METHODOLOGY

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Chemosystematics (chemotaxonomy or chemical plant taxonomy), in which chemical characters are used as aids in taxonomy, owes its origin to the earliest classification of plant kingdom where the algae are grouped based on their color (pigments), storage food and cell wall. Though Helen Abbot predicted, as early as in 1886, that the study on chemical principles of plants from a purely botanical view would become a new field of research, McNair (1929) who screened 300 oils, fats and waxes occuring in 83 families and arranged them in relation to taxonomy may be hailed as the pioneer in this field. Manske's (1944) works on alkaloids and Mirov's (1961) work on volatile oils are other landmarks in the history of chemical plant taxonomy. But it is with the simultaneous release of three books 'Chemotaxonomie der Pflanzen' (Hegnauer, 1962-65), 'Biochemical Systematics' (Alston and Turner, 1963) and 'Chemical Plant Taxonomy' (Swain,1963), this branch of taxonomy is recognised as a legitimate branch of taxonomy.

Swain (1963) defines chemotaxonomy as "the investigation of the distribution of chemical compounds or groups of biosynthetically releated compounds, in series of related or supposedly releated plants". Since the chemical compounds are intermediates between the genes and their morphological expressions, the chemical characters are sometimes considered more important than the characters from other disciplines. The cryptic nature of the chemical characters, in

that they are 'hidden' from the eyes of the 'classical' taxonomist also favoured giving them undue weightage in some treatments. But the critical studies conducted in recent years proved that the chemical characters also are susceptible to all the drawbacks such as parallelism, convergence, reduction, environmental modification and inconsistency, as other taxonomic evidences and, therefore, the present day taxonomists give them equal weightages at par with evidences from other disciplines.

Any chemical compound present in the plant at any stage of growth is a potential taxonomic marker which may be used at higher or lower levels of taxonomic hierarchy. Thus the presence of chlorophyll delimits the green plants from fungi while betacyanins circumscribe the *Centrospermae*. This means that none of the chemical markers can be dismissed because of its wide or narrow distribution. But a good chemical character must be 'variable, stable, unambiguous and not easily, if at all, changeable'. The presence of high incidence of contrasting characters, the ease of determination and low correlation are added advantages of a chemical marker. A high correlation between characters, would be of great phylogenetic interest. The chemical characters have an added advantage over morphological characters in that they can be very exactly described in terms of definite structural and configurational formulae.

Most of the chemical characters are attempted at ordering them into phylogenetic sequences. Though the absence of fossil record may seem an obvious disadvantage, the knowledge of biosynthetic routes definitely gives the chance to judge whether a character is early or late in evolution. An organism having a

long biosynthetic sequence is belived to be more advanced than an organism with a short sequence. Thus if a sequence A->B->C->D is recognised, then it is assumed that an organism with A has fewer enzymes than one with D and the latter is considered more advanced than the former. As an alternative to this, if a compound is shown to be statistically correlated in its occurence with another character already known to be advanced, then the first character also is considered advanced (Sporne, 1956).

The chemical characters available for a chemotaxonomist are legion. David and Heywood (1967) classified them into three groups (1) directly visible substances, (2) serology, electrophoresis of proteins and DNA/RNA analyses and (3) plant products.

Directly visible substances present in plants are starch grains, raphides and other crystals. The form, structure and the mechanism of formartion of starch grains were used by the earlier taxonomists. Recently Czaja (1978) conducted an exhaustive study on the behaviour of starch grains in polarised light and their swelling pattern when placed in water and warmed. Raphides were used as a taxonomic character in *Orchidaceae*, *Scitaminae*, *Rubiaceae* etc. (Gibbs, 1963). Crystals of calcium oxalate, silica and gypsum were used in the classification of *Allium*, Poaceae and Tamaricaceae respectively (David and Heywood, 1967).

Serology, which employes the specificity of antogen-antibody reaction, was extensively used in 1930's and 1940's to

classify higher plants. But the difficulty in distinguishing the different antigen-antibody systems in liquid media prompted the development of Ouchterlony and Elek double diffusion method. Electrophoresis of proteins, peptide finger print data and the amino acid sequences are some other ways of utilising proteins in chemotaxonomy. **DNA homology** *i.e.* the extent to which two DNA molecules pair,is looked upon as the similarity index between the source organisms from which the two molecules come from. This work is extended to **DNA-RNA homology** where the extent of duplex formation between RNA and single-stranded DNA is taken as the affinity index (Alston and Turner, 1966).

Plant products include all the plant metabolites stored in plants for a longer period of time. They are classified into two groups, the primary and secondary metabolites. The **Primary metabolites** include the sugars, polysaccharides, amino acids, peptides, fatty acids and fats. The qualitative analysis of these compounds is more significant than the quantitative, since the quantity may vary according to the environmental conditions. Some of the sugars used in Taxonomy are (1) glycolose-found in the free state in the leaves of *Caprifoliaceae* members, (2) apiose- a cell wall constituent of many aquatic monocotyledons, (3) sorbitol- in the fruits of *Rosaceae* and (4) dulcitol - in *Celastraceae*. Non-protein amino acids having a restricted distribution in plant kingdom are also effectively used in classification. Sulphur-containing amino acids and azetidine-2-carboxylic acid in *Liliaceae* are an examples of their utility. Among the fatty acids, erucic acid in *Brassicaceae*, petroselinic acid in Apiaceae and chaulmoogric acid in *Flacourtiaceae* are

examples of characteristics components of plant groups.

The **secondary metabolites** are all those compounds produced by the plant but are not ultimately involved in basic energy transfer or assimilatory activity of all tissues (Alston and Irwin,1961). They are synthesized by the secondary metabolic pathways which are almost independent of the primary metabolic pathways. The functions of most of these plants are obscure. Earlier, these compounds were considered 'excretory products', 'waste products', or 'side products' and Heslop-Harrison calls them 'luxury diversifications' which reflect the chemical virtuosity of the plant concerned. All these compounds are genetically controlled and probably are fixed at a low selection pressure. But studies in the recent past indicate that most of the secondary metabolites have a positive role in plants, in that, most of them are defensive in nature, greatly important for survival of plants in this planet. Some of the secondary metabolites effectively used in the present study and the methods of analysis employed for them are as follows :

Flavonoids

Flavonoids by far, are the group of natural products which is the most sought after by chemotaxonomists. This group includes all the C_6-C_3-C_6 compounds related to a flavone skeleton. The differrent classes of flavonoids are distinguished by the oxidation level of the C_3 fragment and the various members of each class are recognised by the number and positions of the hydroxyl groups, substitutions and glycosylation. These

compounds are preferred over most of the other low molecular weight constituents like terpenes and alkaloids, for their universal distribution in vascular plants (that also in all organs), structural diversity, stability during extraction procedures and the ease and rapidness of identification. Even herbarium specimens can be screened for these compounds. The facilities required for the isolation and characterisation of the common flavonoids are paper chromatographic set ups and a spectrophotometer.

In one of the most critical studies on the reliability of flavonoids as taxonomic markers, McClure and Alston examined the pattern of flavonoid chemistry in *Spirodela* grown in as many as 58 different culture media. The different treatments included addition of various growth hormones like auxins and gibberellic acids. In almost every instance the flavonoid pattern remained unchanged and this conclusively proves that the formation of flavonoids is controlled completely by intrinsic factors (Alston and Turner, 1966).

All the classes of flavonoids have been effectively utilised in taxonomy. But the most useful characters are those which are visibly colored or those which exhibit fluorescence in ultraviolet light, such as anthocyanidins, flavonols, flavones, chalcones and aurones. Proanthocyanidins (leucoanthocyanins) are also included here because they liberate anthocyanidins which are easily detectable when treated with acid. Most of the flavonoids occur in plants as their glycosides and so a methanolic extraction followed by an aqueous extraction of the

residue (left after distilling off the methanol) would separate out most of these compounds in water. These compounds may either be chromatographed or hydrolysed to determine the aglycone. The aglycones are few in number and are easily identified with the help of R_f value (or R_{st} value, when a standard is used), colour in UV light, fluorescene in UV light, change in fluroscene with ammonia fumes, stability in chromatograms after the papers/plates are sprayed with sodium carbonate solution, colour development after ferric chloride spray and UV spectra. Once the basic skeleton is known, further characterisation is easy because many reagents are available now,which react with the hydroxy or methoxy groups at specfic locations and effect hypsochromic or bathochromic shifts in the methanolic spectra. The identification of compounds is confirmed by co-chromatography (in six solvent systems) with the standards isolated from the known plant sources or procured from commercial sources.

One of the advantages of working on flavonoids is that this data can be interpreted in terms of phylogeny. The chemogenetic series of flavonoids, analogous to morphogenetic series, is identified from the biosynthetic studies and from the correlation of flavonoid structures with progressive specialisation in plants. Harborne (1967) and Swain(1975) have proposed more or less similar evolutionary schemes for flavonoids. Based on this as well as the correlation studies, it is concluded that 3-oxygenation, more hydroxyl groups and simple glycosylation in anthocyanidins, presence of proanthocyanidins (in leaf) and myricetin, absence of methylation, more hydroxyl

groups, hydroxylation at 6-position in flavonols and C-acylation are primitive features in Angiosperms. 3-Deoxygenation, O-methylation, absence of proanthocyanidins (in leaf) and myricetin, presence of flavones, their O-methylation, complex hydroxylation, 6-oxygenation and 2'-hydroxylation, aurones and absence of C-glycosylation, biflavonyls, flavonones and C-acylation are considered advanced. By considering the reduction trends, which involve reverse trends down the biosynthetic series, the presence of seemingly primitive features (according to above dicta) in certain groups,which are otherwise advanced, may be considered highly advanced. These reduction trends may culminate in the ultimate loss of flavonoid skeleton from plants (Gornall and Bohm, 1978).

'Bioflavonoids' are a group of flavonoids exhibiting Pharamacological properties, espicially 'vitamin P' activity. 'Vitamin P' refers to a group of compound which are known to be the 'permeability factors' which increase the capillary resistence and therby used to treat subcutaneous capillary bleeding. Rutin,(3-rutinoside of quercetin), its methylated derivatives and flavonones from *Citrus* fruits formed the principal components of vitamin P. The interest on Physiological effects of flavonoids resulted in a spurt on the research on these compounds and consequently more than 200 preparations were in use in U.S.A in 1970's (Meyers, **et al.,** 1972). It is experimentally established that flavonoids with free hydroxyl groups at the 3',4' positions exert beneficial physiological effects on the capillaries through (1) chelating metals and thus sparing ascorbate from oxidation, (2) prolonging epinephrine

action by the inhibition of O-methyl transferase, and (3) stimulating the pituitary-adrenal axis (De eds, 1968). Srinivasan et al., (1971) presented evidance that flavonoids play another important role in circulatory system by acting on the aggregation of erythrocytes. Most of the flavonoids occur as water soluable glycosides in plants. They are extracted with 70% ethanol or methanol and remain in the aqueous layer, following partition of this extract with solvent ether. Due to the phenolic nature of flavonoids, they change in colour when treated with a base or with ammonia and thus are easily detected in chromatograms or in solutions. Flavonoids contain conjugated aromatic systems and thus show intense absorption bands in UV and visible regions on the spectrum. A single flavonoid aglycone may occur in a plant in several glycosidic combinations and for this reason it is considered better to examine the aglycones present in hydrolysed plant extracts (Harborne, 1984).

Normally the flavonoids are linked to sugar by Ø-glycosidic bonds, which are easily hydrolysed by mineral acids. But there is another type of bonding in which sugars are linked to aglycones by C-C bonds. The latter group of compounds, known as C-glycosides, are generally observed among flavones. They are resistant to normal methods of hydrolysis and will remain in the aqueous layer when hydrolysed extract is extracted with ether to remove aglycones.

The procedures followed in the present work for the extraction, isolation and identification of flavonoids are described below.

Five grams of leaf powder was extracted in a soxhlet with methanol for 48 hrs till the plant material became colourless. The methanolic extract was concentrated to dryness in a water bath. 25-30 ml of water was added to the dry residue and the water soluble phenolic glycosides were filtered out. The filtrate was hydrolysed in a water-bath for one hour using 7% HCl. This hydrolysate was extracted with diethylether/solvent ether, whereby the aglycones got separated into ether fraction (Fraction A). The remaining aqueous fraction was further hydrolysed for another 10 hr to ensure the complete hydrolysis of all the O-glycosides. Aglycones were once again extracted into diethyl ether (Fraction B) and the residual aqueous fraction was neutralized and evaporated for the analysis of glycoflavones.

Ether fractions A and B were combined and analysed for aglycones using standard procedures (Harborne, 1967, 1984; Mabry et al., 1970; Markham, 1982). The combined concentrated extract was banded on whatman No. 1 paper and chromatographed along with quercetin as the reference sample. The solvent system employed were Forestal (Con. HCl : Acetic acid: Water : 3 : 30 :10) or 30% glacial acetic acid. The developed chromatograms were dried in air and the visibly coloured compounds were marked out. These papers were observed in Ultraviolet light (360 nm) and the bands were noted. Duplicate chromatograms were then sprayed with 10% aqueous Na_2CO_3 and 1% $FeCl_3$ and the colour changes were recorded. R_q (R_f relative to quercetin) values were calculated for all the compounds. The bands of compounds were cut out from unsprayed chromatograms and were eluted with spectroscopic grade

methanol. The UV absorption spectra of these compounds were recorded in methanol using 'Shimadzu UV 240' recorder type spectrophotometer. The bathochromic and hypsochromic shifts induced by the addition of various reagents were studied. The reagents used and their preparation are given below :

Sodium methoxide (NaOMe) : Freshly cut sodium metal (2.5 gms) was added cautiously in small portions to dry spectroscopic methanol (100 ml). The solution was stored in a tightly closed glass bottle.

Aluminium chloride ($AlCl_3$) : Five gms of fresh anhydrous AR grade $AlCl_3$ (which appeared yellow-green and reacted violently when mixed with water) were added cautiously to spectroscopic methanol (100 ml).

Hydrochloric acid (HCl) : Concentrated AR grade HCl (50 ml) was mixed with distilled water (100 ml) and the solution was stored in glass stoppered bottle.

Sodium acetate (NaOAc) : Anhydrous powdered AR grade NaOAc was used.

Boric acid (H_3BO_3) : Anhydrous powdered AR grade H_3BO_3 was used.

The concentrations of the sample solution prepared by eluting chromatogram strips were adjusted so that the optical density (OD) fell in the region of 0.6 to 0.8. The methanol spectrum was taken using 2-3 ml of this stock solution. The NaOMe spectrum was measured immediately after the addition of three drops of NaOMe stock solution to the flavonoidal solution

used for methanol spectrum. The solution was then discarded. The $AlCl_3$ spectrum was measured immediately after the addition of six drops of $AlCl_3$ stock solution to 2-3 ml of fresh stock solution of the flavonoids. The $AlCl_3$/HCl spectrum was recorded next, after the addition of 3 drops of the HCl stock solution to the cuvette containing $AlCl_3$. The solution was then discarded. For NaOAc spectrum, excess coarsely powedered anhydrous AR grade NaOAc was added by shaking the cuvette containing 2-3 ml of fresh solutions of the flavonoids, till about a 2 mm layer of NaOAc remained at the bottom of the cuvette. The spectrum was recorded 2 minutes after the addition of NaOAc. NaOAc/H_3BO_3 spectrum was taken after sufficient H_3BO_3 was added to give a saturated solution. The solution was discarded after recording the spectrum.

The structure of a flavonoid is established by its absorption maxima, shape of the curves, shifts (both bathochromic and hypsochromic) with different reagents and colour reactions. The identifications were confirmed by co-chromatography with authentic samples.

The aqueous fraction remaining after the separation of aglycones was neutralized by the addition of anhydrous Na_2CO_3/$BaCO_3$ and concentrated to dryness. When $BaCO_3$ was used barium chloride got precipitated and was filtered out. This filtrate was concentrated to dryness. The alcoholic extract of the dried residue was banded on whatman No.1 paper and the chromatogram was developed with water as solvent system. Glycoflavones were visualized by their colour in UV and with 10%

Na_2CO_3 spray. Further analysis and identification were done using spectroscopic methods as explained before.

Phenolic acids

Phenolic acids are simple phenols, having a functional acid group and varying number of hydroxyl groups at different positions. Acid hydrolysis of plant tissues release a number of ether-soluble phenolic acids, some of which are universal in distribution. These acids occur either associated with lignin or are bound to the glycosides. They are also seen as depsides or as esters in hydrolysable tannins. Phenolic acids which are almost universally distributed in Angiosperms are *p*-hydroxy benzoic acid, vanillic acid and syringic acid, which are the components of lignin. Gentisic acid also is fairly widespread. Salicyclic acid and the related *o*-pyrocatechuic acids are abundent in the Ericaceae. Ellagic acid and gallic acids are located in many plant groups of the Polypetalae. The phenolic acids are extracted in ether alongwith the flavonoid aglycones from the hydrolysed extract (Fraction A and B) of plant materials. They are analysed as follows :

Analysis of phenolic acids in the combined ether fraction (A and B) was carried out by two-dimensional ascending paper chromatography. Benzene : acetic acid : water (6:7:3, upper organic layer) in the first direction and sodium formate : formic acid : water (10:1:200) in the second direction, were used as irrigating solvents. The sprays used to locate the compounds on the chromatograms were diazotised *p*-nitraniline or dizaotised sulphanilic acid and a 10% Na_2CO_3 overspray (Ibrahim and Towers,1960).

Diazotization : Ø.7 gms of *p*-nitraniline/sulphanilic acid was dissolved in 9 ml of HCl and volume made upto 1ØØ ml. Five ml of 1% $NaNO_2$ was taken in a volumetric flask and kept in ice till the temprature was below 4°C. The diazotized sprays were prepared by adding 4 ml of p-nitraniline/sulphanilic acid stock solution to the cooled $NaNO_2$ solution. The volume was made up to 1ØØ ml with ice-cold water.

The various phenolic acids present in the extract were identified based on the specific colour reactions they produce with the spray reagents and the relative R_f values in different solvent systems.

Saponins

Saponins are glycosides which form emulsions with water and possess marked haemolytic properties. They possess steroidal or triterpenoid aglycones. The steroidal saponins are common in monocots, while the triterpenoid saponins are found in dicots. Their taxonomic value is less at a higher level of hierarchy although they may be used as useful chmical characters at lower levels.

About 5 gm of the powedered leaf material was boiled with 5Ø ml water for half an hour. This extract was filtered, the filtrate was taken in a test-tube after cooling and shaken vigorously (to froth) for a minute or two. The formation pf a persistent froth of 1 cm length showed the presence of saponins (Hungund and Pathak, 1971). Foam formation takes place during aqueous extraction if the concentration of the saponins are more in the plant materials (Harborne, 1984).

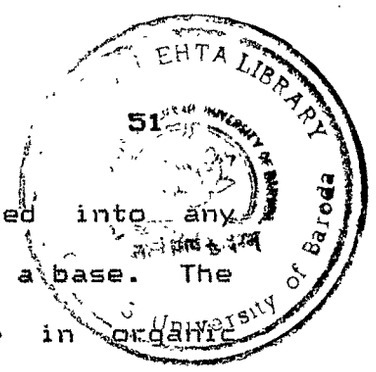
Proanthocyanidins

For testing the presence of proanthocyanidins, about 5 gm of finally chopped (fresh leaf material /2 gm dry powdered material) was taken in 20 ml test-tube and covered with approximately 5 ml of 2N HCl. Extraction was carried out by placing the test-tube in a boiling water bath for half an hour. The extract was decanted after cooling and shaknen with amyl alcohol. Presence of a red or near-carmine colour in the upper alcohol layer denoted a positive reaction for proanthocyanidins. An olive-yellow colour represented a negative reaction (Gibbs, 1974).

Alkaloids

Alkaloids comprise the largest single class of secondary metabolites. They are basic plant products having a nitrogen-containing hetrocyclic ring system and high pharmacological activity. They are restricted to certain groups of plants and therefore, often used as criterion in classification of only those groups of plant which contain them. The prsence of various types of alkaloids are used effectively in classifying various taxa (Manske,1944; Price,1963; Gibbs,1974).

Alkaloids, as a rule, are insoluble in water but soluble in organic solvents. But their salts are soluble in water and insoluble in organic solvents. Alkaloids are normally extracted from plants into weakly acids (1M HCl or 10% acetic acid) or acidic alcoholic solvents and are then precipitated



with concentrated ammonia. They are also extracted into any organic solvent after treating plant material with a base. The base frees the alkaloids and makes them soluble in organic solvents. From the organic solvents, the alkaloids are extracted into acidic solution and tested with specific reagents.

Five grams of powdered leaf material was extracted with 50 ml of 5% ammoniacal ethanol for 48 hours. The extract as concentrated (by distillation) and the residue was treated with 10 ml of 0.1N H_2SO_4. The acid soluble fraction was tested with Mayer's, Wagner's and Dragendorff's reagents (Paech and Tracey, 1965). A precipitate denoted the presence of alkaloids (Amarasingham **et al.**, 1964). The preparation of the reagents were as follows :

Mayer's reagent : (Potassium mercuric iodide) 1.36 grams of $HgCl_2$ were dissolved in 60 ml of distilled water and 5 gms of KI in 10 ml of water. The two solutions were mixed and diluted to 100 ml with distilled water. A few drops only of this reagent wer added, as precipitates of some alkaloids were slouble in excess of the reagent.

Wagner's reagent : (Potassium Iodide) 1.27 grams of I_2 and 2 grams of KI were dissolved in 5 ml of water and the solution diluted to 100 ml. It gave brown flocculent precipitate with most of the alkaloids.

Dragendorff's reagent : (Potassium bismuth iodide) 8 grams of $Bi(NO_3)_3 5H_2O$ were dissolved in 20 ml of HNO_3 (sp.gr.1.18) and 27.2 grams of KI in 50 ml of water. The two solutions were mixed

and allowed to stand when KNO_3 crystallizd out. The supernatant was decanted off and made up to 100 ml with distilled water.

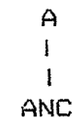
Cladistics :

The construction of Wagner tree is based on the method of Jensen (1981). Two types of Taxonomic units are employed in the preparation of Wagner trees : (1) Operational Taxonomic units (OTU), represents any level of taxonomic hierarchy from phylum to a single species and, (2) Hypothetical Taxonomic unit (HTU). The characters possessing phylogenetic significance are selected. The primitive or plesiomorphic state of the characters is given a score 0 and advanced or apomorphic state is given score 1. The polarity of the chemical characters is done based on the views of Gornall and Bohm (1978) and Harborne (1988). The biosynthetic pathways and correlation studies are also taken into consideration.

A table showing the distribution of these characters in the OTUs is prepared. The first step involves the calculation of Manhattan distances between the Ancestor (an OTU having 0 state for all the characters) and each OTU. This distance is called as the advancement index or AD(I) value. The formula for calculating the AD(I) value is

$$d\ (A\ B) = \sum (A_i - B_i)$$

Where d = distance (the sum overall n characters of the absolute differences between OTUs), A and B are the OTUs and i = a given character. Then OTU, A, with the samllest AD(I) value is taken and connected to the ancestor.



Next the OTU, B, having the smallest remaining AD(I) value is selected. A HTU is constructed to form a node for the attachment of B. The character states of HTUI is determined as the median of the states of each character for A, ancestor and B. The HTUI is placed in the interval of A and ancestor and B is connected to it.

The OTU C, with next smallest AD(I) value is selected and the minimal distance between C and each possible interval is calculated using the formula :

$$d\,[C.\ Int.\ (A.B)] = 1/2\,[d\,(A.C) + d\,(B.C) - d\,(A.B)]$$

In this step 3 possible intervals are available.

$$d\ [C.\ Int.\ (A.\ HTU1)] = a$$

$$d\ [C.\ Int.\ (B.\ HTU1)] = b$$

$$d\ [C.\ Int.\ (ANC.\ HTU1)] = c$$

The HTU2 is constructed as the median of C and nodes XY of the interval that is least distant from C. The OTU C is placed in this interval through HTU2. In this sequence all the OTUs are incorporated and a Wagner tree with all the OTUs is prepared.

In certain cases the minimal distance of more than one interval are found to be the same and therefore the OTU can be placed in any one of the intervals. In such cases all the possible trees are constructed and each tree is subjected to HTU optimization. In this procedure the HTUs are recalculated as the medians of 2 descendants. HTU optimization involves two passes. One downward pass where the direction is top downwards. Each character state is assigned Ø if both the descendants are Ø; (Ø, 1) if one has Ø and other has state 1 or 1 if both the descendants are 1. The second pass is the upward pass. In this pass the HTU immediately above the ancestor is scanned for any ambiguities i.e. character score (Ø, 1). If a character is at ambiguous state, then it is assigned the state found in the ancestor. This is continued till all the ambiguities are eliminated. Of the various trees, one having the shortest length is selected.

Plant Materials

The plant materials for the present work are collected from different parts of India such as Trivandrum (Museum Botanic Garden; Tropical Botanic Garden and Research Institute, Palode), Coimbatore, Banglore (Lalbagh), Mahabaleshwar, Pavagadh and Baroda. Voucher specimens of the plants collected are deposited in the Herbarium, Departmentof Botany, the M.S. University, Baroda. The date and place of collection and the herbarium numbers are given in Appendix I. For extraction, the leaves collected were from 5th node downwards. Care was taken to collect the healthy and uninfected leaves. The leaves were dried

at the place of collection in shade and later dried completely in an oven at 60°C. The dried leaves were powdered and stored in airtight glass bottles or plastic bags. This powder was used for the analysis of almost all the chemical markers. Fresh materials, when available, were used for testing proanthocyanidins.