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Chemical extraction and biomedical importance of secondary organic metabolites from plants – A review

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ABSTRACT

Medicinal plants are the important source of potentially useful chemotherapeutic agents which have made enormous contributions to human health and well-being. The bacteria, fungi, virus, etc have developed resistance against available drugs. The secondary metabolites of the variety of plants can serve as major source for the development of new drugs for the treatment of different diseases and infections. Depending upon the bioactive constituents present in the plant sample, these metabolites are extracted by different methods including most recent soxhlet, microwave, ultrasound, supercritical fluid, etc extractions. The different groups of secondary metabolites with their structural variations, chemical extraction methods and their potent biomedical applications have been discussed in this review.

Keywords: Phytochemistry, Medicinal Plants, Secondary metabolites, Alkaloids, Flavonoids

INTRODUCTION

Over the centuries humans and animals have depended on plants for basic needs of life such as food, clothing, and shelter, all produced directly or manufactured from resources of plant matrices such as leaves, woods, fibers and storage parts (fruits, tubers). Bioactive compounds in plants have also been utilized for additional purposes, namely as arrow and dart poisons for hunting (several Aconitum species), poisons for murder, hallucinogens used for ritualistic purposes, stimulants for endurance, and hunger suppression, as well as inebriants and medicines. The plant chemicals (bioactive compounds) used for pharmacological or toxicological effects are largely the secondary metabolites, which are derived biosynthetically from plant primary metabolites (e.g., carbohydrates, amino acids, and lipids) and are not directly involved in the growth, development, or reproduction of plants i.e. they are not nutrients of plant. These secondary metabolites called bioactive compounds can be classified into several groups according to their chemical classes, such alkaloids, terpenoids, cardiac glycosides, saponins, steroids, limonoid, tannins, flavonoids, and phenolics.[1] These products are not needed for the daily functioning of the plant. Alkaloid may be distributed as 20%, flavonoids as 15%, triterpenes and simple phenolics around 10%, and remaining others below that, with limonoid being the least. The traditional and pharmacological studies suggested that the medicinal plant contains substances that can be used for therapeutic purposes or which are precursors for chemo-pharmaceutical semi-synthesis. The different parts of such plants including leaves, roots, rhizomes, stems, barks, flowers, fruits, grains or seeds, are employed in the control or treatment of a disease and therefore contains chemical components that are medically active.

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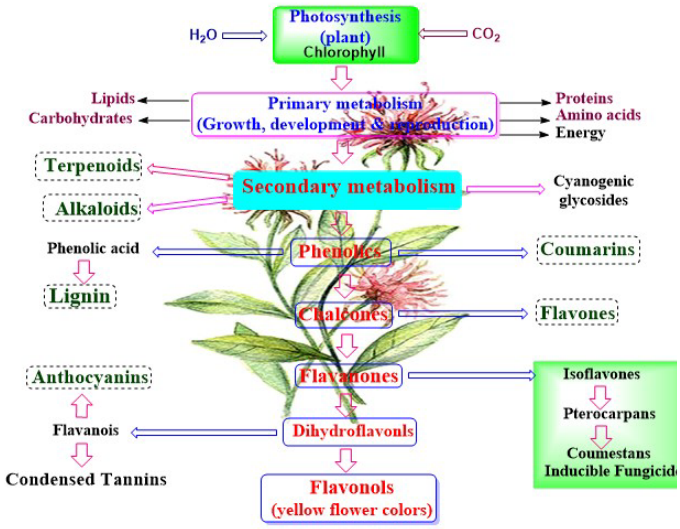

Figure 1: General Biosynthesis route of Primary and Secondary metabolites

Phytogenetically, the secondary bioactive compounds in plants appear to be randomly synthesized (as shown in Figure 1)– but they are not useless junk. Several of them are found to hold important functions in the living plants. e.g. Flavonoids can protect against free radicals generated during photosynthesis. Terpenoids may attract pollinators or seed dispersers, or inhibit competing plants. Alkaloids usually ward off herbivore animals or insect attacks (phytoalexins). Other secondary metabolites function as cellular signaling molecules or have other functions in the plants. Almost all typical plants including the common food and feed plants are capable of producing such compounds. However, the typical poisonous or medicinal plants contain higher concentrations of more potent bioactive compounds than food and feed plants.

Plants have formed the basis of sophisticated traditional medicine (TM) practices that have been used for thousands of years by people in China, India, and many other countries.[2]

Some of the earliest records of the usage of plants as drugs are found in the Artharvaveda, which is the basis for Ayurvedic medicine in India. Before the realization of pharmacologically active compounds presence in medicinal plants, it was used in crude form for the treatment of diseases (without isolation of bioactive compound). For example, goldenrod with a yellow hue was used to cure jaundice, red-colored herbs were used to treat blood diseases, liverworts were used for liver diseases, pilewort's for hemorrhoids, and toothwort's for toothache.[2] In 1805, morphine became the first pharmacologically active compound to be isolated in pure form from a plant.[2] The 19th century marked the isolation of numerous alkaloids from plants (species in parentheses) used as drugs, namely, atropine (*Atropa belladonna*), caffeine (*Coffea arabica*), cocaine (*Erythroxylum coca*), ephedrine (*Ephedra* species), morphine and codeine (*Papaver somniferum*), pilocarpine (*Pilocarpus jaborandi* Holmes), physostigmine (*Physostigma venenosum*), quinine (*Cinchona cordifolia* Mutis ex Humb.), salicin (*Salix* species), theobromine (*Theobroma cacao*), theophylline (*Camellia sinensis*), taxol (*Taxus brevifolia*), 10-deacetylbaccotin III (*Taxus baccata L.*), and (+)-tubocurarine (*Chondodendron tomentosum* Ruiz & Pav.).[2] Following these discoveries, bioactive secondary metabolites from plants were later utilized more widely as medicines, both in their original and modified forms.

BIOACTIVE COMPOUNDS USED IN MEDICINAL CHEMISTRY

In 18th century and before that, the plants served as a major source for the treatment of different diseases and infections. Now a day's some of the plant derived natural products that includes vinblastine, vincristine, taxol, podophyllotoxin, camptothecin, digitoxigenin, gitoxigenin, digoxigenin, tubocurarine, morphine, codeine, aspirin, atropine, pilocarpine, capscicine, allicin, curcumin, artemesinin and ephedrine among others are also used as drug in pure form or in crude form or mixture. Some of them are synthesized synthetically but about 121 (45 tropical and 76 subtropical) major plant drugs have been identified for which no synthetic route is currently available.

Development of new drug is a complex, time-consuming, and expensive process. The time taken for the discovery of a new drug to its reaching in the clinical applications is nearly 12 years, involving more than 1 billion US$ of investments in today's context. The new molecule can be sourced either by chemical synthesis or through the isolation from natural resources. However from the screening of thousands of the molecules, very few of them are selected for clinical and final trials. Cancer and infectious diseases are the two predominant therapeutic areas for which natural product based drugs are of interest to chemist and researcher. But along with them many other therapeutic areas, such as neuropharmacological, cardiovascular, gastrointestinal, inflammation, metabolic, etc are also covered.[3]

There are four major basic natural sources for the new chemical entities (NCE) : botanical sources, fungi, bacteria and marine sources. Out of these sources, botanical sources are of specific importance because of their variety and abundance. The botanical sources are providing following classes of NCEs for the drug discovery process –

A. Bioactive compounds are directly used as drug.
B. Bioactive compound structures themselves may act as lead compounds for the more potent compounds.
C. Bioactive compounds which are not used directly as drug but used for the synthesis of drugs by chemical or biological means.
D. Pure phytochemicals are used as pharmacological tools, markers compounds for the standardization of crude plant extracts or materials.
E. Herbal extracts as botanical drugs.

The use of botanical source as starting point in the investigation of active biomolecules is due to the specific advantages:

A. Selection of the biological source (plant) for the investigation can be done on the basis of long term use by humans (ethnomedicines). This is based on the assumption that the active compounds isolated from

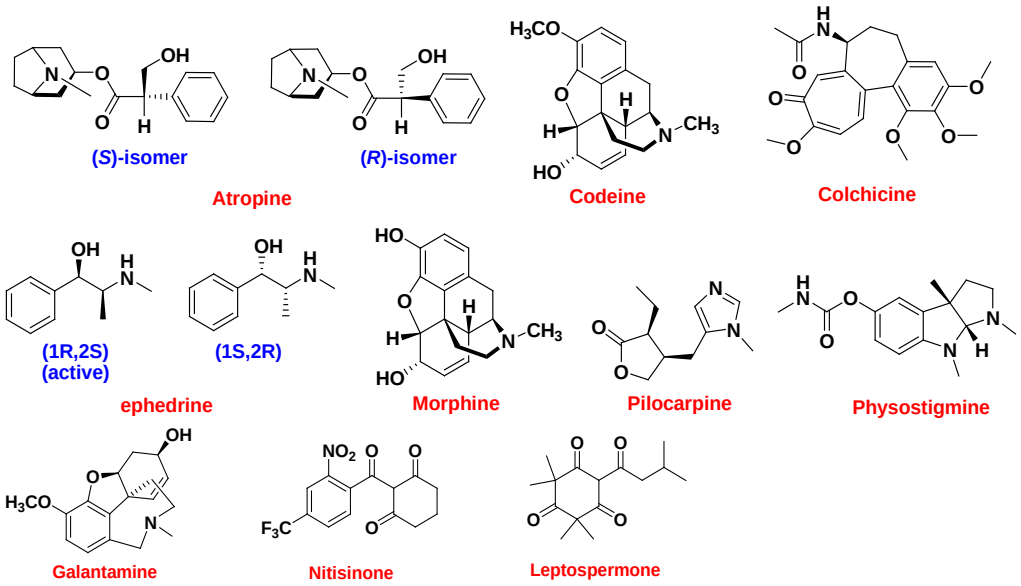

Figure 2: Some natural products used as Drugs

such source are likely to be safer than those derived from plant species with no history of human use. After some period of time, one can synthesize the active molecule and reduce the pressure on the natural resources.

B. Sometimes, such approaches lead to development of novel molecules derived from the other source due to inherent limitations of the original molecule. e.g. ***Podophyllin*** derived from *Podophyllum hexandrum* was faced with dose-limiting toxicities. Such limitations could be overcome to a great extent by semi-synthesis of ***etoposide***, which continues to be used in cancer therapy. Similar was the case with ***camptothecin*** (originally isolated from *Camptotheca sp*. and subsequently from *Mappia sp.*), which led to development of novel anticancer molecules like topotecan and irinotecan.

On the other hand, the natural resources are also associated with certain limitations:

A. It is difficult to commercialize the bioactive molecule and if it is commercialized then it put undesirable impact on the environment. It can be avoided by synthesizing the active molecule, but it is not possible for every molecule because of complex structure. The dependency on the natural resources would decrease to some extent. e.g. The total synthesis of anticancer molecules such as etoposide, paclitaxel, docetaxel, topotecan, and irinotecan depends on the starting material obtained from plant resources.

B. Over a period of time, the intellectual property rights protection related to the natural products is going haywire.

Some of the plant-derived compounds in pure form, such as atropine (anticholinergic), codeine (cough suppressant), colchicine (antigout), ephedrine (bronchodilator), morphine (analgesic), pilocarpine (parasympathomimetic), and physostigmine (cholinesterase inhibitor) are still being used widely as single-agent or combination formulations in prescription drugs,[4] as shown in following Figure 2. Nowadays, the use of plant-based traditional medicines or phytotherapeutics has increased steadily over the last 10 years.

Galantamine was used for the symptomatic treatment of patients with early-onset Alzheimer's disease.[5] Galantamine is an alkaloid that was initially isolated from the snowdrop (*Galanthus woronowii* Losinsk) and has been found in other plants in the family Amaryllidaceae.[6] Due to the limited availability of the plants of origin of this compound, galantamine is now produced by total synthesis. Nitisinone is used for the treatment of hereditary tyrosinemia type 1 (HT-1).[5]

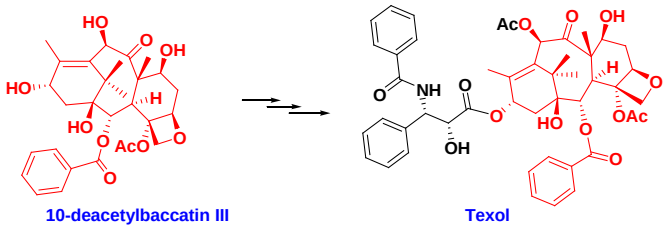

Figure 3: Synthesis of Texol from natural precursor 10-deacetylbaccatin III

HT-1 is a rare pediatric disease caused by a deficiency of fumaryl acetoacetate hydrolase (FAH), an enzyme essential in the tyrosine catabolism pathway. Nitisinone is a derivative of leptospermone, a new class of herbicide from the bottlebrush plant [*Callistemon citrinus* (Curtis) Skeels]. Both nitisinone and leptospermone inhibit 4-hydroxyphenyl pyruvate dioxygenase (HPPD), the enzyme involved in plastoquinone and tocopherol biosynthesis in plants.[7]

Some of the small molecules obtained from the plant sources are not used directly as drug but used as important precursor for the synthesis of compound of interest by converting it into other form by using some chemical modification or fragmentation methods. Such semisynthesis approach were used to resolve the shortage of supply due to low yield of the compounds obtained from plant sources and/or to reduce the high cost of total synthesis. There are several bioactive compounds having complex structures and many chiral centers, protracted methods may be used for their synthesis which may not be economically feasible. For example, a antitumor drug paclitaxel (***Texol***) is isolated from the bark of the slow growing Pacific yew tree, *Taxus brevifolia* but the yield from bark is very low (0.014%)

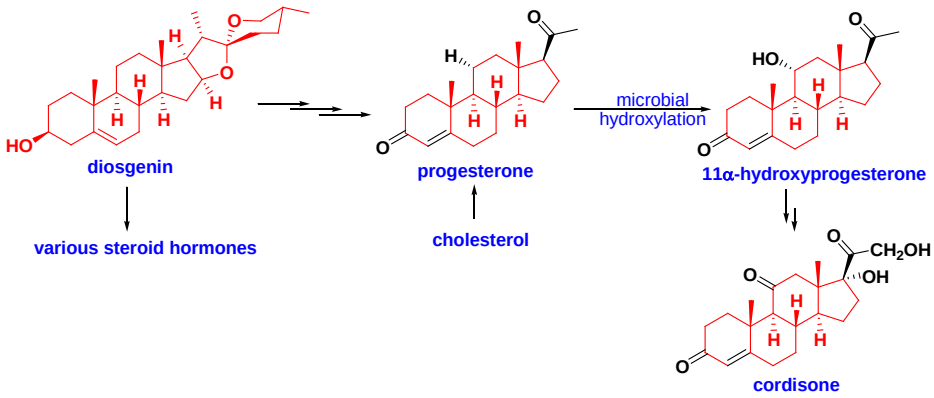

Figure 4: Synthesis of cordisone from diosgenin isolated from *Dioscorea*

which is very less than market demand.[8] This complex molecule was produced by total synthesis from the less active ***10-deacetylbaccatin III*** (Figure 3) which is isolated in large amount from the needles of other related yew species, such as Taxus Baccata L. and can be converted chemically to taxol.[9,10]

A important hormone, ***progesterone*** can be used as a female oral contraceptive,[11] which originally isolated from sow ovaries with very low yield (20 mg from 625 kg of ovaries). It was synthesized from ***cholesterol*** with very low efficiency.[12] A steroidal sapogenin, ***diosgenin*** obtained from the tubers of various *Dioscorea* species found in Maxico can be chemically converted in progesterone by several steps. Progesterone is a key intermediates for the production of – ***cordisone*** (Figure 4), an important anti-inflammatory drug through 11α-hydroxyprogesterone by microbial hydroxylation at C_{11} position.[13,14]

PLANT METABOLITES – BIOACTIVE COMPOUNDS:

The plant produces a broad variety of chemical compounds that have huge economical importance. The bioactive compounds obtained by secondary metabolism are classified according to the different criteria.

An approach based on ***biological effect*** is not used for the classification of molecules because chemically different molecules shows similar clinical and biological effect. A ***botanical categorization*** based on families and genera of the plant producing the bioactive compounds might also be relevant, as closely related plant species most often produce the same or chemically similar bioactive compounds. But there are various examples where genetically less related species produces similar secondary metabolites. Therefore, all these bioactive chemical compounds are categorized according to the ***biochemical pathways and chemical classes***.

There are four major pathways for synthesis of secondary metabolites or bioactive compounds: (a) Shikimic acid pathway, (b) malonic acid pathway, (c) Mevalonic acid pathway and (d) non-mevalonate (MEP) pathway (shown in *Figure 5*).[15] Alkaloids (basic compounds) are produced by aromatic amino acids (come from shikimic acid pathway) and by aliphatic amino acids (come from tricarboxylic acid cycle). Phenolic and acidic compounds are synthesized through shikimic acid pathway and malonic acid pathway. Mevalonic acid pathway and MEP pathway produces terpenes.

The bioactive compounds of plants are broadly divided into three main categories – terpenes & terpenoids (approximately 25000 types), alkaloids (approximately 12000 types) and phenolic compounds (approximately 8000 types). There are about more than 100000 compounds has been known that have been extracted from plants.

The main chemical groups of bioactive compounds in plants:

Alkaloids: The largest group consists of the terpenoids and the second largest group is formed by the alkaloids, comprising many drugs, stimulants, narcotics and poisons.The alkaloids are heterocyclic, nitrogen containing compounds, usually with potent activity and bitter taste. These bases are synthesized from amino acid building blocks with various radicals replacing one or more of the hydrogen atoms in the peptide ring, most containing oxygen. In fact, one or more nitrogen atoms that are present in an alkaloid, typically as 1°, 2° or 3° amines, contribute to the basicity of the alkaloid. Majority of alkaloids exist in solid such as atropine, some as liquids containing carbon, hydrogen, and nitrogen. Most alkaloids are readily soluble in alcohol and though they are sparingly soluble in water, their salts of are usually soluble. The solutions of alkaloids are intensely bitter. Alkaloids having high value but they are found in low concentration in the plant kingdom and

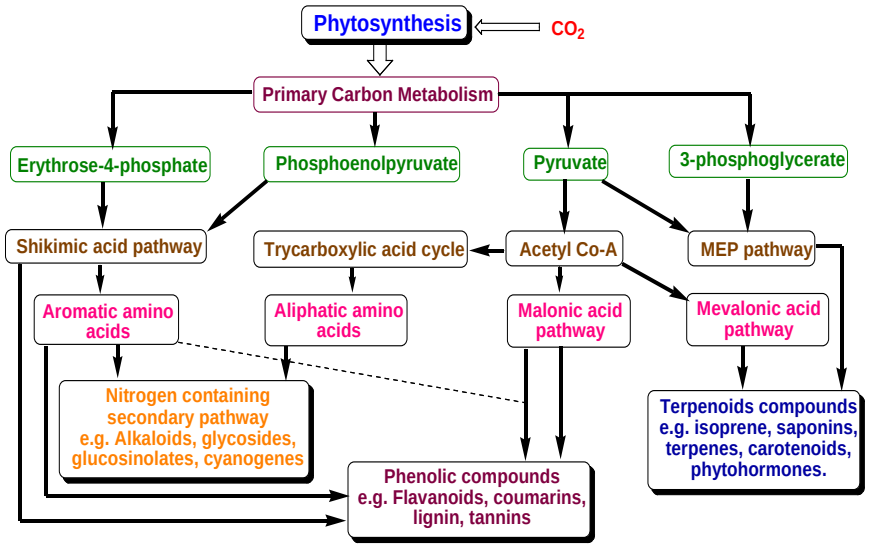

Figure 5: Major pathways for biosynthesis of secondary metabolites

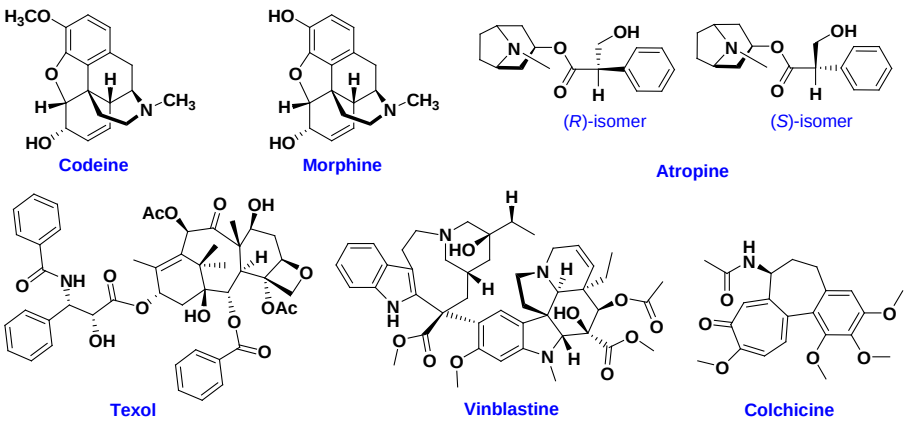

Figure 6: Biologically important alkaloids

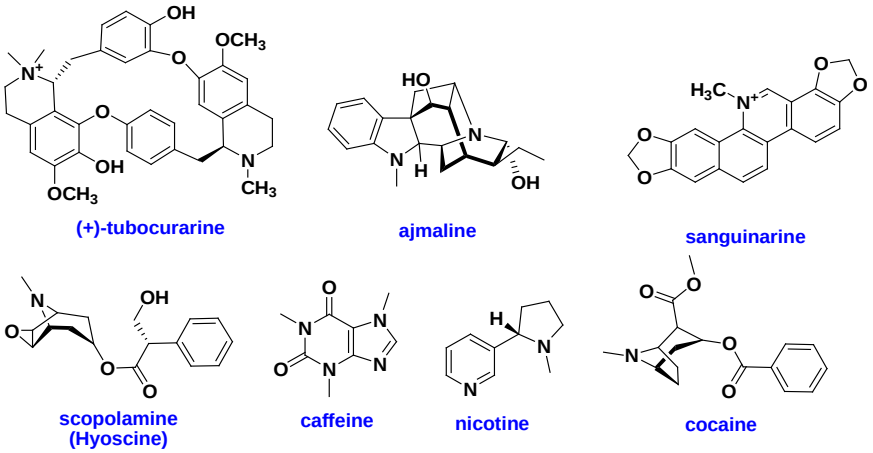

Figure 7: Examples of some biologically important alkaloids

are difficult to purify from plant extract. The various groups have diverse clinical properties. In nature, the alkaloids exist in large proportions in the seeds and roots of plants and often in combination with vegetable acids. The major function of nitrogenous compounds is in the defense of plants against herbivores and pathogens; they are widely used directly as pharmaceuticals, stimulants, narcotics, poisons, etc due to their potent biological activities. These are exist largely in the seeds and roots of the plant.

Some important plant-derived alkaloids (shown in Figure 6, 7) currently in clinical use include the analgesics ***morphine***, ***atropine*** and ***codeine***, the anticancer agents ***vinblastine*** and ***taxol***, the gout suppressant ***colchicine***, the muscle relaxant ***(+)-tubocurarine***, the antiarrythmic ***ajmaline***, the antibiotic ***berberine*** and ***sanguinarine***, and the sedative ***scopolamine***. Other important alkaloids of plant origin include ***caffeine***, ***nicotine***, ***cocaine***, and the synthetic *O,O*-acetylated morphine derivative ***heroin***.***Tropane alkaloids*** have a tropane (C_4N skeleton +) nucleus. They are present in *Solanaceae* (nightshade family) for instance *Atropa belladonna* (deadly nightshade) *Datura* spp (thorn apples) and *Hyoscyamus niger* (henbane). These alkaloids are synthesized as postcursors of pyrrolines. The compounds have anticholinergic activity (muscarine receptor antagonists) and are used medically to reduce smooth muscle spasms, hypersecretion and pain. e.g. atropine, hyoscyamine, tropinone, tropine, littorine, cuscohygrine and scopolamine, and the narcotic tropical anesthetic cocaine and ecgonine (figure 8).

Pyrrolizidine alkaloidsare found in 15 different families, although three plant families are the most important source of it. The major quantity of it is produced in *Asteraceae* (daisy family), particularly in *Senecio* spp. (Ragworts), *orchidaceae*, *fabaceae* family and in *Boraginaceae* (borage family). It was less frequently found in *convolvulaceae* and *poaceae* family. These alkaloids are containing pyrrolizidine nucleus. Their adverse effect in man and animals are hepatotoxicity after bioactivation. The major characteristic of these alkaloids are – they are accumulated in plants as N-oxides, they are poisons and some of them have a bioimpact (e.g. indicine-N-oxide). E.g. triangularine, lycopsamine, heliosupine, echinatine, heliotrine, indicine, lithosenine, scropioidine, myoscorpine, doronine, monocrotaline, etc (Figure 9).

Papaveraceae (poppy family) and *Berberidaceae* (barberry family) produce ***isoquinoline alkaloids*** which have a range of biochemical effects relevant for medical use, as inhibition of various conditions as pain, cancer cells and bacteria, and stimulation of bone marrow leucocytes as well as myocardial contractility. It is large and diverse alkaloid groups with more than 2500 defined structures. e.g. morphine as an analgesic, colchicine as a microtubule disrupter, and (+)-tubocurarine as a neuromuscular blocker suggests that these alkaloids function as herbivore deterrents, sanguinarine is a antimicrobial agent which protect against pathogens. There are some other examples such as berberine and palmatine (figure 10) were specifically shown to confer protection against herbivores and pathogens.[16]

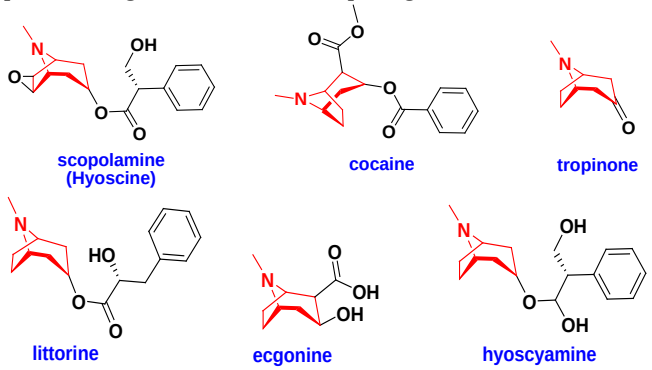

Figure 8: Biologically important Tropane Alkaloids

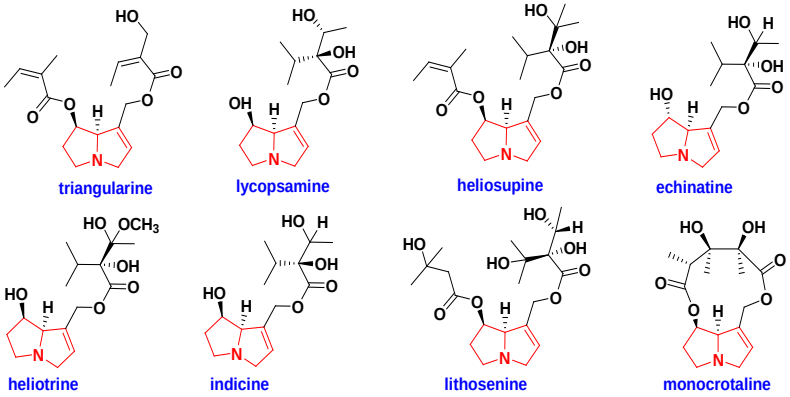

Figure 9: Examples of some biologically important pyrrolizidine Alkaloids

The main producers of ***methylxanthine alkaloids***are *Coffea arabica* (coffee), *Camellia sp*. (consumed as tea) and *Theobroma cacao* (cacao). e.g. caffeine, theobromine, theacrine, aminophylline, theophylline, dyphylline, etc (*Figure 11*). Methylxanthines (theobromine) to a various extent bind to adenosine receptor and elicit neurological effects in man and animals which may be regarded stimulating at low to moderate intake. It inhibits the growth of plant cells. In rodents high intakes of methylxanthines show reduced sperm production and testicular atrophy.

Terpenoid indole or ***secologanin tryptamine alkaloids*** consist of nearly 3000 compounds isolated from natural resources that include the antineoplastic agents vinblastine and camptothecin, the antimalarial drug quinine, the rat poison strychnine, defense of plants against pests and pathogens.[17] It consists of indole moiety provided by tryptamine and a terpenoid component derived from the iridoid glucoside secologanin. The flowering plant *Rauwolfia serpentina* which contains reserpine was a common medicine in India around 1000 BC. These covered simple indole alkaloids (tryptamine, serotonin, psilocin, and psilocybin), β-carboline alkaloids (harmine), terpenoid indole (ajmalicine, catharanthine, tabersonine), quinoline alkaloids (quinine, quinidine, cinchonidine), pyrroloindole alkaloids (serine), and ergot alkaloids (ergotamine) (*Figure 12*).

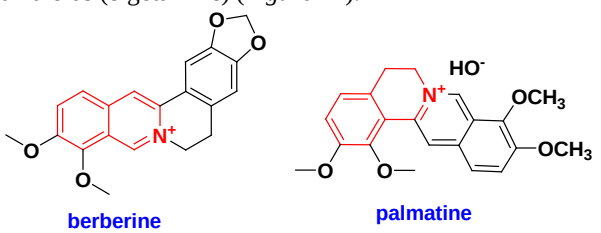

Figure 10: Examples of some biologically important isoquinoline Alkaloids

Figure 11: Examples of some biologically important methyl - xanthine Alkaloids

The plants that are major source of non-isoprenoid indole alkaloids include *harmal* (*Peganum harmala*), which contains harmane, harmine and harmaline, and Calabar bean (*Physostigma venenosum*) containing physostigmine. Some members of the family Convolvulaceae, in particular *Ipomoea violacea* and *Turbina corymbosa*, contain ergolines and lysergamides.[18] Despite the considerable structural diversity, most of monoterpenoid indole alkaloids is localized in three families of dicotyledon plants: Apocynaceae (genera *Alstonia*, *Aspidosperma*, *Rauwolfia* and *Catharanthus*), Rubiaceae (*Corynanthe*) and Loganiaceae (*Strychnos*).[18]

There are number of compounds called ***pseudoalkaloids*** (Figure 13) which have chemical properties close to alkaloids and not originated from amino acids. This group of alkaloids includes terpene-like and steroid-like alkaloids. These are produced by species in *Apiaceae* (carrot family) for instance *Cicutavirosa* (cowbane) and *Conium maculatum* (hemlock), and in *Taxaceae* (yew family) for instance *Taxus baccata* (yew). The pseudoalkaloids in *Cicuta virosa* and *Conium maculatum* have effects on the central nervous system and taxine in yews like *T. baccata* inhibits the ion transport of the hearth. e.g. ephedrine, cathinone, aconitine, delphinine, solasodine, solanidine, veralkamine, batrachotoxin.

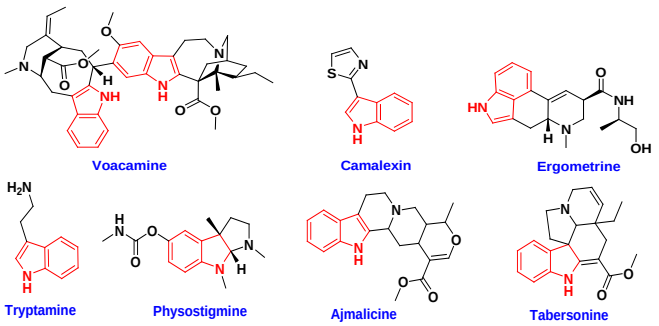

Figure 12: Biologically important Terpenoid indole Alkaloids

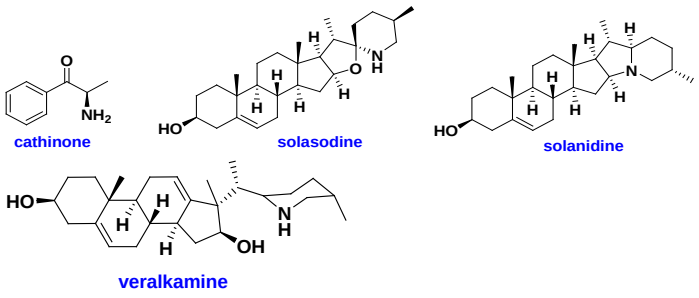

Figure 13: Biologically important pseudoalkaloids Alkaloids

Terpinoids: ***Terpenes*** is the generic term summarizing all kinds of isoprene (C_5H_8) polymers and their derivatives with its general formula of $(C_5H_8)_n$. The terpenoids serves important primary functions as photosynthetic pigments (carotenoids), electron carriers (side chains of ubiquinone and plastoquinone), regulators of growth and development (gibberellins, abscisic acid, strigolactones, brassinosteroids, cytokinins), in protein

glycosylation (dolichols), elements of membrane structure and function (phytosterols), terpenoid metabolites and other biological roles.

These are synthesized via the mavalonate and deoxyxylulose pathway with the use of a five carbon building block called isoprene (*Figure 14*).

Based on the number of the isoprene units contained inside the molecule, the terpenes can be classified into: hemiterpenes, monoterpene, sesquiterpene, diterpene, sesterterpenes, triterpenoids, tetraterpene and polyterpene. There are a large number of terpenes forming various kinds of oxygenated derivatives including alcohols, aldehydes, ketones, carboxylic acids, esters and glycoside forms; nitrogen-containing derivatives as well as a minority of sulfur-containing derivatives presented except terpene hydrocarbons. Monoterpene and sesquiterpene are the major component of volatile oil. Diterpene is the major substance forming the resin; triterpenoid is an important material forming plant saponins and resins, tetraterpene mainly include some fat-soluble pigments widely distributed in the plants.

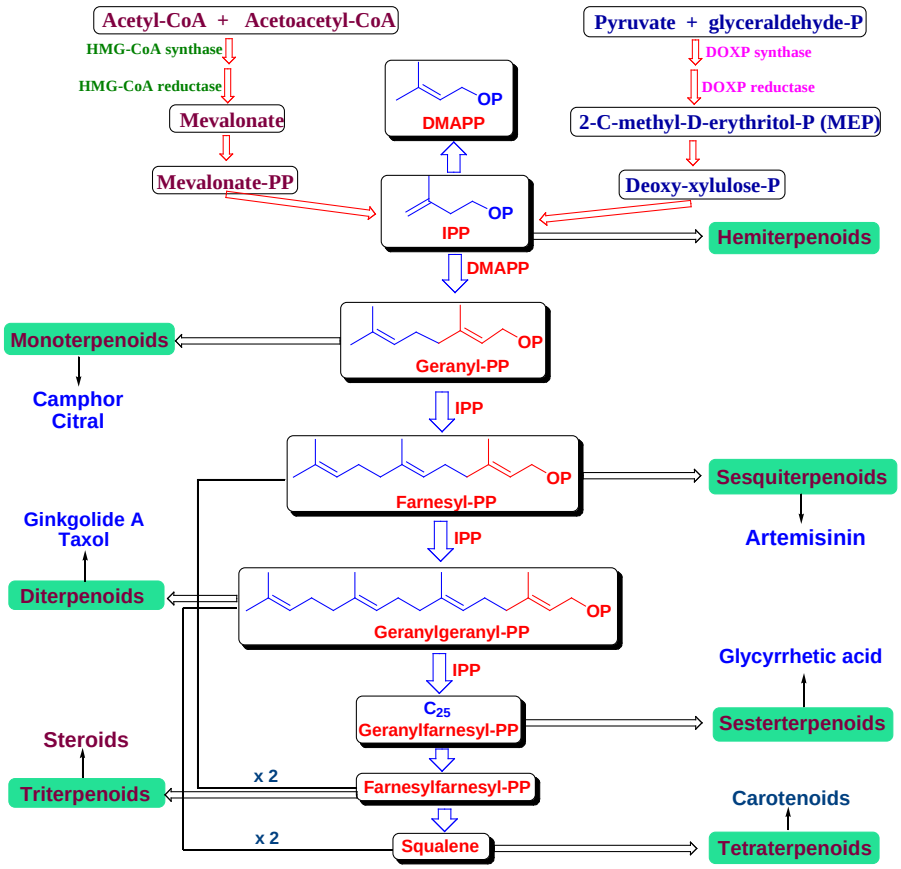

Figure 14: General biosynthesis route of terpenoids

Mono- and sequi-terpenoids, and phenylpropanoids: The terpenoids are synthesized via the five-carbon building block isoprene via two biosynthetic routes. Monoterpenoids consist of two isoprene units (monomeric unit-geranyl-PP) and sesquiterpenoids of three units (monomeric unit-farnesyl-PP). They are referred to as low-molecular-weight terpenoids and represent the most diversely category of plant constituents with more than 25,000 individual compounds identified. The less diverse phenylpropanoid (*Figure 16*) are based on a nine carbon skeleton (less number of carbon atom) and are synthesized via another pathway called as shikimic acid pathway by the way of cinnamic acid (amino acid phenyl alanine). The phenylpropanoids have most of the same properties as mono-terpenoids. Compounds of all three groups are lipophilic and tend also to volatilize readily. They have strong odours and flavours. Many are of them are optically active and different isomers can have completely different properties. Their actions vary greatly, a range of which have been utilized in herbal remedies. These biomolecules are different applications; some of them are antineoplastic, antibacterial, antiviral effects as well as gastrointestinal stimulation. However these terpenoids are not associated with toxicity unless they are concentrated as volatile oils. The plant family best known for these compounds is *Lamiaceae* (thyme family) but is also present in a range of other families.

e.g. α- and β-pinene (anti-inflammatory), caryophyliene (anxiety, depression), δ-3-carene (anti-inflammatory), limonene (antifungal, antibacterial, anti-carcinogenic), linalool (anti-inflammatory), terpineols (anti-oxidant), nerolidol (anti-fungal, anti-malarial), homulene (anti-tumor, anti-bacterial, anti-inflammatory, anorectic), geraniol (anti-oxidant, anti-tumor, neuroprotectant, anti-bacterial, anti-fungal), myrcene (antioxidant, anti-carcinogenic, good for muscle, depression), borneol (analgesic, anti-insomnia, anti-septic), camphene (antibiotic, anti-inflammatory), etc (Figure 15a,b).

Diterpenoids: Diterpenoids are composed of 4 isoprene units (20 carbons; GGPP unit). They are very lipophilic and tend to have strong flavours, but are not volatile and thus, odourless. Much less toxicological information is available on the diterpenoids than on the lower molecular terpenoids even though they are well absorbed orally, given their lipophilicity. Several of the compounds possess antineoplastic activity. Diterpenoids are found in several plants, among them *Coffea arabica* (coffee). Diterpenoids are also typically present in resins in plants. They do not tend to occurs as glycosides. They show wide range of activities such as anti-cancer activity, antineoplastic activities, bitter properties, etc. The isolation and extraction of the plant terpenoids is from certain tissues types as roots, leaves, or flowers. The *Euphorbiaceae* (spurge family), for example, contains many plant species that produce diterpenoids with casbane, lathyrane, jatrophane, tigliane, or ingenane skeletons. Some biological important terpenoids are shown in following *Figure 17*.

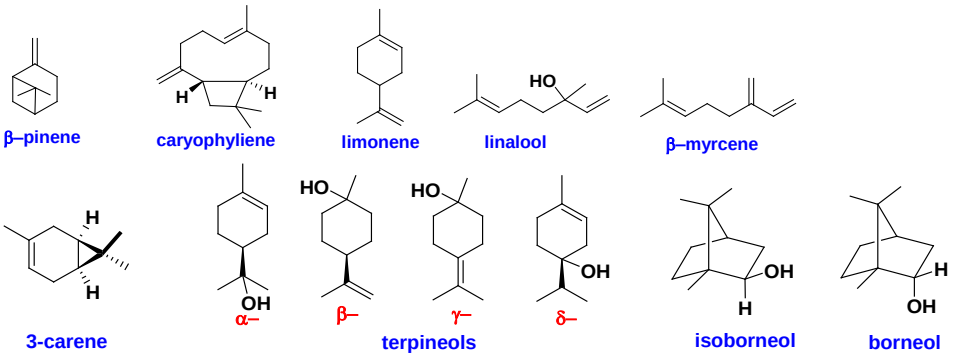

Figure 15a: Some important examples of mono and sequiterpenoids

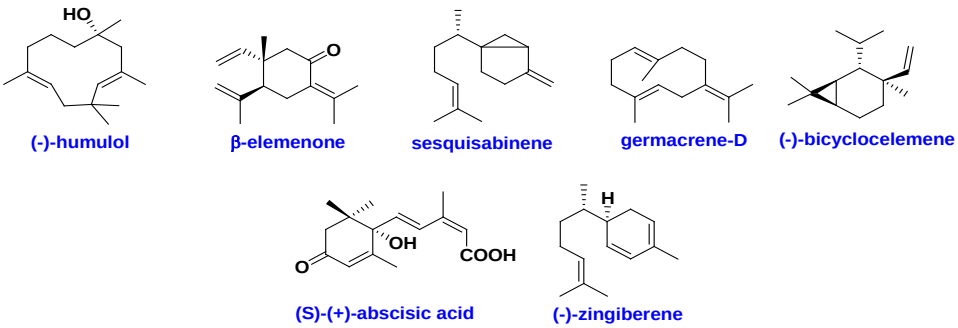

Figure 15b: Some important examples of mono and sequi-terpenoids

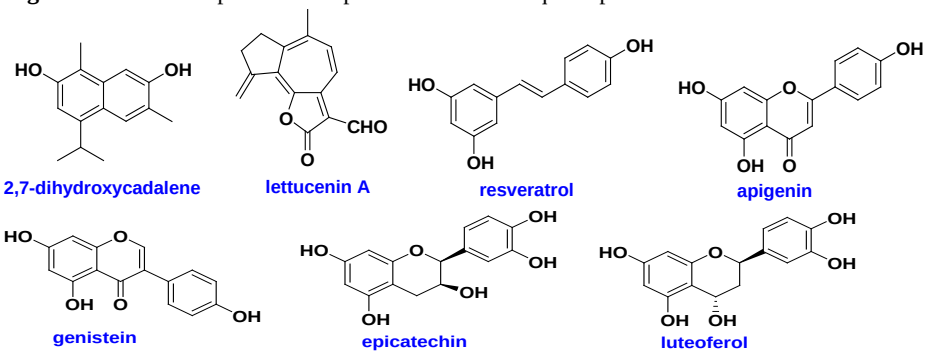

Figure 16: Examples of some *phenylpropanoids*

Ingenol mebutate (*Figure 18*) from *Euphorbia peplus* is used for the treatment of the precancerous skin condition actinic keratosis and in the treatment of superficial basal cell carcinoma,[19] **Resiniferatoxin** (*Figure 18*), a tigliane diterpenoid from *Euphorbia resinifera*, is used for the treatment of intractable pain,[20] **Prostratin** (a tigliane) (*Figure 18*), which is produced by *Homalanthus nutans*, *Euphorbia fischeriana*, and *Euphorbia cornigera*, has shown potential as an adjuvant therapy for the treatment of latent HIV infection,[21a-d] **jatrophane** esters (*Figure 18*), which are produced by a number of *Jatropha* and *Euphorbia* species shown to inhibit p-glycoprotein transporters responsible for efflux of chemotherapeutic agents and useful for the treatment of multidrug resistant cancers,[22a-b] **Jatrophone** (*Figure 18*) from *Jatropha isabelli* has antiplasmodial activity.[23]

Glycosides: The glycosides consist of various categories of secondary metabolites (phenolics, terpenoids, alkaloids, pigments, coumarin, iridoids, sulfur containing compounds, etc) which are bound to a mono- or oligosaccharide or to uronic acid i.e. these are condensation products of sugar. These compounds are on enzymatic or acid hydrolysis forming sugar and non-sugar or genin. The saccharide or uronic acid part is called ***glycone***, and the other part the ***aglycone***. Glycosides are colorless, crystalline, water soluble phytoconstituents found in the cell sap. The main groups of glycosides are cardiac glycosides, cyanogenic glycosides, glucosinolates, saponins and anthraquinone glycosides. Furthermore, flavonoids frequently occur as glycosides.[24] The glycosides are classified on the basis of type of sugar component, chemicals nature of aglycone or pharmaceutical action.

Some of their useful actions include cadiatonic, purgative, analgesic, antirheumatic, demulscent, antiulcer, etc. They are the active constituents of many plants, and their pharmacological activity is basically due to the non-sugar part or aglycone. Some of the important properties of different groups are listed in table below.

Following ingestion the glycosides usually hydrolyse in the colon, and the more hydrophobic aglycone might be absorbed.

Glycosides are classified as per linkage between glycone and aglycone part:-

O-glycosides: These are glycosides in which sugar part is linked with oxygen of alcoholic or phenolic hydroxyl of

Type	Properties	Species
Anthraquinones	Purgatives	Holy peel, Sen
Cardiac glycoside	Diuretic, Heart tonic	Digital
Cyanogenics	Anesthetics, Anti-spasmodic, Hypo-tenser	Cherry tree, Almond tree
Coumarine	Antibacterial, Anti-coagulant, Protection from the sun	Oats
Phenol	Anti-pyretic and febrifuges	Pear tree, willow
Flavonoids	Weak capillaries	Sunflower, rude
Ranunculosides	Irritation of the skin	Berry plants
Saponins	Hemolytic anemia, Emollients, Dermatitis	Fir tree, Maize, Liquorice, Saponaria, Viole
Sulphurates	Antibiotics	Garlic, Onion, Radish

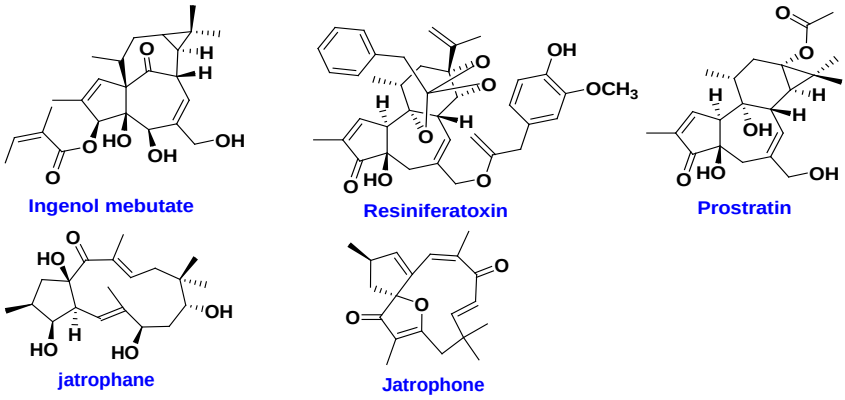

Figure 18: Biologically important diterpenoids

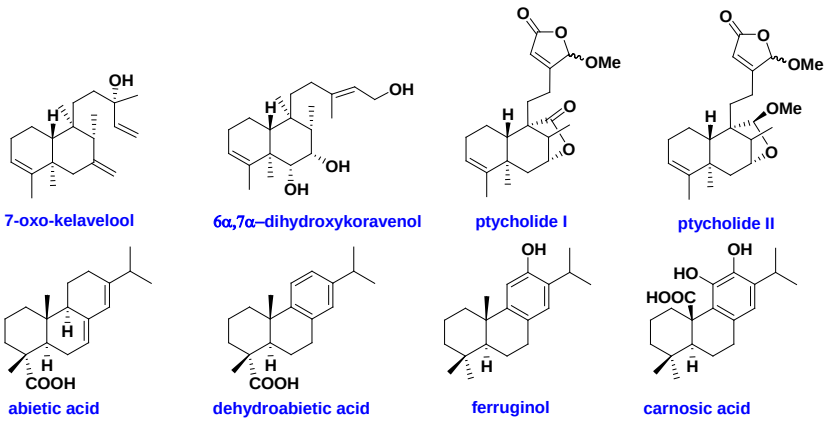

Figure 17: Examples of some biologically important diterpenoids

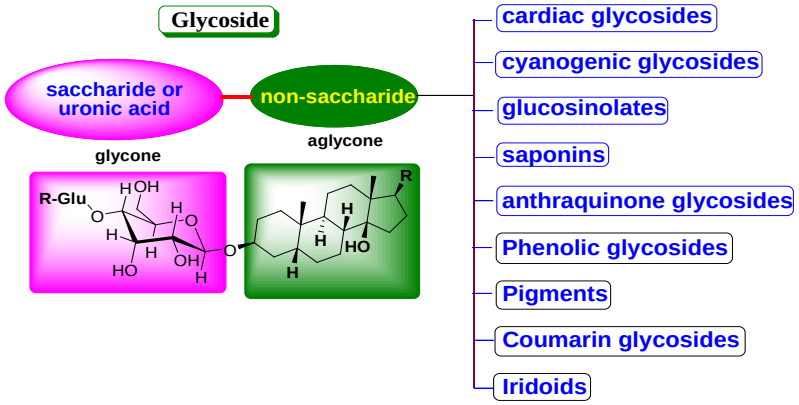

Figure 19: Main groups of glycosides

carboxylic acid group (ether or ester linkage) (Figure 20). The O-glycosides present in the plant sample is hydrolyzed by boiled with HCl/H_2O and an aqueous base to respective aglycones. The formation of pink or violet coloration in the base layer indicates the presence of glycosides in the plant sample.

S-glycosides: These are glycosides in which sugar part is attached to a sulfur atom of aglycone such as sinigrin.

N-glycosides: These are glycosides in which sugar part is linked with nitrogen atom of amino group of aglycone, (*Figure 20*).

C-glycosides: These are glycosides in which sugar part is condensed directly to carbon atom of aglycone like in aloin. These glycosides of the plant sample were hydrolyzed by using $FeCl_3$/HCl and an aqueous base such as NaOH or NH_4OH solution.

Examples of some glycosides - cardiac glycosides (acts on the heart), anthracene glycosides (purgative, and for treatment of skin diseases), chalcone glycoside (anticancer), amarogentin, gentiopicrin, andrographolide, amygdalin - treatment of cancer, ailanthone and polygalin. The plant extracts contain cyanogenic glycosides are used as flavouring agents in many pharmaceutical preparations.

The ***phenolic glycosides*** containing benzene ring with hydroxide group as aglycone *(Figure 21)*. e.g. bark of oak (Quercus sp.), leaves of tea (Thea sinensis, Theaceae), tanning sumac (Rhus coriaria, Anacardiaceae) containing gallic acid.

Another phenolic compounds present in different plants are ellagic acid, salicylic acid, etc.

Iridoids glycosides are consist of ten, nine, or rarely eight carbons in which C_{11} is more frequently missing than C_{10} as aglycone. e.g. Agnuside, amarogentin, aucubin, catapol, loganin, verbenalin, geniposidic acid, etc (*Figure 22*). Iridoid.

Glycosides have laxative and antimicrobal properties, anti-inflammatory properties and anti-inflammatory effect. Some of the iridoid glucosides showed antiviral effects against herpes simplex virus type 1 (HSV-1) and alkaline phosphatase (ALP)-enhancing activity in MC3T3-E1 osteoblast cells.[25,26]

The aglycones of ***cardiac glycosides*** (*Figure 23*) have a steroidal structure. Their effect is inhibition of Na^+/K^+-ATPase-pumps in the cell membranes. These pumps are concentrated in and critical for the functioning of the cardiac cells and the effects from these compounds are very pronounced in the heart, resulting in increased contractility and reduced vertebrate heart rate. The cardiac glycosides are present in plants of

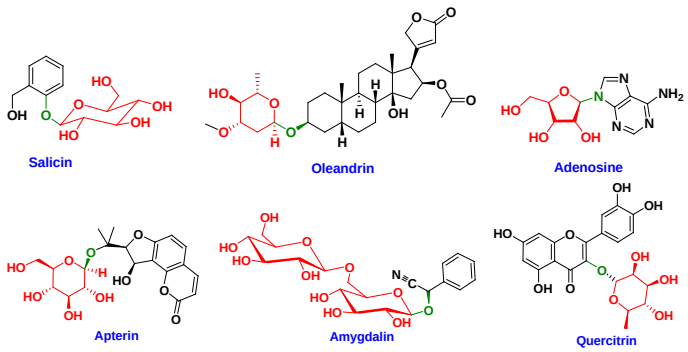

Figure 20: Examples of N- and O-glycosides

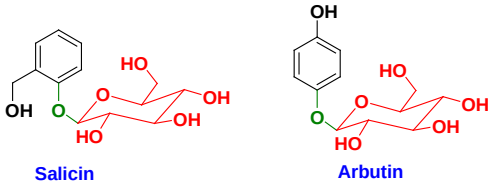

Figure 21: Examples of phenolic glycosides

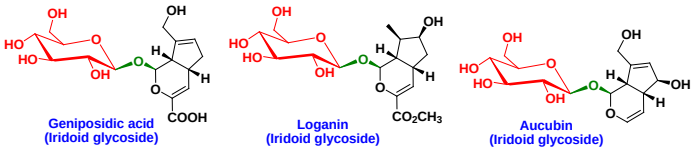

Figure 22: Examples of phenolic glycosides

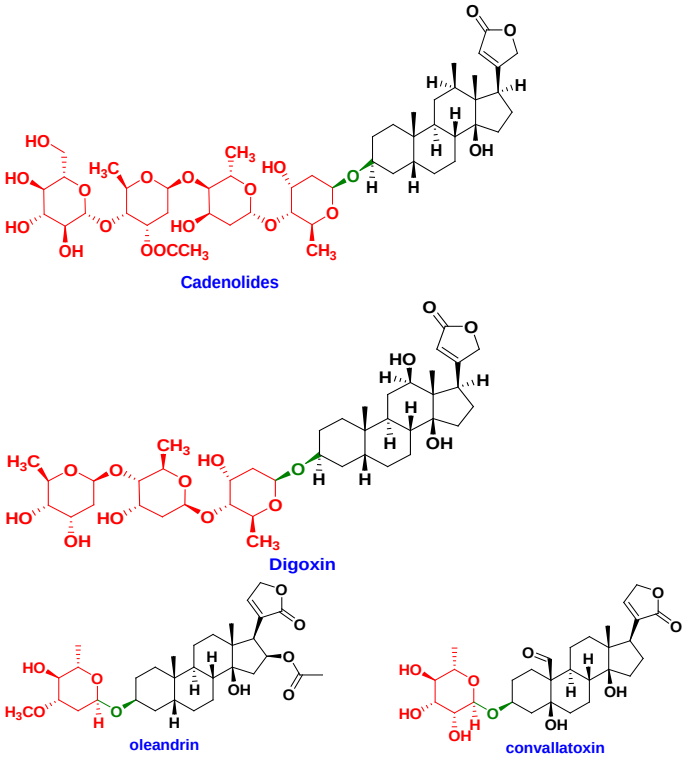

Figure 23: Examples of cardiac glycosides

Scrophulariaceae (figwort family) particularly *Digitalis purpura* (foxglove), *Asclepiadaceae* (milkweeds) and in *Convallariaceae* (convall family) with *Convallaria majalis* (lily of the valley) as a typical example. There two types of cardiac glycosides – **cardenolides** (strophanthin, ubain – strophantus combe, *Erythroxilaceae*; **digitoxin**, **digoxin** – digitalis purpurea, *Scrophullariaceae*; **oleandrin** – nerium oleander, *Apocynaceae*; convalozid, **convallatoxin** – *Liliaceae*; **adonitoxin** – periploca graeca, *Apocynaceae*; periplocinum – periploca graeca, *Apocynaceae*) and **bufadienolides** (helleborine – hellebores odorus, *Ranunculaceae*; scilaren – allium maritimum, *Liliaceae*).

The ***cyanogenic glycosides*** (*Figure 24*) have aglycones derived from amino acids. Several of these compounds can interfere with the iodine utilization and result in hypothyroidism. The other important effect is via their release of hydrogen cyanide after hydrolysis, which is very toxic being

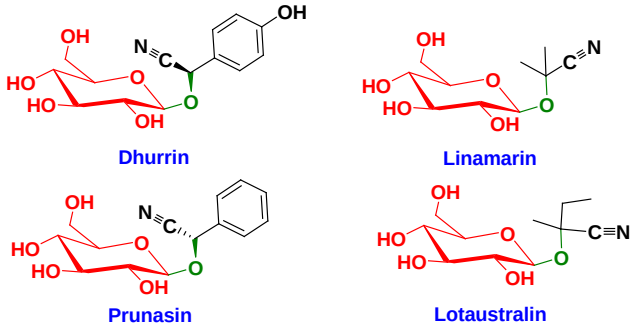

Figure 24: Examples of cyanogenic glycosides

lethal at high dosages. Cyanogenic glycosides are present in species of *Rosaceae* (rose family) in particular in *Prunus* spp. and peas (*fabaceae* family). ***Amygdalin*** (almond, apricot, cherries and peach) and ***dhurrin*** (sorghum) which is toxin of cellular respiration by binding to the Fe-containing heme group of cytochrome oxidase and other respiratory enzymes.The ***glucosinolates***(mustard oil glycosides, (*Figure 25*) contain sulphur-nitrogen containing compounds which are containing pungent amino acid-derived aglycones. The compounds show a complex set of effects on cytochrome P450 isoforms in various cells and tend to decrease hepatic bioactivation of environmental procarcinogens. These have characteristic taste and odor. The glucosinolates can be skin irritating and also induce hypothyroidism and goitre.

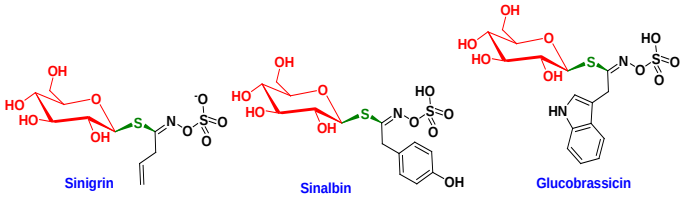

Figure 25: Examples of glucosinolates

The *Brassicaceae* (brassica family – cabbage, broccoli, horseradish, and watercress) is the family mainly associated with glucosinolate production.

Saponins: Most ***saponins*** **–** "soap forming compound"- occur as glycosides containing one or more sugar molecules. The aglycones (also called as sapogenin) consist of either pentacyclic triterpenoids or tetracyclic steroids. They are structurally distinct, but have main functional properties in common.The saponin glycosides are large molecules with a hydrophilic glycone and ahydrophobic aglycone, which give emulsifying properties and can be used as detergents. Saponins show immune modulating, blood cholesterol level, cancer and antineoplastic effects as shown in *Figure 26*.

A common *in vitro* effect is haemolysis of red blood cells. However, this effect does not seem to be an *in vivo* problem. Some saponins induce photosensitisation and jaundice. Saponins are present in a range of plant families – vegetables, beans and herbs. Among them is *Liliaceae* (lily family) with the important sheep toxic plant *Narthesium ossifragum* (bog asphodel). Types of saponins – steroidal saponins (ruskogenin and diosgenin) and triterpenic saponins (ginsenoside, aescin, saporubin, glycyrrhizin, primula saponin, header saponin, panax

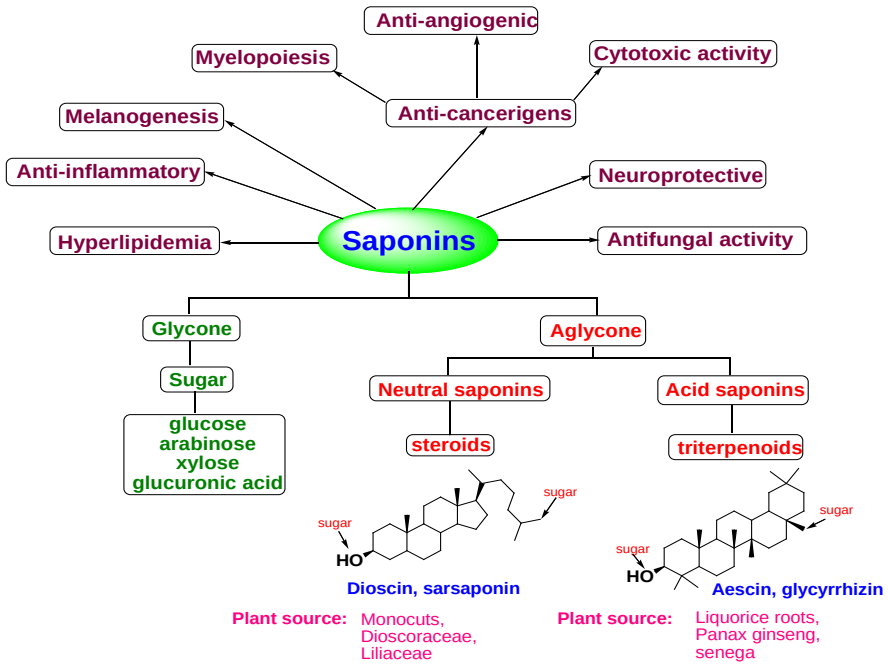

Figure 26: General structural parts of saponins and its applications

saponin) (*Figure 27*). The commercial saponins are extracted from *Yucca schidigera* and *Quillaja saponaria*.

Anthraquinone glycosides(*Figure 28*) show a relatively limited distribution within the plant kingdom containing anthracene and its derivative as aglycone. Anthraquinone glycosides are easily hydrolyzed forming aglycones – chrysophanol, aloe-emodin, rheine, emodin (from frangula and cascara) and physcion (from frangula and cascara). In *Polygonaceae* (dock family) they are present for instance in *Rumex crispus* (curly dock) and *Rheum* spp (rhubarbs). Their primary effect is induction of water and electrolyte secretion as well as peristalisis in colon also act as stimulant cathartics and exert their action by increasing the tone of smooth muscle in wall of the large intestine. e.g. Aloe-emodin (aloe vera-aloe, *liliacear*), Rheine (rheum officinale – *rhubarb polygonaceae*), hypericin (hypericum perforatum – st. John's wort, *hypericaceae*), chrysophanol (senna obtusifolia, *sennafabaceae*).

Flavonoids and proanthocyanidins: Flavonoid was the important group of phytochemicals containing polyphenols widely distributed among the plant flora. These compounds have three rings including aromatic ring along with the groups such as phenolic alcohols, aldehydes, ketones and their glycosides (*Figure 29*). Phenolic substances are water soluble since they are combined with sugar as glycosides and located in cell vacuoles.

Polymeric substances like lignins, melanins and tannins are polyphenols and occasionally phenolic units are encountered in proteins, alkaloids and terpenoids. Phenolics are effective antioxidants and antimicrobial agents. Other actions are diverse – several structures reduce inflammation, carcinogenicity, cardiovascular diseases prevention, neurodegenerative, reduces blood pressure, anti-oxidant effect, and immune system benefits.

Detection of such phenolic compounds was due to their color developing nature such as intense green, purple, blue or black colors in presence of ferric chloride. Majority of phenolic compounds and flavonoids can be detected with their colors of fluorescence in UV (since they are aromatic) light. Phenolic compounds are visibly colored and hence can be easily isolated and purified.

Flavonoids are the groups of phytonutrients founds in almost all fruits and vegetables. It consists of a central three-ring structure. Proanthocyanidins are oligomers of flavonoids. Both groups of compounds can occur as glycosides. Flavonoids are water soluble phenolic compound derived from the parent substance flavone. Flavonoids are present in combination with glycosides and also in Free State. The mixtures of different flavonoid class of compounds which give different colors and fluorescence with chemical and UV treatment. There are several significant groups (*Figure 30*) of flavonoids including – anthocyanidins, flavanols, flavonols, flavones, flavonones and isoflavones (are primarily known as phytoestrogens).

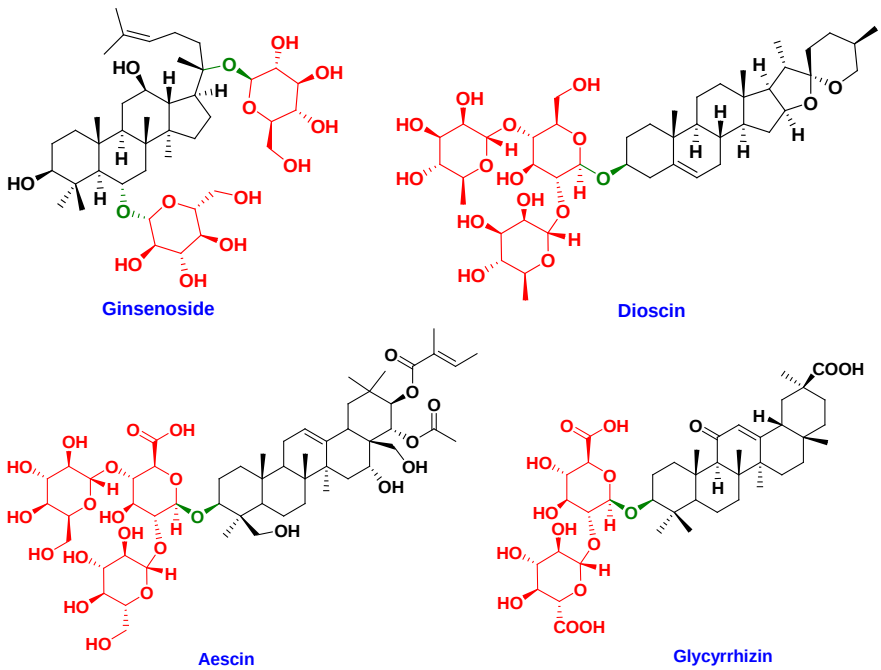

Figure 27: Examples of steroidal and triterpenic saponins

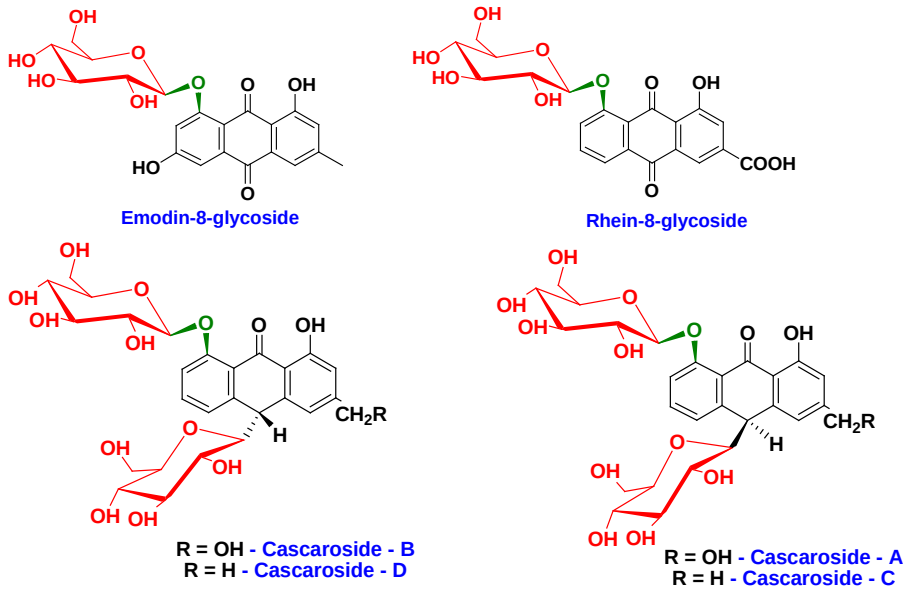

Figure 28: Examples of Anthraquinone glycosides

Flavonoids and proanthocyanidins (*Figure 31*) are all pigments with distinct colors – blue-violet occurring in a long range of plant families (generally in fruit and flowers). These are breaks to sugars in presence of bright light. Isoflavones are produced by species of *Fabaeceae* (bean family). E.g. cyanidin (grapes, blueberries, cherries), malvidin (common mallow), delphinidin (larkspur), pelaegonidin (pelargonium), peonidin (poeny), etc.

Tannins: Tannins are polyphenolic plant secondary metabolites which are widely distributed throughout the plat kingdom, especially legumes and browse. There are three distinct types of tannins – condensed tannins, hydrolysable tannins and derived or complex tannins (tea, quercus, castanea).

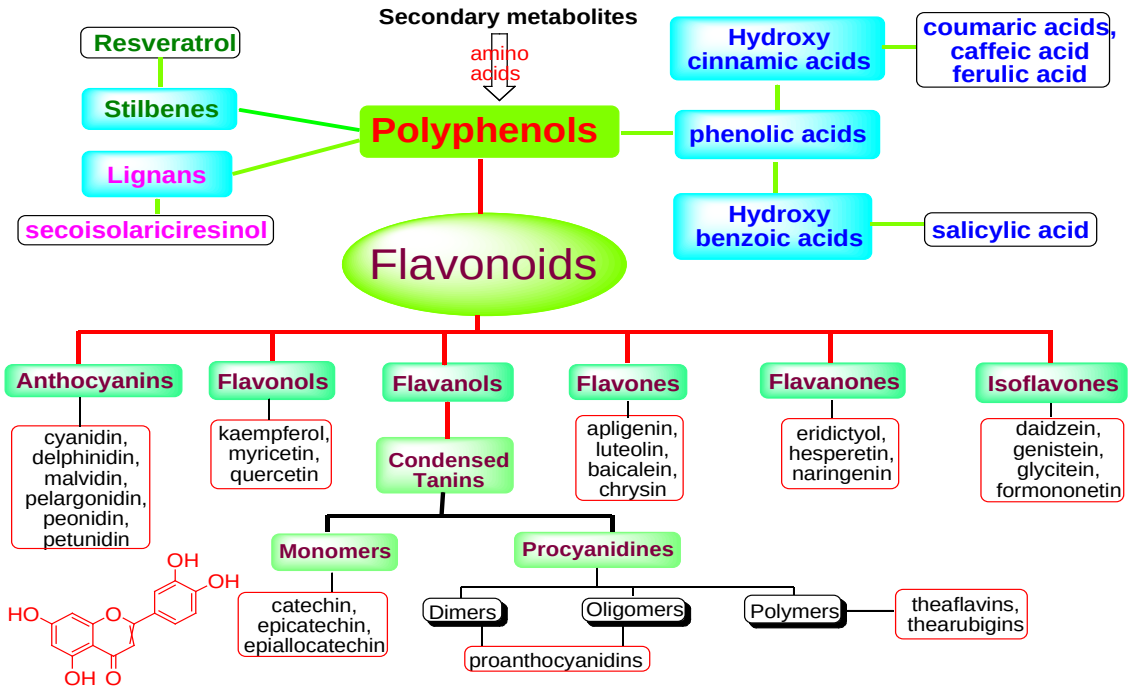

Figure 29: Classification of flavonoids

Condensed tannins (flavonoid, catechin, flavonol-3,4-diol) (*Figure 33*) which are large polymers of flavonoids and hydrolysable tannins (gallic acid, pyrogallol, ellagic acid) (*Figure 32*) which are polymers composed of a monosaccharide core (most often glucose) with several catechin derivatives attached. The two types of tannins have most properties in common, but hydrolysable tannins are less stable and have greater potential to cause toxicity. The water solubility is restricted and decrease in general with the size of the tannin molecule. Tannins indiscriminately bind to proteins and larger tannins are used as anti-oxidant property, carcinogenicity, astringents in cases of diarrhoea, skin bleedings and transudates. Tannins have ability to precipitate solution of gelatin, alkaloids, glycosides, heavy metals, and proteins. Tannins are very widely distributed in the plant kingdom. Examples of plant families associated with presence of tannins are *Fagaceae* (beech family) and *Polygonaceae* (knotweed family).

Tannins has medicinal importance – antidote, antiseptic, algicidals, astringents, and anti-carcinogenic agents.

Tannins: Tannins are polyphenolic plant secondary metabolites which are widely distributed throughout the plat kingdom, especially legumes and browse. There are three distinct types of tannins – condensed tannins, hydrolysable tannins and derived or complex tannins (tea, quercus, castanea). Condensed tannins (flavonoid, catechin, flavonol-3,4-diol) (*Figure 33*) which are large polymers of flavonoids and hydrolysable tannins (gallic acid, pyrogallol, ellagic acid) (*Figure 32*) which are polymers composed of a monosaccharide core (most often glucose) with several catechin derivatives

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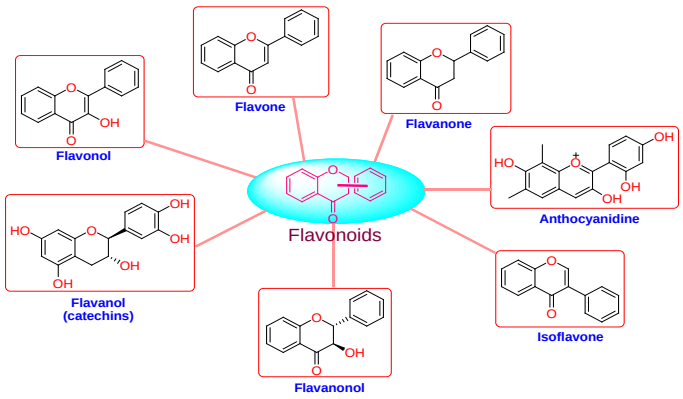


Figure 30: Different groups present in flavonoids

presence of tannins are *Fagaceae* (beech family) and *Polygonaceae* (knotweed family). Tannins has medicinal importance – antidote, antiseptic, algicidals, astringents, and anti-carcinogenic agents.

Lignans:Lignans (*Figure 34*) are composed of two phenylpropanoid units to form an 18-carbon skeleton, with various functional groups connected. Lignan precursors that have been identified in the human diet include pinoresinol, lariciresinol, secoisolariciresinol, matairesinol, and others. They are generally lipophilic and have structural functions within the plant cell membranes. Lignans are polyphenols found in a wide variety of plant based foods including seeds, whole grains, legumes, fruit, and vegetables. Lignans are present at highest concentrations in oil seeds or flaxseeds. The lignans are converted to the enterolignans, enterodiol and enterolactone by the bacteria normally colonize the human intestine. Several lignans show clinical activity as phytoestrogenic, cathartic, anti-oxidants, reduction of risk of cardiovascular disease and osteoporosis, anti-cancer, and antineoplastic effects.

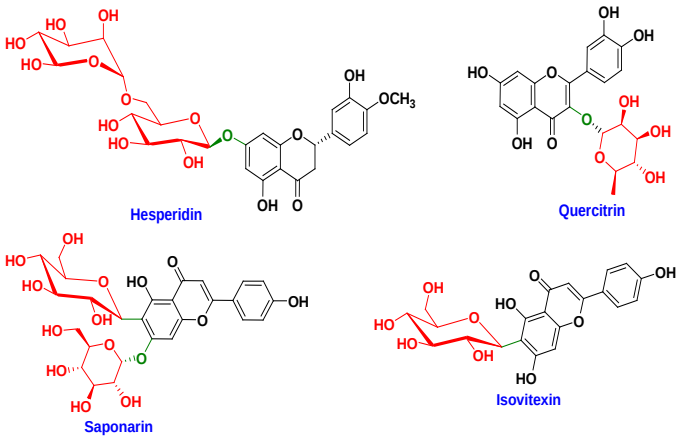


Figure 31: Examples of Flavonoids

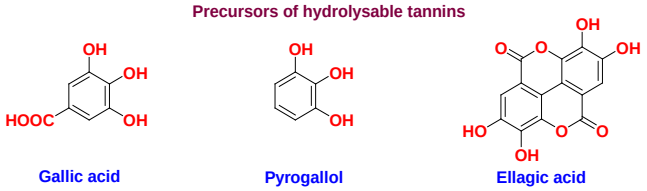


Figure 32: Hydrolysable tannins.

Resins: The resins are complex lipid-soluble mixtures (solid or semisolid, amorphous products) – usually both non-volatile and volatile compounds. The non-volatile fraction may consist of diterpenoid and triterpenoid compounds, and mono- and sequiterpenoids predominate in the volatile fraction. Chemically, resins are complex mixtures of resin acids, resin alcohols (resinol), resin phenols (resinotannols), resin esters and chemically inert compounds known as resenes. Most typical are resins secreted by wood structures, but resins are also present in herbaceous plants in the form of entirely resin (benzoin), oleo-resin (turpentine oil), gum-resin (copaiba resin), oleo-gum-resin (myrrh), balsam (tolu balsam, benzoin, peru balsam, storax), gluco-resin (convolvuaeae family drug). They are all sticky and the fluidity depends on their contents of volatile compounds. All resins are sticky and harden when exposed to air. They are producing fragrant smoke after burning and therefore used as incense. They are insoluble in water and usually insoluble in petroleum spirit, but dissolve more or less completely in alcohol, chloroform and ether. Most resins are analgesic, antineoplastic, antimicrobial, burn and wound healing, but their actions depend on the composition of the chemical mixture. Resins are generally safe, but contact allergy may occur. Resins are used widely in varnishes, lacquers, water-proofing agents, adhesives, and

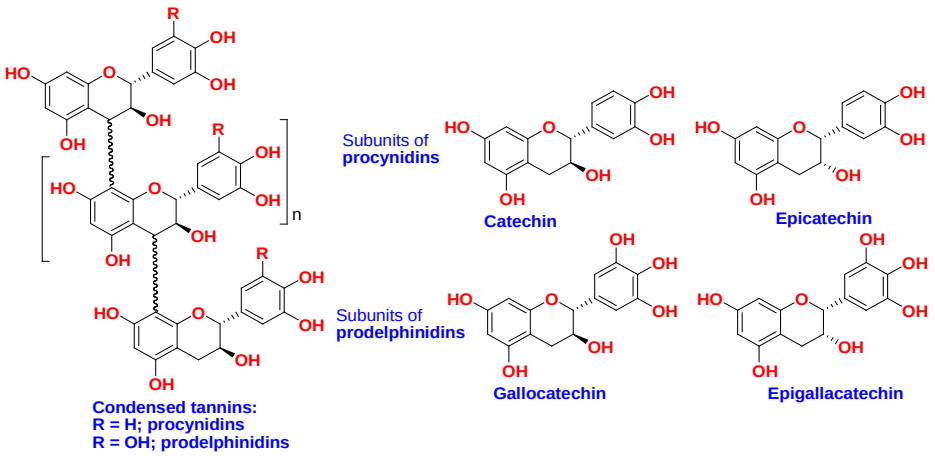


Figure 33: Example and precursor of condensed tannins

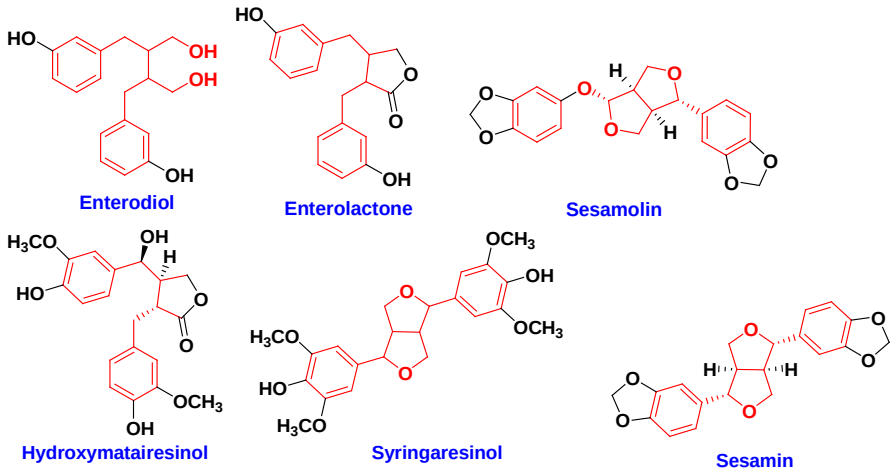


Figure 34: Examples of some biologically important lignans

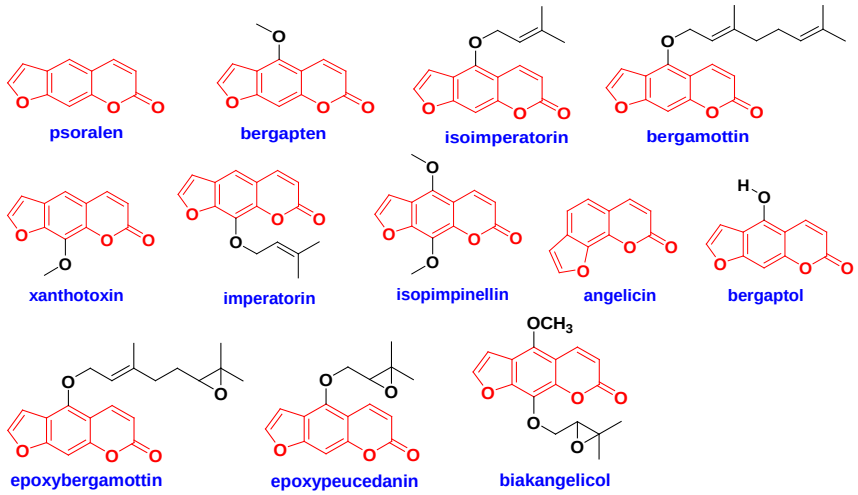


Figure 35a: Examples of Furocoumarin

precursor material for the industrial chemical productions. e.g. Amber is fossilized resin. Balsams (*Myroxylon balsamum var. perierae*) are highly fragrant resins that tend to stay relatively soft at room temperature. Frankincense is the gum resin extracted from *Boswellia serrata* is used as inflammation modulator, for the treatment of asthma and ulcerative colitis. Gums, latex and mucilages are not resins but instead of that they are thick, sticky mixtures of polysaccharides. In most of cases, gums and resins are intermixing to form gum resins, but these two are formed separately and are chemically distinct.

Furocoumarins and naphthodianthrons: Furocoumarins are biosynthesized partly through the phenylpropanoid pathway and the mavalonate pathway. It contains the coumarin ring fused with furan nucleus (*Figure 35a*). e.g. two isomers - psoralen and angelicin, bergamottin, bergapten, imperatorin, xanthotoxin and isoimperatorin. Many furocoumarins are toxic and produced by the plants as a defense mechanism against various types of predators, insecticidal and or fungicidal agents but they are also act as photoactivated carcinogens. Furocoumarins in *Apiaceae* (carrot family) particularly in *Heracleum* spp (cow parsnips) have photosensitizing properties. They are found in a wide variety of plants, but which are present at their highest concentrations in members of the *Umbelliferae* family, particularly parsnips, celery and parsley.

The naphthodianthrons (*Figure 35b*) for instance in *Hypericum* spp (St. John's-worts) of *Clusiaceae* (garcinia family) and in *Polygonaceae* (dock family) e.g. *Fagopyrum esculentum* (buckwheat) have similar effects. The compounds in *Hypericum* spp. have an antidepressant effect.

COLLECTION AND AUTHENTICATION OF PLANT MATERIAL

From the total available species of plants (comprising angiosperms and gymnosperms; approximately 250,000 species), only 6% have been reportedly screened for biological activity and about 15% have been screened for phytochemical activity.[27]

India is a land of immense biodiversity in which two out of eighteen hot spots of the world of plant biodiversity are located. India is also one of the twelve mega biodiversity countries in the world. The total number of plant species of all groups recorded from India is 45,000 (the total number may be even close to 60,000, as several parts of India are yet to be botanically explored). From these, the seed-bearing plants are nearly 15,000–18,000. India enjoys the benefits of varied climate, from alpine in the Himalaya to tropical wet in the south and arid in Rajasthan. Such climatic conditions have given rise to rich and varied flora in the Indian subcontinent. In order to promote Indian herbal drugs, there is an urgent need to evaluate the therapeutic potentials of the drugs as per WHO guidelines. The primary benefits of using plant derived medicines are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment. Initial listing of the candidate species for screening of biological activity is a major task of specific

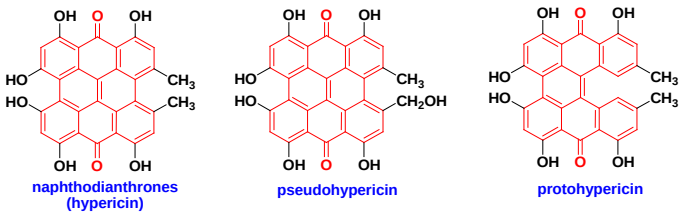


Figure 35b: Examples of naphthodianthrons

importance in itself. Fabricant and Farnsworth,[27] have recommended the following approach for the selection of plant species for the study - Random approach, ethnopharmacology approach, traditional system of medicine approach and zoo pharmacognosy approach.

Random approach: It is very difficult to make a truly random collection of plants which involves collecting of plants without using any specific criteria. A tree or shrub can be selected at random from a specific habitat and making extracts of these plants, which are then exposed to an array of assay (*Figure 36*). One way of the selection of plant for study is the collection of plant present in high proportion that has not yet been phytochemically studied or lack of documented ethnobotanical information or the probability of finding novel substances, bioactive or not, is certainly higher in this type of selection. Another way for random ecological approach, also known as *field observations*, consists in observations of interactions between organisms in their ecological environment, inducing to potential biological activity (antibacterial, antifungal, agrotoxic, pesticide) basis of experience of farmers, woodcutters and tribes about the different plants and herbs.

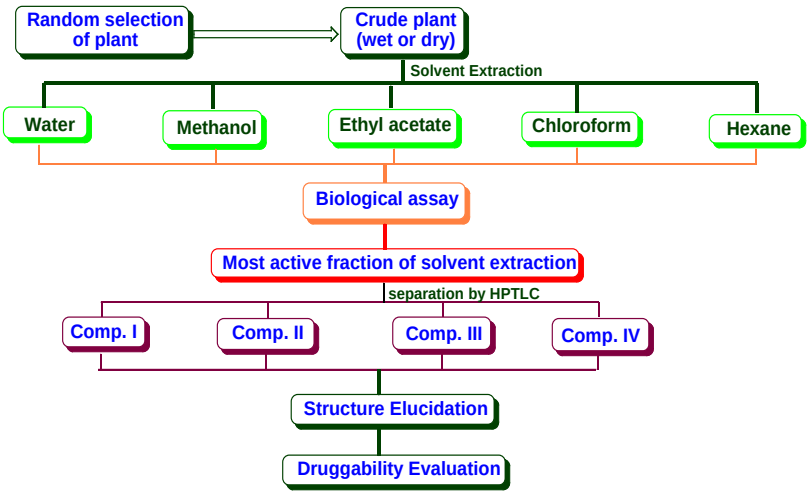


Figure 36: Flowchart for random approach for selection of plant and its study

Ethnopharmacology approach: Ethnopharmacology is a multidisciplinary scientific exploration of biologically active agents traditionally employed or observed by man or interdisciplinary scientific study of materials from animal, vegetable or mineral origin and related knowledge and practices that different cultures use to modify the state of a living organism by therapeutic (curative/prophylactic) or diagnostic purposes. The approach of ethnopharmacology essentially depends on empirical experiences related to the use of botanical drugs for the discovery of biologically active new chemical entity i.e. interfase between pharmaceutical science and phytotherapy. This process involves the observation, description, and experimental investigation of indigenous drugs, and is based on botany, chemistry, biochemistry, pharmacology, and many other disciplines like anthropology, archaeology, history, and linguistics.[28] This approach based on ethnomedicinal usage history has seen some success.

Ethnopharmacological strategies have been widely used to conduct biological screening in various therapeutic areas such as cancer, immunomodulatory drugs, allergy drugs, analgesics, contraceptives, antimalarial, anti-diarrhea, antimicrobial, antiviral, etc,[29]. e.g. *Andrographis paniculata* was used for dysentery in ethnomedicine and the compounds responsible for the activity were isolated as andrographolide. Morphine from *Papaver somniferum*, Berberine from *Berberis aristata*, and Picroside from *Picrorrhiza kurroa* are some examples of this approach. Some of the plants which are not selected on the basis of ethnomedical use also had some success stories, like L-Dopa from *Mucuna prurita* and paclitaxel from *Taxus brevifolia*.

Traditional system of medicine approach:The countries like India and China have a rich heritage of well documented traditional system in the form of Ayurveda, Unani, Kampo, and traditional Chinese medicine. These medicines system is also followed till date in various part of globe where botanical sources are used as medicines. These systems are still in place today because of their organizational strengths, and they focus primarily on multicomponent mixtures,[30]. Even though Western medical science views such systems as lacking credibility, but these system is build up with the empirical practices on strong conceptual foundations of human physiology as well as of pharmacology. During these systems, mixture or crudely extracted juices and decoctions were used for the treatment which was not recognized by advance pharmaceutical science, so standardization of such system is important. Adverse effects from those widely used plants are not well documented in the literature, and efficacy of these plants and plant mixtures is more difficult to assess.

Discovery of artemisinin from *Artemesia alba* for malaria, guggulsterones from *Commiphora mukul* (for hyperlipidemia), boswellic acids from *Boswellia serrata* (anti-inflammatory), reserpine from *Rauwolfia serpentine* and bacosides from *Bacopa monnieri* (nootropic and memory enhancement) was based on the leads from these codified systems of medicine prevailing in China and India. However, it can be stated that such approach for selecting plant for the drug discovery programs has not been adopted much so far. Nonetheless, the approach has a distinct promise in terms of hit rates. From the existing natural medicinal drugs, fairly high percentage of useful plant-derived drugs were discovered from the result of scientific follow-up of well-known plants uses in traditional medicine, and therefore it can be concluded that this is a good approach for discovering other useful new drugs from plants while the other approaches, such as phytochemical screening, massive biological screening of randomly collected plants, and phytochemical examination of plants with the aim of identifying new chemical compounds have not proved to be very helpful in discovering new drugs.[31]

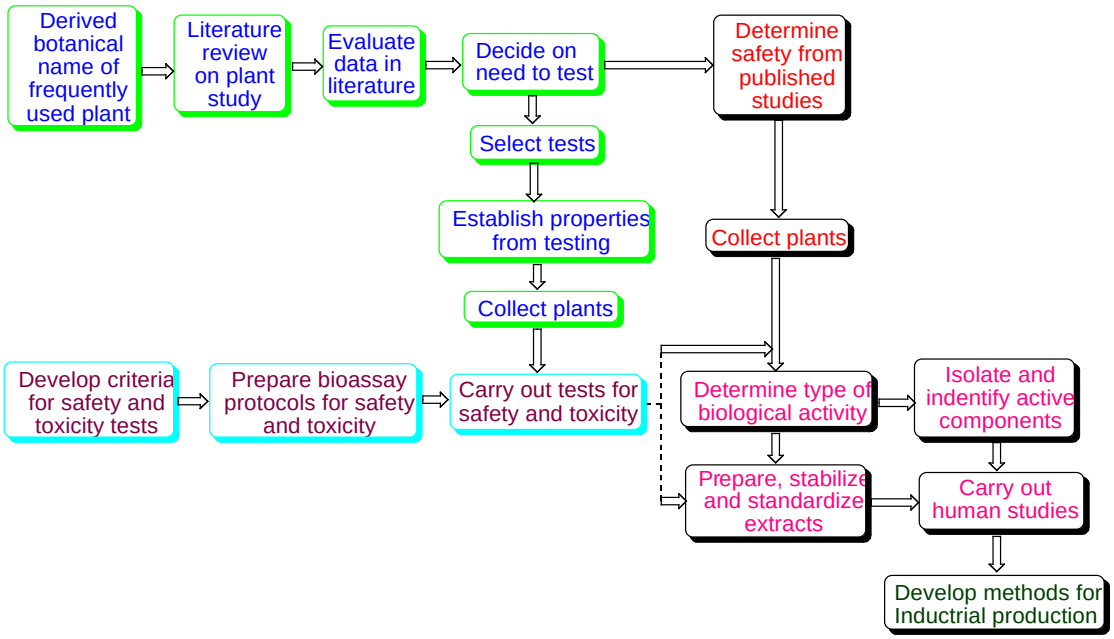


Figure 37: Flowchart for drug development process from traditional system and data

The possibility of drug development in the form of stable, standardized crude extracts and eventual development of the active principles from medicinal plants was envisioned,[31] in following flow chart (*Figure 37*) which focus on initial need to produce safe and effective galenical products.

Zoo pharmacognosy approach:Observation of the behavior of the animals with a view to identify the candidate plants for new drug discovery is not a distant phenomenon. The term '***zoo pharmacognosy***' is the process by which wild animals select and use specific plants with medicinal properties for the treatment of diseases and protection from parasites. The self medication after unusual feeding habits by the animal is observed of their interest. Observation of straight tails linked to cattle grazing habits in certain regions of South America led to identification of a plant *Cestrum diurnum* and three other plant members of family *Solanaceae*, which probably are the only known plant sources of the derivatives of Vitamin D_3. This approach, however, needs close observation and monitoring of the behavior of animals. Chacma baboons (*Papio ursinus*) in South Africa are known to consume each day a little quantity of leaves of specific plants, which are well known for their stimulant property. These include *Croton megalobotrys* (Euphorbiaceae), *Euphorbiaavasmontana* (Euphorbiaceae), *Datura innoxia* and *D. stramonium* (Solanaceae).[32]

SOLVENT USED FOR EXTRACTION:

Successful extraction and determination of bioactive compounds from plant material is depends on the type of solvent used and extraction procedure. Properties of a good extracting solvent in plant extractions includes low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, and inability to cause the extract to complex or dissociate. The factors based on good choice of solvent are - quantity of phytochemicals to be extracted, rate of extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process, and the potential health hazard of the extractants,[33]. But if the plant material is extracted to isolate chemical components without using bioassay, toxicity of the solvent is not important because the solvent. The choice of solvent is influenced by what is intended with the extract. The choice will also depend on the targeted compounds to be extracted,[34]. Different solvents used for extractions of dried plant material with different polarity are – water, ethanol, methanol, acetone, methylene dichloride, dimethylsulfoxide, ethyl acetate, chloroform, carbon tetrachloride, ether, pet ether, hexane, etc.

Plants samples such as leaves, barks, roots, fruits and flowers can be extracted from fresh or dried plants material. The plant material in grinded or powdered form is best for extraction. Lowering particle size increases surface contact between samples and extraction solvents. Grinding resulted in coarse smaller samples; meanwhile, powdered samples have a more homogenized and smaller particle, leading to better surface contact with extraction solvents. Fresh or dried plant material in powdered form can be used as a source for secondary plant components for the extraction because of following reasons - (i) There are fewer problems associated with the large scale extraction of dried plant material than with fresh material because of water content; (ii) the time delay between collecting plant material and processing it makes it difficult to work with fresh material because differences in water content may affect solubility or subsequent separation by liquid–liquid extraction; (iii) the secondary metabolic plant components should be relatively stable especially if it is to be used as an antimicrobial agent; (iv) many, if not most plants are used in the dried form [or as aqueous extract] by traditional healers,[33].

Water	Ethanol	Methanol	Chloroform	DCM	Ether
Anthocyanins	Tannins	Anthocyanin	Terpenoids	Terpenoids	Alkaloids
Tannins	Polyphenols	Terpenoids	Flavonoids		Terpenoids
Saponins	Flavonol	Saponins			Coumarins
Terpenoids	Terpenoids	Tannins			Fatty acids
Polypeptides	Alkaloids	Flavones			
Lectins	Sterols	Polyphenols			

Drying of plant sample by various means as - **Air-drying, microwave-drying, oven-drying and freeze-drying.*Air-drying*** (by natural drying) usually takes from 3-7 days to months and up to a year depending on the types of samples dried (e.g. leaves or seed). Plant samples, usually plants leaves with stem were tied together and hang to expose the plant to air at ambient temperature in hot air oven. This drying method does not force dried plant materials using high temperature (in oven more than 100^0C); hence, heat-labile compounds (volatile) is preserved. The air-drying take longer time in comparison with other drying techniques as - microwave drying and freeze drying and may be subjected to contamination at unstable temperature condition. ***Microwave-drying*** uses electromagnetic radiation that causes simultaneous heating through dipolar rotation and alignment of the molecules possessing a permanent or induced dipole moment (e.g. solvents or samples), and ionic induction, that produce oscillation of the molecules,[35]. Oscillation causes collisions between molecules and resulted in fast heating of the samples simultaneously. This method can shorten drying time but sometimes causes degradation of phytochemicals. ***Oven-drying*** is a pre-extraction method that uses thermal energy to remove moisture from the samples. This sample preparation is considered as one of the easiest and rapid thermal processing that can preserved phytochemicals. Oven-drying at 44.5°C for 4 hours using 80% methanol resulted in highest antioxidants activities in *Cosmos caudatus* extracts and similar result were observed in optimized 80% methanol extracts at 44.12°C for 4.05 hours.[36] Shorter period of extraction time was obtained using this method. However, effect of drying on *Orthosiphon stamineus* showed no significant effect on the antioxidant activity but the bioactive phytochemicals; such as sinensetin and rosmarinic acid content were affected by the oven and sunlight-drying, suggesting the sensitivity of the compounds to temperature,[37]. ***Freeze-drying*** is a method base on the principle of sublimation. Sample is frozen at -80°C to -20°C prior to lyophilisation to solidify any liquid (e.g. solvent, moisture) in the samples. After an overnight freezing, sample is immediately lyophilized to avoid the frozen liquid in the sample from melting. Mouth of the test tube or any container holding the sample is wrapped with needle-poked-parafilm to avoid loss of sample during the process. Most of the time, sample was lost by splattering out into the freeze-flask. Freeze-drying yielded to higher level of phenolic contents compared to air-dying as most of the phytochemicals are preserved using this method. However, freeze-drying is a complex and expensive methods of drying compared to regular air drying and microwave-drying.

Following solvents were used for the extraction -

Water: Water is universal solvent used to extract plant products (polar components) which are used directly for the study of antimicrobial activity. It is a good solvent for the extraction of many types of active constituents such as alkaloidal salts, colouring agents, glycosides, gums, sugars, anthraquinone derivatives and tannins. It can also act as menstruum for many organic acids and small proportions of volatile oils. Water is not a suitable menstruum (Solvent) for constituents like waxes, fats, fixed oil and alkaloidal bases due to their insolubility in water. Water is not selective as it can dissolve a wide range of substances and leads to hydrolysis of many substances. Though traditional healers use primarily water extracts but plant extracts from organic solvents have been found to give more consistent antimicrobial activity compared to water extract. Also water soluble flavonoids (mostly anthocyanins) have no antimicrobial significance and water soluble phenolics only important as antioxidant compound.[38]

Water soluble herbs are aloe, glycyrrhiza, linseed, senna leaves, senna pods, ginger etc.

Acetone: Acetone dissolves many hydrophilic and lipophilic components from the plants used, is miscible with water, is volatile and has a low toxicity to the bioassay used, it is a very useful extractant, especially for antimicrobial studies where more phenolic compounds and flavonols are required to be extracted. A study reported that extraction of tannins and other phenolics was better in aqueous acetone than in aqueous methanol.[33,38] Both acetone and methanol were found to extract saponins which have antimicrobial activity.[39]

Alcohol: Alcohol or ethanol can dissolve a large number of chemical constituents such as alkaloids, alkaloidal salts, glycosides, tannins, anthraquinone derivatives, volatile oils and resins, but constituents like albumin, gums, waxes, fats, fixed oils and sucrose are insoluble in alcohol. Generally dilute alcohols (hydroalcoholic solutions) are used for many extractions, but in some cases stronger alcohol may be used to prevent the extraction of unwanted substances such as gums. The ethanolic extracts contains higher amount of polyphenols as compared to aqueous extracts which indicates that ethanol is more efficient in cell wall and seed degradation. Most of the polyphenols undergoes degradation in water which is also one reason for less content polyphenols in water. The flavonoid content were found to be maximum with 70% ethanol extract,[40]. It is non-toxic in the quantities present in medicinal substances. It is reasonably selective. In a herb containing a number of chemical substances such as alkaloidal salts, glycosides, albumin and gum, water will dissolve all the substances. Whereas dilute alcohol will dissolve only the alkaloidal salts and glycosides but these are insoluble in non-polar solvents. Alcohol soluble herbs are benzoin, asafetida, ginger, valerian, myrrh etc. Each time before extraction, the powdered material is dried in hot air oven below 50^0C.

Many extracts contain significant amount of fatty material than can be interfere with chromatographic separation. The extract can be dissolved in a mixture of methanol-pet ether (1:1) and enough water (about 10%) is added so as to give two phases that are then separated. The aqueous methanol layer can be concentrated and the organic material recovered by back extraction with ethyl acetate. The toluene-methanol, heptane-acetonitrile, heptane-ethylene glycol pairs can also be used.

Ether: Soluble Constituents are alkaloids, terpenoids, coumarins and fatty acids. Highly inflammable produces physiologically effects. Ether soluble herbs are capsicum, male fern, linseed, nutmeg etc. For the extraction of organic bases such as alkaloids in water insoluble solvent usually basification of plant material is required whereas for aromatic acids and phenols acidification may be required.

Chloroform: Soluble constituents are flavonoids, lactones, tannin and alkaloidal bases. It is non-inflammable. The tannin and terpenoids contents were found maximum in chloroform extract than the water extract.

Glycerin: Soluble constituents are tannins. It is non-inflammable and viscous liquid.

Light Petroleum: Soluble constituents are oils, fats, waxes, resins and alkaloidal bases. It is highly inflammable and very volatile.

Fixed Oils: Soluble constituents (Arachis Oil) can act as menstruum for camphor. Non inflammable and viscous.

Propylene Glycol: Soluble constituents are progesterone, phenobarbitone sodium. Clear, colorless, odorless, viscous liquid, miscible with water, alcohol and chloroform.

Dichloromethanol: It is another solvent used for carrying out the extraction procedures. It is specially used for the selective extraction of only terpenoids.[41]

In general, the extraction of different bioactive compounds by using different solvents is summarized in following table.[41]

DIFFERENT METHODS FOR EXTRACTION OF PLANT METABOLITES:

There are variety of plant species available containing great variations in bioactive compounds, it is necessary to build up a standard and integrated approach to screen out these bioactive compounds having human health benefits.Farnsworth,[42]reported an integrated approach showing sequence of steps to study the medicinal plant, which started from name collection of frequently used plants and ended at industrialization or commercialization.

Works of particular order for medicinal plant study and the position of extraction techniques are shown by a above flow chart.

The separation, identification, and characterization of bioactive compounds is only possible after the used of appropriate extraction process. Different extraction techniques should be used in diverse conditions for understanding the extraction selectivity from vaious natural sources. Successful determination of biologically active compound from plant material is largely dependent on the choice and purity of solvent used in the extraction procedure. The main goals for the solvent extraction are related to one or more important properties:

A. High yield: the target compounds are exhaustively or approximately exhaustively recovered with minimum number extracting steps. The target compound should have maximum solubility in extracting solvent.

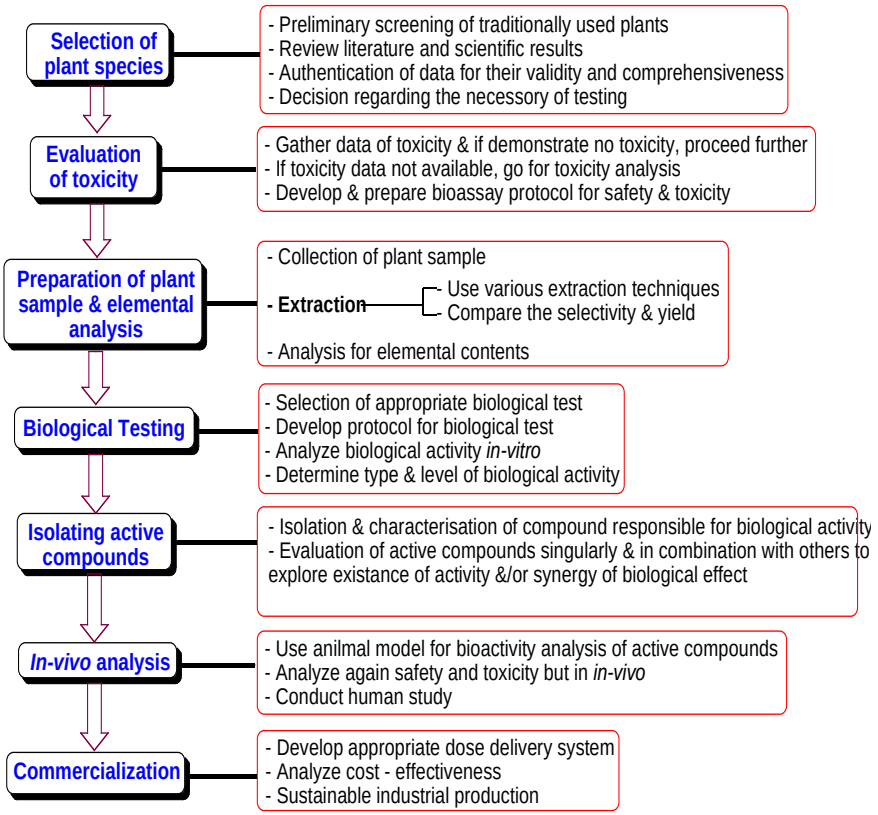
B. High selectivity/purity: the resulting extracts has low amount of interfering or undesirable co-extracted compounds i.e. extract targeted bioactive compounds from complex plant sample by increasing selectivity of analytical technique.

C. High sensitivity: the resulting extract allows for different quantification techniques that produce a high slope in the calibration curves i.e. to increase sensitivity of bioassay by increasing the concentration of targeted compounds.

D. Low limit of detection/quantification: components in the extracts can be detected or quantified at low levels because low noise levels are obtained in the analytical system.

E. To convert the bioactive compounds into a more suitable form for detection and separation.

F. To provide a strong and reproducible method that is independent of variations in the sample matrix.[43]



Variation in extraction methods are usually depend on the length of the

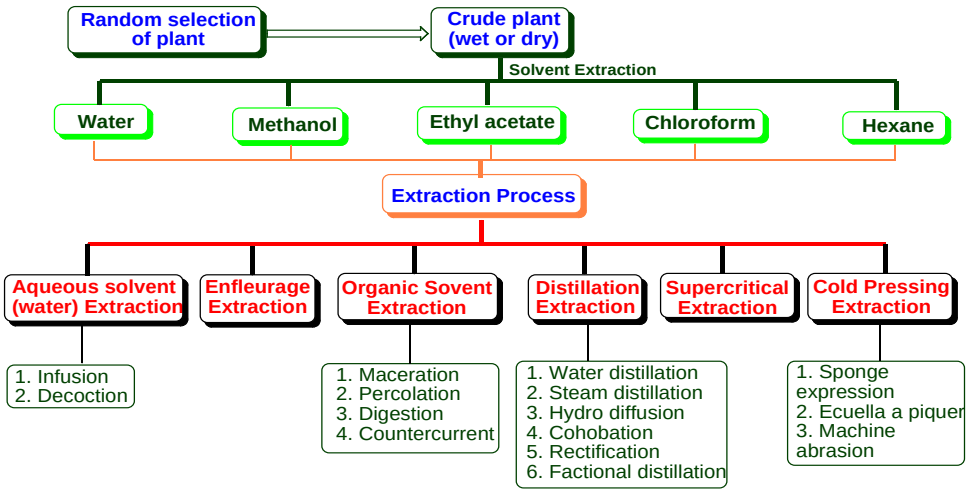


Figure 38: Different solvent extraction techniques used in phytochemistry

extraction period, solvent used, pH of the solvent, temperature, particle size of the plant tissues and the solvent-to-sample ratio. Another common method is serial exhaustive extraction which involves successive extraction with solvents of increasing polarity from a non-polar (hexane) to a more polar solvent (methanol) to ensure that a wide polarity range of compound could be extracted. Other researchers employ soxhlet extraction of dried plant material using organic solvent,[44].

General techniques used for the solvent extraction (*Figure 38*):

- Maceration » Infusion » Percolation » Digestion
- Decoction » Hot continuous extraction (soxhlet)
- Aqueous – alcoholic extraction by fermentation
- Counter – current extraction » Microwave – assisted extraction » Ultrasound extraction (sonication)
- Supercritical fluid extraction
- Phytonic extraction (with hydrofluorocarbon solvents)

Extraction techniques for aromatic *plants (Figure 38):*

- Hydrodistillation techniques (water distillation, steam distillation, water & steam distillation)
- Hydrolytic maceration followed by distillation, expression and effleurage (cold fat extraction)
- Headspace trapping » Solid phase micro extraction
- Protoplast extraction » Microdistillation
- Thermomicrodistillation » Molecular distillation

All the extraction processes are summarized in flowchart Figure 38.

Maceration: In maceration (for fluid extract), whole or coarsely powdered plant material or drug is kept in contact with the extracting solvent in a stoppered container for a defined period of time (minimum 3 days) with frequent agitation until soluble matter is dissolved i.e. for soaking and agitation of plant material and solvent together. This method is best suitable for use in case of the thermolabile drugs,[39]. It is widely used in wine making and widely used in medicinal plant research. During this period of time, the plant's cell wall is soften and breaks to release the soluble phytochemicals. After 3 days, the mixture is pressed or strained by filtration. In this conventional method, heat is transferred through convection and conduction and the choice of solvents will determine the type of compound extracted from the samples.

Infusion:It is a dilute solution of the readily soluble components of the crude drugs. Fresh infusions are prepared by macerating the solids for a short period of time with either cold or boiling water,[45]. Infusion and decoction uses the same principle as maceration; both are soaked in cold or boiled solvent generally in water. However, the maceration period for infusion is shorter and the sample is boiled in specified volume of solvent for a defined time for decoction.

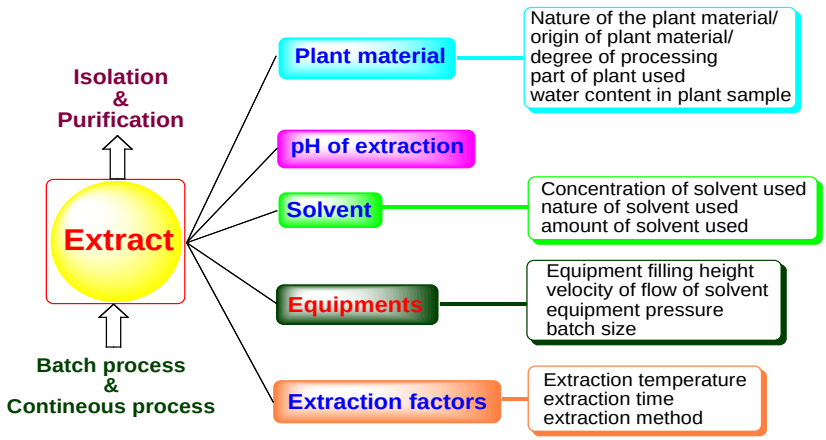


Figure 39: Factor affecting solvent extraction.

Decoction:This method is used for the extraction of the water soluble and heat stable constituents from hard plant sample (e.g. roots and barks) or crude drug by boiling it in water for 15 minutes, cooling, straining and passing sufficient cold water through the sample or drug to produce the required volume,[45].

Percolation:With this method, the plant material is moistened with the appropriate amount of solvent and allowed to swell before being placed in one of a series of percolation chembers. A percolator (a narrow, cone-shaped vessel open at both ends) is generally used. The plant material was repeated rinsed with the solvent until all active ingredients have been removed and the solvent get saturated by reused in repeated cycles. New solvent is used on plant material that is almost completely exhausted, and then reused on subsequently less exhausted batches. This method is more effective in obtaining active ingredients than the above techniques.

Digestion:This is a kind of maceration in which gentle heat is applied during the maceration extraction process provided that temperature does not alter the active ingredients of plant material so there is greater efficiency. The most used temperatures are in between 35^0C to 40^0C (moderately elevated temperature) which is not objectionable and the solvent efficiency of the menstrum is increased thereby,[45]. This process is used for the tougher plant parts or those that contain poorly soluble substances.

Serial exhaustive extraction: It is another common method of extraction which involves successive multiple extraction at room temperature with solvents of increasing polarity from a non polar (hexane) to a more polar solvent (methanol) to ensure that a wide polarity range of compound could be extracted (as per solubility). Some researchers employ soxhlet extraction of dried plant material using organic solvent. This method cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds.[46]

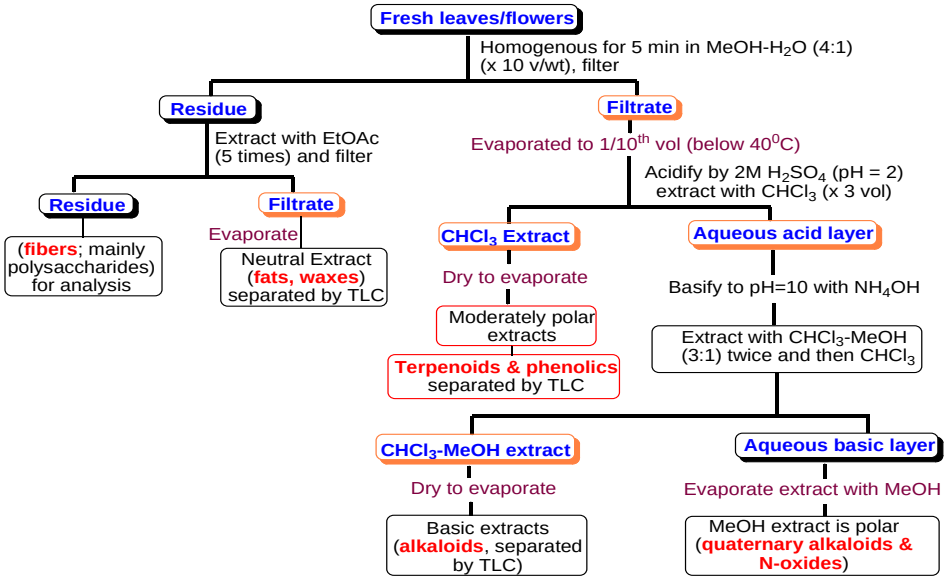


Figure 40: Flowsheet of separation of alkaloids and terpenoids by solvent extraction

Soxhlet extraction: The classical method of obtaining constituents of dried plant tissue is to continuously extract the powdered material in soxhlet apparatus with a range of solvents to obtain wide range of compounds. Soxhlet extractor was first proposed by German chemist Franz Ritter Von Soxhlet in 1879. It was designed mainly for extraction of lipid but now it is not limited for this only. Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a high solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch and small amount of solvent is recycled. This method cannot be used for thermo labile compounds as prolonged heating may lead to degradation of compounds,[47]. This method has some disadvantages such as exposure to hazardous and flammable liquid organic solvents, with potential toxic emission during extraction. This procedure is carrying some pollution problems as compared to advance extraction methods such as supercritical fluid extraction. Extraction efficiency is limited to the dry and finely divided solid and many other factors such as temperature, solvent-sample ratio, and agitation speed need.

Sonication: The procedure involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz according to the nature of sample; this increases the permeability of cell walls and produces cavitation. Although the process is useful in some cases, like extraction of rauwolfia 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.[46]

Microwave assisted extraction (MAE): MAE utilizes microwave energy to separate the analytes from the sample matrix into the solvent.[48] Microwave radiation interacts only with dipoles of polar and polarizable materials of the sample including solvents causes heating near the surface of the materials and heat is transferred by conduction into whole sample. Dipole rotation of the molecules induced by microwave electromagnetic disrupts hydrogen bonding; enhancing the migration of dissolved ions and promotes solvent penetration into the matrix.[49] In non-polar solvents, poor heating occurs as the energy is transferred by dielectric absorption only.[50] MAE can be considered as selective methods that favor polar molecules and solvents with high dielectric constant.

Supercritical fluid extraction (SFE):

Supercritical fluid (SF) or also called as dense-gas is a substance that has the physical properties of both states - gas and liquid at its critical point. Both factors, temperature and pressure are the determinants that push a substance into its critical region. SF behaves more like a gas but have the solvating characteristic of a liquid phase. An example of SF is CO_2 that become SF at above 31.1^0C and 7380 kPa. Interest in Supercritical- CO_2 (SC-CO_2) extraction due to excellent solvent for nonpolar bioactive analytes and CO_2 is readily available at low cost and has low toxicity. Even though SC-CO_2 has poor solubility for polar bioactive compounds, modification such as adding small amount of ethanol and methanol enable it to extracts polar compounds. SC-CO_2 also produces analytes at concentrate form as CO_2 vaporizes at ambient temperature. SC-solvents strength can be easily altered by changing the temperature, pressure or by adding modifiers that lead to reduce

extraction time. e.g. Optimization of SC-CO_2 on Wadelia calendulacea achieved its optimum yield at 25 MPa, 25^{0}C temperature, 10% modifier concentration and 90 minute extraction time.[51] A major drawback of this method is the initial cost of the equipment is very high,[52].

FACTOR AFFECTING EXTRACTION:

Plant based selected natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc i.e. any part of the plant may contain active components. The systematic screening of plant species with the purpose of discovering new bioactive compounds, selective biological activity, separation of complex mixture of components is a routine activity in many laboratories. Therefore, scientific analysis of plant components follows alogical pathway. These plant samples are collected either randomly or by following leads supplied by local healers in geographical areas where the plants are found,[53]. The fresh or dried plant samples (soft or hard) can be used as a source for the extraction of secondary plant components. Many researchers have been reported that plant extract preparation from the fresh plant tissues. The logic behind this is come from the ethno medicinal use of fresh plant materials among the traditional and tribal people. But also many plants are used in the dry form (or as an aqueous extract) by traditional healers and due to differences in water content within different plant tissues in different sessions, plants are usually air dried to a constant weight before extraction. There are different techniques used by the researchers for drying the plants such as drying in the oven at about 40^{0}C for 72 h, by using hot air oven, microwave drying, etc. In most of the reported works suggested the underground parts (roots, tuber, rhizome, bulb etc.) of a plant were used extensively as compared to other above ground parts in search for bioactive compounds possessing antimicrobial properties.[39,44]

Successful determination of biologically active compounds from plant material is largely dependent on the type and polarity of solvent or solvent mixture used in the extraction procedure. Properties of a good solvent in plant extractions includes, low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, inability to cause the extract to complex or dissociate. The factors affecting the choice of solvent are quantity of phytochemicals to be extracted, rate of extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process, potential health hazard of the extractants,[39]. The choice of solvent is depends on what is to be extracted with the solvent. Since the end product will contain traces of residual solvent, the solvent should be non-toxic and should not interfere with the bioassay. The choice will also depend on the targeted compounds to be extracted,[39,44]. Polar components are soluble in polar solvent and non-polar components are soluble in nonpolar solvents so that solvent selection depends on either nature of phytoconstituents directly or extraction of component followed by removal of interference first (example for curcumin extraction defatting is done first and then extraction is done by methanol and chloroform). Therefore, the selection of solvent is depends on the physicochemical properties of the substance and extraction method.

The method of extraction depends on various parameters such as – length of extraction period, solvent used, pH of the solvent, temperature, particle size of the plant sample, and finally solvent to sample ratio (*Figure 39*). The basic requirement to reduce the extraction time, grind the plant material (dry or fresh/wet) which increase the extraction rate. The study shows that the solvent to sample ratio is 10:1 (v/w) for dry sample,[44].

Temperature is one of the most important factor affecting the efficiency of extraction. As temperature of the system increases, higher recovery of the target compounds in plant matrices which may occurs due to increasing the soluble ability of solvents, speeding up the diffusion rate of analyte molecules and then increasing the mass transfer, disturbing the strong solute-matrix interactions and decreasing viscosity & surface tension of solvent. Therefore, for the extraction of thermally stable phytochemicals, the temperature was usually set above the normal boiling point of the solvents used while the extraction of marker compounds those decompose or degrade rapidly are recovered at elevated temperature. e.g. the extraction of glycosinolates such as epiprogoitrin, progoitrin, gluconapin and glucotropacolin in *Isatis tinctoria*, thermal degradation of the analyte was observed above 50^{0}C, where more than 60% of the glycosinolates was lost at 100^{0}C within 10 minutes. A similar observation was found in during the extractions of terpene trilactones (ginkgolides and bilobalide) at room temperature. Some thermal labile compounds such as Z-ligustilide is volatile and unstable compound was changed to other phthalides at higher temperature.

The relatively acidic and basic fractions can be obtained and purified from the extracts by changing the pH. The naturally occurring nitrogenous bases (non-polar or non-ionized alkaloids) can't be extracted into aqueous system from non-polar extract but can be extracted into polar aqueous acid due to their basic nature and salt formation in acid. The aqueous layer also contains non-basic water soluble compounds. The basic compounds can be separated from these by further step consisting of basification of the acidic layer and then extraction with fresh amount of organic solvent. This approach is very commonly used for the extraction of alkaloids. Fatty acids, phenols and other acidic phytochemicals are extracted from organic solvent by using water at alkaline pH and then acidify the alkaline aqueous layer and extract acidic content by suitable organic solvent. The compounds should not break down at employed pH values, e.g. esters are prone to hydrolysis in alkali and glycosides lose the sugar moiety in acid pH of solution,[54]. The organic layers usually contain some amount of water so that it is faintly cloudy appearance. Therefore, the organic layer should be dried by the addition of anhydrous sodium sulphate with good mixing by agitation or swirling.

TLC OF PHYTOCHEMICAL CONSTITUENTS:

There is not a definite solvent system for running TLC of crude extract. The best result was founds for the - $CHCl_3$: Methanol: EtOAc [9:3:5].

Depending up on polarity of the compounds, one can use first less polar solvent pet ether, n-hexane, ethyl acetate, choroform, dicloromethane, chloroform-methanol mixture, etc for separation of constituents of plant extract.

For the less polar: pet.ether: acetone (9:1) or less medium polarity (chloroform: methanol starting with 9.5:0.5) more polar it is necessary to add polar solvent like water / acids as (methanol: chloroform: acetic acid) 66:33:0.1.

- for terpenoids toluene : ethyl acetate (4:1) or benzene : ethyl acetate (5:1)
- for alkaloids $CHCl_3$/MeOH/AcOH (18:1:1, v/v/v), and Dragendorff as a revealing reagent
- for coumarins n-BuOH/AcOH/H_2O (4:1:5, v/v/v), and acetate of lead (5%) and alcoholic KOH (5%) as a revealing reagent
- for flavonoids n-BuOH/AcOH/H_2O (4:1:5, v/v/v), and AlCl3 (0,5 g/100 mL of EtOH) as a revealing reagent while
- for tannins use $FeCl_3$ (10% in MeOH/H_2O, 1:1, v/v)
- for anthocyanins HCl/ formic acid/water, (19.0/39.6/41.4 v/v/v)

FLOW SHEETS FOR EXTRACTION OF PHYTOCHEMICALS:

A general method of extraction of any phytochemical includes –

(i) Breaking the plant cells to release their chemical constituents in the solvent system used for the extraction at suitable conditions;

(ii) Extracting the sample using a suitable solvent or through distillation or trapping of compounds;

(iii) Separating the desired bioactive compound from undesired contents of extracts that confound analysis and quantification; and

(iv) Analyzing the product by an appropriate method e.g., thin layer chromatography (TLC), gas chromatography (GC), or liquid chromatography (LC).

Usually, the last three steps will vary depending on the nature, polarity and size of the target bioactive molecule (*Figure 40*). Appropriate precautions should be taken which give us guarantee that potential active constituents are not lost, altered or destroyed during the preparation and process of the extract.

The chemist should aware that for the extraction of particular components, he should follow the specific processing otherwise most of the bioactive compounds undergoes change or decomposed, therefore a generic extraction scheme is best indicated for the preparation of crude extracts. During this separation chart (*Figure 41*), we use mineral acids and strong bases so the hydrolysis of some phytochemical is possible therefore it is need to use alternative chart for the separation by using tartaric acid and sodium carbonate is given below. Alternatively, alkaloids can also be extracted with 10% acetic acid in ethanol, followed by concentration under vacuum to one quarter to the original volume and then precipitation of the crude alkaloid fraction by drop wise addition of ammonium hydroxide (*Figure 41*).

Plant extracts are prepared by maceration or percolation of fresh green plants or dried powdered plant material in water and/or organic solvents. For hydrophilic compounds, polar solvents such as methanol, ethanol or ethyl-acetate are used.

For extraction of more lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol 1:1

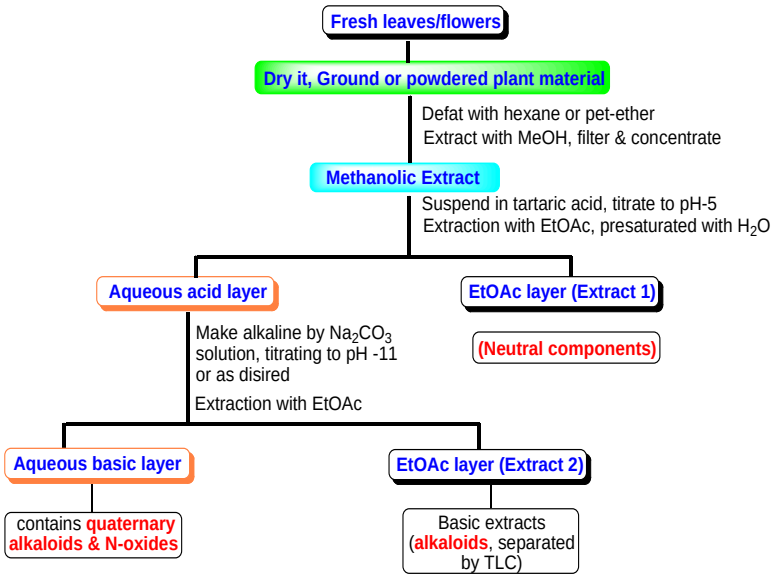


Figure 41: Flowsheet of separation of alkaloids by solvent extraction.

are used (*Figure 42*). In some instances, extraction in hexane is used to remove chlorophyll. A concentration of the extract is usually required and is based on evaporation of the solvent in vacuo. It is advised to extract and evaporate at low temperature not to destroy any thermolabile constituent. Introducing pH differences may further enhance separation of acid, neutral and basic constituents,[55]. e.g. From the air-dried and finely milled P. macrocarpa fruits samples (1000 g of fruits) hexane, chloroform, ethyl acetate and water extraction were performed sequentially as shown in following flow sheet (*Figure 43*),[56]. The extracts were concentrated using a rotary evaporator under reduced pressure at 35^{0}C to avoid decomposition of components of sample. Dried extracts were stored at -4^{0}C and used for further study.

Flow sheet for the extraction of alkaloid from plant material (*Figure 44*):The plant material is dried by using suitable method, then finally powdered and extracted with boiling methanol. The solvent is distilled out under reduced pressure and the resulting residue treated with inorganic acids, when the bases (alkaloids) are extracted in water in their soluble salts. The aqueous layer containing the salt of alkaloids and soluble plant impurities is made basic with suitable base. The insoluble alkaloids are set free and get precipitated out. The

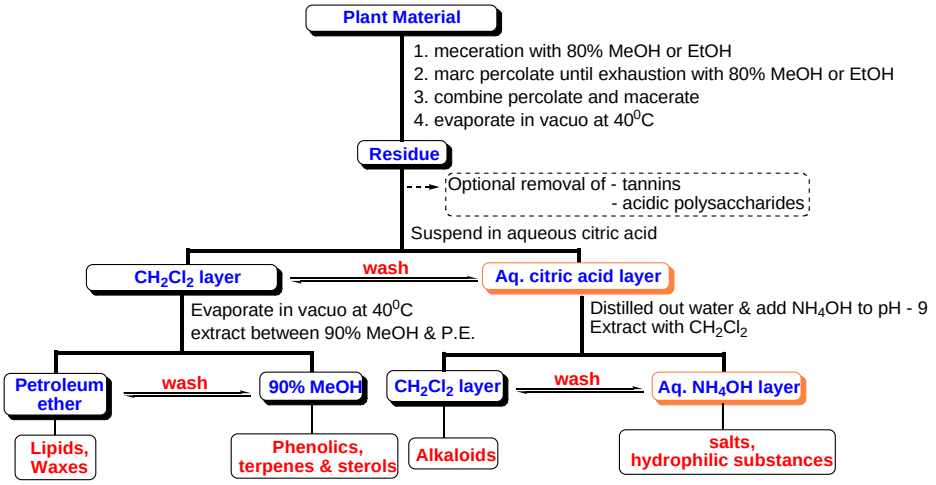


Figure 42: Flowsheet of separation of alkaloids by solvent extraction.

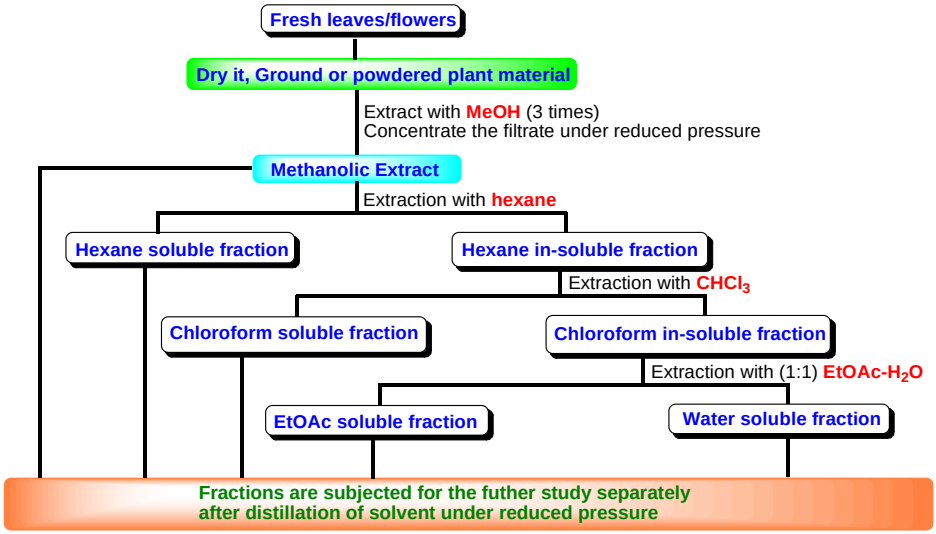


Figure 43: Flowsheet of separation of phytochemicals according to solubility in different

solid mass so obtained is then extracted with ether when alkaloids pass into ether solution.

Dry the extract with anhydrous sodium sulphate and evaporate the solvent under reduced pressure.

The number study suggest that, the rate of extraction by using different acids is – sulfuric acid > HCl > acetic acid but the content of alkaloid extracted in acetic acid than is higher than the aqueous solution of mineral acid. Therefore, it was suggested that, extraction was done by using acetic acid by maceration method.

Flow sheet for the extraction of terpenes from plant material:The extracting protocol used for the separation and extractions of terpenes from plant sample is depends on the nature of molecule - non-polar compounds containing methyl groups, moderately polar including acyl, ketone, ester, etc groups and polar molecules including hydroxyl group, volatile molecules, molecules with higher molecular weight such as glycosides,[57].

Most of the primary terpenes (linear terpenes and cyclized terpenes without any polar functional group) are exclusively composed of hydrocarbons, so these molecules are non-polar. The terpenes with 15 carbons or less may be volatile due to their small size and low polarity which allow emission to the atmosphere. Non-volatile terpenes can be extracted using a very non-polar organic solvent such as hexane.

Using silica as a stationary phase in chromatography is another perfect tool to separate these non-polar terpenes from other compounds in the extract. During silica chromatography, terpenes with more carbons usually elute more slowly than lower molecular weight compounds, and cyclized terpenes can elute faster than the corresponding non-cyclized terpene with the same number of carbons because of their compact size,[57].

Plant samples which having higher content of terpenes was air dried and Wiley milled to 20 mesh particle size. Select better solvent, chose extraction solvent systems were tested to determine the solvent that yielded the maximum amount of terpenes extracted from the sample such as methylene chloride, hexane (H), hexane:acetone (v/v, 1:1), hexane:diethyl ether (v/v, 1:1), and hexane:ethyl acetate (v/v, 1:1), etc. Methylene chloride is a more efficient extraction medium than the non-polar solvents; acceptable extraction efficiency was obtained after shaking for 24 hours (Figure 45). Add suitable amount of solvent to known quantity of powdered sample and place as it for different temperatures and for different time interval to select optimum condition of extraction. The extract solvent containing the terpenoid components was then aliquoted for separate analysis of mono-/sesquiterpenoids and resin acids. To analyze and separate for resin acids, add adequate quantity of 0.1 M aqueous ammonium carbonate into organic extract and shake well. The aqueous layer was removed with a pipette or separation flask and the organic layer was then dried by using suitable dehydrating agent for 1 h. If the organic layer containing any amount of organic bases (alkaloids) are separated by washing the organic layer with aqueous tartaric or acetic acid.

Flow sheet for the extraction of phenolics from plant material: The preparation and extraction of phenolic compounds from this wide range of samples depends mostly on the nature of the sample matrix and the chemical properties of the phenolics, including molecular structure, polarity, concentration, number of aromatic rings and hydroxyl groups. Variation in the chemistry of phenolics in a sample is depends

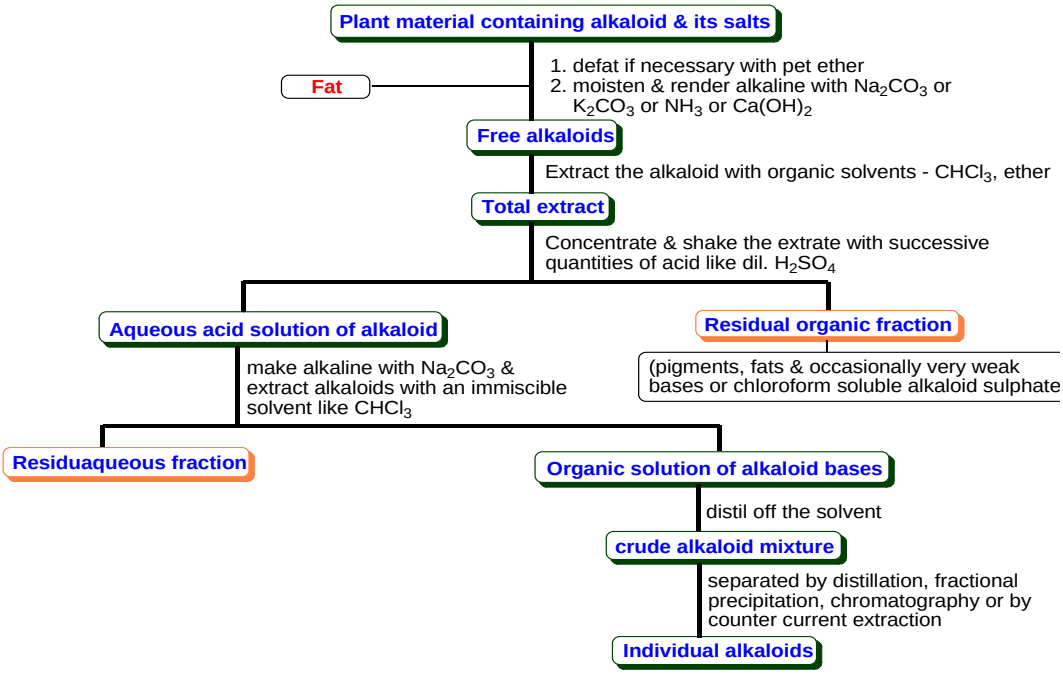


Figure 44a: Flowsheet of separation of alkaloids by solvent extraction

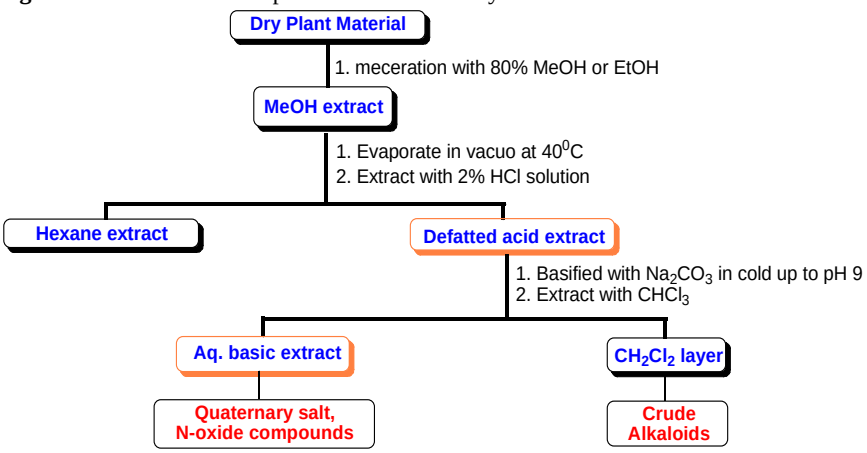


Figure 44b: Flowsheet of separation of alkaloids by solvent extraction

on the concentration of simple and complex polyphenolic compounds and the different proportions of phenolic acids, flavonoids, anthocyanins and proanthocyanins (among others). The samples need to be dried using freeze-drying, air-drying or oven-drying. Dried samples are milled or ground to obtain a certain particle size, whereas liquid samples are treated by centrifugation, filtration and purification using a separation system when required. Defatting processes can be applied to remove oil from lipid-containing samples by using hexane.

The most common techniques to extract phenolics employ solvents, either organic or inorganic. Several parameters may influence the yield of phenolics, including extraction time, temperature, solvent-to-sample ratio, the number of repeat extractions of the sample, as well as solvent type. The choice of extraction solvents such as water, acetone, ethyl acetate, alcohols (methanol, ethanol and propanol) and their mixtures,[58] will influence the yields of phenolics extracted. These differences could be due to the properties of the phenolic components of the plants concerned. Phenolics may bind to other sample elements such as carbohydrates and proteins. Acidic and alkaline hydrolysis is also employed in the isolation of phenolics from plants and plant products and is important for the stability of the phenolics in the extract.

Phenolic acids generally exist in a free, esterified or glycosylated form in plants. The sample is extracted by using alcohol, aqueous alcohol at room temperature followed by centrifugation.

The extract was then treated with 4 M HCl to reduce the pH to 2–3 and the phenolic fraction separated by using ethyl acetate and dried with anhydrous sodium sulfate. The bound or esterified phenolic acids of sample were extracted by removing the free phenolic acids and lipid using 70% ethanol and hexane, respectively. An aqueous suspension of the extract was then prepared and adjusted to pH 2 with 6 M HCl and the free phenolic acids extracted using diethyl ether. Apart from ethanol, mixtures of water with methanol, acetone and chloroform may be used for phenolic acid extraction from plant-based products. Flavonoids are highly bioactive compounds. They are often extracted with methanol, ethanol, acetone, water or mixtures of these solvents using heated reflux extraction methods. Following extraction, the flavonoid glycosides are frequently hydrolyzed into the aglycone forms by applying HCl under N_2 for 2 hrs at 80^0C. Anthocyanins are the most common pigments in nature and can be extracted with acidified solvents like water, acetone, ethanol, methanol or mixtures of aqueous solvents. The acid in the solvents acts to rupture cell membranes and release anthocyanins. It is therefore important to acidify solvents with organic acids (formic or acetic acid) rather than mineral acids such as 0.1% HCl.[59]

Phenolic compounds can exist as nonassociated forms in cell vacuoles (free phenols), or can be bound to cell wall

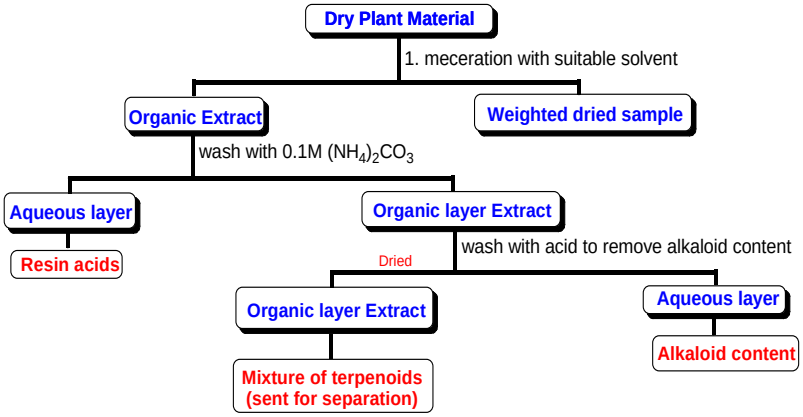


Figure 45: Flowsheet of separation of Terpenoids by solvent extraction

components through ester/ether linkages (bound phenols), or can form the structural matrix of lignin (lignin phenols) and tannins. These phenolic fractions have different biological significance therefore they should separate into three fractions by using following flow sheet diagram,[60]. Free phenols from sample are extracted by shaking with methanol at room temperature for about 3 – 8 hours in rotary shaker (*Figure 46*). The residue was dried at 50^{0}C overnight. For the bound phenol fraction, the residue was hydrolyzed with freshly prepared 1M NaOH under the argon atmosphere at 90^{0}C for 3 hours in water bath. Cool the mixture in ice bath and centrifuge, transfer the supernatant liquid and wash the residue with deionized water, dried the residue and used for lignin analysis. The aqueous layer was acidified by using 50% HCl (to maintain pH to < 2). The solution was centrifuge and discarded the precipitate. The solution is cooled at 40C for 20 minutes and the solution was extracted with ethyl acetate to get bounded phenol fraction. The lignin fraction was oxidatively depolymerized by using base hydrolyzed pellet (500 mg of CuO, 75 mg of $Fe(NH_4)_2(SO_4)_2.6H_2O$ and 5 ml of freshly prepared 2M NaOH in Teflon cup) and incubated at 155^{0}C for 3 hours. Cool the mixture and acidify by using 24N H_2SO_4 was added to adjust the pH to < 2. The depolymerized lignin-derived phenols were recovered using liquid–liquid extraction with 2 ml of ethyl acetate at 4°C.

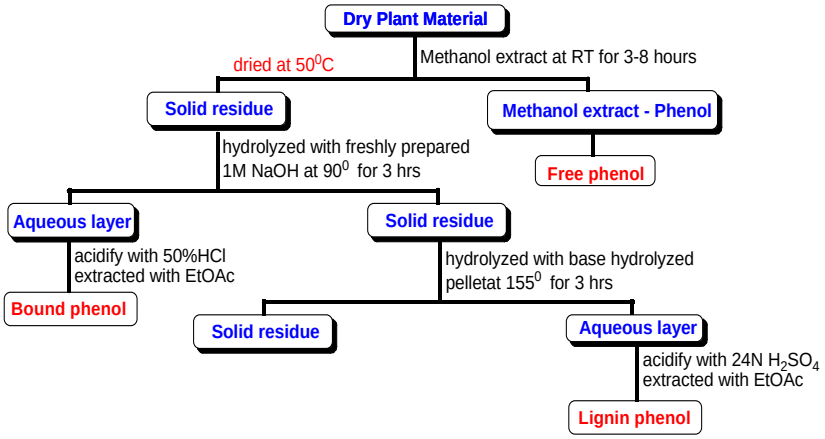


Figure 46: Flowsheet of separation of Terpenoids by solvent extraction

IDENTIFICATION OF CONSTITUENTS BY PHYTOCHEMICAL TEST:

The extracts in different solvents were subjected to qualitative tests for detection of phytoconstituents present in it as alkaloids, carbohydrates, glycosides, phytosterols, fixed oils & fats, phenolic compounds & tannins, proteins and free amino acids, gums & mucilages, flavanoids, lignins and saponins.[2]

ANALYSIS OF PHYTOCHEMICALS AND PLANT MATERIAL/PLANT EXTRACT:

The moisture content of fresh plant material was determined by AOAC method,[61a]. Take in triplicate, each fresh plant sample (100 g) was sliced into tiny pieces and was dried for approximately 24 h to a constant weight at 70^{0}C in a vacuum oven. The percentage of moisture content was calculated as follows:

$$Moisture\ content\ (\%) = \frac{original\ sample\ wt. - final\ sample\ wt.}{original\ sample\ wt.}\ x\ 100$$

Alternatively, the moisture content of the samples was determined by drying 10 g of the sample in an air oven at 105°C until constant weight (16–18 h) was achieved.

The bulk density was measured manually with the aid of measuring cylinder. First, 1 g of powder was added to a 10 ml cylinder. The powder was then added until it filled up every part of the cylinder at an equal level. The volume occupied by 1 g of powder was then measured. Bulk density of sample powder had been calculated from the relationship of mass of the powder and volume of the powder,[61c]. Porosity is a value that expresses the relative amount of pore space in the powder. The porosity was determined after identifying the bulk density and the particle density.

$$Porosity = 1 - \frac{Bulk\ density\ (\frac{g}{ml})}{Particle\ density\ \left(\frac{g}{ml}\right)}$$

.

The flowability of the powders was evaluated in terms of the Carr index (CI),[61b]. The CI was calculated from the bulk and tapped densities of the powder.

$$CI = 1 - \frac{\rho_{tapped} - \rho_{bulk}}{\rho_{tapped}}$$

Protein content was determined by using the A.O.A.C method,[62]. Briefly, powdered plant sample (1 g), add 50 ml of distilled water, 5 g of copper sulphate, and 15 ml of concentrated sulphuric acid were added into a Kjeldahl flask. The flask was partially closed by means of a funnel and the contents were digested by heating the flask at an inclined position in the digester. The mixture was heated for approximately 30 minutes until its volume was reduced to 70%. Standard sulphuric acid (0.1 M) and a few drops of

Sr. No.	Phytochemical	Test of phytochemical	Reagent/Chemicals of test	Observation
1.	Carbohydrates	Molish Test	1% alcoholic α-napthol, conc. H_2SO_4.	Purple or red colour ring at the junction
		Fehling Test	Fehling's A solution	Orange precipitate
		Barfoed's Test	Barfoed's reagent	Scanty brick red ppt
		Osazone Test	Acetic acid, Phenyl hydrazine hydrochloride, sodium acetate	Yellow crystalline solid
		Seliwanoff's Test	Dil HCl and resorcinol	Cherry red colour complex formation
		Benedicts Test	Solution of sodium citrate and sodium carbonate mixed with a solution of copper sulfate	Reddish precipitate
2.	Protein	Biuret Test	5% solution of NaOH, 1% solution of $CuSO_4$.	Pink coloration
		Ninhydrin Test	Ninhydrin reagent	Purple coloration
		Million's Test	Million's reagent	Red coloration
		Xanthoproteic Test	conc. HNO_3 and heat	Yellow precipitate
3.	Alkaloids (with hydrochloride of alkaloids)	Dragendroff's Test	Dragendroff's reagent (Solution of potassium bismuth iodide, potassium chlorate, a drop of hydrochloric acid, evaporated to dryness, and the resulting residue is exposed to ammonia vapour)	Orange or reddish brown ppt
		Mayer's Test	Mayer's reagent (Potassiomercuric iodide solution)	Cream ppt
		Wagner's Test	Wagner's reagent (Iodine in potassium iodide)	Reddish brown ppt
		Hager's Test	Hager's reagent (saturated solution of picric acid)	Yellow ppt
		Tannic acid Test	Tannic acid	Precipitation
4.	Steroids	Salkowski Test	chloroform and conc. sulphuric acid	red color in $CHCl_3$
		Liebermann Burchard Test	Chloroform, acetic anhydride, conc. H_2SO_4.	Bluish-green/pink/ red colour
5.	Saponins	Froth (Foam formation) Test	Water and shake vigorously	Persistent foam
6.	Glycosides	Legal's Test	Sample hydrolyzed with HCl, $CHCl_3$, dil NH_3.	Purple colour in ammoniacal layer
		Kellar Killani Test	Acetic acid, $FeCl_3$ solution, conc. H_2SO_4	Brown or greenish ring or violet ring
		--	Dilute HCl and 2 ml Sodium nitropruside in pyridine and sodium hydroxide solution	Formation of pink to blood red color - Cardiac glycosides
		Borntrager's Test (anthraquinones derivative)	Dil sulphuric acid, filter and extract with chloroform and dilute ammonia	ammonical layer becomes pink to red
		Modified Borntrager's Test	5% ferric chloride and dilute hydrochloric acid, shake with benzene, add NH_3 in organic layer	ammonical layer is pinkish red colour
7.	Starch	Tannic acid Test	20% tannic acid	Precipitate formation
		Iodine test	Iodine solution	Purple-black ppt
8.	Flavanoids	Shinoda Test	Alcohol, Magnesium, conc. HCl	Magenta colour
		Alkaline reagent Test	20% sodium hydroxide solution	intense yellow colour get disappear after acidification
		Lead acetate Test	10% lead acetate solution	yellow precipitate
9.	Tannins & Phenols	Ferric chloride Test	5% Ferric chloride solution	Green/Blue/Violet/ black colour
		Lead acetate Test	10% Lead acetate solution	White precipitate
		Gelatin Test (tannins)	1% solution of gelatin containing 10% NaCl	White precipitate
10.	Terpenoids	Copper acetate Test (diterpenes)	Chloroform, conc. H_2SO_4	Reddish brown ppt
		--	Dissolve in $CHCl_3$& heat to dryness, conc. H_2SO_4	Grayish color
		Salkowki's test	Dissolve in $CHCl_3$& conc. H_2SO_4	Reddish brown coloration at interface
11.	Lignins	--	Alcoholic solution, phloroglucinol, conc. HCl	Red coloration
12.	Quinones	--	conc. HCl, stand for some time	Yellow ppt
13.	Oxalate	--	Ethanoic acid glacial	Greenish black coloration

methyl red indicator is added to the clear solution. The flask was then placed below a condenser and the end of the adapter tube was dipped in the acid. Kjeldahl distillation apparatus was set up and 70 ml of 40% sodium hydroxide was added through the funnel. The funnel was then washed twice with 50 ml of distilled water and distillation was performed for one hour. The distilled ammonia was titrated with 0.1 M standard acid solution until the color changed from yellow to colorless. The experiment was repeated three times. The nitrogen content and protein content in the sample was calculated by using the following relation

$$Percentage\ of\ Nitrogen = \frac{(V_s - V_b) x\ M_A\ x\ 0.140\ x\ 100}{Weight\ of\ sample\ (W)}$$

Protein content = *Percentage of nitrogen* x 6.25 r

Where, V_s – volume in ml of standard acid used in titration of sample; V_b – volume in ml of standard acid used in titration of blank; M_A – molarities of the standard acid solution, W – weight of sample in g.

Fat content was determined using the soxhlet extraction method,[62]. A powdered plant sample (10 g) was placed in a cotton bag which was then placed in a soxhlet extractor. Petroleum ether (50 ml) was then poured into the soxhlet extractor and the extraction was allowed to proceed for 12 hours. After completion of extraction, excess petroleum ether was removed by distillation until the volume was reduced to about 15 ml. The residual petroleum ether removed by drying at 105^0C in an oven until constant weight was obtained. The experiment was repeated three times. The fat content was then calculated by using the following equation:

$$Percentage\ of\ content = \frac{weight\ of\ fat\ obtained\ from\ sample\ x\ 100}{Weight\ of\ sample\ (W)}$$

Crude fiber content was determined according to the AOAC method,[62]. A dried powdered sample was accurately weighed and placed into a conical flask. Hot sulphuric acid (200 ml, 1.25%) was added and the mixture was boiled for half an hour; water was added to maintain a constant level. The mixture was then filtered and washed with distilled water. The residue was transferred into a flask with 200 ml of sodium hydroxide solution (1.25%). It was then gently boiled for half an hour; water was continuously added into the flask to maintain a constant level. The residue was then filtered and washed with boiling water until neutral pH was achieved. It was then washed with alcohol and ether and then dried in an oven at 100^0C until constant weight was achieved. The fiber content was calculated by using the following equation:

$$Percentage\ of\ crude\ fiber = \frac{loss\ in\ weight\ x\ 100}{Weight\ of\ sample\ (W)}$$

Ash determination was performed as described by the AOAC method,[62]. Five grams of the dried, powdered sample was weighed in a crucible and then placed in a furnace at 500^0C until the substance turned into ash. The crucible was then cooled in desiccators and weighed. The procedure was repeated until a constant weight was obtained and the percentage of the total ash was calculated using the following formula,[63]:

$$Percentage\ of\ Ash = \frac{weight\ os\ Ash\ x\ 100}{Weight\ of\ sample\ (W)}$$

The **reducing sugar content** was determined using the Fehling's reducing method of Lane and Eynon,[64]. Weighed samples were placed into 250 ml round-bottomed flasks; add 20 ml 0.5 M sulphuric acid. Reflux the content on sand bath for 2.5 hours. The flask was then cooled and its content filtered with filter paper. The resulting residue was washed with warm distilled water (a total of 100 ml). A total of 10 ml of the solution was then neutralized with sodium carbonate. The mixture was topped up to 100 ml with distilled water. Fehling's solution (5 ml) was placed into a flask, followed by 5 ml of distilled water. The solution was boiled for 15 seconds. Several drops of methylene blue indicator was then added and titrated with the sample solution until the color changed from blue to green. The carbohydrate content was then calculated according to the following equation. **Carbohydrate content** can be calculated by multiplying reducing sugar content by a factor of 0.9.

$$Percentage\ of\ carbohydrate\ content = \frac{5\ x\ 0.005\ x\ 100\ x\ 100}{Weight\ of\ sample\ (W) x\ V\ x\ 10}$$

Where, V = volume of sample solution (titration volume), W= weight of powdered sample.

Reducing sugar content was determined using the Lane and Eynon titration method,[64]. Sample (1 g) was placed in 200 ml of distilled water and shaken for 30 minutes and filtered. Pipette out 20 ml sample filtrate, add 10 ml distilled water and finally add the mixture of Fehling's A (10 ml) and Fehling's B (10 ml) solution. The solution was mixed thoroughly, boiled for 5 minutes, and cooled down to room temperature in an ice bath. This was followed by the addition of 10 ml of 30% potassium iodide solution, 10 ml of 20% sulfuric acid solution and 1 ml of starch indicator solution. Titration was then performed until the blue starch-iodine color disappeared. Reducing sugar content was calculated using the following formula:

$$Percentage\ of\ reducing\ sugar = \frac{\_\_\_\_\ x\ 200\ x\ 100}{Wt\ of\ sample\ (W) x\ 20\ ml\ x\ 1000\ mg/g}$$

The **total phenol content** of the crude methanol extract and its fractions (hexane, chloroform, ethyl acetate, and water) were determined using the Folin-Ciocalteau method with slight modification,[65-68]. 200 μl (20 mg/ml) of each sample was added to 1 ml of 10% Folin-Ciocalteu reagent and 800 μl of 7.5% Na_2CO_3 solution. The mixture was shaken for five minutes and then incubated at 37^0C for 15 minutes, followed by incubation in the dark for 1 hr. Absorbance was then measured at 760 nm against distilled water as a blank. Gallic acid solution was used to construct a standard curve. The amount of total phenol content was calculated as mg/g gallic acid equivalent (GAE). For the gallic acid, the curve of absorbance versus concentration was described by the equation y = 0.00138x - 0.00247 (R^2 = 0.923), where, y = absorbance and x = concentration,[69a]. The total phenolic content of extracts and fractions of sample was calculated using the following formula:

$$Total\ phenol\ content = \frac{Gallic\ acid\ equivalent\ x\ V}{m}$$

Where, GAE is the gallic acid equivalence (mg/ml); V is the volume extract (ml) and m is the weight (g) of the pure plant extract.

Total phenol content (TPC) of the extract was determined using the Folin-Ciocalteau (FC) method modified by McDonald,[69b]. The freeze-dried extract (in any solvent) was dissolved in distilled water to make concentration of 50 mg/ml. A calibration curve was plotted by using standard Gallic acid (0-60 mg/ml) solution. The diluted extract or gallic acid (1.6 ml) was added to 0.2 ml FC reagent (5-fold diluted with distilled water) and mixed thoroughly for 3 minutes. Sodium carbonate (0.2 ml, 10% w/v) was added to the mixture and the mixture was allowed to stand for 30 minutes at room temperature. The absorbance of the mixture was measured at 760 nm by using UV-VIS-spectrophotometer. Total Phenol content was expressed as milligram gallic acid equivalent per gram defatted (mg GAE/g DFLA).

An alternative spectrophotometric method used to determine **total amount polyphenols** (TP) in tea extracts,[69c-d]. 1 ml of tea extract was transferred into a 25 ml volumetric flask to react with 5 ml dyeing solution (1 g ferrous sulfate and 5 g potassium sodium tartrate tetrahydrate dissolved in 1000 ml distilled water), 4 ml distilled water and 15 ml buffer (0.067 M potassium phosphate, pH 7.5). Several minutes were required for color development. Absorbance readings were made at 540 nm by spectrophotometer, using a blank solution prepared with distilled water replacing the tea extract. The total phenol content was calculated by the following equation:

$$Total\ phenol\ content\ (\frac{mg}{g}) = \frac{2A\ x\ 1.957\ x\ L_1\ x\ M}{L_2}$$

Where, L_1 is the total volume of extract solution in ml; L_2 is the volume of the extract solution used for analysis in ml; M is the mass of tea leaves in mg; A is the absorbance at 540 nm; 1.957 is constant, meaning that when absorbance at 540 nm was 0.5 under the earlier conditions, the concentration of TP was 1.957 mg/ml.

The **total flavonoid content** of the methanol extract of plant sample and its fractions was measured with modifications,[65,70,71a]. 1 ml of sample (1 mg/ml) was poured into a centrifuge tube. This was followed by the addition of 0.1 ml of 10% $Al(NO_3)_3$ solution, 0.1 ml of 1 M potassium acetate, and 3.8 ml of methanol. The content of the centrifuge tube was then mixed thoroughly with a vortex mixer for two to three minutes and allowed to stand for 10 minutes at room temperature. Absorbance was then measured at 410 nm. Quercetin was used as a standard compound for the quantification of total flavonoids. Total flavonoid content was calculated as quercetin equivalent (mg/g) using the equation obtained from the curve Y = 0.0869x + 0.15, R^2= 0.9684, where X is the absorbance and Y is the quercetin equivalent. The amount of flavonoids in the sample in quercetin equivalents was calculated using the formula:

$$Flavonoid\ content = \frac{A\ x\ m_0\ x\ 10}{A^0 x\ m}$$

Where: X = flavonoid content in mg quercetin/g; A = the absorbance of sample; A^0 = the absorbance of standard quercetin.

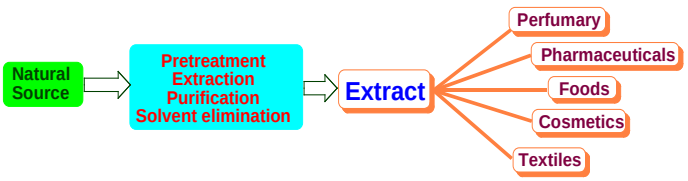
Along with this method, other two colorimetric methods are used for the detection of flavonoid contents – one by the method aluminum chloride ($AlCl_3$) for the quantification of flavones and flavonols (get yellow orange color) that react better with $AlCl_3$ and other by the method of 2,4-dinitrophenylhydrazin (DNP) for flavanones and flavanonols,[71b-c].

The method of Kumaran and Mbaebie,[72,73a] with slight modification was used to measure the **total flavonol content**. Briefly, 1 ml of extract or fractions (1 mg/ml) was added to a centrifuge tube with 2 ml of $AlCl_3$ prepared in ethanol and 3 ml of 50 g/lit sodium acetate solution. This was then mixed thoroughly with a vortex mixer and incubated for 1 hour. Absorbance was then measured at 440 nm with a spectrophotometer. Total flavonol content was calculated as quercetin equivalent (mg/g) using the following equation based on the calibration curve Y = 0.0297x + 0.1288, R^2 = 0.9729, where X is the absorbance and Y is the quercetin equivalent. The amount of flavonol in the sample in quercetin equivalents was calculated using the formula:

$$Flavonol\ content = \frac{A\ x\ m_0\ x\ 10}{A^0 x\ m}$$

Where: X = flavonoids content, mg quercetin/g; A = the absorbance of sample; A^0 = the absorbance of standard quercetin.

An alternative method,[73b]by using $AlCl_3$,was usedfor the determination of **flavonoid content**. A 50 μl aliquot of plant extract appropriately diluted, was mixed with 1250 μl of deionized water and 75 μl of 5 % sodium nitrite, after 6 min, 150 μl of 10 % $AlCl_3$ solution was added and the mixture was



allowed to stand for 5 min; followed by addition of 500 μl of 1 M sodium hydroxide. After 30 min of reaction, absorbance was read at 510 nm. The flavonoid content was assessed by reference to a calibration curve of catechin (0.2–1.0 mg/ml) and expressed as µg of catechin equivalent per mg of extract (µg CE/ mg).

Quantification of **flavanones and flavanonols** was accomplished using a spectrophotometric method,[73c], which was based on the interaction of these compounds with DNP in acid medium, to form phenylhydrazones. 40 μl of sample were dissolved in 80 μl of DNP solution (For 5 ml: 50 mg of DNP in 100 μl of 96 % sulfuric acid and 4850 μl of methanol), mixed and incubated at 50^{0}C for 50 min in a water bath, cooled at room temperature and 280 μl of 10 % potassium hydroxide (KOH) in water were added. Finally, the absorbance was measured at 486 nm. The total content of flavanones was determined using a calibration curve based on pinocembrin

(0.5–5.0 mg/ml) and expressed as µg of pinocembrin equivalent per milligram of extract (µg PNE/mg).

DPPH (1,1-Diphenyl-1-picrylhydrazyl) has a free radical initiator that tends to capture hydrogen from antioxidants compounds. Due to its free radical, the methanolic DPPH is violet and absorbs at 517 nm. **Antioxidant activity** screening of plant sample and their fractions was carried out by determining the DPPH free radical scavenging property using the UV spectrophotometric method,[70,74]. 50 μl of test solutions (different concentrations from dry extracts) were dissolved in water and added to 1.95 ml of DPPH in methanol. The mixtures were vortex-mixed and kept at room temperature, in the dark, for 30 minutes. The increase in absorbance was recorded at 517 nm. Methanol was used as a blank, methanol and DPPH solution was used as a negative control (A_0), and gallic acid, a standard phenolic compound, was used as a positive control. The antioxidant activity was represented as IC_{50}. The antioxidant activity was determined as the final concentration of the tested sample required for the prevention of the generation of DPPH radical by 50%,[75]. The DPPH radical concentration was calculated using the following equation:

$$Scavenging\ effect\ (\%) = \frac{(A_0 - A_1)\ x\ 100}{A_0}$$

Where, A_0 was the absorbance of the control reaction (blank) and A_1 was the absorbance in the presence of the tested extracts. The IC_{50} (concentration providing 50% inhibition) was calculated graphically using a calibration curve in the linear range by plotting the extract concentration versus the corresponding scavenging effect,[75].

A Ferric reducing antioxidant power (FRAP) reagent was prepared in acetate buffer (pH 3.6), 10 mmol 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mmol hydrochlorin acid and 20 mmol iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was freshly prepared. 5 μl of samples at 0.5 to 2 mg/ml diluted with 20 μl of distilled water were added to 150 μl of FRAP reagent,[76]. The absorbance of the mixture was measured using microplate spectrophotometer reader at 593 nm after 8 min. The standard curve was prepared with ascorbic acid (AA) and the results were expressed as µmol AA Equivalent/mg polyphenol-rich extract.

The **reducing power** of the dried extract was investigated by observing the transformation of Fe^{3+} to Fe^{2+},[77]. The extract was diluted with distilled water till obtain the concentration 60-220 mg/ml. The diluted extract (0.5 ml) was mixed with phosphate buffer (2.5 ml, pH 6.6) and potassium ferricyanide (2.5 ml, 1% w/w) in a test tube, followed by incubating in a water bath at 50^0C for 30 minutes. After the tube was removed from the water bath, trichloroacetic acid (2.5 ml, 10% w/v) was added into the tube and centrifuged (13,000 rpm per min, 10 minutes). The supernatant (2.5 ml) was diluted with distilled water (2.5 ml), and freshly prepared ferric chloride (0.5 ml, 0.1% w/w) was added. The mixture was mixed thoroughly and its absorbance was measured at 700 nm using a UV-VIS spectrophotometer.

APPLICATIONS OF SECONDARY METABOLITES:

Medicinal plants are the richest bio-resources of drugs of traditional medicinal systems, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceuticals, intermediate and chemical entitled for synthetic drugs. The primary benefits of the plant derived medicines are that they are relatively safer than synthetic alternatives, therapeutic benefits (fewer side effects) and more affordable treatment. Now in the societies, herbal remedies have become more popular in the treatment of minor ailments and also on account of the increasing costs of personal health maintenance.

Nearly 121 (45 tropical and 76 subtropical) major plant based drugs have been identified for which no synthetic one is currently available. Some of the useful plant based drugs includes - vinblastine, vincristine, taxol, podophyllotoxin, camptothecin, digitoxigenin, gitoxigenin, digoxigenin, tubocurarine, morphine, codeine, aspirin, atropine, pilocarpine, capscicine, allicin, curcumin, artemesinin and ephedrine, etc. In some cases, the crude extract of medicinal plants may be used as medicaments. The active molecule cannot be synthesized economically; then product must be obtained from the cultivation of plant material. It has been estimated that 14 - 28% of higher plant species are used medicinally and that 74% of pharmacologically active plant derived components were discovered after following up on ethno medicinal use of the plants.

ANTI-MICROBIAL ACTIVITY OF PLANTS AND SECONDARY METABOLITES:

Plant derived bioactive compounds have recently become of great interest owing to their versatile applications in medicinal field. Medicinal plants and herbs have been used for centuries as remedies for human infections and diseases which offer a new source of biologically active chemical compound as antimicrobial agent. Medicinal plants and herbs are the richest bio-resources of variety of bioactive molecules which are used as traditional medicinal systems, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceuticals, intermediate and chemical entitled for synthetic drugs. Recently the acceptance of traditional medicine derived from natural source as an alternative form of health care because of development of microbial resistance by the bacteria and fungi to the available medicines and antibiotics. So, for human and animals, the plants natural products having antimicrobial activity are best biorational alternatives to the chemical drugs used today. Medicinal plant based antimicrobial agents represent a vast untapped source of pharmaceuticals and further exploration of plant antimicrobials need to occur for treatment of infectious diseases both in plants and humans while simultaneously for mitigating many of the side effects that are often associated with synthetic antimicrobials. Out of the several hundred thousand medicinal plant and herbs species around the globe, only few of them has been investigated both phytochemically and pharmacologically,[44]. Natural drugs used in modern medicine were used in crude form for traditional or folk healing practices but the benefits of plant derived medicines was that they were relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment. Phytomedicines represent vast untapped

Common name	Scientific name	Compound	Class	Activity
Aloe	*Aloe barbadensis, Aloe vera*	Latex	Extract mixture	*Corynebacterium, Salmonella, Streptococcus*
Basil	*Ocimum basilicum*	Essential oils	Terpenoids	*Salmonella*, bacteria
Bay	*Laurus nobilis*	Essential oils	Terpenoids	Bacteria, fungi
Chili peppers, paprika	*Capsicum annuum*	Capsaicin	Terpenoid	Bacteria
Fava bean	*Vicia faba*	Fabatin	Thionin	Bacteria
Onion	*Allium cepa*	Allicin	Sulfoxide	Bacteria, *Candida*
Turmeric	*Curcuma longa*	Curcumin	Terpenoids	Bacteria, protozoa

sources of drugs effective in treating infectious diseases simultaneously mitigating many of the side effects of synthetic antimicrobials,[78].

A multitude of plant compounds (often of unreliable purity) is readily available over-the-counter from herbal suppliers and natural-food stores, and self-medication with these substances is common place. Medicinal plants are the richest bio-resources of drugs of traditional medicinal systems, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceuticals, intermediate and chemical entitled for synthetic drugs,[79]. The evaluation for antimicrobial agent of plant and herbs origin begins with thorough biological evaluation of plant or herb extracts to ensure efficacy and safety followed by identification of active principles, dosage formulations, efficacy and pharmacokinetic profile of the new drug. Many plants have been used because of their antimicrobial traits and antimicrobial properties of plants have been investigated by a number of researchers worldwide. Ethno pharmacologists, botanists, microbiologists and natural product chemists are searching the world for phytochemicals which could be developed for treatment of infectious diseases. Plants have limited ability to synthesize aromatic secondary metabolites, most of which are phenols, aldehydes, carboxylic acids, ketones or their oxygen-substituted derivatives. Important subclasses of these oxygenated compounds include phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins. These groups of compounds show good antimicrobial effect and serves as plant defense mechanisms against pathogenic microorganisms.[44] Antimicrobial activity of *Cassia alata* was noticed against *E. coli* and fungi. Water extract *of Senna alata* showed antimicrobial activity against *B. subtilis* only but other reports of antimicrobial activity exists.[80]

Now days, resistance in Gram-negative bacteria has been increasing. A methanol extracts of Cameroonian medicinal plants such as *Albizia adianthifolia*, *Alchornea laxiflora*, *Boerhavia diffusa*, *Combretum hispidum*, *Laportea ovalifolia*and *Scoparia dulcis*against was tested against a panel of 15 multidrug resistant Gram-negative bacterial strains and determine the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the extracts. The result shows that the extracts containing the classes of polyphenols and triterpenes, other classes of chemicals. The best antibacterial activities were recorded with bark and root extracts of *A. adianthifolia*as well as with *L. ovalifolia*extract, with MIC values ranging from 64 to 1024 µg/ml on 93.3% of the fifteen tested bacteria. The lowest MIC value of 64 µg/ml was recorded with*A. laxiflora*bark extract against *Enterobacter aerogenes*EA289.[81] The result suggested that, the antibacterial activity of the tested plants is due to mixture of phytochemicals but these mixture phytochemicals, active component is not separated.

The leaves of *Ampelopsis grossedentata* in the form of Rattan tea was potentially perform multiple pharmacological roles, including anti-bacterial, anti-cancer, antioxidant, hepatoprotective and anti-hypertension functions. These beneficial functions of Rattan tea are strongly associated due to the presence of bioactive compound ampelopsin, a major flavonoid compound. The ampelopsin extract exerts strong inhibitory effects on *Staphylococcus aureus* and *Bacillus subtilis*. Ampelopsin, as the predominant compound in Rattan tea, possesses potent anti-inflammatory activity whereby it can inhibit the activation of NF-κB and PI3K/Akt pathways, and inhibit oxidative stress by scavenging free radicals and attenuating lipid oxidation as well as reducing ROS levels,[82].

The phytochemicals of dry leaves extracts of *H. rosa-sinensis* L. in water and methanol subjected for the analysis contains varying amount of alkaloids, tannin, saponins, flavonoids, cardiac glycosides, anthraquinones and phlobatanins, The quantitative analysis of the leaves shows that it contain carbohydrate (CHO) (31.66%), moisture content (2.63%) and high fibre content (3.99%) while the protein value (7.01%). The results show that hot aqueous extraction had maximum zone of inhibition against *Bacillus subtilis*, *Escherichia coli (E. coli)* and *Staphylococcus aureus* while the methanol extract showed zones of inhibitions against *B. subtilis*, *E. coli*, *S.aureus*.[83a] The *Terminalia arjuna* bark is known to have beneficial effect in myocardial disease besides evaluated for antibacterial activity.[83b]

The leaf extracts are high enough in essential nutrients required for optimal physiological performance and the maintenance of good health. This is in line with the report of Hussain et al,[84] that tannins are plant metabolites which are useful in wound healing.

The leaves, flowers and roots of the plant *Hibiscus rosa-sinensis* was collected, washed and dried. The dried sample of roots, leaves and flowers of the plant was extracted with different solvents water, alcohol, acetone, petroleum ether, chloroform and ethyl acetate. All these extracts are subjected for the testing antimicrobial activity against Gram positive and Gram-negative bacteria and fungal strains by measuring zone of inhibition.[85] The leaf extract showed high activity against *Staphylococcus aureus* at very low concentration compared other strains such as *E.coli*, *Bacillus subtilis*, leaf extractshowed high activity against *Candida parapsilosis* compared to *Aspergillus niger*. The root extract showed high activity against all the bacteria and fungi strain *Candida parapsilosis* and *Aspergillus niger*at a very low concentration compared to

Abrus precatorius L.:	Acacia Catechu	Acacia Concinna	Adonis Vernalis L.
Aesculus Hippocastanum L.	Berberis vulgaris	Azadirachta Indica	Ananas Comosus (L.) Merrill
Androqraphis paniculata	Boerhavia diffusa	Camellia Sinensis	Camellia Sinensis (L.) Kuntze
Camptotheca acuminate	Catharanthus roseus	Cinnamomum tamala	Cissampelos Pareira L.
Colchicum autumnale L.	Curcuma Longa	Ficus benghalensis	Crotalaria sessiliflora L.
Erythroxylum coca Lamk	Larrea divaricata Cav.	Leptadenia reticulate	Lawsonia inermis Linn.
Lpomoea carnea Jacg	Nigella sativa	Murraya Koenigii	Mangifera Indica L.
Petroselinum crisoum	Ocimum sanctum	Phyllanthus emblica	Papaver somniferum L.
Rauvolfia canescens L.	Plantago major	Piper Longum L.	Podaphyllum peltatum L.
Salvia macrociphon	Silybum Marianum	Riccinus communis	Stephania sinica Diels
Terminalia arjuna	Swertia Chirata	Taxus brevifolia	Terminalia chebula
Tetraclinis articulate	Tinospora cordifolia	Thymus vulgaris L.	Uncaria tomentosa
Withania Somnifera	Zinziber officinalis		

Trichophyton rubrum. The flowers extract showed strong activity against *E.coli and Staphylococcus aureus* cultures. Flower extract showed high activity against *Candida parapsilosis* and *Aspergillus niger* as compared to *Trichophyton rubrum*.[85]

The *in-vitro* antibacterial activities are reported for methanolic extract of five Cameroonian edible plants namely *Colocasia esculenta*, *Triumfetta pentandra*, *Hibiscus esculentus*, *Canarium schweinfurthii*and *Annona muricata* against a panel of 19 multidrug resistant Gram-negative bacterial strains.[86]

All the extracts of the plant material contained the classes of polyphenols, alkaloids, triterpenes and steroids, other classes of chemicals which are tested by suitable test. *Canarium schweinfurthii* extract showed the best activity while *C. schweinfurthii* displayed the lowest MIC value against all the tested bacteria strains. Moderate or weak activities were recorded for the most of plant extracts. Differences in the antibacterial activities of the extracts are observed due to their chemical composition as well as in the mechanism of action of their bioactive constituents.

The methanolic extract of leaves of *Eucalyptus tereticornis* was tested for antibacterial activity against the bacterial strain *Escherichia coli*.[87]

A fresh plant leaves collected and dried and extract the bioactive components in methanol. At the optimum conditions for the maximum antibacterial activity (zones of inhibition, mm) of *E. tereticornis* extract was scanned and finally the results shows suggest the optimum conditions as - Limonene, 3 ml; 1,8-Cineole, 11 ml; Terpinen-4-ol, 15 ml; pH, 7.0 and temperature, 40^0C. All the concentrations of the plant extract were effective against the bacterial strain. The results suggest that the methanolic extract of *E. tereticornis* could be used as new and effective herbal medicines to combat infections caused by multi-drug resistant microbial strains from community and hospital settings.

The plants showing anti-bacterial are listed in above table.

CONCLUSION

Utilization of different plants and herbs for medicinal purposes in India has been documented long back in ancient literature (Ayurved, Unani) because they containing variety of secondary metabolites which are essential for human survival. Traditional medicinal system is widely distributed in India, especially in villages and Tribes. A major proportion of population mostly belonging to rural areas of the world is still dependent on traditional system of medicines for their various health needs. Therefore, traditional and cultural medical knowledge has a catalyzing effect in meeting health care demands. From the available literature survey, it should clearly show that number plants having different bioactive compounds which we should know, identify and separated by using different separation techniques. That separated compounds now serve as an important source forthe treatment of many infections, diseases, and used in different day to day human and mankind activities. These bioactive compounds were extensively used in an Indian medicine system either in pure form, extract of one plant or mixture of the different plants and natural products. In most of cases, natural products are used as important precursors for the synthesis of different drugs and medicinally important compounds. Plant are useful for the treatment of different infections and diseases such as abdominal troubles, bronchitis, tumors, loss of consciousness, asthma, leucoderma, piles, inflammation, enlargement of spleen, anemia, ulcers, cutaneous diseases, dropsy, dyspepsia, pain, flatulence intermittent fever, fever and in chronic respiratory troubles due to present of different natural products as Euphol, monohydroxy triterpenes, nerifoliol, taraxerol, flavonoids, steroidal saponins, sugar, tannins, alkaloids, β-amyrion, proteins, glycosides, alkaloids, phenolics, etc. in appreciable, moderate and trace amount. Now days, developed society has been successfully used plants and their products or extracts in different forms for the many health problem since a long period of time for the treatment of wide variety of health issues. There is scope for development of newer drug molecule or mixture for the treatment of multiple diseases simply by changing the dosage.

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