Review Article

Preparation of Medicinal Plants: Basic Extraction and Fractionation Procedures for Experimental Purposes

Abdullahi R. Abubakar[1], Mainul Haque[2]

[1]Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Bayero University, Kano, Nigeria, [2]Unit of Pharmacology, Faculty of Medicine and Defence Health, National Defence University of Malaysia, Kuala Lumpur, Malaysia

ABSTRACT

Preparation of medicinal plants for experimental purposes is an initial step and key in achieving quality research outcome. It involves extraction and determination of quality and quantity of bioactive constituents before proceeding with the intended biological testing. The primary objective of this study was to evaluate various methods used in the preparation and screening of medicinal plants in our daily research. Although the extracts, bioactive fractions, or compounds obtained from medicinal plants are used for different purposes, the techniques involved in producing them are generally the same irrespective of the intended biological testing. The major stages included in acquiring quality bioactive molecule are the selection of an appropriate solvent, extraction methods, phytochemical screening procedures, fractionation methods, and identification techniques. The nitty-gritty of these methods and the exact road map followed solely depends on the research design. Solvents commonly used in extraction of medicinal plants are polar solvent (e.g., water, alcohols), intermediate polar (e.g., acetone, dichloromethane), and nonpolar (e.g., *n*-hexane, ether, chloroform). In general, extraction procedures include maceration, digestion, decoction, infusion, percolation, Soxhlet extraction, superficial extraction, ultrasound-assisted, and microwave-assisted extractions. Fractionation and purification of phytochemical substances are achieved through application of various chromatographic techniques such as paper chromatography, thin-layer chromatography, gas 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: *Chromatography, extraction, fractionation, isolation, medicinal plants*

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INTRODUCTION

Medicinal plants are extracted and processed for direct consumption as herbal or traditional medicine or prepared for experimental purposes. The concept of preparation of medicinal plant for experimental purposes involves the proper and timely collection of the plant, authentication by an expert, adequate drying, and grinding. This is followed by extraction, fractionation, and isolation of the bioactive compound where applicable. In addition, it comprises determination of quantity and quality of bioactive compounds.[1-5] Recently, plant as a source of

Address for correspondence: Prof. Mainul Haque, Unit of Pharmacology, Faculty of Medicine and Defence Health, National Defence University Malaysia, Kem Sungai Besi, 57000 Kuala Lumpur, Malaysia.
E-mail: runurono@gmail.com

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medicine is gaining international popularity because of its natural origin, availability in local communities, cheaper to purchase, ease of administration, and perhaps less troublesome. Also, herbal medicine may be useful alternative treatment in case of numerous side effects and drug resistance.[1-5] Extraction of medicinal plants is a process of separating active plant materials or secondary metabolites such as alkaloids, flavonoids, terpenes, saponins, steroids, and glycosides from inert or inactive material using an appropriate solvent and standard extraction procedure. Plant materials with high content of phenolic compounds and flavonoids were found to possess antioxidant properties, and hence are used to treat age-related diseases such as Alzheimer's disease, Parkinsonism, anxiety, and depression.[2,5] Several methods were used in the extraction of medicinal plants such as maceration, infusion, decoction, percolation, digestion and Soxhlet extraction, superficial extraction, ultrasound-assisted, and microwave-assisted extraction. In addition, thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), paper chromatography (PC), and gas chromatography (GC) were used in separation and purification of the secondary metabolites.[1-5] The choice of an appropriate extraction method depends on the nature of the plant material, solvent used, pH of the solvent, temperature, and solvent to sample ration. It also depends on the intended use of the final products.[1-5] This study aimed to assess various solvents of extractions, methods of extraction, fractionation, purification, phytochemical screening, and identification of bioactive compounds in medicinal plants.

Definition of terms

Medicinal plant. It refers to a plant comprising active ingredients or secondary metabolites that possess biological activity. A whole plant may be medicinally active or plant parts.[4,6,7] *Herbal medicine*. These are medicinal preparations comprising active ingredients obtained from the herbal plant. The product can be made from the whole plant or any part. Preparations from by-product herbal plants such as oils, gums, and other secretions are also considered as herbal medicine.[4,6,7] *Menstruum*. It is a liquid or a suitable solvent chosen for an effective extraction process.[2,3] *Marc*. It is an insoluble or inert drug material that is left behind at the end of the extraction process.[2,3] *Micelle*. It is the mixture of both the extracted drug material and the solvent of extraction.[2,3] *Primary plant constituents*. These are mainly nutritional components of plants such as common sugars, amino acid, proteins, and chlorophyll. These have little or no medicinal properties.[6,7] *Secondary plant constituents*. These are also known as secondary metabolites such as alkaloids, terpenoids, saponins, phenolic compounds, flavonoids, and tannins. These are responsible for many biological or pharmacological activities.[6,7] *Bioassay-guided fractionation*. It involves extraction of plant material followed by testing for biological activity. Once the extract tested is found to be biologically active, the next step is to proceed with fractionation. Subsequently, various fractions obtained are tested for biological activity. Also, the most productive portion is then taken for compound isolation. Finally, the compound isolated is identified and tested for biological activity.[1,5,8] *Bioautography*. It is a process that uses both TLC and antimicrobial testing to establish the identity of a compound extracted as well as its antimicrobial activity.[5,9] *Finger printing in medicinal plants*. It involves the use of chromatographic techniques, identification techniques, and chemical analysis to characterize a pharmacologically active compound from a medicinal plant.[4,5] *Immunoassay*. It is a process of identification of bioactive molecule as well as its biological activity via immune reaction, receptor binding, and enzyme-mediated reactions. The extract and low-molecular-weight secondary metabolites first interact with monoclonal antibody to detect drug–receptor binding. This is followed by application of enzyme-linked immunoassay (ELISA) to determine its enzymatic activities.[5]

Solvents of Extraction

The solvent used for the extraction of medicinal plants is also known as the menstruum. The choice of solvent depends on the type of plant, part of plant to be extracted, nature of the bioactive compounds, and the availability of solvent. In general, polar solvents such as water, methanol, and ethanol are used in extraction of polar compound, whereas nonpolar solvents such as hexane and dichloromethane are used in extraction of nonpolar compounds.[3,5,10] During liquid–liquid extraction, the conventional way is to select two miscible solvents such as water–dichloromethane, water–ether, and water–hexane. In all the combinations, water is present because of its high polarity and miscibility with organic solvent. The compound to be extracted using liquid–liquid extraction should be soluble in organic solvent but not in water to ease separation.[11] Furthermore, solvent used in extraction is classified according to their polarity, from *n*-hexane which is the least polar to water the most polar.[3,5,10] The following are 11 various solvents of extractions arranged according to the order of increasing polarity[3,9]:

	Solvents	Polarity
1.	*n*-Hexane	0.009
2.	Petroleum ether	0.117
3.	Diethyl ether	0.117
4.	Ethyl acetate	0.228
5.	Chloroform	0.259
6.	Dichloromethane	0.309
7.	Acetone	0.355
8.	*n*-Butanol	0.586
9.	Ethanol	0.654
10.	Methanol	0.762
11.	Water	1.000

During fractionation, the selected solvent is added according to the order of increasing polarity, starting from *n*-hexane, the least polar to water with the highest polarity.[3,9] If a researcher wishes to select five solvents during fractionation, the usual practice is to choose two solvents with low polarity (*n*-hexane, chloroform), two with medium polarity (dichloromethane, *n*-butanol), and one with the highest polarity (water).

Properties of Solvent of Extractions

(i) *Water*. It is the most polar solvent and is used in the extraction of a wide range of polar compounds.[9,12] *Advantages*. It dissolves a wide range of substances; it is cheap, nontoxic, nonflammable, and highly polar.[9,12] *Disadvantages*. It promotes bacterial and mold growth; it may cause hydrolysis, and a large amount of heat is required to concentrate the extract.[9,12]

(ii) *Alcohol*. It is also polar in nature, miscible with water, and could extract polar secondary metabolites.[9,12] *Advantages*. It is self-preservative at a concentration above 20%. It is nontoxic at low concentration, and as small amount of heat is required for concentrating the extract.[9,12] *Disadvantages*. It does not dissolve fats, gums, and wax; it is flammable and volatile.[9,12]

(iii) *Chloroform*. It is a nonpolar solvent and is useful in the extraction of compounds such as terpenoids, flavonoids, fats, and oils.[3,12,13] *Advantages*. It is colorless, has a sweet smell, and is soluble in alcohols. It is also well absorbed and metabolized in the body.[3,12,13] *Disadvantages*. It has sedative and carcinogenic property.[3,12,13]

(iv) *Ether*. It is a nonpolar solvent and is useful in the extraction of compounds such as alkaloids, terpenoids, coumarins, and fatty acids.[3,12,13] *Advantages*. It is miscible with water, has low boiling point, and is tasteless in nature. It is also a very stable compound and does not react with acids, bases, and metals.[3,12,13] *Disadvantages*. It is highly volatile and flammable in nature.[3,12,13]

(v) *Ionic liquid (green solvent)*. This is a unique solvent of extraction and is highly polar and extremely heat stable. It can remain in a liquid state even at 3,000°C and usable where high temperature is applicable. It has extreme miscibility with water and other solvent and is very suitable in the extraction of polar compounds.[14] *Advantages*. It has excellent solvent that attracts and transmit microwave, and hence it is suitable for microwave-assisted extraction. It is nonflammable and is useful for liquid–liquid extraction and highly polar.[14] *Disadvantage*. It is not ideal for preparation of tinctures.[14]

Factors to be considered in selecting solvents of extraction

Various factors enumerated below should be taken into consideration when choosing a solvent of extraction.[3,9,15] (i) *Selectivity*. The ability of a chosen solvent to extract the active constituent and leave the inert material. (ii) *Safety*. Ideal solvent of extraction should be nontoxic and nonflammable. (iii) *Cost*. It should be as cheap as possible. (iv) *Reactivity*. Suitable solvent of extraction should not react with the extract. (v) *Recovery*. The solvent of extraction should be quickly recovered and separated from the extract. (vi) *Viscosity*. Should be of low viscosity to allow ease of penetration. (vii) *Boiling temperature*. Solvent boiling temperature should be as low as possible to prevent degradation by heat.[3,9,15]

Methods used in Extraction of Medicinal Plants

Quite numbers of procedures were technically used in the extraction of medicinal plants. Some newer methods are still evolving, whereas the existing ones are undergoing modifications.[2,5] The choice of an appropriate way of extraction is very vital, which in some cases depends on the intended use of an extract.

Factors to be considered in choosing extraction method

(a) *Stability to heat*. Heat-stable plant material is extracted using Soxhlet extraction or microwave-assisted extraction, whereas plant materials that are not heat stable are extracted using maceration or percolation.[2,11] (b) *Nature of solvent*. If the solvent of extraction is water, maceration is a suitable method but for volatile solvent percolation and Soxhlet extraction are more appropriate.[2,11] (c) *Cost of the drug*. Cheap drugs are extracted using maceration, whereas costly drugs are preferably extracted using percolation.[2,11] (d) *Duration of extraction*. Maceration is suitable for plant material requiring long exposure to the menstruum, whereas techniques such as microwave- or

ultrasound-assisted extraction are used for a shorter duration.[2,11] (e) *Final volume required*. Large volume products such as tinctures are prepared by maceration, whereas concentrated products are produced by percolation or Soxhlet extraction.[2,11] (f) *Intended use*. Extracts intended for consumption by human are usually prepared by maceration, whereas products intended for experimental testing are prepared using other methods in addition to maceration.[2,11]

Commonly used methods in the extraction of medicinal plants

(i) *Maceration*. This is an extraction procedure in which coarsely powdered drug material, either leaves or stem bark or root bark, is placed inside a container; the menstruum is poured on top until completely covered the drug material. The container is then closed and kept for at least three days.[1-4,11,16] The content is stirred periodically, and if placed inside bottle it should be shaken time to time to ensure complete extraction. At the end of extraction, the micelle is separated from marc by filtration or decantation. Subsequently, the micelle is then separated from the menstruum by evaporation in an oven or on top of water bath.[1-4,11,16] This method is convenient and very suitable for thermolabile plant material.

(ii) *Infusion*. This is an extraction process such as maceration. The drug material is grinded into fine powder, and then placed inside a clean container. The extraction solvent hot or cold is then poured on top of the drug material, soaked, and kept for a short period of time.[1-3,11] This method is suitable for extraction bioactive constituents that are readily soluble. In addition, it is an appropriate method for preparation of fresh extract before use. The solvent to sample ratio is usually 4:1 or 16:1 depending on the intended use.[1-3,11]

(iii) *Digestion*. This is an extraction method that involves the use of moderate heat during extraction process. The solvent of extraction is poured into a clean container followed by powdered drug material. The mixture is placed over water bath or in an oven at a temperature about 50°C.[1,3,11] Heat was applied throughout the extraction process to decrease the viscosity of extraction solvent and enhance the removal of secondary metabolites. This method is suitable for plant materials that are readily soluble.[1,3,11]

(iv) *Decoction*. This is a process that involves continuous hot extraction using specified volume of water as a solvent. A dried, grinded, and powdered plant material is placed into a clean container. Water is then poured and stirred. Heat is then applied throughout the process to hasten the extraction.[1-3,11] The process is lasted for a short duration usually about 15 min. The ratio of solvent to crude drug is usually 4:1 or 16:1. It is used for extraction of water soluble and heat stable plant material.[1-3,11]

(v) *Percolation*. The apparatus used in this process is called percolator. It is a narrow-cone-shaped glass vessel with opening at both ends. A dried, grinded, and finely powdered plant material is moistened with the solvent of extraction in a clean container. More quantity of solvent is added, and the mixture is kept for a period of 4 h. Subsequently, the content is then transferred into percolator with the lower end closed and allow to stand for a period of 24 h.[2,3,11] The solvent of extraction is then poured from the top until the drug material is completely saturated. The lower part of the percolator is then opened, and the liquid allowed to drip slowly. Some quantity of solvent was added continuously, and the extraction taken place by gravitational force, pushing the solvent through the drug material downward.[2,3,11] The addition of solvent stopped when the volume of solvent added reached 75% of the intended quantity of the entire preparations. The extract is separated by filtration followed by decantation. The marc is then expressed and final amount of solvent added to get required volume.[2,3,11]

(vi) *Soxhlet extraction*. This process is otherwise known as continuous hot extraction. The apparatus is called Soxhlet extractor made up of glass. It consists of a round bottom flask, extraction chamber, siphon tube, and condenser at the top. A dried, grinded, and finely powdered plant material is placed inside porous bag (thimble) made up of a clean cloth or strong filter paper and tightly closed.[1-4,11,17,18] The extraction solvent is poured into the bottom flask, followed by the thimble into the extraction chamber. The solvent is then heated from the bottom flask, evaporates, and passes through the condenser where it condenses and flow down to the extraction chamber and extracts the drug by coming in contact. Consequently, when the level of solvent in the extraction chamber reaches the top of the siphon, the solvent and the extracted plant material flow back to the flask.[1-4,11,17,18] The entire process continues repeatedly until the drug is completely extracted, a point when a solvent flowing from extraction chamber does not leave any residue behind. This method is suitable for plant material that is partially soluble in the chosen

solvent and for plant materials with insoluble impurities. However, it is not a suitable method for thermolabile plant materials. *Advantages*. Large amount of drug can be extracted with smaller amount of solvent. It is also applicable to plant materials that are heat stable. No filtration is required, and high amount of heat could be applied.[1-4,11,17,18] *Disadvantages*. Regular shaking is not possible, and the method is not suitable for thermolabile materials.[1-4,11,17,18]

(vii) *Microwave-assisted extraction*. This is one of the advanced extraction procedures in preparation of medicinal plants. The technique uses mechanism of dipole rotation and ionic transfer by displacement of charged ions present in the solvent and drug material. This method is suitable for extraction of flavonoids. It involves the application of electromagnetic radiation in frequencies between 300 MHz and 300 GHz and wavelength between 1 cm and 1 m.[1,4,10,14] The microwaves applied at frequency of 2450 Hz yielded energy between 600 and 700 W. The technique uses microwave radiation to bombard an object, which can absorb electromagnetic energy and convert it into heat. Subsequently, the heat produced facilitates movement of solvent into the drug matrix.[1,4,10,14] When polar solvent is used, dipole rotation and migration of ions occur, increase solvent penetration, and assist extraction process. However, when nonpolar solvent is used, the microwave radiation released will produce only small heat; hence, this method does not favor use of nonpolar solvents.[1,4,10,14] *Advantages*. Microwave-assisted extraction has special advantages such as minimizing solvent and time of extraction as well as increase in the outcome.[1,4,10,14] *Disadvantages*. This method is suitable only for phenolic compounds and flavonoids. Compounds such as tannins and anthocyanins may be degraded because of high temperature involved.[1,4,10,14]

(viii) *Ultrasound-assisted extraction*. This process involves application of sound energy at a very high frequency greater than 20 KHz to disrupt plant cell all and increase the drug surface area for solvent penetration. Consequently, secondary metabolites will be released. In this method, plant material should dry first, grinded into fine power, and sieved properly. The prepared sample is then mixed with and appropriate solvent of extraction and packed into the ultrasonic extractor.[2,3,10] The high sound energy applies hasten the extraction process by reducing the heat requirements. *Advantages*. Ultrasound-assisted extraction is applicable to small sample; it reduces the time of extraction and amount of solvent used, and maximizes the yield.[2,3,10] *Disadvantages*. This method is difficult to be reproduced; also, high amount of energy applied may degrade the phytochemical by producing free radical.[2,3,10]

Phytochemical Screening Methods

Phytochemical screenings are preliminary tests conducted to detect the presence of both primary and secondary metabolites in an extract. Several qualitative analyses described below have been used to detect the presence of alkaloids, flavonoids, tannins, saponins, flavones, sterols, terpenes, cardiac glycosides, protein, carbohydrates, and fats.[3,19-21]

Test for alkaloids

(a) *Dragendorff's test*. 1 mL of extract was taken and placed into a test tube. Then 1 mL of potassium bismuth iodide solution (Dragendorff's reagent) was added and shaken. An orange red precipitate formed indicates the presence of alkaloids.[3,19-21] (b) *Wagner's test*. 1 mL of extract was taken and placed into a test tube. Then 1 mL of potassium iodide (Wagner's reagent) was added and shaken. Appearance of reddish brown precipitate signifies the existence of alkaloids.[3,19-22] (c) *Mayer's test*. 1 mL of extract was taken and placed into a test tube. Then 1 mL of potassium mercuric iodide solution (Mayer's reagent) was added and shaken. Emergence of whitish or cream precipitate implies the presence of alkaloids.[3,19-21] (d) *Hager's test*. 1 mL of solution of an extract was taken and placed into a test tube. Then 1 mL of saturated ferric solution (Hager's reagent) was added and shaken. Formation of yellow-colored precipitate indicates the existence of alkaloids.[3,19-21]

Test for glycosides

(a) *Bontrager's test (modified)*. One gram of the crude extract was first weighed, placed into a test tube, and dissolved in 5 mL of dilute hydrochloric acid. Then 5 mL of ferric chloride (5%) solution was added. The mixture was shaken and placed over water bath. Then the mixture was allowed to boil for 10 min, cooled, and filtered.[3,19-21] Afterward, the mixture was then extracted again with benzene. Finally, equal volume of ammonia solution was added to benzene layer. Appearance of pink color indicates the presence of anthraquinone glycosides.[3,19-21] (b) *Legals test*. 1 mL of an extract was taken, and then an equal volume of sodium nitroprusside was added followed by few quantity of sodium hydroxide solution and shaken. Formation of pink-to-blood-red precipitate signifies the existence of cardiac glycoside.[3,19-21] (c) *Keller–Killiani test*. 2 mL of the extract was taken and diluted with equal volume of water. Then 0.5 mL of

lead acetate was added, shaken, and filtered. Again, the mixture was extracted with equal volume of chloroform, evaporated, and dissolved the residue in glacial acetic acid. Then few drops of ferric chloride was added.[3,19-21] Again, the whole mixture was placed into a test tube containing 2 mL of sulfuric acid. Emergence of reddish brown layer that turns bluish green implies the presence of digitoxose.[3,19-21]

Test for steroids and triterpenoids

(a) *Libermann Burchard's test*. This method is utilized for an alcoholic extract. Extract need to dry out first through evaporation, then extracted again with chloroform. Add few drops of acetic anhydrites followed by sulfuric acid from the side of the test tube. Formation of violet to blue-colored ring at the junction of the two liquids indicated the presence of steroids.[3,19-22] (b) *Salkowski's test*. 1 mL solution of the extract was taken and 2 mL of chloroform was added, shaken, and filtered. Few drops of concentrated sulfuric acid were added to filtrate, shaken, and allowed to stand. Development of golden-yellow precipitate indicates the presence of triterpenes.[3,19-22]

Test for tannins

(a) *Gold Beater's skin test*. A Gold Beater's Skin was obtained from Ox skin. The Gold Beater's Skin was soaked in 2% hydrochloric acid and washed with distilled water. Then it was placed in a solution of an extract for 5 min and washed with distilled water. Finally, it was placed in 1% ferrous sulfate solution. If the Gold Beater's Skin changed to brown or black tannins is present.[3,19-21] (b) *Gelatin's test*. 1 mL of extract was taken and placed in a test tube. Then 1% gelatin solution containing sodium chloride added and shaken. Appearance of white precipitate indicates the presence of tannins.[3,19-22]

Test for flavonoids

(a) *Shinoda's test*. 1 mL of extract was taken and placed into a test tube. Then, few drops of concentrated hydrochloric acid was added followed by 0.5 mg of mRimandoium turnings and shaken. Emergence of pink coloration indicates the presence of flavonoids.[3,19-21] (b) *Lead acetate test*. To detect the presence of flavonoids, 1 mL of extract was taken and placed into a test tube. Then few drops of lead acetate added and shaken. Formation of yellow precipitate signifies the presence of flavonoids.[3,19-21] (c) *Alkaline reagent test*. 1 mL of extract was taken and placed into a test tube. Then few drops of sodium hydroxide solution were added and shaken. Emergence of intense yellow color that turns to colorless after adding dilute acid implies the existence of flavonoids.[3,19-22]

Test for phenols

(a) *Ferric chloride test*. 1 mL solution of an extract was taken and placed into a test tube. Then 1% gelatin solution containing sodium chloride was added and shaken. Formation of bluish-black color indicates the presence of phenols.[3,19-21] (b) *Lead acetate test*. 1 mL solution of an extract was taken and placed into a test tube. Then 1 mL of alcoholic solution was added, followed by dilution with 20% sulfuric acid. Finally, solution of sodium hydroxide was added. Formation of red-to-blue color signifies the occurrence of phenols.[3,19-21] (c) *Gelatin test*. A solution of plant extract was placed into test tube followed by 2 mL of 1% gelatin solution and shaken. Appearance of white precipitate indicates the presence of phenols.[3,19-21] (d) *Mayer's reagent test (potassium mercuric iodide test)*. To a solution of plant extract, 1 mL of Mayer's reagent was added in an acidic solution. Manifestation of white precipitate shows the existence of phenolic compounds.[3,19-21]

Test for protein

(a) *Biuret test*. Some quantity of an extract was taken and 4% sodium hydroxide solution of the drug was produced. This is followed by the addition of 1 % copper sulfite. Appearance of violet color implies the existence of peptide linkage.[3,19-21] (b) *Ninhydrin test*. 1 mL of an extract was taken and placed into a test tube. Then 0.25% of ninhydrin reagent was added and shaken. The mixture was then boiled for few minutes. Formation of blue color signifies the presence of protein. (c) *Xanthoproteic test*. 1 mL of the extract was taken and placed it into a test tube. Then few drops of nitric acid were added and shaken. Emergence of yellow-color indicates presence of protein.[3,19-21]

Fractionation and Purification Methods

Fractionation is a process of separation of plant extracts into various fractions. It further segregates the fractions into portions comprising a number of compounds. The process continues until pure compound is isolated.[4,8,23] When several solvents are required for the fractionation, they should be added according to the order of increasing polarity. Fractionation techniques are basically classified into physical or chemical method.

Chemical methods

This extraction method is based on the type of functional groups possessed by a compound in the given mixture. Separation or purification can be achieved by chemical reactions using appropriate reagents.[4]

Physical methods

Physical methods used in separation of compounds from mixtures include separation funnel method, chromatographic techniques, fractional distillation, fractional crystallization, fractional liberation, and sublimation.[4]

(a) *Separation funnel method*. When four different solvents (*n*-hexane, chloroform, acetone, and *n*-butanol) are selected, fractionation begins by moistening or complete dissolution of crude extract with 250 mL of water. This is followed by transfer into a separating funnel, shaken, and allowed to settle. Furthermore, to 250 mL of *n*-hexane, the least polar solvent was added and shaken. The content can settle, and the bottom of the separating funnel opened to remove the aqueous layer. The remaining content in the separating funnel was poured into a clean container to get *n*-hexane fraction.[1,5,8,24] Equal volume of *n*-hexane was added again, shaken, and separated. The addition continued until after adding *n*-hexane and shaken no reasonable quantity of extract appeared to move into the *n*-hexane portion.[1,5,8,24] Similar cycle was performed for chloroform, acetone, *n*-butanol to get chloroform, acetone, and *n*-butanol fractions. The remaining portion left after the fractionation is termed as residual aqueous fraction (RAF) as the crude extract was first dissolved in water.[1,5,8,24]

(b) *Fractional distillation*. This is a process of separating or purifying compounds from a mixture. It is usually used in separation of hydrocarbons such as crude oil, citral, and eucalyptol. Purification is achieved based on the differences in their boiling points. Fractional distillation apparatus is constructed in such a manner that when heat is applied each compound will evaporate and separates at its boiling point. Consequently, each compound fractionated will condense and collected as a separate entity through several siphons attached to fractional distillation apparatus.[4]

(c) *Fractional crystallization*. Large numbers of compounds that exist naturally in plant extracts are crystal in nature. Separation is achieved via formation of crystals during concentration of an extract using heat or refrigeration.[4]

(d) *Fractional liberation*. This method is suitable for separating compounds that can easily form precipitate from the mixture. The precipitate is usually formed by changing the compounds into their salt form. Fractional liberation is commonly applicable in purification cinnamon alkaloids.[4]

(e) *Sublimation*. This method involves changing from solid to gaseous state without passing through liquid state. Substances such as camphor and volatile oils when heated get separated and converted directly into gas.[4]

(f) *Chromatographic techniques*. These are special techniques used in separation of compounds from mixtures based on their size, shape, and charge. The concept of chromatography involves the use of mobile phase, which is the solvent of extraction and the stationary phase such as silca gel and sephadex mixed with a calcium sulfate as a binder.[1,4,5,23,24] Silica gel is used for parting amino acids, sugars, fatty acids, lipids, and alkaloids. Sephadex is applicable in isolation of proteins and amino acids. Aluminum is useful in separation of vitamins, carotenes, phenols, steroids, and alkaloids. Cellulose powder is used in separation of amino acids, food dyes, and alkaloids. Celite is applicable in separation of organic cations and steroids.[1,4,8,23] Various mechanisms were involved in separation compounds using chromatographic techniques, namely, adsorption, partition, affinity, ion exchange, or size exclusion.[1,4,5,23,24] Chromatographic techniques include PC, TLC, column chromatography (CC), liquid chromatography (LC), GC, and HPLC.[1,4,5,8,23,24]

Mechanisms of separation in chromatography

(i) *Adsorption chromatography*. Separation is performed based on the interaction between compounds to be separated and the stationary phase. In this case, the stationary phase will pull and remove compounds via hydrophobic, non-covalent Van der Waals forces of attraction. The compound that is loosely bound will first be eluted by the mobile phase.[1,5,24]

(ii) *Partition chromatography*. Compounds are separated by addition of two or more immiscible solvents in to the mixture of an extract. Each compound will part away from the mixture by dissolving in the portion of solvent where it is soluble.[1,5,24] Subsequently, the immiscible liquids will be separated using separating funnel to obtain the individual compounds. The partition chromatography is otherwise known as liquid/liquid separation.[1,5,24]

(iii) *Affinity chromatography*. The stationary phase is a ligand positioned in a separating column. The mobile phase applied washed down the compounds that have no affinity for the stationary phase. As such, compounds with high affinity for stationary phase get attracted and separated.[1,5,24]

(iv) *Ion exchange chromatography*. The concept of ion exchange is useful in separation of polar compounds based on the type of charge they possessed. As such like charges attract, whereas unlike charges repelled. Like-charge substances attracted to each other and get separated the mixture or extract.[1,5,24]

(v) *Size exclusion chromatography*. This method considers separating compounds based on their molecular size by application of mesh of different diameters. It is also known as gel filtration or molecular sieving.[1,5,24] A smaller size mesh was first applied followed by medium size, and finally larger pores size mesh.

Chromatographic techniques used in the separation of compounds from a mixture or extracts

(1) *PC*. The mechanism of separation involved in adsorption chromatography. The apparatus comprises a glass chamber and a stationary phase, which is a filter paper made from cellulose. The filter paper is hanged from the top and suspended into the glass chamber.[1,5,24] The mixture to be separated is placed at the bottom of the filter paper. In addition, the solvent is then poured into the bottom of the container to serve as a mobile phase. The mobile phase immediately begins to ascend along with the filter paper; separation is carried out by the upward movement of the mobile phase via capillary action. The compounds that are soluble will move together with the solvent and stick to the filter paper based on their solubility.[1,5,24] The speed of separation depends on the type of filter paper used. Movement of liquid and the separation process is faster when thick filter paper is used, whereas porous filter paper slowed the whole process. Identification of each compound separated is done by calculating the retardation factor, which is the ratio of distance traveled by the compound to the distance traveled by the solvent.[1,5,24] The advantages of this technique include simplicity and cost-effectiveness, very sensitive to small quantity of material. The disadvantages include time-consuming and fragility of paper, which can be destroyed by chemicals.[1,5,24]

(2) *TLC*. This technique also involves the use of adsorption mechanism to separate a compound from a mixture. Separation is based on the interaction between the compounds in a mixture and stationary phase. It is applicable in the separation of compounds with low molecular weight.[1] The stationary phase usually is 100 g of silica gel dissolved in distilled water to make a slurry. Meanwhile, in some instances Sephadex is applicable. The solution of silica gel is then poured into a glass plate with dimension 20 cm × 20 cm to produce a thickness of 1.5 mm. It is then kept for 1 h at 105°C to solidify.[1,23] Afterward, 10 mL of extract is injected into the lower part of the plate and allowed to spread. The plate is then carefully inserted into the separation chamber containing mobile phase and allowed to stand for 30 min. The compounds contained in the mixture will ascend to various positions on the plate based on their solubility. Each compound separated is identified by calculating its retardation factor which is the ratio of distance traveled by the compound to the distance traveled by the solvent and compare it with that of a known compound).[1,5,23,24] The compounds spotted are scrapped at different position using spatula and finally re-extracted using various solvents.[1,23] Advantages include less time-consuming, producing clear spots, and stable to acid as solvent.

(3) *CC*. It involves the use of several mechanisms such as adsorption chromatography, molecular sieve, and ion exchange to achieve the desired outcome.[1] The column is made up of a long glass tube (5–50 mm in diameter, 5 cm–1 m long) with a tap and glass wool filter at the bottom. In addition, silica gel, alumina, cellulose, or Sephadex are used as stationary phase, whereas the mobile phase is liquid. The process begins by packing 30 g of silica gel (70/35) into a transparent glass column (80 cm long, 5 cm diameter) without introducing air bubbles. Subsequently, the extract to be partitioned is added from the top. Least polar solvent (*n*-hexane) was first added as a mobile phase and allowed to stand for 1 h in a closed column. The bottom of the column opened and various fractions of *n*-hexane collected at an interval. In addition to that, other solvents such as chloroform, ethyl acetate, *n*-butanol, and methanol added. Fractions of these solvents were collected individually at different time intervals and finally characterized.[1]

(4) *GC*. The mechanism implicated is partition. Two immiscible solvents are used: one in gaseous form (mobile phase) and the other is in liquid form adsorbed into the surface of inert solid to serve as the stationary phase.[1,4,23] Substances that are soluble in the gaseous phase will leave the liquid, move to the gaseous phase, and get separated. Similarly, compounds that are soluble only in liquid form will remain in the stationary phase.[1,4,23] Inert helium gas was used as mobile phase, at a constant flow rate. The crude extract to be analyzed was first diluted with methanol and injected into the system.[4,17,23] Advantages of this method include ability to separate plant material contaminated with volatile pesticides, also used in quality control testing.

(5) *HPLC*. This technique uses the mechanism of adsorption to achieve effective separation. It is suitable for the partitioning of both organic and inorganic compounds. The mobile phase is a suitable solvent, whereas the stationary phase is solid particles tightly joined together. Separation is initiated via interaction of the compounds in the mixture with the solid particle of the stationary phase.[1,4,5,16,23] The apparatus consists of a

solvent reservoir, sample injector, pressure pump, HPLC tube, and diode detector. The process begins by injecting the mixture to be separated at the bottom of HPLC. In addition, a suitable solvent is poured into the solvent reservoir. The tap is now opened to allow the movement of solvent downward, which is then pushed by a pressure pump to mix up with the injected sample. Finally, the mixture moved into the diode detector, which separated the compounds, removed the waste, and pumped the final content to processing units.[1,4,5,16,23]

Identification Techniques

Several methods were used in the identification of compounds from medicinal plant extracts. It comprised detection of functional group, presence of multiple bonds and rings, hydrogen and carbon arrangement as well as full structural elucidation.[1,4,10,17,23] The techniques used include mass spectroscopy (MS), ultraviolet spectroscopy (UV), nuclear magnetic resonance spectroscopy (NMR), and infrared spectroscopy (IR).

(i) *MS*. This method is useful in the identification of compounds based on chemical structure and molecular weight. The aim is to sequence and identify the unknown compound in a mixture. The substances usually identified include oligonucleotides and peptides.[1,4,10,17,23,25] The process begins by bombarding an organic molecule with an electron and converts it into very energetic charged ions. The signal was first detected using electron ionization energy of 70 eV; also, the sample spectra are detected and recorded as percentage peak. Compounds are identified based on their relative molecular mass and molecular weight. This can be achieved by plotting mass of the fragmented ions against the charges of individual ion.[1,4,10,17,23,25] Notably, MS provides abundant information on organic molecules. Therefore, one of the standard procedures in processing medicinal plant is the combination of MS/HPLC.[4,17,23,25]

(ii) *UV*. This method is suitable for qualitative and quantitative analysis of compounds present in the plant's extract. Various secondary metabolites such as phenols, anthocyanins, tannins, and polymer dyes could be detected at certain frequencies. Total phenolic content and other secondary metabolites can be established using this technique. Specific frequencies were used to identify flavonoids (320 nm), phenolic compounds (280 nm), anthocyanins (520 nm), and phenolic acids (360 nm).[4,10,23,25]

(iii) *NMR*. This technique pays more attention to the physical properties of the bioactive molecule such as number and array of the carbon atom, presence of isotopes of carbon, hydrogen atom, and protons. It also described how atoms are arranged in a molecule.[1,4,10,17,23,25]

(iv) *IR*. This method tries to assess functional groups present in a compound. Knowledge of the functional group helps in defining the physical and chemical properties of a given compound. Also, single, double, and multiple bonds are identified through this process.[1,4,5,10,23,25] The technique involves passing an organic compound through infrared radiation, which is absorbed in certain frequencies. Liquid samples are identified using sodium chloride plates, whereas solids samples are determined using potassium bromide milled together and compressed into a thin pellet. The result is recorded as a spectrum that is percentage transmittance. Lastly, the spectra are analyzed; the peaks obtained at certain wave number are compared with standard reference.[1,4,5,10,23,25]

Conclusion

Several works have been done on medicinal plant either to investigate and prove a reported claim of biological activity or to mimic its traditional medicinal use based on ethnomedicinal survey. Large numbers of medicinal plants have been extracted, fractionated, and compounds isolated successfully. In addition, compounds obtained were tested for biological or pharmacological activity, and in most cases, they were found to be active. Nonetheless, the rate of success and the authenticity of these findings depends on the accuracy in selection of solvents, selection and proper execution of extraction methods, phytochemical screening, fractionation, and identification techniques. Lastly, proper understanding and implementation of these techniques are indispensable. Advancement and modification of these methods periodically will ease research processes and improve the outcome.

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Conflicts of interest

There are no conflicts of interest.

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