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PHYTO ACTIVE COMPOUNDS FROM HERBAL PLANT EXTRACTS: ITS EXTRACTION, ISOLATION AND CHARACTERIZATION

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ABSTRACT

Herbal extracts its pure compounds provide opportunities for new drug discovery. Herbal Plants are widely used in the pharmaceutical industry for herbal drug development due to their structural diversity and presence of various pharmacological activities. The biological active compounds that are present in plants referred as phytochemicals. These phytochemicals derived from different parts of plants and thereby used as sources of lead molecules. The secondary metabolic compounds present in the plants describes the phytochemistry. Naturally occurring chemical compounds are present in plants and contains structurally diverse bioactive molecules. This review article highlights on the analytical methodologies which include the extraction, isolation and characterization of active ingredients from herbal plants. Different techniques of extraction are explained as extraction is the most important first step towards analysis of active constituents. This paper also highlights the isolation of active molecules by chromatographic techniques like TLC, column etc. The most important step towards analysis of bioactive compounds present in the plant extracts is characterization which includes phytochemical screening assays, HPLC(High Performance Liquid Chromatography), HPTLC (High Performance Thin Layer Chromatography), FTIR Fourier Transform Infra-Red spectroscopy (FTIR), NMR (Nuclear Magnetic Resonance), GCMS (Gas Chromatography and Mass Spectrometry) through which

lead molecule structure identification can be done .Thus key challenges in research related to herbal drug development is discussed in this paper.

KEYWORDS: Herbal extracts, Phytochemicals, Extraction, Chromatographic techniques, Herbal drug development.

INTRODUCTION

Plants are natural reservoir of medicinal agents. These are almost free from the side effects.[1] Natural products, such as plants extract, open a new horizon for the discovery of new therapeutic agents. It also provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity.[2] In most developing countries traditional medicine and medicinal plants are used for the maintenance of good health and about 80% of the world's population relies on herbal medicines as better curative measures without or with minimum side effects.[3] Primary health care treatment, most of which involve the use of herbal preparations which include plant extracts containing active constituents.[4,5] Thus Plants contain a wide range of chemical compounds that can be used to treat chronic as well as infectious diseases.[6] Due to the development of adverse effects and microbial resistance to the chemically synthesized drugs, men turned to ethnopharmacognosy. They found literally thousands of phytochemicals from plants as safe and broadly effective alternatives with less adverse effect. According to the World Health Organization, a medicinal plant is any plant which, in one or more of its parts, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi synthesis. Parts of such plants 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 plant chemical compounds or bioactive components are often referred to as phytochemicals ('phyto'- from Greek - *phyto* meaning 'plant') or phytoconstituent. They protect plants against microbial infections or infestations by pests.[7,8] These constituents have been in use by the ancient therapist for the use of various human aliments also. The science of application of these indigenous or local medicinal remedies including plants for treatment of diseases is currently called ethno pharmacology. Many beneficial biological activity such as anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic, antiinflammatory and wound healing activity were reported. In many cases the people claim the good benefit of certain natural or herbal products. So as per current generation and considering standers it is mandatory to

show evidence based approach towards medicine from herbal source. The evidences can only be obtained by performing research and thus when we have pure form of phytochemicals ,the researcher can prove it through characterization and pharmacological activity. According to the World Health Organization (WHO), nearly 20,000 medicinal plants exist in 91 countries including 12 mega biodiversity countries. The process to obtain the biologically active compound from herbal plant are extraction, isolation, characterization of bioactive compound, pharmacological screening, toxicological evaluation and clinical evaluation. This review focus on different available techniques for the extraction of these phytochemicals ,its isolation and characterization for structure identification ,number of hydrogen atom, carbon atom present in the molecule and its molecular weight through phytochemical screening assay, chromatographic techniques, such as NMR, HPLC, HPTLC, GCMS, and FTIR. etc.

Strategies in the search of new natural compounds.[9]

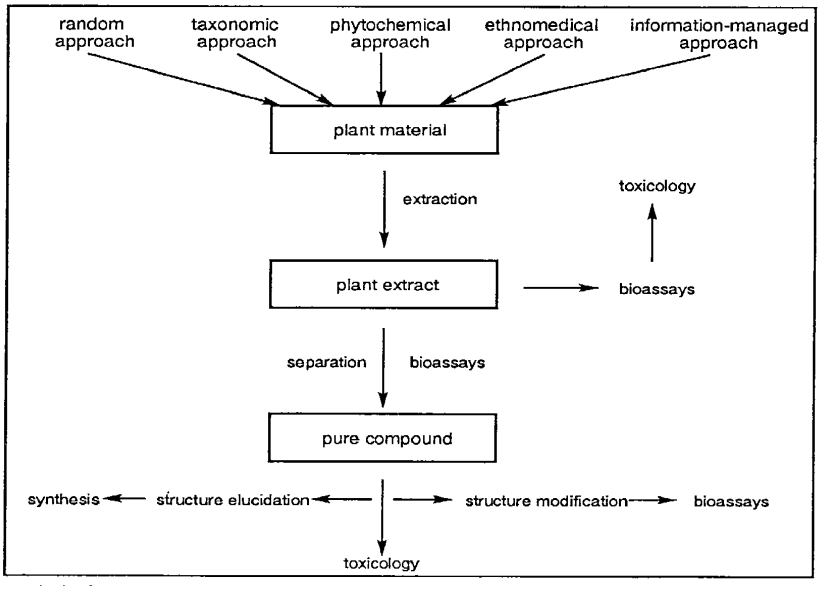


Fig. 1: General procedure for obtaining active principles from plants.

To study medicinal plants, it is first of all necessary to know which plant to select and what type of biological activity to look for. The selection criteria of plants, which potentially contain new biological agents, is based on five principle approaches: the random, the taxonomic, the phytochemical, the ethnomedical and the information-managed approach. In the random approach all available species are collected, irrespective of prior knowledge and experience. In the taxonomic approach, plants of a specific genus or family are deemed to be of interest, and sought from diverse locations. The phytochemical (chemo-taxonomic) approach is based on a particular compound type, which is of biological interest. Plants

anticipated to produce related compounds are collected. Taxonomic and the phytochemical approach are closely related and can not be clearly divided from each other. In the ethnomedical approach, credence is given to information on the medicinal use of the plant. Based on this information, the plant is collected and evaluated.

Practical Aspects of Herbal Drug Discovery[10,11]

The following scheme represents a summary of the stages involved in the development of pure drug from a plant source.

- Collection and identification of the plant and deposition of voucher sample in local and major herbarium.
- Literature survey on the plant species selected for studies.
- Extraction with solvent and preparation of non-polar and polar extracts for initial biological testing.
- Evaluation of plant extract against a panel of biological test methods, as exemplified by receptor biding, enzyme inhibition, and /or cytotoxicity assays.
- Activity guided fractionation on the extract showing activity, by monitoring each chromatographic fraction with bioassay chosen from the panel available to the investigation.
- Structure elucidation of pure active isolates using spectroscopic techniques and chemical methods, if necessary.
- Test each active compound (whether of novel or known chemical structure) in all in vitro and in vivo biological test methods available, in order to determine potency and selectivity of the drug.
- Perform molecular modeling studies and prepare derivatives of the active compound of interest.
- Sometime requires to carry out large scale reisolation of interesting active compounds for toxicological, pharmacological and for mutation studies.
- Clinical trials.

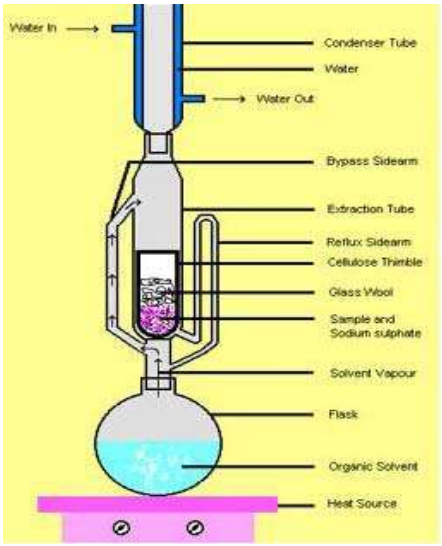
Extraction Methods for Studying Phytochemicals

Extraction is the first important step in the analysis of medicinal plants, because for further separation and characterization it is necessary to extract the desired chemical components from the plant materials. The extraction of plant constituents is essential to isolate biologically active compounds and in understanding their role. Extraction is the first step in

the analysis of medicinal plants. The basic steps included are pre-washing, drying of plant materials or freeze drying, grinding to obtain a homogenous sample. Extraction from the plant is important since different solvents are utilized at varying conditions such as time and temperature of extraction. It must be assured that the potential active constituents are not lost or distorted during the process of extraction. If the plant was selected on the basis of traditional uses,[12] then it is needed to prepare the extract as described by the traditional healer in order to mimic as closely as possible the traditional 'herbal' drug. Different type of solvent systems are available to extract the active compound. The specific solvent system to be used depends on the nature of the bioactive compound which is being targeted. The extraction of hydrophilic compounds uses polar solvents such as methanol, ethanol or ethyl-acetate. For extraction of more lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol in ratio of 1:1 are used. In some instances, extraction with hexane is used to remove chlorophyll.[13] As the target compounds may be non-polar to polar and thermally labile, the suitability of the methods of extraction must be considered. Various methods, such as sonification, heating under reflux, soxhlet extraction and others are commonly used[14-16] for the plant samples extraction. In addition, plant extracts are also prepared by maceration or percolation of fresh green plants or dried powdered plant material in water and/or organic solvent systems. Further fractionation of extracted compounds done on the basis of their acidity, polarity or molecular size. The extraction methods mostly used has been discussed below:

Hot Continuous Extraction (Soxhlet)

In this method, the finely ground crude drug is placed in a porous bag or "thimble" made of strong filter paper, which is placed in chamber E of the Soxhlet apparatus. The extracting solvent in flask A is heated, and its vapors condense in condenser D. The condensed extractant drips into the thimble containing the crude drug, and extracts it by contact. When the level of liquid in chamber E rises to the top of siphon tube C, the liquid contents of chamber E siphon into flask A. This process is continuous and is carried out until a drop of solvent from the siphon tube does not leave residue when evaporated. The advantage of this method, compared to previously described methods, is that large amounts of drug can be extracted with a much smaller quantity of solvent. This effects tremendous economy in terms of time, energy and consequently financial inputs. At small scale, it is employed as a batch process only, but it becomes much more economical and viable when converted into a continuous extraction procedure on medium or large scale.[17]



Soxhlet Apparatus

Microwave-assisted extraction (MAE)

It simply termed as microwave extraction, that combines microwave and traditional solvent extraction. It has application in extraction of high-value compounds from natural sources including phytonutrients, nutraceutical and functional food ingredients and pharmaceutical actives from biomass. Heating the solvents and plant tissue using microwave increases the kinetic of extraction, is called microwave-assisted extraction.[18] The target for heating in dried plant material is the minute microscopic traces of moisture that occurs in plant cells. The heating up of this moisture inside the plant cell due to microwave effect, results in evaporation and generates tremendous pressure on the cell wall. The cell wall is pushed from inside due to the pressure and the cell wall ruptures. Thus the exudation of active constituents from the ruptured cells occurs, hence increasing the yield of phytoconstituents.[19,20] It offers following advantages:[21]

1. Increased purity of crude extracts, improved products, improved stability of marker compounds, possibility to use less toxic solvents.
2. Increased recovery and purity of marker compounds, reduced processing costs, very fast extraction rates, reduced energy and solvent usage.

The other modern extraction techniques include solid-phase micro-extraction, supercritical-fluid extraction, pressurized-liquid extraction, microwave-assisted extraction, solid-phase extraction, and surfactant-mediated techniques, which possess certain advantages. These are the reduction in organic solvent consumption and in sample degradation, elimination of additional sample clean-up and concentration steps before chromatographic analysis,

improvement in extraction efficiency, selectivity, and/ kinetics of extraction. The ease of automation for these techniques also favors their usage for the extraction of plants materials.[22]

Isolation, Identification and Characterisation of Phytochemicals

Combination of various types of bioactive compounds or phytochemicals with different polarities are usually present in different plant extracts. Separation, identification and characterization of bioactive compounds is a big challenging job in herbal drug development process. A number of different separation techniques are used for identification and characterization of these bioactive compounds to obtain pure compounds such as TLC, column chromatography, flash chromatography, HPTLC and HPLC. The pure compounds are then used for the determination of structure and pharmacological activity. Various techniques such as phyto-chemical screening assay, Fourier-transform infrared spectroscopy (FTIR), NMR(Nuclear Magnetic Resonance), GCMS (Gas Chromatography and Mass Spectrometry) are specifically used for the identification of the bioactive compounds through which lead molecule structure identification can become easy.[23]

Phytochemical screening assay[24-29]

Phytochemical screening assay is a simple, quick, and inexpensive procedure that tells about various types of phytochemicals in a mixture and an important tool in bioactive compound analyses. Phytochemical examinations are carried out for all the extracts as per the standard methods.

1. Tests for Carbohydrates

Preparation of test solution: The test solution was prepared by dissolving the test extract with water. Then it was hydrolyzed with 1 volume of 2N HCl and subjected to following chemical tests.

- Molisch's test (General test): To 2-3 ml aqueous extract, added few drops of α-naphthol solution in alcohol, shaken and added concentrated H_2SO_4 from sides of the test tube and then observed for violet ring at the junction of two liquids.
- Fehling's test: 1 ml Fehling's A and 1ml Fehling's B solutions were mixed and boiled for one minute. Added equal volume of test solution. Heated in boiling water bath for 5-10 min. observed for a yellow, then brick red precipitate.

- Benedict's test: Equal volume of Benedict's reagent and test solution in test tube were mixed. Heated in boiling water bath for 5 min. Solution may appear green, yellow or red depending on amount of reducing sugar present in test solution.
- *Barfoed's test*: Equal volume of Barfoed's reagent and test solution were added. Heated for 1-2 min, in boiling water bath and cooled. Observed for red precipitate.

2. Tests for Proteins

- Biuret test (general test): To 3 ml test solution (T.S.) add 4% NaOH and few drops of 1% $CuSO_4$ solution observed for violet or pink colour.
- Million's test (for proteins): Mixed 3 ml T.S. with 5 ml million's reagent, white precipitate. Precipitate warmed turns brick red or precipitate dissolves giving red colour.
- Xanthoprotein test (For protein containing tyrosine or tryptophan): Mixed 3 ml T.S. with 1 ml concentrated H_2SO_4 observed for white precipitate.

3. Tests for Amino acids

- Ninhydrin test (general test): 3 ml T.S. and 3 drops 5% Ninhydrin solution were heated in boiling water bath for 10 min. observed for purple or bluish colour.
- Test for tyrosine: Heated 3 ml T.S. and 3 drops Million's reagent. solution observed for dark red colour.
- Test for tryptophan: To 3 ml T.S. added few drops glycoxalic acid and concentrated H_2SO_4 observed for reddish violet ring at junction of the two layers.
- Test for cysteine: To 5 ml. T.S. add few drops of 40% sodium hydroxide and 10% lead acetate solution. Boil. Black ppt. of lead sulphate is formed.

4. Tests for Steroid and Triterpenoid

- Salkowaski reaction: Mixed 2 ml of extract, 2 ml chloroform and 2 ml concentrated H_2SO_4, Shake well, whether chloroform layer appeared red and acid layer showed greenish yellow fluorescence was observed.
- Liebermann-burchard reaction: Mixed 2ml extract with chloroform add 1-2 ml acetic anhydride and 2 drops concentration H_2SO_4 from the side of test tube observed for first red, then blue and finally green colour was observed.
- Liebermann's reaction: Mixed 3 ml extract with 3 ml acetic anhydride. Heated and cooled. Added few drops concentrated H_2SO_4 observed for blue colour.

Tests for Glycosides

Preparation of test solution: The test solution was prepared by dissolving extract in the alcohol or hydro-alcoholic solution.

A) Tests for cardiac glycosides

- Baljet's test: A test solution observed for yellow to orange colour with sodium picrate.
- Legal's test (For cardenoloids): To aqueous or alcoholic test solution, added 1 ml pyridine and 1 ml sodium nitroprusside observed for pink to red colour.
- Test for deoxysugars (Keller killiani test): To 2 ml extract, add glacial acetic acid, one drop of 5% $FeCl_3$ and concentrated H_2SO_4 observed for reddish brown colour at junction of the two liquid and upper layers bluish green.
- Liebermann's test (for bufadenolids): Mixed 3 ml extract with 3 ml acetic anhydride. Heat and cooled. Added few drops concentrated H_2SO_4 observed for blue colour.

B) Tests for saponin glycosides

- Foam test: The extract was shaken vigorously with water. Persistent foam was observed.
- Haemolytic test: Added test solution to one drop of blood placed on glass slide. Haemolytic zone appears.

C) Tests for anthraquinone glycosides

- Borntrager's test: To 3 ml. extract, add dil.H_2SO_4. Boil and filter. To cold filtrate, add equal volume benzene or chloroform. Shake well. Separate the organic solvent. Add ammonia. Ammoniacal layer turns pink or red.
- Modified borntrager's test: To 5 ml. extract, add 5 ml. 5% $FeCl_3$ and 5 ml. dil. HCl. Heat for 5 min. in boiling water bath. Cool and add benzene, shake well and separate organic layer. Add equal volume dil. ammonia in organic layer. Ammoniacal layer shows pinkish red colour.

Tests for Flavonoids

The flavonoids are all structurally derived from the parent substance called flavone. The flavonoids occur in the free form as well as bound to sugars as glycosides. For this reason, when analyzing flavonoids it is usually better to examine the flavonoids in hydrolyzed plant extracts.

Preparation of test solution

i. To a small amount of extract added equal volume of 2M HCl and heated in a test tube for 30 to 40 min. at 100°C.
ii. The cooled extract was filtered, and extracted with ethyl acetate.
iii. The ethyl acetate extract was concentrated to dryness, and used to test for flavonoids.

- Shinoda test: To extract, add 5 ml 95% ethanol, few drops concentrated HCl and 0.5 g magnesium turnings. Pink colour was observed. To small quantity of residue, acetate solution was added, observed for yellow coloured precipitate. Addition of sodium hydroxide to the residue showed yellow colouration, which was decolourised after addition of dilute hydrochloric acid.
- Ferric chloride test: Test solution with few drops of ferric chloride solution shows intense green colour.
- Alkaline reagent test: Test solution was treated with sodium hydroxide solution shows intense yellow colour which becomes colourless on addition of few drops of dilute hydrochloric acid.
- Lead acetate solution test: Test solution with few drops of lead acetate solution (10%) gives yellow precipitates.

Tests for Alkaloids

- Mayer's test: Test solution treated with mayer's reagent (Potassium mercuric iodide) cream coloured precipitate was not obtained.
- Wagner's reagent: The test solution treated with wagner's reagent (Iodine in potassium iodide) brown precipitate was not obtained.
- Hager's test: The test solution treated with hager's reagent (Saturated picric acid solution) gives yellow precipitate.
- Dragendorff's test: The test solution treated with dragendorff's reagent (Pottasium bismuth iodide) reddish brown precipitate was not obtained.

Tests for Tannins and Phenolic compounds

To 2-3 ml of extract, add few drops of following reagents:

5% $FeCl_3$ solution: deep blue-black colour.

Lead acetate solution: white precipitate.

Gelatin solution: white precipitate

Bromine water*:* decoloration of bromine water.

Acetic acid solution: red colour solution

Dilute iodine solution: transient red colour.

Dilute HNO_3: reddish to yellow colour.

Dilute $KMnO_4$: Pink colour disappears.

Chromatography techniques

Chromatography is a technique where the molecules are separated based on their shape, size and charge.[30] In any extract, there are hundreds of unknown components and many of them are in very low amount. During chromatography analyte in solvent and move through solid phase that acts as a sieving material. As molecule proceeds further through molecular sieve it gets separated. 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 herbal medicine. Thin layer chromatography are the chromatographic techniques which readily provides qualitative information and through which it become possible to obtain quantitative data.

Thin layer chromatography (TLC)

Stahl given the first practical application of thin layer chromatography.[31] Advantage of TLC is its versatility, speedy and sensitivity. TLC is an adsorption chromatography[32] where samples are separated based on the interaction between a thin layers of adsorbent attached on the plate. The technique mostly used for the separation of low molecular weight compounds. Different adsorbent like silica gel, aluminium, cellulose powder, starch etc can be used to separate various compounds like amino acids, alkaloids, phenols, steroids, vitamins etc.

It is being employed extensively for the following reasons:

1) It enables rapid analysis of herbal extracts with minimum sample clean-up requirement.
2) It provides qualitative and semi quantitative information of the separated compounds
3) It enables the quantification of chemical constituents.

Table no 1: TLC mobile phase for important classes of phytoconstitutents.[45]

Plant constituents	Stationary Phase	Mobile phase	Detection
Carbohydrates	Silica gel	Ethyl acetate: toluene(1:1)	10% ethanolic sulphuric acid
Alkaloids/ phenanthrenes	Silica gel	Toluene: Ethyl acetate: diethylamine (7:2:1)	Dragendroff Reagent
Flavonoids	Silica gel	Ethyl acetate:Formic acid:glacial acetic acid:water (10:1.1:1.1:2.6)	UV 254nm or 366nm
Tannins	Silica gel	Ethyl acetate:Formic acid:glacial acetic acid:water (7.5:0.3:0.2:2)	Vanillin Sulfuric acid reagent
Saponin glycoside	Silica gel	Chloroform:cglacial acetic acid: Methanol: water (6.4:3.2:1.2:0.8)	Vanillin Sulfuric acid reagent
Specific Mobile phases			
Betasitosterol	Silica gel	Benzene: Ethylacetate (9:1)	Vanillin Sulfuric acid reagent
Rutin	Silica gel	Ethyl acetate:Formic acid:glacial acetic acid: water (10:1.1:1.1:2.6)	UV 254nm or 366nm
Curcumin	Silica gel	Chloroform:methanol(9.8:0.2)	Visible light
Gingerol	Silica gel	Toluene: ethylacetate (9.3:0.7)	Vanillin Sulfuric acid reagent
Stigmasterol	Silica gel	Petroleum ether:ethyl acetate (7:3)	Vanillin Sulfuric acid reagent

High performance thin layer chromatography (HPTLC)

HPTLC is a more powerful separation tool for quantitative analysis and it uses the technique in a more optimized way. High performance thin layer chromatography (HPTLC) is a planar chromatography where separation of sample components is achieved on high performance layers with detection and data acquisition. These high performance layers are pre-coated plates coated with a sorbent of particle size 5-7 microns and a layer thickness of 150-200 microns. The reduction in thickness of layer and particle size results in increasing the plate efficiency as well as nature of separation. HPTLC gives chromatogram i.e. separated samples after chromatography can be inspected by the eyes only in case of HPTLC. The procedure used is as follow:[33] A silica gel 60 F254 pre-coated plate (20 x 10 cm) are used with any developed solvent system. Different extracts are to be spotted on pre-coated HPTLC plates. Spots of different concentration in (l micro lit) was applied on HPTLC plates to study the exact separation of spots. Saturation time will be 20 minutes and room temperature 25^{o} C + 2^{o} C. TLC Plates was developed upto 8 cm. After air drying, a plate was heated at 110^{o} C for 2-3 minutes. In TLC fingerprinting, the data that can be recorded using a high performance TLC (HPTLC) scanner includes the chromatogram, retardation factor (Rf) values, the colour of the separated bands, their absorption spectra, λ max 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.

Column chromatography (CC)

Column chromatography involves ion exchange, molecular sieves, and adsorption phenomenon. The flushing in conventional chromatography greatly dilutes the material, and the fractions usually require another step for concentration. A newer method called displacement chromatography elute with some compounds that has great affinity for the adsorbent. Fractions of elute materials can be more concentrated than the original solution applied to column. The column was prepared using silica for column chromatography. The fraction was dissolved in smallest possible volume of solvent and it was mixed with 2 gms of silica for column chromatography. The mixture was dried to obtain free flowing powder and it was added to column. Then the column was eluted with solvent of various proportions. The eluent was collected in properly cleaned test tube.[34]

High performance liquid chromatography (HPLC)[35]

High performance liquid chromatography (HPLC) is a versatile, robust, and widely used technique for the isolation of natural products. Currently, this technique is gaining popularity among various analytical techniques as the main choice for fingerprinting study for the quality control of herbal plants. The biologically active entity is often present only as minor component in the extract and the resolving power of HPLC is ideally suited to the rapid processing of such multicomponent samples on both an analytical and preparative scale. HPLC instruments now are modular in design and comprise a solvent delivery pump, a sample introduction device such as an auto-sampler or manual injection valve, an analytical column, a guard column, detector and a recorder or a printer. Chemical separations can be accomplished using HPLC by utilizing the fact that certain compounds have different migration rates given a particular column and mobile phase. The extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase. Generally the identification and separation of phytochemicals can be accomplished using isocratic system (using single unchanging mobile phase system). Gradient elution in which the proportion of organic solvent to water is altered with time may be desirable if more than one sample component is being studied and differ from each other significantly in retention

under the conditions employed. Identification of compounds by HPLC is a crucial part of any HPLC assay. In order to identify any compound by HPLC, a detector must first be selected. Once the detector is selected and is set to optimal detection settings, a separation assay must be developed. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels which the assay will be performed. UV detectors are popular among all the detectors because they offer high sensitivity and also because majority of naturally occurring compounds encountered have some UV absorbance at low wavelengths (190-210 nm). The high sensitivity of UV detection is bonus if a compound of interest is only present in small amounts within the sample. Besides UV, other detection methods are also being employed to detect phytochemicals among which is the diode array detector (DAD) coupled with mass spectrometer (MS). Liquid chromatography coupled with mass spectrometry (LC/MS) is also a powerful technique for the analysis of complex botanical extracts. It offers accurate determination of molecular weight of proteins, peptides. Isotopes pattern can also be detected by this technique. Recent advances includes electro spray, thermo spray, and ion spray ionization techniques which offer unique advantages of high detection sensitivity and specificity.[36,37] It provides abundant information for structural elucidation of the compounds when tandem mass spectrometry (MSn) is applied. Therefore, the combination of HPLC and MS facilitates rapid and accurate identification of chemical compounds in medicinal herbs, especially when a pure standard is unavailable. The processing of a crude source material to provide a sample suitable for HPLC analysis as well as the choice of solvent for sample reconstitution can have a significant bearing on the overall success of natural product isolation. The source material, e.g., dried powdered plant, will initially need to be treated in such a way as to ensure that the compound of interest is efficiently liberated into solution. In the case of dried plant material, an organic solvent (e.g., methanol, chloroform) may be used as the initial extractant and following a period of maceration, solid material is then removed by decanting off the extract by filteration. The filtrate is then concentrated and injected into HPLC for separation. The usage of guard columns is necessary in the analysis of crude extract. Many natural product materials contain significant level of strongly binding components, such as chlorophyll and other endogenous materials that may in the long term compromise the performance of analytical columns. Therefore, the guard columns will significantly protect the lifespan of the analytical columns. HPLC is useful for compounds

that cannot be vaporized or that decompose under high temperature, and it provides a good complement to gas chromatography for detection of compounds.[38]

METHODS OF DETECTION

Fourier-transform infrared spectroscopy (FTIR)

FTIR has proven to be a valuable tool for the characterization and identification of compounds or functional groups (chemical bonds) present in an unknown mixture of plants extract.[39,40] It helps for identification and structure determination of the molecule. In addition, FTIR spectra of pure compounds are usually so unique that they are like a molecular "fingerprint". For most common plant compound, the spectrum of an unknown compound can be identified by comparison to a library of known compounds. Samples for FTIR can be prepared in a number of ways. For liquid samples, the easiest is to place one drop of sample between two plates of sodium chloride. The drop forms a thin film between the plates. Solid samples can be milled with potassium bromide (KBr) to and then compressed into a thin pellet which can be analyzed.[41] The region in IR spectrum above 1200cm-1 shows spectral bands or peaks due to the vibrations of individual bonds or functional groups under examination. The region below 1200 cm-1 indicates bands due to the vibrations of the whole molecule and because of its complexity is known as the 'Fingerprint region'. Intensities of the various bands are recorded subjectively on a simple scale as being either strong (S), medium (M) or weak (W).[42] And as per new techniques developed, the advanced instruments of company bruker, jasco has made easier by application of one drop or pinch of sample on the instruments and this softerware will give the results and samples can be reused.

Mass spectrometry (MS)

Mass spectrometry is a powerful analytical technique for the identification of unknown compounds, quantification of known compounds and to elucidate the structure and chemical properties of molecules. Through MS spectrum the molecular weight of sample can be determined. The value of the technique is that it requires only microgram amounts of material, that it can provide an accurate molecular weight and that it may yield a complex fragmentation pattern which is often characteristic of that particular compound.[42] This technique works successfully for the structural elucidation of organic compounds, for peptide or oligonucleotide sequencing and for monitoring the existence of previously characterizes compounds in complex mixtures with a high specificity by defining both the molecular weight and a diagnostic fragment of the molecule simultaneously. Gas chromatography

equipment can be directly coupled with rapid scan mass spectrometer (GCMS) of various types. High resolution analysis can be performed due to coupling of equipments.

Liquid Chromatography- Mass Spectroscopy (LC-MS) offers accurate determination of molecular weight of proteins, peptides. Isotopes pattern can also be detected by this technique. Recent advances includes electro spray, thermo spray, and ion spray ionization techniques which offer unique advantages of high detection sensitivity and specificity.[43,37]

Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear Magnetic Resonance Spectroscopy gives physical, chemical and biological properties of matter. C^{13} NMR is used to identify the types of carbon are present in the compound. H1- NMR is used to find out types of hydrogen are present in the compound and to find out how the hydrogen atoms are connected. Proton NMR spectroscopy is basically provides a method for determining the structure of an organic compound by measuring the magnetic moments of its hydrogen atoms. In most compounds, hydrogen atoms are attached to different groups (as -CH2-, -CH-, -CHO, -NH2, -CHOH-, etc.) and the proton NMR spectrum provides a record of the number of hydrogen atoms in these different situations. However, it cannot give any direct information on the nature of the carbon skeleton of the molecule; this can only be obtained by carbon 13 NMR spectroscopy. 13C-NMR spectroscopy is complementary to proton NMR and the combination of the two techniques provides a very powerful means of structural elucidation for new terpenoids, alkaloids or flavonoids. It is useful in the analysis of glycosides, in indicating the linkage between sugar moieties and their configurations. Both proton and 13C-NMR measurements have been successfully applied to structural and other analyses of proteins and other macromolecules.[42]
Liquid Chromatography- Nuclear Magnetic Resonance (LC-NMR) is a combination of chromatographic separation technique with NMR spectroscopy. It 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.[44]

CONCLUSION

With growing interest towards herbal drugs development with minimum side effects there are better opportunities to explore the medicinal and other biological properties of previously inaccessible natural products. To establish its usefulness, it is mandatory to focus on visualization and identification of unused herbal plants over the world. Then it is emphasized

on extraction, its isolation and characterization of phytochemicals which is gift of the nature in rational and scientific way. There is an unmet need for utilization of the natural products for the benefit of human kind and development of new lead for drug discovery. Once the phytochemical is obtained this can be used for the further exploration through QSAR studies, molecular modeling, animal studies followed by clinical trial.

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