MATERIALS AND METHODS

The present work on "Phytochemical, Pharmacological and Electrochemical Investigation of Aerial Roots of *Rhaphidophora aurea* (Linden ex Andre) Intertwined over four different Host Trees" is carried out in the following phases:

- Collection of plant material
- Extraction of plant material by different extraction techniques
- Proximate analysis of plant material
- Phytochemical analysis of plant extracts
- Isolation of compounds from plant extracts using SSF, SF, PTLC and CC isolation methods
- Pharmacological, biological and electrochemical studies of plant extracts and its compounds
  - Antioxidant assay of plant extracts using *in vitro* and voltammetric methods
  - Antimicrobial activity of plant extracts using *in vitro* methods
  - Acute toxicity study of plant extract using Swiss Albino mice
  - Wound healing study of prepared herbal and extract formulation using wistar rats
  - Anti-inflammatory activity of plant extracts using Swiss Albino mice
  - Anticancer study of plant extracts against MCF-7 cell line by MTT assay method
  - Larvicidal and pupicidal activity of plant extracts viz *Cluex quinquefasciatus*
  - Electrochemical sensor for detection of sterols in plant samples

3.1 Plant Materials

Aerial roots of *Rhaphidophora aurea* (Linden ex Andre) intertwined over *Lawsonia inermis* (MM) and *Azadirachta indica* (MN) were collected from Coimbatore District and that intertwined over *Areca catechu* (MB) and *Cocos nucifera* (MC) was collected from Palakkad District. The botanical identification of aerial roots of *Rhaphidophora aurea* was carried by Dr. G.V.S. Murthy, Joint Director, Botanical survey of India, Coimbatore-641003 (Authentication number - BSI/SC/5/23/09-10/Tech- 1534 (Appendix 1)).

3.2 Materials

3.2.1 Chemicals

All the solvents used in the present research work were of analytical grade. The solvents used for extraction and isolation namely petroleum ether, hexane, acetone, ethyl acetate,
chloroform, ethanol and methanol were double distilled according to standard procedures (Vogel, 2004).

3.2.2 Instruments

The equipments and instruments used for extraction, characterization and different analysis during the study period are Ajay heating Mantle, PCITM Ultrasonic bath Sonicator, Biologic Inc Ultrasonic Homogenizer model 300 V/T, LG Microwave oven, Equitron rotary flash evaporator, Systrons PC 2202 UV Double beam spectrophotometer, Hitachi UV lamp-F8T5, Shimadzu FT-IR (4000-400) spectrophotometer, NMR Bruker Avance III 500MHZ, Thermo TSQ 8000 GC-MS, JEOL GCMATE II GC-MS, Potentiostat (Solatron 1284), PARSTAT 2273, Eutech instrument pH tutor, Spectrophotometer- Model In-31, INSTRON universal Tensile 5500R/6021, Light microscope (LABOMED, German), Cuber ELISA ROI Micro plate reader and Muffle furnace.

3.3 Extraction of plant sample

3.3.1 Sequential solvent extraction of the aerial roots of MM, MB, MC and MN

The aerial roots of Rhaphidophora aurea (Linden ex Andre) intertwined over Lawsonia inermis (MM), Areca catechu (MB), Azadirachta indica (MN) and Cocos nucifera (MC) were defatted using petroleum ether (PE) and the defatted plant residue was then sequentially extracted by conventional refluxing with solvents acetone (AC), chloroform (CHCl₃), ethyl acetate (EA), ethanol (EtOH) and aqueous (Aq) for 12h (Scheme-1 and 2) and also the MM and MB were directly extracted with ethanol (DOH) for 12h (Table 1). The variation in plant weight was due to the restricted availability of the plant samples.

<table>
<thead>
<tr>
<th>Plant material</th>
<th>Weight (g)</th>
<th>Solvents</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM</td>
<td>570</td>
<td>Petroleum ether, Acetone, Chloroform, Ethyl acetate, Ethanol, Water</td>
<td>12h soaking and 12h refluxing</td>
</tr>
<tr>
<td>MB</td>
<td>740</td>
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<tr>
<td>MC</td>
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<tr>
<td>MN</td>
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</tbody>
</table>

3.3.2 Different methods of extraction (Scheme-3)

3.3.2.1 Conventional method-refluxing

The defatted MM and MN plant residue (30g) were extracted with 120ml ethyl acetate in a heating mantle for three hours, after which it was filtered and the filtrate distilled to yield extract. The same procedure was adopted for ethanol and aqueous extraction.
3.3.2.2 Extraction using sonic waves in an Ultrasonic bath

The defatted MM and MN plant residue (30g) was sonicated with 120ml ethyl acetate in a sonic bath at the intervals of 15 minutes for three hours, after which it was filtered. The filtrate on distillation yielded the corresponding extract. The same procedure was adopted for ethanol and aqueous extraction.

3.3.2.3 Extraction using sonic waves in an Ultrasonic homogenizer

The defatted MM and MN plant residue (30g) was treated with 120ml ethyl acetate and sonicated at a pulse rate of 10 seconds, at intervals of 10min for three hours in an ultrasonic homogenizer. Distillation of the filtrate yielded ethyl acetate extract. Similar procedure was adopted for ethanol and aqueous extraction.
Sequential extraction of MC and MN

(360g MC /290g MN)

Petroleum ether $\Delta$ 12h/12h soaking

Petroleum ether residue

Petroleum ether extract → Distillation

Ethyl acetate $\Delta$ 12h/12h soaking

Ethyl acetate residue

Ethyl acetate extract → Distillation

Ethanol $\Delta$ 12h/12h soaking

Ethanol residue

Ethanol extract → Distillation

Water $\Delta$ 12h/12h soaking

Aqueous extract → Distillation

Aqueous residue
Sequential extraction of MM and MB

(570g MM /740g MB)

Petroleum ether \( \Delta \) 12h/12h soaking

Petroleum ether extract

Plant residue

Acetone \( \Delta \) 12h/12h soaking

Distillation

Petroleum ether extract

Acetone

Plant residue

Distillation

Ethyl acetate \( \Delta \) 12h/12h soaking

Acetone residue

Ethyl acetate extract

Chloroform \( \Delta \) 12h/12h soaking

Distillation

Ethyl acetate residue

Chloroform extract

Plant residue

Distillation

Chloroform residue

Ethanol \( \Delta \) 12h/12h soaking

Distillation

Ethanol residue

Plant residue

Distillation

Water \( \Delta \) 12h/12h soaking

Ethanol residue

Aqueous extract

Plant residue

Distillation

Aqueous residue
3.3.2.4 Extraction of aerial roots of *R. aurea* using microwaves

The MM and MN plant residue (20g) priorly defatted with PE was extracted with 90ml of distilled water in a microwave oven for 90 seconds. The solvent was distilled off and the residue was weighed, to get the yield of extract.

3.4 Proximate evaluation of MM and MB (Rajurkar and Hande 2011)

3.4.1 Determination of dry matter and moisture content

Accurately weighed pulverized MM (1g) and MB (1g) in a pre-weighed china dish and heated at 105°C for 12h in an oven and thereafter cooled in a desiccator. The dishes were then weighed in triplicate and the percentage of the dry matter was calculated as given below:
3.4.2 Determination of ash value

The pulverized MM and MB were analyzed for total ash, acid insoluble ash and water soluble ash according to standard procedures as follows:

3.4.2.1 Total ash

Pulverized MM (6g) and MB (6g) were weighed accurately and kept into a previously ignited and dry silica crucible. The material was evenly spread and ignited via Bunsen flame for 30min. The crucible was placed in a Muffle furnace and the temperature was gradually increased to 600°C till it became white, indicating the absence of carbon, then placed the dishes in a desiccator for cooling and weighed. The total ash percentage was calculated as below and expressed as mg per gram air-dried sample.

\[
\text{Ash} \% = \frac{(\text{Weight of crucible + ash}) - \text{Weight of crucible}}{\text{Weight of fresh sample}} \times 100
\]

3.4.2.2 Acid insoluble ash

Calculated quantities of ash (500mg) was treated with 2N HCl (15ml) covered with a watch glass, boiled gently for 5min and filtered. The insoluble matter was collected and washed with hot water until the filtrate became neutral. The filter paper was then transferred to a pre-weighed crucible, dried on a hot plate and ignited to constant weight. The residue was allowed to cool in a desiccator for 30 minutes and then weighed. The acid insoluble ash percentage was calculated as below and expressed as mg per g air-dried sample.

\[
\text{Solubility} \% = \frac{\text{Weight loss of sample} \times 100}{\text{Weight of sample taken}}
\]

3.4.2.3 Water soluble ash

Calculated quantities of ash (500mg) was treated with 15ml of water, boiled for 5 minutes and filtered. The insoluble ash matter was collected in a crucible, washed with hot water and ignited in a silica dish for 15 minutes at 320-350°C in an oven. The water soluble ash percentage was calculated as bellow and expressed as mg per g of air-dried sample.

\[
\text{Solubility} \% = \frac{\text{Weight loss of sample} \times 100}{\text{Weight of sample taken}}
\]
3.5 Isolation of secondary metabolites from MM and MB (Rajurkar and Hande 2011)

3.5.1 Extraction

Pulverized MM (5g) was taken in a round bottomed flask, added 150ml methanol: water (4:1) and refluxed for 12 hours, cooled and filtered using Whatmann filter paper no: 41. Similar procedure was adopted for MB extraction. The filtrates were refrigerated for further use. The plant residue was used for determining the fiber content.

3.5.1.1 Determination of crude fiber

The plant residue obtained from the above extraction was further extracted with 125ml ethyl acetate (12h soaking + 2h refluxing) and filtered. The plant residue consisting of plant fiber and the filtrate was used for determining the fatty matter.

3.5.1.2 Determination of fat and wax

The filtrate from the above procedure was allowed to evaporate on a water bath (45°C) and placed in a desiccator for cooling. The weight of the residue gives the quantity of fat and wax.

3.5.1.3 Determination of phenolics and terpenoids

The filtrate obtained from the extraction was allowed to evaporate to 1/10th of the volume at 70°C on a water bath, acidified with 2M H₂SO₄ and extracted with chloroform (75ml). The procedure was repeated till the chloroform layer was colourless. Collected both the aqueous and chloroform layer in a beaker, allowed the chloroform layer to evaporate and placed in a desiccator for removing its moisture content. Then the chloroform layer containing phenolics and terpenoids was weighed to get the yield.

3.5.1.4 Determination of alkaloids

The aqueous layer obtained from the above procedure was neutralized with 2M NaOH and extracted with 60ml of 3:1 chloroform: methanol, followed by extraction with 40ml of chloroform. The procedure was repeated till the chloroform layer become colourless. Both the aqueous and chloroform layers were separated using a separatory funnel. Chloroform was then distilled off and the alkaloid fraction obtained weighed, to get the yield.

3.5.1.5 Determination of quaternary alkaloids and N-Oxides

Aqueous layer obtained from the above procedure was allowed to evaporate on a water bath at 70°C, dried and weighed to get the yield of quaternary alkaloids.

3.5.2 Determination of flavonoids (Bohm and Kocipal, 1974)

Pulverized (2g) plant material (MM/MB) was extracted with 20ml of 80% aqueous: methanol via soaking (12h) and filtered. The residue and the filtrate were allowed to evaporate and weighed to get the yield of flavonoids.

3.6 Phytochemical screening of MM, MB, MC and MN

Phytochemical analysis of the plant extracts were carried out using standard procedures (Table 2).
Table 2. Phytochemical tests of MM, MB, MC and MN

<table>
<thead>
<tr>
<th>Name of the tests</th>
<th>References</th>
</tr>
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<td>Test for alkaloids</td>
<td>Mayer’s test</td>
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<td>Ayoola et al, 2008</td>
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3.7 Isolation of compounds

A crude extract is most of the time an extremely complicated mixture of several compounds with varying physical and chemical properties. Hence four different methods were taken for isolation viz (i) Solvent-Solvent Fractionation isolation (SSF), (ii) Solvent Fractionation isolation (SF), (iii) Preparative Thin Layer Chromatography isolation (PTLC) and (iv) Column Chromatographic isolation (CC).
3.7.1 Isolation by Solvent – Solvent fractionation (SSF)

In this method, compounds distribute between two immiscible solvents according to their partition co-efficients. In the large-scale separation of compounds from crude extracts this step is very crucial (Sarker et al, 2006). In the present study 1g of petroleum ether extract PEMM was partitioned between chloroform and water. The organic layer and aqueous layers were separated and solvent distilled off to get a residue which was treated with distilled alcohol and refrigerated. The solid thrown out (PEMMEtOH) on refrigeration was filtered and the filtrate further fractionated to yield two compounds (PEMMCH and UK) (Scheme-4).

![Scheme-4](image)

3.7.2 Isolation by Solvent Fractionation (SF)

Isolation of compounds from crude extracts through solvent fractionation minimizes the usage of solvents and the procedure involves use of only one solvent. This method is based on hydrophobicity/ hydrophilicity of the chemicals taken for study (Tobias, 2009). Choice of solvent is thus important in isolation and separation of compounds. In the present study the petroleum ether extracts of MB, MC, MN and the ethanol extract of MM, MB and MC were taken for SF (Scheme-5 to 10).
Scheme-5
SF of petroleum ether extract of MB

PEMB (120mg)
Acetone (10ml) Steam bath (5-10min) & filtered
Residue Filtrate
Refrigerated filtered

Solid Solid
MBPEAC (35mg) MBPEAC1 (20mg)

Scheme-6
SF of petroleum ether extract of MC

MCPE (65mg)
Repeated crystallization with Ethanol

MCPE2 (14mg) MCPE3 (31mg)

Scheme-7
SF of ethanol extract of MC

MCEtOH (150mg)
Repeated crystallization with Ethanol

MCETOHPE (10mg) Cream colour solid (130mg)

Scheme-8
SF of ethanol extract of MM and MB

MMEtOH (1.9g) / MBEtOH (2.1g)
Concentrated & refrigerated
Filtered Filtered
Colourless crystals Colourless crystals
MMOH (110mg) MBOH (143mg)
3.7.3 Preparative thin layer chromatographic isolation (PTLC)

Petroleum ether extracts of MB, MN and direct ethanol extract of MM were taken up for PTLC isolation of compounds.

Petroleum ether extracts of MB (20mg) were applied as a horizontal band with a capillary tube onto a pre-coated TLC sheets (20×10 cm). After elution with suitable solvent system petroleum ether: ethyl acetate: ethanol (4:1:1), the separated bands were visualized in a UV chamber (356 nm). The residue showed intense green blue fluorescence band at Rf 0.5 and non-fluorescence band at Rf 0.2. Selected bands were cut into strips and extracted with acetone. Crystals thrown out of acetone were filtered off, dried and the compound purity checked by TLC (Scheme-11).
PEMN extract (15mg) was dissolved in petroleum ether and it was applied to the plate as a thin layer and eluted with the developing solvent system petroleum ether: ethyl acetate: ethanol (4:1:1). The separated bands were visualized in a UV chamber (356 nm). The residue showed intense green fluorescence band at Rf 0.32. Selected bands were cut into strips and extracted with acetone. Crystals thrown out of acetone were filtered off, dried and the compound purity checked by TLC (Scheme-11a).

The direct ethanol extract of MM (2g) was extracted with acetone (25ml). The acetone soluble portion was evaporated and the solid obtained fractionated by PTLC in petroleum ether: ethyl acetate (4:1) to yield MDP1 (Rf 0.3) and MDP2 (Rf 0.56) (Scheme-12).
3.7.4 Open column chromatography of ethanol extract of MB

Column chromatography isolation is a standard method for isolation of compounds. In this method the glass column was packed with 400 g silica gel. The ethanol extract slurry of MB (22g) was carefully poured over silica gel. The separation of ethanol crude extract was dispensed by different mobile system (Petroleum ether, ethyl acetate, ethanol and methanol). Totally 807 fractions (250 ml/fraction) were collected and distilled. The fractions were spotted onto a TLC and eluted with suitable mobile phase. The TLC spots of the fractions were visualized in a UV chamber at 356nm. The iodine absorption of the fraction spots was monitored in an iodine chamber. Based on the TLC observations the fractions were combined and labeled as in table 3.

<table>
<thead>
<tr>
<th>Solvent polarity</th>
<th>Combined fractions</th>
<th>Labeled As</th>
<th>Solvent polarity</th>
<th>Combined fractions</th>
<th>Labeled As</th>
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<tbody>
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<td>541-551</td>
<td>P23</td>
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Based on TLC comparison, analysis and yield of solid residue obtained, the fractions P4, P8, P9, P11, P31, P33, P41, P42 and P43 were further worked up, to isolate and purify the solids from the residue. Fractions which were of low yield and which failed to separate on repeated trials were not taken up for further study.

3.7.5 Characterization of isolated compounds

The isolated compounds were characterized through recording UV (Systronics PC based double beam spectrophotometer-2202), IR (Shimadzu 87005), GC-MS/MS (Thermo) and 1D NMR and 2D NMR (Bruker 500).

3.8 Pharmacological, Biological and Electrochemical Studies

3.8.1 Antioxidant assay of the solvent extracts of MM, MB, MC and MN

The petroleum ether, ethyl acetate, ethanol and aqueous extract of MM, MB, MC and MN were evaluated for their antioxidant activity by DPPH radical scavenging and reducing power assay and the results were compared with ascorbic acid. The extracts codes used in this study are indicated below:

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<tr>
<th>Solvents</th>
<th>MM</th>
<th>MB</th>
<th>MC</th>
<th>MN</th>
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<td>Ethanol</td>
<td>M3</td>
<td>B3</td>
<td>C3</td>
<td>N3</td>
</tr>
<tr>
<td>Aqueous</td>
<td>M4</td>
<td>B4</td>
<td>C4</td>
<td>N4</td>
</tr>
</tbody>
</table>

3.8.1.1 Preparation of stock solution

The extracts and ascorbic acid were weighed (1mg) and made up to 10ml with distilled methanol. Various concentrations (10, 20, 30, 40, 50 and 60 μg/ml) of sample were prepared for the study by appropriate dilution of the stock solution.

3.8.1.2 DPPH radical scavenging assay

The hydrogen donating ability of the extracts was measured by DPPH assay by standard procedure (Blois, 1958). Methanolic solution of DPPH (2ml, 0.1Mm) was added to various concentrations of extract (1ml) and the absorbance was measured in a Spectrophotometer at 517nm. The percentage of antioxidant activity was calculated as:

\[
\text{% of DPPH scavenged} = \frac{B_0 - B_1}{B_0} \times 100
\]

where, \(B_0\) = Absorbance of the control, \(B_1\) = Absorbance of the sample

3.8.1.3 Reducing power assay

The reducing power assay of the extract was determined by Oyaizu method (Oyaizu, 1986). The extract (1ml) was added to 2.5ml of phosphate buffer (0.2M monobasic sodium phosphate, 0.2M dibasic sodium phosphate) and 2.5ml of one percent potassium ferricyanide.
The mixture was incubated at 50ºC for 20min. Trichloroacetic acid (10%, 2.5ml) was added to the mixture and centrifuged. The supernatant (1ml) was treated with 3ml of purified water and 0.5ml of ferric chloride. Absorbance value of both the extracts and standard were measured at 700nm.

3.8.1.4 Determination of IC\textsubscript{50} and EC\textsubscript{50}

*Masterplex* 2010 software was used to calculate the half maximal inhibitory concentration, effective concentration and for linear regression analysis.

3.8.1.5 Antioxidant activity index (AAI)

The antioxidant activity index (AAI) was calculated by using the IC\textsubscript{50} value of the extract and standard (*Scherer and Godoy*, 2009).

\[
\text{AAI} = \frac{\text{DPPH (µg/ml)}}{\text{IC}_{50} (µg/ml)}
\]

3.8.3 Antimicrobial studies of the solvent extracts of MM, MB, MC and MN

The plant extracts were tested for their antimicrobial activity against 3 different bacteria and 2 fungi, reported to cause human disease. Table below gives the pathogenic effect of the selected microbes.

<table>
<thead>
<tr>
<th>Micro organism</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella sp</em></td>
<td>An etiologic agent of enteric fever and food poisoning (<em>Abdullahi</em>, 2010)</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>Affects the immune system of cancer patients (<em>Gershman et al</em>, 2008)</td>
</tr>
<tr>
<td><em>Klebsiella sp</em></td>
<td>Urinary tract infections (<em>Uzoigwe and Agwa</em>, 2011)</td>
</tr>
<tr>
<td><em>Candida tropicalis</em></td>
<td>Nosocomial candidaemia (<em>Kothavade et al</em>, 2010)</td>
</tr>
<tr>
<td><em>Aspergillus fumigates</em></td>
<td>Infections in human (respiratory, lung etc.,) (<em>Latge</em>, 1999)</td>
</tr>
</tbody>
</table>

3.8.3.1 Preparation of culture media

3.8.3.1.1 Mueller-Hinton Medium for bacteria

**Composition of Mueller – Hinton medium**

<table>
<thead>
<tr>
<th>Contents</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid Casein Peptone (H)</td>
<td>17.50</td>
</tr>
<tr>
<td>Starch</td>
<td>1.50</td>
</tr>
<tr>
<td>Beef Infusion</td>
<td>2</td>
</tr>
<tr>
<td>Bacteriological Agar</td>
<td>17</td>
</tr>
</tbody>
</table>

Mueller–Hinton medium (38g) was suspended in one litre of purified water, heated with frequent agitation and boiled for one minute to completely dissolve the medium. It was then
autoclaved at 121°C for 15min and cooled to room temperature (Ananthanarayan and Paniker, 1978).

3.8.3.1.2 SDA medium (Sabouard Dextrose Agar) for fungi

<table>
<thead>
<tr>
<th>Contents</th>
<th>g/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDA</td>
<td>65</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Calculated quantities of Sabouard Dextrose Agar (65.0g) was dissolved in100ml distilled water and boiled to dissolve the medium. This medium was preserved and used for the study.

3.8.3.2 Antimicrobial testing

3.8.3.2.1 Disc diffusion method (Antibacterial)

Paper discs of 4mm diameter were sterilized in an autoclave and dried at 100°C in an oven. The discs were them soaked in test solutions at the rate of 50µg (dry weight) per disc for antibacterial analysis. One drop of bacterial suspension was taken in a sterile petri dish of 90mm diameter. Approximately 20ml of sterilized melted nutrient agar (~45°C) was poured into the plate and spread uniformly.

The paper discs after soaking with test chemicals were placed at the center of the pour plate. A control plate was also maintained in each case with alcohol. First, the plates were maintained at low temperature (4°C) for 4h. The plates were then incubated at 35°C for growth of test organisms and were observed at 24h intervals for two days. The activity was expressed in terms of zone of inhibition in mm. Each test was carried out in triplicate. Gentamicin was used as a positive control and compared with test extracts under identical conditions (Ananthanarayan and Paniker, 1978).

3.8.3.2.2 Streak plate isolation method (Antifungal)

The required amount of SDA medium was taken in a conical flask separately and was sterilized in autoclave (at 121°C and 15Psi) for 15min. Liquefied SDA was dispensed into the petri dish. The plate was rotated gently for uniform distribution of the medium. The inoculating loop was held at a 60°C angle in the hottest part of the Bunsen burner flame. The entire tube was heated to redness. The loop was allowed to cool for 15 to 20 seconds before it touches the culture. A small amount of the culture was taken from the tube with a sterilized inoculating loop and the microorganisms were streaked in a plate following a quadrant pattern. The stock solutions were equipped following the quadrant by dissolving the compounds in ethanol. The process of inoculation was done under aseptic condition and the spores were vaccinated in the medium and keep warmed for five days. A clear zone or ring on the SDA plate develops, the diameter of which is measured as the zone of inhibition. The antimicrobial activities of the
compounds were recorded by photographing the petri dishes (Ananthanarayan and Paniker, 1978). Fluconazole was used as a positive control.

3.8.3.3 Activity index

The measured zone of inhibition (ZOI) was compared with that of standard namely Gentamicin in antibacterial studies and Fluconazole in antifungal studies. Activity index of each extract was calculated using the formula given by Singariya et al., 2012.

\[
\text{Activity index (AI) = ZOI of sample / ZOI of standard}
\]

3.8.4 Acute toxicity, wound healing and anti-inflammatory studies

Acute toxicity study was performed to elucidate the possible toxic effect of the MM ethanol extract on Swiss mice. Mice were orally administrated a single dose of 100, 250, 500, 750, 1000 and 2000 mg/kg of ethanol extract and the mortality was observed for 14 days post treatment of extract. Ointments prepared with ethanol extract were prepared according to standard procedures and the wound healing efficiency was evaluated on the Albino Wistar rats through incision wound models. The parameters studied include rate of period of wound contraction, skin breaking strength, period of epithelialization and histopathological study of the rat tissue to know the extent of collagen formation in the wound tissue. Anti-inflammatory study of MM and MB ethanol extracts was carried out to determine the inflammation inhibition of crude extract.

Ethical clearance: KMCH College of Pharmacy, Coimbatore-48: KMCRET/PhD03/2010-11 (Appendix 2).

3.8.4a Housing and feeding of animals

Animals used for all the three studies were facilitated with a standard temperature-controlled environment (23 ± 2°C; 12h: 12h (light: dark cycle)). The standard laboratory animal food pellets with water ad libitum feed was supplied to animals during the study period.

3.8.4.1 Acute toxicity studies of the ethanol extract of MM

3.8.4.1.1 Animals

Healthy young adult Swiss Albino mice (25-30g), nulliparous, non-pregnant female animals were kept in groups of 6 per cage.

3.8.4.1.2 Experimental groups

In order to study the possible toxic effect or changes in normal behaviour, six groups (each group containing 6 mice) of mice were used in this experiment. Before commencing the experiment, each animal was assigned a unique identification by marking with paint like head, tail, body, head and body and no mark.

3.8.4.1.3 Procedure
The extract was administered as a single dose using a specially designed mice oral pine needle. Animals were deprived of foodstuff 3h prior to dosing. Subsequent to the period of fasting, the animals were weighed and distributed into six treated groups. Group 1, 2, 3, 4, 5 and 6 were orally administered the dose of 100, 250, 500, 750, 1000 and 2000 mg/kg body weight respectively, of test substance.

After extract administration, foodstuff was withheld for 2 hours. Parameters like tremors, convulsions, lethargy, diarrhoea, salivation, coma and sleep were the direct observation parameters and additional parameters like skin, fur, eyes, mucous membrane, respiratory, circulatory, autonomic, somatomotor activity, central nervous systems and behaviour pattern were also observed. The animals were observed at least once during the first 30min, periodically during the first 24h, with individual attention given in the first 4h and daily thereafter, for a total of 14 days.

3.8.4.2 Wound healing activity of the ethanol extract of MM and a herbal formulation

3.8.4.2.1 Preparation of formulation and topical ointment

Fresh roots of turmeric and flowers of Tanners cassia (Aavaram poo) were shade dried, crushed and powdered. Sandal powder was prepared from original sandal wood and fresh aloe vera was used for the formulation.

**Formulation F1**: 1.6g of MM ethanol extract was mixed with 120mg of Tanners cassia flower powder, 120mg of sandal wood powder, 120mg of aloe vera and 40mg of turmeric powder. This mixture was stored in a proper container and preserved.

**Formulation F2**: 240mg of Tanners cassia flower powder, 240mg of sandal wood powder, 240mg of aloe vera and 80mg of turmeric powder were mixed together to make up the formulation F2.

3.8.4.2.3 Animals and experimental groups

Healthy Albino Wistar rats (young adult) weighing 180 to 220g were randomly separated into 5 groups of 4 animals. Each animal was assigned a unique identification by marking.

- **Group I (G1)** – Control
- **Group II (G2)** – Wound + Standard (Betadine)
- **Group III (G3)** – Wound + Vehicle
- **Group IV (G4)** – Wound + F1
Group V (G5) – Wound +F2

3.8.4.2.4 Wound creation

The predetermined area for an inflicted wound at the back of the animal was prepared for surgery by removing hairs. Group 1 animals did not receive any treatment. Before wound creation G2, G3, G4 and G5 group rats were anaesthetized with ketamine chloride. The skin part of the animal was shaved before incision.

3.8.4.2.5 Incision wound model

Skin incisions of about 5cm length were made through the skin with a sterile scalpel blade. The parted skin was sutured with surgical thread at 1cm intervals using a curved needle (no: 42). The thread was tightened for good closure of the wounds and the wounds were left undressed. The ointments were administered topically to the animals of respective groups until 11th day. The animals were sacrificed on eleventh day and the skin breaking strength of the healed wound was calculated using INSTRON universal tensile testing system.

3.8.4.2.6 Evaluation

Wound contraction, which contributes to wound closure is expressed as a reduction in percentage of the original wound size studied, starting from the day of operation until the day of compete epithelialization and evaluated to calculate the degree of wound healing.

3.8.4.2.7 Percentage of wound contraction

The progressive reduction in the wound area was monitored and the percentage of wound healing was computed at the beginning of experiment and after 4, 7 and 9 days.

\[
\text{\% of wound area} = \frac{\text{Wound area on X}^\text{th day}}{\text{Wound area on the first day}} \times 100
\]

where X = day 1, day 4, day 7 and day 9

\%

of wound healing = 100 - \% of wound area

3.8.4.2.8 Skin tensile strength

The animals were sacrificed on the 14th day and healed tissue was cut out from the animal in strips of 70mm length. The strips were preserved in normal saline and loaded between the upper and lower holder of the INSTRON universal tensile testing machine. The total breaking strength was measured in gram force. The tensile strength was calculated using the following formula.

\[
\text{Tensile strength} = \frac{\text{Total breaking load}}{\text{Cross-sectional area}}
\]

3.8.4.2.9 Histopathological studies
The tissues removed from the animals were preserved separately in 10% formalin and dehydrated with alcohol - xylene and embedded in paraffin mixed paraffin wax (melting point 55-57°C). Serial section of 5µm were cut and stained with hematoxylin and eosin stains. The section was examined under light microscope (LABOMED, Germany) and photomicrographs were taken.

3.8.4.2.10 Statistical analysis
All the data were expressed as mean ± S.E.M. The data on percentage wound healing, wound contraction and tensile strength was statistically analyzed by two-way analysis of variance (ANOVA) followed by Dunnet’s test. The values of \( P < 0.05, P < 0.01 \) and \( P < 0.001 \) were considered statistically significant.

3.8.4.3 Anti-inflammatory evaluation of the ethanol extract of MM and MB

3.8.4.3.1 Animals
Healthy young Swiss Albino male mice (18-32g) were kept in groups of 3-4 per cage.

3.8.4.3.2 Anti-inflammatory studies
Anti-inflammatory study was carried out by adopting the method of Nuhu et al, 2010 with slight modifications. Swiss Albino mice were divided into four groups of 4 mice each. The groups were treated intraperitoneally; group 1 (positive control) received 10mg of ketoprofen per kg body weight of mice, group 2 received 1ml normal saline per kg body weight of mice (negative control), group 3 received 100 mg MM per kg body weight of mice and group 4 received 100mg MB per kg body weight of mice respectively. After thirty minutes, formalin was injected to all the four mice groups and the variation in diameter of the right paw and left hind paw was measured using a Vernier caliper at regular intervals of 1 hour up to 6 hours. The percentage inhibition of the expansion of oedema was calculated as:

\[
\text{% inhibition} = \frac{(\text{St-Sc}) \text{ control} - (\text{St-Sc}) \text{ treated}}{(\text{St-Sc}) \text{ control}} \times 100
\]

St-the mean paw size for each group after treatment
Sc-the mean paw size obtained for each group before injection

3.8.4.3.3 Statistical analysis
All measurements in this study were recorded as mean ± standard error of the mean (SEM) and statistical analysis was done using one way ANOVA using Dunnett’s test values with \( P<0.05 \) considered significant.

3.8.6 Larvicidal and pupicidal activity of the ethanol extracts of MM, MB, MC and MN

3.8.6.1 Larvicidal and pupicidal activity
A 1% stock solution was prepared with 200mg of plant residue in 20ml ethanol and was kept in a screw-cap vial covered with aluminum foil. The stock solution was then serially diluted.
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Phytochemical, Pharmacological and Electrochemical Investigation of Aerial Roots of Rhaphidophora aurea (Linden ex Andre) Intertwined over four different Host Trees

ten-fold in methanol (2ml solution to 18ml solvent) and test concentrations were obtained by adding 0.1–1.0ml of the appropriate dilution to 100ml distilled water (WHO, 2005). One gram of the plant residue was dissolved in 100ml of methanol (stock solution) and considered as 1% stock solution. From this stock solution different concentrations were prepared ranging from 30, 50, 100, 125 and 150ppm respectively.

3.8.6.2 Mosquito rearing and maintenance

*Culex quinquefasciatus* was used to test the larvicidal and pupicidal activity. The eggs were collected from in and around Coimbatore district with the help of ‘O’ type brush and maintained at 27 ± 2°C and at 80% ± 5 relative humidity less than 12 L: 12 D cycles. These eggs were brought to the laboratory and transferred to 18 X 13 X 4 cm size enamel trays containing 500 ml of water for larval hatching. The mosquito larval and pupal culture was maintained in the laboratory. Plastic jars were kept in a 90 X 90X 90 cm size mosquito cage for adult emergence. A wooden cage enclosed with polythene sheets on 4 sides (2 laterals, one back and other one upper) and muslin cloth on the front portion was used in the study. Sugar solution (10%) was kept in the cage for a period of three days. The mosquitoes (adult female) were allowed to feed on the blood of a rabbit (exposed on the dorsal side) for two days. Later, blood feeding trays with water were positioned in the cage for the adults to lay eggs.

3.8.6.3 Larvicidal activity

A laboratory colony of *Culex quinquefasciatus* larvae was used for the study. Twenty-five numbers of first, second, third and fourth instar larvae were kept in a 500ml glass beaker containing 249ml of dechlorinated water and 1ml of desired concentration of plant extracts. Larval food was prearranged for the test larvae. At all tested concentrations (two to five trials) were made each in triplicate. The control was setup by mixing 1ml of acetone with 249ml of dechlorinated water. The larvae exposed to dechlorinated water without acetone served as control. The control mortalities were corrected using Abbott’s formula.

\[
\text{Corrected mortality} = \frac{\text{Observed mortality in treatment} - \text{Observed mortality in control}}{100 - \text{Control mortality}} \times 100
\]

\[
\text{Percentage mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \times 100
\]

3.8.6.4 Pupicidal activity

A laboratory colony of *Culex quinquefasciatus* pupae was used for analyzing the pupicidal activity. Twenty numbers of freshly emerged pupae were kept in a 500ml glass beaker containing 249ml of dechlorinated water and 1ml of desired plant extract concentration. Five replicates were setup for all test concentrations. The control sample was setup by mixing 1ml
acetone with 249ml dechlorinated water. The control mortality was corrected using Abbott’s formula.

\[
\text{Corrected mortality} = \frac{\text{Observe mortality in treatment} - \text{Observe mortality in control}}{\text{100} - \text{Control mortality}} \times 100
\]

\[
\text{Percentage mortality} = \frac{\text{Number of dead pupae}}{\text{Number of pupae introduced}} \times 100
\]

3.8.6.5 Repellent test

The repellent dose - protection time response method was used (WHO, 1996). Approximately 1 hour prior to the start of the test, 3-4 day-old blood-starved female Culex quinquefasciatus (100) were placed into a net cage (45 cm W x 45 cm H x 45 cm L). Then, both arms of a human test subject were washed with ethanol and allowed to air dry. Three doses of MM, MB, MC and MN were tested (1, 2.5 and 5 mg/cm²). A single dose was applied to the forearm skin of a test subject in each test (Venkatachalam et al, 2002). The other forearm was used as a negative control. At the beginning of the test, the control and treated arms were introduced simultaneously into the cage. The number of mosquitoes that landed on the exposed skin on each arm in 3 minutes was recorded at 30 minute intervals between 6.00 pm and 6.00 am. Each dose of plant extract was tested for repellency 5 times. The effectiveness of the extract was assessed by determining the percentage protection provided by the extract against mosquito landing on the treated arm compared with the untreated arm.

3.8.6.6 Statistical calculation

\( \text{LC}_{50}, \text{ LC}_{90}, \) regression equation and 95% confidence limit of lower confidence of limit (LCL) and upper confidence limit (UCL) were calculated using probit analysis (Finney, 1971).