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EXTRACTION, AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM PLANT EXTRACTS A REVIEW

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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 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. Botanicals and herbal preparations for medicinal usage contain various types of bioactive compounds. The focus of this paper is on the analytical methodologies, which include the extraction, isolation and characterization of active ingredients in botanicals and herbal preparations. The common problems and key challenges in the extraction, and characterization of active ingredients in botanicals and herbal preparations are discussed. As extraction is the most important step in the analysis of constituents present in botanicals and herbal preparations, the strengths and weaknesses of different extraction techniques are discussed. The analysis of bioactive compounds present in the plant extracts involving the applications of common phytochemical screening assays, chromatographic techniques such as HPLC and, TLC as well as non-chromatographic techniques such as immunoassay and Fourier Transform Infra Red (FTIR).

INTRODUCTION

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 [1-2].

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 [3].

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Digestion

This is a form of maceration in which gentle heat is used during the process of extraction. It is used when moderately elevated temperature is not objectionable. The solvent efficiency of the menstruum is thereby increased.

Decoction

In this process, the crude drug 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, heatstable constituents. This process is typically used in preparation of Ayurvedic extracts called“quath” or “kawath”. 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 its original volume by boiling during the extraction procedure. Then, the concentrated extract is filtered and used as such or processed further.

Percolation

This is the procedure used most frequently to extract active ingredients in the preparation of tinctures and fluid extracts. A percolator (a narrow, cone-shaped vessel open at both ends) is generally used. The solid ingredients are moistened with an appropriate amount of the specified menstruum 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 menstruum 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 menstruum 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 menstruum is added to produce the required volume, and the mixed liquid is clarified by filtration or by standing followed by decanting [3].

Hot Continuous Extraction (Soxhlet)

In this method, the finely ground crude drug is placed in a porous bag or "thimble" made of strong filter paper, which is placed in chamber E of the Soxhlet apparatus (Figure 2). The extracting solvent in flask A is heated, and its vapors condense in condenser D. The condensed extractant drips into the thimble containing the crude drug, and extracts it by contact. When the level of liquid in chamber E rises to the top of siphon tube C, the liquid contents of chamber E siphon into fl ask A. This process is continuous and is carried out until a drop of solvent from the siphon tube does not leave residue when evaporated. The advantage of this method, compared to previously described methods, is that large amounts of drug can be extracted with a much smaller quantity of solvent. This effects tremendous economy in terms of time, energy and consequently financial inputs. At small scale, it is employed as a batch process only, but it becomes much more economical and viable when converted into a continuous extraction procedure on medium or large scale [4].

Aqueous Alcoholic Extraction by Fermentation

Some medicinal preparations of Ayurveda (like *asava*and *arista*) adopt the technique of fermentation for extracting the active principles. The extraction procedure involves soaking the crude drug, in the form of either a powder or a decoction (*kasaya*), for a specified period of time, during which it undergoes fermentation and generates alcohol in situ; this facilitates the extraction of the active constituents contained in the plant material. The alcohol thus generated also serves as a preservative. If the fermentation is to be carried out in an earthen vessel, it should not be new: water should first be boiled in the vessel. In large-scale manufacture, wooden vats, porcelain jars or metal vessels are used in place of earthen vessels [5].

Counter-current Extraction

In counter-current extraction (CCE), wet raw material is pulverized using toothed disc disintegrators to produce a fine slurry. In this process, the material to be extracted is moved in one direction (generally in the form of a fine slurry) within a cylindrical extractor where it comes in contact with extraction solvent. The further the starting material moves, the more concentrated the extract becomes. Complete extraction is thus possible when the quantities of solvent and material and their flow rates are optimized. The process is highly efficient, requiring little time and posing no risk from high temperature. Finally, sufficiently concentrated extract comes out at one end of the extractor while the marc (practically free of visible solvent) falls out from the other end [6].

This extraction process has significant advantages

i) A unit quantity of the plant material can be extracted with much smaller volume of solvent as compared to other methods like maceration, decoction, percolation.
ii) CCE is commonly done at room temperature, which spares the thermolabile constituents from exposure to heat which is employed in most other techniques.
iii) As the pulverization of the drug is done under wet conditions, the heat generated during comminution is neutralized by water. This again spares the thermolabile constituents from exposure to heat.
iv) The extraction procedure has been rated to be more efficient and effective than continuous hot extraction.

Ultrasound Extraction (Sonication)

The procedure involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz; this increases the permeability of cell walls and produces cavitation. Although the process is useful in some cases, like extraction of rauwolfia root, its large-scale application is limited due to the higher costs. One disadvantage of the procedure is the occasional but known deleterious effect of ultrasound energy (more than 20 kHz) on the active constituents of medicinal plants through formation of free radicals and consequently undesirable changes in the drug molecules [7].

Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is an alternative sample preparation method with general goals of reduced use of organic solvents and increased sample throughput. The factors to consider include temperature, pressure, sample volume, analyte collection, modifier (cosolvent) addition, flow and pressure control, and restrictors. Generally, cylindrical extraction vessels are used for SFE and their performance is g good beyond any doubt. The collection of the extracted analyte following SFE is another important step: significant analyte loss can

occur during this step, leading the analyst to believe that the actual efficiency was poor [8].

There are many advantages to the use of CO2 as the extracting fluid. In addition to its favorable physical properties, carbon dioxide is inexpensive, safe and abundant. But while carbon dioxide is the preferred fluid for SFE, it possesses several polarity limitations. Solvent polarity is important when extracting polar solutes and when strong analyte-matrix interactions are present. Organic solvents are frequently added to the carbon dioxide extracting fluid to alleviate the polarity limitations. Of late, instead of carbon dioxide, argon is being used because it is inexpensive and more inert. The component recovery rates generally increase with increasing pressure or temperature: the highest recovery rates in case of argon are obtained at 500 atm and 150° C. The extraction procedure possesses distinct advantages:

i) The extraction of constituents at low temperature, which strictly avoids damage from heat and some organic solvents.

ii) No solvent residues.

iii) Environmentally friendly extraction procedure.

The largest area of growth in the development of SFE has been the rapid expansion of its applications. SFE finds extensive application in the extraction of pesticides, environmental samples, foods and fragrances, essential oils, polymers and natural products. The major deterrent in the commercial application of the extraction process is its prohibitive capital investment.

Phytonics Process

A new solvent based on hydrofluorocarbon-134a and a new technology to optimize its remarkable properties in the extraction of plant materials offer significant environmental advantages and health and safety benefits over traditional processes for the production of high quality natural fragrant oils, flavors and biological extracts. Advanced Phytonics Limited (Manchester, UK) has developed this patented technology termed "phytonics process". The products mostly extracted by this process are fragrant components of essential oils and biological or phytopharmacological extracts which can be used directly without further physical or chemical treatment. The properties of the new generation of fluorocarbon solvents have been applied to the extraction of plant materials. The core of the solvent is 1, 1, 2, 2-tetrafluoroethane, better known ashydrofluorocarbon-134a (HFC-134a). This product was developed as a replacement for chlorofluorocarbons. The boiling point of this solvent is -25° C. It is not flammable or toxic.Unlike chlorofluorocarbons, it does not deplete the ozone layer. It has a vapor pressure of 5.6 barat ambient temperature. By most standards this is a poor solvent. For example, it does not mix with mineral oils or triglycerides and it does not dissolve plant wastes [9].

The process is advantageous in that the solvents can be customized: by using modified solvents with HFC-134a, the process can be made highly selective in extracting a specific class of phytoconstituents. Similarly, other modified solvents can be used to extract a broader spectrum of components. The biological products made by this process have extremely low residual solvent. The residuals are invariably less than 20 parts per billion and are frequently below levels of detection. These solvents are neither acidic nor alkaline and, therefore, have only minimal potential reaction effects on the botanical materials. The only utility needed to operate these systems is electricity and, even then, they do no consume much energy. There is no scope for the escape of the solvents. Even if some solvents do escape, they contain no chlorine and therefore pose no threat to the ozone layer. The waste biomass from these plants is dry and "ecofriendly" to handle.

Parameters for Selecting an Appropriate Extraction Method

i) Authentication of plant material should be done before performing extraction. Any foreign matter should be completely eliminated.

ii) Use the right plant part and, for quality control purposes, record the age of plant and the time, season and place of collection.

iii) Conditions used for drying the plant material largely depend on the nature of its chemical constituents. Hot or cold blowing air flow for drying is generally preferred. If a crude drug with high moisture content is to be used for extraction, suitable weight corrections should be incorporated.

iv) Grinding methods should be specified and techniques that generate heat should be avoided as much as possible.

v) Powdered plant material should be passed through suitable sieves to get the required particles of uniform size.

vi) Nature of constituents:

a) If the therapeutic value lies in non-polar constituents, a non-polar solvent may be used. For example, lupeol is the active constituent of Crataevanurvalaand, for its extraction, hexane is generally used. Likewise, for plants like Bacopamonnieriand Centella*asiatica*, the active constituents are glycosides and hence a polar solvent like aqueous methanol may be used.

b) If the constituents are thermolabile, extraction methods like cold maceration, percolation and CCE are preferred. For thermostable constituents, Soxhlet extraction (if nonaqueous solvents are used) and decoction (if water is the menstruum) are useful.

c) Suitable precautions should be taken when dealing with constituents that degrade while being kept in organic solvents, e.g. flavonoids and phenyl propanoids.

d) In case of hot extraction, higher than required temperature should be avoided. Some glycosides are likely to break upon continuous exposure to higher temperature.

e) Standardization of time of extraction is important, as:

• Insufficient time means incomplete extraction.

• If the extraction time is longer, unwanted constituents may also be extracted. For example, if tea is boiled for too long, tannins are extracted which impart astringency to the final preparation.
f) The number of extractions required for complete extraction is as important as the duration of each extraction.
vii) The quality of water or menstruum used should be specified and controlled.
viii) Concentration and drying procedures should ensure the safety and stability of the active constituents. Drying under reduced pressure (e.g. using a Rotavapor) is widely used. Lyophilization, although expensive, is increasingly employed.
ix) The design and material of fabrication of the extractor are also to be taken into consideration.
x) Analytical parameters of the final extract, such as TLC and HPLC fingerprints, should be documented to monitor the quality of different batches of the extracts.

Circulatory Extraction

The efficiency of extraction in a maceration process can be improved by arranging for the solvent to be continuously circulated through the drug. Solvent is pumped from the bottom of the vessel to the inlet where it is distributed through spray nozzles over the surface of the drug. The movement of the solvent reduces boundary layers, and the uniform distribution minimizes local concentration in a shorter time [10].

Multiple Stage Extraction

Like the normal maceration process, however, extraction is incomplete, since mass transfer will cease when equilibrium is set up. This problem can be overcome by using a multistage process. The equipment needed for this method is a vessel for the drug, together with a circulating pump and spray distributors, and a number of tanks to receive the extracted solution. The extractor and tanks are connected with piping and valves so that anyone of the tank may be connected to the extractor for the transfer of solution. Examination of these procedures showed that each batch of drug is treated several times with solvent and that, once a cycle is in process, the receivers contain solution with the strongest in receiver 1 and the weakest in receiver 3 [11].
Advantages:

- The drug is extracted as many times as there are receivers – in this case, three. If more extraction stages are required, it is only necessary to have more receivers.
- The last treatment of the drug before it is discharged is with fresh solvent, giving maximum extraction.
- The solution is in contact with fresh drug before removal for evaporation, giving the highest possible concentration.

Procedure

- Fill extractor with drug, add solvent and circulate. Run off to receiver 1.
- Refill extractor with solvent and circulate. Run off to receiver 2.
- Refill extractor with solvent and circulate. Run off to receiver 3.
- Remove drug from extractor and recharge. Return solution from 1 to extractor. Remove for evaporation.
- Return solution from 2 to extractor and circulate. Run off to receiver 1.
- Return solution from 3 to extractor and circulate. Run off to receiver 2.
- Add fresh solvent to extractor and circulate. Run off to receiver 3. Remove drug from extractor and recharge. Repeat cycle.

Fractional Crystallization

Crystallization is an old method but a very important method of purification of the compounds from the mixture. Crystallization mostly depends upon the inherent character of the compound which forms crystals at the point of supersaturation in the solvent in which it is soluble. Many phytopharmaceuticals and natural products are crystalline in nature which tends to crystallize even in the mixtures. Compounds such as sugars, glycosides, alkaloids, triterpenoids, flavonoids etc show the crystalline nature in exception cases. The processes such as concentrations, slow evaporation, and refrigeration are used for crystallizing the products. In case of sugars, osazone formation leads to the crystallization of derivatives in the form of various types of crystals enabling the analysis of the sugars [12].

MICROWAVE ASSISTED EXTRACTION

Microwave assisted is one of the simple, fast, clean, eco-friendly and efficient method. It is economic in saving energy, fuel and electricity. A very short response time and better yield of the product are main advantages of microwave heating.

Microwave assisted extraction (MAE) has received increasing attention as a potential alternative to conventional method for the extraction of secondary metabolites from plants. Extraction is one of the crucial points in analytical chain in the effort of achieving a complete recovery of target compounds. Recently, microwave energy is being used for extraction of phytoconstituents from plants. Microwave extraction follows the same principle as maceration or percolation but the speed of breaking up of the plant cell, plant tissue much higher. This reduced processing time is an economic advantage and also there is less risk of decomposition and oxidation of the valuable plant constituents. Microwave assisted extraction requires shorter time, less solvents, higher extraction rate and better products with lower loss.2,3 The reduced time is not just of economic advantage but also there is less risk of decomposition and oxidation of phytoconstituents. For heat sensitive materials microwave would be a better option. The speed of breaking up of plant cells is much higher. The penetration of

microwave into the plant tissues depends on the dielectric properties of the plant. The energy required for dense materials is higher than that for leaves [13].

Characterization techniques

Gas Chromatography - Mass Spectrometry (GC/MS) analysis For the identification of metabolites the samples were subjected to GC-MS analysis. The identification of metabolites in the samples was carried using a QP2010 gas chromatography with Thermal Desorption System TD 20 coupled with mass spectroscopy (Shimadzu). The ionization voltage was 70 eV. Gas chromatography was conducted in the temperature programming mode with a Restek column (0.25 mm, 60 m; XTI-5). The initial column temperature was 800C for 1 min, then increased linearly at 7 0C min−1 to 220 0C, hold for 3 min followed by linear increased temperature 100C min−1 upto 2900C hold for 10 min. The temperature of the injection port was 290 0C and the GC/MS interface was maintained at 2900C. The samples were introduced via an all-glass injector working in the split mode, with helium carrier gas flow rate was 1.2 ml min−1. The identification of components was accomplished by comparison of retention time and fragmentation pattern, as well as with mass spectra in the NIST spectral library stored in the computer software (version 1.10 beta, Shimadzu) of the GC-MS. The relative percentage of each extract constituent was expressed as percentage with peak area normalization [14].

Fourier-transform infrared spectroscopy

FTIR has proven to be a valuable tool for the characterization and identification of compounds or functional groups (chemical bonds) present in an unknown mixture of plants extract . In addition, FTIR spectra of pure compounds are usually so unique that they are like a molecular "fingerprint". For most common plant compounds, the spectrum of an unknown compound can be identified by comparison to a library of known compounds. Samples for FTIR can be prepared in a number of ways. For liquid samples, the easiest is to place one drop of sample between two plates of sodium chloride. The drop forms a thin film between the plates. Solid samples can be milled with potassium bromide (KBr) to and then compressed into a thin pellet which can be analyzed. Otherwise, solid samples can be dissolved in a solvent such as methylene chloride, and the solution then placed onto a single salt plate. The solvent is then evaporated off, leaving a thin film of the original material on the plate [15].

HPLC

Natural products are frequently isolated following the evaluation of a relatively crude extract in a biological assay in order to fully characterize its properties. The resolving power of HPLC is ideally suited to the rapid processing of such multi component samples on both an analytical and preparative scale. Several authors describe the use of HPLC for characterization and quantification of secondary metabolites in plant extracts, mainly phenol compounds, steroids, flavonoids, alkaloids .Reversed-phase chromatography is the most commonly used separation technique in HPLC due to its broad application range. It is estimated that over 65% of all HPLC separations are carried out in the reversed phase mode. The reasons for this include the simplicity, versatility, and scope of the reversed-phase method as it is able to handle compounds of a diverse polarity and molecular mass for example, to identify secondary plant metabolites. In addition, the colloquial term used for the mobile phases in reversed phase chromatography is "buffer". However, there is little buffering capacity in the mobile phase solutions since they usually contain strong acids at low pH with large concentrations of organic solvents. Adequate buffering capacity should be maintained when working closer to physiological conditions . In order to identify any compound by HPLC, a detector must first be selected. The processing of a crude source material to provide a sample suitable for HPLC analysis as well as the choice of solvent for sample reconstitution can have a significant bearing on the overall success of natural product isolation. The source material, e.g., dried powdered plant, will initially need to be treated in such a way as to ensure that the compound of interest is efficiently liberated into solution. In the case of dried plant material, an organic solvent (e.g., methanol, chloroform) may be used as the initial extracting and following a period of maceration, solid material is then removed by decanting off the extract by filtration. The filtrate is then concentrated and injected into HPLC for separation. The usage of guard columns is necessary in the analysis of crude extract. Many natural product materials contain significant level of strongly binding components, such as chlorophyll and other endogenous materials that may in the long term compromise the performance of analytical columns. Therefore, the guard columns will significantly protect the lifespan of the analytical columns [16].

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 R_f of a compound is compared with the R_f 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 [17].

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 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 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 .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. Other problems are the need for complete removal of residual low volatile solvents, such as *n*-BuOH, trifluoroacetic acid and ammonia and the transfer of the active compounds from the stationary phase into the agar layer by diffusion .Because bio-autography allows localizing antimicrobial activities of an extract on the chromatogram, it supports a quick search for new antimicrobial agents through bioassay-guided isolation The bioautography agar overlay method is advantageous in that, firstly it uses very little amount of sample when compared to the normal disc diffusion method and hence, it can be used for bioassay-guided isolation of compounds. Secondly, since the crude extract is resolved into its different components, this technique simplifies the process of identification and isolation of the bioactive compounds.

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