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Review Article

A review on identification strategy of phyto constituents present in herbal plants

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ABSTRACT

Plants have been used as medicine in ancient. Now day's Pharmaceutical companies start processing of medicinal and aromatic plants in their formulation by using extraction of active components. Extraction of plant components like volatile, Essential or ethereal oils and mixtures composed of volatile liquid and solid compounds depend upon their composition and their boiling point. Now days there are several processes like distillation, enfleurage, maceration, expression, solvent extraction and fluid extraction are available for extraction of plant components. This review also summarizes the characters of phytoconstituents, choice of solvents, influence of solvents, extraction strategy, procedures for extraction of herbal drugs and treatment of drug residue after extraction.

Keywords: Secondary metabolites; Phytoconstituents; Enfluerage

1. INTRODUCTION

Natural products are secondary metabolites which are derived from herb or animal sources. Natural products are chemical compounds found in nature and they have pharmacological and biological activity. Natural products are generally used in drug discovery and drug design. Separation of a single molecular entity is very difficult from complex mixtures contain fats, oils, alkaloid, tannins and glycoside. In 1803 the first alkaloid, nicotine and then morphine, strychnine, emetine and many others were separated. This review compiles the recent literature with special focus on various approaches for extraction. This review also summarizes choice of solvent, interfering compound and strategies involve in extraction.

Medicinal plants have been the mainstay of traditional herbal medicine amongst rural dwellers worldwide since antiquity to date. The therapeutic use of plants certainly goes back to the Sumerian and the Akkadian civilizations in about the third millenium BC. Hippocrates (ca. 460–377 BC), one of the ancient authors who described medicinal natural products of plant and animal origins, listed approximately 400 different plant species for medicinal purposes. Natural products have been an integral part of the ancient traditional medicine systems, e. g. Chinese, Ayurvedic and Egyptian (Daffre et al., 2008). Over the years they have assumed a very central stage in modern civilization as natural source on chemotherapy as well as amongst scientist in search for alternative sources of drugs. About 3.4 billion people in the developing world depend on plant-based traditional medicines. This represents about 88 per cent of the world's inhabitants, who rely mainly on traditional medicine for their primary health care. (Doughari et al., 2009; Yadav et al., 2003).

According to the World Health Organization, a medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi synthesis. Such a plant will have its parts including leaves, roots, rhizomes, stems, barks, flowers, fruits, grains or seeds, employed in the control or treatment of a disease condition and therefore contains chemical components that are medically active. These non-nutrient plant chemical compounds or bioactive components are often referred to as phyto chemicals ('phyto-' from Greek - *phyto* meaning 'plant') or phytoconstituents and are responsible for protecting the plant against microbial infections or infestations by pests. (Liu, 2004) The study of natural products on the other hand is called phytochemistry. Phytochemicals have been isolated and characterized from fruits such as grapes and apples, vegetables such as broccoli and onion, spices such as turmeric, beverages such as green tea and red wine, as well as many other sources. (Bohlin et al., 1993) The science of application of these indigenous or local medicinal remedies including plants for treatment of diseases is currently called ethno pharmacology but the practice dates back since antiquity. Ethno pharmacology has been the mainstay of traditional medicines the entire world and currently is being integrated into mainstream medicine.

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Different catalogues including *De Materia Medica, Historia Plantarum, Species Plantarum* have been variously published in attempt to provide scientific information on the medicinal uses of plants. The types of plants and methods of application vary from locality to locality with 80% of rural dwellers relying on them as means of treating various diseases. For example, the use of bearberry (*Arctostaphylos uva-ursi*) and cranberry juice (*Vaccinium macrocarpon*) to treat urinary tract infections is reported in different manuals of phytotherapy, while species such as lemon balm (*Melissa officinalis*), garlic (*Allium sativum*) and tee tree (*Melaleucaalternifolia*) are described as broad-spectrum antimicrobial agents. A single plant may be used for the treatment of various disease conditions depending on the community. Several ailments including fever, asthma, constipation, esophageal cancer and hypertension have been treated with traditional medicinal plants. The plants are applied in different forms such as poultices, concoctions of different plant mixtures, infusions as teas or tinctures or as component mixtures in porridges and soups administered in different ways including oral, nasal (smoking, snoffing or steaming), topical (lotions, oils or creams), bathing or rectal (enemas). Different plant parts and components (roots, leaves, stem barks, flowers or their combinations, essential oils) have been employed in the treatment of infectious pathologies in the respiratory system, urinary tract, gastrointestinal and biliary systems, as well as on the skin. (Cowan, 1999)

Medicinal plants are increasingly gaining acceptance even among the literates in urban settlements, probably due to the increasing inefficacy of many modern drugs used for the control of many infections such as typhoid fever, gonorrhoea, and tuberculosis as well as increase in resistance by several bacteria to various antibiotics and the increasing cost of prescription drugs, for the maintenance of personal health. Unfortunately, rapid explosion in human population has made it almost impossible for modern health facilities to meet health demands all over the world, thus putting more demands on the use of natural herbal health remedies.

Current problems associated with the use of antibiotics, increased prevalence of multiple-drug resistant (MDR) strains of a number of pathogenic bacteria such as methicillin resistant *Staphylococcus aureus, Helicobacter pylori,* and MDR *Klebsiela pneumonia* has revived the interest in plants with antimicrobial properties. In addition, the increase in cases of opportunistic infections and the advent of Acquired Immune Deficiency Syndrome (AIDS) patients and individuals on immunosuppressive chemotherapy, toxicity of many antifungal and antiviral drugs has imposed pressure on the scientific community and pharmaceutical companies to search alternative and novel drug sources.

2. CLASSES OF PHYTOCONSTITUENTS

2.1 ALKALOIDS

These are the largest group of secondary chemical constituents made largely of ammonia compounds comprising basically of nitrogen bases synthesized from amino acid building blocks with various radicals replacing one or more of the hydrogen atoms in the peptide ring, most containing oxygen. The compounds have basic properties and are alkaline in reaction, turning red litmus paper blue. 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. The degree of basicity varies considerably, depending on the structure of the molecule, and presence and location of the functional groups (Daffre et al., 2008) They react with acids to form crystalline salts without the production of water.

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. These nitrogenous compounds function in the defence of plants against herbivores and pathogens, and are widely exploited as pharmaceuticals, stimulants, narcotics, and poisons due to their potent biological activities. In nature the alkaloids exist in large proportions in the seeds and roots of plants and often in combination with vegetable acids. Alkaloids have pharmacological applications as anesthetics and CNS stimulants. More than 12, 000-alkaloids are known to exist in about 20% of plant species and only few have been exploited for medicinal

Table 1: Methods for detection of alkaloids

Reagents	Composition of the reagent	Result
Meyer's reagent	Potassium mercuric iodide solution	Cream precipitate
Wagner's reagent	Iodine in potassium iodide	Reddish-brown precipitate
Tannic acid	Tannic acid	Precipitation
Hager's reagent	A saturated solution of picric acid	Yellow precipitate
Dragendorff'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 precipitate (except with caffeine and a few other alkaloids

purposes. The name alkaloid ends with the suffix –*ine* an plant-derived alkaloids in clinical use include the analgesics morphine and codeine, the muscle relaxant (+) -tubocurarine, the antibiotics sanguinafine and berberine, the anticancer agent vinblastine, the antiarrythmic ajmaline, the pupil dilator atropine, and the sedative scopolamine. Other important alkaloids of plant origin include the addictive stimulants caffeine, nicotine, codeine, atropine, morphine, ergotamine, cocaine, nicotine and ephedrine. Amino acids act as precursors for biosynthesis of alkaloids with ornithine and lysine commonly used as starting materials.

2.2 GLYCOSIDES

Glycosides in general, are defined as the condensation products of sugars (including polysaccharides) with a host of different varieties of organic hydroxy (occasionally thiol) compounds (invariably monohydrate in character), in such a manner that the hemiacetal entity of the carbohydrate must essentially take part in the condensation. Glycosides are colorless, crystalline carbon, hydrogen and oxygen-containing (some contain nitrogen and sulfur) water-soluble phytoconstituents, found in the cell sap. Chemically, glycosides contain a carbohydrate (glucose) and a non-carbohydrate part (aglycone or genin). Alcohol, glycerol or phenol represents aglycones. Glycosides are neutral in reaction and can be readily hydrolyzed into its components with ferments or mineral acids. Glycosides are classified on the basis of type of sugar component, chemical nature of aglycone or pharmacological action. The rather older or trivial names of glycosides usually has a suffix 'in' and the names essentially included the source of the glycoside, for instance: strophanthidin from *Strophanthus*, digitoxin from *Digitalis*, barbaloin from *Aloes*, salicin from *Salix*, cantharidin from *Cantharides*, and prunasin from *Prunus.* However, the systematic names are invariably coined by replacing the "ose" suffix of the parent sugar with "oside". This group of drugs are usually administered in order to promote appetite and aid digestion.

Glycosides are purely bitter principles that are commonly found in plants of the Genitiaceae family and though they are chemically unrelated but possess the common property of an intensely bitter taste. The bitters act on gustatory nerves, which results in increased flow of saliva and gastric juices. Chemically, the bitter principles contain the lactone group that may be diterpene lactones (e. g. *andrographolide*) or triterpenoids (e. g. *amarogentin*). Some of the bitter principles are either used as astringents due to the presence of tannic acid, as antiprotozoan, or to reduce thyroxine and metabolism. Examples include cardiac glycosides (acts on the heart), anthracene glycosides (purgative, and for treatment of skin diseases), chalcone glycoside (anticancer), amarogentin, gentiopicrin, andrographolide, ailanthone and polygalin reported that extracts of plants that contain cyanogenic glycosides are used as flavouring agents in many pharmaceutical preparations. (Cowann, 1999) Amygdalin has been used in the treatment of cancer (HCN liberated in stomach kills malignant cells), and also as a cough suppressant in various preparations. Excessive ingestion of cyanogenic glycosides can be fatal. Some foodstuffs containing cyanogenic glycosides can cause poisoning (severe gastric irritations and damage) if not properly handled. To test for O-glycosides, the plant samples are boiled with HCl/H_2O to hydrolyse the anthraquinone glycosides to respective aglycones, and an aqueous base, e. g. NaOH or NH_4OH solution, is added to it. For C-glycosides, the plant samples are hydrolysed using $FeCl_3$/HCl, and and an aqueous base, e. g. NaOH or NH_4OH solution, is added to it. In both cases a pink or violet colour in the base layer after addition of the aqueous base indicates the presence of glycosides in the plant sample.

2.3 Flavonoids

Flavonoids re important group of polyphenols widely distributed among the plant flora. Stucturally, they are made of more than one benzene ring in its structure (a range of C15 aromatic compounds) and numerous reports support their use as antioxidants or free radical scavengers. (Yadav et al., 2003) The compounds are derived from parent compounds known as flavans. Over four thousand flavonoids are known to exist and some of them are pigments in higher plants. Quercetin, kaempferol and quercitrin are common flavonoids present in nearly 70% of plants. Other group of flavonoids include flavones, dihydroflavons, flavanflavonols, anthocyanidins, proanthocyanidins, calchones and catechin and leuco anthocyanidins.

2.4 Phenolics

Phenolics, phenols or polyphenolics (or polyphenol extracts) are chemical components that occur ubiquitously as natural colour pigments responsible for the colour of fruits of plants. Phenolics in plants are mostly synthesized from phenylalanine via the action of phenylalanine ammonia lyase (PAL). They are very important to plants and have multiple functions. The most important role may be in plant defence against pathogens and herbivore predators, and thus are applied in the control of human pathogenic infections. (Liu, 2004) They are classified into (i) phenolic acids and (ii) flavonoid polyphenolics (flavonones, flavones, xanthones and catechins) and (iii) non-flavonoid polyphenolies. Caffeic acid is regarded as the most common of phenolic compounds distributed in the plant flora followed by chlorogenic acid known to cause allergic dermatitis among humans. Phenolics essentially represent a host of natural antioxidants, used as nutraceuticals, and found in apples, green-tea, and red-wine for their enormous ability to combat cancer and are also thought to prevent heart ailments to an appreciable degree and sometimes are anti-inflammatory agents. Other examples include flavones, rutin, naringin, hesperidin and chlorogenic.

2.5 Saponins

The term saponin is derived from *Saponaria vaccaria* (*Quillaja saponaria*), a plant, which abounds in saponins and was once used as soap. Saponins therefore possess 'soaplike' behaviour in water, i. e. they produce foam. On hydrolysis, an aglycone is produced, which is called sapogenin. There are two types of sapogenin: steroidal and triterpenoidal. Usually, the sugar is attached at C-3 in saponins, because in most sapogenins there is a hydroxyl group at C-3. *Quillaja saponaria* is known to contain toxic glycosides quillajic acid and the sapogenin senegin. Quillajic acid is strenutatory and senegin is toxic. Senegin is also present in *Polygala senega*. Saponins are regarded as high molecular weight compounds in which, a sugar molecule is combined with triterpene or steroid aglycone.

There are two major groups of saponins and these include: steroid saponins and triterpene saponins. Saponins are soluble in water and insoluble in ether, and like glycosides on hydrolysis, they give aglycones. Saponins are extremely poisonous, as they cause heamolysis of blood and are known to cause cattle poisoning. They possess a bitter and acrid taste, besides causing irritation to mucous membranes. They are mostly amorphous in nature, soluble in alcohol and water, but insoluble in non-polar organic solvents like benzene and n-hexane. Saponins are also important therapeutically as they are shown to have hypolipidemic and anticancer activity. Saponins are also necessary for activity of cardiac glycosides. The two major types of steroidal sapogenin are diosgenin and hecogenin. Steroidal saponins are used in the commercial production of sex hormones for clinical use. For example, progesterone is derived from diosgenin. The most abundant starting material for the synthesis of progesterone is diosgenin isolated from *Dioscorea* species, formerly supplied from Mexico, and now from China. (Cowann, 1999) Other steroidal hormones, e. g. cortisone and hydrocortisone, can be prepared from the starting material hecogenin, which can be isolatedfrom Sisal leaves found extensively in East Africa.

2.6 Tannins

These are widely distributed in plant flora. They are phenolic compounds of high molecular weight. Tannins are soluble in water and alcohol and are found in the root, bark, stem and outer layers of plant tissue. Tannins have a characteristic feature to tan, i. e. to convert things into leather. They are acidic in reaction and the acidic reaction is attributed to the presence of phenolics or carboxylic group. They form complexes with proteins, carbohydrates, gelatin and alkaloids. Tannins are divided into hydrolysable tannins and condensed tannins. Hydrolysable tannins, upon hydrolysis, produce gallic acid and ellagic acid and depending on the type of acid produced, the hydrolysable tannins are called gallotannins or egallitannins. On heating, they form pyrogallic acid. Tannins are used as antiseptic and this activity is due to presence of the phenolic group. Common examples of hydrolysable tannins include theaflavins (from tea), daidezein, genistein and glycitein Tanninrich medicinal plants are used as healing agents in a number of diseases. In Ayurveda formulations based on tannin-rich plants have been used for the treatment of diseases like leucorrhoea, rhinnorhoea and diarrhea.

2.7 Terpenes

Terpenes are among the most widespread and chemically diverse groups of natural products. They are flammable unsaturated hydrocarbons, existing in liquid form commonly found in essential oils, resins or oleoresins. Terpenoids includes hydrocarbons of plant origin of general formula (C5H8) n and are classified as mono-, di-, tri- and sesquiterpenoids depending on the number of carbon atoms. Examples of commonly important monterpenes include terpinen-4-ol, thujone, camphor, eugenol and menthol. *Diterpenes (C20)* are classically considered to be resins and taxol, the anticancer agent, is the common example. The t*riterpenes (C30)* include steroids, sterols, and cardiac glycosides with anti-inflammatory, sedative, insecticidal or cytotoxic activity. Common triterpenes: amyrins, ursolic acid and oleanic acid s*esquiterpene (C15*) like monoterpenes, are major components of many essential oils (Martinez *et al.,.,* 2008). The sesquiterpene acts as irritants when applied externally and when consumed internally their action resembles that of gastrointestinal tract irritant. A number of sesquiterpene lactones have been isolated and broadly they have antimicrobial (particularly antiprotozoal) and neurotoxic action. The sesquiterpene lactone, palasonin, isolated from *Butea monosperma* has anthelmintic activity, inhibits glucose uptake and depletes the glycogen content in *Ascaridia galli.* Terpenoids are classified according to the number of isoprene units involved in the formation of these compounds.

2.8 Anthraquinones

These are derivatives of phenolic and glycosidic compounds. They are solely derived from anthracene giving variable oxidized derivatives such as anthrones and anthranols. Other derivatives such as chrysophanol, aloe-emodin, rhein, salinos poramide, luteolin and emodin have in common a double hydroxylation at positions C-1 and C-8. To test for free anthraquinones, powdered plant material is mixed with organic solvent and filtered, and an aqueous base, e. g. NaOH or NH_4OH solution, is added to it. A pink or violet colour in the base layer indicates the presence of anthraquinones in the plant sample. (Cowann, 1999).

2.9 Essential oils

Essential oils are the odorous and volatile products of various plant and animal species. Essential oils have a tendency evaporate on exposure to air even at ambient conditions and are therefore also referred to as

volatile oils or ethereal oils**.** They mostly contribute to the odoriferous constituents or *'essences'* of the aromatic plants that are used abundantly in enhancing the aroma of some spices. Essential oils are either secreted either directly by the plant protoplasm or by the hydrolysis of some glycosides and structures such as directly Plant structures associated with the secretion of essential oils include: Glandular hairs (Lamiaceae e. g. *Lavandula angustifolia*), Oil tubes (or vittae) (Apiaceae eg. *Foeniculum vulgare*, and Pimpinella anisum (Aniseed), modified parenchymal cells (Piperaceae e. g. *Piper nigrum* - Black pepper), Schizogenous or lysigenum passages (Rutaceae e. g. *Pinus palustris* - Pine oil. Essential oils have been associated with different plant parts including leaves, stems, flowers, roots or rhizomes. Chemically, a single volatile oil comprises of more than 200 different chemical components, and mostly the trace constituents are solely responsible for attributing its characteristic flavour and odour. (Liu, 2004) Essential oils can be prepared from various plant sources either by direct steam distillation, expression, extraction or by enzymatic hydrolysis. Direct steam distillation involves the boiling of plant part in a distillation flask and passing the generated steam and volatile oil through a water condenser and subsequently collecting the oil in florentine flasks. Depending on the nature of the plant source the distillation process can be either water distillation, water and steam distillation or direct distillation. Expression or extrusion of volatile oils is accomplished by either by sponge method, scarification, rasping or by a mechanical process. In the sponge method, the washed plant part e. g. citrous fruit (*e. g.,* orange, lemon, grape fruit, bergamot) is cut into halves to remove the juice completely, rind turned inside out by hand and squeezed when the secretary glands rupture. The oozed volatile oil is collected by means of the sponge and subsequently squeezed in a vessel. The oil floating on the surface is separated. For the the scarification process the apparatus Ecuelle a Piquer (a large bowl meant for pricking the outer surface of citrus fruits) is used. It is a large funnel made of copper having its inner layer tinned properly. The inner layer has numerous pointed metal needles just long enough to penetrate the epidermis. The lower stem of the apparatus serve two purposes; *first*, as a receiver for the oil; and *secondly*, as a handle.

Now, the freshly washed lemons are placed in the bowl and rotated repeatedly when the oil glands are punctured (scarified) thereby discharging the oil right into the handle. The liquid, thus collected, is transferred to another vessel, where on keeping the clear oil may be decanted and filtered. For the rasping process, the outer surface of the peel of citrus fruits containing the oil gland is skilfully removed by a grater. The 'raspings' are now placed in horsehair bags and pressed strongly so as to ooze out the oil stored in the oil glands. Initially, the liquid has a turbid appearance but on allowing it to stand the oil separates out which may be decanted and filtered subsequently. The mechanical process involves the use of heavy duty centrifugal devices so as to ease the separation of oil/water emulsions invariably formed and with the advent of modern mechanical devices the oil output has increased impressively.

The extraction processes can be carried out with eithervolatile solvents (e. g hexane, petroleum ether or benzene) resulting into the production of 'floral concretes'- oils with solid consistency and partly soluble in 95% alcohol, or non volatile solvents (tallow, lard or olive oil) which results in the production of perfumes. Examples of volatile oils include amygdaline (volatile oil of bitter almond), sinigrin (volatile oil of black mustard), and eugenol occurring as gein (volatile oil of *Geum urbanum*).

2.10 Steroids

Plant steroids (or steroid glycosides) also referred to as 'cardiac glycosides' are one of the most naturally occurring plant phytoconstituents that have found therapeutic applications as arrow poisons or cardiac drugs. (Liu, 2004) The cardiac glycosides are basically steroids with an inherent ability to afford a very specific and powerful action mainly on the cardiac muscle when administered through injection into man or animal. Steroids (anabolic steroids) have been observed to promote nitrogen retention in osteoporosis and in animals with wasting illness. Caution should be taken when using steroidal glycosides as small amounts would exhibit the much needed stimulation on a diseased heart, whereas excessive dose may cause even death. Diosgenin and cevadine (from *Veratrum veride*) are examples of plant steroids.

3. ISOLATION OF NATURAL PRODUCTS

As time passes new separation techniques and analysis are being introduced to separate different nature of compounds like alkaloids, glycosides, steroids, saponins, tannins, flavonoids etc. Carbohydrates, fats, and proteins are considered more valuable as they are having dietetic importance. (Bohlin, 1998), (Rahman, 1989) Many starches and gums are used in pharmacy but lack any marked pharmacological action; are used as binder, viscosity builder and as hydrocolloid to increase the stability of emulsions and suspensions.

After GATT there is a great surge to find out immense potential of traditional and other herbal drugs to prevent and cure diseases. Several extraction, fractionation/separation and isolation methods are developed after which isolation of the active moiety and their chemical examination is performed. Microwave extraction, sonication, lyophilization, spray drying and vacuum drying have also been employed with good results. Generally the extraction is based on pharmacological activity rather than chemical nature of compounds. Further studies are carried out after the isolation of the active moiety to confirm the compound. (Abo et al., 1991) The phytochemical investigation of a

plant involves the selection, collection, identification and authentication, extraction of the plant material (first fractionation), fractionation/separation (second fractionation) and isolation (third fractionation) of the constituents, characterization of the isolated compounds and investigation of the biosynthetic pathways of particular compound, quantitative evaluations and pharmacological activities.

3.1 PRINCIPLE

Extraction processes for drugs can depends on the partition of component between solvent phase and solid residual and dependent on diffusion of component. Solvent volume is used such as the final concentration gradient between micelle and residue has become zero which is an equilibrium stage. (Firn, 2010) The position of the equilibrium depends on properties of the drug nature and type of drug, quantity, degree of comminution, solvent selectivity, solvent quantity and moisture content. Factors which affect the extraction are quantity and nature of drug, degree of size reduction, moisture content, volume and nature of solvents, mixing ratios of solvents, method for preparation of solution from intact cells, method for preparation of solution from lysed cells, imbibition by solvent, rate of equilibrium establishment, temperature, pH of the extracting solvent, interaction between dissolved components, polarity of the solvent mixture, process governing separation, mixture ratio of solvent and herb, dissolution from lysed cells, penetration of solvent and swelling of drug plant material, movement of constituents out from intact cells and interaction of dissolved constituents with insoluble support material of plants.

4. IDENTIFICATION STRATEGY

Strategy for identification is divided into following stages:

1. Pre-extraction investigations
   - Collection, identification, selection and authentication of the plant material
   - Nature of constituents or secondary metabolites
   - Solvents for extraction
   - Interfering compounds
2. Extraction method
   - General
   - Miscellaneous methods
   - Extraction of alkaloid, sesquiterpene lactone and cardiac glycoside, flavonoids, other polyphenols, sterols, saponins, carbohydrate.
3. Isolation methods
4. Solvent recycling

4.1 Pre-extraction investigations

4.1.1 Selection, collection and identification and authentication of the plant material

The plant material should be properly authenticated to get desired component with high yield and reproducible results. The running of drug-discovery programs on randomly collected/selected plants is less economic.

Selection of plant

Generally plant selection involves a deep literature survey of the floristic diversity. Selection of plant material is performed by different approaches

1. Totally random selection.
2. Specific selection using ethnopharmacological reports.
3. By restricting the plants of interest to group based on chemotaxonomic, geographical, or compound structural - type preferences.
4. Computer-based selection method or Literature lnformation Selection Technique (L. I. S. T) using the NAPRALERT database (Novel approach involves correlation of biological activity, botanical facts, and chemotaxonomic information. (Doughari et al., 2009).

Collection

During collection of plant, it should be kept in mind that the specimens to be studied should be healthy. The microbial growth or other microbial infections may change the metabolites produced by the specimen, e. g. by phytoalexin formation. (Rahman, 1989), (Abo et al., 1991).

Factors Influencing the Concentration Levels and Kinds of Secondary Metabolites

Some of the factors that affect the concentration levels and kinds of secondary metabolites are site altitude, plant age, climate, soil type, time of collection, species etc. Chemical composition of herms also varies depending on species and environmental factors. (Adedpo et al., 2005).

Identification and authentication

After collection, the plant material should be identified or authenticated by a taxonomist. At least three specimens should be prepared. One of these samples should be deposited in a local national herbarium, and the others should be deposited in a specialist museum or herbarium and kept in an appropriate protected place for future reference.

Following details should be mentioned on herbarium

- Place
- Altitude
- Environment

- Characteristics
- Chemical constituents
- Part of plant taken
- Season

Above information is of vital importance in those cases where a recollection of the plant material is necessary and it is beneficial for researchers to reproduce their work in future. An erroneous identification of a plant sample may lead to confusion in scientific literature.

4. 1. 2 Characters of Phytoconstituents

The basic knowledge of nature and characteristics of phytoconstituents is essential to select method and solvent for extraction. Nature of phytoconstituents involves polarity, pH, thermostability etc.

Polarity

As a doctorine "like dissolves like". Polar components are soluble in polar solvent and non-polar components are soluble in nonpolar solvents. 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). It is rudimentary to study about the relationship between the extraction method applied and the physicochemical properties of the substances to be extracted. (Madziga et al., 2010).

pH

The pH of solvents affects the extent of extraction of components. Non- polar alkaloids can't be extracted into aqueous system but can be extracted into polar aqueous acid due to their basic nature and salt formation in acid. Fatty acids, phenols and other acidic phytochemicals are extracted using solvents at alkaline pH. (Firn, 2010) 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. (Sarker et al., 2007).

Thermostability

Normally solubility increases with raise in temperature. Higher temperatures facilitate penetration of the solvent into the cellular structures of herbs. Thermolabile components are sensitive to to higher temperatures. The artifacts may arise in presence of solvent/components on heating which may degrade the biologically active moiety or may exert toxicity or may create separation problem. (Fennell et al., 1994).

4. 1. 3 Choice of solvents

Usually secondary metabolites have different degrees of polarity so the solvent (s) should be chosen for the extraction should be considered carefully to ensure dissolution of secondary metabolites under study.

Solvent should have following properties:

- Easy to remove
- Inert
- Nontoxic
- Not easily inflammable
- No interaction or less chemical interaction

Solvent (mixture of solvent) is employed to dissolve the secondary metabolite and finally to diffuse out the dissolved solute into bulk solvent phase.

Solvents employed are:

- Polar: Water
- Non-polar: Petroleum ether, chloroform, Diethyl ether
- Semipolar: Ethanol, Acetone
- Azetropic mixtures

Polar Solvents

The polar components like polysaccharides, phenols, aldehydes, ketones, amines, and other oxygen containing compounds dissolve in water due to formation of hydrogen bonding. The solubility of aliphatic alcohol increases the solubility of the compound in water decreases. Additional polar groups are present in the molecule, as found in propylene glycol, glycerin, and tartaric acid, water solubility increases greatly due to addition of polar groups. Branching of the carbon chain reduces the nonpolar effect and leads to increased water solubility (tertiary butyl alcohol is miscible in all proportions with water, whereas n-butyl alcohol dissolves to the extent of about 8 g/100 ml of water at 20°C). The polar solvents such as water act as solvents according to the following mechanisms:

- Normally polar solvents have high dielectric constant which reduces the force of attraction between oppositely charged ions in crystals such as sodium chloride or molecule. Polar solvent like water has a dielectric constant of 80 while which dissolve polar component rapidly than non-polar solvent chloroform, which has a dielectric constant of 5 and due to low dielectric constant, ionic compounds are practically insoluble in non-polar organic solvents.
- Polar solvents break covalent bonds of potentially strong electrolytes by acid-base reactions since these solvents are amphiprotic. For example, water brings about the ionization of HCl as follows:
  - Weak organic acids are not ionized appreciably by water.
  - Their partial solubility is attributed instead to the hydrogen bond formation by with water. Phenols

and carboxylic acids, however, are readily dissolved in solutions of strong bases.

- Polar solvents has property of dipole interaction forces, particularly hydrogen-bond formation dut to which solvating molecules and ions become soluble and which leads to the solubility of the compound. The solubility of sodium salt of oleic acid and water is due to ion-dipole interaction. (Madziga et al., 2010).

Non-polar Solvents

Non-polar solvents have low dielectric constants and dissolve non-polar solutes with similar internal pressures through induced dipole interactions. Ionic and polar solutes are insoluble or slightly soluble in non-polar solvents. Weak Van-Der-Waals and London type of forces are responsible for the solubility of molecules.

Semi-polar Solvents

Semi-polar solvents like ketones and alcohols can induce a certain degree of polarity in non-polar solvent like benzene is readily polarizable, becomes soluble in alcohol. Semi-polar compounds act as intermediate solvents which bring about miscibility of polar and non-polar liquids.

Azeotropic Mixtures

Azeotropes are mixture of different solvent with varying polarity which has near boiling points. These are usually either binary or ternary mixtures with the ratio of their mixture (wt %) of component 1 soluble with wt % of component 2 and ternary azeotropic mixtures with the respective concentration of their mixture (mol %) of component 1 miscible with mol % of component 2 and component 3. (Doughari et al., 2009) They affect the dissolution properties and degree of extraction of extractable matters. To utilize this phenomenon fully it is recommended that the composition of the menstrum be chosen so that a binary or ternary azeotropic mixture is produced. This has the advantage that upon concentration of the extracts, the solvent boils constantly and the condensate, perhaps after a small correction by replacement of components preferentially retained in the drug residue, can be reused. Azeotropic mixtures have great potential to extract active phytochemical metabolites from the crude drugs depending on their varied chemical nature as they can extract large numbers of constituents based on its nature.

Influence of Solvents

A component may behave like strong electrolyte or non-electrolyte, depending on pH of solution. Precipitation of components occurs, when the pH of solution is adjusted to such a value at which un- ionized molecules are produced in sufficient concentration to exceed its solubility. (Adedapo et al., 2005).

Solvent-Solute interactions

Polar solvents like water is a good solvent for salts, sugars etc while non-polar solvents like mineral oil and benzene are often solvents for substances that are normally only slightly soluble in water. It proves the doctorine of “like dissolves like" (Fennell et al., 1994).

Combined Effect of pH and Solvents

The solvent affects the solubility of a weak electrolyte in a buffered solution in two ways,

I. The addition of alcohol to a buffered aqueous solution of a weak electrolyte increases the solubility of the un-ionized species by adjusting the polarity of the solvent to a more favourable value.

II. Being less polar than water, alcohol decreases the dissociation of a weak electrolyte, and the solubility of the drug goes down as the dissociation constant is decreased (pKa is increased). (Yadav et al., 2003).

Solvents, Problems and Limitations

The secondary metabolites must dissolve in solvent chosen for the extraction. The solvent chosen for the extraction must have following qualities,

- It must dissolve the secondary metabolites
- It should be easy to remove.
- It should be inert.
- It should be nontoxic.
- It should not be easily flammable.
- It should not form any type of unstable substance during extraction or mixing.
- Solvents should be distilled or even double distilled prior to use if they are of low or unknown quality.
- Solvent must be free from plasticizers like dialkyl phthalate, tri-n-butyl acetyl citrate and tri-butyl phosphate which are commonly found as impurities in solvents and mat impart stability problem.

Effect of Solvent, Solvent Mixtures and Solution on Extraction

Solvent or the extraction agents used in the preparation of phyto pharmaceuticals must be suitable for dissolving the important therapeutic drug constituents and thus for separating them from the substances containing the drugs which are to be extracted. (Jakhetia et al., 2010) In pharmaceutical technology, the extraction agent or solvent is known as menstrum and the extract solution separated from the residual insoluble drug plant material is called miscella. Menstrum are therefore solvents which readily dissolve and often also have certain selectivity for the extracted substances. For example, bitter principles, mucin, pigments, resins, etc. should (if at all possible) not be dissolved in the extraction of vegetable oils. (Doughari et

al., 2009), (Fennell et al., 1994) According to the customary definition in this technology solvents are, under normal conditions, volatile, usually organic liquids capable of dissolving other gaseous, liquid or solid substances without either themselves or the dissolved substance being chemically altered. Ln pharmaceutical technology, this is not always the case, as solvents such as oils that are not volatile under normal conditions are also used. (Yadav, 2003) The only inorganic solvents used in pharmacy are water and CO_2, although liquids such as ammonia, sulphur dioxide and hydrocyanic acid solutions are used for special purposes in pharmaceutical processing. (Vlietinck, 2010) Water, pure organic liquids and mixtures of organic liquids with water or with other organic\ liquids are used as extraction solvents. These organic liquids are nearly always hydrocarbons and their derivatives such as halogenated hydrocarbons, alcohol, esters, ketones, ethers, oils, etc. Selectivity, ease of handling, econorny, protection of the environment and safety are major factors to be considered in the choice of a suitable solvent or of a mixture of several solvents. (Firn, 2010) The last two factors in particular are becoming increasingly important due to the legislators sharpened attention to them. This has become so critical that many manufacturers are making serious attempts to design extraction processes so that the smallest possible number of 'safe' liquids, e. g. water, lower alcohol and in special areas, super critical gases are used. It must not be forgotten here that traditional plant extracts must satisfy the requirements of the pharmacopoeias. This is usually possible only when the specified menstrum is used. A degree of choice is offered by using cheaper methanol instead of expensive ethanol (on which duties have to be paid) if its polarity is equal to that of the required ethanol-water mixture. As a rule of thumb, a 10% higher methanol concentration roughly corresponds to the required ethanol concentration. (Heinrich et al., 2004) One essential consideration in the choice of suitable solvents is the question of whether the menstrum remains wholly or partly in the end product. lf this is the case, physiologically harmless solvents must be used. lf the end-product is free of solvent, as is the case with dried extracts, or if it still contains only harmless components from the originally used solvent mixture after further processing of the miscella, then for reasons of selectivity and economy the choice can be made without considering the physiological properties of the mixture used.

4. 1. 4 Interfering Compounds

Many naturally occurring compounds may interfere with the isolation and purification of a desired bioactive plant constituent like lipids, pigments, tannins, plasticizers, silicon etc.

4. 2 EXTRCTION METHOD

Extraction of plant material

There is no single perfect method for extraction, purification and isolation of compound. The first fractionation step is general extraction of secondary metabolite which is done either by using single “all-purpose” solvents such as methanol or by using petroleum ether to defatted the material. Methanol dissolves most of the secondary metabolites and enhancing their release from cellular matrix/cell surface. (Fennelll et al., 1994), (Jakhetia, 2010) Filtration or centrifugation is the first basic technique used to separate insoluble material and filtrate that contain dissolved secondary metabolite. The choice of extraction procedure and extraction solvent depends on the physicochemical nature of secondary metabolite, nature of plant material (fresh parts, dried parts) and their physical state (particle size). Fresh plants (leaves) are first homogenized or macerated with alcohol prior to extraction. (Yadav et al., 2003) An idea about properties of interfering compound provides best criteria for solvent selection for extraction and to select method of extraction. So prior to extraction a major emphasis must be given on nature of interfering compounds.

The 3 stages are basic

1. First step of extraction involves solvent penetration into herb cells/tissues, solubilization of secondary metabolites and finally release the dissolved secondary metabolites in solvent of extraction. Solvents of varying polarity are used alone or in combinations for extraction depend on component. So a large proportion of the unwanted material is removed. (Maceration, Digestion, Decoction, Soxhlet extraction, Supercritical chromatography, Hydro-distillation, Enflurage, Eculle, Supercritical fluid chromatography).

2. Second step is fractionation with subsequent analysis: Either open silica column or counter current distribution/liquid-liquid extraction is used to separate or fractionation of components. (Distillation, Sublimation, Evaporation, Fractional crystallization, fractional distillation, Sublimation, Fractional crystallization, fractional distillation, GC, CCD).

3. The third final stage is achieved by HPLC or TLC which involves separation of desired component in adequate purity (TLC, GC, GLC, Mass, NMR, UV, Fluorimerty)

Extraction

Extraction is followed either by

- Powdered dried material is directly extracted to achieve extract.
- First defeat the material and then extraction of desired component.
- Fresh plants (e. g. leaves) can be homogenized or macerated with alcohol.

Selection of extraction method depends on

- Nature of component
- Nature of material to be used
- Solvent system available

Techniques used to enhance extraction

- Ultrasound may enhance the extraction process for some plant materials, eg., the preparation of a 50% ethanolic solution of opium for the assay of alkaloid. Use of microwaves can also enhance extraction.
- By altering pH
- By stirring
- By reducing the particle size
- By changing the polarity of solvents

During the extraction of the herbal drugs the rinsing of extractive substances out of disintegrated plant cells, swelling of the drug plant material in order to increase the permeability of the cell walls, Penetration of the solvent into the plant cells and swelling of the cells, Dissolution of the extractive substances, Diffusion of the dissolved extractive substances out of the plant cell and finally the dissolution of extractive substances out of intact plant cells by diffusion take place. It has been found that the liquorice root when extracted with the solvent (0.25% ammonia solution) it penetrates into the roots more rapidly and this process is accelerated by raising the temperature. The steeping and swelling process is strongly influenced by particle size and is more evident radically. Upon penetration into the plant material, the solvent becomes enriched with extractive substances and hence the highest content of extractive substances is found on the solvent front. Some general points should be considered in terms of the extraction process, such as the overall characteristics of the secondary metabolites to be extracted (e. g. some glycosides are thermolabile or pH-sensitive). Although the normal practice is 'to apply 2iitanOarO technique to obtain a crude extract from a plant material, e. g. an acid-base shakeout to prepare an alkaloidal extract, because of the structural diversity within a given natural product group and their possible special requirements, it is advisable to consult specific review papers, and books in order to prevent the avoidable loss of desired bioactive metabolites caused by the use of an inappropriate extraction technique. The most simple extraction processes employed may be classified as follows: extraction with organic solvents: percolation, maceration, and extraction using a Soxhlet apparatus; and extraction with water: infusion, decoction, and steam distillation. (Voravuthikunchai et al., 2003), The most popular method of extraction is to use a liquid solvent at atmospheric pressure, possibly with the application of heat. Other methods include steam distillation, supercritical fluid extraction and the use of liquefied gases under moderate pressure. The choice of method depends on the factors listed above as well as the intrinsic advantages and disadvantages of the procedures. (Kar, 2007).

Supercritical fluid extraction

The process of separation of one component which is extracting from matrix by using supercritical fluid is known as supercritical fluid separation. Supercritical fluids showed property intermediate between those of the liquid and gaseous phases, for any substance it is a condition above the critical temperature and pressure.

SFE offers many advantages as follows,

- It leads to lower solvent usage
- Controllable selectivity
- Cleaner extracts and less thermal degradation as compared to conventional solvent extraction and steam distillation methods,

Super critical carbon dioxide (SCO2) - with its particularly attractive properties such as non-toxicity, non-flammability, non-corrosiveness, chemical inertness, low critical temperature (304"K), moderately low critical pressure (73 atm), easy availability, co-effectiveness and environmental acceptability is the preferred solvent for many super critical extractions. Liquid carbon dioxide is completely miscible with components of essential oils like aldehyde, ketones, esters and alcohols. At same time, proteins, starches, mineral salts and water are insoluble in liquid carbon dioxide. Essential oils obtained by liquid carbon dioxide extraction are superior to that obtained through steam distillation and solvent extraction. Extraction of several natural products such as pyrethrins from chrysanthemum flower, essential oils from anise, caraway, clove, star anise, cinnamon and ginger are increasingly done by this process. (Heinrich et al., 2004), (Abo et al., 1991).

Solid phase extraction

Solid phase extraction is process of separation of dissolve and suspended component from liquid mixture by using another component in the mixture according to their physical and chemical property. (Fennell et al., 1994).

Distillation

Distillation may be defined as separation of components of a mixture of two or more liquids by virtue of difference in their vapor pressure. There are three systems of distillation-

- Hydro distillation
- Hydro-steam distillation
- Steam distillation

Hydro distillation

Hydro distillation is the oldest method being used for separation of essential oil. In this method plant material is contact with boiling water in a crude metallic

distillation unit. This process use principle of osmotic press principle of osmotic pressure to diffuse oil from the oil glands. The essential oil of a plant consists of many compounds which generally boil between 150° to 300° C. The vapors pass through a coiled tube contained in a water bath and condensate is obtained at the bottom of the condenser tube. The disadvantages are that the heat is difficult to control and hence the rate of distillation is variable. Also the possibility exists for local overheating and "burning" of the charge which can lead to poorer quality oil.

Hydro-steam distillation

To overcome the drawback of water distillation, modifications in techniques was developed. In this technique plant material is supported on a perforated grid or screen inserted at some distance above bottom of still. Water filled below the grid is heated which produce saturated and wet steam; produced steam pass through plant material and vaporized essential oil.

Steam distillation

A process of extracting essential oils from plant products through a heating and evaporation process is known as steam distillation. Steam distillation is a popular method for the extraction of volatile oils (essential oils) from plant material. This can be carried out in a number of ways. One method is to mix the plant material with water and to heat to boiling (distillation with water). The vapors are collected and allowed to condense, and the oil separated from the water.. It resembles hydro-steam distillation except that no water is kept in bottom of still. This method is efficient and gives higher yields. However, it is not generally employed to delicate flowers. To maximize the yields of the oils, precautions must be taken to ensure efficient condensation of the steam and vaporized oil and collection of the condensate in such a way as to prevent loss of the volatile material. However, to avoid risk of explosion, a completely closed system must not be used. The advantages of this type of "dry" steam distillation are that it is relatively rapid, therefore charging and emptying the still is much faster and energy consumption is lower. The rapid distillation is also less likely to damage those oils which contain reactive compounds, e. g. esters. However, it cannot be used where the oil contains hydrolysable components such as esters or those that are easily. (Doughari et al., 2009), (Vlietinck, 2010).

Maceration (Extraction with hot fat)

Maceration is process of extraction with hot oil or fat. In maceration, oil cells of fragrant flowers are ruptured by immersion in a hot fat or oil at 60-70°C which in turn absorbs essential oils. Fat is separated from spent flowers and reused for absorbing fragrance from next batch of fresh flowers. Fat retained by flowers is recovered by hydraulic pressing. Resultant perfumed pomade is frequently marketed as such but is often extracted with strong alcohol to yield extracts. This is very much the same technique used in solvent extractions, where solvents are used instead of the hot oil as used in maceration. (Heinrich et al., 2004) 0, (Kar, 2007).

Enfleurage (Extraction with cold fat)

Enfleurage is the process of extraction of fragrance by absorbing it from flowers in contact with cold fats. This process is adopted for fragrant flowers of jasmine and tuberose, which continue to manifest their characteristic fragrance even in plucked condition. Fats should be saturated and odorless to prevent entrance of fat odors. Refined lard or beef suet are preferred. Fat is thinly layered on both sides of a glass plate supported on a rectangular wooden frame or chassis. Fresh fragrant flowers are lightly layered on fat coated chassis. Enfleurage gives a much greater yield of flower oil than other methods. Despite this advantage, enfleurage has lately been replaced by extraction with volatile solvents because enfleurage is a very delicate and lengthy process requiring much experience and labour. (Adedapo et al., 2005), (Doughari et al., 2009).

Extraction with volatile solvents

Principle of extraction with volatile solvents is simple. Fresh flowers are charged into specially constructed extractors and extracted systematically at room temperature, with a carefully purified solvent usually petroleum ether. Solvent penetrates flowers and dissolve natural flower perfume together with some waxes and albumins and colouring matter. Solution is subsequently pumped into an evaporator and concentrated at a low temperature. After the solvent is completely driven off in vacuum, concentrated flower oil is obtained. Thus, temperature applied during entire process is kept at a minimum; live steam as in case of distillation, does not exert its action upon delicate constituents of flower oil. Compared with distilled oils, extracted flower oils more truly represent natural perfume as originally present in flowers. (Jakhetia et al., 2010).

Expression

It is physical process in which pressure is applied to squeeze the oil out of the material or juice from plant. This was usually achieved by a tincture press. This method is employed when essential oils are thermo sensitive. It is used for isolating essential oils from lemon and orange peels. In general, expression involves squeezing any plant material at great pressures to press out oils or other liquids. The process is carried out by hand-operated presses or crushes in isolated rural areas or by gigantic mechanical presses in industrial centres. (Fennell et al., 1994), (Voravuthikurchai et al., 2003).

Infusion

Infusions are prepared by soaking a drug in water for a specialized period of time. The process can be either hot or cold, depending upon the type of the ingredients present as decomposition may occur at higher temperatures. Infusions are generally prepared for immediate use, as preservatives are absent. In some cases preservatives like alcohol are used and the infusions concentrated by boiling. The term infusion are used for the preparations prepared from soft tissues like petals, leaves etc.

Percolation (Exhaustive Extraction)

Percolation in usually one of the most widespread methods for plant extraction since it does not require much manipulation or time. It is a continuous process in which the saturated solvent is constantly being displaced by fresh menstrum. Normally, percolation is not used as a continuous method because sample is steeled in solvent in the percolator for 24hrs (for up to three times), and then the extracted materials are collected and pooled. (Fennell et al., 1994).

In general process of percolation, particularly in the manufacture of concentrated preparations like liquid extracts, the following problems may arise:

- If the active substances are thermo-labile, evaporation of large volume of dilute percolate, may result in partial loss of the active constituents
- In the case of alcohol- water mixture, evaporation results in preferential vaporization of alcohol leaving behind an almost aqueous concentrate which may not be able to retain the extracted matter in solution and hence get precipitated.

In such cases the modification in general process of percolation is required as given below,

Reserved Percolation

In this case the extraction is done through the general percolation procedure. At the last, the evaporation is done under reduced pressure in equipment like a Climbing evaporator to the consistency of a soft extract (semi solid) such that all the water is removed. This is then dissolved in the reserved portion which is strongly alcoholic and easily dissolves the evaporated portion with any risk of precipitation. (Madziga et al., 2010).

Decoction

Decoctions are prepared in a similar manner to that of infusions but the ingredients are boiled with that of water for a specified period of time or till a definite volume is attained. The term decoction is used when the preparation is prepared using hard plant parts like root, bark, wood etc. Decoctions are usually the method of choice when working with tougher and more fibrous plants, barks and roots (and which have water soluble chemicals). Depending on the type of plant material used, strong decoctions are prepared in two general ways. The first involves boiling the mixture longer. This is usually indicated when working with larger woody pieces of bark. Longer boiling time, up to 2 hours or more, is sometimes necessary to break down, soften, and extract the larger pieces. Alternatively, when smaller woody pieces are used yet a stronger remedy is wanted, the decoction is prepared as above (boiling 20 minutes), then it is allowed to sit/soak overnight before straining out the herb. When straining, again, make sure to press on the cut herb pieces in the strainer to get as much moisture/decoction out of the herb pieces. (Kamboj, 2010).

Ultrasound Extraction

Extraction of intracellular compounds by cell-lysis is done by using ultrasound. When sound wave propagate in liquid media results in high-pressure and low-pressure cycle. The main effects of ultrasound extraction can be summarized as:

- To increase the permeability of the cell walls
- To produce cavitations i. e. the spontaneous formation of bubbles in a liquid belowS its boiling point resulting from strong dynamic stressing.
- To increase mechanical stressing of the cells so called interface friction Treatment with the ultrasound plays a major role e. g. decomposition of the alkaloids in jaborandi leaves is observed after 30 s ultrasound treatment on the laboratory scale at 20KHz but in the case of foxglove leaves the content of digitalis glycosides fell when an ultrasound output representing the optimum formation of hydrogen peroxide during the extraction. (Kar, 2007), (Jakhetia et al., 2010).

Hot continuous extraction (Soxhlet extraction)

Soxhlet extraction method described by soxhlet in 1837. In this methods fat and oil from solid material is extracted by repeated washing with organic solvent under reflux. Organic solvent commonly used are hexane and petroleum ether. Disadvantage of this process are-polar lipid, long time involved large volumes of solvents, hazards of boiling solvents. (Kar 2007).

In gel digestion

The in-gel digestion is part of the sample preparation for the mass spectrometric identification of proteins in course of proteomic analysis. The in-gel digestion primarily comprises the four steps destaining, reduction and alkylation (R&A) of the cysteines in the protein, proteolytic cleavage of the protein and extraction of the generated peptides.

Destaining

Proteins which were separated by 1D or 2D PAGE are usually visualized by staining with dyes like Coomassie Brilliant Blue (CBB) or silver. (Yadav et al., 2003) Alkyla-

tion (r&a) of the cystines or cysteines potentially embodied in the protein. Hereby the disulfide bonds of the proteins are irreversibly broken up and the optimal unfolding of the tertiary structure is obtained. The reduction to the thiol is accomplished by the reaction with chemicals containing sulfhydryl or phosphine groups such as dithiothreitol (DTT) or tris-2-carboxyethylphosphine hydrochloride (TCEP).

Digestion

The protein is cut enzymatically into a limited number of shorter fragments. These fragments are called peptides and allow for the identification of the protein with their characteristic mass and pattern. The serine protease trypsin is the most common enzyme used in protein analytics. (Rahman, 1989), (Adekunle et al., 2009).

Extraction

After finishing the digestion the peptides generated in this process have to be extracted from the gel matrix. This is accomplished by one or several extraction steps. Drawbacks for the in-gel digestion are the extended time need and the multiple processing steps making the method error-prone in respect to contaminations.

Extraction by Electrical Energy

In this method, electrical energy is used in the form of an electric field to accelerate extraction and improve the yield of the extraction. Extraction of scopolamine from the seeds and capsules of Indian thorn apple has been reported by this process. (Vlietinick 2010).

Vertical or Turbo Extraction

Vertical or turbo-extraction is used. Here the drug to be extracted is stirred in the menstrum with a high-speed mixer or homogenizer. The shredding and shearing forces break down the drug material to a particle size, which is smaller than that of the material when it is first put in the mixer. The cells become highly disintegrated. The diffusion of extractive substances through the cell membranes is largely replaced by washing out from the destroyed cellular tissues. These result in substantially faster establishment of the maceration equilibrium and hence in a considerable saving of time. (Bohlin L, 1998), (Abo et al., 1991).

Counter Current Extraction

In continuous countercurrent extraction, a moving solution, emulsion, suspension or solid mass is extracted by a liquid phase flooring against it. In relative counter current extraction, on the other hand, only one phase (as a rule the extraction solvent) is in motion, the other phase (usually the solid) remains stationary. (Kar, A 2007).

5.2.2 Miscellaneous Methods

Following are miscellaneous method used for extraction.

Expression

This method is use to obtain fixed oils from plant material. This involves disruption of the cellular structure by the application of pressure to the material and allows oil to flow out of the material. This method is frequently used for soya oil, sunflower oil and olive oil. The rupture in the cell kernel causes the elution of oil in this method. (Adekunle et al., 2009).

Pervaporation

This method is currently being developed and its success will depend on the generation of new membranes which show selective binding for particular chemical groups. Hydrophilic membranes may be used to remove polar materials, including water from organic solvents and hydrophobic membranes can be used to remove organic compounds from an aqueous phase. This method has been used to remove aroma compounds from fruit juices. (Yadav et al., 2003).

Sublimation

In this process some substances, change from solid to gas or vice versa without passing through a liquid state on heating or cooling. This method can be used to obtain the substance from dried plant material or a dry crude extract. Caffeine of high purity can be obtained by this method from dry tea leaves.

4.2.3 Extraction of alkaloid, sesquiterpene lactone and cardiac glycoside, flavonoids, other polyphenols, sterols, saponins, carbohydrate

Solvent extraction is the most popular method of extraction. The main groups of compound to be considered are fixed oils, fats and waxes, volatile or essential oils, carotenoids, alkaloids, glycosides, aglycones, phenolic compounds, polysaccharides and proteins. Polarity and pH are two important factors. A general outline of the solvents that would be appropriate for extraction depends on above classes of compounds. The methods given in this section are general ones based on the common properties of broad classes of phytochemicals. (Kamboj, 2010).

Alkaloids

All alkaloids contain at least one nitrogen atom and the compound is basic. This means that salt formation can occur in the presence of acid. This fundamental property of alkaloids is used in their extraction and further clean-up. Two methods may be used for alkaloid extraction. One is to basify the plant material using diethylamine or ammonia and extract with an organic solvent. (Bohlin L, 1998).

Carotenoids

Carotenoids are responsible for red, orange and yellow pigments observed in the plant and animal kingdoms. They are generally tetraterpenoid and can be divided into hydrocarbons and oxygenated forms known as xanthophylls. Hydrocarbon tetraterpenoid are less po-

lar and can be extracted into petroleum ether. Xanthophylls are more polar therefore be extracted into ethanol or mixtures of ethanol and less polar solvents such as chloroform. (Fennell et al., 1994).

Glycosides

Glycosides are relatively polar in nature and its polarity depends on both number and type of sugar the structure of the aglycone. Most glycosides can be extracted with polar solvents such as acetone, ethanol, methanol, water or mixtures of these. However, cardiac glycosides have bulky steroidal aglycone, which shows appreciable solubility in chloroform. When extracting into water, and sometime enzymatic breakdown also possible. This will not occur if boiling water is used or if significant proportions of alcohol or ammonium sulphate are added to the extract. In some cases, it may be the aglycone rather than the glycoside that is to be extracted, and this requires hydrolytic separation of the aglycone and sugar before or after extraction. (Abo et al., 1991).

Phenolic Compounds

These can exist as free phenols or in glycosidic form. Due to the multiplicity of hydroxyl functions, phenols tend to be relatively polar and dissolve in aqueous alcohols. As they are weak acids, they may also be extracted or partitioned into aqueous alkali as phenolate salts. A problem encountered with phenolic compounds is that they can undergo extensive polymerization a reaction by the action of polyphenol oxidizes. This reaction will responsible for the development of brown coloration in damaged plant material when exposed to the air and in certain extracts. The polymerization reaction is catalyzed by acid. (Kamboj, 2010).

Proteins

Due to the presence of free carboxylic, amino and phenolic groups on amino acid side- chains in proteins, most can be ionized at high or low pH values. The pH at which no net charge is carried is known as the isoelectric point and this will vary with each protein depending on the constituent amino acids. At pH values above the isoelectric point, the protein carries a net negative charge, and the pH values below the isoelectric point, a net positive charge is carried. As a result of this, most proteins can be extracted with water, buffers, dilute acid or base or simple salt solutions. However, more lipophilic proteins require the use of 70-80% alcohol. Selective precipitation of groups of proteins in a crude protein extract can be achieved by gradual addition of acetone, ethanol or ammonium sulphate. Conversely, for proteins with a greater solubility in salt solutions that water, e. g. globulins, a crude protein mixture can be extracted in 10% sodium chloride solution and the globulins precipitates by addition of water. For prolamines, extraction in 70-80% alcohol can be followed by precipitation by dilution with water. The precipitation step can be followed by resolubilization and further separation using ultra filtration, gel filtration, ion-exchange chromatogramphy or electrophoresis. (Doughari et al., 2009), (Jakhetia et al., 2010).

Polysaccharides

Polysaccharides are polymers of sugars or sugar derivatives. Generally, there are three types of sugar polymers - those that are completely water soluble, those that partially dissolve in water and swell to form gels and lastly, those that are water insoluble. Polysaccharides that totally or partially dissolve in water can be extracted using cold or warm water.

Volatile Oils

Volatile oils are the odorous principals found in various plant parts. Because they evaporate in air at ordinary temperatures, they are called volatile oils, ethereal oils or essential oils.

4.3 ISOLATION METHODS

While different techniques have evolved for the extraction of the phytoconstituents so as to obtain crude extracts (complex mixtures), fractions (simpler mixtures) and isolated (pure) components from natural sources. These are usually then subjected to a number of further analytical investigations in order to obtain more information on the properties of their constituent substances.

Broadly, the nature of further investigations after extraction of a crude drug is of three types:

1. Qualitative chemical analysis - determination of the nature of the constituents of a mixture or the structure of an isolated compound.

2. Quantitative chemical analysis - determination of the purity of an isolated substance or the concentration of a single substance or group of substances in a mixture

3. Bioassays - determination of the biological or pharmacological activity of substances and the dose range over which they exert their effects.

The amount of fraction available is possibly the most important factor in making decisions about its future treatment and analysis. Investigative methods can be either non-destructive or destructive. Non-destructive methods mean that the sample can be recovered and used for other tests. Some physico-chemical procedures, e. g. chemical tests, analytical chromatography, mass spectrometry and all biological testing procedures are destructive i. e. they ule up the sample and generally it cannot be recovered. Another important factor is that th6 different types of analysis require the sample to be present in different types of medium. lt is always desirable to supply the fractions as solids, which can be weighed accurately and reconstituted in the appropriate solvents. The solid forms are usually produced from solutions by evaporation under reduced pressure to minimize decomposition or, in the case of

aqueous solutions, by freeze-drying, sometimes called lyophilisation. (Heiurich, et al., 2004), (Kamboj, 2010).

Isolation techniques

1) Sublimation

2) Distillation

3) Evaporation

4) Fractional liberation

5) Fractional crystallization

6) Fractional distillation

7) Chromatography

Fractionation

All separation processes involve the division of a mixture into a number of discrete fractions. These fractional may be physically discrete fraction. In which successive partition into diethyl ether, chloroform and ethyl acetate will often afford in turn flavonones and flavonols, methoxylated flavonoids and flavonoid monoglycosides. Di- and polyglycosylated flavonoids will remain in the residual aqueous phase. Saponins are water soluble compounds, and the crude saponin fractions are water soluble compounds, and the crude saponin fractions may be obtained. The alkaloids are basic compounds may be extracted either into aqueous acid solution after removal of neutral impurities with an organic solvent, or by treating the groups wet plant material with an alkaline substance such as CaCO3 powder and extracting with diethyl ether after standing overnight. Specific literature should be consulted for specific plant extraction problems. (Voravuthikurchai, et al., 2003).

Sublimation

Sublimation is used for isolation of caffeine from tea, for its purification of materials present in a crude extract and for separation of camphor. (Kamboj, 2010).

Distillation

Steam distillation is much used to isolate volatile oils and hydrocyanic acid form plant material. The TAS oven is used for steam distillation on a semi micro scale for the direct transfer of volatile materials from a powdered drug to thin layer plate. (Heinrich, et al., 2004).

Fractional liberation

Some compounds are separated by fractional liberation from a mixture. A mixture of alkaloid salts in aqueous solution, when treated with aliquots of alkali, will give first the weakest base in the free salt followed by base liberation in ascending order of basicity. If the mixture is shaken with an organic solvent after each addition, then a fractionated series of base will be obtained. A similar procedure is used for organic acids soluble in water immiscible solvents. In this case, starting with a mixture of the acid salts, it is possible to fractionally liberate the acids by addition of mineral acids. Sodium salts of acids on treatment with dilute HCl yield free organic acids. (Kar, A 2007).

Fractional crystallization

The method is used on the difference in solubility of the components of a mixture in a particular solvent. Frequently, derivatives of the particular components are used (picrates of alkaloids, osazones of sugars). (Yadav, et al., 2003).

Chromatography

Chromatography represents a group of techniques for separation of compounds of mixtures by their continuous distribution between two phases, one of which is moving past the other.

Chromatographic fingerprinting and marker compound analysis

Chromatographic fingerprint of an Herbal Medicine (HM) is a chromatographic pattern of the extract of some common chemical components of pharmacologically active and or chemical characteristics. This chromatographic profile should be featured by the fundamental attributions of “integrity” and “fuzziness” or “sameness” and “differences” so as to chemically represent the HM investigated. It is suggested that with the help of chromatographic fingerprints obtained, the authentication and identification of herbal medicines can be accurately conducted (integrity) even if the amount and/or concentration of the chemically characteristic constituents are not exactly the same for different samples of this HM (hence, “fuzziness”) or, the chromatographic fingerprints could demonstrate both the “sameness” and “differences” between various samples successfully.

Thus, we should globally consider multiple constituents in the HM extracts, and not individually consider only one and/or two marker components for evaluating the quality of the HM products. However, in any HM and its extract, there are hundreds of unknown components and many of them are in low amount. Moreover, there usually exists variability within the same herbal materials. Hence it is very important to obtain reliable chromatographic fingerprints that represent pharmacologically active and chemically characteristic components of the HM. In the phytochemical evaluation of herbal drugs, TLC is being employed extensively for the following reasons:

- it enables rapid analysis of herbal extracts with minimum sample clean-up requirement,
- it provides qualitative and semi quantitative information of the resolved compounds and
- it enables the quantification of chemical constituents. Fingerprinting using HPLC and GLC is also carried out in specific cases.

In TLC fingerprinting, the data that can be recorded using a highperformance TLC (HPTLC) scanner includes the chromatogram, retardation factor (Rf) values, the colour of the separated bands, their absorption spectra, λmax and shoulder inflection/s of all the resolved bands. All of these, together with the profiles on derivatization with different reagents, represent the TLC fingerprint profile of the sample. The information so generated has a potential application in the identification of an authentic drug, in excluding the adulterants and in maintaining the quality and consistency of the drug. HPLC fingerprinting includes recording of the chromatograms, retention time of individual peaks and the absorption spectra (recorded with a photodiode array detector) with different mobile phases. Similarly, GLC is used for generating the fingerprint profiles of volatile oils and fixed oils of herbal drugs. Furthermore, the recent approaches of applying hyphenated chromatography and spectrometry such as High-Performance Liquid Chromatography–Diode Array Detection (HPLC–DAD), Gas Chromatography–Mass Spectroscopy (GC–MS), Capillary Electrophoresis- Diode Array Detection (CE-DAD), High- Performance Liquid Chromatography–Mass Spectroscopy (HPLC–MS) and High- Performance Liquid Chromatography–Nuclear Magnetic Resonance Spectroscopy (HPLC– NMR) could provide the additional spectral information, which will be very helpful for the qualitative analysis and even for the on-line structural elucidation.

Liquid chromatography

a. Preparative high performance liquid chromatography

There are basically two types of preparative HPLC. One is low pressure (typically under 5 bar) traditional HPLC, based on the use of glass or plastic columns filled with low efficiency packingmaterials of large particles and large size distribution. A more recent form PLC, Preparative High Performance Liquid Chromatography (Prep. HPLC) has been gaining popularity in pharmaceutical industry. In preparative HPLC (pressure >20 bar), larger stainless steel columns and packing materials (particle size 10-30 μm are needed. The examples of normal phase silica columns are Kromasil 10 μm, Kromasil 16 μm, Chiralcel AS 20 μm whereas for reverse phase are Chromasil C18, Chromasil C8, YMC C18. The aim is to isolate or purify compounds, whereas in analytical work the goal is to get information about the sample. Preparative HPLC is closer to analytical HPLC than traditional PLC, because its higher column efficiencies and faster solvent velocities permit more difficult separation to be conducted more quickly In analytical HPLC, the important parameters are resolution, sensitivity and fast analysis time whereas in preparative HPLC, both the degree of solute purity as well as the amount of compound that can be produced per unit time i. e. throughput or recovery are important. This is very important in pharmaceutical industry of today because new products (Natural, Synthetic) have to be introduced to the market as quickly as possible. Having available such a powerful purification technique makes it possible to spend less time on the synthesis conditions.

b. Liquid Chromatography- Mass Spectroscopy (LC-MS)

In Pharmaceutical industry LC-MS has become method of choice in many stages of drug development. Recent advances includes electro spray, thermo spray, and ion spray ionization techniques which offer unique advantages of high detection sensitivity and specificity, liquid secondary ion mass spectroscopy, later laser mass spectroscopy with 600 MHz offers accurate determination of molecular weight proteins, peptides. Isotopes pattern can be detected by this technique. (Cowan et al., 1999).

c. Liquid Chromatography- Nuclear Magnetic Resonance (LC-NMR)

The combination of chromatographic separation technique with NMR spectroscopy is one of the most powerful and time saving method for the separation and structural elucidation of unknown compound and mixtures, especially for the structure elucidation of light and oxygen sensitive substances. The online LC-NMR technique allows the continuous registration of time changes as they appear in the chromatographic run automated data acquisition and processing in LC-NMR improves speed and sensitivity of detection. The recent introduction of pulsed field gradient technique in high resolution NMR as well as three-dimensional technique improves application in structure elucidation and molecular weight information. These new hyphenated techniques are useful in the areas of pharmacokinetics, toxicity studies, drug metabolism and drug discovery process.

Gas chromatography

a. Gas Chromatography Fourier Transform Infrared spectrometry

Coupling capillary column gas chromatographs with Fourier Transform Infrared Spectrometer provides a potent means for separating and identifying the components of different mixtures.

b. Gas Chromatography-Mass Spectroscopy

Gas chromatography equipment can be directly interfaced with rapid scan mass spectrometer of various types. The flow rate from capillary column is generally low enough that the column output can be fed directly into ionization chamber of MS. The simplest mass detector in GC is the Ion Trap Detector (ITD). In this instrument, ions are created from the eluted sample by electron impact or chemical ionization and sortored in a radio frequency field; the trapped ions are then ejected from the storage area to an electron multiplier detector. The ejection is controlled so that scanning on the basis of mass-to-charge ratio is possible. The ions

trap detector is remarkably compact and less expensive than quadrapole instruments. GC-MS instruments have been used for identification of hundreds of components that are present in natural and biological system (Madsen, et al., 1995).

Supercritical Fluid Chromatography (SFC)

Supercritical fluid chromatography is a hybrid of gas and liquid chromatography that combines some of the best features of each. This technique is an important third kind of column chromatography that is beginning to find use in many industrial, regulatory and academic laboratories. SFC is important because it permits the separation and determination of a group of compounds that are not conveniently handled by either gas or liquid chromatography. These compounds are either non-volatile or thermally labile so that GC procedures are inapplicable or contain no functional group that makes possible detection by the spectroscopic or electrochemical technique employed in LC. SFC has been applied to a wide variety of materials including natural products, drugs, foods and pesticides.

Other Chromato-Spectrometric studies

The NMR techniques are employed for establishing connectivity between neighbouring protons and establishing C-H bonds. INEPT is also being used for long range hetero nuclear correlations over multiple bonding. The application of Thin Layer chromatography (TLC), High Performance Chromatography (HPLC) and HPLC coupled with Ultra violate (UV) photodiode array detection, Liquid Chromatography- Ultraviolet (LC-UV), Liquid Chromatography-Mass Spectrophotometry (LCMS), electrospray (ES) and Liquid Chromatography-Nuclear Magnetic Resonance (LC-NMR) techniques for the separation and structure determination of antifungal and antibacterial plant compounds is on the increase frequently. Currently available chromatographic and spectroscopic techniques in new drug discovery from natural products Currently, computer modelling has also been introduced in spectrum interpretation and the generation of chemical structures meeting the spectral properties of bioactive compounds obtained from plants. The computer systems utilise 1H, 13C, 2D-NMR, IR and MS spectral properties. Libraries of spectra can be searched for comparison with complete or partial chemical structures. Hyphenated chromatographic and spectroscopic techniques are powerful analytical tools that are combined with high throughput biological screening in order to avoid re-isolation of known compounds as well as for structure determination of novel compounds. Hyphenated chromatographic and spectroscopic techniques include LC–UV–MS, LC–UV–NMR, LC–UV–ES–MS and GC–MS.

4. 4 Solvent Recycling

For both economical and ecological reasons the recovery of solvent is important. Use of single solvent is much better rather than azeotropic mixture which is difficult to separate into individual components. Complex distillation procedure is used for solvent recovery. An adequate care must be taken in disposing of solvents in a way that causes least damage to the environment. Most scientific establishments have standard procedures for the disposal of organic solvents. (Kamboj, 2010).

Treatment of Drug Residue after Extraction

Further treatment of the drug residue is very essential and more important in the context of extraction of herbal drugs. This is necessary for several reasons as follows:

- The drug residue contains considerable quantities of absorbed solution with valuable extractive substances
- The solvents used should be recovered.
- There will be further uses of the drug residue, which necessitate removal of the solvents which must not be equated with recovery of the solvents)

During extraction, the drug material, depending on its nature and the species of plant from which it is obtained, absorbs varying quantities of solvent and hence swells up to varying degrees. The drug residue has a species-dependent retention capacity for solvents, which is known as the 'absorption capacity'. The solvent can, in principle, be removed in two ways,

(1) Expression of the drug residue

(2) Expulsion of the solvent by warming with or without pressure reduction. (Voravuthikunchai et al., 2003)

5. CONCLUSION

Natural product drug discovery program a long term capital-intensive program. Several extraction, fractionation/ separation and isolation methods are developed after which isolation of the active moiety and their chemical examination is performed. Microwave extraction, sonication, lyophilization, spray drying and vacuum drying have also been employed with good results. Generally the extraction is based on pharmacological activity rather than chemical nature of compounds. Further studies are carried out after the isolation of the active moiety to confirm the compound. The phytochemical investigation of a plant involves the selection, collection, identification and authentication, extraction of the plant material (first fractionation), fractionation/separation (second fractionation) and isolation (third fractionation) of the constituents, characterization of the isolated compounds and investigation of the biosynthetic pathways of particular compound, quantitative evaluations and pharmacological activities. Thus, this review compiles the recent literature with special focus on various approaches for extraction& isolation methods of phytoconstituents present in plants.

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