CHAPTER - II

MATERIALS & METHODS

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The plant materials for the present work were collected from different parts of India, particularly Kerala, Tamil Nadu Maharashtra and Gujarat. The plant materials were identified and voucher specimens have been deposited in the herbarium Department of Botany, The M.S.University of Baroda, BARODA (BARO) Table - 6). 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 air-tight 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 proanthocyanins. A brief account of the various methods followed in the extraction of chemical compound and the characterisation is presented below.

2.1 FLAVONOIDS

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 with ammonia and thus are easily detected in chromatograms or in solution. 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 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 hours till the plant material become 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 filterate was hydrolysed in a water bath for one hour using 7% HCl. This hydrolysate was extracted with diethylether/solvent

ether, whereby the aglycone got separated into ether fraction (Fraction A). The remaining aqueous fraction was further hydrolysed for another 10 hours 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 neutralised 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 were Forestal (Conc. HCl : Acetic acid; Water, 3 : 30 : 10) or 30% glacial acetic acid. The developed chromatograms were dried in air and the visibly coloured compounds were marked out. These papers were observed in ultraviolet light (360 nm) and the bands were noted. Duplicate chromatograms were then sprayed with 10% aqueous Na_2CO_3 and 1% $FeCl_3$ and the color 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 using Carl-Zeiss VSU 2 P Spectrophotometer. The NaOMe spectrum was measured immediately after the addition of 3 drops of NaOMe stock solution to the flavonoid solution used for methanol spectrum. The solution was then discarded. The $AlCl_3$ spectrum was measured immediately after the addition of six drops of $AlCl_3$

stock solution to 2-3 ml of fresh stock solution of the flavonoids. The $AlCl_3$/HCl spectrum was recorded next, after the addition of 3 drops of 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 absorption maxima, shape of the curves, shifts (both bathochromic and hypsochromic) with different reagents and reactions. The identifications were confirmed by co-chromatography with authentic samples.

The aqueous fraction remaining after the separation of aglycones was neutralised 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 using spectroscopic method as explained before. Preparation of reagent stock solutions for spectral analysis is as follows:

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 tightly closed glass bottle.

Aluminium chloride ($AlCl_3$): Five grams of fresh anhydrous a 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 AR grade NaOAc was used.

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

2.2 Phenolic acids

Phenolic acids were extracted in ether along with 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 chromatography. Benzene : acetic acid : water (6 : 7 : 3, upper organic layer) in the first direction and sodium formate: formic acid : water (10 : 1 : 100) in the second direction were used as irrigating solvents. The sprays used to locate

the compounds on the chromatograms were diazotised p-nitraaniline or dizotised sulphanilic acid and 10% Na_2CO_3 over spray (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 was below 4°C. The diazotised sprays were prepared by adding 4 ml of p-nitraaniline/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.

2.3 Tannins

Tannins are extracted in water and are treated by treating them with protein solution. The formation of white or milky precipitate on addition of 20% freshly prepared gelatin solution to aqueous plant extract indicated the presence of tannins in the plant material (Gibbs, 1974; Harborne, 1984).

2.4 Proanthocyanins

The presence of proanthocyanins were tested following Gibbs (1974). 5 gm of finely chopped plant material was

hydrolysed in a test tube in a boiling water bath for half an hour. The extract was decanted after cooling and shaken with amyl alcohol. Presence of red or near carmine colour in the upper alcoholic layer denoted a positive reaction for proanthocyanidins. An olive yellow color represented a negative reaction.

2.5 Iridoids

The plants were surveyed for iridoids by a simple procedure described by Wieffering (1966) based on the Trim-Hill Color test (Trim-Hill, 1952). Fresh or dry powdered leaf material (1 gm) was placed in a test tube with 5 ml of 1% aqueous Hydrochloric acid. After 3-5 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% $CuSO_4 \cdot 5H_2$ in water and 0.5 ml Conc. HCl). When the tube was heated for a short time in a flame, a color was produced, if iridoids are present (Asperulose, Aucubin and monotropein give blue colors, Herpagide a red-violet; Harborne, 1984).

2.6 Alkaloids

Alkaloids, as a rule are insoluble in water but soluble in organic solvents. But their salts are soluble in water but 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% ammoniacal ethanol for 48 hours. 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's reagents (peach and Tracey, 1955). A white precipitate denoted the presence of alkaloids (Amarasingham et al. 1964). The preparation of the reagents were as follows.:

Mayers' 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 $Bi(NO_3)_3.5H_2O$ 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 and made up to 100 ml with distilled water.

Table - 6. Details of voucher specimens deposited in the herbarium Department of Botany, The M.S. University of Baroda, belonging to the family Euphorbiaceae.

Sr.No.	Name of the plant	Place of collection	Date of collection	Voucher specimen No.
1.	Bridelia cernulata Roxb.	Marathwada	18-9-1983	BARO AN 501
2.	Cleistanthus collinus Benth.	"	"	BARO AN 502
3.	Aporusa lyndeliana (Wight) Baillon.	Kerala	26-12-1982	BARO AN 525
4.	Dryostes roxburghii wall.	Vaghai	10-9-1982	BARO AN 526
5.	Securinega virosa (Willd.) Baillon	Marathwada	18-9-1983	BARO AN 527
6.	S.leucopyrus (Willd.) DC.	"	"	BARO AN 528
7.	Breynia nivosa (Bull.) pax & Hoffn.	"	"	BARO AN 529
8.				
8.	Breynia rhamnoides Retz.	"	"	BARO AN 530
9.	B.retusa (Bennst.) Alston	Baroda	15-10-1983	BARO AN 601
10.	Emblia officinalis Gaertn.	Kerala	26-12-1982	BARO AN 602
11.	Cicca acida L.	Billimora	15-3-1983	BARO AN 603
12.	Phyllanthus virgatus Forst.	Tiruchirappalli	20-4-1983	BARO AN 638
13.	Phyllanthus fractaranus Webster	"	"	BARO AN 639
14.	P.madaraspatensis L.	"	"	BARO AN 705

Table - 6 (Contd.)

Sr.No.	Name of the plant	Place of collection	Date of collection	Voucher specimen No.
15.	Chrozophora prostrata Dalz.	Baroda	11-8-1982	BARO AN 706
16.	C.rottleri (Geisler) Sprengel	"	"	BARO AN 707
17.	Ricinus communis L.	"	"	BARO AN 739
18.	Macaranga indica Wight. Icon.	Kerala	26-12-1982	BARO AN 740
19.	M.peltata (Roxb.)Muell Arg.	"	"	BARO AN 788
20.	Acalypha ciliata Forsk.	Baroda	15-10-1983	BARO AN 807
21.	Acalypha hispida Burm.	"	"	BARO AN 789
22.	Acalypha wilkesiana Muell.	"	"	BARO AN 790
23.	A.godseffiana L.	Trivandrum	26-12-1982	BARO AN 801
24.	A.indica L.	Baroda	15-10-1983	BARO AN 802
25.	Trewia nudiflora L.	Waghai	10-9-1982	BARO AN 803
26.	Mallotus philippianensis Muell Arg.	Pavagarh	3-1-1984	BARO AN 804
27.	Tragia involucrata L.	Bhavnagar	25-12-1983	BARO AN 805
28.	T.hildbrandtii Muell Arg.	"	"	BARO AN 806
29.	Dalechampia scandens L.	Vasad	15-10-1983	BARO AN 925
30.	Hevea brasiliensis Muell Arg.	Kerala	26-12-1982	BARO AN 927

Table - 6 (Contd.)

Sr.No.	Name of the plant	Place of collection	Date of collection	Voucher specimen No.
31.	Manihot esculenta cranz.	Kerala	26-12-1982	BARO AN 926
32.	Jatropha gossypifolia L.	Baroda	15-10-1983	BARO AN 928
33.	J.curcas	"	"	BARO AN 929
34.	J.panduraefolia L.	Trivandrum	26-12-1982	BARO AN 986
35.	J.multifida L.	"	"	BARO AN 987
36.	J.podagrica Hook.	"	"	BARO AN 988
37.	Croton bonplandianus Baill.	Narmi	8-1-1983	BARO AN 1005
38.	Croton tiglium L.	Trivandrum	26-12-1982	BARO AN 1006
39.	C.oblongifolius Roxb.	Narathwada	18-9-1983	BARO AN 1007
40.	Kirganelia reticulata(Poir.) Baill.	Baroda	15-10-1983	BARO AN 1008
41.	Sebastiania chamaelia Muell. Arg.	Tiruchirappalli	20-4-1983	BARO AN 1025
42.	Daliospermum montanum(Willd.)	Pavagarh	3-1-1984	BARO AN 1028
43.	Excoecaria bicolor Hassk.	Trivandrum	26-12-1982	BARO AN 1029
44.	Sapium sebiferum (L.)Roxb.	Vaghai	10-9-1982	BARO AN 1030
45.	S.insignie (Royle.)Benth	"	"	BARO AN 1035
46.	Hura crepitans L.	Trivandrum	26-12-1982	BARO AN 1038
47.	Euphorbia hirta L.	Baroda	15-10-1983	BARO AN 1039
48.	E.fulgens karn ex Klotzsch	Saputhara	10-9-1982	BARO AN 1051

Table - 6 (Contd.)

Sr.No.	Name of the plant	Place of collection	Date of collection	Voucher specimen No.
49.	Euphorbia Graecunculoides Lemk.	Baroda	15-10-1983	BARO AN 1052
50.	E.heyneana sprengel	Harni	8-1-1983	BARO AN 1053
51.	E.geniculata orteg.	Baroda	15-10-1983	BARO AN 1054
52.	E.milii Des.moul.	"	"	BARO AN 1055
53.	E.lasta Heyne ex Roth.	"	"	BARO AN 1056
54.	E.tirucalli L.	"	"	BARO AN 1057
55.	E.lactea Haw.	"	"	BARO AN 1058
56.	E.pulcherrima Willd	"	"	BARO AN 1059
57.	E.nerrifolia L.	"	"	BARO AN 1060
58.	E.prostrata Ait.	Vaghai	10-5-1982	BARO AN 1089
59.	E.antiquorum L.	"	"	BARO AN 1090
60.	E.coccinea Roth.	"	"	BARO AN 1091
61.	E.thymifolia Burm.	Tiruchirappalli	20-4-1983	BARO AN 1092
62.	E.heterophylla L.	"	"	BARO AN 1093
63.	E.elegans L.	"	"	BARO AN 1094
64.	E.parviflora L.	" "	"	BARO AN 1095
65.	Pedilanthus tithymaloides L. Poit	Baroda	15-10-1983	BARO AN 2005

Table - 6 (Contd.)

Sr.No.	Name of the plant	Place of collection	Date of collection	Voucher specimen No.
66.	P. tithymaloides var. Variegatus L. poit	Baroda	15-10-1983	BARO AN 2006
67.	P. tithymaloides var. nanus L. poit	"	"	BARO AN 2007