Chapter 2
Materials and Methods

The plant materials were collected from in and around Baroda, different parts of Gujarat and from Himachhal Pradesh, Madhya Pradesh. 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. Pharmacognostic studies (including organoleptic characterization, macroscopical and micromorphological studies) and physico - chemical analysis (The ash values and extractive values) were done by using standard methods (Anon.2004).

A. Phytochemical studies

Flavonoids:

Flavonoids are the most widely distributed group of polyphenols which include all the C_6-C_3-C_6 compounds related to a flavone skeleton .The flavone may be considered, consisting of (1) a C_6-C_3 fragment that contains the "B" ring and (2) a C_6 fragment the "A' ring, both these units being of different biosynthetic origin. The flavonoids are subdivided as anthocyanidins, flavones, flavonols, chalcones, etc. based on oxidation level of C_3 fragment of the phenyl propane unit (Geissman, 1962). These pigments sometimes completely replace the carotenoids as the yellow flower/fruit pigment. Anthocyanidins are the purple/blue pigments while chalcones and aurones are yellow in colour. Flavonols and flavones though classified as colourless flavonoids, are responsible for the white, cream or ivory colours of the flowers. All these pigments absorb strongly in ultraviolet & thus may be responsible for attracting those pollinators (e.g. bees) whose vision extends in ultraviolet region (Harborne & Smith, 1978). Flavonols, dihydroflavanols, biflavonyls, dihydrochalcones, isoflavones & proanthocynidins are the minor flavonoids since they have a restricted distribution.

'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 flavanones from citrus fruits formed the principle 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'- positions exert beneficial physiological effects on the capillaries through (1) chelating metals and thus sparing ascorbate from oxidation, (2) prolonging epinephrine action by the inhibition of O-methyl transferase and (3) stimulating the pituitary-adrenal axis (De Eds, 1968). Srinivasan *et al.*, (1971) presented evidence that flavonoids play another important role in circulatory system by acting on the aggregation of erythrocytes.

Most of the flavonoids occur as water soluble glycosides in plants. They are extracted with 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 (esp. ammonia) and thus are easily detected in chromatograms or in solutions. Flavonoids contain conjugated aromatic system and thus show intense absorption bands in UV and in the visible regions on 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 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 (glycoflavones), are generally observed among flavones. They are resistant to normal methods of hydrolysis and will remain in the aqueous layer when the 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.

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, 1967, 1984; Mabry *et al*; 1970; 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.

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.

Phenolic acids:

Phenolic acids are simple phenols, having a functional acidic group and varying number of hydroxyl groups at different position. Acid hydrolysis of plant tissue 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. Ellagic acid and gallic acid are located in many plant groups of the Polypetalae. The phenolic acids are extracted in ether along with the flavonoid agylcones from the hydrolysed extract (fraction A and B) of plant materials. They are analysed as follows:

Analysis of phenolic acids in the combined ether fraction (A and B) was carried out by two-dimensional ascending paper chromatography. Benzene: acetic acid: water (6:7:3, upper organic layer) in the first direction and sodium formate: formic acid: water (10:1:200) in the second direction were used as irrigating solvents. The sprays used to locate the compounds on the chromatograms were 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.

Quinones:

They are aromatic diketones, which form the largest class of natural coloring 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-, naptha- and anthraquinones depending on the mono-, bi- or tricyclic ring system they contain. In plants their function is not properly understood. It is assumed that they play some role in oxidation-reduction processes.

For extraction of quinones, approximately 5-10 gm of dried, powdered 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.

Proanthocyanidins:

The proanthocyanidins are condensed tannins which yield anthocyanidins on hydrolysis. 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). The colored hydrolysate is extracted with amyl alcohol and this extract was chromatographed in Whatman No.1 paper using Forestal or 30% HOAc. The anthocyanidins which separate as colored bands were eluted with acidic methanol and the absorption spectra were measured in the range between 500-600nm. The different anthocyanidins were identified by their visible colors, Rf values and $\lambda_{max.}$

Alkaloids:

Alkaloids comprise the largest single class of secondary metabolites. They are basic plant products having nitrogen-containing heterocyclic ring system & a high pharmacological activity. 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 weakly acids (1M HCl or 10% acetic acid) or acidic alcoholic solvents and are then precipitated with alcoholic ammonia. They are also extracted into any organic solvents 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 regents.

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. 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.

The extract which showed presence of alkaloids were concentrated and this fraction was spotted in Whatman No.1 chromatographic papers along with standard alkaloids such as ephedrine, berberine, quinine etc. and developed in BAW. The developed chromatograms were seen in UV light and the fluorescent regions were marked. The chromatograms were then sprayed with Dragendorff's reagent

Gums and Mucilages

Gums and mucilages include all the hydrocolloids obtained from plants and are polysaccharides consisting of more than one type of monosaccharide residues. The gums are considered as pathologic products, produced in response to injury by a process known as " gummosis", whereby the cell walls and their ingredients are dissolved to form a colloid which serves as a protective layer over the wounded tissue and later occurs as exudates from the various plant parts especially the trunk. Mucilages, on the other hand, are classified as natural plant products produced by the plant for the imbibition and retention of water. But from a chemical point of view gums and mucilages are almost identical and it is nearly impossible to draw a line

demarcating one from the other.

The solutions of gums and mucilages are laevorotatory. On hydrolysis they yield sugars like arabinose, galactose, glucose, mannose and xylose alongwith various uronic acids and methyl sugars. The sugar acids when present in appreciable amounts, tend to lower the pH of the solution enabling the gums to occur frequently as salts of sodium, potassium, calcium or magnesium. In some cases, the sugar components are methylated (gum Tragacanth) or acetylated (Karraya gum). The trace amounts of nitrogen (0.08-5.6%), at times encountered in certain samples of gum, are considered to be due to the presence of proteins or sugar amines like glucosamine.

Gums containing linear polysaccharides are found to be less soluble in water, producing very viscid solutions. They tend to precipitate in course of time because of the inter- molecular hydrogen bonding facilitated by the parallel alignment of molecules. The solutions of branched polysaccharides are more soluble in water and form colloidal gels-sols-possessing low surface tensions and therefore act as important protective colloids and stabilising agents.

B. Pharmacognostic studies

1. Organoleptic characterization

The organoleptic evaluation comprise macroscopic and sensory characteristics of drugs. The purview of study comprises morphological origin, condition, shape, size, colour, texture, taste, odour, hardness and fracture (Wallis, 1957).

2. 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 leaf, as well as T.S., T.L.S and R.L.S of stem

and roots were taken by free hand and were stained in Toluidine blue (0.5%) and mounted in 50% glycerine. The slides were examined under the microscope and Camera Lucida sketches were drawn at 400x magnification and the size was 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.*

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

3. Powder study

powdered drugs explain cellular element of respective morphological parts and their inclusions. The powder of the whole drugs consists of the elements of all the morphological parts included in the drugs. The finely powdered drug was scanned under 400x magnification for recording the cell elements.

C. Physico-chemical analysis

There are certain physico-chemical parameters viz. Total Ash, Acid Insoluble Ash, Alcohol Soluble Extractive and Water Soluble Extractives. The procedures followed for the determination of the various parameters during proximate analysis are as follows (Anon.2004).

1. Total ash content

A silica crucible was heated to red heat for 30 minutes and was allowed to cool in a dessicator. The crucible was weighed. Accurately 2 grams of the air-dried plant powder was weighed into the crucible and was incinerated at a temperature not exceeding 450°C until free from carbon. The crucible was cooled and weighed. The percentage of total ash formed was calculated with respect to the air-dried plant powder.

2. Acid insoluble ash content

The total ash obtained from the above procedure was boiled with 25 cc of 2M HCl solution for 5 minutes. The insoluble matter was collected on an ashless filter paper, washed with hot water, ignited in a crucible and cooled in a dessicator. The weight

was noted. The percentage of the acid insoluble ash was calculated with reference to the air dried plant powder.

3. Alcohol soluble extractive

Five grams of air-dried plant material was macerated with 100cc of alcohol in a closed flask for 24 hours, shaking frequently during the first 6 hours and allowed to stand for the remaining 18 hours. Thereafter, filtration was done rapidly taking precautions against loss of ethanol. Twenty five cc of the filtrate was evaporated to dryness in a tared flat bottomed shallow dish, dried at 105°C and weighed. The percentage of the ethanol soluble extractive was calculated with respect to the air-dried plant powder.

4. Water soluble extractive

Chloroform water was prepared by dissolving 2.5cc of chloroform in 900cc of distilled water and diluting upto 1000cc with water. Using the prepared chloroform water, the procedure followed was similar to that of the ethanol soluble extractive. The percentage of the water soluble extractive was calculated with respect to the air-dried plant powder.

Chromatographic Characterization

Chromatography is the science which studies the separation of molecules based on differences in their structure and/or composition. In general, chromatography involves moving a test preparation of the materials to be separated, over a stationary support. The molecules in the test preparation will have different interactions with the stationary support leading to separation of similar molecules. Test molecules which display tighter/ stronger interactions with the support will tend to move more slowly through the support than those molecules with weaker interactions. In this way, different types of molecules can be separated from each other as they move over the support material. Chromatographic separations can be carried out using a variety of supports, including immobilized silica on glass plates (thin layer chromatography), very sensitive High Performance Thin Layer Chromatography (HPTLC), volatile gases (gas chromatography), paper (paper chromatography), and liquids which may incorporate hydrophilic, insoluble molecules (liquid chromatography).

D. HPTLC finger-printing Analysis

Regulatory agencies recommend fingerprint chromatography as the basis for proper identification of herbal drugs, herbal drug preparations and herbal medicinal products. In general, the fingerprint of one sample (unknown) is compared with that of another sample (i.e. reference material). The reference material can either be of the same kind as the unknown (herbal drug or preparation thereof) or be a solution of any number of chemically defined substances. Fingerprints are compared with respect to number, sequence, position and colour of the separated zones. The fingerprint can be optimized for certain target compounds. Even if some components migrate with the solvent front and others remain at the application position, the fingerprint always represents the sample in its entirety.

A balance must be found between the number and type of compounds extracted, and those of interest, because unwanted substances can disturb the analysis. Proteins, lignans and sugars can constitute an undesirable matrix. In cases where the active principles are known, they can serve as markers. If no active principle has been determined, any of the secondary metabolites like essential oils, flavonoids, alkaloids, or others which are in appreciable quantities, may be selected. Even amino acids, plant acids or sterols can provide plant-specific profiles. There could be cases where little or nothing is known about the chemical constituents of a given herbal drug. In such cases, generating multiple fingerprints is very valuable. Multiple fingerprints can be obtained either from the same plate by multiple detections or from the same sample looking at different fingerprints representing various substance classes.

The traditional way of describing an HPTLC fingerprint chromatogram is comparison of the sequence, colour and intensity of the separated zones of the sample, with that of the reference. The use of chemically defined compounds as reference material may be preferred, because such compounds are readily available in suitable purity. Only against such substances can zones of the HPTLC fingerprint be identified, unless the unknown is isolated and externally analyzed. Such reference materials also provide the option of estimating the quantitative composition of the sample.

Here in this work HPTLC analysis done qualitatively aim to develop HPTLC fingerprint for individual drugs for comparison and to distinguish genuine drugs from their substitutes/adulterants.

Preparation of extracts for HPTLC analysis

One gm of coarse powder material were extracted by refluxing in 5ml of methanol at 60°C in a water bath for 30 min. Extracts were filtered, concentrated and re-suspended in 1ml of methanol and used directly for HPTLC analysis.

Table 1: Optimized chromatographic conditions for HPTLC analysis.

HPTLC Sample applicator	Linomat 5 (CAMAG)
Make of syringe	Hamilton
Capacity of syringe	100µL
Development chamber	CAMAG twin trough chamber (10x10cm)
Stationary phase	Precoated $60F_{254}$ silica plates (Merck)
Size of plate used	10x10 cm
Sample applied	10 µl
Distance between tracks	12 mm
Band length	8mm
Solvent front	90 mm
Mobile phase	Toluene :Formic acid : Ethyl formate (5 : 1 : 4) (v/v/v)
Mode of visualization	Short wave UV light (UV 254nm) Long wave UV light (UV 366nm)
HPTLC scanner	TLC Scanner 3 (CAMAG)
Radiation source	Deuterium lamp
Software	WinCats