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Techniques for Extraction and Isolation of Natural Products: A Comprehensive Review

V. N. Raje[1], S. S. Lokhande[2], S. S. More[3]
[1]*Principal, Department of Pharmacy, G. E. S. College of Pharmacy, Satara, India*
[2,3]*Lecturer, Department of Pharmacy, G. E. S. College of Pharmacy, Satara, India*

***Abstract*: Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. Due to an increasing demand for chemical diversity in screening programs, seeking therapeutic drugs from natural products, interest particularly in edible plants has grown throughout the world. The aim of this work is to provide choices in the field of drug discovery and allied research areas on the optimal techniques that can be employed. Through a basic literature review method on the techniques that are systematically employed in drug discovery from natural sources, students, researchers and project managers can make an informed choice of the techniques to employ in their projects.**

***Keywords*: Bioactive compound, Plant Extraction, Isolation, Phytochemical evaluation of, herbal drugs.**

1. Introduction

Drug discovery from natural sources demands a pursuit of multifaceted approach to scientific investigation. It requires a good understanding of the ethno uses, chemistry, pharmacology, biology, and toxicology, among other disciplines, all to understand drug or drug-like activities [1] They found literally thousands of phytochemicals from plants as safe and broadly effective alternatives with less adverse effect. Many beneficial biological activity such as anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic and wound healing activity were reported. In many cases the people claim the good benefit of certain natural or herbal products. However, clinical trials are necessary to demonstrate the effectiveness of a bioactive compound to verify this traditional claim. Clinical trials directed towards understanding the pharmacokinetics, bioavailability, efficacy, safety and drug interactions of newly developed bioactive compounds and their formulations (extracts) require a careful evaluation. Clinical trials are carefully planned to safeguard the health of the participants as well as answer specific research questions by evaluating for both immediate and long-term side effects and their outcomes are measured before the drug is widely applied to patients [2].

Extraction, as the term is used pharmaceutically, involves the separation of medicinally active portions of plant or animal tissues from the inactive or inert components by using selective solvents in standard extraction procedures. The products so obtained from plants are relatively impure liquids, semisolids or powders intended only for oral or external use. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts and powdered extracts. Such preparations popularly have been called galenicals, named after Galen, the second century Greek physician [3]. The purposes of standardized extraction procedures for crude drugs are to attain the therapeutically desired portion and to eliminate the inert material by treatment with a selective solvent known as *menstruum*.

The extract thus obtained may be ready for use as a medicinal agent in the form of tinctures and fluid extracts, it may be further processed to be incorporated in any dosage form such as tablets or capsules, or it may be fractionated to isolate individual chemical entities such as ajmalicine, hyoscine and vincristine, which are modern drugs. Thus, standardization of extraction procedures contributes significantly to the final quality of the herbal drug [4].

Many beneficial biological activities such as anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic and wound healing activity were reported. In many cases the people claim the good benefit of certain natural or herbal products. However, clinical trials are necessary to demonstrate the effectiveness of a bioactive compound to verify this traditional claim. Clinical trials directed towards understanding the pharmacokinetics, bioavailability, efficacy, safety and drug interactions of newly developed bioactive compounds and their formulations (extracts) require a careful evaluation. Clinical trials are carefully planned to safeguard the health of the participants as well as answer specific research questions by evaluating for both immediate and long-term side effects and their outcomes are measured before the drug is widely applied to patients.

According to the World Health Organization (WHO), nearly 20,000 medicinal plants exist in 91 countries including 12 mega biodiversity countries. The premier steps to utilize the biologically active compound from plant resources are extraction, pharmacological screening, isolation and characterization of bioactive compound, toxicological evaluation and clinical evaluation.

A brief summary of the general approaches in extraction, isolation and characterization of bioactive compound from plants extract can be found in Figure 1. This paper provides details in extraction, isolation and characterization of bioactive

compound from plants extract with common phytochemical screening assay, chromatographic techniques, such as HPLC, and HPLC/MS and Fourier Transform Mass Spectrometry (FTMS) [5].

2. Extraction

Extraction is the first step to separate the desired natural products from the raw materials. Extraction methods include solvent extraction, distillation method, pressing and sublimation according to the extraction principle. Solvent extraction is the most widely used method. The extraction of natural products progresses through the following stages:

1) the solvent penetrates into the solid matrix;
2) the solute dissolves in the solvents;
3) the solute is diffused out of the solid matrix;
4) the extracted solutes are collected.

Any factor enhancing the diffusivity and solubility in the above steps will facilitate the extraction. The properties of the extraction solvent, the particle size of the raw materials, the solvent-to-solid ration, the extraction temperature and the extraction duration will affect the extraction efficiency. The selection of the solvent is crucial for solvent extraction. Selectivity, solubility, cost and safety should be considered in selection of solvents. Based on the law of similarity and intermiscibility (like dissolves like), solvents with a polarity value near to the polarity of the solute are likely to perform better and vice versa. Alcohols (EtOH and MeOH) are universal solvents in solvent extraction for phytochemical investigation.

Generally, the finer the particle size is, the better result the extraction achieves. The extraction efficiency will be enhanced by the small particle size due to the enhanced penetration of solvents and diffusion of solutes. Too fine particle size, however, will cost the excessive absorption of solute in solid and difficulty in subsequent filtration [6]-[10].

3. Extraction methods

Plant material extraction is a crucial process in the isolation of natural plant compounds and their purification. Plant matrices naturally are complex, containing a wide range of compounds that have various physical and chemical properties [11]. It is therefore imperative to carefully, isolate from the rest of the plant, matrices and make pure, compounds of interest in plants for their characterization. There are several ways extraction methods can be categorized [12]. In this chapter, they have been categorized based on the temperatures they work under.

A. Low or room Temperature methods

1) Cold extraction method

The method has been described in literature [13]. Briefly, dried plant parts samples (Cut, crushed or milled) are put in various solvents for seven days, with shaking every 24 hours. The samples are then filtered using a Whatman filter paper and dried under vacuum at room temperature on pre-weighed watch glasses and the mass of yield determined by difference. One common example of cold extraction is maceration. In this method, coarsely powdered or whole plant part material is stored in contact with a solvent for some time with regular shaking. This releases soluble matter dissolved in the solvent [14].

2) Enzyme Assisted Extraction (EAE)

This method employs solvents with various enzymes selected depending on the environments they perform the best and the pathway in mind that the scientist needs the compounds to be catalyzed. Some of the enzymes generally used in the extraction are protease, lipase and phospholipase and they effectively reduce the use of solvents [15]. Specifically, for essential oils, pectinase and α-amylase are the mostly used enzymes. The method is non-degrading to compounds but the setup is costly. It is also too demanding in terms of nutrients required, oxygen presence and temperature optimization.

3) Plant tissue homogenization

Fresh, wet or dried parts of plants are ground and soaked in solvents. The mixture is either vigorously shaken for 5 to 10 minutes or let stand for 24 hours with regular shaking and then the extract is filtered. The extract filtrates may be centrifuged for clarified concentration then dried. Sometimes the filtrates may be directly dried under reduced pressure and then re-dissolved using solvents of interest [16].

4) Ionic Liquid Extraction

In this method, organic salts in liquid form interacts selectively with polar and nonpolar compounds using π-stacking interactions, hydrogen bonding, ion exchange and hydrophobic interactions [17]. It is a very good method that recovers organic and inorganic ligands in high yields. Due to the ionic interactions, the quality and efficiency of the extraction is very high.

5) Maceration

In this process, the whole or coarsely powdered crude drug is placed in a stoppered container with the solvent and allowed to stand at room temperature for a period of at least 3 days with frequent agitation until the soluble matter has dissolved. The mixture then is strained, the marc (the damp solid material) is pressed, and the combined liquids are clarified by filtration or decantation after standing.

6) Infusion

Fresh infusions are prepared by macerating the crude drug for a short period of time with cold or boiling water. These are dilute solutions of the readily soluble constituents of crude drugs [18].

B. Chromatographic Techniques

1) Thin-layer chromatography (TLC) and Bio-autographic methods
2) Paper chromatography
3) Gas chromatographic techniques

1) Thin-layer chromatography (TLC) and Bio-autographic methods

TLC is a simple, quick, and inexpensive procedure that gives

the researcher a quick answer as to how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture when the Rf of a compound is compared with the Rf of a known compound. Additional tests involve the spraying of phytochemical screening reagents, which cause color changes according to the phytochemicals existing in a plants extract; or by viewing the plate under the UV light.

This has also been used for confirmation of purity and identity of isolated compounds.

Bio-autography is a useful technique to determine bioactive compound with antimicrobial activity from plant extract. TLC bioautographic methods combine chromatographic separation and *in situ* activity determination facilitating the localization and target-directed isolation of active constituents in a mixture. Traditionally, bioautographic technique has used the growth inhibition of microorganisms to detect anti-microbial components of extracts chromatographed on a TLC layer [19]. This methodology has been considered as the most efficacious assay for the detection of anti-microbial compounds (Shahverdi, 2007). Bio-autography localizes antimicrobial activity on a chromatogram using three approaches: (i) direct bio-autography, where the micro-organism grows directly on the thin-layer chromatographic (TLC) plate, (ii) contact bio-autography, where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact and (iii) agar overlay bio-autography, where a seeded agar medium is applied directly onto the TLC plate (Hamburger and Cordell, 1987;

Rahalison et al., 1991). The inhibition zones produced on TLC plates by one of the above bioautographic technique will be use to visualize the position of the bioactive compound with antimicrobial activity in the TLC fingerprint with reference to *Rf* values (Homans and Fuchs, 1970). Preparative TLC plates with a thickness of 1mm were prepared using the same stationary and mobile phases as above, with the objective of isolating the bioactive components that exhibited the antimicrobial activity against the test strain. These areas were scraped from the plates, and the substance eluted from the silica with ethanol or methanol.

Eluted samples were further purified using the above preparative chromatography method. Finally, the components were identified by HPLC, LCMS and GCMS. Although it has high sensitivity, its applicability is limited to micro-organisms that easily grow on TLC plates [20].

2) Paper chromatography

A special sheet of paper is used to provide an inert platform for separation. Test samples are place gently near the bottom of the paper, then the paper is placed in a chromatographic chamber containing a solvent which moves upwards by capillary action carrying respective soluble molecules along. The paper need to have high porosity for high rate of capillary action and thick to accommodate a reasonably higher amount of samples better [21]. The advantage of paper chromatography is that it is relatively cheaper and has a considerable reproducibility of retention factor (RF) values right on the paper [22].

3) Gas chromatographic techniques

This technique is used to separate volatile and stable compounds, where species are distributed between the gas (Mobile) and liquid (stationery) phases. Samples are vaporized and injected into a chromatographic column where it gets transported by an inert gas. The stationery phase is embedded onto an inert solid material. The distribution of species in the test sample gives a measure of separation, where some gets well mingled into the stationery phase and delay or does not elute with the gas phase at all and those that distribute well into the gas phase will elute as the gas does. Gas chromatography has three most common categories based on phase states and mechanism. The gas-bonded phase has already been described in bonded phases section. The second one are gas-liquid, where a liquid adsorbed onto a solid acts as the stationery phase and equilibrium is reached through partition between gas and liquid. The last one is gas-solid technique, where a solid is the stationery phase and equilibrium is reached by adsorption [23].

C. Non-chromatographic techniques

1) Immunoassay

This method employs monoclonal antibodies against low molecular mass bioactive natural compounds and drugs They provide very high sensitivity for receptor binding analyses, qualitative and quantitative analyses and enzyme assays. Compared to chromatography, Enzyme linked immunosorbent assay (ELISA) are more sensitive in most cases [24].

2) Phytochemical screening assay

Phytochemicals are chemicals derived from plants and the term is often used to describe the large number of secondary metabolic compounds found in plants. Phytochemical screening assay is a simple, quick, and inexpensive procedure that gives the researcher a quick answer to the various types of phytochemicals in a mixture and an important tool in bioactive compound analyses [25].

4. Qualitative techniques for the determination of phytochemicals

A. Alkaloids

1) Mayer's test

Two drops of Mayer's reagent are added along the sides of test tube in to few amount of plant extract. The presence of alkaloids is indicated by a white creamy precipitate [26].

2) Wagner's test

A few drops of Wagner's reagent are added to a few amount of plant extract and a reddish brown precipitate depicts the presence of alkaloids [26].

3) Dragendroff's test

The addition of few drops of Dragendroff's reagent into the extract gives red precipitate if alkaloids are present in the sample [27].

4) Hager's test

A small amount of Hager's reagent is added to the extract. The formation of yellow precipitate indicates the presence of alkaloids [27].

B. Carbohydrates

The 100 mg of extract is dissolved in 5 ml of distilled water and filtered [28].

1) Molish's test

Two drops of alcoholic solution of α–naphthol are added to 2 ml of filtrate and 1 ml of concentrated sulpuric acid is added slowly along the sides of test tube. A violet ring indicates the presence of carbohydrates.

2) Fehling's test

An equal volume of Fehling solution A and B are added to and equal volume of filtrate and it should boil in a water bath. The formation of red precipitate indicates the presence of sugar.

3) Barfoed's test

An equal volumes of filtrate and Barfoed's reagent are mixed and heat in a water bath. A red precipitate confirms the presence of sugar.

4) Benedict's test

A mixture of plant extract and the Benedict reagent is heated on water bath for 2 minutes and a characteristic colored precipitate indicates the presence of sugar. For detection of glycosides, the plant extract is hydrolyzed with concentrated hydrochloric acid and the filtrate should be subjected to following tests.

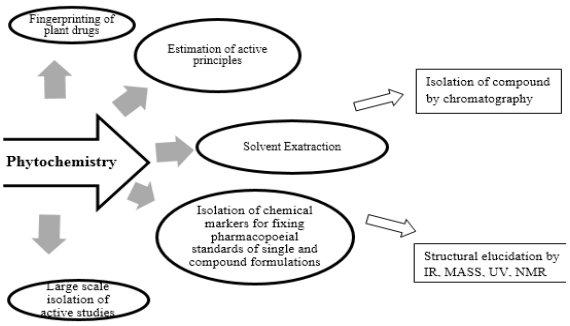

Fig. 1. Phyto chemical evaluation of herbal drugs

5. Conclusion

Phytochemicals can be screened using different qualitative techniques. But some advanced methods can be used to discover them qualitatively as well as quantitatively at once without performing several individual tests. Since bioactive compounds occurring in plant material consist of multi-component mixtures, their separation and determination still creates problems. Practically most of them have to be purified by the combination of several chromatographic techniques and various other purification methods to isolate bioactive compound(s).

The phytochemical examination of a plant involves the selection, collection, identification and authentication, extraction of the plant material. Cost of some operational apparatus and equipment may be a limiting factor in extraction and isolation. A combination of simpler and cheaper methods could overcome this limitation. Due to a variety of compounds in plants for potential drug development, a single method may not be ideal to extract and to isolate them. Sometimes, efficiency may be achieved when two or more methods.

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