Chapter 3
MATERIALS AND METHODS

3.1. Helminth Parasites

Native bred live fowls (Gallus domesticus Linnaeus, 1758) were used as the source of gastrointestinal parasitic helminths and were readily available from the local abattoirs in Aizawl, Mizoram. The fowls were sacrificed and on autopsy, live worms such as cestodes and nematodes were collected from the intestines. The cestode, Raillietina echinobothrida Megnin, 1880, and the nematode, Ascaridia galli Schrank, 1788, were the two major helminths recovered; thus, a representative species of each phylum of Platyhelminthes and Nematoda, respectively, were obtained. Only adult worms were selected and collected in 0.9 % phosphate buffered saline (PBS: 8 g of NaCl, 0.34 g of KH₂PO₄ and 1.21 g of K₂HPO₄ dissolved in 1 litre of distilled water, pH 7-7.3) and were kept in a glass-chambered incubator (Digital BOD T-801, Biocraft & Scientific Industries, India) maintained at the physiological temperature of 37±1°C.

3.2. Plant Materials

Two perennial climbing shrubs, Millettia pachycarpa Bentham (family Fabaceae; synonym: Millettia taiwaniana Hayata), called ‘Rulei’ by the Mizo natives, and Acacia oxyphylla Graham ex Bentham (family Mimosaceae; synonyms: A. caesia Linnaeus, A. intsia Willdenow), named ‘Khangngo’ by the natives, are two indigenous anthelmintic plants (Fig. 3.1) among the Mizo tribes inhabiting the remotest region of north-east India. These two plants were collected from the nearby forest of Aizawl,
which are predominantly found near the banks of streams, and the barks of the desired plant parts were carefully peeled off.

### 3.3. Preparation of Plant Extracts

The procured barks of *A. oxyphylla* stem and *M. pachycarpa* root were thoroughly washed with distilled water, and then chopped off into fine pieces and dried in a hot air oven at 50°C. A known quantity of each plant was nicely ground using mortar and pestle and separately immersed in beakers containing ethanol (100g/l). They were kept for several days with three changes of the alcohol to garner maximum extract. The resulting ethanolic solutions were refluxed at 60°C for 8 hours. Refluxing was repeated three times. The solutions were filtered through sterilized cotton pad to remove large debris, and then through Whatman filter paper (No. 1). The supernatants were evaporated to complete dryness at 50°C in a hot air oven. The crude ethanolic extracts were obtained as a deep brown precipitate for *M. pachycarpa* root and as a deep green precipitate for *A. oxyphylla* stem. The resultant materials were made to fine powder, which were then refrigerated at 4°C for further processing and experimental use. The net yield from such extraction was 4.04% for *A. oxyphylla* and 7.2% for *M. pachycarpa*.

### 3.4. Fractionation of Plant Extracts

A portion of the crude alcoholic extracts of both the plants were further processed as follows:
Predetermined weights of the crude extracts of both the plants were mixed with methanol in a fractionating flask, with several changes of the solvent and constant vigorous shaking for 24 hours. After the crude extracts completely dissolved, the resultant solutions were filtered through Whatman filter paper (No. 1) and refluxed at 60°C for 8 hours. Refluxing was repeated three times. The solutions were again filtered through Whatman filter paper (No. 1). The solutions obtained were then evaporated to complete dryness at 50°C in a hot air oven. After complete evaporation of the solvent, solid precipitates were obtained, which were marked as methanol extracts and then refrigerated at 4°C. The total yield was 1.35% for *A. oxyphylla* stem bark and 2.5% for *M. pachycarpa* root bark.

Another portion of the crude extract of each plant material was mixed with acetone. Similar processing, refluxing, filtration and evaporation as before yielded the acetone extracts of 0.72% for *A. oxyphylla* and 1.28% for *M. pachycarpa*. The extracts were refrigerated at 4°C.

The same procedure of extraction was applied for benzene, diethyl ether and ethyl acetate, separately for both the plant materials. Unfortunately, neither of the crude extracts of the two plants was soluble in these solvents; the filtrate did not yield any precipitates after complete evaporation. Therefore, no amount of extracts could be prepared for these organic solvents.

### 3.5. Reagents and Chemicals

All the chemicals used in the experiments were of standard analytical grades, procured either from Merck or Central Drug House (CDH), India, except where
otherwise stated. Ethanol was supplied by Bengal Chemicals, Kolkata, India, and the pharmaceutical drugs, albendazole and piperazine hydrates are the products of Glaxo-SmithKline Pharmaceutical Limited, India. Albendazole is a broad spectrum anthelmintic effective against all classes of parasitic helminths, whereas piperazine is specific for nematodes and conventionally employed for poultry ascarids. Rotenone is a product of Sigma-Aldrich Corporation, St. Louis, Missouri, USA. Rotenone is a known constituent of *M. pachycarpa* (Singhal *et al.*, 1981) but unknown in terms of activity on helminths. The chemicals *p*-nitrophenol and *p*-nitrophenyl phosphate were obtained from S.D. Fine-Chemicals Limited, Mumbai, India. Haematoxylin and eosin were purchased from Qualigens Fine Chemicals, Mumbai, India.

3.6. **In Vitro Test for Anthelmintic Activity**

1 h prior to experimental assay, solutions of varying concentrations such as 0.5, 1, 2, 5, 10 and 20 mg/ml, of the three extracts, viz ethanol, methanol and acetone, of each of *A. oxyphylla* and *M. pachycarpa*, and rotenone were prepared in separate petri dishes by dissolving them in 0.9% PBS supplemented with 1% dimethylsulfoxide (DMSO). Similar concentrations were also prepared for broad-spectrum anthelmintic drugs albendazole and piperazine as standard references.

The freshly collected cestode and nematode worms were immediately incubated at 37±1°C. Batches of 3 worms each of cestode and nematode with approximately the same size were introduced into each concentration of all the three extracts of both *A. oxyphylla* and *M. pachycarpa*. Each incubation medium consisted of 5 replicates. Similar batches of both cestodes and nematodes were maintained in media containing only PBS with 1% DMSO to serve as control experiment. Same dosages and
treatments of cestodes were performed for albendazole and of nematodes for piperazine as the standard reference drugs.

Anthelmintic efficacies of the preparations were assessed in terms of motility, survival and histomorphological alterations, if any, of the worms. Motility and paralysis were determined visually by physical provocation of the worms. Complete immobilization and death were substantiated by dipping the worms in tepid PBS (40-50°C), which induced movement in sentient worms. Parasite death was defined as no further motor activity observable after such stimulation. The time duration between the introductions of the worms to the solutions and their respective paralysis and death were recorded in order to evaluate the differential survivability of worms in the different media.

3.7. Data Analyses

Results recorded were subjected to statistical analyses using Microsoft Excel 2003 and Biostat 2007, a product of Analyst-Soft, Vancouver, Canada. All the data were presented as means plus or minus the standard error (SE) of the mean. Comparison of the mean values of the experimental treatments against those of the control groups was made using unpaired Student’s t-test, and the level of significant probability considered at $P < 0.05$.

3.8. Histological Processing for Light Microscopy

From each group, a set of worms taken directly from the experiment was fixed in Bouin’s fluid overnight. After completely removing the fixative under running tap
water, the specimens were dehydrated through a series of graded alcohols up to absolute ethanol. After complete dehydration, they were treated with a mixture of xylene and clove oil, and cleared in pure xylene. After complete infiltration with molten wax and embedding in solid paraffin, they were trimmed into rectangular blocks for microtomy. Sections were cut at 7-9 µm thickness using Erma Japan type Rotary microtome (Biocraft & Scientific Industries, India). The sections were then deparaffinized with pure xylene followed by another round of complete dehydration, double staining with eosin and haematoxylin, and finally mounted on glass slides with DPX for histological observation. Photomicrographs of the sections were taken with Zeiss image analyzer HBO 50.

3.9. Morphological Preparation for Scanning Electron Microscopy (SEM)

Another set of worms from each group was thoroughly washed in 0.85% normal saline and then fixed in 4% or 10% neutral phosphate buffered formaldehyde at 4°C at least for 24 h. After post fixation in 1% buffered osmium tetraoxide for 1 h, the worms were washed with PBS. Subsequent dehydration was carried out through ascending concentration of acetone up to pure acetone. Following the standardized scanning electron microscopic methods specific for helminth parasites described by Dey et al. (1989) and Roy and Tandon (1991), the specimens were treated with tetramethylsilane (TMS: [CCH₃]₄Si, boiling point 26.3°C, surface tension 10.3 dynes/cm at 20°C) for 10 minutes and then allowed to dry at room temperature (25°C). The dried materials were placed on metal stubs according to the required orientation and sputter-coated with gold in a fine-coat ion sputter, JFC-1100 (JEOL). The gold-coated specimens were
observed under scanning electron microscope (LEO 435 VP) at an electron accelerating voltage of 20 kV.

3.10. Biochemical Analyses

For all biochemical tests, only *R. echinobothrida* treated with 20 mg/ml each of albendazole and the different extracts of the two plants were used for comparison with the control worms since the most profound anthelmintic effects were noted at this concentration.

3.10.1. Trace metals

Fresh worms in control group were directly taken for analyses of their trace metal contents. Cestodes in different treatment groups were harvested immediately after they succumbed to paralytic state, and thoroughly washed with deionised double distilled water. Whole worms were quick-dried in an incubator set at 50°C. The dried worms were finely pulverized to powder. The powdered tissue (2 g) was digested in 10 ml of concentrated HNO₃ in an air-tight corked conical flask for overnight at 50°C. The fully digested solution was transferred to and kept on a hot plate at 70°C for ~2 h to allow complete evaporation of the acid. After cooling down, 10 ml of deionised water was then added, vigorously mixed with magnetic stirrer, and filtered through Whatman filter paper (110 mm Φ). The volume was finally made to 100 ml by adding more deionised water to the filtrate. The final solution was directly used for quantitative measurement of the trace elements using a single beam atomic absorption spectrophotometer (Chemito AAS-201, India) at the absorbance wavelengths of 422.6
nm for calcium, 285.2 nm for magnesium, 589.0 nm for sodium, and 766.5 nm for potassium.

3.10.2. Acid phosphatase (AcPase)

The AcPase activity was estimated using p-nitrophenol product from an enzyme source following the method as described by Plummer (1988) with slight modification in the concentration of the buffer and substrate.

1 g of the cestode tissue directly taken after incubation was homogenized in 10 ml of ice-cold (0±2°C) buffer composed of 125 mM sodium acetate and glacial acetic acid at pH 4.5 using a Potter-Elvehjem motor-driven glass homogenizer fitted with a teflon pestle (REMI motor, India). This 10% (w/v) tissue homogenate was filtered through cotton pad and the filtrate was centrifuged at 5,000 rpm at 4°C for 20 minutes in a cooling centrifuge (REMI C4, India). The supernatant obtained was used as enzyme source for estimation of AcPase.

Standard nitrophenol (100 mM) was prepared by dissolving 96.75 mg of p-nitrophenol in 5 ml of double distilled water. 1 ml of the substrate mixture contained:

\[
\begin{align*}
\text{Sodium acetate, pH 4.5} & \quad 125 \ \mu\text{mole} \\
\text{p-Nitrophenyl phosphate} & \quad 62.5 \ \mu\text{mole}
\end{align*}
\]

A set of the substrate mixture was preincubated for 5 minutes at 37±1°C before the tissue extract was added to initiate the reaction. 0.2 ml of the 10% tissue extract was then added and thoroughly mixed. 5 ml of 0.02 N NaOH solution was immediately added to one set to stop the reaction to be used as zero time assay (blank). The other set was incubated for 10-15 min at 37°C after which the reaction was stopped by
adding 5 ml of 0.02 N NaOH solution. The absorbance of both the blank and incubated solutions was measured at 405 nm in UV-VIS Spectrophotometer (Systronics model 119, India). The amount of p-nitrophenol produced was calculated from a linear standard graph plotted for increasing concentrations (0.1-1.0 mM) of standard nitrophenol solution.

Total protein was estimated following the method of Lowry et al. (1951) using Folin-Ciocalteau reagent and bovine serum albumin as the standard protein.

One unit of AcPase activity was defined as that amount which catalyzed the formation of 1 mM of p-nitrophenol/h at 37°C. The enzyme activity was expressed as the total activity (units/g wet wt of tissue) and specific activity (units/mg protein).

### 3.10.3. Alkaline phosphatase (AlkPase)

The AlkPase activity was estimated following the same method of Plummer (1988). 1 g of the helminth tissue directly collected after incubation was homogenized in 10 ml of ice-cold (0±2°C) buffer composed of 50 mM Sodium glycine using a Potter-Elvehjem motor-driven glass homogenizer fitted with a teflon pestle (REMI motor, India). This 10% (w/v) tissue homogenate was filtered through cotton pad and the filtrate was centrifuged at 5,000 rpm at 4°C for 20 minutes in a cooling centrifuge (REMI C4, India). The supernatant was used as enzyme source for estimation of AcPase.

1 ml of the reaction mixture contained:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
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<tbody>
<tr>
<td>Sodium glycine, pH 10.5</td>
<td>0.1 μmole</td>
</tr>
<tr>
<td>p-Nitrophenyl phosphate</td>
<td>31.25 μmole</td>
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The assay procedure was similar to that for AcPase. 0.1 ml of 10% tissue extract was used. Incubation was carried out at 37±1°C and the reaction was stopped by adding of 0.02 N NaOH. The decrease or increase in absorbance at 405 nm was recorded and the amount of p-nitrophenol produced was calculated from linear standard graph prepared using different concentrations (0.1-1.0 mM) of standard nitrophenol solution.

Total protein was estimated following the method of Lowry et al. (1951) using Folin-Ciocalteau reagent and bovine serum albumin as the standard protein.

One unit of AlkPase activity was defined as that amount which catalyzed the formation of 1 mM of p-nitrophenol/h at 37±1°C. The enzyme activity was expressed as the total activity (units/g wet wt of tissue) and specific activity (unit/mg protein).
Fig. 3.1. Photographs of the plant materials used.
