

8.10.1 MICROSCOPY

8.10.1.1 Normal control

Section studied shows pancreatic lobules separated by connective tissue septa. The pancreatic lobules consist largely of the exocrine acini and their intralobular ducts. Most of the lobules show small, round, light-staining islets of...
Langerhans. The center of islet cells consist of aggregates of small β-cells (75%, Short-arrow), while the periphery comprises large α-cells (20%, Long-arrow). Intervening these cells are seen thin walled capillaries.

**Important highlights:**

- β cells – 75%, α cells – 20%

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### 8.10.1.2 Positive control (Diabetic control)

Section studied shows pancreatic lobules separated by fibrovascular septa. The pancreatic lobules consist of intact acinar cells with their intralobular ducts. The number of islets appears to have reduced in number [compared to standard group]. The center of islet cells consists of quantitative decrease in β-cells (35%, Fig. Short-arrow), while the periphery comprises α-cells (60%, Fig. Long-arrow). Also seen are some degenerated β-cells within the islets.

**Important highlights:**

- β cells – 35%, α cells – 60%

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### 8.10.1.3 ME-CMRH (250 mg/kg b.wt)
Section studied shows pancreatic lobules separated by fibrovascular septa. The pancreatic lobules consist of intact acinar cells with their intralobular ducts. The number of islets appears quantitatively reduced in number [compared to standard group]. The center of islet cells consists of $\beta$-cells (55%, Fig. Short-arrow), while the periphery comprises $\alpha$-cells (40%, Fig. Long-arrow).

Fig. 8.11 Section of the pancreas-after treatment with ME-CMRH (250 mg/kg b.wt) Fig., H and E, x400

$\beta$ cells – 55%, $\alpha$ cells – 40%.

8.10.1.4 ME-CMRH (500 mg/kg b.wt)

Section studied shows pancreatic lobules separated by fibrovascular septa. The pancreatic lobules consist of intact acinar cells with their intralobular ducts. The number of islets appears quantitatively reduced in number [compared to standard group]. The center of islet cells consists of $\beta$-cells (70%, Fig. Short-arrow), while the periphery comprises $\alpha$-cells (25%, Fig. Long-arrow).
8.10.1.5 Standard (Gliclazide 25 mg/kg b.wt)

Section studied shows pancreatic lobules separated by fibrovascular septa. The pancreatic lobules consist of intact acinar cells with their intralobular ducts. The number of islets appears quantitatively increased in number [compared to control group]. The center of islet cells consists of $\beta$-cells (65%, Fig. Short-arrow), while the periphery comprises $\alpha$-cells (30%, Fig. Long-arrow).
8.11 DISCUSSION

The present study with ME-CMRH revealed that it was not toxic to *Wistar albinorat*s when a single dose up to 2000mg/kg body weight was administered orally. This would indicate that the extract has low toxicity and was safe at least up to the maximum tested dose.

As per the OECD 423 guidelines, thus the LD$_{50}$ (cut off value) was fixed as 2000 mg/kg body weight and was classified as category 4.

The possible antidiabetic potential was evident from the enhancement of glucose uptake induced by the extract, particularly at 500 µg/ml dose in isolated rat hemidiaphragm. This was comparable with insulin given at a dose of 0.62 ml of 0.4 U/ml.

The finding that the extract prevented the elevation of blood glucose level in treated diabetic rats compared with diabetic control rats also supports the *in vitro* result.

Treatment with the extract higher dose has significantly reduced the creatinine kinase (CK) as well as lactate dehydrogenase (LDH) levels compared with the diabetic control group.

The elevation of CK could be attributed to possible damage to skeletal muscle. Treatments have reversed the cell damage and was indicated by the decrease in CK level.

Serum LDH values have risen significantly in the diabetic control animals compared to the normal control. The treatments with the extract and the standard drug have reversed the rise in LDH levels.

The rise in LDH level could be due to cell damage induced by streptozotocin. Therefore it is possible that, the treatments have reduced the cell damage induced by streptozotocin.

These studies therefore lend credence to claims in folk medicine on the utility of the plant as a medicine for diabetes.

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