CHAPTER - IV

MATERIALS AND METHODS
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4.1 STUDIES ON THE DIVERSITY OF MOSSES

A systematic collection was made periodically (2003-2006) covering different seasons, during and after monsoon showers so as to facilitate completeness in survey and inclusive of various stages of life-cycle.

For the collection of mosses in general, no special technique is required. However for a proper collection certain precautions were taken, to collect with delicate handling of fragile plant parts. Plants are gently scraped out from the substrates with the help of sharp edged knife, since some mosses adhere very firmly to their substratum, as patches or tufts. A chisel was used to cut or peel patches of moss colonies from hard substrata of earth, rock or logs. Specimens were collected avoiding weeds, since many species are mixed with herbaceous weeds. The collected materials were washed gently to remove the soil adhering to them, without damaging the fragile parts like calyptra, operculum of gemmae. Finally the collected parts are carefully squeezed or pressed out without damaging the sporophyte.

Epiphytic species were collected by climbing up the trees. The canopy species, which were not easily accessible, were collected from the fallen branches. The plants which were strongly adherent to the substratum
were collected with a portion of the bark and rock respectively. The materials were collected in the polythene bags. The geographical distribution, biological features, edaphic factors, climatic conditions, water and light interests were recorded in the field note. Photographs were taken whenever necessary to show their habit and habitat.

The collected specimens were teased lose from each other carefully and preserved without any damage to the specimens. The herbaria were prepared by air drying at room temperature; highly wet materials were kept spread between sheets of blotting paper without applying weight or pressure to avoid rupture and destruction of sporophyte and other critical morphological features of the specimens. These blotting papers were changed in quick intervals to quicken the drying.

The dried specimens were stored in brown paper packs of dimensions 5" × 4". Poisoning is not necessary as they are good self repellants. However naphthalene balls were used to keep the sporophyte from insect attacks. The packets were labeled with the informations like the name of genus, species, habit including substratum, elevation, vegetation type in which it occurred, the host plant, geographical locality, name of the district, state etc. collector’s name, date of collection and collection number.

For studies and observations the materials were soaked in hot water for about 10 to 15 minutes to retain turgidity. Identifications were made with the help of Gangulee’s ‘Mosses of Eastern India and Adjacent Regions’ (1969-1980) and other related works and also by comparing with
protologues, types and or authenticated specimens. The final identification and confirmation were done in consultation with Bryologists of the University of Calicut, Kerala.

Individual species were described after careful dissections and observation made under stereo binocular microscope for the analysis of leaf, peristome and capsule characters. Every attempt has been made to note the gametophyte characters. Many species are not always available with sporophytes and some do not produce sporophyte at all. Protographs are provided for most species as far as possible. In addition diagrammatic illustrations have been made for all the species. The herbarium packets are kept in small containers and deposited in the Rapinat Herbarium, St. Joseph’s College (Autonomous), Tiruchirappalli, Tamil Nadu, India.

4.2 **ANTIMICROBIAL STUDIES**

*Plant materials*

Plant materials were collected from the localities as mentioned above and identified. After thorough washing, they were shade dried at room temperature. The whole plant was used for the study.

*Extraction procedure*

The air dried plant materials were crushed into fine pieces, not made into fine powder. From this 50 gm were taken in the soxhlet apparatus and allowed for the percolation of solvents in the ratio 1:6 (w/w) at the boiling temperature of the solvents used. This process was continued for 48 hours.
The extract was collected and condensed using vacuum rotary evaporator at 60° C. The final pure extract was collected in labelled sterile universal bottles and kept in refrigerator at 4° C. The solvents used for the extraction were water, ethanol, chloroform and ethyl acetate.

Microorganisms

The following microorganisms were used for the screening. Bacterial strains include both Gram-positive and Gram-negative, Bacillus cereus, Streptococcus faecalis and Staphylococcus aureus of Gram-positive and Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Pseudomonas aeruginosa and Proteus vulgaris of Gram-negative bacteria. The fungal strains include non-dermatophytic fungi such as Aspergillus niger, A. flavus, A. parasiticus and A. fumigatus, a dermatophytic fungi Microsporum gypseum, a plant pathogen Geotrichum candidum and one yeast species Candida albicans.

These microorganisms were obtained from microbial type culture centre, Chandigarh, Punjab. The bacterial strains were maintained in Nutrient Agar (Hi-media) Laboratories Pvt. Ltd., Mumbai and the fungal strains were maintained on Potato Dextrose Agar Slants (Hi-media). The strains were subcultured bimonthly. The cultural strains were stored at 5° C for further analysis after which it was allowed to grow for a week.
Bioassay procedures

The antimicrobial activity of the aqueous and other solvents extracts were analysed by means of different assays against bacteria and fungi. They were Streak Plate Disc Diffusion and Agar Well Diffusion methods.

4.2.1 Antibacterial Assays

4.2.1.1 Stroke Method (Orzechowski, 1972)

Principle

The inhibitory effect of plant extract can be easily rated by this stroke method with unaided eye. The result of this test was usually rated as strong inhibition where no growth of the bacterium occurs, partial inhibition where less growth of the bacterium occurs, partial inhibition where less growth of the bacterium than the normal occurs and no inhibition where full growth of the bacterium occurs. Once an extract showed a definite inhibitory effect, further testing was carried out by means of disc diffusion assay of agar well diffusion assay.

Procedure

Nutrient agar medium was prepared and 8 ml of the molten agar medium and 2 ml of the plant extract were mixed thoroughly and the mixture was poured into a sterile petridish under aseptic condition. It was cooled at room temperature. After cooling, the bacterial culture was taken (24 hr. old) and using an inoculation needle, the bacterial culture was applied on the surface of the agar medium in the form of parallel strokes.
(streaks). For each bacterium triplicates were used. The plates were incubated at 37° C for 24 hr. After 24 hr, the results were rated as strong inhibition (if no growth of the test bacterium) partial inhibition (if less growth of the bacterium than the normal) and no inhibition (if full growth of the test bacterium). Control plates without the plant extract were also maintained for reference.

4.2.1.2 Disc-diffusion Test (Maruzzella and Henry, 1958)

The disc diffusion method provides a simple and reliable test in routine clinical microbiology in order to find out the effect of a particular substance on specific bacterium. This method consists of impregnating small circular discs of standard filter paper with given amount of chosen concentration of substance. The discs are placed on the plates of culture medium previously spread with bacterial inoculums to be tested. After incubation the degree of sensitivity is determined by measuring the incubation zone produced by diffusion of the antibiotic substances from the discs of the surrounding medium.

**Preparation of discs**

Discs usually consisted of absorbent paper impregnated with the compound (plant extract). It is most convenient to use Whatman No 1 filter paper for preparing the discs. Dry discs of 6 mm diameter were prepared from Whatman No 1 filter paper and sterilized in an autoclave. These dry discs were used for the assay.
Procedure

Circular discs of 6 mm diameter were prepared from Whatman No 1 filter paper and sterilized in an autoclave. These paper discs were impregnated with test compounds (plant extract) in the respective solvents for overnight and placed on nutrient agar plates seeded with the test bacterium. The plates were incubated at 37° C for 24 hr. After 24 hr the zone of inhibition around each disc was measured and the diameter was recorded. Gentamycin (10 mcg/disc) was used as the reference. A negative control was prepared using only the solvent used for extraction and kept for comparison. The tests were repeated 4 times to ensure reliability of the result.

4.2.1.3 Agar Well Diffusion Method (Perez et al., 1990)

Agar well diffusion method is also known as Hole Plate Diffusion Method (Brantner et al., 1993) or Cup Diffusion Method (Vikas Dhingra et al., 1999).

Principle

It is an important method for studying the inhibitory effect of any compound (plant extract or antibiotics) on the growth and multiplication of a particular bacterium. Here well or cups are made using a sterilized cork borer on the seeded nutrient agar in a petridish to which the test compound is added. The treated petridishes are incubated at 37° C for 24 hr. The inhibition zone formed around each well indicates the antimicrobial activity.
**Procedure**

Nutrient agar was used as the culture medium for this assay. The molten nutrient agar was dispensed in pre-sterilized petridishes (25 ml each) and allowed to cool. These agar plates were homogenously inoculated with the test bacterium previously suspended in tryptose broth ($10^6$ cells/ml). The plates were allowed to solidify. After solidification holes/wells (cups) of 6 mm diameter were punched into the agar with the help of flamed cork borer. Five wells were prepared for each plate. Of these five, three holes were filled with 0.2 ml of the plant extract and the fourth hole was filled with 0.2 ml of standard antibiotic solution (Gentamycin, 500 µg/ml) and the fifth hole was filled with blank (extracting solvent alone). The petridishes were incubated at 37° C for 24 hr. After this incubation period the diameter of the inhibition zone formed around each hole (well/cup) was measured and the values were recorded. The antimicrobial activity was expressed as the ratio of the inhibition zone produced by the plant extract and the inhibition zone caused by the standard. Two sets of control were used. One control was the organism control where standard antibiotic solution was used and the other control was the blank where only the extracting solvent was used. This was just to ensure the validity of the test. Testing was carried out for each bacterium in quadruplicates.

4.2.2 Antifungal Assays

The antifungal activity of the plant extract was determined by the following methods.
4.2.2.1 Streak Plate Method (Orzectowski, 1972)

Principle

The inhibitory effect of plant extract can be easily rated by this stroke method with unaided eye. The result of this test was usually rated as strong inhibition where no growth of the fungus occurs, partial inhibition where less growth of the fungus than the normal occurs and no inhibition where full growth of the fungus occurs. Once an extract showed a definite inhibitory effect, further testing was carried out by means of disc diffusion assay or agar well diffusion assay.

Procedure

Two millilitre of different concentration of plant extracts using various solvents (0%, 25%, 50%, 75% and 100%) were taken into 5 petridishes. Potato dextrose agar medium about 15-20 ml were poured in each petridish containing plant extracts using different solvents and various concentrations. They were mixed thoroughly. Medium was allowed for solidification. With the help of inoculation loop dipping in the particular fungal strain, streak was made in each petridish on the solidified potato dextrose agar medium. They were incubated at room temperature. After 48 hours, observations were recorded.
4.2.2.2 Disc Diffusion Method (Maruzzella and Henry, 1958)

**Principle**

The disc diffusion method provides a simple and reliable test in routine clinical bacteriology in order to find out the effect of a particular substance on a specific fungus. This method consists of impregnating small circular discs of standard filter paper with given amount of a chosen concentration of substance. The discs are placed on plates of culture medium previously spread with a fungal inoculum to be tested. After incubation the degree of sensitivity is determined by measuring the inhibition zone produced by the diffusion of the antibiotic substances from the discs into the surrounding medium.

**Preparation of Discs**

Discs usually consisted of absorption paper impregnated with the compound (plant extract). It is most convenient to use Whatman No 1 filter paper for preparing the discs. Dry discs of 6 mm diameter were prepared from Whatman No 1 filter paper and sterilized in an autoclave. These dry discs were used for the assay.

**Procedure**

Autoclaved liquid potato dextrose agar was taken into each petridish about 15-20 ml. The medium was allowed to solidify. One ml of each fungal strain (diluted in distilled water) was taken and put on the solidified medium. Using ‘L’ rod, each strain was spread over the medium. Three submerged discs were thus taken using sterilized forceps and inoculated on
the medium. They were incubated at room temperature (37° C) for 48 hr. The observations were recorded.

4.2.2.3 Agar Well Diffusion Method (Perez et al., 1990)

**Principle**

It is an important method for studying the inhibitory effect of any compound (plant extract or antibiotics) on the growth and multiplication of a particular bacterium. Here wells or cups are made using a sterilized cork border on the seeded potato dextrose agar in a petridish to which the test compound is added. The treated petridishes are incubated at 37° C for 48 hr. The inhibition zone formed around each well indicates the antifungal activity.

**Procedure**

About 0.2 ml of fungal cultures was added to 15 ml of molten potato dextrose agar mixed well and poured into a sterile petridish and allowed to set. A sterile cork borer (6 ml dia was used to make wells in the set agar). 0.2 ml of plant extracts were incubated overnight at 37° C for 48 hr. Antifungal activity was recorded only when a zone of growth inhibition around the agar well was greater than 6 mm. Any extract showing inhibition was tested in duplicate.

In this method petridish were inoculated with selected microbes along with culture medium. After solidification three wells made in the agar plate each in the depth. The well was filled of 2 cm and width 6 mm in diameter.
The well was filled with plant extract. The antifungal activity of the plant extract was evaluated by measuring the inhibition zone diameter observed around the well.

4.3 PHYTOCHEMICAL STUDIES

4.3.1 Infrared Spectra

IR spectroscopic analysis is a very useful tool in the detection of functional groups of bio-molecules, thus aiding in their structural elucidation.

Principle

IR spectroscopy is based on the study of the reflected, absorbed or transmitted radiant energy in region of electromagnetic spectrum ranging from wavelength 0.8 to 500 nm. A more commonly used measurement is the frequency and is expressed in wave number. The IR spectrum is usually divided into three regions namely near IR (12500 to 4000 cm\(^{-1}\)) mid IR (4000 to 400 cm\(^{-1}\)) and far IR (400 to 20 cm\(^{-1}\)). Only the mid IR region is usually referred to as Infra red and is widely used in the analysis of pharmaceuticals.

Procedure

IR spectra of the plant substance was measured in an automatic recording IR spectrophotometer either in solution (in chloroform or carbon tetrachloride, 1-5%) as a mull with nujol oil or in the solid state, mixed with potassium bromide. In the latter case, a thin disc was prepared under
anhydrous conditions from a powder containing about 1 mg of material and 10-100 mg potassium bromide, using a mould and press. The range of measurement was from 4000 to 667 cm\(^{-1}\) (or 2.5 to 15 \(\mu m\)) and the spectrum was recorded within 3 minutes. IR spectroscopy is most frequently used in phytochemical studies as a fingerprinting device for comparing a natural with a synthetic sample, which is very important in the complete identification of many types of plant constituent. It can also usefully contribute to structural elucidation, when new compounds are encountered in plants.

4.3.2 Ultraviolet and Visible Spectra

The visible and ultraviolet spectra of organic compounds are associated with transitions between electronic energy levels. The transitions are generally between a bonding or lone-pair orbital and an unfilled or anti-bonding orbital. The highest energy in separation is found when electrons are in \(\sigma\)-bonds. The absorption is in the 120-200 nm.

4.3.3 Measurement of Spectrum

The ultraviolet or visible spectrum is usually taken from a very dilute solution. An appropriate quantity of the compound is often about 1 mg when the compound has a molecular weight of 100-200. The solvent is weighed accurately then made up to for instance 100 ml. A portion of this is transferred to a silica cell. The cell is so made that the beam of light passes through 1 cm thickness (the value \(l\) in Eq. 1.2). In most spectrometers there
are two sources. One is of white ultraviolet and another one is of white visible light, which have to be changed. The spectrum is plotted automatically on most machines as a log 10 (I0/I) ordinate and \( \lambda \)-abscissa. It is measured by the area under the absorption peak then by the intensity of the maximum of the peak.

4.3.4 **Nuclear Magnetic Resonance Spectroscopy (NMR)**

NMR is the branch of spectroscopy dealing with the absorption of radio-frequency radiation by substances held in a magnetic field. Absorption results from interaction of radiation with magnetic movement of nuclei in the sample and it occurs at different frequencies for nuclei with chemically different environments within a molecule. NMR may be of two types proton NMR \(^1\text{H} \text{NMR}\) and carbon \(^{13}\text{C-NMR}\).

4.3.5 **Proton NMR Spectroscopy \(^1\text{H}-\text{NMR}\)**

**Principle**

Proton NMR spectroscopy essentially provides a means of determining the structure of an organic compound by measuring the magnetic moments of its hydrogen atoms. In most compounds, hydrogen atoms are attached to different groups (as CH\(_2\), CH\(_3\), CHO, etc.) and the proton NMR spectrum provides a record of the number of hydrogen atoms in these different situations.
Procedure

The sample (5-10 mg of the substance) was placed in an inert solvent [deuterochloroform (CDCl₃), deuterium oxide (D₂O) carbon tetrachloride (CCl₄) or deuterated dimethyl sulphoxide (DMSO)] and the solution was placed between the poles of a powerful magnet. The different chemical shifts of the proton according to their molecular environments within the molecule was measured in the NMR apparatus relative to a standard, usually tetra-methyl-silane (TMS), an inert compound which can be added to the sample solution (this does not cause any chemical reaction). Chemical shifts are measured in their δ or τ = 10 δ and δ = ΔV × 10⁶ / radio frequency. ΔV being the difference in absorption frequency of the sample and the reference compound (TMS) in Hertz units. Since total radio frequency is usually 60 mega hertz (60 million hertz) and shifts are measured in Hertz units, they are often referred to as PPM. Also the intensity of the signals may be integrated to show the number of protons resonating at any one frequency.

The major use of proton NMR is for structural determination in combination with other spectral techniques.

4.3.6 Carbon-13 NMR (¹³C-NMR) Spectroscopy

¹³C-NMR spectroscopy is essentially complementary to proton-NMR on the combination of the two techniques provides a very powerful means of structural elucidation for new terpenoids, alkaloids or flavonoids. It is
useful in the analysis of glycosides in indicating the linkage between sugar moieties and their configuration.

**Principle**

The detection of signals from carbon atoms in the NMR apparatus is possible due to the presence of small amounts of carbon-13 along with carbon-12 in natural plant substances. The smaller magnetic moment generated by $^{13}$C compared to that of a proton means that the signals is much weaker. The weaker signals are amplified and the technical advances of pulsed NMR actually makes the $^{13}$C NMR possible for spectroscopy.

**Procedure**

The procedure is similar to that of proton NMR. Similar solvents were used as in proton NMR, but the range of $^{13}$C resonances was much greater namely 0-200 ppm down field from TMS compared with a range from 0-10 ppm for proton resonances. Thus $^{13}$C NMR spectra are more highly resolved and in most cases, each carbon within the molecule can be assigned to a separate signal. As with proton NMR differently substituted carbon atoms gives shifts within specific ranges, for example aliphatic carbon atoms gives shifts between 0-30 ppm, aromatic carbons between 110 and 150 ppm and ketonic carbons between 160 and 230 ppm.

Both proton and $^{13}$C NMR measurements have been successfully applied to structural and other analysis of proteins and other macromolecules.
4.3.7 Gas Chromatography - Mass Spectrum Study (GC-MS)

In this study to identify the compounds from the plant extract GC-MS technique is used.

Plant Sample Extraction

Twenty grams of powdered plant leaf material is soaked in 50 ml of absolute alcohol overnight and then filtered through Whatman filter paper No 41, along with 2 gm sodium sulphate to remove the sediments and traces of water in the filtrate. Before filtering the filter paper along with sodium sulphate is wetted with absolute alcohol. The filtrate is then concentrated by bubbling nitrogen gas into the solution and reduce the volume to one ml. The extract contains both polar and non-polar phytocomponents of the plant material and the plant extract is injected in the Gas Chromatography - Mass Spectrometer.

One micro litre of the filtrate was injected into the Gas Chromatography column. The sample gets evaporated and carried away by carrier gas helium. It gets segregated into individual components. The sample fraction coming out of the column was led into mass detector and the mass spectrum of each component was recorded. The mass spectrum of unknown component was compared with the known spectrum of NIST library and the components were identified.
Principle and application of GC-MS Detector in Phytochemical analysis

GC-MS plays a key role in the analysis of unknown components of plant origin. GC-MS ionizes compounds and measures their mass numbers. Ionization is typically, the C.I. (Chemical ionization) and E.I. (Electron Ionization). The E.I. method provides good results for quantitative analysis as well of the compounds and it is a highly selective method for interfering components. Gas chromatography technique involves the separation of volatile components in a test sample using suitable capillary column coated with polar and non-polar or intermediate polar, chemicals.

Elite-1 column (100% Dimethyl poly siloxane) is a non-polar column used for analysis of phytocomponents in medicinal plants and pesticide residues. Elite-5 column (5% phenyl and 95% methyl polysiloxane) is an intermediate column used for the estimation of pesticide residues in soft drinks and food grains. Elite wax (polyethylene glycol) is a polar column used in the estimation of fragrances in rice, alcohol, flowers and fatty acid profile of edible oils. An inert gas such as hydrogen or nitrogen or helium is used as a carrier gas.

The components of test sample is evaporated in the injection port of the GC equipment and segregated in the column by adsorption and adsorption technique with suitable temperature programme of the oven controlled by software. Different components are eluted from the column based on the boiling point of the individual components. The GC column is
heated in the oven between 60 to 270° C. The time at which each component eluted from the GC column is termed as retention time (RT).

The eluted component is detected in the mass detector. The spectrum of the unknown component is compared with the spectrum of the known components stored in the NIST library and ascertains the name, molecular weight and structure of the components of the test materials in GC-MS study. Food grain fragrances, floral fragrances, pesticide residues, terpenes, steroids, alkaloid and fatty acids are some of the useful components analyzed in the GC-MS study.