

Isolation, Characterization, and Identification of Bioactive Compounds in Six Medicinal Plants

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Abstract: *Natural Products from medicinal plants either as pure or as a standardized extracts provide unlimited opportunities for new drugs because of the unmatched availability of chemical constituents. Since ancient times, natural products have been utilized to treat and cure chronic diseases like cancer, diabetes, asthma, anti-inflammatory, analgesic and as alternatives for hormone replacement therapy worldwide. According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. Despite their widespread existence, the variety of bioactive natural products in natural medicines are not sufficient. Today, it is very crucial to develop effective and selective methods for the extraction and isolation of new natural products. The focus of this review paper is to provide a comprehensive view on the analytical methodologies, which include extraction, isolation and characterization of the natural products from medicinal plants and common phytochemical screening assays.*

Keywords: *Bioactive Compounds, Extraction Methods, Medicinal plants, Natural Products.*

I. INTRODUCTION

Throughout history, humankind has always been interested in naturally occurring compounds from prebiotic, microbial, plants and animals sources. Various extracts of different parts of plants have been widely used in folk medicines and perfumes as well as in food flavor and preservatives and are more commonly utilized in chronic as well as common diseases. Plants contain several active compounds such as alkaloids, steroids, tannins, glycosides, volatile oils, fixed oils, resins, phenols and flavonoids that are deposited in their different parts. The beneficial of the medicinal effects of the plant result from the combination of these active compounds [1]. Various extracts of different parts of the plants have been widely used as folk medicines and perfumes as well as food flavor and preservatives. Bioactive natural products more commonly utilized in chronic as well as infectious diseases [2] like cancer, diabetes and asthma, anti-inflammatory, analgesic, antipyretic solutions and as alternatives for hormone replacement therapy [3,4,5]. It also uses as a remedy for the treatment of gastropathy, hepatitis, nephritis, edema, chest pain, fever and cough of pneumonia, bronchitis, and arthritis [6]. Today, natural medicines not only provide the primary health-care needs for the majority of the population in developing countries but also have attracted more and more attention in the developed countries due to the high health care cost and low or no side effect. In the USA, approximately 49% of the population has tried natural medicines for the prevention and treatment of diseases. According to the World Health Organization (WHO),

More than 80% of the world's population relies on traditional medicine for their primary healthcare needs, most of which involve the use of plant extracts[7]. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, nutraceuticals, food supplements, pharmaceutical intermediates, chemical entities for crude and synthetic drugs [8]. Despite their widespread existence and importance, the bioactive natural products are not sufficient. Nowadays there is an urgent need to develop effective and selective methods and stimulate natural products research. This review paper takes a comprehensive look at the different extraction methods of the bioactive natural products. It mostly focused on the analytical methodologies, which includes the extraction methods, isolation and characterization of the natural products present in the plant extracts.

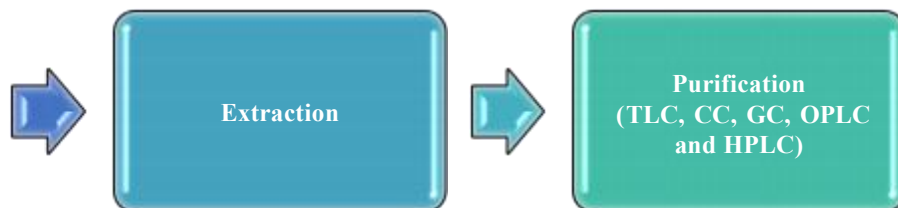
II. EXTRACTION METHODS

Extraction is the separation of medicinally active portions of plant using selective and standard procedures. It is the crucial first step in the analysis of medicinal plants, because it is necessary to extract the desired chemical components from the plant materials for further separation and characterization [9]. There are several extractions methods for the separation of natural product from plants present. These methods can be called conventional (long been used) and modern (developed more recently). Conventional techniques are the one using organic solvents or water and are carried out generally at atmospheric pressure while modern techniques using pressure and / or elevated temperatures [10]. 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 [11]. For the extraction procedures, solvents such as water, ethanol, chloroform, dichloromethane, hexane, ethyl acetate, methanol etc. are most commonly used. The conventional extraction methods generally use organic solvents and require a large volume of solvents and long extraction time [12]. The modern extraction methods have also been applied in natural products extraction and they offer some advantages such as lower organic solvent consumption, shorter extraction time and improve extraction yield [13]. Several of the commonly used extraction methods (conventional and modern) from medicinal plants are discussed below:

A. Maceration

In this process, solid plants parts are placed in a stoppered container with the whole of the solvent and allowed to stand for a period of at least 3 days (3 - 7 days) with frequent agitation, until soluble matter is dissolved. The mixture is

then strained (through sieves / nets), the marc pressed and the combined liquids clarified (cleaned by filtration) or by decantation, after standing. When the solvent is water and the period of maceration is long, a small quantity of alcohol maybe added to prevent microbial growth [14].



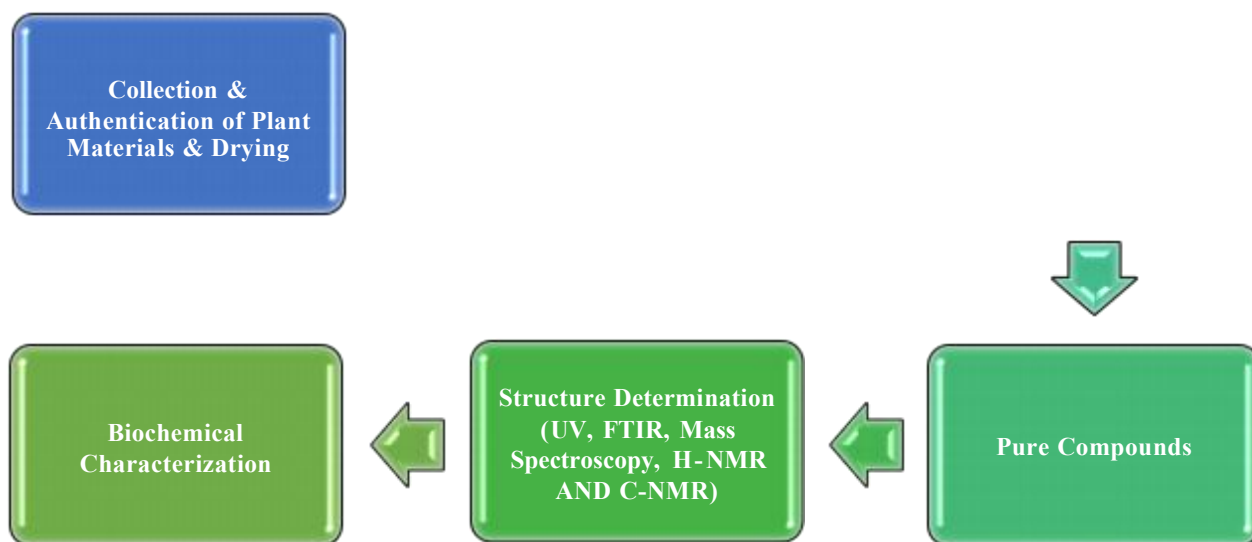


Fig. 1. A Brief Summary in Extraction, Isolation and Characterization of Natural Products from Medicinal Plant.

B. Percolation

This is the procedure used most frequently to extract active ingredients in the preparation of tinctures and fluid extracts. The plant material is taken in a percolation tube plugged with cotton or fitted with a filter and a stopcock [15]. Solvent is added into the plant material allowed to stand for approximately 4 hour in a well closed container, after which the mass is packed and the top of the percolator is closed. The whole system is kept for 24 hour at room temperature and the solvent along with the extracted material is collected by opening the stopper below and mixed liquid is clarified by filtration or by standing followed by decanting [14].

C. Digestion

This is a form of maceration in which gentle heat (40-60°C) is applied during the process of extraction. It is used when moderately elevated temperature is not objectionable [16]. The process may be modified by mixing the material with the solvent using magnetic stirrer, mechanical stirrer or by shaking occasionally by hand. After 8 to 12 hours, the extract is filtered and fresh solvent is added and the process repeated till all the desired products are extracted.

D. Infusion

In this extraction process, the plant material is macerated for a short period of time with either cold or boiling water [17]. It is a dilute solution of the readily soluble components of the crude drugs.

E. Decoction

In this process, the powdered plant materials is boiled in a specified volume of water for a defined time; it is then cooled and strained or filtered. This procedure is suitable for extracting water-soluble, heat-stable constituents. This process is typically used in preparation of Ayurvedic extracts called —quathl or —kawathl. The starting ratio of

crude drug to water is fixed, e.g. 1:4 or 1:16; the volume is then brought down to one-fourth to its original volume by boiling during the extraction procedure. Then, the concentrated extract is filtered and used as such or processed further [17].

F. Reflux

In this hot extraction process, the material is treated with boiling solvent. The solvent vapor is recycled by a condenser fitted on top of the container, preferentially a round bottomed flask. It cannot be used for the extraction of thermolabile natural products [11].

G. Tincture

It is the extract of plant material in alcohol. Usually the plant material (fresh) and ethyl alcohol are taken at the ratio of 1:5. Because of the alcohol content, the tinctures can be stored at room temperatures without being decomposed.

H. Pressurized Liquid Extraction (PLE)

The method is also known as accelerated solvent extraction system (ASE) or enhanced solvent extraction system (ESE). The method uses elevated pressure and temperature, where the increased temperature accelerates the extraction process by increasing the diffusivity of the solvent, whereas the increased pressure keeps the organic solvent in liquid state without boiling and also forces the solvent to penetrate the matrix pores [18].

I. Soxhlet Extraction

Named after „Franz Ritter von Soxhlet“, a German agricultural chemist, it is the best method for the continuous extraction of a solid by a hot solvent [19]. Soxhlet apparatus is a specialized glass refluxing unit mainly used for organic solvent extractions.

O. Ultrasonic Extraction

The powdered solid material is placed in a thimble made up of filter paper and is placed inside the soxhlet apparatus. The apparatus is fitted to a round bottomed (RB) flask containing the solvent and to a reflex condenser. The solvent in the RB flask is boiled gently, the vapor passes up through the side tube, condensed by the condenser and falls into the thimble containing the material

In this process, natural compounds are liberated from the plant tissues by high frequency sound, which damage the cell wall. Ultrasound assisted extraction can be used with mixtures of immiscible solvents such as hexane with methanol/water. The process creates heat so that heat labile compounds may decompose [22]. In such cases the extraction container is placed in ice bath to reduce the

and slowly fills the soxhlet. When the solvent reaches the top of the attached tube it siphons over into the flask, thus removes the portion of the substance, which it has extracted.

J. Steam Distillation

It is the standard process employed for the isolation of volatile oil from crude plant material [11]. Steam distillation is simple vaporization achieved by passing steam directly through the material. Here the steam volatile essential oil is recovered by condensation, where oil separates out from water.

K. Hydro Distillation

This is the widely used process for isolation of essential oils. The plant material is soaked in water and boiled using a heating mantle. Due to the influence of hot water the essential oil is freed from the oil glands in the plant tissues and passes along with the steam. By using a typical glass apparatus known as Clevenger apparatus, the steam oil mixture is condensed and oil is separated from water and the condensed water is recycled [20].

L. Expression

Expression, also referred to as cold pressing, is a method of extraction specific to citrus essential oils. In older times, expression was done in the form of sponge pressing, which was literally accomplished by hand. The oil release in this process is absorbed by sponge and it was recovered back by squeezing the sponge. It is reported that oil produced this way contains more of the fruit odor character than oil produced by any other method [9].

M. Enflourage

This technique is employed for the extraction of delicate fragrances from flowers. The flower petals are spread over a layer of refined fat that picks up the odor of the flowers and the saturated fat is treated with a solvent, usually alcohol in which the fragrant components are soluble. The residual fat dissolved in alcohol may be removed by cooling the alcohol extract to 20°C, when fat separates out. The alcohol is evaporated under reduced pressure and pure oils are obtained [9].

N. Supercritical Fluid Extraction (SFE)

This is the most technologically advanced extraction system Supercritical Fluid Extraction (SFE) involves use of gases, usually CO₂, and compressing them into a dense liquid. This liquid is then pumped through a cylinder containing the material to be extracted. From there, the extract-laden liquid is 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, no solvent residues left in it as CO₂ evaporates completely [21].

Besides that, non-chromatographic techniques uses such as immunoassay, which use monoclonal antibodies (MAbs), phytochemical screening assay. The pure compounds are then used for the determination of structure and biological activity [11]. Several of the commonly used separation techniques of the natural products are discussed below:

P. Microwave Assisted Extraction (MAE)

It simply termed as microwave extraction, that combines microwave and traditional solvent extraction. Revolution in organic compound synthesis has been promoted by microwave assisted organic syntheses (MAOS) by which small molecules are built up into large polymers in a fraction of time [23]. Heating the solvents and plant tissue using microwave increases the kinetic of extraction to facilitate partition of analytes from the sample matrix into the solvent [24]. Microwave radiation interacts with dipoles of polar and polarizable materials causes heating near the surface of the materials and heat is transferred by conduction. Dipole rotation of the molecules induced by microwave electromagnetic disrupts hydrogen bonding; enhancing the migration of dissolved ions and promotes solvent penetration into the matrix [25]. In non-polar solvents, poor heating occurs as the energy is transferred by dielectric absorption only [26].

Q. Solid Phase Extraction (SPE)

This rapid, economical and sensitive technique uses different types of cartridges and disks, with a variety of sorbents, where the solute molecules are preferentially attached over the stationary phase. Sample preparation and concentration can be achieved in a single step [27]. Normal phase, reverse phase and ion exchange solid phase extraction units are available. For example, with _Sep-Pak C18 cartridges (reverse phase) it is possible to remove polar components whereas the retained low polar ones can be eluted later.

III. ISOLATION AND PURIFICATION

The components in the extracts from the above methods are complex mixture and contains various type of natural products with different polarities. To obtain pure bioactive compound involves further separation and purification. Their separation remains a big challenge for the process of identification and characterization of pure bioactive natural product. Purification and isolation of natural products has undergone new development in recent years [28]. Many bioactive natural products have been isolated and purified by using different separation techniques such as TLC, HPTLC, Paper chromatography, Column chromatography, Gas chromatography, OPLC and HPLC. Column chromatography and thin-layer chromatography (TLC) are still mostly used due to their convenience, economy, and availability in various stationary phases [29].

detector must first be selected, The extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase. Modern HPLC uses a non-polar solid phase, like C18 and a polar liquid phase, generally a mixture of water and another solvent. High pressure up to 400 bars is required to elute the analyte through column before they pass through a diode array detector (DAD). A

A. Thin Layer Chromatography (TLC)

TLC is the most commonly used planar chromatographic method in natural product research. This is the easiest and cheapest technique and can be applied in the analysis, isolation and setting the parameters for column chromatography [9]. Usually, silica or alumina (more polar) is used as the stationary phase and organic solvents (less polar) are used as the mobile phase. This situation is categorized as normal phase chromatography. In contrast to this, reverse phase TLC is available, in which stationary phase is alkyl bonded silica or alumina (less polar) and mobile phase is polar solvent like water, alcohol etc.

B. Column Chromatography (CC)

Column chromatography is the most effective technique used in separation of crude plant extracts into its components in pure form. This is a preparative chromatographic method and the stationary phase (silica gel) is packed in a column and then the mobile phase (eluent) is passed through the column after loading the extracts on the top of the stationary phase. The mobile phase carries the natural products present in the mixture at different rate based on their affinities to the stationary and mobile phase. Separated compounds can be collected along with the mobile phase [9].

C. Gas Chromatography (GC)

It is an analytical technique for separating compounds based primarily on their volatilities. GC provides both qualitative and quantitative information for individual compounds present in a sample. The gas phase is flowing and the liquid phase is stationary. The rate of migration for the chemical species is determined through its distribution in the gas phase. For example, a species that distributes itself 100% into gas phase will migrate at the same rate as the flowing gas, whereas, a species that distributes itself 100% into stationary phase will not migrate at all. Species that distribute themselves partly in both phases will migrate at an intermediate rate [30]. Gas chromatography involves a sample being vaporized and injected onto the head of the chromatographic column. The sample is then transported through the column by the flow of inert, gaseous mobile phase. The column itself contains a liquid stationary phase, which is adsorbed onto the surface of an inert solid.

D. High Performance Liquid Chromatography (HPLC)

It is a versatile, robust, and widely used technique for the isolation of natural products. HPLC is an analytical technique for the separation and determination of organic and inorganic solutes in any samples especially biological, pharmaceutical, food, environmental, industrial etc. Currently, this technique is gaining popularity among various analytical techniques as the main choice for fingerprinting study for the quality control of medicinal plants. In order to identify any compound by HPLC, a

The structure of the natural compound can be identified. Scientists mainly use spectra produced from either three or four regions—Ultraviolet (UV), Visible, Infrared (IR), Radio frequency (FTIR), and electron beam for structural clarification [26].

important role in separation and characterization of different natural products. Practically most of them have to be purified by the combination of several chromatographic as well as non-chromatographic techniques and various other purification methods to isolate natural products.

A. UV-Visible Spectroscopy

UV-visible spectroscopy can be performed for qualitative analysis and for identification of certain classes of compounds in both pure and biological mixtures. Preferentially, UV-visible spectroscopy can be used for quantitative analysis because aromatic molecules are powerful chromophores in the UV range. Natural compounds can be determined by using UV-visible spectroscopy [31]. Moreover, spectroscopic UV-Vis techniques were found to be less selective and give information on the composition of the total polyphenol content. This technique is not time-consuming, and presents reduced cost compared to other techniques [32].

B. Fourier Transform Infrared Spectroscopy (FTIR)

Fourier-transform infrared spectroscopy is a valuable tool for the identification of functional groups present in the plant extract. It helps for identification and structure determination of the molecule [32]. It is a high-resolution analytical tool to identify the chemical constituents and elucidate the structural compounds. FTIR offers a rapid and nondestructive investigation to fingerprint herbal extracts or powders.

C. Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear Magnetic Resonance Spectroscopy gives physical, chemical and biological properties of matter. One dimensional technique is routinely used but the complicated structure of the molecules could be achieved through two dimensional NMR techniques. Solid state NMR spectroscopy is used for the determination of molecular structure of solids. Radiolabeled ^{13}C NMR is used to identify the types of carbon are present in the compound. ^1H -NMR is used to find out types of hydrogen are present in the compound and to find out how the hydrogen atoms are connected [30].

D. Mass Spectroscopy

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. This method mostly employed for the structural elucidation of organic compounds, for peptide or oligonucleotide sequencing and for monitoring the existence of previously characterized compounds in complex mixtures with a high specificity by defining both the molecular weight and a diagnostic fragment of the molecule simultaneously [30].

V. CONCLUSION

Since natural products from plant extracts usually contain various component mixtures with different polarities, their separation creates a big challenge for the process of identification and characterization. Extraction plays an

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