Chapter 2.

Materials and Methods

The plant materials were collected from in and around Baroda, Kerala, Ooty, Goa etc. The voucher specimens of these plants are deposited in BARO, the herbarium of Department of Botany, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat. Plant materials were washed, shade dried and later completely dried by keeping in an oven at 60 °C. The dried materials were powdered and stored in airtight plastic bags. This powder was used for the analysis of all the chemical constituents.

A. Phytochemical studies

1. Flavonoids:

The procedures followed in the present work for the extraction, isolation and identification of flavonoids are described below.

Fifty grams of leaf power was extracted in a Soxhlet's apparatus with methanol for 48hrs 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 hours to ensure the complete hydrolysis of all the O-glycosides. Aglycones were once again extracted into diethyl ether (fraction B) and the residual aqueous fraction was neutralized and evaporated for the analysis of glycoflavones.

Ether fractions A and B were combined and analysed for aglycones using standard procedures (Harborne, 1984; Mabry *et al*; Markham, 1982). The combined concentrated extract was banded on Whatman No. 1 paper & chromatographed along with quercetin as the reference sample. The sample system employed were Forestal (Con.HCl: acetic acid: water; 30:30:10) or 30% glacial acetic acid. The developed

chromatograms were dried in air and the visibly color compounds were marked out. These chromatograms were observed in the ultra-violet light (360 nm) and the bands were noted. Duplicate chromatograms were then sprayed with 10% Na_2CO_3 and 1% $FeCl_3$ and the color changes were reported. Rq (Rf relative to quercetin) values were calculated for all the compounds. The bands of the 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 'Perkin-Elmer Lambda 25 UV/Vis' spectrophotometer. The bathochromic & hypsochromic shifts induced by the addition of various regents were studied. The reagents used and there preparation are given below (Mabry *et al,* 1970).

Sodium methoxide (NaOMe): Freshly cut sodium metal (2.5) gm was added cautiously in small portion to spectroscopic methanol (100ml). The solution was stored in a tightly closed glass bottle.

Aluminium chloride ($AlCl_3$): Five grams of fresh anhydrous AR grade $AlCl_3$ (which appeared yellow-green and reacted violently when mixed with water) were added cautiously to spectroscopic methanol(100ml), formed initially, dissolved after about 24 hrs.

Hydrochloric acid (HCl): Concentrated AR grade HCl (50ml) was mixed with distilled water (100ml) 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 5 ml of this stock solution. A reference solution was prepared by extracting a piece of blank chromatographic paper from the same chromatogram with spectroscopic methanol. The NaOMe spectrum was measured immediately after the addition of three drops of NaOMe stock solution to the flavonoidal solution used for methanol spectrum. The solution was then discarded. The $AlCl_3$ spectrum was then measured immediately after the addition of six ml of $AlCl_3$ stock solution to 5 ml of fresh stock solution of the flavonoids. $AlCl_3$ /HCl spectrum was recorded next, after the addition of 3 drops of the HCl solution to

the solution containing $AlCl_3$. The solution was then discarded. For NaOAc spectrum, excess coarsely powdered anhydrous AR grade NaOAc was added by shaking the cuvette containing 5 ml of fresh solution of the flavonoids, till about a 2mm layer of NaOAc remained at the bottom of the cuvette. The spectrum was then recorded 2 minutes of 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 of flavonoid was established by its absorption maxima (λ_{max}), shape of the curves, shifts (both bathochromic & hypsochromic) with different reagents $AlCl_3$, $AlCl_3$ /HCl, NaOAc/H_3Bo_3, NaOMe (Mabry et al; 1970), color reactions and Rf values. The identification was confirmed by co - chromatography with authentic samples.

Normally, a flavonoid is linked to a 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 aglycone by C-C bonds. The latter group of compounds, known as C-glycosides (glycoflavones) are generally observed among flavones. They are resistant to normal methods of hydrolysis and will remain in the aqueous layer when hydrolysed extract was extracted with ether to remove aglycones. The procedures followed for isolating glycoflavones are described below:-

The aqueous fraction remaining after the separation of agylcones 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. To this dried residue, ethanol was added to dissolve the glycoflavones. The alcoholic filtrate was concentrated, and was banded on Whatman No.1 paper and the chromatogram was developed in water as solvent system. Glycoflavones were visualized by their colour in UV & with 10% aqueous Na_2CO_3 spray. Further analysis and identification were done by measuring the λ max and spectral shifts and co=chromatography with authentic samples. Biflavones were extracted with other flavonoids in methanol. But they were fractionated from the aqueous extract (prepared after concentrating the methanolic extract) using solvent ether or ethyl acetate. The individual biflavones were separated in paper using 30% acetic acid and Forestal or 15% acetic acid.

2. Phenolic acids:

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 diazotized *p*-nitraaniline or diazotised sulphanilic acid and a 10% Na_2CO_3 overspray (Ibrahim and Towers, 1960). Diazotization: 0.7gms of *p*-nitraniline/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*-nitraniline/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 presents in the extract were identified based on the specific colour reactions they produce with the spray reagents and the relative Rf values in the different solvent system.

3. Quinones:

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

The neutral fraction was also chromatographed over silica gel TLC plates using the same solvent system. The various quinones (Anthra-, Benzo-, Napthaquinones) were visualized by their colours in visible/UV light, colour reactions after spraying

with 2% magnesium acetate or 10% aqueous NaOH (the quinones give purple/pink/orange-yellow colours) and the absorption spectra.

4. Proanthocyanidins:

For testing the presence of proanthocyanidins, about 5 gm 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. 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 color in the upper alcohol layer denoted a positive reaction for proanthocyanidins. An olive yellow color represented a negative reaction (Gibbs, 1974).

5. Steroids:

The ethyl acetate fraction of the plant extract was analysed by thin layer chromatography using chloroform: carbon tetrachloride: acetone (2:2:1) as the solvent system. The plate was spread with Libermann-Burchard reagent or 50% sulphuric acid and heated at 85-90°C for 15 minutes. The Libermann-Burchard was prepared by mixing 1 ml of concentrated sulphuric acid (H_2SO_4), 20 ml acetic anhydride and 50 ml of chloroform. Steroids give characteristic colours which indicate their substitutions.

6. Alkaloids:

Five grams of powdered plant material was cold 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 0.1N H_2SO_4. The acid soluble fraction was tested with Mayer's, Wagner's and Dragendorff's regents (Paech and Tracey, 1955). The white precipitate denoted the presence of alkaloids (Amarasingham et *al.*, 1964). The acid soluble fraction was spotted on TLC (Toluene: EtOAc: Diethylamine; 7:2:1) and the Rf values are measured. The preparation of the reagents was as follows:

Mayer's reagent: (Potassium mercuric iodide) 1.36gm of $HgCl_2$ were dissolved in 60 ml of distilled water and 5gm of KI in 10ml of solvent. 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 gm of I_2 and 2gm of KI were dissolved in 5 ml of water and the solution diluted to 100 ml. It gave brown flocculent precipitate with most of the alkaloids.

Dragendorff's reagent: (Potassium bismuth iodide) 8gm of $Bi(NO_3)_3.5H_2O$ were dissolved in 20 ml of HNO_3(sp. gr. 1.18)and 27.2 gm 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 100 ml with distilled water.

Pharmacognostic studies

1. Micromorphology and Anatomy

Micromorphological and anatomical studies were carried out on fresh materials. Fresh leaves were washed and small fragments of leaves were taken from the middle region of the mature leaves. Washed leaf fragments were first boiled in 90% alcohol for about 3-5 minutes to remove chlorophyll, then washed 2-3 times in water, then again boiled with 10% KOH solution (Wallis, 1957) for 2-3 minutes and washed 4-5 times in water and kept in clean water to remove all traces of the clearing agent. Both the epidermal layers were stripped off gently from the mesophyll tissue with their help of pointed needle and forceps. The epidermal peels were washed in water, stained with Toluidine blue (0.5%) prepared in aqueous borax (Trump, 1961) and mounted in 50% glycerine; the margins of the cover slips were sealed with DPX (Johansen, 1940). Transverse Sections of leaves and fruits as well as T.S., Tangential Longitudinal sections and Radial Longitudinal Sections of stems and barks were taken by free hand and were stained in Toluidine blue (0.5%)/ safranin and mounted in 50% glycerine. The slides were examined under the microscope and Camera Lucida sketches were drawn at 400x magnification and the sizes were measured using

an ocular micrometer. The quantitative data were based on the average of 20 readings.

Leaf constants such as stomatal index/mm^2 and trichome index/mm^2 were calculated. Stomata index (SI) was calculated as defined by Salisbury (1927, 1932) *viz.*

$$SI = \frac{S}{S + E} \times 100$$

Trichome index (TI) was calculated as defined by Salisbury (1927, 1932) *viz.*

$$TI = \frac{T}{T + E} \times 100$$

2. Powder study

The finely powdered drug was scanned under 400x magnification for recording the cell elements.