Chapter -4

EXPERIMENTAL INVESTIGATIONS
## CHAPTER – 4 EXPERIMENTAL INVESTIGATIONS

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CHAPTER - 4
EXPERIMENTAL INVESTIGATIONS

4.1 PLANT MATERIAL

Leaves of *Flemingia chappar* was obtained from Madhya Pradesh and bark of *Gyrocarpus asiaticus* was collected from Tirumala forest Andhra Pradesh, India, in April 2010 Authentified by Prof. B. Prathiba Devi, Department of Botany, Osmania University, Hyderabad. A.P. Specimen Vouchers (Ref No: GPWKDP/YP/2010-196 and 197) were deposited at Department of Botany for further reference.

4.2 PREPARATION OF EXTRACTS

Shade dried crude drugs were pulverized, sieved (10/44) and stored in air-tight containers. About 5000 grams of crude drugs were extracted using AR grade solvents petroleum ether (60-80°C), Benzene, Chloroform, Acetone, Ethylacetate and Ethanol (95%) by successive sohxlation method until the phytoconstiuents were completely exhausted. All the extracts were concentrated by using Rota-vaccum evaporator (Buchi type Mumbai, India) until a semisolid extract is obtained, dried at less than 50°C, comminuted in a ball mill and preserved in air tight containers, kept in dessicators prior to its studies.

4.3 QUALITATIVE PHYTOCHEMICAL STUDIES

Phytochemical screenings of extracts was carried out as follows
### Table: 4.1 Phytochemical tests performed

<table>
<thead>
<tr>
<th>Detection of Alkaloids</th>
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<tr>
<td>S.No.</td>
<td>Test</td>
<td>Reagent</td>
<td>Observation</td>
</tr>
<tr>
<td>1</td>
<td>Mayer’s Test</td>
<td>Potassium mercuric iodide</td>
<td>Yellow ppt.</td>
</tr>
<tr>
<td>2</td>
<td>Dragendorff's test</td>
<td>Potassium bismuth iodide</td>
<td>Red ppt</td>
</tr>
<tr>
<td>3</td>
<td>Wagner’s test</td>
<td>Iodine in Potassium iodide</td>
<td>Brown/Reddish ppt</td>
</tr>
<tr>
<td>4</td>
<td>Hager’s Test</td>
<td>Saturated Picric acid solution</td>
<td>Yellow ppt.</td>
</tr>
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<table>
<thead>
<tr>
<th>Detection of Carbohydrates</th>
<th></th>
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<tbody>
<tr>
<td>1</td>
<td>Molish Test</td>
<td>Alcoholic alpha-Naphthol solution</td>
</tr>
<tr>
<td>2</td>
<td>Fehling’s Test</td>
<td>Fehlings Solution A &amp; B</td>
</tr>
<tr>
<td>3</td>
<td>Benedict’s Test</td>
<td>Benedict’s reagent</td>
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<tr>
<th>Detection of Anthraquinone glycosides</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>1</td>
<td>Modified Borntrager’s Test</td>
<td>FeCl₃ followed by Benzene, Benzene layer</td>
</tr>
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<table>
<thead>
<tr>
<th>Detection of Saponins</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Froth Test</td>
<td>Diluted with water shaken for 15 min.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Detection of Flavonoids</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ferric chloride test</td>
<td>Ferric chloride solution</td>
</tr>
<tr>
<td>2</td>
<td>Shinoda test</td>
<td>Magnesium turnings and conc.</td>
</tr>
<tr>
<td>3</td>
<td>Lead acetate test</td>
<td>Lead acetate solution</td>
</tr>
<tr>
<td></td>
<td>Alkaline reagent test</td>
<td>Sodium hydroxide solution</td>
</tr>
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</tbody>
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**Detection of Phenolics and Tannins**

<table>
<thead>
<tr>
<th></th>
<th>Gelatin test</th>
<th>1% gaelatin solution containing Sod.chloride</th>
<th>White ppt.</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Vanillin hydrochloric acid test</td>
<td>Vanillin hydrochloric acid reagent</td>
<td>Pinkish red colour</td>
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**Detection of Proteins and aminoacids**

<table>
<thead>
<tr>
<th></th>
<th>Ninhydrin test</th>
<th>Ninhydrin reagent</th>
<th>Blue colour</th>
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<tr>
<td></td>
<td>Xanthoproteic test</td>
<td>Conc.HNO₃</td>
<td>Yellow colour</td>
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**Detection of Fixed oils and fats**

<table>
<thead>
<tr>
<th></th>
<th>Stain test</th>
<th>Contrainment of extract between two filter papers</th>
<th>Staining on filter paper</th>
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<tbody>
<tr>
<td></td>
<td>Saponification test</td>
<td>Add alcoholic Potassium hydroxide solution, Phenophthalein, heated</td>
<td>Neutralization of alkali</td>
</tr>
</tbody>
</table>

**Detection of Phytosterols and Triterpenoids**

<table>
<thead>
<tr>
<th></th>
<th>Libermann-Burchard’s Test</th>
<th>Treated with CHCl₃, Acetic anhydride Conc.H₂SO₄</th>
<th>Formation of brown ring at the junction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Salkowski test</td>
<td>Treated with CHCl₃, Acetic anhydride Conc.H₂SO₄</td>
<td>Golden yellow colour</td>
</tr>
</tbody>
</table>
4.4. ELEMENTAL ANALYSIS

4.4.1. Elemental analysis using Inductively coupled plasma Optical Emission Spectrometer (ICP-OES):

Elements like Mg, As, Na, K, Pb, Pd and Hg were analysed by ICP-OES\textsuperscript{226}.

3 grams of pulverized extract was taken into a crucible and ignited gradually until a colourless ash was obtained. Added 1 g of the ash to 10 ml Conc. HNO\textsubscript{3} and heated at 150\textdegree C until the volume of nitric acid was reduced to 5 ml. HNO\textsubscript{3} was added again and heated at 250\textdegree C till the volume of nitric acid was reduced to 5 ml. It was cooled to room temperature and heated with 3 ml perchloric acid. The dense fumes formed were passed through inductively coupled plasma Optical Emission Spectrometer (ICP-OES).

4.4.2. Elemental analysis using Atomic Absorption Spectrometer (AAS):

Elements like Mg, As, Na, K, Pb, Pd and Hg have been analyzed by AAS\textsuperscript{227}. Aliquot sample was directly introduced into the flame and the absorbing, emitting or fluorescing at characteristic wavelength was measured.

4.5 ACUTE TOXICITY STUDIES

Ethanolic extracts of leaves of Flemingia chappar and bark of Gyrocarpus asiaticus used for toxicity studies as given\textsuperscript{228}. 
4.5.1. Acute toxicity studies: OECD Guidelines No. 420:

Female wistar rats (nulliparous and non-pregnant) of 8 to 10 weeks old weighing 200 – 250gms supplied by National Institute of Nutrition, Hyderabad, India, were individually housed in polypropylene cages lined with husk renewed every 24 h in well-ventilated rooms at 22±3°C and RH between 50 to 60, under artificial lighting12:12 h light and dark cycle in hygienic condition for at least five days prior to the study. The rats were fed with standard laboratory pellet diet (Hindustan lever) and water ad libitum. The studies were performed according to OECD Guidelines 420 and the protocol was approved by the Institutional Animal Ethics Committee (Reg. No. VNCP/1472/PO/a/CPCSEA).

4.5.1.1. Sighting study:

Animals were fasted over-night prior to dosing and weighed. The test substance was administered to single animals in a sequential manner following the flow charts in Annex 2 of OECD 420. The starting dose for the sighting study was selected from the fixed dose levels of 300 mg/kg (as there is no evidence from in vivo and in vitro data). The next dose used for this study was 2000 mg/kg. The Test substances were administered in a constant volume of 2 ml/100g body weight in the form of suspension. After the substance has been administered, food was withheld for a further 3-4 h. A period of at least 24 hours was allowed between the dosing of each animal. All animals were observed for at least 14 days.
4.5.1.2. Main study:

A total of five female wistar rats were used for each dose level investigated and the animals were made up of one animal from the sighting study dosed at the selected dose level together with an additional four animals. The time interval between dosing at each level was 3 or 4 days.

4.5.2. Acute toxicity studies: OECD Guidelines No. 425:

Animals were divided into two groups of 3 animals each. Group I was treated with vehicle (distilled water) and was kept as a control. Group II was treated with 5000 mg/kg dose according to their body weight. Blood and tissue were collected on 14th day. Haematological and biochemical parameters were measured in treated group as well as in control group. The organs were quickly blotted and weighed in a digital balance. Gross necropsy of heart, liver and kidney were observed.

4.5.3. Sub acute toxicity studies: OECD Guidelines No. 407:

The plant extract at the dose of 250, 500 and 1000 mg/kg body weight were administered orally to 4 groups of six rats respectively to every 24 h for 28 days and control received vehicle at the same volume. The toxic manifestation such as body weight, mortality, and food and water intake was monitored. After 28 days all surviving animals were fasted overnight and anaesthetized with ether. The
heparinised blood samples were collected for determining haematological parameters and the serum from non-heparinised blood was carefully collected for determining clinical blood chemistry. Animals were sacrificed after blood collection and the internal organs were removed and weighed to determine the relative organ weights and observed for gross lesions. The internal organs were preserved in 10% buffered formaldehyde solution for histological examination.

4.6 ISOLATION OF ACTIVE PRINCIPLES

4.6.1. Isolation of active principles from ethanolic extract of Flemingia chappar:

Column chromatography was conducted using silica gel (60-120) packed using hexane. The column was run by gradient elution technique using hexane, chloroform and methanol. TLC was used to monitor the eluates. A total of 158 eluates were collected and similar fractions were pooled together.

Further purification was carried out using preparative TLC. The unsaponifiable matter dissolved in chloroform and spotted on TLC plates precoated with silica gel 60 and placed in the TLC chamber consisting of specific solvent system. The individual spots were viewed in UV light as well as 5% sulphuric acid in methanol. Through several pilot experiments it was found that the compounds were best separated by the solvent system of chloroform: ethanol (9.8:0.2).
Spots were identified, scraped, separated, dissolved and concentrated in ethanol. Eluted fractions were filtered, dried and again chromatographed with standard markers. The eluted fractions were then crystallized separately with chloroform. The crystallized materials were subjected to its IR, $^1$HNMR, $^{13}$CNMR and Mass spectral analysis$^{229}$.

**4.6.1.2 Spectroscopic characterization:**

Different spectroscopic methods were used to elucidate the structure of isolated compounds. Among the spectroscopic techniques IR, $^1$H-NMR, $^{13}$C-NMR and GC-MS were carried out. The IR Spectrum was recorded on FTIR, $^1$H-NMR and $^{13}$C-NMR spectra were recorded using CDCl$_3$ as solvent on Bruker Advance II 400 NMR spectrometer and GC-MS Spectra were recorded at high resolution on a mass spectrometer and the data are given in m/z values$^{229}$.

**4.7 BIOLOGICAL STUDIES**

**4.7.1 Analgesic activity**

**4.7.1.1 Hot plate method:**

Mice that showed nociceptive responses within 10 sec on placing the Eddy’s hot plate ($55 \pm 0.5 \degree C$) were used in this study. Eight groups of mice (6 in each group) were administered as follows$^{230}$. 
Group I – 2% v/v, aq. Tween 80, 10 ml/kg, p.o.

Group II – Morphine 2mg/kg, p.o.

Group III – FCE (100 mg/kg) in 2% v/v.aq. Tween 80, 10 ml/kg.p.o.

Group IV – FCE (200 mg/kg) in 2% v/v.aq. Tween 80,10 ml/kg.p.o.

Group V – FCE (400 mg/kg) in 2% v/v.aq. Tween 80, 10 ml/kg.p.o.

Group VI – GAE (100 mg/kg) in 2% v/v.aq. Tween 80, 10 ml/kg.p.o.

Group VII– GAE (200 mg/kg) in 2% v/v.aq. Tween 80, 10 ml/kg.p.o.

Group VIII– GAE (400 mg/kg) in 2% v/v.aq. Tween 80, 10 ml/kg.p.o.

Inhibition% = \frac{\text{Reaction time (Control)} - \text{Reaction time (Treated)}}{\text{Reaction time (Control)}} \times 100

After 30 min of treatment, each mouse was placed on the hot plate (55 ± 0.5°C) and the reaction time was noted (n=6).

4.7.1.2 Tail Immersion Method:

The animals were grouped similarly in the previous method. The tail of each mouse was marked at 3 cm length and immersed in the water bath (51°C). The withdrawal time was noted after a gap of 5 min and the mean values were calculated (n=6).231

4.7.1.3 Tail flick method:
The animals were grouped similarly and after 30 min of the treatment the basal reaction time for each mouse was noted by placing the tip of the tail on the radiant heat source of the analgesiometer and the withdrawal time of tail (Flicking response) was noted (n=6)\textsuperscript{231}.

**4.7.1.4 Tail clip method:**

All the mice were screened by applying a metal artery clip with its jaw sheathed with thin rubber tubing to the base of the tail. The animals were treated and grouped similarly and the time to dislodge the clip of each mouse was recorded (n=6)\textsuperscript{231}.

**4.7.1.5 Acetic acid induced writhing test:**

The animals were treated and grouped similarly as described in method. Except group II was treated with Aspirin 20 mg/kg.p.o. After 30 min after the above treatment each mouse was injected with 10 ml/kg of 0.7% aqueous acetic acid i.p and was placed in a plastic transparent observation care and number of abdominal constrictions was cumulatively counted from 5 to 15 min. Results were expressed as percent inhibition of analgesia\textsuperscript{231}.

**4.7.2 Anti-inflammatory activity**

**4.7.2.1 Carrageenan-induce rat paw edema:**

Albino Wistar rats (200-250 g) were housed in a room at temp \((22\pm 1^\circ C)\), RH (60-70\%) at 12 h light and dark cycle provided with...
standard diet. They were fasted for 24 h prior to test but had free access to water\textsuperscript{232}.

Group I – 2% v/v, aq. Tween 80, 10 ml/kg, p.o.

Group II – Indomethacin 20 mg/kg, p.o.

Group III – FCE (100 mg/kg) in 2% v/v.aq. Tween 80, 10 ml/kg.p.o.

Group IV – FCE (200 mg/kg) in 2% v/v.aq. Tween 80,10 ml/kg.p.o.

Group V – FCE (400 mg/kg) in 2% v/v.aq. Tween 80, 10 ml/kg.p.o.

Group VI – GAE (100 mg/kg) in 2% v/v.aq. Tween 80, 10 ml/kg.p.o.

Group VII– GAE (200 mg/kg) in 2% v/v.aq. Tween 80, 10 ml/kg.p.o.

Group VIII– GAE (400 mg/kg) in 2% v/v.aq. Tween 80, 10 ml/kg.p.o.

One h after administration, 0.1 ml of 1% freshly prepared suspension of carrageenan in normal saline by subplantar injection (left hind paw) to all the animals. The volume of paws was measured using plethysometer.

\textbf{4.7.2.2 Histamine induced rat paw edema:}

Edema was induced by subplantar injection of 0.05ml of 1\% w/v, histamine in normal saline to all animals, which were grouped
and treated similarly and the volume of paws were measured using plethysmometer\textsuperscript{233}.

**4.7.2.3 Dextran induced rat paw edema:**

In this model, edema was induced by subplantar injection of 0.05 ml of freshly prepared 1% w/v solution of dextran to the hind paw of the rats, which were grouped and treated similarly\textsuperscript{233}.

**4.7.2.4 Chronic test:**

In this model, the rats were anaesthetized using ether and 10 mg of sterile cotton pellets were inserted into the axilla of each rat. The treatment was continued for seven consecutive days from the day of cotton pellets implantation. The animals were anaesthetized on 8\textsuperscript{th} day and the cotton pellets were surgically removed and dried at 60\textdegree C until constant weight was obtained. The increment in the dry weight of the cotton pellets was taken as a measure of granuloma formation\textsuperscript{233}.

**4.7.3 Hepato protective activity:**

Wistar rats (200-250g) housed in standard cages maintained at temperature (22±2\textdegree C) at 12:12 h light dark cycle. The experimental procedures were conducted out in strict compliance with the ethical guidelines for investigations of experimental pain in conscious animal framed by the Animal Ethical Committee rules and regulation in this institute\textsuperscript{234}. 
A toxic dose of carbon tetrachloride/paracetamol was administered to induce liver damage in experimental animals. In the present investigation, rats (n=6) were randomized into following groups.

Group I    -    2% v/v, aq. Tween 80, 10ml/kg, p.o. (control)
Group II   -    CCl₄ (2 ml/kg), i.p
Group III  -    Paracetamol (2 g/kg), p.o.
Group IV   -    CCl₄ (2 ml/kg), i.p + FCE (100mg/kg), p.o.
Group V    -    CCl₄ (2 ml/kg), i.p + FCE (200mg/kg), p.o.
Group VI   -    CCl₄ (2 ml/kg), i.p + FCE (400mg/kg), p.o.
Group VII  -    Paracetamol (2g/kg), p.o. + FCE (100mg/kg), p.o.
Group VIII -    Paracetamol (2g/kg), p.o. + FCE (200mg/kg), p.o.
Group IX   -    Paracetamol (2g/kg), p.o. + FCE (400mg/kg), p.o.
Group X    -    CCl₄ (2 ml/kg), i.p + Silymarin (50mg/kg), p.o.
Group XI   -    Paracetamol (2g/kg), p.o. + N-acetyl -1-cystine (100mg/kg), p.o.

After 21 days of such treatment, rats were sacrificed by cervical dislocation. Blood was subjected for determination of biochemical parameters like AST, ALT, ALP, total proteins and total bilirubin. A part of the liver was used for histopathological studies²³⁵-²³⁸.

The hepatoprotective activity of GAE was also assessed in the similar fashion.

4.7.4 Anti-diabetic activity
Diabetes mellitus is a chronic metabolic disorder due insufficient synthesis of insulin from pancreas characterized by hyperglycemia, glycosuria.

4.7.4.1 Oral glucose tolerance test\textsuperscript{239}:

The experimental procedure was approved by the Institutional Animal Ethics Committee (Reg. No. 1423/PO/a/CPCSEA). Wistar rats (8 - 10 weeks) weighing 200 - 250 g were housed in polypropylene cages in well-ventilated rooms at 22 ± 3°C and % RH about 50 to 60, under 12:12 h light and dark cycle for five days prior to the study.

- Group I - Glucose (2 g/kg)
- Group II - Glibenclamide (2 mg/kg) + glucose (2 g/kg). p.o.
- Group III - FCE (100 mg/kg), p.o. + glucose (2 g/kg). p.o.
- Group IV - FCE (200 mg/kg), p.o. + glucose (2 g/kg). p.o.
- Group V - FCE (400 mg/kg), p.o. + glucose (2 g/kg). p.o.
- Group VI - GAE (100 mg/kg), p.o. + glucose (2 g/kg). p.o.
- Group VII - GAE (200 mg/kg), p.o. + glucose (2 g/kg). p.o.
- Group VIII - GAE (400 mg/kg), p.o. + glucose (2 g/kg). p.o.

The animals were fasted overnight and treated with above dosage schedule orally. The FCE, GAE and glibenclamide were administered half an hour before administration of glucose solution. Blood glucose levels were assessed at 0 (before glucose challenge), 30, 90 and 150\textsuperscript{th} min after glucose administration.

4.7.4.2 Anti-diabetic activity\textsuperscript{240}:
The rats were divided into nine groups each consisting of six animals.

Group I - 1% w/v NaCMC, p.o (control)
Group II - STZ (60 mg/kg), i.p
Group III - STZ (60 mg/kg), i.p + Glibenclamide (2mg/kg), p.o.
Group IV - STZ (60 mg/kg), i.p + FCE (100mg/kg), p.o.
Group V - STZ (60 mg/kg), i.p + FCE (200mg/kg), p.o.
Group VI - STZ (60 mg/kg), i.p + FCE (400mg/kg), p.o.
Group VII - STZ (60 mg/kg), i.p + GAE (100mg/kg), p.o.
Group VIII - STZ (60 mg/kg), i.p + GAE (200mg/kg), p.o.
Group IX - STZ (60 mg/kg), i.p + GAE (400mg/kg), p.o.

The study was carried for the period of 21 days by dosing once daily.

Blood sample were collected from the animals prior to the treatment with above schedule and after 30 min of glibenclamide/ethanol extracts administration on 7th, 14th and 21st day. Blood obtained from the retro orbital venous plexus of rats under ether anaesthesia using a glass capillary tube and was centrifuged (2500 rpm/10 min) to separate serum. The collected serum was used for biochemical analysis of blood glucose, triglycerides, total cholesterol, HDL-cholesterol, SGOT, SGPT and ALP. After 21 days all the animals were anaesthetized by overdose of ether anaesthesia, liver and skeletal muscle tissues were collected for the assessment of glycogen content.
4.7.5 Antipyretic activity

4.7.5.1 Effect on normal body temperature in rats:

Seven groups of six animals in each group were used in the study. The initial rectal temperatures were recorded by inserting a thermocouple to a depth of 2 cm into the rectum. Rectal temperatures were recorded at 1, 2, 3, 4 and 5 h.

Group I    -     2% v/v, aq. Tween 80 (10 ml/kg), p.o.
Group II    -    FCE (100 mg/kg + 2% v/v aq. Tween 80, p.o.
Group III   -    FCE (200 mg/kg) +2% v/v aq. Tween 80, p.o.
Group IV   -    FCE (400 mg/kg) +2% v/v aq. Tween 80, p.o.
Group V    -    GAE (100 mg/kg) +2% v/v aq. Tween 80, p.o.
Group VI   -    GAE (200 mg/kg) +2% v/v aq. Tween 80, p.o.
Group VII   -   GAE (400 mg/kg) +2% v/v aq. Tween 80, p.o.

4.7.5.2 Effect on yeast induced pyrexia in rats:

A 20% suspension of 20 ml/kg Brewer’s yeast in normal saline was given subcutaneously to induce fever. 18 h post challenge; the rise in rectal temperature was recorded. Animals that showed an increase of 0.3-0.5°C in rectal temperature were selected for further studies. Eight groups of six animals in each group were taken. Rectal temperatures were recorded at 1, 2, 3, 4 and 5 h post dosing.

Group I    -     2% v/v aq. Tween 80 solution (10 ml/kg), p.o.
Group II    -    Paracetamol (100 mg/kg), p.o.
Group III - FCE (100mg/kg) + 2% v/v aq. Tween 80 solution
Group IV - FCE (200mg/kg), p.o. + 2% v/v aq. Tween 80 solution
Group V - FCE (400mg/kg), p.o. + 2% v/v aq. Tween 80 solution
Group VI - GAE (100mg/kg), p.o. + 2% v/v aq. Tween 80 solution
Group VII - GAE (200mg/kg), p.o. + 2% v/v aq. Tween 80 solution
Group VIII - GAE (400mg/kg), p.o. + 2% v/v aq. Tween 80 solution