CHAPTER – 7

Phytochemical Studies

Extraction- The precise mode of extraction naturally depends on the texture and water content of the plant material being extracted and on the type of substance that is being isolated. In general, it is desirable to 'kill' the plant tissue, i.e. prevent enzymatic oxidation or hydrolysis occurring, and plunging fresh leaf or flower tissue, suitably cut up wherever necessary, into boiling ethanol is a good way of achieving extraction. Alcohol, in any case, is a good all purpose solvent for preliminary extraction. The classical chemical procedure for obtaining organic constituents from the dried plant tissue (leaf, root, seed etc.) is to continuously extract powdered material in a Soxhlet apparatus with a range of solvents (usually in the increasing order of polarity) under controlled temperature. [23]

Preparation of Extracts- Sufficient quantity of *Ammania baccifera* whole plant and *Bergenia ciliata* leaves were shade dried and grounded separately to get each of 2 kg coarse powder. Successive extracts were prepared from 1 kg powder by soxhlet extraction method at 50^0c using different solvents in the increasing order of their polarity (petroleum ether (40-60^0C), chloroform, ethyl acetate, n-butanol and finally with ethanol (95%)). The marc left behind after each extraction was pressed and dried completely at room temperature before using it for next extraction with another solvent. Each extract was concentrated to a small volume in a rotary flash evaporator and allowed to dry in a dessiccator over sodium sulfite. After drying, the respective extracts were weighed and stored in refrigerator till use.

Another quantity of both powders (100g each) was extracted only with ethanol (95%) separately, by soxhlet method at 50^0c to prepare ethanol extract. The remaining marc was considered as aqueous extract. Extract was concentrated to a

small volume in a rotary flash evaporator and allowed to dry in a dessiccator over sodium sulfite, weighed & stored in refrigerator till use. [23-25]

Phytochemical screening of extracts:

All the prepared extracts were subjected to primary qualitative general chemical tests to detect the presence of different class of phytoconstituents.

Preparation of test solution- An appropriate amount of all the prepared extracts of both the plants, were dissolved in appropriate volume of methanol, shook well and filtered. The filtrate was used as test solution/extract solution.

Tests for Carbohydrates

Molisch's test- Treat the extract solution with few drops of alcoholic α-napthol. Add 0.2 ml of concentrated H_2SO_4 slowly through the sides of the test tube, development of purple to violet color ring at the junction indicates the presence of carbohydrates.

Tests for proteins & amino acids

Millon's Test- Extract solution + 2 ml of Millon's reagent (Mercuric nitrate in nitric acid containing traces of nitrous acid) white precipitate appear which turns red upon gentle heating.

Ninhydrin Test- Amino acids and proteins when boiled with 0.2% solution of Ninhydrin (Indane 1, 2, 3 trione hydrate) produces violet color appear.

Tests for Sterols and Triterpenoids

Libermann-Burchard test- Treat extract with few drops of acetic anhydride, boil cool and concentrated sulphuric acid through the sides of the test tube, a brown ring at the junction of two layers and the upper layer turns green indicates the presence of sterols and formation of deep red color indicates the presence of triterpenoids.

Salkowski's test- Treat extract in chloroform with few drops of concentrated Sulfuric acid, shake well and allow to stand for some time, red color appears in the lower layer indicates the presence of sterols and formation of yellow colored lower layer indicating the presence of triterpenoids.

Tests for Glycosides

Test I- Extract 200 mg of the drug by warming in a test tube with 5 ml of dilute (10%) sulphuric acid on a water bath at 100°C for two minutes, centrifuge or filter, pipette out supernatant or filtrate. Neutralize the acid extract with 5% solution of Sodium hydroxide (noting the volume of NaOH added). Add 0.1 ml of Fehling's solution A and B until alkaline (test with pH paper) and heat on a water bath for 2 minutes. Note the quantity of red precipitate formed and compare with that formed in Test II.

Test II- Extract 200 mg of the drug using 5 ml of and boil on water bath. After boiling add equal volume of water to the volume of NaoH used in the above test. Add 0.1 ml of Fehling's A and B until alkaline (red litmus changes to blue) and heat on water bath for two minutes. Note the quantity of the red precipitate formed.

Compare the precipitates of Test II with Test I. If the precipitate in Test-II is greater than in Test-I, then Glycoside may be present. Since Test I represent the amount of free reducing sugar already present in the crude drug, whereas Test-II represents the Glycoside after acid hydrolysis.

Tests for Alkaloids

Test solution was additioned with 2 ml of dilute HCL, mixed well and then used.

Mayer's test-To the extract/sample solution, add few drops of Mayer's reagent (Potassium mercuric iodide solution), creamy white precipitate is produced

Dragendroff's test- To the extract/sample solution, add few drops of Dragendroff's reagent (Potassium bismuth iodide solution) , reddish brown precipitate is produced.

Wagner's test- To the extract/sample solution, add few drops of Wagner's reagent (Solution of Iodine in Potassium Iodide), reddish brown precipitate is produced.

Hager's Test- To the extract/sample solution, add few drops of Hager's reagent (Saturated solution of Picric acid), yellow precipitate is produced.

Tannic acid test- To the extract/sample solution, add few drops of Tannic acid solution, buff colored precipitate is produced.

Tests for phenolic compounds

Ferric chloride test- To the extract solution add few drops of $Fecl_3$, gives blue-green / bluish black colored precipitate is produced.

Test for flavonoids

Shinoda Test (Magnesium hydrochloride reduction test)- To the extract solution, add few fragments of magnesium ribbon and concentrated hydrochloric acid drop wise, yellowish, yellow- orange occasionally orange color appears after few minutes.

Zinc-hydrochloride reduction test- To the extract solution, add a mixture of Zinc dust and conc. hydrochloric acid. It gives yellowish, yellow- orange occasionally orange color appears after few minutes.

Alkaline reagent test- To the extract solution, add few drops of sodium hydroxide solution; formation of an intense yellow color that turns to colorless on addition of few drops of dilute acetic acid indicates the presence of flavonoids.

Tests for Tannins

Ferric chloride test- To the extract solution add few drops of $Fecl_3$, blue-green / bluish black colored precipitate is produced.

Gelatin test- To the extract solution add few drops of 1% gelatin solution containing 10% sodium chloride, white precipitate develops.

Vanillin hydrochloride test- Extract solution treated with few drops of vanillin hydrochloride reagent, purple red color develops. [26-29]

Thin Layer Chromatographic studies of extracts:

Thin Layer Chromatography studies were done, using different solvent systems and suitable detecting reagents, to substantiate the presence of constituents detected in chemical tests & to know how many compounds are present in each extract. TLC plates were prepared manually and activated as per the standard procedure. [30-31] Precoated TLC plates were also used in the study. R_f value (s) were determined using formula

$$\text{Resolution Factor } (R_f) = \frac{\text{Distance travelled by the solute from the origin}}{\text{Distance traveled by the solvent front from the origin}}$$

A brief general procedure followed for TLC studies is tabulated below. [31-34]

Table No.3

TLC studies for phytoconstituents

Class of compound	Solvent system	Detecting reagent	Observation
Flavonoids	Ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26)	Ammonia vapours	Appearance of blue, yellow-orange, yellow-green indicates flavonols or flavones
Bitter principles	Ethyl acetate:methanol: water (77:15:8)	Vanillin sulphuric acid reagent	Red/yellow or brown/blue-green zones after heating at 100^0C for 5-10min.
Terpenoides	Benzene: Ethyl acetate (2:1)	Vanillin sulphuric acid reagent	Yellow zone indicates presence of terpenes