

# A GUIDE TO PHYTOCHEMICAL ANALYSIS

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## Abstract

*Plants are a very important source of nutrients and a very important part in the human diet. They provide us carbohydrates, protein, vitamins, cholesterol lowering compounds, antioxidants and other important sources of biologically active substances. Many nutritional values of plants have been discussed in the literature but there is very limited research in the biologically active compounds that are present in them. These biologically active compounds are called as phytochemicals. These phytochemicals are derived from every part of the plant including roots, stem, leaves, flowers, fruits, seeds etc. These phytochemicals are sometimes used as such and in some cases they form the raw materials for a variety of other medicinally important compounds. In this paper we will be discussing about the different methods for the collection, processing, extraction of the phytochemicals from the different plants and also the qualitative and quantitative analysis of the phytochemicals that are present in the same.*

**Keywords:** *phytochemical analysis, biochemistry, plant metabolites, phytochemicals, quantitative, qualitative.*

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## 1. INTRODUCTION

The chemicals that are produced by plants are called as phytochemicals. These are produced by the plant's primary and secondary metabolism. These phytochemicals are important for the plants to thrive or thwart other plants, animals, insects and microbial pests and pathogens. They also help plants and protect them from disease and damage caused by environmental hazards like pollution, UV, stress and draught. They are used as traditional medicine and as poisons from ancient days.

Phytochemicals are not the essential nutrients they are rather than the essential nutrients because there is no proof for them to cause any possible health effects in humans are not still established. It is known that they have roles in the protection of human health. More than 4,000 phytochemicals have been catalogued and are classified by protective function, physical characteristics and chemical characteristics.

The phytochemicals are generally classified into the following types; they include carotenoids and polyphenols which include phenolic acids, stilbenes/ lignans. Which further have classifications like flavonoids are further classified into flavones, anthocyanins, isoflavones and flavanols.

Medicinal plants are a gift to us from the nature as they provide a number of health benefits to us. In India these medicinal plants are used for about centuries for their properties and are still used to this date. India has a variety of traditional medical systems like Ayurveda, siddha, unani and a huge class of ethnomedicine. This knowledge of medicine was disappeared due to the western culture that has been on us on the past and is reappearing again as their importance have been realized and lack of side effects are also an important aspect in these types of traditional medicine.

As per a report by World Health Organization (WHO), over 80% of the people of developing countries are relying on the traditional medicines that are extracted from the plants for their primary health needs. Use of these traditional medicines for the preparation of modern medical preparations is indispensable and thus 'Phytomedicines' are a link between the traditional and modern medicine.

Medicinal plants are very important in health care of individuals and communities in many developing countries. Medicinal plants are believed to be much safer and are used in treatment of various ailments. The

plants provide the basic nutrients needed for the growth of animals and humans like proteins, carbohydrates, fats, vitamins and oils minerals. The phytochemicals are majorly classified as primary and secondary metabolites. The primary metabolites are responsible for the basic development of the plant which includes the sugars, amino acids, proteins, nucleic acids, chlorophyll, etc.

Secondary metabolites are those which are needed for the survival of the plants in a harsh environment. They forms the smell, colour and taste of the plants and secondary metabolites such as flavonoids, tannins, saponins, alkaloids, steroids, phytosterols are found to have other commercial applications like they can be used as colouring agents, as drugs as flavouring agents, insecticides, pesticides, anti-bacterial and antifungal products. Moreover they can also be used to protect humans from many diseases like cancer, diabetes, cardiovascular diseases, arthritis and aging etc.

## **2. STEPS IN PREPARATION OF PLANT SAMPLE**

### **2.1. Selection of plant samples**

Proper selection and identification is important for any phytochemical research any defect in this could severely affect the research and may reduce the value of the study.

1. Traditionally used plants by humans for food, medicine or poison based on literature or other sources can be investigated.
2. A random or systematic collection of plants over a large biodiverse area regarding secondary metabolite production can be used
3. Other plant species that are phylogenetically related to the species known to produce a compound of interest
4. Species based on the reports of biological activity in the literature.

### **2.2. Collection of plant samples**

Plant collection can be done on either from wild forests or from herbariums. But in the case of wild plants there is a risk of plants that are been incorrectly identified. They have an advantage that they do not contain any pesticides or herbicides. After collection they are processed soon to prevent the deterioration of secondary metabolites present in the samples.

### **2.3. Identification of plant samples**

The collected samples must be identified elsewhere.

1. Reviewing the flora of the region to compile a list of the plants that are in interest and to separate them from the plants that are to be avoided
2. Field identification must be done. They must at least be identified to their level of genus
3. To aid the identification, taxonomic experts should identify the plant species with a permanent scientific record or in case of a voucher specimen, the plant with the reproductive organs must be submitted to the major institutions or herbaria of the source country.

### **2.4. Cleaning of plants**

Proper cleaning of plants is an important step after collection. This process involves the following steps of washing, peeling, stripping leaves from the stems. This is usually done in hands to have better results.

### **2.5. Drying**

The plant materials are dried to remove the water content and thus after the removal of water so that they can be stored. This process should be done immediately as soon as the plants are collected so that it is prevented from spoilage. There are two methods in drying the plants,

#### **2.5.1. Natural process**

This process include sun-drying. In this the plants are kept in the shades and are air dried in sheds. This process takes few weeks for complete drying of the moisture. This time depends on the temperature and humidity

### 2.5.2. Artificial drying

Artificial drying is done using the help of artificial driers. This process will reduce the time consumed to few hours or minutes. The common method used for the drying of medicinal plants is warm-air drying. This is done using the hot air oven on which warm air is blown. This method is applicable for drying of succulent parts of plants and fragile flowers. The drying must be done in lower temperature to prevent the thermolabile compounds being disintegrated.

### 2.6. Grinding of plant materials

After complete drying of moisture the plant samples are to be powdered for the further analysis. There are different types of powdering, they include the following

1. Grinding can be done by milling in an electric grinder or by a spice mill or can also be in mortar and pestle.
2. Grinding increases the efficiency of the extraction due to increased surface area of the plants. The decrease in the surface area can lead to dense packing of the material.
3. Milling the plants into a fine powder is always ideal but if they are too fine this affects the solvent's flow and also produces more heat which could degrade some thermolabile compounds.

## 3. CHOICE OF SOLVENT

The solvent that is being used for the extraction process is very important in determining the biologically active phytochemicals from the plants. These solvents must be less toxic, easy to evaporate in less heat, should preserve the compounds in it and should not dissociate it. The various solvents commonly used for extraction include:

1. **Water:** It is a universal solvent; the plant extracts with anti-microbial activities are usually extracted with water. But the organic solvents give consistent results in anti-microbial activities when compared to water. Water soluble compounds in the extract cannot give significant results.
2. **Alcohol:** These alcoholic extracts of plants show more activity than aqueous extracts due to the presence of higher amounts of polyphenols. This is because of the higher cell wall and seed degradation by the alcohols which releases the polyphenols which will be degraded in the case if aqueous extracts. But Ethanol is more microbicidal than water. More bioactive compounds are extracted in 70% ethanol than pure ethanol. Ethanol is also found easier to extract intracellular ingredients from plant materials. Polar solvents like methanol, ethanol and their aqueous mixtures are used for extraction of phenolic compounds. Addition of water to alcohol will improve the rate of extraction. Methanol is more polar but it is unsuitable for extraction due to its cytotoxic nature.
3. **Acetone:** Acetone dissolves many hydrophilic and lipophilic compounds from the plants and it is miscible with water. It is low toxic and volatile and it is used for extracting antimicrobial activities. Extracting tannins and other phenolic compounds are done with acetone. They are also used to extract saponins.
4. **Chloroform:** Terpenoid lactones are obtained from barks by extraction with chloroform. Tannins and Terpenoids are treated with less polar solvents.
5. **Ether:** They are used for the extraction of coumarins and fatty acids.

## 4. METHODS OF EXTRACTION:

### 4.1. Homogenization

This method is one of the most widely used methods for extraction. This is either done by dried or wet extraction method. In this dried extraction method the dried plant samples are finely powdered and added to the solvent mixed for few minutes and kept in an orbital shaker for about 24 hours. In wet extraction process, the parts of the plants are cut into small pieces, grinded in a mortar and pestle and are added to a solvent and shaken in an orbital shaker for 24 hours and then filtered. The filtrate can be used for the further analysis.

### 4.2. Serial Exhaustive Extraction

It is done with a variety of solvents from a non-polar solvent like hexane to more polar solvent like methanol to extract a wide polarity range of compounds. The disadvantage is that thermolabile compounds cannot be extracted due to the high heat which leads to the degradation.

#### 4.3. Soxhlet Extraction

It is used when the compound is less soluble in the solvent and the impurities are soluble in the solvent. If the desired compound is highly soluble in the solvent the impurities can be removed by simple filtration. The advantage is that the solvent is recycled in this method and hence there is less wastage of the solvent. Similar to the above method, thermolabile compounds cannot be extracted in this method

#### 4.4. Maceration

In this method, whole plant or the powder can be kept in the solvent for a certain period with frequent agitation until the soluble compounds are dissolved. This method is the best suitable method for the thermolabile compounds

#### 4.5. Decoction

In this method heat stable and water soluble compounds are extracted. This is extracted plant materials are boiled in the water for about 15 minutes and are cooled, filtered and are used for further analysis

#### 4.6. Infusion

It is done by diluting the compounds in the solvents. It is prepared by macerating the compounds for a short period in cold or boiling water

#### 4.7. Digestion

This is a process where the extraction is done as maceration with a gentle heat applied. It is used when the elevated temperature does not interfere the solvent efficiency or the compounds.

#### 4.8. Percolation

For this process an instrument called percolator is used which is a narrow, cone shaped Vessel with open ends. The ingredients are moistened with an appropriate amount of the specified menstrum and allowed to stand for approximately 4 h in a well closed container, after which the mass is packed and the top of the percolator is closed. Additional menstrum is added to form a shallow layer above the mass, and the mixture is allowed to macerate in the closed percolator for 24 h. The outlet of the percolator then is opened and the liquid contained therein is allowed to drip slowly. Additional menstrum is added as required, until the percolate measures about three quarters of the required volume of the finished product. The marc is then pressed and the expressed liquid is added to the percolate. Sufficient menstrum is added to produce the required volume, and the mixed liquid is clarified by filtration or by standing followed by decanting.

#### 4.9. Sonication

In this method the ultrasound with higher frequencies of 20 kHz – 2000 kHz are used which will disrupt the cells and releases the constituents. Although the process is useful in some cases, like extraction of rauwolfia a root, its large-scale application is limited due to the higher costs. One disadvantage of the procedure is the occasional but known deleterious effect of ultrasound energy (more than 20 kHz) on the active constituents of medicinal plants through formation of free radicals and consequently undesirable changes in the drug molecules.

### 5. QUALITATIVE ANALYSIS OF PRIMARY METABOLITES:

#### Test for carbohydrates

1. Benedict's test: About 0.5 ml of the filtrate was taken to which 0.5 ml of Benedict's reagent is added. This mixture was heated for about 2 minutes in a boiling water bath. The appearance of red precipitate indicates the presence of sugars

2. Molisch's test: To about 2ml of the sample, 2 drops of alcoholic solution of  $\alpha$ -naphthol was added and to the mixture after being shaken well. Few drops of conc.  $H_2SO_4$  were added along the sides of the test tube. A violet ring indicates the presence of sugars

#### Test for Starch



To about 5 ml of distilled water, 0.01g of iodine and 0.075 g of potassium iodide were added and this solution was added to about 2-3 ml of the extract. Formation of blue colour indicates the presence of starch

#### **Test for proteins**

1. Biuret test: 2ml of filtrate was taken to which 1 drop of 2% copper sulphate solution was added; 1ml of 95% ethanol was added. Then it was followed by excess addition of KOH. The appearance of pink colour indicates the presence of protein.
2. 2ml of extract was mixed with 2ml of water and about 0.5% of conc.  $\text{HNO}_3$  was added. The appearance of yellow colour indicates the presence of proteins.
3. To about 2 ml of the extract, 2ml of miller's reagent was added white precipitate which turns red on heating will confirm the presence of proteins.

#### **Test for amino acids**

1. To 1ml of the extract, few drops of ninhydrin reagent (10mg of ninhydrin in 200ml of acetone) were added. The appearance of purple colour indicates the presence of amino acids.
2. To 2ml of extract few drops of nitric acid were added along the sides of the tube the appearance of yellow colour indicates the presence of protein and free amino acids.

#### **Test for fatty acids**

1. 1 ml of the extract was mixed with 5 ml of ether. These extracts were allowed to evaporate on a filter paper and the filter paper was dried. The appearance of transparency indicates the presence of fatty oils

#### **Miscellaneous compounds**

##### **Test of resins**

1. Precipitation test: about 0.2 g of extract was extracted with 15ml of 95% ethanol. The alcoholic extract was then poured into a beaker containing about 20ml of distilled water.
2. 1ml of extract was taken and to this few ml of acetic anhydride was added to this 1ml of conc.  $\text{H}_2\text{SO}_4$  was added. The appearance of orange to yellow colour indicates the presence of resins

##### **Test of fixed oils and fats**

1. Spot test: small quantity of the extract was taken and pressed between 2 filter papers. The appearance of spots indicates presence of oils
2. Saponification test: To the extract, few drops of 0.5N alcoholic KOH and few drops of phenolphthalein were added. This mixture was heated for about 2 hours. The formation of soap or partial neutralization of alkali indicates the presence of fixed oils or fats

##### **Gums and mucilage**

To 1ml of extract, distilled water, 2ml of absolute ethanol was added with constant stirring white or cloudy precipitate indicates the presence of gums or mucilage

##### **Carboxylic acids**

1. To 1ml of extract a pinch of sodium bicarbonate is added. The production of effervescence indicates the presence of carboxylic acids
2. 2ml of alcoholic extract was taken in warm water and filtered. The filtrate was then tested with litmus paper and methyl orange. The appearance of blue colour.

## **6. QUALITATIVE ANALYSIS OF SECONDARY METABOLITES**

##### **Test for anthraquinones**

To 5ml of extract, few ml of conc.  $\text{H}_2\text{SO}_4$  was added and 1ml of diluted ammonia was added to it. The appearance of rose pink confirms the presence of anthraquinones

**Test for quinones**

To 1ml of extract, alcoholic KOH is added the presence of red to blue colour indicates the presence of quinones

**Test for alkaloids**

1. Mayer's test: to a few ml of filtrate, 2 drops Mayer's reagent was added a creamy or white precipitate shows a positive result for alkaloids.
2. Wagner's test (iodine – potassium iodine reagent): To about an ml of extract few drops of Wagner's reagent were added. Reddish – brown precipitate indicates presence of alkaloids.
3. To 5ml of extract 2ml of HCl was added. Then 1 ml of Dragendroff's reagent was added an orange or red precipitate shows a positive result for alkaloids.

**Test for glycosides**

1. Borntrager's test: to 2ml of filtrate, 3ml of chloroform is added and shaken. The chloroform layer is separated and 10% ammonia solution was added. The pink colour indicates the presence of glycosides
2. 5ml of extract was hydrolysed with 5ml of conc. HCl boiled for few hours in a boiling water bath, small amount of alcoholic extract was dissolved in 2ml of water and 10% of aqueous 10% NaOH was added the presence of yellow colour was a positive result for the glycosides.
3. 2ml of extract is mixed with about 0.4 ml of glacial acetic acid containing traces of ferric chloride and 0.5 of conc.  $H_2SO_4$  was added the production of blue colour is positive for glycosides.

**Test for cardiac glycosides (Keller-Killani test)**

1. 5ml of solvent extract was mixed with 2ml of glacial acetic acid and a drop of ferric chloride solution was added followed by the addition of 1ml of conc.  $H_2SO_4$ . A brown ring in the interface indicates the presence of deoxy sugars of cardenoloides. A violet ring may appear beneath the brown ring while acetic acid layer a green ring may also form just gradually towards the layer.

**Test for phenol**

1. Gelatine test: To 5ml of extract 2ml of 1% solution of gelatine containing 10% of NaCl is added. Appearance of white precipitate indicates the presence of phenol
2. Lead acetate test: To 5 ml of extract 3ml of 10% lead acetate solution was added and mixed gently. The production of bulky white precipitate is positive for phenols.

**Test for polyphenols**

1. To the 3ml of extracts 10ml of ethanol was added and were warmed in a water bath for 15 minutes. To this few drops of ferric cyanide (freshly prepared) was added. The formation of blue – green colour indicates presence of polyphenols.
2. To 1ml of extract few drops of 5% solution of lead acetate was added. The appearance of yellow precipitate indicates the positive results for polyphenols
3. To the 5ml of ethanolic extract 3ml of 0.1% gelatine solution was added. The formation of precipitate was positive for polyphenols

**Test for tannins**

1. To 5ml of extract few drops of neutral 5% ferric chloride solution was added, the production of dark green colour indicates the presence of tannins

**Test for Flavonoids**

1. To the aqueous solution of the extracts 10% ammonia solution is added and is heated. The production of fluorescence yellow is positive for flavonoids.
2. 1ml of extract was taken and 10% of lead acetate was added. The yellow precipitate is positive inference for the flavonoids
3. The extract is treated with concentrated  $H_2SO_4$  resulting in the formation of orange colour indicates the positive result for flavonoids.

4. To 5ml of dilute ammonia the plant extract is added and shaken well. The aqueous portion is separated and concentrated  $H_2SO_4$  is added. The yellow colour indicates the presence of flavonoids.

**Test for phytosterols**

1. The extract is dissolved in 2ml of acetic anhydride and to which 1 or 2 drops of concentrated  $H_2SO_4$  is added along the sides an array of colour change indicates the presence of phytosterols.
2. The extract was refluxed with alcoholic KOH and saponification takes place. The solution was diluted with ether and the layer was evaporated and the residue was tested for phytosterols. It was dissolved in diluted acetic acid and few drops of concentrated  $H_2SO_4$  are added. The presence of bluish green colour indicates the presence of phytosterols.

**Test for phlobatannins**

1. Aqueous extract was boiled with diluted HCl leading to the deposition of reddish precipitate indicates the presence of phlobatannins

**Test for saponins**

1. 0.5 mg of extract was vigorously shaken with few ml of distilled water. The formation of frothing is positive for saponins
2. The froth from the above reaction is taken and few drops of olive oil is added and shaken vigorously and observed for the formation of emulsion.

**Test for steroids**

2ml of extract with 2ml of chloroform and 2ml of concentrated  $H_2SO_4$  are added, the appearance of red colour and yellowish green fluorescence indicates the presence of steroids

**Test for xanthoproteins**

1ml of extract is taken and to this few drops of nitric acid and ammonia are added. Reddish brown precipitate indicates the presence of xanthoproteins

**Test for chalcones**

2ml of ammonium hydroxide is added to 0.5 g of extract. The appearance of red colour indicates the presence of chalcones

**Test for Terpenoids (Salkowski test)**

3ml of the extract was taken and 1ml of chloroform and 1.5 ml of concentrated  $H_2SO_4$  are added along the sides of the tube. The reddish brown colour in the interface is considered positive for the presence of terpenoids

**Test for triterpenoids**

To 10 mg of extract 1ml of chloroform is added and is mixed to dissolve it. 2ml of concentrated  $H_2SO_4$  is added followed by 1ml of acetic anhydride. Formation of reddish violet colour is positive for the presence of triterpenoids.

**Test for anthocyanins**

2ml of aqueous extract was taken to which 2N HCl was added and it was followed by the addition of ammonia, the conversion of pink-red turns blue-violet indicates the presence of anthocyanins.

**Test for Leucoanthocyanins**

To 5ml of extract dissolved in water, 5ml of Isoamyl alcohol is added. The red appearance of the upper layer indicates the presence of Leucoanthocyanins

**Test for Coumarins**

To 2 ml of the extract, 3 ml of 10% aqueous solution of NaOH is added. The production of yellow colour indicates the presence of coumarins

**Test for emodins**

To 5ml of extract, 2ml of NH<sub>3</sub>OH and 3ml of benzene are added. The production of red colour indicates the presence of emodins

**7. QUALITATIVE ANALYSIS OF VITAMINS****Test for Vitamin – A**

In 5 ml of chloroform, 250mg of the powdered sample is dissolved and it is filtered, to the filtrate, 5ml of antimony trichloride solution is added. The appearance of transient blue colour indicates presence of vitamin-A

**Test for vitamin – C**

In 5ml of distilled water, 1ml of the sample was diluted and a drop of 5% sodium nitroprusside and 2ml of NaOH is added. Few drops of HCl are added dropwise, the yellow colour turns blue. This indicates the presence of vitamin- C

**Test for vitamin – D**

In 10 ml of chloroform, 500mg of powdered extract is dissolved and filtered. 10ml of antimony trichloride is added, the appearance of pinkish-red colour indicates the presence of vitamin – D

**Test for vitamin – E**

Ethanoic extract of the sample was made and filtered (500mg in 10ml), few drops of 0.1% ferric chloride were added and 1ml of 0.25% of 2'-2'dipyridyl was added to 1ml of the filtrate. Bright-red colour was formed with a white background.

**8. QUALITATIVE AND QUANTITATIVE**

Analysis Qualitative and quantitative analysis of phytochemicals can be done using Gas Chromatography Mass Spectroscopy (GCMS). GCMS can be applied to solid, liquid and gaseous samples. First the samples are converted into gaseous state then analysis is carried out on the basis of mass to charge ratio. High Performance Liquid Chromatography is applicable for compounds soluble in solvents. High performance thin layer chromatography is applicable for the separation, detection, qualitative and quantitative analysis of phytochemicals.

**8.1. Gas Chromatography**

Volatile compounds are analysed using gas chromatography. In this method, there is a gas and a liquid phase. The liquid phase is stationary where the gas phase is a mobile phase. These compounds to be analysed are also in the mobile phase with a carrier gas which is usually helium, hydrogen or argon. The chemicals are separated depending on the migration rate into the liquid phase. Higher percentage of the chemical will lead to faster migration in the liquid phase. This is widely used in qualitative and quantitative phytochemical analysis.

**8.2. High Performance Liquid Chromatography: (HPLC)**

HPLC is also known as High- Pressure Liquid Chromatography. This method involves the interaction of liquid solvent in the tightly packed solid column or a liquid column. These acts as the stationary phase while the liquid (solvent) acts as the mobile phase, high pressure enables the compounds to pass to the detector. As HPLC compounds are analysed after vaporisation, thermolabile compounds cannot be analysed with this technique.

**8.3. High Performance Thin Layer Chromatography: (HPTLC)**

This method is modified form of thin layer chromatography. It is a type of planer chromatography where the separation is done by high performance layers with detection and the sample components are acquisition using an advanced work- station. The reduction of the thickness of the layer will increase the efficiency of the



separation and hence HPTLC is more advanced method for qualitative, quantitative and micro-preparative chromatography.

#### 8.4. Optimum Performance Laminar Chromatography: (OPLC)

OPLC combines the advantages of TLC and HPLC. The system separates about 10-15 mg samples, with simultaneous processing of up to 4 or 8 samples at a time depending on the model. In OPLC a pump is used to force a liquid mobile phase through a stationary phase, such as silica or a bonded-phase medium.

### 9. CONCLUSION:

Plants are an important source of phytochemicals which are an important source of drug and medicine. These phytochemicals have extraordinary properties like antibacterial, antifungal, anti-cancerous, antioxidant, anti-inflammatory, anti-diabetic activities etc. The identification of this compound relies on the tools of phytochemical analysis and hence the knowledge about these techniques is important. This article will be helpful in the collection, identification, extraction and analysis of phytochemicals that are extracted from the plants. The methods followed for this analysis should be standard and following non-standard protocols could lead to the false results that are not reproducible. The above article will help in the qualitative analysis of the phytochemicals.

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