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Review article

Plant secondary metabolites, their separation, identification and role in human disease prevention

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Abstract

Phytoconstituents are the most essential non-nutritive components that are secreted by the plants. They are also known as secondary metabolites which are not utilized by the plants, but used for protective or disease preventive properties to the human. There are many phyto-constituents that isolated and used as drugs. Perhaps, some are also used as a marker compounds which are isolated through fingerprinting techniques and acts as phytochemical standards. Scientific studies have revealed that the isolated phytoconstituents are more effective than crude extracts for therapeutic activity. Hence, the demand is towards primary identification as well as separation of the constituents through various solvent systems, using thin layer chromatographic (TLC) technique. Various chemical tests for identification of functional group of compounds, followed by the TLC solvent system as well as spraying reagents will clearly separate and detect the constituents. These studies will show the further step towards the discovery of the new drug molecules for effective therapeutic efficacy.

Key words: Biomarker, chromatographic techniques, fingerprinting, isolation, identification, phytoconstituents

1. Introduction

God has created a natural plant kingdom which is having super power nature for curing and treating many human diseases. It was well known that based on the diseases of human, remedies are also available by the plants and for that we have to recognize or identify the correct one. The plants provide almost everything that man needed and the nature has been influencing to make use of available resources since antique (Payum, 2017). According to World Health Organization (WHO), more than 80% of the population in the World is believe on medicinal plants because they play an important role for the development of health in mankind (Alves and Rosa, 2005). It is now established and fully believed that phytoconstituents obtained from the medicinal plants serve as primary and important molecules in the modern medicines (Neube *et al*., 2008; Rajeshwari *et al*. 2013; Manoharachary and Nagaraju, 2016; Majeed, 2017; Dang, 2018) and many people still depend on the traditional medicine for their preliminary healthcare and treatment (Bannerman *et al*., 1986). All these treatments are mainly depend on the phytoconstituents present in the plants.

In early ages about 60 thousand years ago, people used raw plants directly for curing diseases but in many cases, these plants showed adverse effects due to misinterpretation, or reactions because of unkown chemicals or due to toxic effects as a result nausea, vomiting, diarrhea or other toxic reactions and even also death (Fabricant and Farnsworth, 2001). Then, gradually traditional medicine system came in herbal field and based on the applications of traditional medicines, many branches are established such as Ayurveda, Siddha, Unani, Homeopathy and also Chinese medicines. These systems are used natural plants in the curing and diagnosis of physical and mental illness of humans as per the basic concept of the above various said systems.

The most interesting and challenging concepts of these systems are curing diseases with the help of plants without or may be very negligible industrial processing and, hence till now they are continuing their importance in the herbal field to treat chronic diseases in root level. In this traditional medicine system, plants are in combinations or single used from therapeutic efficacy, but was not much scientific evidences that which plant chemicals are responsible for curing those particular diseases. These concepts forced to discover modern medicines in early 19th century where plants are scientifically proved their efficacy due to which particular chemical present. Hence, the drug discovery from the plant sources gains maximum momentum and importance with the era of modern medicine system by the concept of ethanobotanical experience of traditional medicines.

Natural plants have a vast range of biodiversity and due to that one plant contains more than one chemical component. This concept brought new challenges to the phytochemists to isolate, identify and characterization of plant constituents, followed by establishment of pharmacological and therapeutic efficacy. The first phytochemical morphine from opium (1804) (Serturner, 1806),

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followed by emetine from Ipecac (1817) (Grollman and Jarkovsky, 1975), strychnine from *Nux vomica* (1818), quinine from cinchona bark (1820) (Pelletier and Caventou, 1818) and thereafter, nicotine (alkaloid) was isolated from the tobacco plant in 1828 (Posselt and Reimann, 1828). From then, scientists are conducted extensive research in discovery of drugs with the help of modern analytical chemistry, combinatorial chemistry with the help of pharmaceutical industries to establish the characterization of drugs. Scientific paper evident that any therapeutic efficacy showed by plants is based on the groups of compounds that is present in the plant body. They are known as plant secondary metabolites which are stored in various plant parts that are not utilized by the plants, but are essential for humans to get therapeutic effects. Such secondary metabolites are glycosides, alkaloids, carbohydrate, proteins, lipids, tannins, flavonoids, terpenoids, steroids, polyphenols, phytosterols, resins, glycoalkaloids, *etc*. Hence, various extraction methods followed by details phytochemical screening methods are developed to know the complete history of the plant constituents' especially preliminary identification of the phytogroups.

For extraction of plant constituents, particular solvents are used based on the solubility of the plant constituents. Further, various chemical tests are carried out to identify the group of the constituents. General chemical tests are carried out to detect the secondary metabolites whereas individual chemical tests are performed to identify particular constituents.

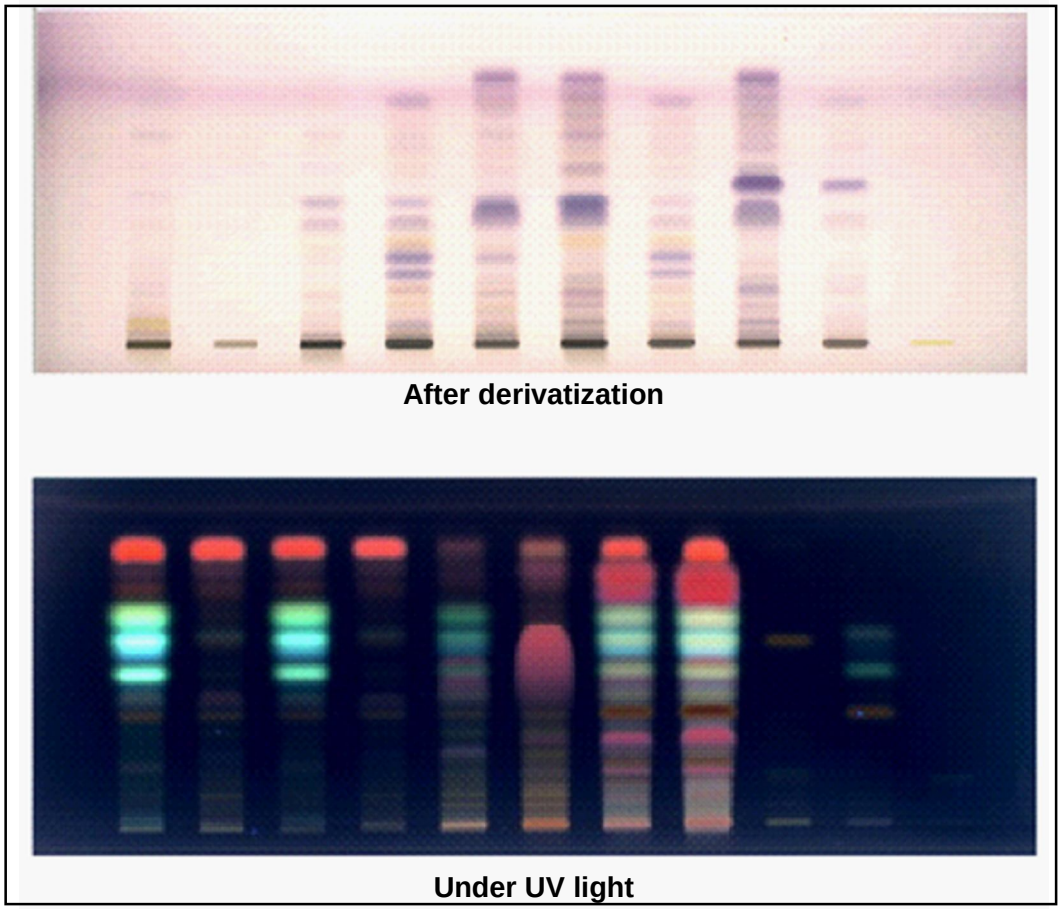

Figure 1: HPTLC fingerprinting for plant extract.

Furthermore, thin layer chromatography (TLC) method is performed to get separation and identification of constituents that are present in the particular plant extracts. Identified constituents are further isolated by using column chromatography or by preparative HPLC or HPTLC methods. This method is also known as fingerprinting, used for quality control of herbals, by which plant based markers are isolated. This is known as biomarkers. They are the compounds preferably present in 'detectable quantities' in plants. These are chemically defined constituents of a herbal drug that are used for control purposes independent of whether they have any therapeutic activity or not. A fingerprint comprises of scanning, in UV, fluorescence, ultraviolet spectra and photographic images in ultraviolet light (254 and 366 nm) and occasionally using visible light after derivatization (Figure 1). HPTLC fingerprint is obtained at low cost and high speed, satisfy the need of a modern quality control method. Hence, HPTLC is the most powerful tools linking the botanical identity to the chemical constituent profile of the plant as well as employed for quantitative determination of such marker compounds. Based on these studies, further revenues opened in modern era, such as nutraceuticals, nano herbal technology, molecular phytopharmacology, plant robotics, *etc*.

Therefore, it is necessary to know the detail preliminary study of phytoscreening technology with respect to various chemical tests as well as TLC study on the relationship among natural products,

traditional medicines and modern medicine to explore the possible concepts and methodologies from plant products to further discovery of new drug molecules.

2. Secondary plant metabolites

Plants produce a vast number of different chemical compounds which are broadly categorized by primary metabolites and secondary metabolites. Primary metabolites are essentials for the plants for their survival likely: sugar, proteins, amino acid, *etc*. Thereafter, secondary metabolites are the products that are considered as waste substances of plants. These secondary metabolites are not essential, but stored in various parts of the plant bodies and provide protections to the plants from attacks of microorganisms, attractors for pollinators, *etc*. These secondary metabolites are relatively produced in low quantities in plants but have significant economic and medicinal value to the humans. High concentration of secondary metabolites present in plants indicates more resistant plants. Various scientific research evidences proved that the therapeutic efficacy and pharmacological actions are mainly by the action of secondary metabolites and hence, plant secondary metabolites are gaining importance to natural products chemists. They are classified on the basis of chemical structure (Example: having rings, containing a sugar), as per composition (containing nitrogen or not), their solubility in various solvents, as well as the pathway by which they are synthesized (*e.g*., phenyl propanoid, which produces tannins). Basically, they are classified into four major groups like nitrogen containing compounds, glycosides, phenolics and terpenes. Detail list of classification is tabulated in Figure 2.

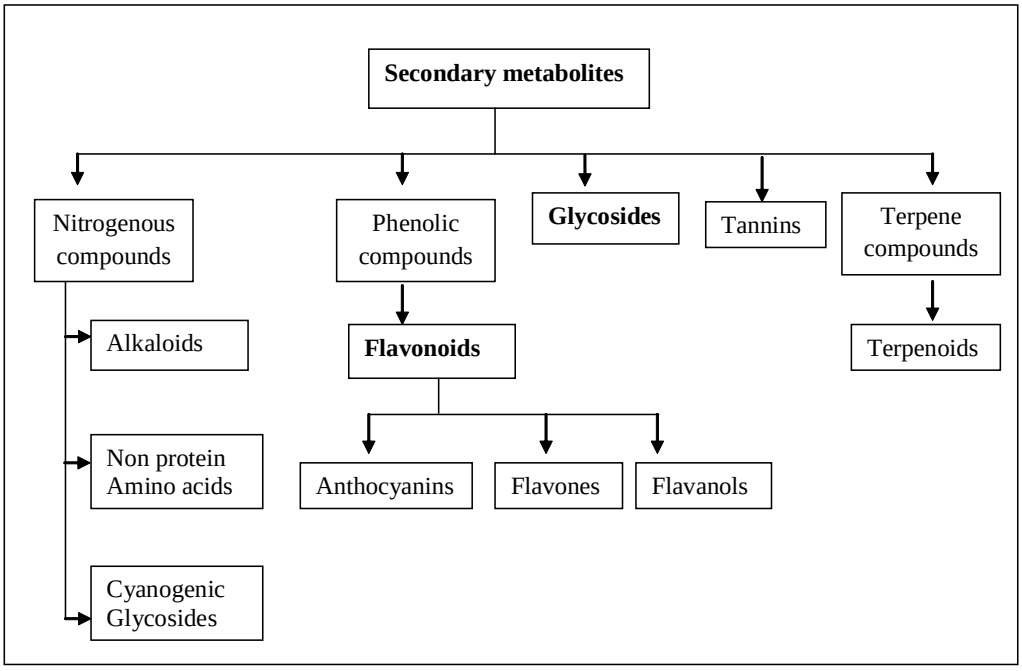

Figure 2: Broad classification of plant secondary metabolites.

Apart from the therapeutic applications, these secondary metabolites are also useful in preparation of various dyes, useful for preparation of agrochemical insecticides, availability of raw materials in industries, useful in perfumes and cosmetics, useful in coloring as well as flavoring agents. Some of the important characters of secondary metabolites are discussed below:

Alkaloids

The name alkaloid indicates “alkali like” which are important plant secondary metabolites, primarily composed of nitrogen. They are also highly toxic, but show medicinal activity in low doses. About 5000 types of alkaloids are present in 15% of plants in the land which distributed in more than 150 families. These important families are like Apocyanaceae, Papaveraceae, Ranunculaceae, Rubiaceae, Solanaceae, Rutaceae, Loganiaceae, *etc*. In all these plants, alkaloids are present as a slat form of organic acid. They are sparingly soluble in water and extracted with alcohol. They are generally white crystalline solid, but some alkaloid is volatile like nicotine. Alkaloids are colorless, but some are colorful like nicotine (brown), berberine (yellow), canadine (orange). They react with heavy metal iodide and form precipitate. They are reserved substances that supply nitrogen. As per the position of the nitrogen in ring structure, alkaloids are broadly classified into three groups’, *viz*., true alkaloids, proto alkaloids and pseudo alkaloids.

True alkaloids contain nitrogen in their heterocyclic ring and they are synthesized from amino acids. These alkaloids are giving general chemical tests for alkaloids. (Examples: morphine, nicotine, atropine). Proto alkaloids are also known as amino alkaloids because they contain nitrogen in outside of the heterocyclic ring but they derived from amino acids. They give positive reactions for all general tests for alkaloids (Example: ephedrine, colchicines, taxol). Finally, pseudo alkaloids are the group of alkaloids that did not derive from amino acids and have nitrogen in a heterocyclic ring but do not give a positive response towards general tests for alkaloids. Examples are caffeine, conessine (Evans, 2009; Kokate *et al*., 2009).

Alkaloids further classified based on their biosynthetic origin (Examples: indole alkaloids from tryptophan, tropane alkaloids from ornithine, *etc*.), based on taxonomic nature and based on their pharmacological action (Examples: poisons, stimulant, astringents, anti-inflammatory, antihypertensive, analgesics, expectorants, *etc*.).

Non-protein amino acids

Non-protein amino acids are neither a part in any proteinic molecule nor involved in protein metabolism. They are not coded for by the DNA. They are generally formed by metabolic intermediates, pharmacological agents, post translational modifications, *etc*. (Ambrogelly *et al*., 2007). They are mainly found in the food and fodder plants. They provide protection to the plants against pathogens, predators but are toxic to the humans. They are chemically incorporated in to peptides for protecting the functional groups. Examples are tryptophan and tyrosine residue in some proteins, are substituted with 4-fluorotyrptophan and m-fluorotyrosine, respectively, the 19 Fnuceli is used as magnetic resonance without any effects on protein functions. All non-protein amino acids are broadly classified as alpha and non-alpha amino acids. Alpha amino acids are like ornithine, thyroxine, homocysteine, ovathiol, *etc*., and non-alpha amino acids are like beta alanine, gama amino butyric acid, beta amino isobutyric acid. Some general functions of non-protein amino acids are like, tryptophan is used as a precursor of neurotransmitter serotonin, tyrosine is the precursor of neurotransmitter dopamine, arginine is a precursor of nitric oxide.

Cyanogenic glycosides

Cyanogenic glycosides are natural plant toxin, widely distributed in edible plants belongs to the family Leguminosae, Fabaceae, Rosaceae, Linaceae, Rubiaceae and Compositae. They are the group of nitrile containing plant secondary compounds that yields cyanide. Upon hydrolysis of cyanogenic glycoside by the beta-glucosidase enzyme, cyanide is formed and occurs during consumption or crushing and processing of the edible plant materials (Harborne, 1993; Vetter, 2000). The reaction is known as cyanogenesis.

Some of the important plants that contain high level of HCN are included white clover, linum, almond, sorghum, the rubber tree, plum, cashews, bitter almond, cassava, *etc*. Intentional or unintentional exposure or consumption of cyanide from cyanogenic glycosides leads to acute intoxications. Cyanide toxicity is dependent on the release of hydrogen cyanide and occurs in humans at doses between 0.5 and 3.5 mg HCN per kilogram body weight which are characterized by neurological symptoms, resulting from tissue damage in the CNS and also included other symptoms likely vomiting, stomach ache, diarrhea, convulsion, and even death (WHO, 1993). Detoxification of cyanogenic glycosides occurs based on processing methods that reduces the risk of cyanide poisoning. In the human body; cyanide is detoxified by the enzyme rhodanase with the help of sulfur containing amino acids and converted to thiocyanate, which is nontoxic and further excreted through the urine (Banea *et al*., 2012). They play pivotal roles in plants as chemical defense and in plant insect interactions (Ganjewala *et al*., 2010). Some of the important edible cyanogenic glycosides are, *viz*., amygdalin (*Prunus amygdalus*), Dhurrin (*Sorghum album*), prunasin (*Prunus avium*), *etc*.

Phenolic compounds

This is most important secondary metabolite that became very popular due to their health benefits. They consist of a hydroxyl group (–OH) attached to an aromatic ring and are found abundantly in all parts of the widely distributed plant species. The first group of phenols is the flavonoids. Flavonoids are water soluble pigments which are found in the vacuoles of the plant cells. They are having C_6-C_3-C_6 basic structural unit, contains two phenyl rings along with fused one heterocyclic ring. They are widely distributed in plants as important secondary metabolites which are mainly found in the members of family Rutaceae. Flavonoids are further divided into many sub-groups which are depicted in Figure 3.

All the flavonoids are involved in many important therapeutic activities like antiallergic, anti-inflammatory, antioxidant, antimicrobial, antiviral, anticancer, *etc*. (Choi *et al*., 2007; Ravishankar *et al*., 2013; Gomes *et al*., 2012;). Anthocyanins exist in various colored compounds such as red, blue and purple and mostly found in grapes, berries. They are water soluble glycoside of polyhydroxyl and polymethoxyl derivatives of 2-phenyl benzopyrylium. They act to protect against heart disease, diabetes, cancer and even reduce ageing as skin care products (Hollman and Katan, 1999; Ghosh and Konishi, 2007). Thereafter, flavones are red or purple colored and flavanols are white to colorless compounds, whereas flavonols are yellow colored compounds. Furthermore, plant lignins are also phenolic type of structural compounds that provides strength to the cell walls. This secondary metabolite is broadly distributed in plants. They are classified in six major subgroups, *viz*., chalcones, flavones, flavonols, flavandiols, anthocyanins, and proanthocyanidins or condensed tannins. All these groups of plant constituents are beneficial in protection against ultraviolet (UV) radiation and phytopathogens, signaling during nodulation, male fertility, auxin transport, as well as the coloration of flowers as a visual signal that attracts pollinators (Winkel-Shirley, 2002; Bradshaw and Schemske, 2003). Overall, the biological functions of flavonoids are linked to their potential cytotoxicity and their capacity to interact with enzymes through protein complexation.

Glycosides

Glycosides are important plant secondary metabolites, chemically contains a carbohydrate component linked by an ether bond to a non-carbohydrate nucleus. Carbohydrate part is known as glycone or sugar part which is water soluble whereas non-carbohydrate part is known as aglycone part or genin part. Due to presence of genin part, glycosides are showing therapeutic efficacy like laxative, purgative, antirheumatic, demulcent, cardiotonic, *etc*. They are abundantly found in higher plant containing Fabaceae, Scrophulariaceae, Liliaceae, Rhamnaceae, Polygonaceae, Rosaceae, Apocynaceae, Apiaceae families. They are generally crystalline or amorphous substances. They are water soluble but insoluble in

organic solvents like chloroform, ether, *etc*. They are hydrolyzed by acids or enzymes and separates sugar and non-sugar parts. They are optically active but do not reduce Fehling's solution until they undergone hydrolysis.

Terpenes

Terpenes constitute a large group of hydrocarbons occur in many plants. They are hydrocarbons and components of resins. They are derived from turpentine. Terpenes occur by the combination of two molecules of acetic acid to give mevalonic acid ($C_6H_{12}O_4$) and afterwards converted in to isopentenyl pyrophosphate, which contains the five-carbon isoprene skeleton. The term 'terpene' was given to the compounds isolated from terpentine of pine trees. They are useful active ingredients of natural pesticides, not only that they are also used in food, cosmetics, pharmaceutical and biotechnological industries (Thimmappa *et al*., 2014).

Terpenes are chemically modified by oxidation or rearrangement of the carbon skeleton and form terpenoids, *i.e.*, they are combined hydrocarbons and their oxygenated derivatives. They are also known as isoprenoids because they are the primary constituents of the essential oils. Terpenoid hydrocarbons have the general formula $(C_5H_8)_n$ which is known as isoprene unit. They are classified on the basis of the value of the number of carbon atoms present in the basic isoprene structure. They are basically colorless, fragrant liquids and are lighter than water, hence they are insoluble in water but soluble in organic solvents. They are also optically active. They are found in mainly distributed in Apiaceae, Fabaceae, Lamiaceae, Zingiberaceae, Solanaceae, Pinaceae families and uses as carminative, stimulant, counter irritant, anti rheumatic, perfuming and flavoring agents.

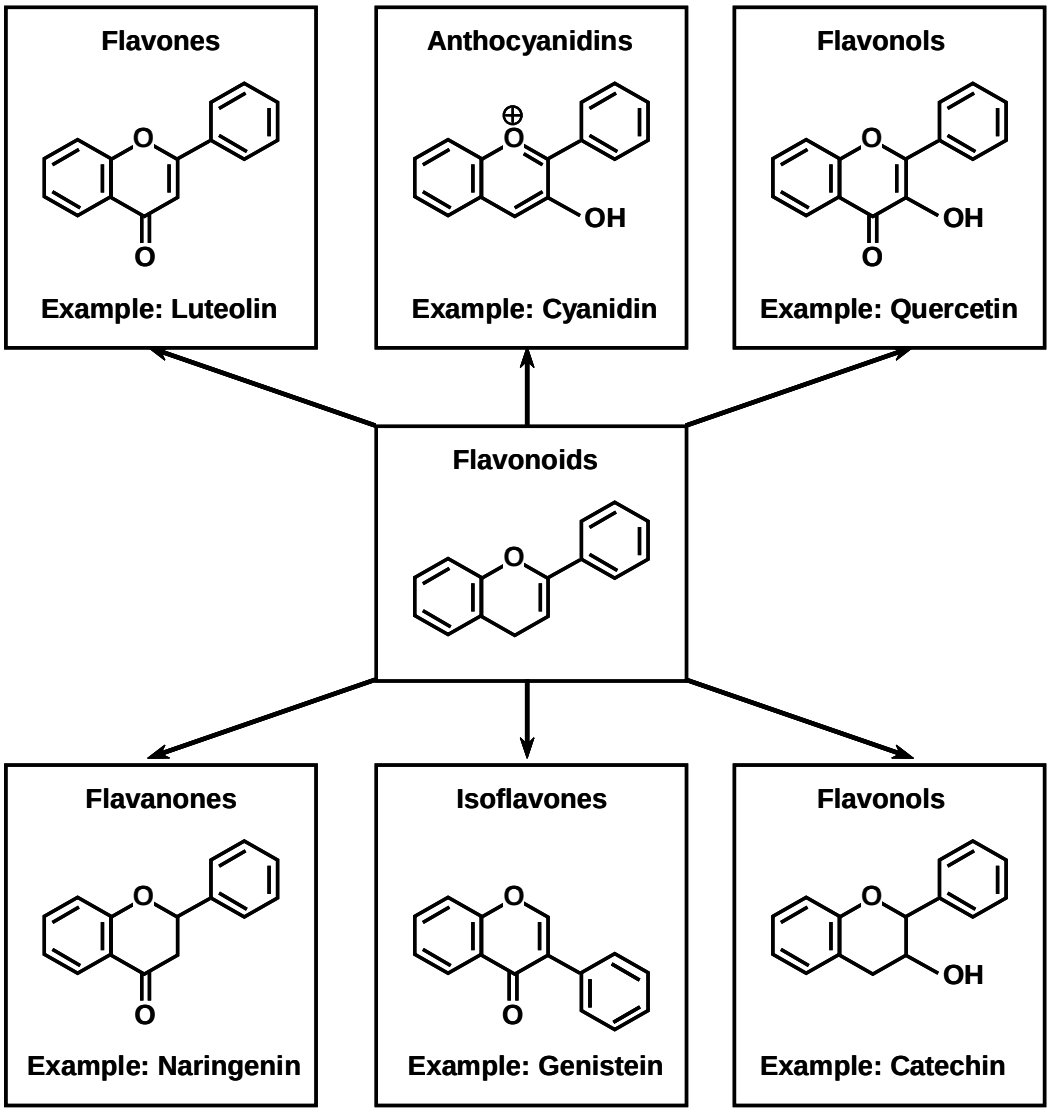

Figure 3: Sub-classification of flavonoids.

Tannins

Tannins are another important plant secondary metabolite that is present in a wide range of higher plants containing Combretaceae, Fagaceae, Fabaceae, Rubiaceae families. They are also phenolic compounds without nitrogen in their ring structure. They are higher molecular weight compounds and mainly utilize as plant defense mechanisms towards mammalian herbivores and insect. They are also used as astringents, against diarrhoea, as diuretics, duodenal tumours, anti-inflammatory, antiseptic and haemostatic. They can precipitate heavy metals and alkaloids (except morphine) and also used in poisonings with these substances. Basically, they are of two types: hydrolysable and condensed tannins. They form colloidal solution with water but soluble in alcohols, dilute alkalies, *etc*. (Kokate *et al*., 2007).

All these important secondary metabolites are present in crude extracts which are separated and identified by color reactions using chemical tests. Chemical tests are a qualitative or quantitative procedure used to identify, quantify, or characterize a chemical compound or chemical group. In natural plant science, chemical tests are qualitative identification where group of chemicals as well as separation of individual phytoconstituent is carried out for further processing, *i.e.*, for isolation of the plant constituents. In this section, some general chemical tests are discussed for easy identification of the constituents.

3. Separation and identification of secondary metabolites

3.1 Chemical test (Edeoga *et al*., 2005; Trease and Evans, 1989**)**

3.1.1 Test for general alkaloids

3.1.1.1 Precipitation reactions

Dragendorff's reaction, Mayer's reaction, Wagner's reaction, Hager's reaction, Marme's reaction, Kraut's reaction, Scheibler's reaction, Tannic acid reaction, Reineckate salt reaction, Sonnenschein's reaction.

Dragendorff's reaction (Potassium iodide + Bismuth nitrate)

Principle: Bismuth nitrate reacts with potassium iodide in an acidic solution and forms potassium bismuth iodide ($K[BiI_4]$) solution which reacts with alkaloids and forms a dark orange precipitation.

Preparation: 0.5 g of bismuth nitrate poured into a beaker and about 10 ml of distilled water is added. The mixture should be like a suspension. Then the solution made acidic by added 10 ml of concentrated hydrochloric acid. Stirred the solution and kept aside. Further, in another beaker, 4 g of potassium iodide is dissolved in little quantity of water. Then, both the solutions are mixed together to get a dark orange solution.

Reaction: Dragendorff's solution + Alkaloid containing extract solution.

Observation: Formation of amorphous orange-red precipitation.

Mayer's reaction (Potassium mercuric iodide)

Principle: Most alkaloids are precipitated from neutral or slightly acidic solution by this reagent to give a creamy color except purine bases, ephedrine, colchicine, ricinine.

Preparation: 1.36 g mercuric chloride and 5 g potassium iodide are separately dissolved in 60 ml and 10 ml distilled water respectively. Then, both solutions were mixed, and with distilled water volume was made up to 100 ml.

Reaction: Mayer's solution + Alkaloid containing extract solution.

Observation: Formation of amorphous white or cream yellow precipitation.

Wagner's reaction (Iodine potassium iodide)

Principle: Alkaloids are precipitated by this reagent to give a reddish brown color.

Preparation: 1.30 g iodine and 2.0 g potassium iodide are dissolved in 100 ml of distilled water.

Reaction: Wagner's solution + Alkaloid containing extract solution.

Observation: Formation of brown or reddish brown precipitation.

Hager's reaction (Saturated solution of picric acid)

Principle: Alkaloids are precipitated by this reagent to give a yellow color.

Preparation: 1.0 g picric acid is dissolved in 100 ml of water.

Reaction: Hager's solution + Alkaloid containing extract solution.

Observation: Formation of yellow precipitation.

Marme's reaction (Cadmium potassium iodide)

Principle: Alkaloids are precipitated by this reagent to give a white or yellow color which further dissolved by added dilute acid.

Preparation: 2.0 g cadmium iodide is mixed to a boiling solution of 4.0 g of potassium iodide in 12 ml of water and then mixed with 12 ml of saturated potassium iodide solution.

Reaction: Marme's solution + Alkaloid containing extract solution + dilute H_2SO_4.

Observation: Formation of white or yellow precipitation which dissolved in dilute H_2SO_4.

Kraut's reaction (Modified Dragendorff's reagent)

Principle: Alkaloids are precipitated by this reagent to give an orange red color.

Preparation: 8.0 g of bismuth nitrate is dissolve in 20 ml of nitric acid then 27.2 g of potassium iodide is added to that solution and mixed well. Finally, volume made up to 100 ml with distilled water.

Reaction: Kraut's solution + Alkaloid containing extract solution.

Observation: Formation of orange red precipitation

Scheibler's reaction (Phosphotungstic acid reagent)

Principle: Alkaloids are precipitated by this reagent which was observed by the appearance of yellowish or brownish yellow color.

Preparation: 20.0 g sodium tungstate and 70.0 g disodium phosphate are dissolved in 100 ml of distilled water containing little nitric acid.

Reaction: Scheibler's solution + Alkaloid containing extract solution.

Observation: Formation of yellowish or brownish yellow precipitation

Tannic acid reaction

Principle: Alkaloid solution reacted with 5% tannic acid solution and form buff color precipitation.

Procedure: 5.0 g tannic acid is dissolve in 100 ml distilled water.

Reaction: Tannic acid solution + Alkaloid containing extract solution $+NH_3$

Observation: Formation of buff precipitation which is soluble in dilute ammonium

Reineckate salt reaction

Principle: Alkaloid solution reacted with ammonium reineckate solution and form red colored precipitate.

Procedure: 1.0 g of ammonium reineckate and 0.3 g of hydroxylamine hydrochloride mixed together in 100 ml of ethanol.

Reaction: Reineckate solution + Alkaloid containing extract solution

Observation: Formation of red colored precipitate

Sonnenschein's reaction (Phosphomolybdic acid)

Principle: Alkaloid solution reacted with Sonnenschein solution and form yellowish or brownish-yellow solid precipitate.

Procedure: 1.0 g of phosphomolybdic acid in 100 ml of ethanol.

Reaction: Sonnenschein solution + Alkaloid containing extract solution

Observation: Formation of yellowish or brownish-yellow solid precipitate.

3.1.1.2 Color reactions

These color reactions are specific for individual alkaloidal plant containing secondary metabolites. Some important phytoconstituents those identified by using color reactions are discussed in this section:

Chemical test for quinine and quinidine

i. Thalleoquine test: Aqueous solution of alkaloid + Bromine water + Ammonium hydroxide ⟶ Emerald green color.

ii. Rosequin test: Aqueous solution of alkaloid + dilute HCl + Bromine water + chloroform + Potassium ferrocyanide + Ammonium hydroxide ⟶ Chloroform layer become red color.

Chemical test for ergot alkaloids

i. Keller test: Aqueous solution of alkaloid + Glacial acetic acid + Ferric chloride + slowly added concentrated sulphuric acid ⟶ Blue color form in the junction.

ii. Van urk test: Aqueous solution of alkaloid + Van Urk reagent (0.12 g para dimethyl amino benzaldehyde + 0.1 ml Ferric chloride + 15% sulphuric acid to make up volume 100 ml) ⟶ Deep blue color.

Chemical test for ipecac alkaloids

i. Alkaloid solution in dilute HCl + Ca-hypochlorite ⟶ Orange color.

ii. Alkaloidal solution + Sulphuric acid + Sodium molybdate ⟶ Bright green color (Presence of Emetine) pale green color (Presence of Psychotrine).

iii. Alkaloidal solution + Conc. Sulphuric acid + Nitric acid ⟶ Cherry red color (Presence of Psychotrine).

iv. Liebermann test: Alkaloidal solution + Potassium nitrite + Conc. Sulphuric acid ⟶ Black color (Emetine).

v. Alkaloid sample solution + dilute HCl + Potassium chlorate ⟶ Yellow changes to red (Presence of Emetine).

Chemical test for opium alkaloids

i. Plant seed sample ⟶ Soak in water ⟶ Filtrate + Ferric chloride solution ⟶ Deep red purple color (Presence of Opium).

ii. Seed sample + Nitric acid ⟶ Orange red color (Morphine present).

iii. Sample solution + Potassium ferricyanide + Ferric chloride ⟶ Bluish green color (Morphine present).

iv. Sample solution + Potassium ferricyanide + HCl ⟶ Lemon yellow color (Papaverine present).

Chemical test for ephedrine

i. Sample solution + dil HCl + Sodium hydroxide + Copper sulphate ⟶ Violet color + Solvent ether ⟶ Organic layer purple color ⟶ Aqueous layer blue color.

Chemical test for tropane alkaloids

i. Vitali morin test: Sample solution + Fuming nitric acid ⟶ Evaporate to dryness + Methanolic potassium hydroxide + Acetone solution of nitrated residue ⟶ Violet color form.

Chemical test for caffeine

i. Murexide test: Sample + Conc. HCl + Potassium chlorate evaporate to dryness + Expose to ammonia vapour ⟶ Purple red color form.

Chemical test for colchicine

i. Sample + 70% sulphuric acid ⟶ Yellow color.

ii. Sample + Alcohol solution + Ferric chloride ⟶ Red color form.

Chemical test for strychnine and brucine

i. Sample + Sulphuric acid + Potassium dichromate ⟶ Violet color (Strychnine).

ii. **Micro chemical tests**

(a) Transverse section of sample + Sulphuric acid + Ammonium vanadate ⟶ Endosperm become purple color (Presence of Strychnine).

(b) TS of sample + Conc Nitric acid ⟶ Endosperm become yellow (Brucine).

Chemical test for steroidal moiety

i. **Salkowski reaction:** Extract solution + Chloroform + conc. Sulphuric acid + shake well ⟶ Chloroform layer shows red color and acid layer greenish yellow.

ii. **Libermann-Burchard reaction:** Extract solution + Chloroform + conc. Sulphuric acid + Acetic anhydride ⟶ Red to blue finally green color.

iii. **Libermann's reaction**: Extract solution + Acetic anhydride + Heat followed by cool + conc. Sulphuric acid ⟶ Blue color form.

Chemical test for reserpine

i. Sample solution + Vanillin – Sulphuric acid ⟶ Violet red color + conc. Nitric acid ⟶ Red color form.

3.1.2 General test for anthraquinone

i. Borntrager's test: Sample powder + Benzene soaked for 10 minutes and then filtered + 10% ammonia solution ⟶ shaken vigorously ⟶ Pink, violet, or red color form in ammonia layer.

3.1.3 General test for glycoside

i. Keller-kiliani test: Aqueous extract sample + A solution of glacial acetic acid + 2.0% $FeCl_3$ + conc. H_2SO_4 ⟶ A brown ring formed between the layers (Cardiac steroidal glycosides).

ii. Kedde reaction: Aqueous extract sample + Chloroform + 1 drop of 90 % alcohol and 2 drops of 2 % 3, 5 dinitrobenzoic acid in 90 % alcohol + 20 % sodium hydroxide ⟶ Purple color form.

3.1.4 General tests for flavonoids

i. Shinoda test: Aqueous crude extract + Pieces of magnesium ribbon + conc. HCl ⟶ Pink color form.

ii. Alkaline reagent test: Aqueous crude extract + 2.0% NaOH ⟶ Concentrated yellow color was produced + Diluted acid ⟶ colorless.

iii. Lead acetate test: Extracts + few drops of lead acetate solution ⟶ yellow color form.

iv. H_2SO_4 test: Extracts + few drops of H_2SO_4 ⟶ Orange color form.

3.1.5 General test for terpenoids

i. **Salkowski's test:** Extract sample + Chloroform + conc. H_2SO_4 ⟶ Reddish brown color in the inner face.

3.1.6 General test for phenols

i. Ferric chloride test: Extract sample + few drops of ferric chloride solution ⟶ Bluish black color form.

ii. Lead acetate test: Extract sample + few drops of lead acetate ⟶ Yellow color ppt form.

3.1.7 General test for saponins

i. Foam test: Extract sample + Water ⟶ shaken ⟶ Formation of frothing.

3.1.8 General test for tannins

i. Extract + water ⟶ heated on a water bath ⟶ filtered + ferric chloride was added to the filtrate ⟶ A dark green color form.

ii. **Modified prussian blue test:** Extract solution + Potassium ferricyanide + Ferric chloride + HCl ⟶ blue color form.

3.1.9 Detection of diterpenes

i. **Copper acetate test:** Extract solution + few drops of copper acetate ⟶ Emerald green color form.

After these chemical tests, the next step is separation of phytoconstituents which generally done by chromatographic method in particular by thin layer chromatography.

3.2 Thin layer chromatography (TLC)

Chromatography is the separation technique where two or more compounds or ions are distributed between two phases, one which is moving and the other which is stationary. These two phases are mainly solid-liquid, liquid-liquid or gas-liquid. Among these, TLC is a chromatographic separation technique where solid-liquid phase is used. In this method, the components of a mixture are separated using a thin stationary phase supported by an inert backing (polar absorbent). It acts on adsorption principle where a compound has different affinities for the mobile and stationary phases, and this affects the speed at which it migrates and finally separated in various spots. Finally, the distance travelled by the spot is determined by retention factor (R_f) (Hassan *et al*., 2015). This method is efficient, easy, reliable and reproducible (Moffat, 2001).

3.2.1 Retention factor

After separation is completed, individual compounds appear as spots separated vertically and calculated as per the distance migrated over the total distance covered by the solvent. It is calculated as:

$$R_f = \frac{\text{Distance travelled by the spot}}{\text{Distance travelled by the solvent}}$$

R_f is the fraction and, hence it has no unit. R_f value is always <1.

3.2.2 Adsorbent used in TLC

Silica gel is the most important adsorbent in TLC. Silica gel has various grades in which silica gel G is standard grade silica gel 60 where "G" stands for gypsum which act as a binder for silica gel in the plate. Silica gel N is also is standard grade silica gel 60 but without binder, silica gel G-HR which is high purity grade silica gel with gypsum binder, silica gel N-HR which is high purity grade silica gel without binder, silica P which is preparative grade silica gel with organic binder and silica gel PG which is preparative grade silica gel with gypsum binder. Apart from that, aluminium oxide (grade G and N), cellulose, kieselguhr, *etc*., are used as adsorbent for TLC. But, based on conditions, adsorbents are used like:

- For lipophilic substances: aluminium oxide, silica, acetylated cellulose, polyamide,
- For hydrophilic substances: cellulose, cellulose ion exchangers, kieselguhr, polyamide and the modified reversed phase silica.

3.2.3 Determination of solvent system

It is one of the most important parameters based on that clear separation of phytoconstituents. Not only that, it is also used for purification and isolation of compounds and based on polarities of the compounds to be isolated; the solvent systems are selected. In non-polar solvents like pentane and hexane, most polar compounds will not travel but non-polar compounds will do and the same way, polar solvents will usually move non-polar compounds to the solvent front and push the polar compounds off of the baseline. Some standard solvents and their polarity are listed below:

- **Very polar solvents:** Methanol > Ethanol > Isopropanol.
- **Moderately polar solvents:** Acetonitrile> Ethyl Acetate> Chloroform> Dichloromethane > Diethyl Ether > Toluene.
- **Non-polar solvents:** Cyclohexane > Petroleum Ether > Hexane > Pentane.

Some of the important solvent systems for separation of phytoconstituents are described below (Wagner and Bladt, 2001):

1. **General alkaloids separation**

   Solvent system: Toluene: Ethyl acetate: Diethyl amine (70:20:10)

   Spraying reagents: i. Spray with a solution of 37% formaldehyde in conc. sulfuric acid (1:10), ii. Dragendorff's solution, iii. Spray with a freshly prepared mixture of 3 ml hexachloroplatinic and iv. acid solution (10%) in 97 ml/min water and 100 ml aqueous potassium iodide solution.

2. **Individual or group of alkaloids separations**

   Solvent system: Ethylacetate: Methanol: Water (100: 13.5: 10)

   i. **Identification and separation:** Xanthine derivatives, Colchicum, Rauwolfia, *etc*.

      Solvent system: Toluene: Chloroform: Ethanol (28.5: 57: 14.5).

   ii. **Identification and separation:** Ephedrine, ergotamine.

      Spraying reagent: Ninhydrine or Van Urk reagent.

      Solvent system: n-butanol: Glacial acetic acid: Water (40: 40:10) and Ethylacetate: Methanol (90:10).

   iii. **Identification and separation:** Vinca alkaloids.

      Spraying reagent: Dragendorff's solution.

      Solvent system: Butanol: glacial acetic acid: Water (14: 3:4) and Ethyl acetate: Butanol: Formic acid: Water (50: 30: 12: 10).

   iv. **Identification and separation:** Berberine.

      Spraying reagent: Dragendorff's solution.

      Solvent system: n-butanol: Glacial acetic acid: Water (40:10:10).

   v. **Identification and separation:** Reserpine, papaverine.

      Spraying reagent: Dragendorff's reagent.

      Solvent system: Toluene: Ethyl acetate: Diethyl amine (70:20:10).

   vi. **Identification and separation:** Strychnine, Brucine, Emetine, Morphine, Codeine, Aconite, Solanaceous drugs.

      Spraying reagent: Dragendorff's reagent.

      Solvent system: Chloroform: Diethyl amine (90:10).

   vii. **Identification and separation:** Quinine.

      Spraying reagent: 10% sulphuric acid followed by Iodoplatinate reagent.

      Solvent system: Ethyl acetate: Methanol: Water (100:13.5: 10).

   viii. **Identification and separation:** Caffeine.

      Spraying reagent: Iodine-Potassium iodide-HCl reagent.

3. **General glycosides separation**

   Solvent system: Ethyl acetate: Methanol: Water (100: 13.5: 10) n-propanol: Ethyl acetate: Water: Glacial acetic acid (40:40:29:1)

   i. **Identification and separation:** Aloes, Cascara.

      Spraying reagents: Ethanolic KOH.

      Solvent system: n-propanol: Ethyl acetate: Water: Glacial Acetic acid (40:40:29:1).

   ii. **Identification and separation:** Senna

      Spraying reagents: HNO_3- KOH

4. **General better glycosides separation**

   Solvent system: Ethyl acetate: Methanol: Water (77:15:8).

   i. **Identification and separation:** Bitter glycoside.

      Spraying reagents: Vanillin-H_2SO_4 reagent.

5. **General cardiac glycoside separation**

   Solvent system: Ethyl acetate: Methanol: Water (100: 13.5: 10).

   i. **Identification and separation:** Digitalis, Strophanthus.

      Spraying reagents: Kedde reagent.

6. **General flavonoids separation**

   Solvent system: Ethyl acetate: Formic acid: Glacial acetic acid: Water (100: 11:11: 26)

   i. **Identification and separation:** Rutin, Quercetrin.

      Spraying reagents: Polyethylene glycol.

7. **General terpenoids separation**

   Solvent system: Toluene: Ethyl acetate (93:7).

   i. **Identification and separation:** Essential oils, terpenes, coumarins.

      Spraying reagents: Vanillin-Sulphuric acid reagent, some of the general spraying reagents that are commonly used for detection of plant secondary metabolites are given in Table 1.

Table 1: General common spraying reagents for TLC

Spraying reagent	Composition	Detected constituents
Aluminium chloride	1% ethanolic solution of aluminum chloride	Flavonoids
Ethanolamine diphenylborate	1% solution of ethanolamine diphenylborate in methanol	Flavonoids
Antimony trichloride	10% solution of antimony trichloride in chloroform	Flavonoids, steroids, and terpenes
4-Aminoantipyrine/ potassium hexacyanferrate (III)	Solution I: 1 g aminoantipyrine (4-aminophenazone) in 100 ml 80% ethanol solution II: 4 g potassium hexacyanoferrate (III) in 20 ml water, fill to 100 ml with ethanol.	Phenols
p-Anisaldehyde–sulfuric acid	Freshly prepared 0.5 ml p-anisaldehyde in 50 ml glacial acetic acid and 1ml 97% sulfuric acid.	Phenols, steroids, and terpenes
Chloranil reagent	1% tetrachloro-p-benzoquinone in toluene	Phenols
Gibb's reagent	Solution of 3% 2,6-dibromo-N-chloro-p-benzoquinone imine in toluene or methanol	Phenols
Dichlorodicyanobenzoquinone reagent	2% 2,3-dichloro-5,6-dicyano-1,4-benzoquinone in toluene	Phenols
p-Dimethylaminobenzaldehyde / hydrochloric acid reagent (Ehrlich's reagent)	1% p-dimethylamino benzaldehyde in conc. hydrochloric acid/methanol (2:2)	Amines, Indole derivatives
Diphenyl amine	10 ml 10% diphenylamine in ethanol, 100 ml HCl and 80 ml glacial acetic acid	Glycoside
Dragendorff's reagent	Solution (1) 1.7 g basic bismuth nitrate and 20 g tartaric acid in 80 ml water solution, (2) 16 g potassium iodide in 40 ml water	Alkaloids
Formaldehyde / phosphoric acid	0.03 g formaldehyde in 100 ml of 85% phosphoric acid	Steroid alkaloids, Steroid sapogenins
Iodoplatinate reagent	3 ml hexachloroplatinic (IV) acid solution (10%) in 97 ml/min water and 100 ml aqueous potassium iodide solution	Alkaloids
Lead tetraacetate / 2,7-dichlorofluorescein	Solution I: 2% (w/v) lead tetraacetate in glacial acetic acid Solution II: 1% (w/v) 2,7-dichlorofluorescein in ethanol	Glycosides, Phenols
Orcinol (Bials reagent)	0.1 g orcinol in 40.7 ml conc. HCl, add 1ml 1% ferric (111) chloride, and dilute to 10 ml	Glycosides
Phosphoric acid–bromine	Spray solution I: 10% aqueous phosphoric acid solution Spray solution II: 2 ml saturated aqueous potassium bromide, 2 ml saturated solution aqueous potassium bromate and 2 ml 25% hydrochloric acid.	Digitalis

4. Role of secondary metabolites in diseases

Medicinal plants are a vital economic resource of naturally active compounds, used extensively from ancient time through worldwide for many ailments and from then people have identified and used safe positive components of traditional medicine in their health systems (Shahriar *et al*., 2012). The safe positive components are no other than secondary metabolites which came in our attention through vast scientific research activities. Plant secondary metabolites play a vital role in economic society. They have a wide range of chemical structures as well as therapeutic and biological activities. They are derived by unique biosynthetic pathways from primary metabolites and intermediates in the plant bodies. They are produced in plant body in low quantities and also are not essential to the plant's survival but beneficial to the humans. Hence, there is need to proper standardization methods to establish effective and safety products. In this section, preliminary screening was carried out through various chemical tests for identification of the group of phytoconstituents, followed by general TLC for identification as well as separation of constituents.

Qualitative determination of plant secondary metabolites through chemical tests is well known and easy procedure to establish the primary knowledge about presence of important phytoconstituents. Some of the important secondary metabolites are identified as alkaloids, glycosides, flavonoids, steroids, terpenoids, saponins, tannins and phenols. All these constituents have their own therapeutic efficacy. Literature survey revealed that the alkaloids have antitumors, antituberculosis, antimicrobial, antidiarrheal activities and also used to treat nocturnal leg cramps caused by vascular spasms, calming effects on psychotic or hypertensive patients, treat psychiatric and palpitation (Onike, 2010). Glycosides are small organic molecules which are used to suppress and soothe irritant dry coughs. They are effective sedative and relaxant agent on the heart and muscles in low doses. They are also acts as laxative, purgative and diuretic agent (Sharma *et al*., 2011). The flavonoids are a large group of naturally occurring phenolic compounds which are present in wide range of natural plant species with multiple therapeutic properties. Mainly, they possess antioxidant activity, anticancer, reduce risk of coronary heart diseases, acts as phytoestrogens (Samanta *et al*., 2011). Steroids are organic compounds which are used as antistress, reduce cholesterol levels, activate immune system, enhance memory and learning and to treat tumor cells (Panda and Kar, 1997). Terpenoids are small molecular products synthesized by plants, show significant pharmacological activities, such as antiviral, antibacterial, antimalarial, anti-inflammatory, inhibition of cholesterol synthesis and anticancer activities (Mahato and Sen, 1997). Saponins are heterogeneous group of natural products, found in many plant-derived foods and medicinal plants. There are two types of saponins: triterpenoids and steroidal saponins. They show biological and pharmacological activities such as anti-inflammatory, anti-hepatotonic, wound healing, veinotonic, expectorant, spasmolytic, hypoglycemic, antimicrobial and antiviral (Onike, 2010). Tannins are members of polyphenol chemical family, are produced analgesic and anti-inflammatory activities. Apart from this, tannins promote the healing of wounds and inflamed mucous membrane with the property of astringency (Okwu and Josiah, 2006). Phenolic compounds are plant secondary metabolites and widespread groups of substances in plants (Onike, 2010). Phenols are antiseptic and reduce inflammation. Polyphenols act as antioxidants, deactivate the substances that promote the growth of tumors (Sharma *et al*., 2011).

These activities indicate the necessity of the phytoconstituents and, hence individual plant constituents are needed to separate and isolate for discovery of new drug molecules. This is possible primarily through chromatographic fingerprinting analysis where each and every individual component is identified, separated and isolated through stationary and mobile phase. This technique plays an important role in the quality control of herbal medicines (Gong *et al*., 2005) because the current scenario appears to demand for plant drugs only based on their safety and efficacy (Ahmed and Rao, 2013). TLC is the preliminary step to identify the phytochemical constituents in a sample. It has some special advantages like this method is versatile, easy to perform, more speed, sensitivity is good and any number of samples and solvents are applied for analysis. Hence, it is well known method for preliminary identification and separation of phytoconstituents.

5. Conclusion

Medicinal and aromatic plants are rich in secondary metabolites, and widely used in herbal medicine to combat and cure various health hazards. Many bioactive chemical constituents including, alkaloids, glycosides, steroids, terpenoids, saponins, tannins, poly phenols are the important plant secondary metabolites which showed remarkable health related benefits such as anti-inflammatory, antispasmodic, antianalgesic, antioxidant, antimicrobial, anti-tuberculosis, anticancer, and diuretic effects. Further, screening of phytoconstituents, TLC and HPTLC fingerprinting of plant constituents are the primary steps for development of new drugs, biomarkers and for commercialization.

Abbreviations

TLC = Thin layer chromatography; HPTLC = High performance thin layer chromatography; HPLC = High performance liquid chromatography; R_f = Retention factor; HCN = Hydrocyanic acid

Conflict of interest

We declare that we have no conflict of interest.

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