Chapter - III

Materials and Methods
MATERIALS AND METHODS

3.1.0 PLANT COLLECTION AND THEIR IDENTIFICATION

Plant collection and their identification was based on literatures as evident by Ethnovet Heritage (Jayvir et al., 2004), Axamar Gos Gosoni (Dutta, 2004), Swachitra Bonohsadhi (Sarma, 1991) and Dictionary of Economic and medicinal plants (Dutta, 1985) and authenticity was confirmed from Botany Department, Gauhati University, Guwahati, Assam.

3.1.1 Preparation of crude plants

Leaves of plant having known or unknown medicinal value were collected, thoroughly washed in distilled water and dried over a plastic net at room temperature. Then the leaves were kept in a hot air oven maintained at 40°C. When the leaves became crisped, they were grinded and sieved through a fine plastic net to get fine powder. The fine leaf powder of each plant was packed in a sterile plastic jar, plugged with a cover, labeled and kept in the deep freeze (-20°C) until use.

3.1.2 Preparation of methanolic crude plant extracts

Crude extract of each plant was prepared by extracting 10 g leaf powder with 100ml of methanol in a screw capped glass bottle. The suspension
was frequently stirred and left for 48 hours at room temperature. It was then filtered through Whatman (No.1) filter paper. The residue was treated with fresh methanol for several times till the filtrate was clear and colourless. The filtrate was evaporated at room temperature under the laminar flow. The residual methanol extract was weighed, suspended in triple distilled water (100 ml) and transferred to a sterile glass bottle, plugged and labeled and kept in a deep freeze (-20°C) until further use. Such water extracts, subsequently referred as “plant extract” were used for screening tests.

3.1.3 Preparation of discs for antimicrobial screening.

Sterile discs of 6 mm diameter (HI-MEDIA, Mumbai) were employed for soaking a measured quantity of freshly prepared plant extract. Each sterile disc was soaked in approximately 20 μl of plant extract containing approximately 200 μg of crude plant extracts. The soaked discs of individual plant extract were kept in an incubator for 30 minutes at 45°C for drying. The dried discs were transferred into sterile bottle of 10 ml capacity, then plugged with sterile lid, labeled and kept in refrigerator (4°C) for further use. The testing of the disc was completed within a month.

3.1.4 Microbial strains for screening antimicrobial activities

The extract of individual plant was screened against 10 pathogenic micro-organisms isolated from various infectious conditions of livestock and
birds. These isolates were purified and identified through the courtesy of Microbiology Department, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam. The micro-organisms included in this study were *Escherichia coli* (0157), *Salmonella enterica gallinarum*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Clostridium perfringens*, *Bacillus anthracis* (Vaccine seeds), *Pasteurella multocida* (Vaccine seeds-P-52), *Diplococcus pneumonae* and *Aeromonas arginosa*. The pure cultures were maintained on Nutrient Agar slants by subculturing at an interval of 6 weeks.

3.1.5 Screening of antimicrobial activities

The agar disc diffusion method was employed for the determination of antimicrobial activities of plant extracts as per method of NCCLS 1999 (National Committee for Clinical Laboratory Standards, 1999). Briefly, bacterial strains were cultured overnight in Mueller Hinton broth (MHB, HIMEDIA) at 37°C. About 0.1 ml of specific fresh culture was transferred to a sterile test tube containing 2 ml of sterile MHB. The inoculum of the specific microbial strain was then spread on the solid Mueller Hinton Agar (MHA) medium in Petri-dishes.

The filter paper discs impregnated with extracts of individual plant were placed on the inoculated plates of MHA with proper identification mark.
After keeping at 4°C for 2 hours, the plates were incubated at 37°C for 24 hours aerobically with the exception of clostridial organisms which were incubated anaerobically with anaerobic gas pack (Himedia, LEO 02A) having indicator in anaerobic jar (3.5 L). Standard antibiotic discs (HIMEDIA) of gentamicin, enrofloxacin, cephalexin and ampicillin were also used in parallel experiments. All the tests were performed in duplicate and the diameter of the zone of inhibition were measured in millimeters using a standard scale (Hi- Media).

3.1.6 Measurement of antimicrobial disc susceptibility of individual plant extract

The antimicrobial disc susceptibility (ADS) of the individual test organism was accessed on the basis of the extent of the diameter of the inhibitory zone produced by the test plant extracts as per method of the National committee for clinical laboratory standards (NCCLS, 1998) as follows:

<table>
<thead>
<tr>
<th>Diameter of the Inhibitory zone in mm</th>
<th>Susceptibility</th>
<th>Reading</th>
<th>Scoring</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inhibitory zone</td>
<td>(−) sensitivity</td>
<td>Nil</td>
<td>0</td>
</tr>
<tr>
<td>10 mm or &lt;10 mm zone size</td>
<td>(+) sensitivity</td>
<td>Low sensitivity</td>
<td>1</td>
</tr>
<tr>
<td>&gt;10 mm to 14 mm</td>
<td>(++) sensitivity</td>
<td>Medium sensitivity</td>
<td>2</td>
</tr>
<tr>
<td>15 mm and more</td>
<td>(+++) sensitivity</td>
<td>High sensitivity</td>
<td>3</td>
</tr>
</tbody>
</table>
In addition to it, a cumulative numerical score of the individual test plant against each test micro-organism-organism was assigned to group the test plants into the following categories:

<table>
<thead>
<tr>
<th>Cumulative scoring of the test plant</th>
<th>Group of the antimicrobial plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Plant with no antimicrobial activity</td>
</tr>
<tr>
<td>1 to 10</td>
<td>Plant with Low antimicrobial activity</td>
</tr>
<tr>
<td>11 to 14</td>
<td>Plant with medium antimicrobial activity</td>
</tr>
<tr>
<td>15 and more</td>
<td>Plant with high antimicrobial activity</td>
</tr>
</tbody>
</table>

3.1.7 **Selection of the most potent antibacterial plant extracts**

The most potent antibacterial plant extract was selected on the basis of the highest scores which was obtained from the antibacterial screening of the selected plants.

3.1.8 **Selection of some parts of the most potent antibacterial plant.**

Different plant parts at different stages of growth of one most potent antibacterial plant were subjected to antimicrobial screening against selected strains of micro-organisms (Para 3.1.4) as per the method mentioned in para 3.2.5. Different plant materials includes: immature and mature leaves, fruits and barks.
3.1.9 Fractionization of the most potent antibacterial plant extracts and follow-up screening of the fractions for antibacterial activities

The methanolic extract of the most potent plant extracts was immediately suspended in triple distilled water (1:2) and partitioned with chloroform (CH Cl3) (2:1) to obtain water soluble (polar) and water insoluble (non-polar, chloroformic) sub-fractions and evaporated to obtain desired concentration (i.e. 200 mcg) in triple distilled water and dimethyl sulphoxide (DMSO) respectively and stored at 4°C. Each subfraction was screened for antimicrobial activities by following standard procedure as described in para 3.1.3 to 3.1.5.

3.1.10 Determination of minimum inhibitory concentration (MIC) of subfraction having antibacterial activities

The minimum inhibitory concentration (MIC) was determined for the subfraction of the most potent plant extracts as per method as recommended by NCCLS (National Committee for Clinical Laboratories Standards, 1999). All test were performed in Mueller Hinton broth (MHB), supplemented with Tween 80 (final concentration of 0.5%, v/v). Bacterial strains (described in Para 3.1.4) were cultured overnight at 37°C in Mueller Hinton agar (MHA) Test strains were suspended in MHB to obtain a final density of 5 x 10^5 cfu/ml which was confirmed by viable counts. Dilution of the selected subfraction of the plant extract ranging from 0.162 mg/ml to 40 mg/ml (membrane filtered) were
prepared in 52 tubes, including one growth control (MHB + Tween 80) and one sterility control (MHD + Tween 80 + test fraction). The tubes were incubated at 37°C for 24 hours (except clostridial culture). The clostridial cultures were incubated in anaerobic jar at 37°C for 24 hours. The bacterial growth was indicated by the presence of a white pellet in the bottom of the culture tube. Bactericidal growth was confirmed by stick culture on MHA plates of the corresponding serial dilution.

3.1.11 *In vivo* safety test of the most potent fraction

*In vivo* safety test was conducted on rabbits, calves and chicks as per method described by Diechmann and Le Blanc (1943).

**Rabbits** : Twelve (12) rabbit of either sex weighing 400-450 gm body weight

**Calves** : Twelve (12) bull calves weighing 30-40 kg body weight under 6 months old.

**Chicks** : Twelve (12) chicks of either sex weighing 200-250 g body weight of 6 weeks old.

The rabbit, calves and chicks were maintained under standard operative procedure and allowed standard feed and water as per norms of farm practices and kept for 7 days on quarantine. Following quarantine period, each type of animal was randomly divided into 2 groups of 6 individuals. Group 1 of each species comprising 6 animals was kept as control. Group 2 of each species comprising 6 animals received selected subfraction of one plant extracts (5 g/kg
body weight) daily for 9 days. The effect of the selected subfraction on body posture, appetite, food intake, thirst, motility, urination and defecation were observed for 7 days of last receiving the subfraction.

3.1.12 Study of cytotoxic effect of the most potent fraction on PK-15 Cell

The cytotoxic effect of the most potent subfraction of the antimicrobial plants was studied in continuous cell line i.e. PK-15 cell. The cell line was maintained at National Fellow Laboratory of the Department of Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara and it was selected because of its adoptability and availability.

The cells were grown in 24 wells tissue culture plates (NUNC). Confluently grown cells were washed with Eagle’s minimum essential medium containing antibiotics (Streptomycin + benzyl penicillin + fungigone) and without foetal calf serum. The subfraction of one antimicrobial plant was diluted in PBS and 1 µl of diluted subfraction having concentration of 100 to 300 mcg was added to 12 of the tissue culture wells and 12 wells were kept as control for each range. The subfraction was allowed to adsorb for 30 minutes after which the Eagle’s minimum essential medium containing 2% foetal calf serum was added to each of the wells. The cells were observed daily for 5 days using a binocular inverted microscope to record any change in growth and morphology of the cells in the treated as well as control set.
3.1.13 *In vivo* potency study of the most potent plant extracts

**Test animals:**

Eighteen (18) mice of either sex weighing 15-18 g body weight.

**Test organisms:**

One species of test organisms known to be pathogenic for mice with known MIC was utilized for *in vivo* test.

**Test plant extract:** Selected fraction of one most potent plant extract.

**Preparation of inoculum:**

Two sets of culture of test organism were prepared by adding 0.1 ml of $10^8$ cells/ml to test tubes containing nutrient broth, one set received selected fraction of plant extract corresponding to the amount in MIC (determined in para 3.3.3), while the other set have only test organism in nutrient broth. The 2 sets (one having NB + Organisms + selected fraction of plant extracts and other having NB + organisms) were incubated at 37°C for 24 hours.

**Inoculation:**

Eighteen (18) Nos. of mice were randomly grouped into 3 having 6 mice in each group as per method of Diechmann and Le Blanc (1943). One group (Group 1) received 0.3 ml of normal saline i/p and served as control, while the other group (Group 2) received 0.3 ml of 24 hours growth culture s/c and the
last group (Group 3) received 0.3 ml of culture containing selected fraction of one plant extracts. All the groups were observed for 5 days and any mortality was recorded.

3.1.14 *In vivo* immunomodulatory effect of the most potent fraction

Twelve (12) calves were utilized for studying *in vivo* immunomodulatory effect. These included 6 male and 6 female calves under 1-2 years old. The animals were randomly divided into 2 groups with equal proportion of age and sex. One group (Group 1) was kept as control while other group (Group 2) received 25 g of dried leaves powder equivalent to 1 g of selected fraction daily for 7 days. On 8th day all the animals of both the groups were vaccinated using standard antigen of *Pasteurella multocida* (P-52) as per recommended doses prescribed by IVB, Khanapara. Blood from individual calf was collected on 28th day after vaccination and serum was separated for estimation of antibody titre. The indirect ELISA was performed as per the method of Yu *et al.* (1988) and modified by Sarma and Sarma (1995). Briefly the ELISA plates (Nunc Polysorp) were coated with *Pasteurella multocida* antigen (vaccine antigen) using 1:50 dilution in carbonate/bicarbonate buffer and kept at 4°C overnight. The coated plates were washed with phosphate buffer saline containing 0.05% Tween 20 (PBST). The test serum samples were diluted starting from 1:10 to 1:1280 in the coated plates as well as in antigen uncoated ELISA plates using blocking buffer (PBST containing 5% Horse serum) and
incubated at 37°C for 1 hour. The plates were washed and antibovine immunoglobulin peroxidase conjugate (Sigma) was added using dilution of 1:2000 in the blocking buffer and incubated for 1 hour at 37°C. The plates were again washed. Hydrogen peroxide (H₂O₂) and Orthophenylene diamine dihydrochloride (OPD) mixture was then added and the reaction was stopped after 10 minutes using 1M H₂SO₄. The reading of the plates was taken in ELISA reader (Dynatech). The highest dilution of serum which showed OD value of 0.1 or above after subtracting the OD value of serum in the corresponding dilution in the antigen coated plates was taken as the titre.

3.1.15 In vivo challenge test of the most potent selected fraction

Eighteen (18) nos. of 10 days old chicks of either sex were utilised for studying the in vivo challenge effect. The chicks were randomly divided into 3 groups of 6 chicks. All the 3 groups received standard feed and filter sterilized drinking water and housed in proper hygienic condition.

Group 1 was kept as control. Group 2 received ad libitum drinking water containing selected fraction of plant extract (2.5 mg/ml) while Group 3 received only plain drinking water. On 3rd day 5 ml of a 24 hours old mixed cultures containing pathogenic Escherichia. coli (0157), Salmonella enterica gallinarum and Diplococcous pneumonaæ were mixed with the daily ration (feeds and drinking water) of Group 2 and Group 3 for 3 consecutive days.
Following 7\textsuperscript{th} day onward the Group 2 were supplemented with the selected fraction of plant extract in every alternate day for another one week (upto 14 thdays). All the chicks were observed upto 21 days of post infection. Any clinical onset of disease, mortality and postmortem findings were recorded.

### 3.1.16 Presentation of the most potent fraction of one antibacterial plant extracts

The selected fraction of the plant extract was presented in two preparations.

(a) One liquid preparation of 50 ml bottle containing 100 mg/ml selected fraction of the plant extract for oral use.

(b) One lotion preparation in liquid paraffin containing 5\% selected fraction of the plant extract for topical use.

### 3.1.17 Clinical trials

Clinical trial of the selected fraction were done on animal and man as per indication of the presented sample by adopting procedure of treating outpatient on the basis of clinical symptoms and results were recorded. Written permission was obtained from the patients before starting the treatment with the plant extract.