II. CHEMOSYSTEMATIC STUDIES

MATERIALS AND METHODS

MATERIALS AND METHODS

The plant materials for the present work were collected from different parts of India, particularly, Kerala, Tamilnadu, Gujarat and Kashmir. Most of the members of Caprifoliaceae and species of Galium were procured from Kashmir and Ooty while most of the Rubiaceae members were collected from Gujarat and Kerala.(The voucher numbers of the herbarium as well as the date and the place of collection of the plants screened in the present study are given in Appendix-1). The leaves from the 5th node downwards were used for extraction. 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 and this powder was used for the analysis. 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 are presented below.

FLAVONOIDS

Flavonoids are C_6-C_3-C_6 compounds related to a flavone skeleton considered as consisting of (i) a C6- C_3 fragment (Phenyl propane unit) that contains the 'B' ring and

(ii) a C_6 fragment the 'A' ring, both are of different biosynthetic origin. These polyphenols are subdivided based on the oxidation level of C_3 fragment of the phenyl propane unit, as anthocyanins, flavones, flavonols, aurones, chalcones etc., (Geissman,1962).

Flavonoids are the most sought after phytochemical characters useful to the classification of plants. The flavonoid data alongwith data from other chemical markers are used in schemes of Angiosperm classification (Harborne, 1977). Flavonoids are present in almost all vascular plants, but some classes of these compounds, such as isoflavones and biflavones are found to have a restricted occurance while flavones and flavonols are more widely distributed.

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, 1972). There is a tendency to resort to 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 while herbaceous taxa elaborate 6-hydroxy flavones (Harbone, 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 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 and so 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 absorbtion bands in UV and in the visible regions of the spectrum. A single flavonoid aglycone may occur in several glycosidic combinations in the same plant 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, are generally observed among flavones. They are resistant to hydrolysis and will remain in the aqueous layer when normal methods of 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 extract 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, 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 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 metallic sodium (2.5 gms) was added cautiously in small portions to dry spectroscopic methanol (100 ml). The solution was stored in a tighly closed glass bottle.

Aluminium chloride ($Al Cl_3$): 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.

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

The concentrations of the sample solution prepared by eluting chromatogram strips were adjusted so that the optical density (OD) fell in the region of o.6 to o.8. The methanol spectrum was taken using 4ml 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 2-3ml 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 a fresh solution of the flavonoids, till about a 2 mm layer 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 the above solution to make it saturated . The solution was discarded after recording the spectrum.

The structure of the flavonoids were elucidated with the help of the absorption maxima, shape of the curves, shifts with different reagents(both bathochromic and hypsochromic) 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 visualised by their colour in UV and with 10% Na_2CO_3 spray. Further analysis and identification were done with spectroscopic methods and co-chromatography.

PHENOLIC ACIDS

Phenolic acids are aromatic compounds having a functional carboxylic 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, formed during the hydrolysis 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 in free form or as bound to sugar in hydrolysable tannins. 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, 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 with a 10% Na_2CO_3 over spray (Ibrahim and Towers, 1960). These sprays are prepared by dissolving 0.7 gms of p-nitraaniline/sulphanilic acid in 9 ml of HCl and the volume made upto 100ml. Five ml of 1% $NaNO_2$ was taken in a volumetric flask and kept in ice till the temperature was below 4°C. The diazotisation was carried out by adding 4 ml of p-nitraaniline/sulphanilic acid stock solution to the cooled $NaNO_2$ solution. The volume was made upto 100ml 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.

COUMARINS

The hydroxy coumarin scopoletin, also is identified from the aglycone fractions. The identity of this compound was confirmed by spectral measurements and co-chromatography with authentic samples.

SUGAR ALCOHOLS

Sugar alcohols were extracted from fresh plant materials using 80% ethanol under reflux. The extract was concentrated under reduced pressure and the residue was dissolved in minimum water. Sugar alcohols were separated on paper using n-propanol-ethyl acetate-water (7:1:2) and were visualised with

alkaline Ag NO_3. The silver nitrate was prepared as a saturated solution in water and 1 ml of this was mixed with 200ml acetone. The chromatogrames were dipped into this solution and allowed to dry. The dry papers were then dipped into 0.5% ethanolic solution of NaOH. Sugar alcohols developed as drak brown spots. Their identity were confirmed by co-chromatography with standard samples.

ORGANIC ACIDS

The Organic acids were extracted by boiling plant materials in alcohol. The alchoholic extract was concentrated and spotted on whatman No.1 chromatographic papers. For separation of organic acids n-butanol-formic acid-water (4:1:5, organic layer) solvent system was used. The acids were visualised as blue spots on an yellow background when the chromatograms were sprayed with bromothymol blue (0.04 g in 100ml 0.01M NaOH). Co-chromatography with authentic samples confirmed the identity of these compounds (Harborne, 1984).

TANNINS

Tannins are polyphenolic compounds which combine with protein producing 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 flavan 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 reaction is used for the detection of condensed tannins. Hydrolysable tannins are mostly gallotannins or ellagitannins depending on whether gallic acid or ellagic acid is present esterified with glucose. They yield the corresponding phenolic acids and glucose on hydrolysis.

Tannins are extracted in water and are tested by treating them with 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 milky precipitate indicated 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, after cooling, was taken in a test-tube 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 plant materials are rich in saponins (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 10 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 cooled, decanted, 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 skelton with hydroxyl group at C_3 and two methyl groups at C_{10} and C_{13}. Cholesterol is the simplest sterol. 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 form 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 Liebermann-Burchard's reagent (1 ml of Conc. H_2SO_4, 20 ml of acetic anhydride and 50ml of chloroform were 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 R_f values.

IRIDOIDS

Iridoids are a group of monoterpenoid glycosides present in a number of advanced dicotyledons. The presence of these compounds in a given taxon 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 gram) was placed in a test-tube with 5ml 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% $CuSO_4.5H_2O$ in water and 0.5 ml Conc. HCl). When the test tube was heated for a short time in a flame, a colour was produced, if iridoids were 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 within the cell.

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. This acidified solution, then was extracted with diethyl ether and separated again into two layers. The lower layer was discarded, while upper acidic fraction was chromatographed over 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- and naphthaquinones) were visualized by their colour in visible, UV light and colour reactions after spraying with 2% magnesium acetate or aqueous NaOH(10%). The quinones give 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, 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 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, Wanger's and Dragendorf's reagents (Paech and Tracey, 1955). A white precipitate denoted the presence of alkolids (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 5ml of water and the solution diluted to 100ml. It gave brown flocculent precipitates with most of the alkaloids.

Dragendorf'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 crystallized out. The supernatant was decanted off and made up to 100ml with distilled water.

RESULTS

RESULTS

The Rubiaceae

The distribution of various flavonoids, alkaloids, iridoids, saponins, tannins, quinones, organic acids, sugar alcohols, coumarins, steroids and phenolic acids in leaves of 64 plants of Rubiaceae is presented in Table-1. The plants are arranged following Schumann (1891).

Out of 64 plants of Rubiaceae screened 52 contained various flavonoids. The types of flavonoids located are flavones, flavonols, glycoflavones and proanthocyanidins. The plants which were devoid of leaf flavonoids were Wendlandia lawii, Oldenlandia alata, Pentas carnea, Gardenia gummifera, Xeromphis spinosa, Knoxia corymbosa, Coffea liberica, Morinda tinctoria, Rubia cordifolia, Galium palustre, Galium asperifolium and Galium aparine. Apigenin, the only flavone located was confined to Gardenia florida. Flavonols formed the dominent phenolic pigments having been located in 48 members (75%). The various flavonols located were the derivatives of kaempferol and quercetin. Between the two flavonols, quercetin (with its derivatives) was more prevalent than kaempferol, the former being identified in 38 and the latter (with its derivatives) in 29. Kaempferol and quercetin co-occured in 19 plants. Methoxylated derivatives of kaempferol were rare while such derivatives of quercetin were more frequent. Only one plant Ophiorrhiza contained 6-hydroxy flavonols. Glycoflavones were located in three plants i.e. Anthocephalus cadamba, Serissa foetida and Spermadictyon suaveolens.

TRIBE-OLDENLANDIEAE.

1. Dentella repens Forst.	+		+	+	
2. Oldenlandia alata Koen.					
3. O. corymbosa Linn.			+		
4. O. auricularia Schum.	+		+		
5. O. herbacea Roxb.	+				
6. O. gracilis DC.			+		
7. O. stylosa O.Kze.	+ +			+	
8. Ophiorrhiza mungos Linn.			+	+	
9. Pentas carnea Benth.					

TRIBE-RONDELETIEAE.

10. Wendlandia lawii Hook.f.					
11. W. notoniana Wall.			+		
12. Rondeletia speciosa Lodd.					+

TRIBE-CINCHONEAE.

13. Cinchona calisaya Wedd.		+	+	+	
14. C. officinalis Hook.			+	+	
15. Hymenodictyon excelsum Wall.	+		+		

TRIBE-NAUCLEEAE.

16. Anthocephalus cadamba Miq.	+		+	+	
17. Adina cordifolia(Roxb.) Benth. & Hook. f.			+		
18. Stephegyne parvifolia Korth.	+		+		+
19. S. tubulosa Hook. f.	+		+		+

Table - 1 Continued.

Sr No.	Name of the plant	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	TRIBE-MUSSAENDEAE.															
20.	Mussaenda frondosa Linn.			+						+		+				+
21.	M. erythrophylla Schum. & Thonn.															+
22.	M. luteola Delile															+
23.	M. laxa Hutch.			+						+						
	TRIBE-GARDENIEAE.															
24.	Randia macrophylla r.				+											
25.	R. rugulosa Thw.													+		
26.	R. dumetorum Lamk.			+	+						+					
27.	Gardenia latifolia At.											+		+		+
28.	G. lucida Roxb.									+	+			+		
29.	G. florida Linn.	+									+					+
30.	G. gummifera Linn.															
31.	Hamelia patens Jacq.			+	+					+				+		+
32.	Catesbaea spinosa Lin.			+												
33.	Xeromphis uliginosa(etz.) Maheshari.											+				
34.	Xeromphis spinosa(Thnb.) Keay															
	II.SUBFAMILY-COFFEOIEAE.															
	TRIBE-KNOXIEAE.															
35.	Knoxia corymbosa Wild.															
	TRIBE-VANGUERIEAE															
36.	Plectronia didyma Kuz.				+											
37.	P. parviflora Bedd.			+	+											
38.	Meyna laxiflora Robns			+						+						+

Table - 1 Continued.

Sr No.	Name of the plant	1	2	3	4	5	6	7	8	9	10	11	12	13	1
	TRIBE IXOREAE														
39.	Ixora parviflora vahl.			+											
40.	I. cocinea Linn.			+						+					
41.	I. nigricans Br.			+				+							
42.	Pavetta indica Linn.											+			
43.	P. indica Linn. Var. tomentosa Roxb.												+		
44.	Coffea robusta Linden														
45.	C. liberica Hiern														
	TRIBE PSYCHOTRIEAE														
46.	Psychotria flavida Talb.			+		+									
47.	P. elongata Wight.				+										
	TRIBE-PAEDERIEAE														
48.	Spermadictyon suaveolens Roxb.		+	+	+					+		+			
	TRIBE-ANTHOSPERMEAE														
49.	Serissa foetida commers.		+												
	TRIBE-MORINDEAE.														
50.	Morinda tinctoria Roxb.														
51.	M. reticulata Gamble			+								+			
52.	M. citrifolia Linn.			+						+	+				
	TRIBE-SPERMACOCEAE.														
53.	Spermacoce latifolia Aubl.			+	+										
54.	Borreria hispida Schum.			+						+			+		
55.	B. eradii Ravi													+	
56.	B. articularis(L.F.)F.N.Will.			+						+				+	
57.	B. verticillata Meyr.			+						+					
58.	Mitracarpum verticillatus (Schum. & Thonn.) Vatke									+				+	

Table-1 Continued.

Sr No.	Name of the plant	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	TRIBE-GALIEAE.														
59.	Rubia cordifolia Linn.														
60.	Galium palustre Linn.														
61.	G. asperifolium Wall.														
62.	G. verum Linn.										+				
63.	G. parisiense Linn.									+					
64.	G. aparine Linn.														

1. Apigenin
2. Glycoflavones
3. Kaempferol
4. 4'-OMe Kaempferol
5. 5 -OMe Kaempferol
6. 7 -OMe Kaempferol
7. 7, 4'- diOMe Kaempferol
8. 3, 7- diOMe Kaempferol
9. Quercetin
10. 3' - OMe Quercetin.
11. 4' - OMe Quercetin.
12. 7-OMe Quercetin.
13. 3', 4'- diOMe Quercetin
14. Herbacetin
15. Proanthocyanidins
16. Scopoletin.
17. Quinones
18. Alkaloids
19. Iridoids
20. Sugar alcohols
21. Saponins
22. Steroids
23. Tannins
24. Organic acids

Proanthocyanidins were located in 17 plants. Iridoids were present in 23, and 35 plants contained saponins in their leaves. Only 10 plants elaborated alkaloids; the hydroxy-coumarin scopoletin was identified in five plants while quinones were seen in 10 taxa. Mannitol, the sugar alcohol was located in seven plants. Only Ixora coccinia contained tannins in its leaves. Phenolic acids present among the members of Rubiaceae were, p-hydroxybenzoic acid, gentisic acid, protocatechuic acid, vanillic acid, syringic acid, β-resorcylic acid, 3-hydroxy, 5-methoxy benzoic acid, melilotic acid, phloretic acid, p-coumaric acid, o-coumaric acid, ferulic acid and sinapic acid. All the plants screened contained steroids in them.

The two subfamilies of Rubiaceae, the Cinchonoideae and the Coffeoideae, show distinct chemical differences between them.

The subfamily Cinchonoideae contained higher frequency of quercetin (71%) and proanthocyanidins (35%) and lesser incidence of kaempferol (41%) and iridoids (26%).

On the contrary, the subfamily Coffeoideae showed lesser incidence of quercetin (47%) and proanthocyanidins (17%). This taxon has a higher percentage of occurence of kaempferol (50%) and iridoids (47%).

The Cinchonoidea

Within the subfamily Cinchonoideae the tribes Cinchoneae and

Naucleeae were closely related to each other in their chemical contents. The tribe Oldenlandieae was distinct in eliminating proanthocyanidins and the Mussaendeae in not containing iridoids.

In tribe Oldenlandieae nine plants belonging to four genera have been screened. Seven plants contained flavonols. Oldenlandia alata and Pentas carnea were devoid of flavonoids. Glycoflavones and proanthocyanidins were absent from all plants. Three plants screened produced both kaempferol and quercetin. Four plants contained kaempferol and one contained its methylated derivatives. Five plants contained quercetin and three plants contained its derivatives.

Of the four genera screened, Ophiorrhiza is unique in having the 6-hydroxy flavonol herbacetin confined to itself. Pentas also is distinct in eliminating the flavonoids in leaves. Dentella contained scopoletin as its characteristic marker. Oldenlandia is similar to Ophirrhiza in containing alkaloids, saponins and similar flavonoids.

The tribe Rondeletieae, wherebthree plants belonging to genera Wendlandia and Rondeletia have been screened, was distinct from the other tribes described hitherto in the absence of kaempferol. Two plants contained flavonols and proanthocyanidins, while Wendlandia lawii was devoid of flavonoids. Wendlandia and Rondeletia are different from each other chemically. Even the two species of Wendlandia are chemically distinct.

In the tribe Cinchoneae three plants belonging to the genera Cinchona and Hymenodictyon have been screened. All the plants screened were found to possess flavonols. Only two plants showed proanthocyanidins. Two plants showed co-occurence of kaempferol and quercetin. In this tribe, Hymenodictyon was found to be strikingly different from Cinchona, with which it was grouped once, in not having alkaloids and in containing scopoletin, quinones, iridoids, mannitol, saponins and phenolic acids like gentisic acid, protocatechuic acid and p-coumaric acid which were not produced by the latter genus. The two species of Cinchona also were distinguishable chemically. Cinchona calisaya was distinct from C. officinalis in having methylated derivatives of kaempferol and proanthocyanidins in its leaves.

The tribe Naucleeae, where four plants belonging to three genera screened, showed a uniform distribution of flavonoids and was similar to Cinchoneae in possessing flavonols in all the members. Glycoflavones were present in one plant i.e. Anthocephalus cadamba. Stephegyne parvifolia, and Stephegyne tubulosa contained kaempferol. Four plants showed quercetin alongwith its methoxylated forms. Proanthocyanidins were seen in two plants. Kaempferol and quercetin co-occured in two plants. In this tribe Anthocephalus stands distinct from other taxa in containing glycoflavones quinones and alkaloids, and in the absence of proanthocyanidins. Adina was different from Stephegyne in not containing kaempferol and in having saponins. Between the two species of Stephegyne, S. parvifolia can be identified due to its proanthocyanidins.

The tribe Mussaendeae, where in four species of the genus Mussaenda have been screened, is distinct being free of iridoids. Two plants contained flavonols, and three members showed proanthocyanidins. Two members, Mussaenda luteola and M.erythrophylla were devoid of flavonols. None possessed glycoflavones. Kaempferol and quercetin co-occurred in M. frondosa and M. laxa where flavonols were present. M. laxa differs from other species in not producing proanthocyanidins and saponins.

In tribe Gardenieae 11 plants belonging to five genera have been screened. Nine plants showed flavonols and one member showed flavone in its leaves. Two members which were devoid of flavonoids, were Gardenia gummifera and Xeromphis spinosa. None produced glycoflavones; proanthocyanidins were present in only three. Kaempferol and quercetin co-occurred in Hamelia patens and Randia dumetorum. One member of this tribe viz. Gardenia floria contained a flavone, apigenin, which is not encountered in any member of the family screened. In this tribe only Randia, Hamelia and Catesbaea contained kaempferol, while proanthocyanidins were restricted to Gardenia and Hamelia. Iridoids also were more prevalent in the genus Randia. Alkaloids were present in Hamelia and Catesbaea.

The Coffeoideae

Among the tribes of Coffeoideae, the knoxieae is distinct in the absence of flavonoid system, the Vanguerieae in not

producing iridoids, and the Psychotrieae in not containing quercetin and its derivatives. The absence of proanthocyanidins keeps the Paederieae, Morindeae, Spermacoceae and Galieae together.

In tribe knoxieae, only one plant has been screened viz. Knoxia corymbosa and flavonoids were found to be absent in this plant.

In tribe Vanguerieae, three plants belonging to two genera Plectronia and Meyna have been screened. All the three, showed flavonols in their leaves. Meyna is distinct from Plectronia in containing quercetin and proanthocyanidins while the later genus in having kaempferol confined to it.

In tribe Ixoreae seven plants belonging to three genera have been screened. Flavonols are present in five plants and three plants contained proanthocyanidins. Both the species of Coffea were devoid of flavonols. Between these two species C. robusta elaborated proanthocyanidins in leaves. All the three species of Ixora characteristically contained kaempferol which is absent from Pavetta.

In tribe Psychotrieae, two plants belonging to the genera Psychotria have been screened. Both plants contained flavonols. Psychotria flavida contained proanthocyanidins. Co-occurence of kaempferol and quercetin were not observed in members screened.

In tribe Paederieae, only one plant has been screened viz. Spermadictyon suaveolens. It showed flavonols, kaempferol and quercetin co-occuring. It also contained glycoflavones and iridoids as its marker characters.

In tribe Anthospermeae only one plant viz. Serissa foetida has been screened. This plant elaborated glycoflavones and phloretic acid, and was devoid of flavonols.

In tribe Morindeae three plants belonging to the genus Morinda have been screened. Morinda reticulata and M. citrifolia contained flavonols; quercetin and kaempferol co-occuring while Morinda tinctoria was devoid of flavonoids. Proanthocyanidins and glycoflavones were absent from all the three plants screened but quinones were seen in them.

In tribe Spermacoceae six plants belonging to three genera have been screened. All the six plants contained flavonols. Glycoflavones and proanthocyanidins were absent from all the plants. Three plants showed co-occurence of quercetin and kaempferol. Borreria contained more of quercetins while Spermacoce contained more of kaempferol. Alkaloids, iridoids, and saponins were confined to Borreria only. Mitracarpum was devoid of kaempferol.

In tribe Galieae six plants belonging to the genera Rubia and Galium were screened. Only two plants, Galium parisiense and Galium verum showed flavonols in them, while all other plants

were devoid of flavonols. Kaempferol and proanthocyanidins also were absent from the plants of this tribe. The iridoids and saponins were omnipresent. Quinones were seen to replace flavonoids in species belonging to Rubia and Galium.

The Caprifoliaceae

The distribution of various flavonoids, proanthocyanidins, saponins, scopoletin, and phenolic acids in leaves of eight members of Caprifoliaceae is presented in table II. The plants are arranged following Schumann (1891).

Out of eight plants screened, all contained various flavonoids in the leaves. The types of flavonoids located are flavones, flavonols, and proanthocyanidins, Proanthocyanidins was confined to Viburnum cottinifolium. Flavones and flavonols were equally (50%) distributed in all the plants screened. The flavones identified were apigenin, acacetin, and 3'-,4'-, and 7-methoxylated luteolins. Among flavones, acacetin was more prevalent than other (5/8). The flavonols present were kaempferol, quercetin and their mono-and dimethoxylated derivatives. Flavonols were the only phenolic pigments in Sambuceae, but they were absent from the Lonicerae. Flavones were widely distributed in the tribe Lonicerae but absent from the Sambuceae. Viburnum cottinifolium contained both flavones and flavonols. Iridoids and alkaloids were absent in the plants

Table - II DISTRIBUTION OF FLAVONOIDS, SCOPOLETIN, SAPONINS AND PHENOLIC ACIDS IN EIGHT MEMBERS OF THE CAPRIFOLIACEAE

Sr No.	Name of the plant	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
	TRIBE - SAMBUCEAE																						
1.	Sambucus Wightiana Wall.									+					+	+	+		+			+	
	TRIBE VIBURNEAE																						
2.	Viburnum cottinifolium Don		+			+			+				+		+	+			+	+			
3.	V. foetens Dcne.						+	+						+	+				+	+	+		
4.	V. tinus Linn.									+	+	+			+		+	+	+	+	+	+	+
	TRIBE LONICEREAE.																						
5.	Abelia triflora Br.		+												+		+		+				+
6.	Lonicera bicolor Klotzsoh	+	+												+				+	+			
7.	L. Japonica Wall.	+	+		+										+				+	+	+		
8.	L. obovata Royle		+	+											+	+	+		+			+	+

screened while saponins were omnipresent. The hydroxy coumarin scopoletin was located only in Viburnum foetens. Phenolic acids present among the members of the Caprifoliaceae were p-hydroxybenzoic acid, gentisic acid, protocatechuic acid, vanillic acid, syringic acid, p-coumaric acid, melilotic acid, and ferulic acid. Syringic acid was uniformly distributed in the Viburneae but absent from the Sambuceae.

The three tribes, Sambuceae, Viburneae and Lonicereae, show a number of chemical differences among them. The tribe Sambuceae is characterised by flavonols, the tribe Lonicereae by flavones and the tribe Viburneae by both flavones and flavonols.

In the tribe Sambuceae where only one member, Sambucus wightiana, was screened contained the flavonol quercetin only, while in the tribe Viburneae where three members have been screened, Viburnum cottinifolium was found to contain the flavones acacetin and methylated derivatives of luteolin along with the flavonol kaempferol. It also showed proanthocyanidins. Viburnum foetens and Viburnum tinus also showed chemical differences in having kaempferol and its methylated derivatives in the former and quercetin and its methylated forms in the latter. These two were devoid of proanthocyanidins. Viburnum foetens produced scopoletin, which was absent in the other members screened.

In tribe Lonicereae where four plants belonging to two genera have been screened, all the members elaborated flavones as the sole phenolic pigments and flavonols were absent from all. Abelia triflora was distinct in containing only acacetin, where as Lonicera bicolor and Lonicera japonica contained both apigenin and acatetin. But Lonicera japonica also contained a methoxylated derivative of luteolin. In Lonicera obovata, apigenin was absent and acacetin and luteolin were found present.

DISCUSSION

DISCUSSION

The Rubiaceae

The family Rubiaceae appears to be a chemically homogeneous taxon. The predominance of flavonols such as quercetin and kaempferol is the major character binding the members together. The near absence of flavones, glycoflavones, 6-hydroxy flavonoids and complete absence of highly hydroxylated flavonol myricetin are the other characters of the family. As reported earlier (Gibbs, 1974) this family is a rich storehouse of alkaloids and cardiac glycosides. A number of taxa are found to elaborate hydroxyquinones in their roots. The presence of these compounds, bring the family closer to the Apocynaceae and Asclepiadaceae with which it has been grouped recently. The reduction of proanthocyanidins and absence of myricetin do not allow to consider the family a very primitive taxon. But the absence of advanced characters such as flavones does not bring the family at a higher level either, although the occurence of iridoids in at least 23 (36%) plants can be cited indicating the evolutionary trends operating within the family.

In containing flavonols, proanthocyanidins and indole alkaloids, Rubiaceae seems to be closer to the Apocynales sensu lato (Daniel & Sabnis, 1987). Aucubin and Asperuloside are the two iridoids common between Apocynales and Rubiales.

The Rubiaceae differ from the Gentianales sensu lato (Daniel & Sabnis, 1982) within which this family is included by some workers in producing hydroxyquinones especially anthraquinones in great variety and in not synthesising flavones (Exacaceae), glycoflavones, xanthones (Gentianaceae), and loganin (Loganiaceae). The proposed relation with Dipsacales, does not seem to get much support from chemical grounds. The characters which are frequent in family of Dipsacales such as flavones (Caprifoliaceae) and monoterpenoid alkaloids (Valerianaceae) are not seen in any of the member of Rubiaceae. It is therefore logical to support the concept of the order Rubiales as done by Cronquist (1981). Rubiales appear to occupy a position intermediate between the Gentianales and the Apocynales but closer to the latter order. The possible relationships are presented in Fig-1. Such a treatment gains support from recent studies from cytology and palynology of the Rubiaceae (Mathew and Philip, 1983).

The two subfamilies Cinchonoideae and Coffeoideae appear to be natural groups. The former contains more of primitive characters such as quercetin and proanthocyanidins while the latter subfamily is comparatively richer in advanced characters like iridoids and kaempferol (between quercetin and kaempferol latter is considered to be advanced). This evidently keep the Cinchonoideae as primitive and Coffeoideae as advanced.

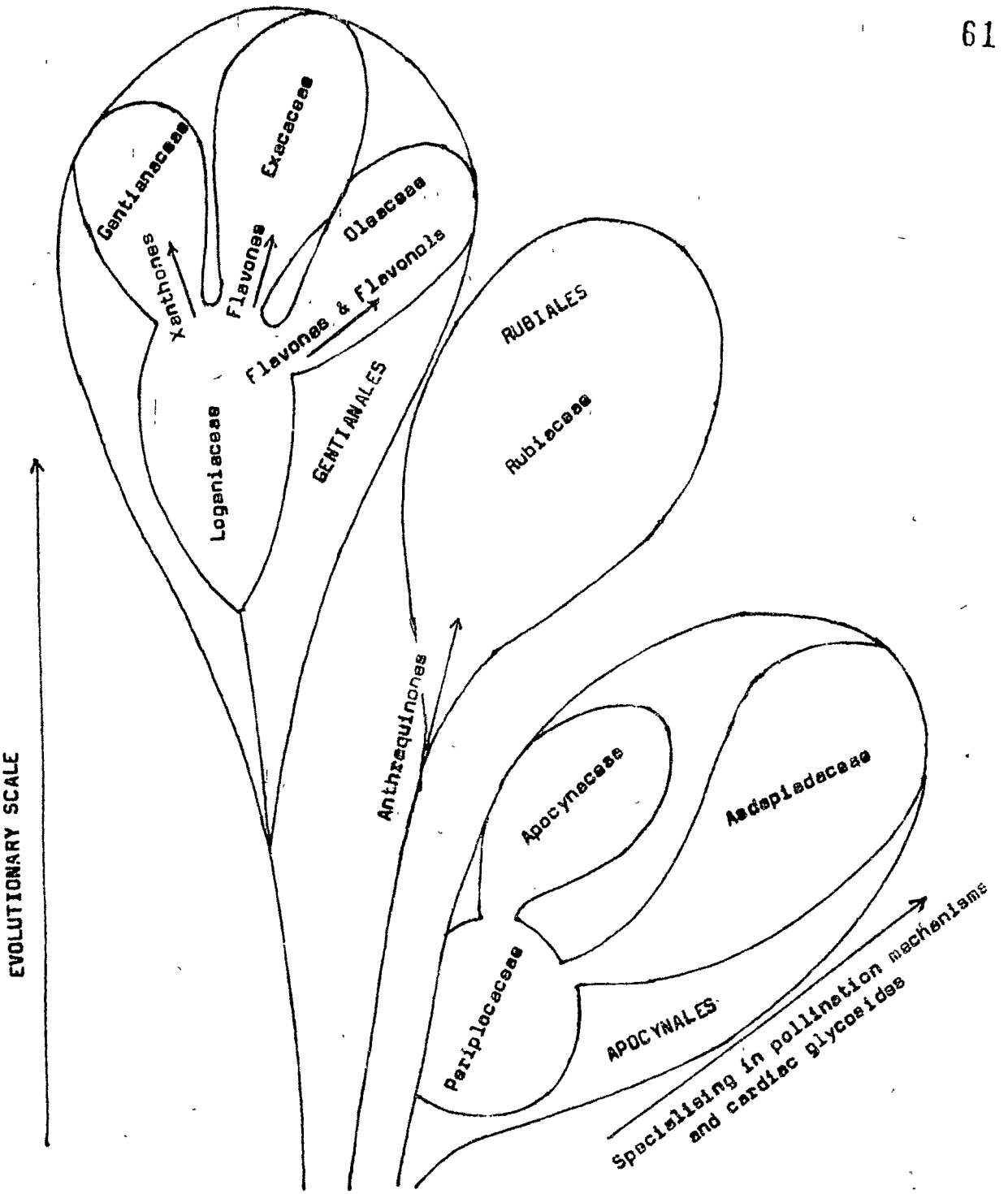

FIG.1. The probable relationships of the Rubiaceae with the families of the Apocynales and Gentianales.

Subfamily Cinchonoideae

Within the tribe Oldenlandieae, Ophiorrhiza is unique in having 6-hydroxyflavonol herbacetin confined to it. It does not possess iridoids and kaempferol which are otherwise common in the tribe. This chemical distinctness gives validity to the treatment of this genus as a separate tribe Ophiorrhizeae by Bremekamp (1966). Palynologically also Ophiorrhiza is peculiar in having a very exceptional phenomenon of pollen bud formation during the development of the male gametophyte (Mathew and Philip, 1975). With the exclusion of Ophirrhiza the tribe Oldenlandieae becomes a homogeneous taxon. The absence of proanthocyanidins and the prevalence of iridoids keep this tribe at an advanced level. This contention get support from cytological and palynological observations.

The tribe Rondeletieae appear to be a natural taxon chemically. The absence of kaempferol and prevalence of proanthocyanidins keep this tribe comparatively primitive.

The close chemical similarity existing between the tribes Naucleae and Cinchoneae makes it difficult to comment on the treatment and reshuffling given to these tribes. Rather it is difficult to distinguish these tribes. Such similarities are also reflected in cytological and palynological characters also. The suggestion of keeping Cinchona in a monotypic tribe (Mathew and Philip, 1983) may get some support from chemistry

in that none of the other genera in these tribes elaborate quinoline type of alkaloids such as quinine. The separate status of Hymenodictyon away from Cinchona is also clear chemically.

In tribe Mussaendeae where in only one genus Mussaenda was screened appear to be primitive in containing proanthocyanidins and in the absence of iridoids. In containing the above mentioned characters this tribe is closer to Cinchoneae-Naucleae.

The tribe Gardenieae appear heterogeneous with a sporadic distribution of flavone and flavonol, proanthocyanidins and iridoids. Saponins are farely prevalent in this tribe. This tribe occupies an intermediate position between Cinchoneae, Naucleae and Oldenlandieae.

Subfamily Coffeoideae

The tribe Knoxieae in which only one member is screened shows the absence of flavonoid system. Though the loss of flavonoid system is considered a highly advanced character, this character may not be taken into consideration here due to the minimum number of plants screened.

The tribe Vanguerieae contains kaempferol distributed throughout. Between the two genera Plectronia and Meyna, the latter appears to be primitive in containing proanthocyanidins.

In the tribe Ixoreae, Coffea is distinct in loss of flavonoids while Ixora and Pavetta retain their chemical identity. The absence of proanthocyanidins and prevalence of kaempferol than quercetin keep the tribes Psychotrieae, Paederiae, Anthospermeae, Morindeae, and Spermacoceae as advanced. With the available chemical data a distinction between Spermacoce and Borreria are not possible. This does not anyway rule out the separate generic status of these two taxa.

Evidently Galieae is the most advanced tribe of the subfamily and also of the Rubiaceae. The reduction in the flavonols, the absence of proanthocyanidins and the uniform distribution of iridoids keep this tribe at a very high evolutionary level. This placement of Galieae is also supported by the cytological and palynological observations of Mathew and Philip. The evolutionary levels achieved by the various tribes of the Rubiaceae are graphically represented in Fig-2.

The presence of polyhydroxy and polymethoxy flavonols in most of the taxa screened is significant. These compounds which are known as 'bioflavonoids' are known to exert many beneficial efforts, such as increasing resistance to the capillary walls, (reducing the capillary fragility) and increasing the blood circulation (by reducing the agglutination of R.B.C. seen at the time of illness). The main plants which can be considered as sources of bioflavonoids are given in table-III.

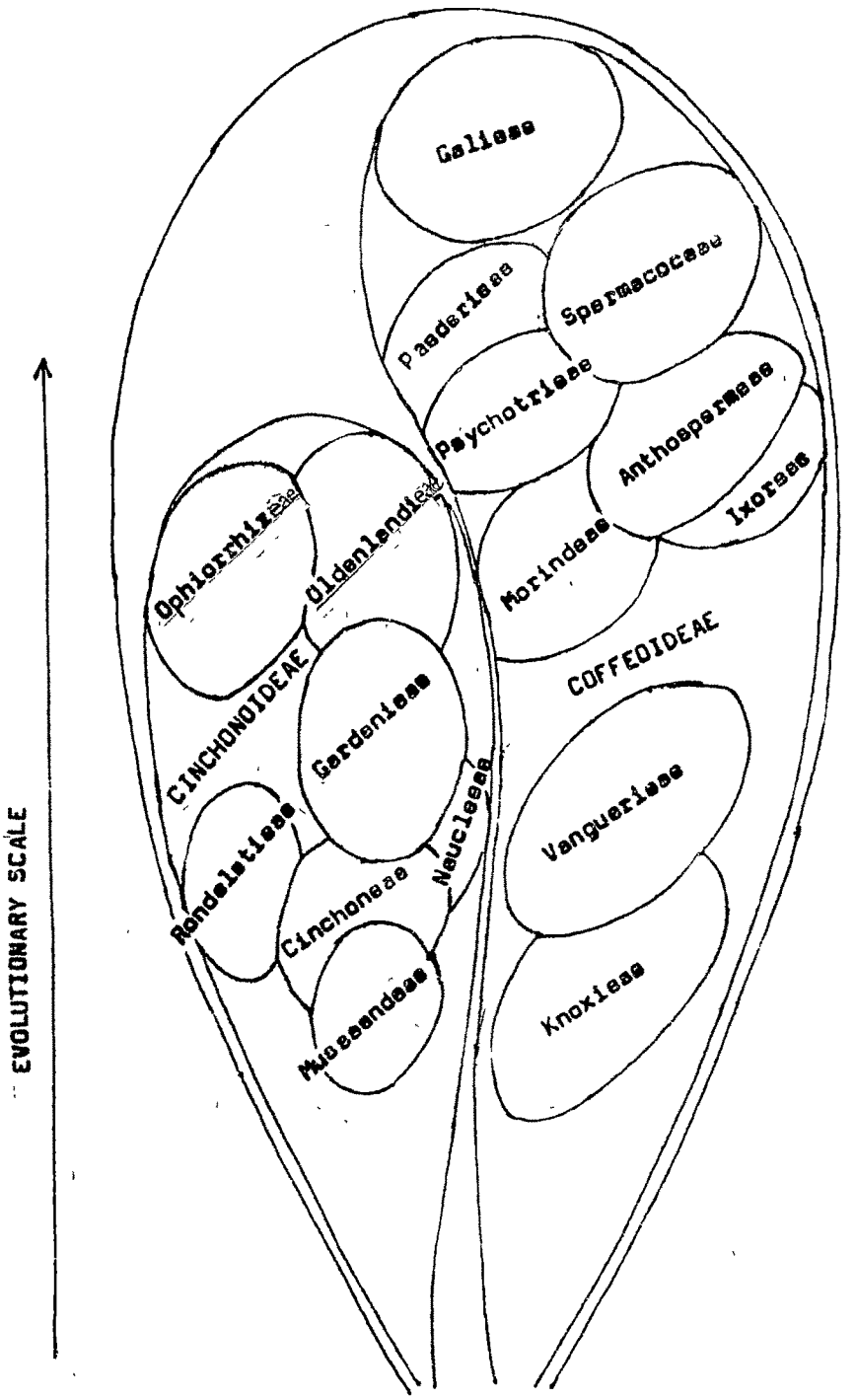

FIG. 2. The evolutionary levels achieved by various tribes of the Rubiaceae.

TABLE - III

Plants which can be considered as sources of bioflavonoids

1. Adina cordifolia (Roxb.) Benth. & Hook. f.
2. Anthocephalus cadamba Miq.
3. Borreria articularis (L.F.) F.N. Will.
4. B. eradii Ravi
5. B. hispida Schum.
6. B. verticillata Meyr.
7. Cinchona calisaya Wedd.
8. C. officinalis Hook.
9. Dentella repens Forst.
10. Galium parisiense Linn.
11. Galium verum Linn.
12. Gardenia florida Linn.
13. G. Latifolia Ait.
14. G. Lucida Roxb.
15. Hamelia patens Jacq.
16. Hymenodictyon excelsum Wall.
17. Ixora coccinea Linn.
18. Meyna laxiflora Robyns
19. Mitracarpum verticillatus (Schum. & Thonn.) Vatke
20. Morinda citrifolia Linn.
21. M. reticulata Gamble

Table - III Contd.

22. Mussaenda frondosa Linn.
23. M. laxa Hutch.
24. Oldenlandia auricularia Schum.
25. O. corymbosa Linn.
26. O. gracillis DC.
27. O. stylosa O. Kze.
28. Ophiorrhiza mungos Linn.
29. Pavetta indica Linn.
30. P. indica Linn. var. tomentosa Roxb.
31. Randia dumetorum Lamk.
32. R. rugulosa Thw.
33. Rondeletia speciosa Lodd.
34. Spermadictyon suaveolens Roxb.
35. Stephegyne parvifolia Korth.
36. S. tubulosa Hook. f.
37. Wendlandia notoniana Wall.
38. Xeromphis uliginosa (Retz.) Maheshwari

The Caprifoliacea

The family Caprifoliaceae is peculiar in containing both flavones and flavonols equally distributed among its members. The tribes Sambuceae, Viburneae and Lonicereae are chemically distinct and chemical nature of the taxa screened shows that the family is a heterogeneous assemblage. This family is distinct from the Rubiaceae in not having alkaloids and having predominance of saponins and flavones (5/8). The reduction in frequency of occurrence in the flavonols (4/8) and proanthocyanidins (1/8) are the other characters distinguishing the Caprifoliaceae from the Rubiaceae. The chemical features do not reflect any similarity between the Rubiaceae and the Caprifoliaceae which were once considered closely related. The colleters which are characteristic features of Rubiaceae are absent in the Caprifoliaceae. In addition the latter family possesses a cellular pattern of endosperm development as against nuclear pattern of endosperm development of the former family. Therefore the apparent similarities between these two families may be considered due to convergence as suggested by Wagenitz (1959) rather than any close affinity. Thus the Caprifoliaceae find a better place in Dipsacales than in Rubiales. In containing flavones and in the absence of proanthocyanidins, the Caprifoliaceae are chemically very close to the families of the Dipsacales.

The tribe Sambuceae is characterised by the presence of flavonols and absence of proanthocyanidins. These characters keep the tribe primitive.

The tribe Viburneae possessing scopoletin, flavones, flavonols, and proanthocyanidins forms the intermediate taxon and is more closer to Rubiaceae than any other tribe of the Caprifoliaceae. Both Sambucus and Viburnum are considered standing somewhat apart from the rest of the Caprifoliaceae on morphological grounds(Cronquist, 1981). This is true of its chemistry also. Both these plants contain flavonols and thus are different from the remaining Caprifoliaceae members which are flavone-rich.

The tribe Lonicereae is the advanced tribe of the family due to the absence of flavonols and in having flavones uniformly distributed.