MATERIALS AND METHODS

MATERIALS AND METHODS

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 Caryophyllaceae and the Polygonaceae members were procured from Kashmir, while most of the Chenopodiaceae and the Amaranthaceae members were collected from Gujarat. 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 a group of polyphenols which include all the C_6-C_3-C_6 compounds related to a flavone skeleton which 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. These units are of

of different biosynthetic origin. The flavonoids are subdivided based on the oxidation level of C_3 fragment of the phenyl propane unit, as flavones, flavonols, chalcones, and aurones etc. (Geissman,1962).

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 diciplines into phylogenetic schemes of Angiosperm classification (Harborne, 1977). Flavonoids are present in almost all vascular plants, but some classes of these compounds, such as flavones and flavonols, are more widely distributed than isoflavones and biflavones which are found to have a restricted occurrence.

Much can be inferred from the general distribution pattern of flavonoids. 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). There is a tendency to introduce flavones in advanced taxa. O-Methylation of flavone 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. Scutellarin) (Harborne, et al., 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 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 (Meyers, et.al.1972). It is experimentally established that flavonoids with free hydroxyl groups at the 3',4'-position 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 flavonoids occur as water soluble glycosides in plants. They are extracted with a 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 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 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 a 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, 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.

5 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 hrs to ensure the complete hydrolysis of all the O-glycosides. Aglycones were once again extracted into diethyl ether (Fraction B) and 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 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 Ultra violet 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. Rq (Rf 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 metal sodium (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 (5 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 soltuion 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 2-3 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 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 method as explained before.

PHENOLIC ACIDS

Pheholic acids are simple phenols, having a functional acid group and varying number of hydroxyl groups at different positions. Acid hydrolysis of plant tissues releases a number of other 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 and 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 is also fairly widespread. Salicylic 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-nitroaniline or diazotised sulphanilic acid and a 10 % Na_2CO_3 over spray (Ibrahim and Towers, 1960).

Diazotization: 0.7 gms of p-nitroaniline/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 was below 4°C. The diazotized sprays were prepared by adding 4 ml of p-nitroaniline/ 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 Rf values in different solvent systems.

TANNINS

Tannins are polyphenolic compounds which combines with protein, forming water insoluble and non-putrescible leather.

There are two main types of tannins; the condensed tannins and the hydrolysable tannins. The condensed tannins (Proanthocyanidins) universally occur in ferns and gymnosperms and are widespread among the woody angiosperms. In contrast, hydrolysable tannins are limited to dicotyledonous plants and are only found in a relatively few families. Tannins are correlated well with other primitive characters and thus the presence of these compounds is considered primitive. The highly advanced herbaceous taxa are generally devoid of these compounds.

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 flavon unit to the next by a 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 a protein solution.

To the water extract prepared by boiling 5 gm plant material in about 50 ml water, 2% freshly prepared gelatin solution was added. The formation of a white (or milky) precipitation 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 steroid 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 chemical characters at lower levels.

About 5 gm 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 vigorously (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 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 gms of finely 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 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).

STEROIDS

Steroids possess a cyclopentanoperhydrophenanthrene skeleton with hydroxyl group at C_3 and two methyl groups at C_{10} and C_{13}. Simplest sterol is cholesterol. It is an animal sterol. β-Sitosterol and stigmasterol are plant sterols. Tetracyclic triterpenoids also possess a steroidal skeleton. But here the number of methyl groups will be more e.g. Lanosterol. The plant sterols occur freely in waxes, cutins and resins or in glycosidic forms as saponins.

Steroids were analysed using the combined ether fraction A and B, which was spotted on T.L.C. plates and allowed to run in chloroform : Carbontetrachloride : acetone (2:2:1). The sprays used to detect the different steroids were 50% sulphuric acid or Liberman Burchard's reagent (1 ml of conc. H_2SO_4, 20 ml of acetic anhydride and 50 ml of chloroform are mixed together and the sprayed plates were heated at 110°C for 5 minutes.) The various types of steroids were located by specific colour reactions with spray reagents and the

Rf values.

IRIDOIDS

Iridoids are a group of monoterpenoid glycosides present in a number of dicotyledons. The presence of these compounds in a given taxa is considered by many (Hegnar, 1966b, 1969, 1971; Kubitzki, 1969; Meeuse, 1970; Bate-Smith, 1972; Bate-Smith and Swain, 1966; Jensen et al., 1975) to be a valuable phylogenetic significant chemical character. The plants were surveyed for iridoids by a simple procedure described by Wichtering (1966) based on the Trim-Hill colour test (Trim and Hill, 1952). Fresh or dry powdered leaf material (1 gram) 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 in to another tube containing 1 ml of Trim-Hill reagent (made up from 10 ml acetic acid, 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. (Asperulose, 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-,Naphtha-, 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 gm of dried, powdered, leaf material was exhaustively extracted with hot benzene for 3 x 12 hrs and 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 precipitates settled down. The acidified solution in turn, was extracted with diethyl ether and separated again into two layers. The lower layer was discarded, while 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 quinone compounds (Anthra-, Benzo-, Naphthaquinones) were visualized by their colour in visible, UV light and colour reactions after spraying magnesium acetate (concentration?) or aqueous NaOH. The quinones gives purple/pink/orange yellow colours.

ALKALOIDS

Alkaloids comprise the largest single class of secondary metabolites. They are basic plant products having a nitrogen containing hetrocyclic ring system and a high pharmacological activity. They are restricted to certain group of plants and therefore, often used as a criterion in classification 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; Price, 1963; 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 ~~but~~ and insoluble in organic solvents. Alkaloids are normally extracted from plants into weakly acids (1 M 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% 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 Dragendroff reagents (Paech and

Tracey, 1955). A white 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 were added, as precipitates of some alkaloids were soluble 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 precipitates with most of the alkaloids.

Dragendroff's reagent: (Potassium bismuth iodide) 8 grams of $Bi(NO_3)_3 \cdot 5H_2O$ 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 crystallized out. The supernatent was decanted off and made up to 100 ml with distilled water.