2. Methodology

The plant materials for the present work were collected from different parts of India, particularly, Kerala, Tamil Nadu, Madhya Pradesh, Gujarat and Kashmir. Most of the Aceraceae, Hippocastanaceae, Geraniaceae and Celastraceae members were procured from Kashmir, while many of the Anacardiaceae, Sapindaceae, Rutaceae and the Burseraceae members were collected from Himachel pradesh, Kerala and Madhya Pradesh. The leaves used for extraction were from the 5th node downwards. Care was taken in collecting only the healthy leaves. The leaves were dried at the place of collection in shade and later completely dried by keeping 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, whenever available, were used for testing iridoids and proanthocyanidins. A brief account of the chemical compounds used as markers and the various methods followed in their extraction and characterisation is presented below.

Flavonoids

Flavonoids are polyphenols which include all the C_6 - C_3 - C_6 compunds related to a flavone skeleton. The flavone may be considered as consisting of (i) a C_6 - C_3 fragment (phenyl propane unit) that contains the 'B' ring and (ii) a C_6 fragment the 'A' ring both these units being of different biosynthetic origin. The flavonoids are subdivided based on the oxidation level of C_3 fragment of the phenyl propane unit, as anthocyanidins, flavones, flavonols, chalcones, and aurones etc. (Geissman, 1962). These pigments sometimes completely replace the carotenoids as the yellow flower/fruit pigments. Anthocyanidins are the purple/blue pigments while chalcones and aurones are yellow in colour. Flavonols and flavones, though classified as colorless flavonoids, are responsible for the white, cream, or ivory colors of the flowers. All these pigments absorb strongly in ultraviolet

and thus may be responsible for attracting those pollinators (e.g. bees) whose vision extends into ultraviolet region (Harborne and Smith, 1978). Flavanones, dihydroflavonols, biflavonyls, dihydrochalcones, isoflavones and proanthocyanidins are the "minor flavonoids" since they have a restricted distribution.

Flavonoids have been one of the most exploited phytochemical characters in relation to the classification of plants. The flavonoid data are being incorporated together with data from other disciplines into phylogenetic schemes of angiosperm classification (Dahlgren, 1980; Cronquist, 1981; Thorne, 1981).

Different groups of flavonoids can successfully be correlatd with phylogenetically significant morphological characters. Flavonols, especially quercetin and myricetin, as well as proanthocyanidins characteristically occur in primitive woody plants, and they gradually disappear from more advanced herbaceous families (Bate-Smith, 1962). Flavones appear late in evolution and therefore are found in advanced taxa. O-Methylation of flavones is another advanced feature. Substitution of an extra hydroxyl group in the 'A' ring of flavonoids seem to follow a similar pattern : i.e. woody plants have 8-hydroxy flavonols (e.g. Gossypetin) while herbaceous taxa elaborate 6-hydroxy flavones (e.g. Scutellarein) (Harborne and williams, 1971).

'Bioflavonoids' are a group of flavonoids exhibiting pharmacological properties, especially 'Vitamin P' activity. 'Vitamin P' refers to a group of compounds which are known to be the 'permeability factors' which increase the capillary resistance and thereby used to treat subcutaneous capillary bleeding. Rutin (3-rutinoside of quercetin), its methylated derivatives and flavanones 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 (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 evidence that flavonoids play another important role in circulatory system by acting on the aggregation of erythrocytes.

Most of the flavones and flavonols occur as water-soluble 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 bases (esp. ammonia) and thus are easily detected in chromatograms or in solutions. Flavonoids contain conjugated aromatic systems and thus show intense absorbtion bands in UV and in the visible regions of 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 O-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 (glycoflavones), 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.

Biflavones are flavone-dimers, mostly of apigenin and its methoxylated derivatives, in which the two monomers are linked by C-C linkages 8-8" (cupressoflavone), 5'-8" (amentoflavone), 5'-6" (robustaflavone) or 3-8" (garciniaflavone) or O-linkages such as 3'-6" (hinokiflavone) or 4'-3" (ochnaflavone). In addition to the flavone dimers, flavone-flavanone dimers (agathisflavone and rhusflavone) also are reported, they occur mostly as aglycones though some may occur as glycosides. The biflavones have a very restricted distribution reported from most of the gymnosperms except the Pinaceae, a few pteridophytes (**Psilotum**, **Selaginella**) and a few angiosperms (the Anacardiaceae, Caprifoliaceae, Casuarinaceae). Their omnipresence in gymnosperms may be correlated with the primitive nature of the group and therefore in angiosperms the biflavones are considered to be a primitive feature. The concept is now questioned and it is suggested that the biflavonoids developed independently in gymnosperms and angiosperms.

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's extractor 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 souluble phenolic glycosides were filtered out. The filtrate was hydrolysed in water bath for one hour using 7% HCl. This hydrolysate was extracted with diethylether, whereby the aglycones got separated into the ether fraction (Fraction A). The remaining aqueous fraction was further hydrolysed for another 10 hrs 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 a reference sample. The solvent systems 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 mm) and the bands were noted. Duplicate chromatograms were than 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 compunds 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 (Mabry, et al., 1970)

Sodium methoxide (NaOMe): Freshly cut sodium metal (2.5 g) 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 grams 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), formed initially, dissolved after about 24 hrs.

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

Sodium acetate (NaOAc) : Anhydrous powered AR grade NaOAc was

used.

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

The concentrations of the sample solution prepared 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 5 ml of this stock solution. A reference solution was prepared by extracting a piece of blank chromatographic paper from the same chromatogram with spectroscopic methanol. 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 6ml of $AlCl_3$ stock solution to 5 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 powdered anhydrous AR grade NaOAc was added by shaking the cuvette containing 5 ml of fresh solution 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 was established by the absorbtion maxima (λ max), shape of the curves, shifts (both bathochromic and hypsochromic) with different reagents, colour reactions and R_f values. 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 which 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 above. Biflavones are extracted with other flavonoids in methanol. But they are fractionated from the aqueous extract (prepared after concentrating the methanolic extract) using solvent ether or ethyl acetate. The individual biflavones are separated in paper using 30 % acetic acid and forestal (in which solvents they have higher R_f values) or 15 % acetic acid. They appear as dark spots in UV light and give typical color reactions of flavonoids. In TLC (silica gel) Toulene : Formic acid : Ethyl formate (5:1:4) is used. Identification is done by spectral measurements, shifts with the reagents and co-chromatography with standard samples isolated from known sources.

Coumarins

Coumarins are phenylpropanoids possessing 2H-benzopyran-2-one nucleus. They are the internal lactones having a limited distribution in angiosperms. These compounds are common in the Rutaceae, Apiaceae, Solanaceae and Lamiaceae. They get extracted in the ether fraction taken after hydrolysis, though some remain in the aqueous layer. Coumarins are highly mobile in typical solvents used to separate aglycones and therefore either 15 % acetic acid or water are employed in PC to isolate them. Almost all the coumarins are intensely fluorescent in UV and many of them exhibit characteristic abosrption spectra.

Phenolic acids

Phenolic acids are simple phenols, having a functional acidic group and varying number of hydroxyl groups at different

positions. Acid hydrolysis of plant tissue releases a number of ether-soluble phenolic acids, some of which are universal in distribution. These acids occur either associated with lignin or 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 derived from the phenylpropane components of lignin. Gentisic acid is also fairly widespread. Salicylic acid and the related O-pyrocatechuic acid are abunadant in the Ericaceae. Ellagic acid and gallic acid 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-nitraaniline or diazotised sulphanilic acid and a 10 % Na_2CO_3 overspray (Ibrahim and Towers, 1960).

Diazotization : 0.7 gms of p-nitraaniline/sulphanilic acid was dissolved in 9 ml of HCl and the volume made upto 100 ml. Five ml of 1 % $NaNO_2$ was taken in a volumetric flask and kept in ice till the temperature lowered below 4°C. The diazotized sprays were prepared by adding 4 ml of p-nitraaniline/sulphanilic acid stock solution to the cooled $NaNO_2$ solution. The volume was made up to 100 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.

Tannins

Tannins are polyphenols of high molecular weight which have the property of combining with protein, forming water insoluble and non-putrescible leather. Based on their reaction with mineral acids two main types of tannins are recognised, the condensed tannins and the hydrolysable tannins. The condensed tannins, which polymerise on hydrolysis, universally occur in ferns and gymnosperms and are widespread among the woody angiosperms. In contrast, hydrolysable tannins, which get broken up to simpler units on acid treatment, are limited to dicotyledonous plants and are found in a relatively few families. Tannins are correlated well with other primitive characters and thus the presence of these compounds is considered primitive. Between the two groups, the hydrolysable tannins are advanced. The highly advanced herbaceous taxa are generally devoid of these compunds.

Condensed tannins or flavolans can be regarded as being formed by the condensation of catechin or gallocatechin molecules and flavan-3, 4-diols to form dimers and higher oligomers with carbon-carbon bonds linking one flavan unit to the next by 4-8 or 6-8 linkage. The name proanthocyanidins is used alternately for condensed tannins because, on treatment with hot acids, some of the carbon-carbon linking bonds are broken and anthocyanidins are released. This property is used for the detection of condensed tannins. Hydrolysable tannins are mostly gallotannins and ellagitannins depending on whether gallic acid or ellagic acid is present esterified with glucose. They yield the corresponding phenolic acid and glucose on hydrolysis.

Tannins are extracted in water and are tested by treating them with protein solution when leather precipitates.

To the water extract prepared by boiling 5 g plant material in about 50 ml water, 2% freshly prepared gelatin solution was added. The formation of a white (or milky) precipitate showed the presence of tannins in the plant material (Hungund et al., 1971).

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 higher levels of hierarchy although they may be used as useful chemical characters at lower levels.

About 5 g of the powdered leaf material was boiled with 50 ml water for half an hour. This extract was filtered, the filtrate was taken in a test-tube after cooling and shaken vigourously (to froth) for a minute or two. The formation of a persistant froth of 1 cm length showed the presence of saponins (Hungund et al., 1971). Foam formation takes place even during extraction with aqueous solvents if the concentration of the saponins are more in the plant materials (Harborne, 1984).

Proanthocyanidins :

Though proanthocyanidins are condensed tannins, the nature of components in these complex molecules often throws light on the phylogeny. Procyanidin and prodelphinidin, which correspond to quercetin and myricetin, are ofter considered primitive due to the more hydroxyl groups they possess while propelargonidin or other proanthocyanidins yielding methylated anthocyanidins (peonidin, malvidin, hirsutidin) are deemed advanced.

For testing the presence of proanthocyanidins, about 5

g of finely chopped (fresh) leaf material/2 g 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 shaken 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).

Iridoids

Iridoids are a group of monoterpenoid glycosides present in a number of dicotyledons. The presence of these compounds in a taxon is considered by many (Hegnauer, 1971; Bate-Smith and Swain, 1966; Jensen et al., 1975) to be a valuable phylogenetically significant chemical character. The plants were surveyed for iridoids by a simple procedure described by Weiffering (1966) based on the Trim-Hill colour test. Fresh or dry powdered leaf material (1 g) was placed in a test-tube with 5 ml of 1% aqueous HCl. After 3-6 hours, 0.1 ml of the macerate was decanted into another tube containing 1 ml of 0.2% $CuSO_4 . 5H_2O$ in water and 0.5 ml Conc. HCl. When the tube was heated for a short time in a flame, a colour was produced, if iridoids are present (asperuloside, aucubin and monotropein give blue colours, herpagide a red-violet; Harborne, 1984).

Quinones

They are aromatic diketones, which form the largest class of natural colouring matters. They are generally known from higher plants and fungi. In higher plants they play a subsidiary or a secondary role. They are generally present in the bark or underground parts. In leaves their color is

masked by other pigments. They are classified into Benzo-, Naptha-, and Anthraquinones depending on the mono-, bi- or tricyclic ring system they contain. In plants their function is not properly understood. It is assumed that they play some role in oxidation reduction processes.

For extraction of quinones, approximately 5-10 g of dried, powdered, leaf material was exhaustively extracted with hot benzene for 3 x 12 hrs and the extract then concentrated to a dry residue. The residue was dissolved in solvent ether and segregated into acidic and neutral fractions by repeatedly shaking with 2N Na_2CO_3 solution. The Na_2CO_3 soluble fraction was acidified with ice-cold 2N HCl dropwise till the precipitate formed settled down. The acidified solution, in turn, was extracted with diethyl ether and separated again into two layers. The lower layer was discarded, while the upper acidic fraction was chromatographed over TLC (silica gel G) plates using petroleum ether-benzene (9:1) as the solvent system (Joshi et al., 1973).

The neutral fraction was also chromatographed over silica gel TLC plates using the same solvent system. The various quinones (Anthra-, Benzo-, Napthaquinones) were visualized by their colours in visible/UV light, colour reactions after spraying with 2% magnesium acetate or 10% aqueous NaOH (the quinones give purple/pink/orange yellow colours) and the absorption spectra.

Alkaloids :

Alkaloids comprise the largest single class of secondary metabolites. They are basic plant products having a nitrogen containing heterocyclic ring system and high pharmacological activity. They are restricted to certain groups of plants and therefore, often used as a criterion in classi-

fication of only those groups of plants which contain them. The presence of various types of alkaloids are used effectively in classifying various taxa (Manske, 1944; Gibbs, 1974; Daniel and Sabnis, 1979).

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 materials with a base. The bases free the alkaloids and makes them soluble in organic solvents. From the organic solvents, the alkaloids are extracted into acidic solutions and tested with specific reagents.

Five grams of powdered leaf material was extracted with 50 ml of 5 % ammonical ethanol for 48 hrs. The extract was concentrated (by distillation) and the residue was treated with 10 ml of 0.1 N H_2SO_4. The acid soluble fraction was tested with Mayer's, Wagner's and Dragendorff's reagents (Paech and Tracey, 1955). A white/colored precipitate denoted the presence of alkaloids (Amarasingham *et al.*, 1964). The preparation of the reagents are as follows :

Mayer's reagent : (Potassium mercuric iodide) 1.36 g of $HgCl_2$ were dissolved in 60 ml of distilled water and 5 g 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 were added, as precipitates of some alkaloids are soluble in excess of the reagent.

Wagner's reagent : (Potassium Iodide) 1.27 g of I_2 and 2 g of KI were dissolved in 5 ml of water and the solution

diluted to 100 ml. It gave brown flocculent precipitates with most of the alkaloids.

Dragendorff's reagent : (Potassium bismuth iodide) 8 g of $Bi(NO_3)_3 . 5H_2O$ were dissolved in 20 ml of HNO_3 (sp. gr. 1.18) and 27.2 g of KI in 50 ml of water. The two solutions were mixed and allowed to stand when KNO_3 crystallised out. The supernatent was decanted off and made up to 100 ml with distilled water.

Cladistics :

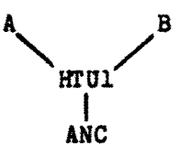
The construction of Wagner tree is based on the method of Jensen (1981). Two types of Taxonomic units are employed in the preparation of Wagner trees : (1) Operational Taxonomic Units (OTU), represents any level of taxonomic hierarchy from phyla to a single species and, (2) Hypothetical Taxonomic Unit (HTU). The characters possessing phylogenetic significance are selected. The primitive or plesiomorphic state of the characters is given a score 0 and the advanced or apomorphic state is given score 1. The polarity of morphological and embryological characters is determined by following Hutchinson (1969), Cronquist (1968), Takhtajan (1980), Eames (1964) and Maheswari (1961). The scoring of the chemical characters is done based on the views of Gornall and Bohm (1978), Harborne (1988), and Waterman and Grundon (1983). 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

$$d\ (A\ B) = \sum_{i=1}^{n} (A_i - B_i)$$

Where d = distance (the sum overall n characters of the absolute difference between OTUs), A and B are the OTUs and i = a given character. Then OTU, A, with the smallest 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 HTU1 is determined as the median of the states of each character for A, ancestor and B. The HTU1 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 distances 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 median of 2 descendants. HTU optimization involves two passes. One downward pass where the direction is top downwards. Each character state is assigned 0 if both the descendents are 0 ; (0, 1) if one has 0 and other has state 1 or 1 if both the descendents 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 (0, 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.

The Wagner tree thus constructed is superimposed on a Wagner 'Bull eye' chart and the taxa are positioned on the semicircles corresponding to their AD(I) values. Based on the branching pattern, a dendogram also is prepared.