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Review article

A Review on Methods Associated with Investigation of Bioactive Compounds from Plant Extracts

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ABSTRACT:

Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unequaled accessibility of chemical diversity. Due to an increasing request for chemical diversity in screening programs, seeking therapeutic drugs from natural products, interest particularly in edible plants has grown throughout the world. Herbal preparations for medicinal usage contain various types of bioactive compounds. In general, extraction procedures include maceration, digestion, decoction, infusion, percolation, Soxhlet extraction, superficial extraction, ultrasound-assisted, and microwave assisted extractions. Fractionation and purification of active ingredients in plant extract are achieved through application of various chromatographic techniques such as paper chromatography, thin-layer chromatography and high-performance liquid chromatography. Finally, compounds obtained are characterized using diverse identification techniques such as mass spectroscopy, infrared spectroscopy, ultraviolet spectroscopy, and nuclear magnetic resonance spectroscopy. Subsequently, different methods described above can be grouped and discussed according to the intended biological testing to guide young researchers and make them more focused.

Keywords: Active ingredients, extraction, chromatography, spectroscopy.

1. 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 used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases [1]. Much beneficial biological activity such as anticancer, antimicrobial, antioxidant, anti-diarrheal, analgesic and wound healing activities were reported [2, 3, 4]. In many cases the people claim the good benefit of certain natural or herbal products. 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 [5]. 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), nearly 20,000 medicinal plants exist in 91 countries including 12 mega biodiversity countries [6]. 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. This review paper provides details in extraction, isolation and characterization of bioactive compound from plants extract with chromatographic techniques, such as paper chromatography, thin-layer chromatography, column chromatography HPLC and spectroscopic techniques such as mass spectroscopy, infrared spectroscopy, ultraviolet spectroscopy, and nuclear magnetic resonance spectroscopy.

2. EXTRACTION

The method of removing/separation of one or more constituent from a solid, liquid or gas by means of a solvent or mixture of solvents is called extraction. Conventional and modern techniques have been used to extract the natural plant products, which include

2.1. Conventional Extraction Techniques

In Conventional extraction techniques more times are required to as compared to modern extraction techniques. Conventional extraction techniques are as follows:

2.1.1 Percolation

Percolation is a method of extraction achieved by the downward displacement of soluble extractive by a suitable solvent through a suitably comminuted drug plant. In percolation, the powered plant material is soaked initially in a percolator. Addition solvent is then poured on the top of

the percolator. Additional filtration of the extract is not required because there is a filter at the outlet of percolator. Percolation is adequate for both initial and large scale extraction [7].

2.1.2 Maceration

In maceration the sample is placed in a stoppered container and is in contact with the solvent. This allows the solvent to penetrate into the cellular structure in order to dissolve the soluble Compounds [7]. Its efficiency may be increased by occasionally shaking the container or by using a mechanical or magnetic stirrer to homogenize the final solution and saturate the solvent. As maceration is a discontinuous method, the solvent should be renewed until the plant material is exhausted; this requires filtration steps that may result in the loss of solvent, analytes, and/or plant material.

2.1.3 Soxhlet Extraction

Soxhlet extraction is only required 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 [8].

Normally, a solid material containing some of the desired compound is placed inside a thimble made from thick filter paper, which is loaded into the main chamber of the soxhlet extractor. The soxhlet extractor is placed onto a flask containing the extraction solvent. The soxhlet is then equipped with a condenser. The solvent is heated to reflux. The solvent vapour travels up a distillation arm, and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapour cools, and drips back down into the chamber housing the solid material.The chamber containing the solid material slowly fills with warm solvent. Some of the desired compound will then dissolve in the warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This cycle may be allowed to repeat many times, over hours or days [7].

2.1.4 Infusion

In the Infusion technique, boiling or cold water is added to the milled sample [7].

2.1.5 Decoction

In Decoction the sample is boiled for about 15 minutes in water. Extraction with pure water, however, is seldom used for plant material as hydrophilic compounds are usually extracted with methanol-water or ethanol-water mixture [7].

2.1.6 Steam distillation

Steam distillation is an old extraction method that is primarily used to obtain essential oils from plant material. In this method, a packed bed of plant material is continuously flushed with steam and the volatile organic compounds present in the material are taken up by the vapor phase due to their low partial vapor pressure. Compounds carried by the vapor stream are then separated after decreasing the temperature of the vapor by forced condensation [9].

2.2 Modern Extraction Techniques

In Modern extraction techniques less time are required as compared to conventional extraction method.

2.2.1 Ultrasonic Extraction

Ultrasonic extraction takes advantage of the very high effective temperatures (which increase solubility and diffusivity) and pressures (which favor penetration and transport) at the inter-phase between the solvent solution and a solid matrix, subjected to ultrasonic energy combined with the oxidative energy of radicals created during sonolysis resulting in high extractive power [10]. Ultrasonically assisted extraction methods have been employed for a great number of different plant materials, e.g. *Salvia officinalis* L., *Valeriana officinalis* L [11]. Compound groups that have been obtained by ultrasonic extraction include polysaccharides, volatile oils, fatty acids and their esters, stigmasterol derivatives.

2.2.2 Microwave-assisted Extraction

Microwave-assisted extraction consists of heating the solvent in contact with the sample by means of microwave energy. The process involves disruption of hydrogen bonds, as a result of microwave-induced dipole rotation of molecules, and migration of the ions, which enhance penetration of the solvent into the matrix, allowing dissolution of the components to be extracted [11]. The main advantages of microwave assisted extraction over the conventional extraction techniques are reduced solvent consumption, shorter operational times, moderately high recoveries, good reproducibility and minimal sample manipulation for extraction process [12].

2.2.3 Pressurized liquid Extraction

Pressurized liquid extraction commonly known as accelerated solvent extraction; ASE [13]. The method uses an organic solvent at high pressures and temperature above the boiling point [14]. The main reasons for the enhanced performance of PLE are the higher solubility of analytes in solvent at higher temperatures, higher diffusion rate as a result of higher temperatures, and disruption of the strong solute matrix interaction caused by van der Waals forces, hydrogen bonding and dipole-dipole attractions between solute molecules and active sites on the matrix. The PLE technique is well suited for the extraction of various types of compound from different plant materials because parameters other than temperature can be varied and the polarity of the extraction solvent can be chosen from a wide range and adapted to the respective matrix.

2.2.4 Supercritical fluid Extraction

In recent years, the extraction method that has received increasing attention and many industrial applications in the isolation of natural products is supercritical fluid extraction (SFE). SFE has several advantages over the conventional liquid-liquid and solid-liquid extraction techniques, e.g. the elimination of most of the organic solvents that may pose a

safety risk during extraction, elimination of carry-over of the more or less toxic solvents in the final extracts, and the possibility of avoiding the detrimental effects of these solvents on the environment [15, 16].

The disadvantages of SFE include the low polarity of the most commonly used fluid, i.e. carbon dioxide, possible problems caused by the presence of water, unpredictability of the matrix effect and the need for specialized/expensive equipment. The effects of pressure and temperature were evaluated on the SFE efficiency of stevioside at 20.4 and 34.0 MPa of 40, 60, and 80^{o} C. Unfortunately, stevioside was extracted as a trace amount under all SFE condition evaluated because CO_2 was too non polar. Among the methods used to enhance the polarity of CO_2, the addition of small amount of organic modifiers to the CO_2 was used in this experiment. The extraction yield was dramatically improved when methanol was added at 20% *(v/v)* and was comparable to conventional organic solvent extraction. After evaluation of the effect of methanol, another modifier, ethanol was added to the CO_2 .Although 20% *(v/v)* of ethanol greatly improved the SFE efficiency of stevioside compared to pure CO_2, it extracted stevioside at only 60% of organic solvent extraction. This result suggested that methanol is a more effective modifier than ethanol. Recent reports on the SFE of natural products described that the addition of a small amount of water could greatly enhance the extraction yield from a plant, e.g., baicalin, baicalein, and wogonin from Scutelariae radix and cocaine from a hair matrix [17]. It may be due to the fact that water could freely release the target compounds from the plant matrix. For stevioside, as shown in Table I, the extraction yield was also more enhanced by a mixture of methanol and water (8:2) than neat methanol. Actually, there was no distinguishable difference in the solubilities of stevioside in CO_2-methanol and CO_2-methanol-water (unpublished result). The results suggest that the supercritical fluid extractability of stevioside is not only limited by the analyte solubility, but also by desorption of stevioside from the plant matrix. In addition to the dramatic improvement in the extractability, SFE with a mixture of methanol and water (8:2) as amodifier could selectively extract stevioside from co-extractants from *S. rebaudiana* leaves such as dulcoside-A, rebaudicoside-A, and rebaudicoside-C.

2.2.5 Medium-Pressure Solid-Liquid Extraction (MPSLE)

Medium pressure solid-liquid extraction (MPSLE), is an extraction technique based on the principles of the diffusion-dissolving processes of parametric pumping. Changes in temperature, pressure, pH or electrical field, result in a reversible differential alteration of the distribution of components between the solid and the liquid phases. In MPSLE the extraction column, i.e. a medium pressure liquid chromatographic (MPLC) column is filled with fine powdered plant material, and the extraction solvent is pumped through the stationary bed. This method constitutes the relative counter-current extraction, and results in exhaustive and rapid extraction. The method can be used for the rapid extraction of various substance classes occurring as complex solid matrices [18]. The exhaustive extraction of 100 - 3000 g of finely powdered plant material of can be performed with automated equipment within a few hours. The same principals as in column chromatography, e.g. the geometry of the column, physicochemical properties of the solvent, flow rate and amount of solvent, pressure, equilibrium time, sample particle size, compactness and amount of extracted material, are valid in MPSLE.

2.2.6 Rotation Planar Extraction (RPE)

Rotation planar extraction (RPE) is a technique, in which the extraction solvent migrates mainly through the action of centrifugal force [19]. A novel, multi-functional separation instrument prototype ExtraChrom® enables the rotation planar extraction of complex matrices because a planar column can be attached to it and filled with the material to be extracted. Factors affecting the RPE process are basically the same as in MPSLE [20]. The instrument enables extraction of materials of small particle size, resulting in particle-free extracts. The extraction takes place in a closed chamber, and it is possible to extract the material successively with solvents of different polarity [21]. The RPE method seems to be well suited for screening purposes when the number of biogenic samples is fairly high and 20-50 g of the material are to be extracted at a time. One disadvantage of ExtraChrom® compared to MPSLE extraction is that it lacks the possibility of scaling up the procedure.

3. ISOLATION AND SEPARATION

Chromatographic techniques are used for purification and separation of various compounds from different fractions of the crude extract of the plant.

3.1 Chromatography

Chromatography (from Greek “chromes” colour and “grafein” to write) is the techniques for the separation of mixtures. It involves passing a mixture dissolved in a “mobile phase” through a stationary phase, which separates the analyte to be measured from other molecules in the mixture and allows it to be isolated.

3.1.1 Adsorption chromatography

Adsorption chromatography is one of the oldest types of chromatography around.it utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase. The equilibration between the mobile and stationary phase accounts for the separation of different solutes.

3.1.2 Partition chromatography

It is a technique in which small mixtures of substances are separated by means of partitions between a moving solvent and a stationary phase by following techniques:

3.1.2.1 Paper chromatography

Paper chromatography is an analytical technique for separating and identifying mixtures that are or can be colored, especially pigments. Paper chromatography is a

technique that involves placing a small dot of sample solution onto a strip of chromatography paper. The paper is placed in a jar containing a shallow layer of solvent and sealed. As the solvent rises through the paper it meets the sample mixture which starts to travel up the paper with the solvent. Different compounds in the sample mixture travel different distances according to how strongly they interact with the paper. After development, the spots corresponding to different compounds may be located by their colour, ultraviolet light, ninhydrin (Triketohydrindane hydrate) or by treatment with iodine vapors. The paper remaining after the experiment is known as the Chromatogram. This allows the calculation of an R_f value and can be compared to standard compounds to aid in the identification of an unknown substance.

3.1.2.2 Column chromatography

In column chromatography, the stationary phase, a solid adsorbent, is placed in a vertical glass (usually) column and the mobile phase, a liquid, is added to the top and flows down through the column (by either gravity or external pressure). Column chromatography is generally used as a purification technique: it isolated desired compounds from a mixture. The mixture to be analyzed by column chromatography is applied to the top of the column. The liquid solvent (the eluent) is passed through the column by gravity or by the application of air pressure. Equilibrium is established between the solute adsorbed on the adsorbent and the eluting solvent flowing down through the column. Because the different components in the mixture have different interactions with the stationary and mobile phases, they will be carried along with the mobile phase to varying degrees and a separation will be achieved. The individual components, or elutants, are collected as the solvent drips from the bottom of the column [22].

Column chromatography is separated into two categories, depending on how the solvent flows down the column. If the solvent is allowed to flow down the column by gravity, or percolation, it is called gravity column chromatography. If the solvent is forced down the column by positive air pressure, it is called flash chromatography. The term “flash chromatography” was coined by Professor W. Clark Still because it can be done in a “flash.”

3.1.2.3 Thin layer chromatography

Thin layer chromatography (TLC) is a chromatography technique used to separate mixtures. It involves a stationary phase consisting of a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose immobilized onto a flat, inert carrier sheet. A liquid phase consisting of the solution to be separated is then dissolved in an appropriate solvent and is drawn up the plate via capillary action, separating the experimental solution based on the polarity of the components of the compound in question. compounds. It also permits the optimization of the solvent system for a given separation problem. In comparison with column chromatography, it only requires Thin Layer chromatography (TLC) is a very commonly used technique in synthetic chemistry for monitoring the chemical reaction and purity of small quantities of the compound and is much faster as well.

3.1.2.4 Gas chromatography

Gas chromatography – specifically gas-liquid chromatography – involves a sample being vaporized and injected onto the head of the chromatographic column. The sample is 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 [23].

3.1.2.5 Liquid chromatography

Liquid chromatography (LC) was the first type of chromatography to be discovered and, in the form of *liquid-solid* chromatography (LSC) was originally used in the late 1890s by the Russian botanist, Tswett to separate and isolate various plant pigments. The colored bands he produced on the adsorbent bed evoked the term chromatography (color writing) for this type of separation.

3.1.2.6 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is a chromatographic separation technique where in the separation is accomplished by partitioning between a mobile phase (solvent) and a stationary column material. HPLC differs from other types of liquid chromatography, in that packing materials of small, uniform particle are utilized. The small size of particlesgive high column efficiencies which also results in high pressure drop across the columns, and hence, higher pressures are utilized to achieve desired flow rates. Hence it is also called high-pressure liquid chromatography [24].

3.1.2.7 Exclusion chromatography

Size exclusion chromatography (SEC) is also known as gel permeation chromatography or gel filtration chromatography and separates particles on the basis of size. Smaller molecules enter a porous media and take longer to exist the column, whereas larger particles leave the column earlier. It is generally a low resolution chromatography and thus it is often reserved for the final, “polishing” step of purification. It is also useful for determining the tertiary structure and quaternary structure of purified proteins, especially since it can be carried out under native solution conditions.

3.1.2.8 Ion exchange chromatography

Ion exchange chromatography utilizes ion exchange mechanism to separate analytes. It is usually performed in columns but the mechanism can be benefited also in planar mode. Ion exchange chromatography uses a charged stationary phase to separate charged compounds including amino acids, peptides, and proteins. In conventional methods the stationary phase is an ion exchange resin that carries charged functional groups which interact with oppositely charged groups of the compound to be retained.

4. STRUCTURE ELUCIDATION

4.1 Spectroscopic techniques

The Different spectroscopic techniques used for the characterization of compounds are as follows:

4.1.1 Ultraviolet and Visible (UV- Vis) Spectroscopy

Range (200 – 800 nm)

This is a spectroscopic technique that refers to the absorption of light for a given molecule in the Ultra Violet region of the electromagnetic spectrum. UV spectrum is a plot of wavelength of absorption vs. absorption intensity. Here transition is associated with electronic levels of atoms and molecules, so it is also called Electronic Spectroscopy [25].

4.1.2 Infrared (IR) Spectroscopy

Range (4000-667 cm^{-1})

This is a spectroscopic technique that involves measurement of the absorption spectrum of a given molecule in the infrared region of the electromagnetic spectrum. It refers to the absorption frequencies of single bonds to hydrogen, triple bonds, and cumulated double bonds and bonds depicting functional groups such as, carbonyl bonds, aromatic bonds, and miscellaneous bonds for such molecules. IR spectrum is a plot of percentage transmittance vs. frequency in wave number. Therefore, absorbance band appears as dips rather than peaks [26].

4.1.3 Nuclear Magnetic Resonance (NMR) Spectroscopy

This is a spectroscopic technique used to characterize the nuclear properties of elements, proton chemical shifts in substituent on molecules or protons associated with double bonds in cyclic and non-cyclic hydrocarbons, and chemical shifts of isotopes (e.g., carbon, nitrogen, phosphorus, boron, silicon and fluorine). Nuclear Magnetic Resonance is a branch of spectroscopy in which radio frequency waves includes transition between magnetic energy levels of nuclei in a magnetic field. Without the magnetic field the spin states of nuclei are degenerates i.e. posses the same energy and energy level transition is not possible. When a magnetic field is applied, the separate levels and radio frequency radiation can cause transitions between these energy levels. Protons having identical chemical or electronic environment resonate at one particular radio frequency and gives signals (peaks) with respect to internal standard i.e. tetramethylsilane (TMS) [23].

4.1.4 Mass spectroscopy

Micro analytical technique mass spectrometry has become one of the most valuable and efficient tools in the structure elucidation of natural products. Mass spectra can be recorded from very low sample quantities (10^{-6}-10^{-10}g) and a great deal of structural information is received from them it deals with the examination of the characteristic fragments (ions) arising from the breakdown of organic molecule when it is exposed to a beam of high energy electron .A mass spectrum is the plot of relative abundance of ions against their mass to charge ratio .it is unlike the other forms of spectroscopy, in that it is not concerned with non–destructive interactions between molecules and electromagnetic radiation. Instead it involves the production and separation of ionized molecules and their ionic decomposition products and finally the measurement of the relative abundances of different ions produced. It is, thus a destructive technique in that the sample is consumed during analysis [26]. The mass spectrum of each compound is unique and can be used as a Chemical Fingerprint to characterize the sample. Sufficient volatility of a compound, however, is necessary if, the most common method, the electron bombardment mass spectrometry, is applied. Polar functional groups and high molecular weights of natural products often prevent recording their mass spectra with this method. The volatility, however, can be often enlarged by simple chemical modifications like methylation , trimethylsilytation or trifluroacetylation. In contrast to conventional mass spectrometry a more recently developed method, field desorption mass spectrometry , allows study of polar molecules of much high molecular weight and is therefore most attractive for natural product chemistry [23].

All the above-mentioned spectroscopic techniques provide useful information for disclosing the identity natural compound/molecule.

5. CONCLUSION

Since natural products from plant extracts usually contain various constituent mixtures with different polarities, their separation creates a huge challenge for the process of identification and characterization. Extraction plays an vital role in separation and characterization of diverse natural products.

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