WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.990
Volume 4, Issue 5, 2703-2717. Review Article ISSN 2277– 7105

EXTRACTION, ISOLATION AND CHARACTERIZATION OF PHYTOCHEMICALS

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Article Received on
16 March 2015,

Revised on 07 April 2015,
Accepted on 01 May 2015

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INTRODUCTION

Plants are natural reservoir of medicinal agents. These are almost free from the side effects.[1] Nearly 75- 80% of the world's population relies on traditional medicines for primary health care, most of which involve the use of plant extracts their active constituents.[2,3] Since ancient times medicinal plants have been the mainstay of traditional herbal medicine amongst rural dwellers. 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.[4,5] 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. Today we are living in the era of evidence based medicine. The evidences can only be obtained when we have pure form of phytochemicals. This review is to focus different available techniques for the extraction of these phytochemicals. The major steps involved in utilizing the biologically active compound from plant resources are extraction, pharmacological screening, isolation and characterization of bioactive compound, toxicological evaluation and clinical evaluation.

Various classes of phytochemicals are[6]

1. Alkaloids
2. Glycosides
3. Flavonoids
4. Saponins
5. Terpenes
6. Tannins
7. Phenolics
8. Anthraquinones
9. Essential oils
10. Steroids

Extraction from the plant is an initial step in which different solvents are utilized under a variety of conditions such as time and temperature. After extraction, the bioactive component then has to be separated from the co extractives. Further purification steps may involve simple crystallization of the compound from the crude extract, further solvent partition of the co extractives or chromatographic methods in order to fractionate the compounds based on their acidity, polarity or molecular size. Final purification, to provide compounds of suitable purity for such structural analysis, may be accomplished by appropriate techniques such as recrystallization, sublimation, or distillation.

EXTRACTION

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. It must be assured that the potential active constituents are not lost or distorted during the process of extraction. 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 general techniques of medicinal plant extraction include.[7]

1. Maceration
2. Infusion
3. Percolation
4. Digestion

5. Decoction
6. Hot continuous extraction (soxhlet)
7. Aqueous-alcoholic extraction by fermentation
8. Counter-current extraction
9. Ultrasound extraction (sonication)
10. Microwave-assisted extraction
11. Supercritical fluid extraction
12. Phytonic extraction (with hydrofluorocarbon solvents).

For aromatic plants, hydrodistillation techniques (water distillation, steam distillation, water and steam distillation), hydrolytic maceration followed by distillation, expression and enfleurage (cold fat extraction) may be employed. Some latest extraction methods for aromatic plants include.[7]

1. Headspace trapping,
2. Solid phase micro-extraction,
3. Protoplast extraction,
4. Microdistillation,
5. Thermomicrodistillation and
6. Molecular distillation.

There are mainly three parameters which govern extraction.

1. Part of the plant to be used
2. Nature and concentration of solvent as well as
3. The techniques for extraction.

The extracted phytochemical depends on nature and origin of plant material, degree of processing, physical characters like moisture and particle sizes. Other factors which influence the varioation in extracted compound are timing of extraction, temperature, etc.

SELECTION OF PLANT MATERIAL & CHOICE OF SOLVENT

Bioactive components can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc.

Properties of a good solvent in plant extractions includes, low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, inability to cause the extract to complex or dissociate

The factors affecting the choice of solvent are

1. Quantity of phytochemical to be extracted
2. Rate of extraction
3. Diversity of different compounds extracted
4. Ease of subsequent handling of the extracts
5. Toxicity of the solvent in the bioassay process
6. Potential health hazard of the extractants

The choice also depends upon the bioactive target which is to be extracted.[8]

The various solvents used for extraction are

1. Water
2. Acetone
3. Alcohol
4. Acetone
5. Ether
6. Dichloromethane etc.

Variation in extraction methods usually depends upon

1. Length of the extraction period,
2. Solvent used and its pH.
3. Temperature.
4. Particle size of the plant tissues
5. The solvent-to-sample ratio

VARIOUS PROCEDURES OF EXTRACTION

1) Plant tissue homogenization

Dried or wet, fresh plant parts are grinded in a blender then put in a certain quantity of solvent and shaken vigorously for 5 - 10 min or left for 24 h after which the extract is filtered. The filtrate thus obtained then may be dried under reduced pressure and redissolved in the

solvent to determine the concentration. However the filtrate may also be centrifuged for clarification of the extract.[9]

2) Serial exhaustive extraction

In this method successive extraction with solvents of increasing polarity from a non polar (hexane) to a more polar solvent (methanol) is done. It ensures that a wide polarity range of compound could be extracted. This method cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds.[9]

3) Soxhlet extraction

This method is used where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a high solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. This method cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds.[10]

4) Maceration: In maceration (for fluid extract), whole or coarsely powdered plant-drug is kept in contact with the solvent in a container for a defined period with frequent agitation until soluble matter is dissolved. This method is best suitable for use in case of the thermolabile drugs.[11]

5) Digestion: In this method gentle heat is applied during the maceration extraction process. It is used when moderately elevated temperature is not objectionable and the solvent efficiency of the menstrum is increased thereby.[12]

6) Infusion: It is a dilute solution of the readily soluble components of the crude drugs. Fresh infusions are prepared by macerating the solids for a short period of time with either cold or boiling water.[12]

7) Decoction: This method is used for the extraction of the water soluble and heat stable constituents from crude drug by boiling it in water for 15 minutes, cooling, straining and passing sufficient cold water through the drug to produce the required volume.[12]

8) Sonication: In this procedure ultrasound with frequencies ranging from 20 kHz to 2000 kHz is used. This increases the permeability of cell walls and produces cavitations. Its large-scale application is limited due to the higher costs.[13]

9) Percolation: This is procedure used mostly to extract active ingredients in the preparation of tinctures and fluid extracts. In this a percolator is used. The solid ingredients are moistened with an appropriate amount of the specified menstrum and allowed to stand for approximately 4 h in a well closed container, after which the mass is packed and the top of the percolator is closed. Additional menstrum is added to form a shallow layer above the mass, and the mixture is allowed to macerate in the closed percolator for 24 h. The outlet of the percolator then is opened and the liquid contained therein is allowed to drip slowly. Additional menstrum is added as required, until the percolate measures about three-quarters of the required volume of the finished product. The marc is then pressed and the expressed liquid is added to the percolate. Sufficient menstrum is added to produce the required volume, and the mixed liquid is clarified by filtration.[13]

NEWER METHODS OF EXTRACTION

Supercritical fluid extraction (SFE)

It is one of the most advanced extraction system. It involves use of gases, usually CO2, and compressing them into a dense liquid.[14] This dence liquid is then pumped through a cylinder containing the material to be extracted. The extract-laden liquid is then pumped into a separation chamber where the extract is separated from the gas and the gas is recovered for re-use. Solvent properties of CO2 can be manipulated and adjusted by varying the pressure and temperature. The advantages of SFE are, the versatility it offers in pinpointing the constituents to be extracted from a given material and the fact that yourend product has virtually no solvent residues left in it (CO2 evaporates completely). But this technology is quite expensive. There are many other gases and liquids that are highly efficient as extraction solvents when put under pressure.

a)Coupled SFE-SFC System in which a sample is extracted with a supercritical fluid which then places the extracted material in the inlet part of a supercritical fluid chromatographic system. The extract is than chromatographed directly using supercritical fluid.[14]

b)Coupled SFE-GC and SFE-LC System in which a sample is extracted using a supercritical fluid which is then depressurized to deposit the extracted material in the inlet part or a column of gas or liquid chromatographic system respectively. Advantages are robustness of sample preparation, reliability, less time consuming, high yield and also has potential for coupling with number of chromatographic methods.[14]

Microwave-Assisted extraction

It is anewer microwave-assisted solvent-extraction technology known as Microwave-Assisted Processing (MAP). It has application in extraction of high-value compounds from natural sources including phytonutrients, nutraceutical and functional food ingredients and pharmaceutical actives from biomass. It offers following advantages:

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.

To deliver desired product attributes and optimize process economics many variables like microwave power and energy density etc. can be tuned accordingly. Examples include antioxidants from dried herbs, carotenoids from single cells and plant sources, taxanes from taxus biomass, essential fatty acids from microalgae and oilseeds, phytosterols from medicinal plants, polyphenols from green tea, flavour constituents from vanilla and black pepper, essential oils from various sources, and many more (Patil & Shettigar, 2010).

Solid phase extraction

In this method absorption of solutes from a liquid medium onto a solid adsorbent is done so that molecules are retained on chromatographic stationary phases. The adsorbents, like chromatographic media, come in the form of beads or resins. These can be used in column or in batch form. Solid phase extraction media include reverse phase, normal phase, and ion-exchange media. This is method for sample purification that separates and concentrates the analyte from solution of crude extracts by adsorption onto a disposable solid-phase cartridge. The analyte is normally retained on the stationary phase, washed and then evaluated with different mobile phase. If an aqueous extract is passed down a column containing reverse phase packing material, everything that is fairly non-polar will bind, whereas everything polar will pass through (Patil & Shettigar, 2010).

ISOLATION, IDENTIFICATION AND CHARACTERISATION OF PHYTOCHEMICALS

Plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities. Their separation is a big challenge for the process of identification and characterization of bioactive compounds. 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, Sephadex chromatography and HPLC, should be. The pure compounds are then used for the determination of structure and biological activity. Various non-chromatographic techniques such as immunoassay, which use monoclonal antibodies (MAbs), phyto-chemical screening assay, Fourier-transform infrared spectroscopy (FTIR), can also be used for the identification of the bioactive compounds.

I) Phytochemical screening assay

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.

Detection of alkaloids

- **Mayer's Test**:- Filtrate is treated with Mayer's reagent (Potassium Mercuric Iodide). If there is formation of a yellow coloured precipitate it indicates the presence of alkaloids.
- **Wagner's Test**. – In this test filtrate is treated with Wagner's reagent (Iodine in Potassium Iodide). If there is formation of brown/reddish precipitate, it indicates the presence of alkaloids.
- **Dragendroff's Test**- Filtrates treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of alkaloids.
- **Hager's Test**- Filtrates treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow coloured precipitate

Detection of carbohydrates

- **Molisch's Test**- For the detection of carbohydrates filtrate is treated with 2 drops of alcoholic α-naphthol solution in a test tube. Formation of the violet ring at the junction indicates the presence of Carbohydrates.

- **Benedict's Test** - In this test filtrate is treated with Benedict's reagent and heated gently. If there is formation of orange red precipitate, it indicates the presence of reducing sugars.
- **Fehling's Test –** In this test filtrate is first hydrolysed with dil. HCl, neutralized with alkali and then heated with Fehling's A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

Detection of glycosides

- **Modified Borntrager's Test**: This test is done for the detection of glycosides. Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. After this the mixture is cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammoniacal layer indicates the presence of anthranol glycosides.
- **Legal's Test**- Extract is treated with sodium nitroprusside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

Detection of saponins

- **Froth Test**- Extract is diluted with distilled water to 20ml and this is shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.
- **Foam Test**- In this test 0.5 gm of extract is shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

Detection of phytosterols

- **Salkowski's Test**- Extracts is treated with chloroform and filtered. The filtrates is then treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.
- **Libermann Burchard's Test –** Extract is treated with chloroform and filtered. The filtrate is treated with few drops of acetic anhydride, boiled and cooled. Conc. Sulphuric acid is then added. Formation of brown ring at the junction indicates the presence of phytosterols.

Detection of phenols

- **Ferric Chloride Test**-Extract is treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

Detection of tannins

- **Gelatin Test**- In this test we add 1% gelatine solution containing sodium chloride to the extract. Formation of white precipitate indicates the presence of tannins.

Detection of flavonoids

- **Alkaline Reagent Test**- Extract is treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.
- **Lead acetate Test**- Extracts is treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of Flavonoids.

Detection of proteins and aminoacids

- **Xanthoproteic Test**- Extract is treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.
- **Ninhydrin Test**- 0.25% w/v Ninhydrin reagent is added to the extract and boiled for few minutes. Formation of blue colour indicates the presence of amino acid.

Detection of diterpenes

- **Copper acetate Test**- In this test we dissolve the extracts in water and then treat with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes.

II) Chromatographic techniques

In any extract, there are hundreds of unknown components and many of them are in very 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 herbal medicine.

Thin-layer chromatography (TLC)

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 resolved compounds

3) It enables the quantification of chemical constituents.

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.

Advanced chromatographic techniques

1. Liquid chromatography

a. **Preparative high performance liquid chromatography**- its higher column efficiencies and faster solvent velocities permit more difficult separation to be conducted more quickly.[15,16]

b. **Liquid Chromatography- Mass Spectroscopy (LC-MS)** - 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.[15,16]

c. **Liquid Chromatography- Nuclear Magnetic Resonance (LC-NMR)** - It 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.[17]

2. Gas chromatography

a) **Gas Chromatography Fourier Transform Infrared spectrometry**

It provides a potent means for separating and identifying the components of different mixtures.[18]

b) **Gas Chromatography-Mass Spectroscopy** - Gas chromatography equipment can be directly coupled with rapid scan mass spectrometer of various types. Simplest type is Ion Trap Detector (ITD).[18]

3. Supercritical Fluid Chromatography (SFC)

It combines some of the best features of both gas and liquid chromatography. It permits the separation and determination of a group of compounds that are not conveniently handled by either gas or liquid chromatography.

Other modified Chromato-Spectrometric studies

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.[19] Computer modelling has also been introduced. 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.

VARIOUS ASSAY METHODS

Antimicrobial assay

Common methods used are

- Agar diffusion method.[20]
- The dilution method[21]
- Turbidimetric and impedimetric monitoring of microbial growth.[22]

Antioxidant assays

- DPPH(2, 2'-diphenyl-1-picrylhydrazyl) radical scavenging system[23]
- Phenolics content and reducing power of extracts is often determined using the Folin-Ciocalteu method.
- For determination of reducing power of plant extracts, the ferric reducing/antioxidant power (FRAP) assay method can be applied.[24]

CONCLUSION

With the increasing technical advancement and growing interest towards drugs obtained from plant sources because of lesser toxicity, easier techniques, less time consuming and less

expensive methods, there are better opportunities to explore the medicinal and other biological properties of previously inaccessible natural products. To establish its usefulness, it is emphasised to focus on standardised methods of extraction as non- standardised methods may lead to degradation of phytochemicals, so that results obtained could be consistent, comparable and reproducible. There is an unmet need for utilization of the natural products for the benefit of human kind. This can only be achieved by using the gift of the nature in rational and scientific way. For validating and confirming the effectiveness and safety of the compound we need the pure form of the chemical which can only be obtained by the use of proper extraction techniques. Once the pure phytochemical is obtained this can be used for the further exploration in animals followed by clinical trial.

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