MATERIALS AND METHODS

CHAPTER - III

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The plant materials for the present work were collected from different parts of India such as Quilon, Trivandrum (Kerala), Ooty (Tamil nadu), Pachmarhi (Madhya Pradesh), Mahabaleshwar, Poona (Western Ghats), Gujarat and Kashmir. All the plants were properly identified and the voucher specimens are deposited in the herbarium of the M.S. University of Baroda, Baroda (BARO). The voucher specimens and the place of collection of the plant materials are presented in Appendix. 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 for 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 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, 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 disciplines 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. 6-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 flavone (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 experimentaly established that flavonoids with free hydroxyl groups at the 3',4'-position exert beneficial physiological effects on the capillaries through (1) chelating metals and then sparing ascorbate from oxidation, (2) prolonging epinephrine action by the intribution 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 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 combination 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 an O-glycosidic bond, 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 fractionated with ether to remove aglycones.

The procedure 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 diethyl ether/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 fraction 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 was 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. The 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. R_q (Rf relative to quercetin) values were calculated for all the compounds. The bands of compounds were cut out from unsprayed duplicate 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 for such studies and their preparations are given below:

Sodium Methoxide (NaOMe):

Freshly cut metallic 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 gm of fresh anhydrous AR grade $AlCl_3$ (which appeared yellow green and reacted violently when mixed with water) was 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 (NaOAe)

Anhydrous powdered AR grade NaOAc was used.

Boric acid(H_3BO_3)

Anhydrous powdered AR grade H_3BO_3 was used.

The concentration 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 6 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 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 strcture 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 methods as explained before.

Quinones

Quinones include all the aromatic diketones. They are also extracted with their flavonoids in the methanol extract. Aglycones as well as the C-glycosides, are isolated in papers or thin layers in the same way as flavonoids are separated. The presence of quinones in a chromatogram is indicated by the persistent pink or red colour when the chromatogram is sprayed with 10 % sodium carbonate solution.

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 releases 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 is also fairly widespread. Salicylic acid and the related o-pyrocatechuic acids are abundant in the Ericaceae. Ellagic acid and gallic acids are located in many plant groups of the Polypetalae. The phenolic acids are extracted in ether along with the flavonoid aglycones from the hydrolysed extract (fraction A & 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 chromatogram were diazotised p-nitra-aniline or diazotised sulphanilic acid, followed by an over spray of 10% Na_2CO_3 (Ibrahim and Towers, 1960).

Diazotization: 0.7 gm Of p-nitra-aniline/sulphanilic acid was dissolved in 9 ml of HCl and the volume made up to 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-nitra-aniline/sulphanilic acid stock solution to the cooled $NaNO_2$ solution. The volume was made upto 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 combine 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 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 flavon 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 alternatively 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 acids 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 emulsion 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 (or 2 gm dry powdered material) was taken in 20 ml test tube and covered with approximately 5 ml of 2N HCl. Hydrolysis 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 given taxa is considered by many (Hegnauer, 1966 ⍜, 1969, 1971; Kubitzki, 1969; Meeuse,1970; Bate-Smith, 1972; 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 Wieffering (1966) based on the Trim-Hill colour test (Trim and Hill, 1951). Fresh or dry powdered leaf material (1 gm) 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 Trim-Hill reagent (made up from 10 ml acetic acid, 1 ml of 0.2% $CuSO_4$. 5 H_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).

ALKALOIDS

Alkaloids comprise the largest single class of secondary metabolites. They are basic plant products having a nitrogen containing heterocyclic 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 and insoluble in organic solvents. Alkaloids are normally extracted from plants into weak acid solutions (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 the 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 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, 1965). The formation of a precipitate denoted the presence of alkaloids. The preparation of these reagents was as follows:

Mayer's reagent: (Potassium mercuric iodide) 1.36 grams of $HgCl_2$ was 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 on excess of the reagent.

Wagner's reagent: (Potassium iodide) 1.27 gms 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.

Dragendorff's reagent: (Potassium bismuth iodide) 8 Grams of $Bi(NO_3)_3 \cdot 5H_2O$ (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 supernatant was decanted off and made upto 100 ml with distilled water.