CHAPTER-2

MATERIAL AND METHODS

Material Methods :

For the phytochemical studies, both the species i.e., *Calotropis gigantea* and *Calotropis procera* of Genus *Calotropis* were collected from the selected sites of Sagar and nearby areas in the month of January 1996 to December 1996. They were subjected for phytochemical analysis to test the presence of carbohydrates, alkaloids, glycosides, aminoacids, flavonoids, phenolic compounds, saponins, tannins and steroids etc. The collection of plants should be done after the completion of flowering and fruiting stages. All the plant parts were dried in shade for about 48 hrs. and then for some time in oven at 60°C and powdered separately with the help of grinder. After that the plant parts were stored in air tight polythene bags at room temperature. The fractionation of plant parts was done with the help of continuous liquid extraction procedure.

Preparation of Extracts : By continuous liquid extraction procedure :

It was carried out according to the scheme/method of Brain and Turner (1975) with slight modifications. The air dried finely powdered material of both species was defatted with the help of petroleum ether (B.P. 60-80°C) in the thimble of the soxhlet apparatus. After defattation the extracts were filtered and remaining residues were then extracted with 500ml, 95% ethanol and after extraction of ethanol soluble consituents, the residue were removed.

The filterates were then solidified by evaporation. These ethanolic extract samples were kept in refrigerator for further analysis.

For the separation of plant constituents in their respective solvents the ethanolic extracts were then successively fractionated with various solvents according to the scheme given in Figs.(1,2,3,4,5,6,7,8). Each successively fractionated sample was concentrated to about dryness and stored in screw cap glass tube under refrigeration until used. These fractions then tested further for the analysis of different chemical constituents.

Fig.(1) Procedure followed for the extraction of Methanol

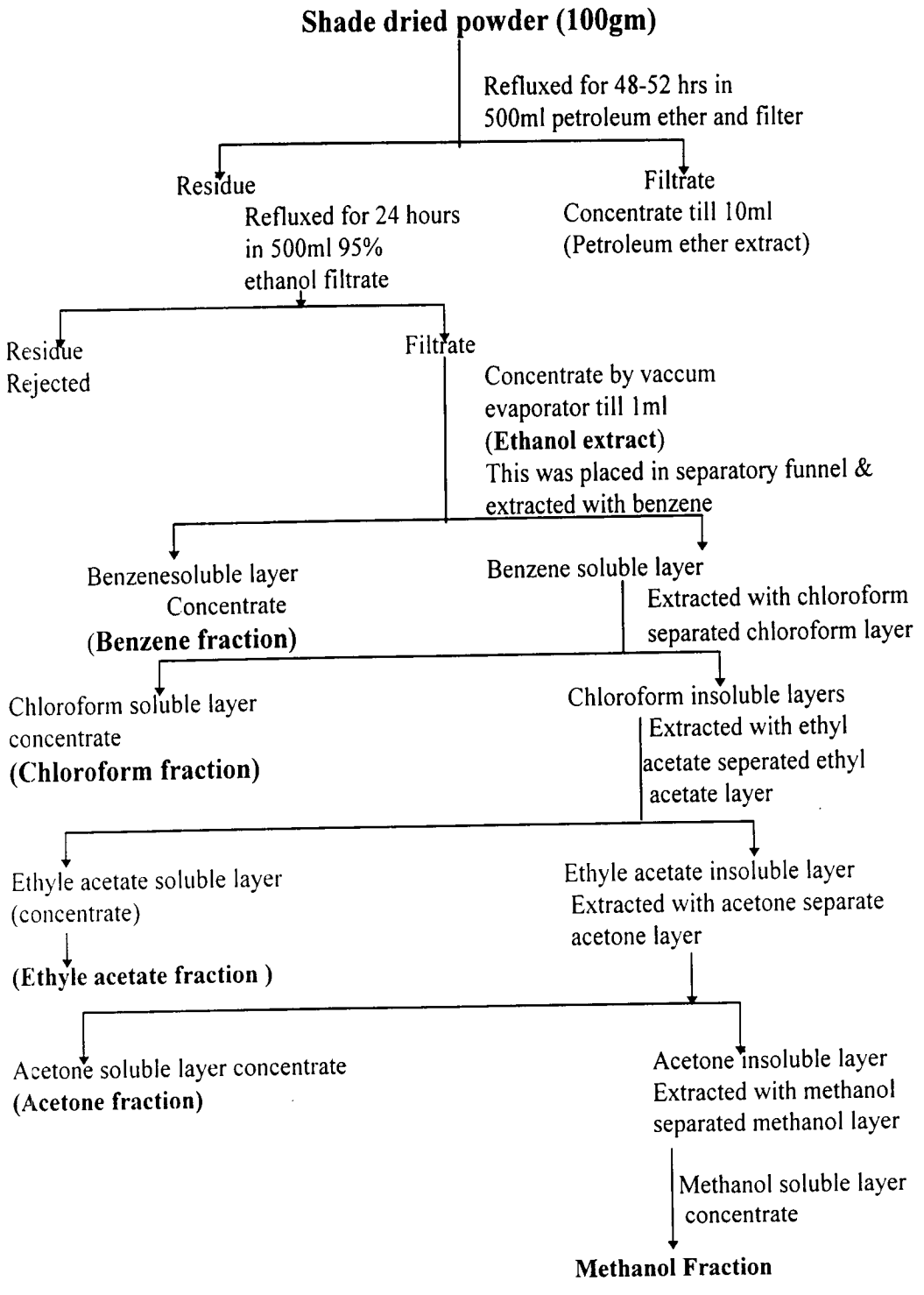


Fig.(2) : Procedure followed for extraction of Glycosides

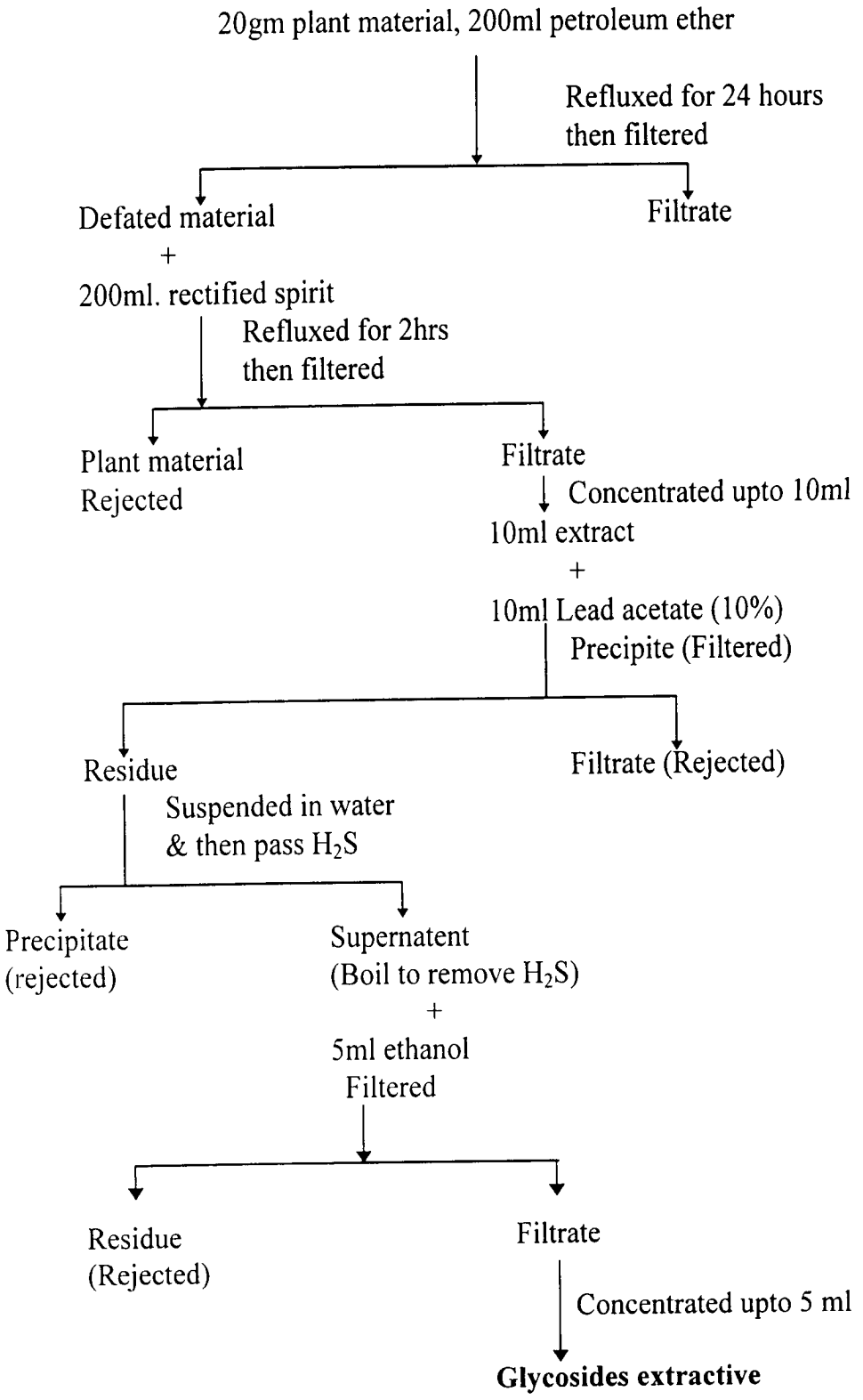


Fig.(3) Procedure followed for the extraction of Alkaloids :

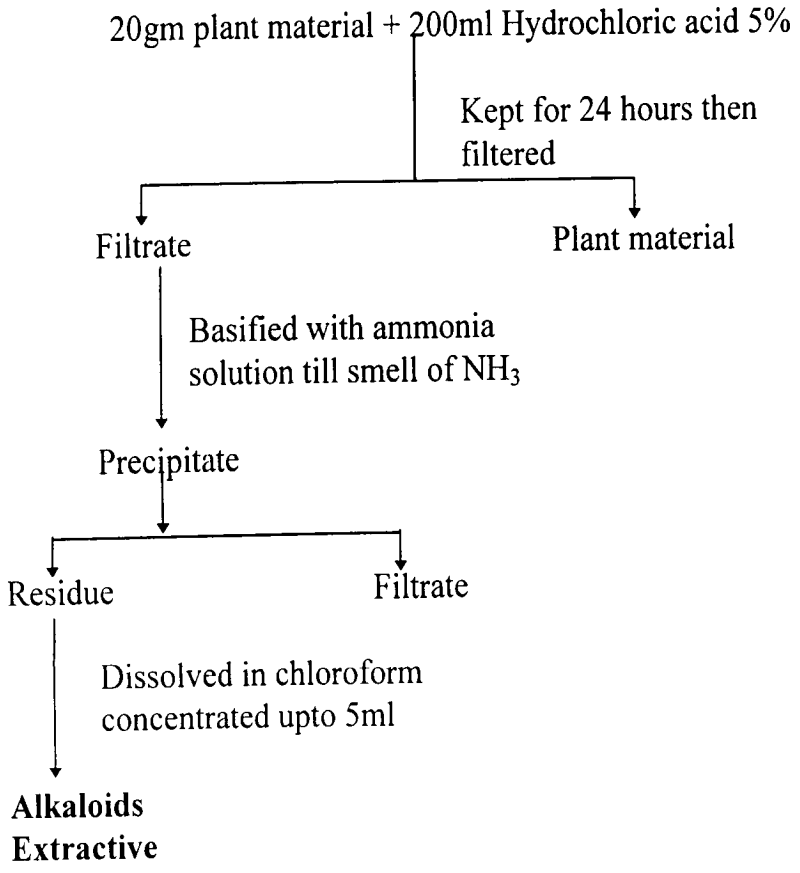


Fig.(4) Procedure followed for the extraction of Flavonoids :

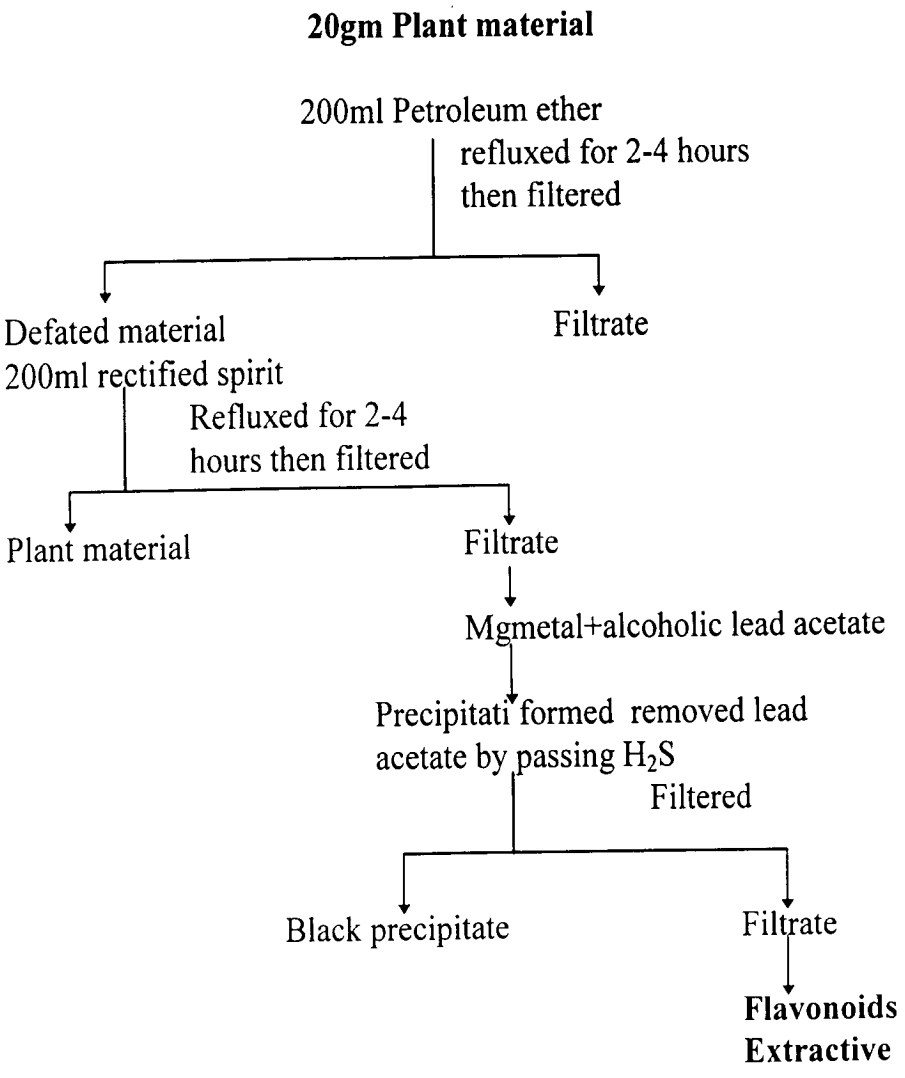


Fig.5. Procedure followed for the extraction of Tannins :

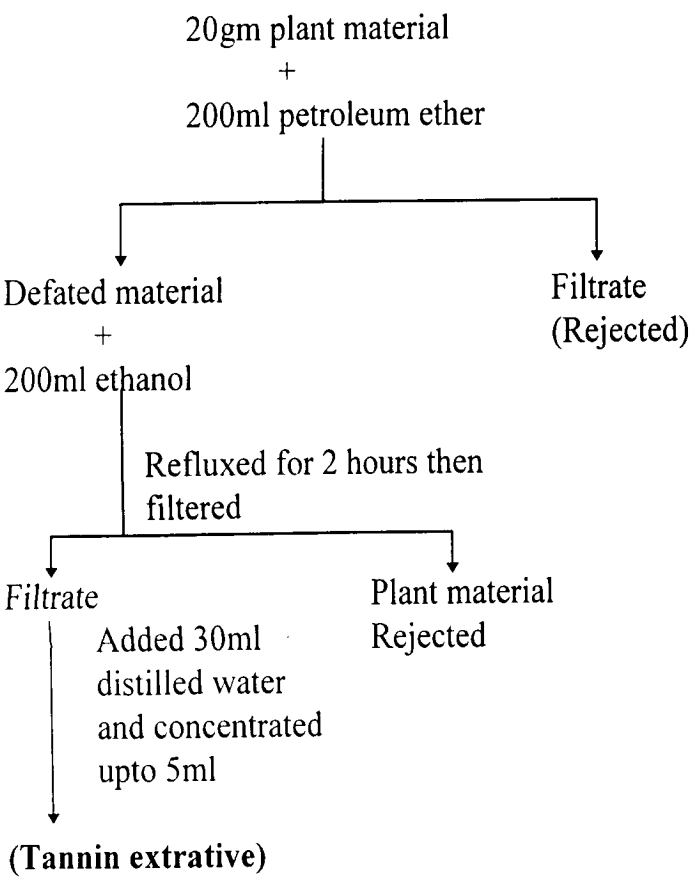


Fig. 6 : Procedure followed for the extraction of Saponins

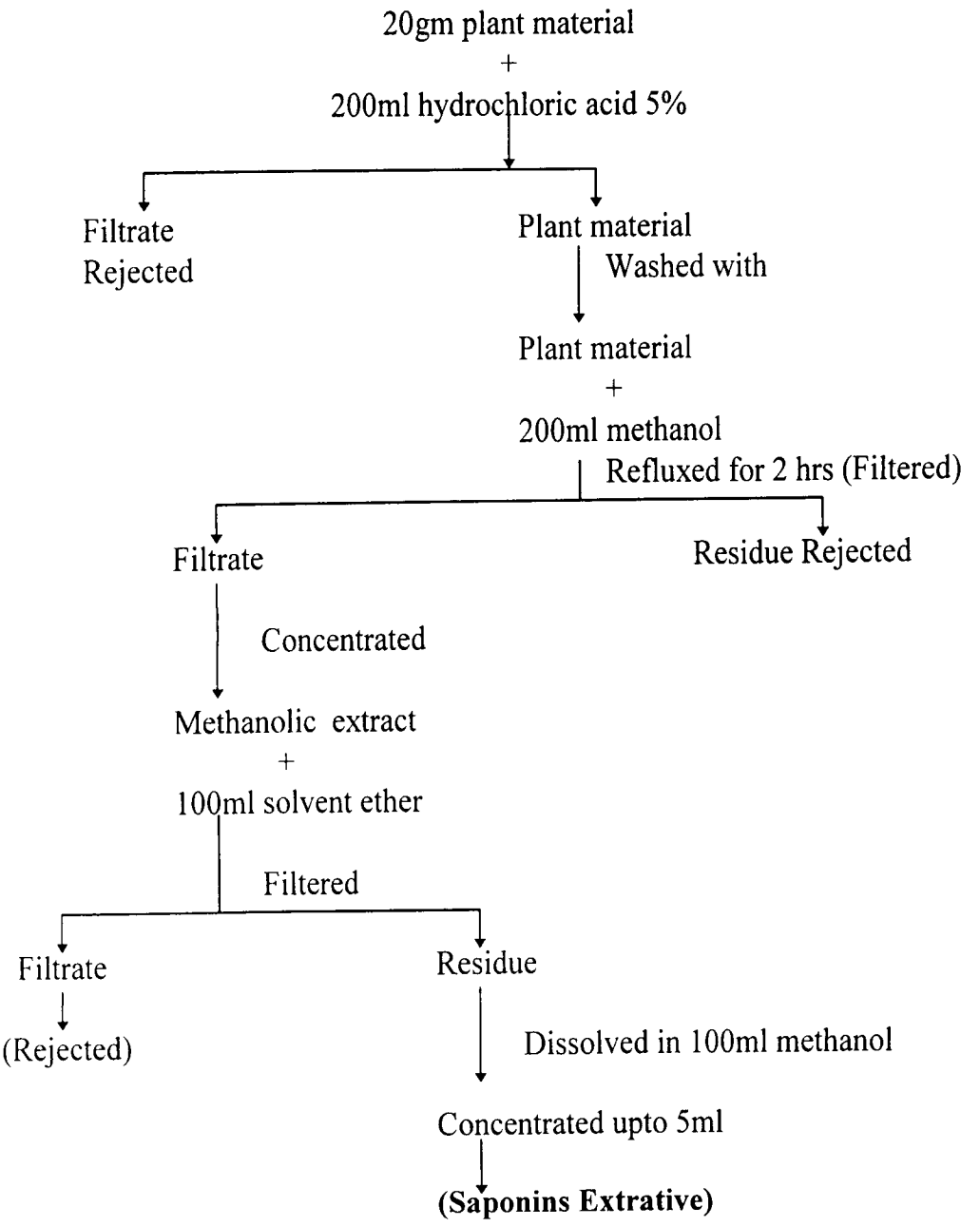


Fig. 7 : Procedure followed for the extraction of Free Sugars and A glycones

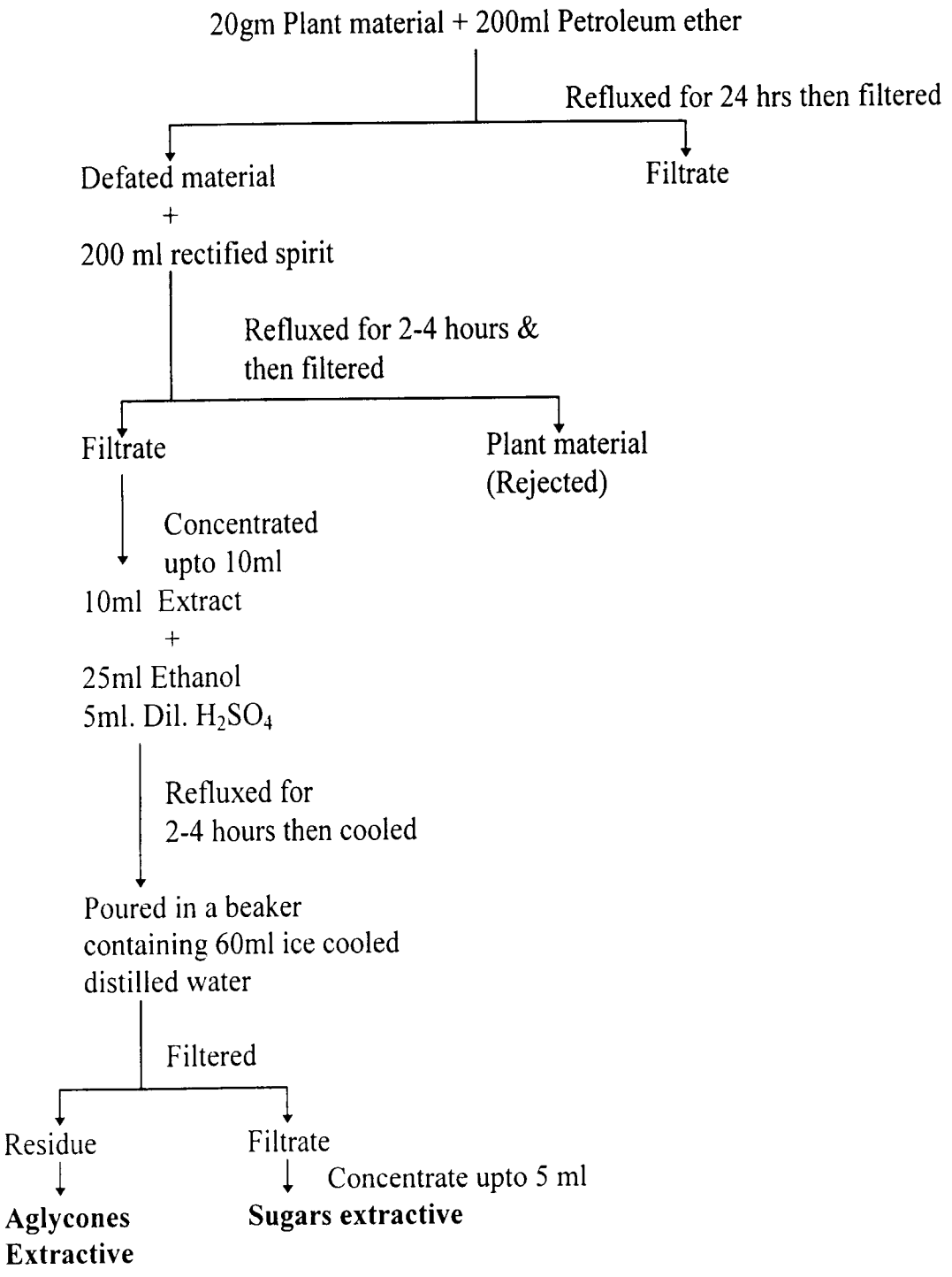
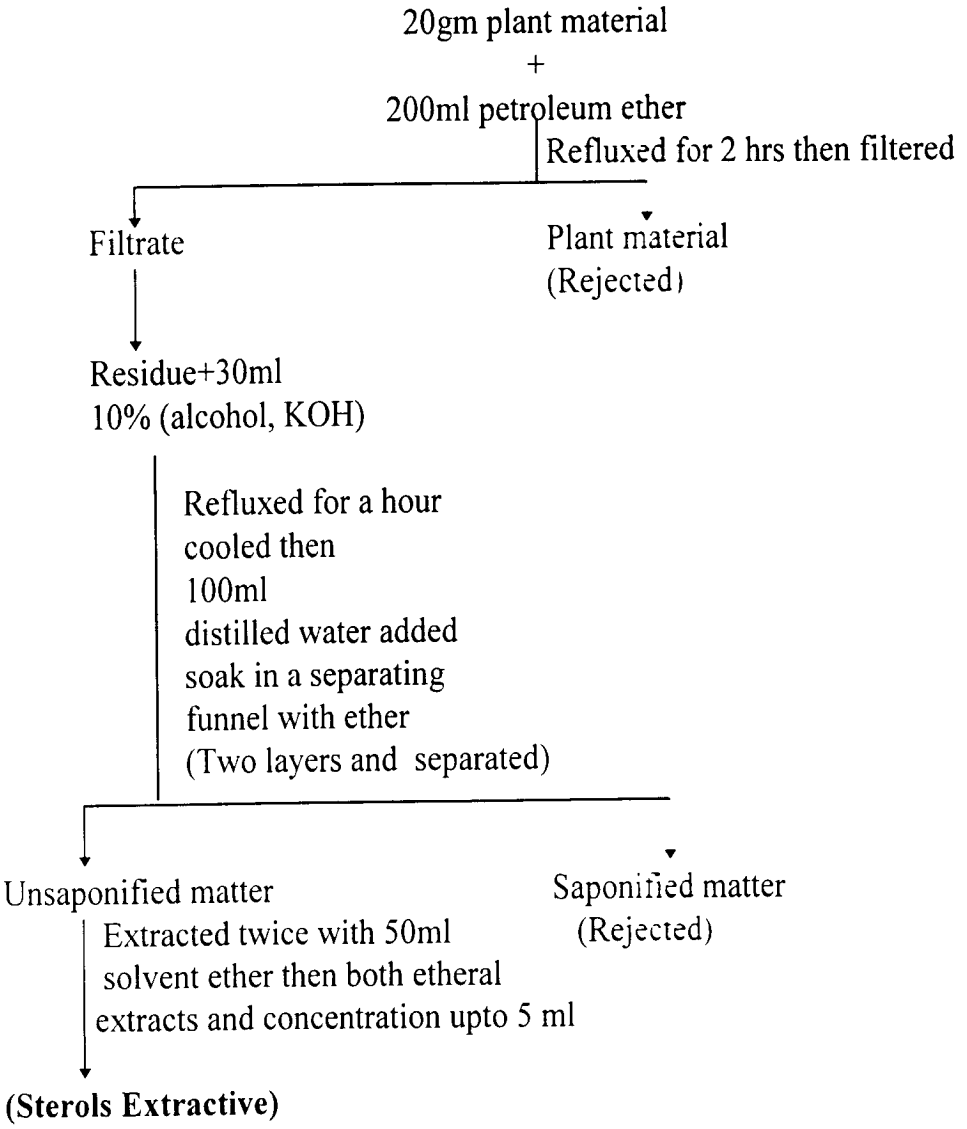


Fig. 8 : Procedure followed for the constituents of sterols



Qualitative chemical test or analysis for the presence of various constituents in the present study :

The presence of the different chemical constituents viz. carbohydrates, amino acids, phenolic compounds, glycosides, alkaloids etc. present in experimental plant extracts were observed by following chemical tests (with the help of reagents) as suggested by Rosenthaler (1930), Vogel (1958), Finar (1959), Robinson (1965), Harborne (1973), Saxena (1983) etc. and by thin layer chromatography. A few milligram of each dried extracts was dissolved in suitable solvents and tested for the presence of common plant constituents.

Carbohydrates :

The presence of carbohydrate was indicated by Molish test, Fehling test Tollens test.

(a) Molish test : Small quantity from the respective extract was dissolved in ethanol and added a few drops of 20% w/w solution of napthol in ethanol (Molish reagent) shaken well about 1ml of concentrated H_2SO_4 was allowed to flow carefully by the side of test tube. A redish violet ring on the junction of the two layer indicate the presence of carbohydrates.

(b) Fehling test : Dissolved small quantity of extract in 1ml of Fehlings solution (mixed A&B together solution, A is the solution of potassium tartrate and B is copper sulphate solution). Then placed it in boiling water bath for a few

minutes. Appearance of brick red precipitate of cuprous oxide indicated the presence of carbohydrates.

(c) Tollens Test : A small fraction of water solution of extract was taken, added a few drops of ammonical silver nitrate solution (Tollens reagent), to the extract and kept in the boiling water bath for 5 minutes. Appearance of silver mirror along the side of the test tube indicates the presence of reducing sugar.

Glycosides : By (1) Legal Test
(2) Killer killiani Test
(3) Raymond Test

(1) Legal test : A little fraction of extract was taken in water and made alkaline, added few drops of sodium nitropruside soluton. Blue colour indicates the presence of glycosides.

(2) Killer killiani test : Extract dissolved in ethanolic added 1ml of glacial acetic acid, containing a trace of ferric chloride and shaken. Added 1ml of concentrated H_2SO_4 by the wall of the test tube. Blue colour in acetic acid layer and red colour at the junction of two liquids indicates the presence of glycosides.

(3) Raymond test : Small quantity of extract dissolved in 50% ethanol. Then added 1ml (1%) or 2.3 drops of 20% NaOH solution, appearance of violet colour indicates the presence of glycosides.

Tannin : Approximately 10mg of ethanolic and aqueous extract were separately dissolved in water and filtered. The filterate was tested with the following reagents.

(1) Ferric-chloride solution : Appearance of blue to bluish green or bluish black colour indicates the presence of tannin.

(2) Lead acetate test : Took a small quantity of extract, dissolved in ethanol and about 1ml of lead acetate solution was added appearance of brownish yellow precipitate indicates the presence of tannins.

(3) Potassium chromate test : Appearance of orange yellow precipitate indicate the presence of tannins.

Saponins : (a) Taken a small quantity of extract in test tube and added about 10ml of distilled water and shaken well. The formation of foam indicate the presence of saponins.

(1) Haemolysis test : Plant extract was diluted with normal saline solution and added 0.5 to 1ml of rabbit blood. Haemolysis of blood shows the presence of saponin in the extract.

Alkaloids : For alkaloid test the following reagent in prepared as follows.

Dragendorff reagent :

(a) Dissolved 0.6gm Bismuth subnitrate in 2ml of concentrated HCl and 10ml water.

(b) Dissolved 6gm potassium iodide in 10ml water. Then this solution was diluted with 400ml water.

Test : Taken a small quantity of extract and dissolved in ethanol- added about 1ml of Dragendorffs, reagent and heated for 5 minutes in water bath. Formation of reddish precipitate indicates the presence of alkaloids.

Flavonoids :

(a) A small fraction of extract was taken in ethanol and added about 2-3 drops of conc. H_2SO_4 and megnesium turnings. Development of pink or red colour indicates the presence of flavonoids.

(b) A small quantity of extract in ethanol and added 5% alcoholic aluminium chloride, development of a characteristic yellow green colour indicates the presence of flavonoids.

Steroids :

(a) Libermann's reagent : A small portion of each extract was taken in 1ml of acetic anhydride and dissolved by warming. The contents were cooled and a few drop of conc. H_2SO_4 were added in case by the sides of test tubes Appearance of blue colour indicates the presence of steroids.

(b) Evaporated the extract fully and dissolved in chloroform, made two fractions added acetic acid and acetic anhydride respectively. Then added conc. H_2SO_4 in both. Formation of brown ring a brown colour respectively confirms the presence of steroids.

Phenolic compounds : A small quantity of extract dissolved in ethanol and added 1% aqueous or alcoholic solution of ferric chloride. Intense green purple or blue colour shows the presence of phenolic compounds.

Thin layer chromatography : It is also known as surface chromatography or open column chromatography. This technique was first introduced by Izamailov and

Shraiber (1983). This layer chromatography was carried out on silica gel plate with the view that it is a simple, faster and the separated compounds can easily be recovered by scraping the adsorbent layer and dissolved in a suitable solvent. It constitutes a versatile analytical tool for separating number of compounds like amino acids, alkaloides etc. (Fahny et al., 1961; Iran, 1969 and Kricohner, 1967).

Glass plates of about 10 x 20 cm size were taken and spread with a 20% silica gel slurry in water of with the help of a glass rod to get a uniform layer of about 25 mm thickness upon drying. The plates were activated at 110-120°C for an hour and then used the follwoing combination of various solvent and sprayer systems were used for the sprayer systems were used for the separation of various components. Detection was done by spraying the plates with concern reagents and heating them at 100-110°C for 5 minutes to make the black spot visible. About 80 to 100 mg of extract was dissolved in 2-5 ml of petrolium ether (60-80°C) and was used for T.L.C. examination.