



## **An Overview of Methods of Extraction, Isolation and Characterization of Natural Medicinal Plant Products in Improved Traditional Medicine Research**

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*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

Herbal plants are very important in traditional community use and enrich our plant biodiversity and conservation. Natural products are vital substances of traditional knowledge systems in complementary and alternative medicine, nutraceutical, food supplements, and pharmaceutical bioactive metabolites of new chemical entities. Bioactive secondary metabolites from herbal plants of different forms are main sources and provide major opportunities for drug active pharmaceuticals due to the diverse flora and fauna biodiversity that produces the necessary available chemical diversity. There has been an increasing popularity in phytochemical research within the high through put (HTS) screening programs in search of lead. Phytochemicals of herbal extracts for traditional uses contain various types of bioactive metabolites of pharmaceutical and pharmacotherapeutic nature, and many phytomedicines for different therapeutic areas have been derived from herbal products. This paper is aimed at giving an insight into the extraction, isolation, and characterization of the rich medicinal plant biodiversity of potential pharmaceutical importance and the major drawbacks and challenges in the extraction, isolation, and characterization of phytochemicals in plant extracts. Phytochemicals in medicinal plants have been studied with more emphasis on the extraction process which is a vital stage in the analysis of bioactive compounds in medicinal plant research. The advantages and disadvantages of the different extraction methods is important to discuss following the regulatory guidelines and different pharmacopoeia. The analysis of bioactive molecules in herbal products involves the applications of various phytochemical screening methods, and the use of chromatographic techniques such as TLC and HPLC, including in some cases the non-chromatographic methods like Fourier Transform Infra-Red (FTIR), immunoassay. This paper has been motivated by the challenges faced by most pharmacy students in data mining of information on phytochemical screening and testing of biological activities in projects related to herbal plants research. This write up is also geared towards providing students with information on the preclinical drug discovery process towards the formulation of an improved traditional medicine/ phytomedicine.

*Keywords: Phytochemicals; bioactive secondary metabolites; plant extraction; natural products.*

## 1. INTRODUCTION

Medicinal plants have gained a significant consideration due to their contributions as one of the main sources of phytochemicals that are starting points of hits or lead compound selection for the development of new chemical entities. Most phytochemicals from herbal plant sources such as the flavonoids, coumarins, and phenolics are known to possess biological and pharmacological activities on health-related diseases [1]. Modern nutraceuticals, food supplements and dietary approaches to stop hypertension (DASH) contain studied phytochemicals incorporated with rich diets derived from fruit and vegetable, sources of antioxidants [2,3]. The focus on the use of natural products in the discovery, development and formulation of new compounds, as an alternative to complement the conventional drugs and synthetic products, adds value to medicinal plant research and industrial manufacturing of phytomedicine [4]. Studies show that high concentration of phenolic compounds and flavonoids in natural products are associated with biological activities such as anti-inflammatory,

antioxidant, antimicrobial and anticancer. These active metabolites can play a role in the treatment and prevention of age-related disease development, particularly those caused by oxidative stress [5]. Considering the significant benefits of bioactive metabolites in medicinal plants and the orientation towards research and development in pharmaceutical and nutraceutical industries, medicinal plants' use is also an important source of conventional drugs.

The study of medicinal plants takes its course from the pre-extraction and extraction procedures, which are important steps in the processing of the phytochemical's constituents from natural products. Traditional and ancestral extraction methods such as maceration and Soxhlet extraction are mostly used for small-scale medicinal research settings or Small Manufacturing Enterprises (SME). A lot of progress has been made in the process of natural product-extraction focusing on increasing the yield at minimal production. This progress has been demonstrated in the following techniques; supercritical fluid extraction (SFE), ultrasound-assisted extraction (UAE), and

microwave-assisted extraction (MAE) [1,5]. There are still further ongoing modifications on the methods for optimum extraction and yield. Since there are many different methods of natural product extractions, the selection of proper extraction methods needs a better understanding of the ethnobotanic information, type of plant materials and the types of extraction products envisaged.

### 1.1 Pre-extraction Preparation of Medicinal Plant Samples

The initial stage involved in the study of natural products starts with the preparation of the plant samples to conserve and preserve the phytochemicals in the plants before the extraction process. Plant samples collected from leaves, barks, roots, fruits and flowers can be extracted from fresh or dried plant materials. Other pre-preparation processes of plant materials such as grinding and drying may have an influence on the preservation of bioactive secondary metabolites in the final extracts [6].

**Fresh and dried plant samples:** Both the fresh and dried herbal plant samples may be used in medicinal plants studies. Generally, dried samples are most commonly used taking into consideration the time factor for experimental design [2,7]. Studies show that the interval between harvest and experimental work can be a maximum period of 3 hours in order to maintain the freshness of the sample as fresh samples can be fragile and have a faster deterioration than the dried samples [8]. A comparative study between fresh and dried *Moringa oleifera* leaves for example, showed no significant effect in total phenolic contents but the dried sample showed higher flavonoid yields [5,9].

### 1.2 Relevance of Pre-extract Preparation

Pre-extraction preparation of natural products allows for efficient extraction to occur. It also allows the solvent to be in contact with the target analytes and therefore the particle size less than 0.5 mm is suitable for efficient extraction [6]. This particular particle size has been reported by Sulaiman et al, for preparing vegetable samples that were ground to 400  $\mu\text{m}$  (0.4 mm) in size. Conventional mortar and pestle or electric blenders and mills are generally used for reducing particle size of herbal samples. Extraction involves the separation of phytochemicals from natural products from the inactive components through the use of selected solvents used in standard extraction procedures

[10]. The products obtained from herbal plants are in some cases relatively impure liquids, semisolids or powders that are for either oral or external use.

**Ground and powdered samples:** The reduction of particle size increases the surface area between samples and extraction solvents. Grinding can result in smaller coarse samples; whereas, powdered samples have a more homogenized and smaller particle size, thus providing a better surface contact with the extraction solvents [7,11]. Although this type of pre-preparation extraction permits an efficient extraction to take place, it is also necessary for the solvent to be in contact with the target analytes and the particle size smaller than 0.5 mm ideal for efficient extraction [6,10]. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, semisolid extracts and powdered extracts. Such preparations popularly have been called galenicals, named after Galen, the second century Greek physician [7,12]. The focus of standardized extraction procedures for phytochemicals is to obtain the therapeutically desired concentrations and to eliminate the inert material by treatment with a selective solvent known as menstruum [7]. Particle size has been a major factor when using enzyme-assisted extraction. The use of pectinolytic and a cell wall polysaccharide degrading enzyme in sample preparation is influenced mainly by the particle size as smaller particles enhance enzyme action [13].

**Air-drying, microwave-drying, oven-drying and freeze-drying (lyophilisation) of plant samples:** Air-drying generally takes between 3-7 days, a month and at times up to a year, depending on the types of samples to be dried (eg. leaves or seed). The leaves and stem samples can be air dried together by exposing to air at room temperature. This drying method does not require drying of plant materials using high temperature; thus, preserving heat-labile compounds. However, air-drying takes a longer time in comparison to microwave drying and freeze drying and may be predisposed to contamination at unstable temperature conditions [4,14].

Microwave-drying is conditioned to use electromagnetic radiation to provide both electric and magnetic fields. The electric field causes at same time heating by dipolar rotation; alignment on the electric field of the molecules possessing a permanent or induced dipole moment (e.g.

solvents or samples), and ionic induction, that can produce free movement of the molecules [8]. Oscillation causes collisions between molecules and can result in rapid heating at the same time of the samples. The microwave drying method is adopted to reduce drying time but with a drawback sometimes of causing breakdown of bioactive molecules.

Oven-drying is one of the pre-extraction methods that uses thermal energy to remove or reduce moisture from the prepared samples. This sample preparation is considered one of the easiest and most rapid thermal processes that can preserve phytochemicals. Oven-drying at 44.5°C for 4 hours using 80% methanolic extracts has been shown to produce the highest antioxidant properties in the herbal *Cosmos caudatus* extracts and correlated results have been shown in optimized 80% methanolic extracts at 44.12°C for 4 hours [9]. However, the effect of drying on another plant *Orthosiphon stamineus* reported no significant effect on the antioxidant activity but the bioactive metabolites such as sinensetin and rosmarinic acid content were affected by the oven- and sunlight-drying, indicating a sensitivity of the phytochemicals to temperature [10,15].

Freeze-drying is a method based on the principle of sublimation, which is the process by which a solid changes state into the gas phase without the necessity to enter the liquid phase. The herbal sample is frozen at -80°C to -20°C before lyophilization to solidify any remaining liquid such as solvent moisture contained in the samples. After freezing for about 12 hours or overnight, the sample is quickly lyophilized to avoid the frozen liquid in the sample from melting. The mouth of the test tube or any container holding the sample is wrapped with needle-poked-parafilm to prevent the loss of the sample during the process [15]. Generally, a sample can be lost by splattering it out into the freeze-flask as shown in Fig. 1. Freeze-drying can yield a greater quantity of phenolic compounds concentration compared to air-drying as most of the bioactive metabolites are preserved using this method [1,16]. However, freeze-drying can be complex and a more expensive method of drying when compared to regular microwave-drying and air drying. Therefore, the use is restricted to delicate, thermal-sensitive materials of high value.

### 1.3 Extraction

Extraction is the process by which a solute is transferred from one phase to a new phase.

There are different types of extraction methods that include:

Liquid-liquid extraction, also known as solvent extraction and partitioning. This is a method used for separating phytochemicals based on their relative solubilities in two different immiscible liquids. The solvents are usually made of water and an organic solvent [16]. The process that involves the transfer of dissolved substances from one phase to another phase, which are immiscible or restrictedly miscible, is called liquid-liquid –partition or partition between two phases of liquids as illustrated in Fig. 1 a,b,c [9,17]

Extraction is a critical first step in the analysis of herbal/medicinal plants, since it is necessary to extract the desired phytochemicals from the plant materials for future separation and characterization. The operation involves, the stages such as pre-washing, drying of plant samples or freeze- drying, grinding to obtain a uniform sample in order to improve the kinetics of analytic extraction and also to increase the surface area of contact of the sample to the solvent system [18]. Well standardized procedures are necessary to guarantee that major bioactive metabolites attain maximum during extraction, and are not altered during the extract process from plant samples, especially if the plants are selected on the basis of ethnobotanical and ethnopharmacological information and traditional uses [19,20]. Plant selection based on traditional use, requires the extract be prepared as described by the traditional healer, as a way to mimic as closely as possible, the traditional concoction [21]. The selection of a solvent system highly depends on the specific nature of the phytochemicals being targeted for extraction. Different solvent systems are required to extract bioactive compounds from natural products. The extraction of hydrophilic compounds uses polar solvents such as methanol, ethanol or ethyl-acetate. For extraction of more lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol in ratio of 1:1 are used. In some cases, extraction with hexane is used to remove chlorophyll [22,23].

### 1.4 Some Modern Extraction Techniques

Other modern extraction techniques include;

Solid-phase micro-extraction, surfactant-mediated techniques, pressurized-liquid extraction, the supercritical-fluid extraction, microwave-assisted extraction, solid-phase

extraction [24]. The techniques have some advantages such as the reduction in the consumption of organic solvent, sample degradation, elimination of extra sample clean-up and concentration steps before chromatographic

analysis. There is also the improvement in extraction efficiency, selectivity, and the kinetics of extraction [25]. The technique is automated and gives an added factor that enhances their use in the extraction of plants materials [26,27].



Fig. 1 a. Process of transfer of a dissolved substance from one phase to another phase, which are immiscible or restrictedly miscible, is called liquid-liquid –partition or partition between two phases of liquids.

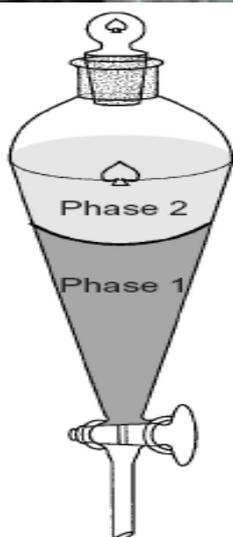


Fig. 1 b. Separatory funnel used for liquid-liquid extraction.

Schematic of a separatory funnel showing two immiscible liquids, where 1 is any phase less dense than 2. Phase 1 is typically an organic solvent and 2 an aqueous phase.



Fig. 1 c. Laboratory-scale liquid-liquid extraction. Photograph of a separatory funnel in a laboratory scale extraction of 2 immiscible liquids: liquids are a diethyl ether upper phase, and a lower aqueous phase.

Fig. 1a,b,c. showing liquid-liquid extraction technique

The Partition law of Nernst – Shilov shows that the relationship of dissolved substance concentration in both phases at constant temperature is constant and does not depend on concentration of the dissolved substance:

$$D = C_{Aorg} / C_{Aaqu}$$

Where D = the distribution ratio remains constant if there are no processes: dissociation or association, polymerisation or other transformations of the dissolved substance

$$\text{Distribution constant } K_{T/D} = \frac{(ML_n)_o}{(ML_n)_B}$$

KT-The ratio of substance activity in one form in organic solvent phase to its activity in a water phase is named a distribution constant.

The distribution ratio and constant are connected with substance solubility. The conditions of a choice of solvent which should be used as extragents are that they should not mix up with water, be selective, should have a significant capacity in relation to extract. The density of extragents should be different from water density, should have the minimum viscosity, low cost, and cannot be explosive [28].

## 2. CLASSIFICATION OF EXTRACTION PROCESSES

### 2.1 Periodical Extraction

Periodical extraction is the process by which a separatory funnel (which contain substance with extragent) is shaken. There is continuous extraction and counter current extraction.

### 2.2 Types of Extraction Systems

1. Halogenides with covalent linkage:  $HgCl_2$ ,  $HgJ_2$ ,  $SbJ_3$ ,  $AsBr_3$ ,  $GeCl_4$ , element iodine etc.
2. Intracomplex salts: dithizonate, dithiocarbamates, oxyquinolines, oxygens,  $\beta$ -diketonate, and di-(2-ethylhexyl)-phosphates actinoids, rare-earth and some other elements, etc.
3. Complex metal acids:  $HFeCl_4$ ,  $HInBr_4$ ,  $HSbCl_6$ , etc. They are coordinatively not solvated (a), Salts tetraphenyl arsonium, tetraphenyl phosphonium and coordinatively solvated (b) salts: The

compounds which are formed at extraction uranyl nitrate and nitrate of thorium by tributyl phosphate from nitrate solutions [29]

4. Heteropoly compounds of phosphorus, arsenic, silicon, vanadium, molybdenum, tungsten etc.
5. Most widely used in extraction processes are the intracomplex salts, complex metallo halogenide acids and coordinatively solvated (b) salts.

The main organic reagents used in extraction method are 8-oxyquinoline which reacts with more than 50 elements.

Acetylacetonate which forms compounds with more than 60 elements and Thionyl trifluoride acetone is used for extraction and separation of actinoids.

Dithizon is used for the determination of Pd, Au, Hg, Ag, Cu, Bi, Pt, In, Zn, Cd, Co, etc.

Sodium diethyl dithiocarbamate reacts with several elements. It is of great importance in toxicological analysis especially in *In vivo* animal studies.

For increase of selectivity extraction, it is important to create an optimum pH medium, use masking (reactions of complexation, oxidation-reduction, and precipitation)

### 2.3 Usage of Extraction in the Drug Analysis

#### 2.3.1 Extraction is very useful in drug analysis for the following

1. Separation of elements
2. Concentrating impurities
3. Clearings of the basic component from impurities in the process of drug substance synthesis.
4. Definition of the basic component from impurities in the process of drug substance synthesis
5. For identification and quantitative definition of chemical agent or substances-markers in the process of the analysis of phyto genesis drugs
6. Increase of sensitivity and selectivity of reactions
7. Studying of formation constant of complex
8. Studying of substance condition in a solution (a charge, polymerisation degree)

## 2.4 Role of Extraction in Concentration and Definition

Absolute concentration is attained at usage of smaller volume of an organic phase in relation to initial volume of water solution. Relative concentrating is an increase in impurity concentration in relation to the main component. Especially important role extraction is by connection with physical and physical-chemical methods of the analysis – hybrid methods of the analysis which have such advantages: High sensitivity, Selectivity, Specificity and rapid analysis method [30].

## 2.5 Stages of Medicinal Herb Extraction

Drying (sometimes it is unnecessary), Crumbling up, Sifting, Selection of optimum extragent, Choice of an optimum extraction technique. Extraction (moisten, passage extragent through pores, dissolution of substances in the middle of the cell, diffusion of substance molecules through cellular covers, mass-carrying extraction substances from a surface of particles in extragent) [31]

## 2.6 Analytical Techniques of Reception of an Extract of Medicinal Herb Analysis on High Quality: These Include

- ✓ Extraction to a full attrition (percolation with 1 g medicinal herbs through burette).
- ✓ Single extraction of raw material shot (by boiling 1 g raw materials with extragent)
- ✓ Equilibrium extraction (for 4-5-hour balance between internal extragent in medicinal herbs and the external extract, analyzes an extract part). In the biochemical and toxicological analysis, extraction is used for removal of chemical substances from animal and herb tissues from fresh and dried samples.
- ✓ Galenicals: This includes classes of preparations such as the decoctions, infusions, macerations, potential fluid extracts, some tincture extracts and in most cases powdered extracts [32]

## 3. THE PHARMACOPEIAL STANDARDS

The pharmacopeial standards involve a cascade of complex processes and analysis necessary for phytomedicine efficacy, safety and quality evaluation. The Standardization parameter for plant drugs is summarized in Fig. 2. The

standardization is relevant for regulatory norms alignment of the development of improved traditional medicine of phytomedicine from medicinal plants of pharmaceutical importance [7,33].

## 3.1 Selection Approach for a Suitable Extraction Method

- i) The authentication and validation of herbal plant material needs to be done before the start of performing any extraction. All foreign materials need to be removed.
- ii) The suitable part of the plant must be used and the age, time, place, time of collection and season of harvest should be recorded.
- iii) The conditions put in place for drying the plant material generally depend on the nature of its desired phytochemicals. The application of hot or cold blowing air flow for drying is ideal for drying. There is need for suitable weight corrections when the crude herbal product with high moisture content has to be used for extraction.
- iv) The grinding methods need to be specified. Any techniques that produce heat should be avoided. Powdered plant material should be passed through sieves in order to obtain the required particles of homogenous size.
- v) Nature of phytochemicals:
  - a) When the therapeutic value falls in non-polar constituents, it is advisable to use a non-polar solvent. For instance, lupeol is the active principle of *Crataeva nurvala* and, for its good extraction, hexane is commonly used as solvent. Similarly, for natural herbal products plants like *Centella asiatica*, and *Bacopa monnieri* the phytochemicals are mostly glycosides and thus require the use of a polar solvent such as aqueous methanol.
  - b) If the bioactive compounds are heat sensitive, the extraction methods such as cold maceration, counter current extraction (CCE) and percolation are preferred. For thermostable compounds, it is suitable to use the Soxhlet extraction method when there is a nonaqueous solvent, and the decoction extraction when water is the main solvent.
  - c) There is need to take precautionary measures when dealing with phytochemicals such flavonoids and phenyl propanoids that easily degrade in organic solvents.

- d) For the case of hot extraction, it is advisable to avoid temperatures that are higher than normal as some glycosides for example may likely break when exposed to higher temperature. There is need for the standardization of extraction time as insufficient time means incomplete extraction. Unwanted bioactive constituents may also be extracted with longer extraction time. Generally, if tea is boiled for a longer duration, tannins can be extracted which gives astringency to the final product preparation.
- e) The number of extractions required for complete extraction is as important as the duration of each extraction.
- vi) The quality of water or menstruum that are used for extraction must be specified and well controlled.
- vii) The concentration and drying procedures of products should ensure that there is safety and stability of the bioactive metabolites. Drying under reduced pressure such as using a Rotavapor is generally used and lyophilization although costly is commonly used.
- viii) Consideration should be taken on the design and fabrication material of the extractor.

- ix) The analytical parameters of the final extraction products, such as thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC), fingerprints, must be well documented to monitor the quality of the different batches of the extracts [35]

### 3.2 Methods of Extraction of Medicinal Plants

#### 3.2.1 Maceration

Maceration is a technique commonly applied in wine making and now adopted and generally in usage for natural products/medicinal plants research. The process involves soaking plant materials usually in powder or coarse form in a stoppered container with a defined solvent. The setup is left to stand at room temperature ( $\pm 25^{\circ}\text{C}$ ) for a minimum of 72 hours with constant shaking [11,36]. The process is conditioned to soften and breakup the plant's cell wall in order to liberate the soluble bioactive metabolites. After 3 days, the whole mixture is pressed and sieved by filtration using Whatman no 1 filter paper. In Maceration, heat is transferred by convection and conduction and the choice of the solvent is crucial to determine the type of phytochemical extracted from the samples [37].

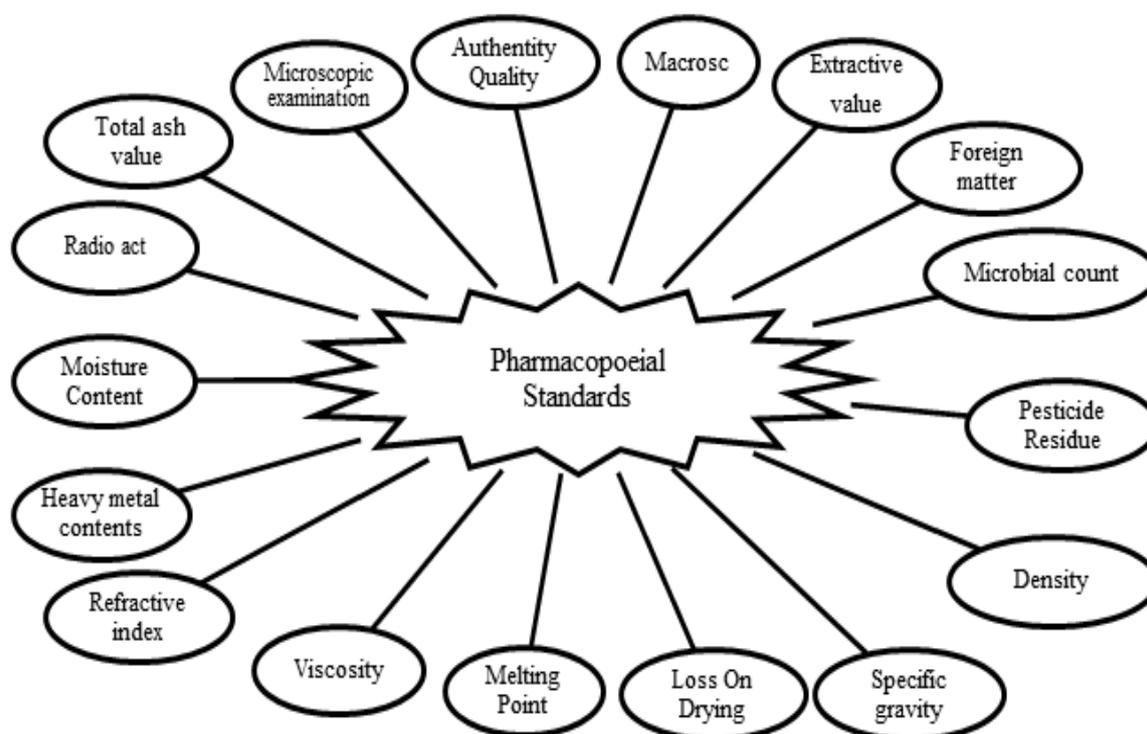


Fig. 2. Standardization parameter for plant drugs [9,34]

### 3.2.2 Infusion

Fresh infusions are prepared by macerating the crude drug for a short period with cold or boiling water. These are dilute solutions of the readily soluble constituents of crude drugs. Infusion and decoction use the same principle as maceration; both are soaked in cold or boiled water. However, the maceration period for infusion is shorter and the sample is boiled in a specified volume of water (eg. 1:4 or 1:16) for a defined time for decoction [10,38,39].

### 3.2.3 Decoction

In decoction, the harvest crude product is boiled in a specified volume of water for a defined period and cooled down then strained or filtered. This procedure is applicable for the extraction of water-soluble, heat stable metabolites. The starting ratio of crude product to water is fixed, such as 1:4 or 1:16; the volume is then brought down to one-fourth its original volume through boiling during the extraction process. Next, the concentrated extract is filtered and processed further [32,40]. Decoction is generally suitable for extracting heat-stable compounds, hard plant parts such as the roots and barks, and the results give more oil-soluble phytochemicals compared to maceration and infusion. The percolator which is another method that shares similar fundamental principles is used in percolation. Dried powdered samples are packed in the percolator, followed by adding boiling water and macerated for 2 hours. The percolation process is generally conducted at moderate rates such as using 6 drops /min, until the extraction is completed before evaporation to obtain a concentrated extract [12,41].

### 3.2.4 Percolation

This is the most frequently used method to extract phytochemicals for the preparation of tinctures and fluid extracts. A percolator made of a narrow cone-shaped vessel open at both ends is generally used. The solid ingredients are moistened with a specified amount of the defined menstruum and allowed to stand for approximately 4 hours in a closed container. Next, the mass is packed and the top of the percolator is closed and an additional menstruum is added to form a shallow layer above the mass, while the mixture is allowed to macerate in the closed percolator for 24 h [42]. The outlet of the percolator is then opened and the liquid contained therein is allowed to drip out slowly. An additional menstruum is added as required, until

the percolate measures about three-quarters of the volume needed of the finished product. The macerated product 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 [18,43].

### 3.2.5 Digestion

This is a maceration method in which there is application of gentle heat during the process of extraction. It is used when a moderately elevated temperature is needed. The solvent efficiency of the menstruum is generally enhanced [44-46].

## 3.3 The Properties of a Good Solvent in Plant Extractions

The properties of a good solvent for herbal plant extraction include; low toxicity, inability to cause the extract to form complexes, the ease of evaporation, preservative action, and promotion of rapid physiological absorption of the extract. [47].

The main solvents used in plant herbal extraction include water, acetone, chloroform, ethanol, ether and methanol as shown in Table 1. The table also illustrates bioactive metabolites that can be isolated using specific solvents such as tannins, anthocyanins, polyphenols, flavonoids, saponins, coumarins, terpenoids etc.

### 3.3.1 Hot continuous soxhlet extraction

In this method, the finely ground crude extract is placed in a porous bag or "thimble" made of strong filter paper, which is placed in the chamber of the Soxhlet apparatus. The extracting solvent in flask A is heated, and its vapour condenses. The condensed extractant drips into the thimble containing the crude drug-extract, and extracts by active contact. When the level of liquid in chamber rises to the top of siphon tube, the liquid contents of chamber siphon into flask A. This process is continuous and is carried out until a drop of solvent from the siphon tube is unable to leave residue when evaporated. The advantage of this method, with respect to the other extraction methods is that large amounts of bioactive metabolites can be extracted with a very small amount of solvent, saving time, energy, and financial costs. On a small scale, it is used mainly as a batch process, it can become much more economical and viable when converted into a continuous extraction procedure on a medium or large-scale extraction [49-50].

**Table 1. Solvents used for active component extraction [48,34]**

<b>WATER</b>	<b>ETHANOL</b>	<b>METHANOL</b>	<b>CHLOROFORM</b>	<b>ETHER</b>	<b>ACETONE</b>
Polyphenols	Tannins	Anthocyanins	Terpenoids	Alkaloids	Phenol
Tanins	Polyphenols	Tanins	Flavonoids	Fatty acids	flavonols
Lectins	Sterols	Totarol		Coumarins	
Terpenoids	Polyacetylenes	Terpenoids		Terpenoids	
Anthocyanins	Terpenoids	Saponins			
	Flavonol	Xanthoxyllines			
Starches	Alkaloids	Quassinoids			
Saponins		Phenones			
		Polyphenols			
		Lactones			
		Flavones			

### 3.3.2 Aqueous alcoholic extraction by fermentation

Some herbal preparations use the technique of fermentation to extract phytochemicals. This process involves soaking the crude product in the form of either a powder or a decoction for a specific duration, during which it undergoes fermentation and produces alcohol *in situ*. This process facilitates the extraction of the bioactive metabolites from the plant extract [51,52]. The alcohol produced usually serves as a preservative. If the fermentation is carried out in an earthen container, it is advisable not to be a new container and water should first be boiled in the vessel before use. In large-scale manufacture, wooden vats, porcelain jars or metal vessels are generally used in place of earthen vessels [53-55]. Fermentation method is not yet standardized but, with progress in fermentation technology, there is great progress to standardize this technique for herbal product phytochemical processing.

### 3.3.3 Counter-current extraction

In counter-current extraction (CCE), the wet raw material is pulverized using toothed disc disintegrators to produce a fine slurry. In this process, the material for extraction is moved in one direction generally in the form of a fine slurry, within a cylindrical extractor as it comes in contact with the extraction solvent [56]. The further the movement of the starting material the more concentrated the extract will become. Complete extraction is achieved when the quantities of solvent, crude material and their flow rates are optimized. The process has minimal risk from high temperatures, very efficient and requires little time. At the end of the process, sufficient concentrated extract is produced at one end of the extractor while the

waste that is free of visible solvent comes out from the other end.

#### 3.3.3.1 Advantages of the counter current extraction process

- i) A unit quantity of the plant herbal 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 crude product is done under wet conditions, the heat generated during combustion is neutralized by water. This reduces the thermolabile constituents from exposure to heat.
- iv) This extraction procedure has been rated to be more efficient and effective than continuous hot extraction [10,20].

### 3.3.4 Ultrasound (sonication) extraction method

This method involves the use of ultrasound with application of frequencies ranging from 20 kHz to 2000 kHz., to enhance the permeability of cell walls and produces cavitation. Although the process is applicable in some cases such as in the extraction of rauwolfia root, the use in a large-scale is limited due to costs. One of the disadvantages of this method is the harmful effect of ultrasound energy of over 20 kHz, on the bioactive metabolites of the medicinal plants caused by the formation of free radicals which interacts to give undesirable effects in the bioactive metabolites [57-59].

### 3.3.5 Supercritical fluid extraction

Supercritical fluid extraction (SFE) constitutes an alternative sample preparation method with a general objective of reducing the use of organic solvents and increased sample throughput [60]. The factors to consider in the method include pressure, sample volume, temperature, analyte collection, modifier or cosolvent addition, restrictors flow and pressure control [7,61]. In most cases, cylindrical extraction vessels are used for SFE and their output has been reported to be good. The collection of the extracted analyte following SFE is an important and critical step as significant analyte loss may occur during this step.

There are many advantages to the use of carbon dioxide as the extracting fluid. Apart from its favourable physical properties, being safe and abundant, CO<sub>2</sub> is not expensive. However, while carbon dioxide is the preferred fluid for SFE, it possesses several polarity limitations. Solvent polarity is critical when extracting polar solutes and when strong analyte-matrix interactions are present [62,63,27]. Organic solvents are regularly added to the CO<sub>2</sub> extracting fluid to solve the problem of polarity limitations. Recently argon has been used as a substitute for carbon dioxide, because it is inert and less expensive. The component recovery rates generally increase with increasing pressure or temperature and the highest recovery rates for argon are obtained at 500 atm and 150°C [14,64]. The SFE procedure possesses a distinct advantage such as the possibility of extraction of constituents at low temperature, which reduces damage from heat and some organic solvents. There is no occurrence of solvent residues and the extraction procedure is environmentally friendly.

The largest growth area in the development of SFE has been the rapid expansion of its applications. SFE is widely used in the extraction of pesticides, essential oils, polymers, foods and fragrances, environmental samples, and natural products. The major drawback in the commercial use of the extraction process is its restrictive capital investment [65].

### 3.3.6 The phytonic process

This involves a new solvent based on hydrofluorocarbon and a new technology used to optimize its remarkable properties in the extraction of plant materials to offer a great environmental advantages and health and safety benefits over traditional processes, for the

production of high quality flavours, natural fragrant oils, and biological extracts [66]. Advanced photonics Limited in Manchester, UK has successfully developed this patented technology called "the phytonic process". The products generally extracted by this process are fragrant compounds of essential oils and phytochemicals which can be used directly without further physical or chemical treatment [24,67].

The properties of the new generation of fluorocarbon solvents have been applied to the extraction of plant materials. The core of the solvent usually has the chemical name 1,1,2,2-tetrafluoroethane, well known as hydrofluorocarbon-134a (HFC-134a) that was developed as a substitute for chlorofluorocarbons. The boiling point of this solvent is -25°C and it is neither flammable nor toxic. Unlike chlorofluorocarbons, it does not deplete the ozone layer and has a vapour pressure of 5.6 bar at room temperature ±25°C. By all standards this is a poor solvent as it does not mix with mineral oils or triglycerides and does not dissolve plant waste [42,68].

The phytonic process is advantageous in that the solvents can be adopted and customized: by using modified solvents with HFC-134a. The process can also be made highly selective in extracting a specific class of phytochemicals and other modified solvents can be used to extract a broader spectrum of components. The biological products made by this process have an extremely low residual solvent [5,69]. The residuals are generally less than 20 parts per billion (PPB) and are known to be below undetectable levels. These solvents are neither acidic nor alkaline in nature and, therefore, have only minimal potential reaction effects on the herbal materials. The sample crude plant is sealed so that the solvents can be constantly recycled and well recovered at the end of each reaction cycle. The only utility needed to operate these systems is electricity and, even then, there is low consumption of energy [70]. There is no room for loss 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 the herbal plant product is dry, environmentally friendly and easier to manage.

#### 3.3.6.1 Advantages of the phytonic process

The phytonic process is cool and gentle compared to other processes that require high

temperatures and its products are not destroyed by exposure to temperatures that exceed room temperature. No vacuum stripping is required as in other processes that can lead to the loss of precious volatiles. The process is carried out at neutral pH and, in the absence of oxygen, the products never predisposed to acid hydrolysis damage or oxidation. The technique is very selective, and offers a choice of operating conditions and a choice of end products. It is environmentally friendly, requires a minimum amount of electrical energy and releases very little harmful emissions into the atmosphere. The resulting waste products are innocuous and pose no problem of waste disposal. The solvents used in the technique are not flammable, toxic or ozone depleting and they ease complete recycling within the system [12,71].

### 3.3.6.2 The use of the photonics process

The photonics process can be used for extraction in biotechnology such as in the production of antibiotics, in the food and herbal drug industry as well as in essential oil, flavour industries, and in the production of other pharmacologically active products [71]. Generally, in most cases, it is used in the production of pharmacologically active intermediates, top-quality pharmaceutical-grade extracts, phytopharmaceuticals, antibiotic extracts. However, the fact that it is used in all these areas in no way prevents its use in other areas. The technique is also used in the extraction of high-quality essential oils, oleoresins, natural food, colours, flavours and aromatic oils from different herbal plant materials [8,72]. The technique is well adopted for refining crude products obtained from other extraction processes. It provides extraction without waxes or other contaminants and helps in the removal of many biocides from contaminated biomass [72].

### 3.3.7 The microwave assisted extraction (MAE)

MAE uses microwave energy to enhance partitioning of analytes from the sample matrix into the solvent [22]. Microwave radiation interacts with dipoles of polar and polarizable materials such as solvents and sample causing heating near the surface of the materials and heat transferred through conduction. Dipole rotation of the molecules induced by microwave electromagnetic disrupts hydrogen bonding; facilitating the migration of dissolved ions and promotes solvent penetration into the matrix

[8,73]. In non-polar solvents, poor heating can occur as the energy is transferred mainly by dielectric absorption [10]. MAE is a selective method that favour more polar molecules and solvents with high dielectric constant (Table 2).

### The advantage and disadvantage of MAE:

This technique can reduce extraction time and solvent volume when compared to the other conventional methods such as maceration & Soxhlet extraction. Improved recoveries of analytes and reproducibility are well observed in the MAE method but it is done with caution of using proper conditions in order to avoid thermal degradation [8]. However, this method is limited to small-molecule phenolic compounds such as phenolic acids (gallic acid and ellagic acid), isoflavin, quercetin, and trans-resveratrol due to the fact that these molecules are stable under microwave heating conditions up to 100°C for 20 minutes. Additional cycles of MAE (e.g. from  $2 \times 10$  s to  $3 \times 10$  s) can lead to a drastic decrease in the yield of phenolics and flavanones, mainly caused by the oxidation of phytochemicals [22,74]. Tannins and anthocyanins are not likely suitable for MAE as they are easily subjected to degradation at high temperature [74]. The dielectric constant of some commonly used solvents for toluene, acetone, methanol, hexane, and others are illustrated in Table 2.

**Table 2. Illustration of dielectric constant of some commonly used solvents [22]**

Solvent	Dielectric constant (20°C)
Water	78.5
Hexane	1.89
Toluene	2.4
Dichloromethane	8.9
Acetone	20.7
Ethanol	24.3
Methanol	32.6

### 3.3.8 The accelerated solvent extraction (ASE)

ASE is an efficient form of liquid solvent extraction as compared to maceration and Soxhlet extraction. The method uses a small amount of solvent. The sample is packed with inert material such as sand in the stainless-steel extraction cell to prevent the sample from aggregating and further blocking the system tubing [6,30,75]. Packed ASE cell includes layers of sand-sample mixture in between cellulose filter paper and sand layers. This automated

extraction technology is well adapted to control the temperature and pressure for each sample and takes less than an hour for extraction. Compared to other solvent techniques, ASE also critically depends on the solvent types. Cyclohexane acetone solution at the ratio of 6:4 v/v with 5-minute heating (50°C) has been showed to yield the highest bixin from *Bixa orellana* with 68.16% purity [30,76].

According to the World Health Organization (WHO), almost 20,000 medicinal plants exist and have been screened in 91 countries including 12 mega biodiversity countries. The first steps to utilize the phytochemicals from plant resources are extraction, pharmacological screening, isolation and characterization of bioactive compounds, toxicological evaluation and clinical evaluation. A summary of the general approaches in the extraction, isolation and characterization of bioactive compounds from plant extracts is shown on Fig. 1. This provides details in extraction, isolation and characterization of bioactive compound from plant extracts with common phytochemical screening assay, chromatographic techniques, such as HPLC, and HPLC/MS and Fourier Transform Mass Spectrometry (FTMS). A summary of the experimental conditions for various methods of extraction for plant material can be understood from Table 3. This table demonstrates the different extraction methods and conditions of extraction.

### 3.3.9 Reflux extraction

Reflux extraction is more efficient than percolation or maceration and requires less extraction time and solvent. It cannot be used for the extraction of thermolabile natural products. Refluxing with 70% ethanol provides the highest yield of the natural bio-active metabolites root among the extracts prepared by different extraction methods (sonication, reflux, Soxhlet, maceration and percolation) [79].

### 3.3.10 The pressurized liquid extraction (PLE)

PLE is also known as accelerated solvent extraction, enhanced solvent extraction, accelerated fluid extraction, pressurized fluid extraction, and the high-pressure solvent extraction as described by different research groups. PLE applies high pressure in extraction. High pressure keeps the solvents in a liquid state

above their boiling point thus resulting in a high solubility and high diffusion rate of lipid solutes in the solvent, and a high penetration of the solvent in the matrix. PLE significantly decreased the consumption of extraction time and solvent and had better repeatability compared to other methods. Pressurized liquid extraction has been successfully applied in extracting many types of natural products including saponins, flavonoids and essential oil from TCM [80,81,82,83]. PLE in some cases could not be used to extract thermolabile compounds due to the high extraction temperature, while others believed it could be used for the extraction of thermolabile compounds because of the shorter extraction time used in PLE [84].

### 3.3.11 Pulsed electric field (PEF) extraction

Pulsed electric field extraction significantly increases the extraction yield and decreases the extraction time because it can increase mass transfer during extraction by destroying membrane structures. The effectiveness of PEF treatment depends on several parameters including field strength, specific energy input, pulse number and treatment temperature. PEF extraction is a non-thermal method and minimizes the degradation of the thermolabile compounds. The highest yield of ginsenosides (12.69 mg/g) is obtained by PEF following conditions of 20 kV/cm electric field intensity, 6000 Hz frequency, 70% ethanol–water solution, and 150 l/h velocity. The yield of the ginsenosides of the PEF extraction method is higher than those of MAE, heat reflux extraction, UAE and PLE [77].

### 3.3.12 Enzyme assisted extraction (EAE)

The structure of the cell membrane and cell wall, micelles formed by macromolecules such as polysaccharides and protein, the coagulation and denaturation of proteins at high temperatures during extraction are the main barriers to the extraction of natural products. Extraction efficiency is enhanced by EAE due to the hydrolytic action of the enzymes on the components of the cell wall and membrane and the macromolecules inside the cell which facilitate the release of the natural product. Cellulose,  $\alpha$ -amylase and pectinase are generally employed in EAE. The polysaccharide yield under the optimized EAE condition using glucose oxidase increased more than 250% compared with that from non-enzyme treated methods [85,78].

**Table 3. A summary of various extraction methods for natural products [77,78]**

<b>Method</b>	<b>Solvent</b>	<b>Temperature</b>	<b>Pressure</b>	<b>Time</b>	<b>Volume of organic solvent consumed</b>	<b>Polarity of natural products extracted</b>
Maceration	Water, aqueous and non-aqueous solvents	Room temperature	Atmospheric	Long	Large	Dependent on extracting solvent
Percolation	Water, aqueous and non-aqueous solvents	Room temperature, occasionally underheat	Atmospheric	Long	Large	Dependent on extracting solvent
Decoction	Water	Under heat	Atmospheric	Moderate	None	Polar compounds
Reflux extraction	Aqueous and nonaqueous solvents	Under heat	Atmospheric	Moderate	Moderate	Dependent on extracting solvent
Soxhlet extraction	Organic solvents	Under heat	Atmospheric	Long	Moderate	Dependent on extracting solvent
Pressurized liquid extraction	Water, aqueous and non-aqueous solvents	Under heat	High	Short	Small	Dependent on extracting solvent
Supercritical fluid extraction	Supercritical fluid (usually S-CO <sub>2</sub> ), sometimes with modifier	Near room temperature	High	Short	None or small	Nonpolar to moderate polar compounds
Ultrasound assisted extraction	Water, aqueous and non-aqueous solvents	Room temperature, or under heat	Atmospheric	Short	Moderate	Dependent on extracting solvent
Microwave assisted extraction	Water, aqueous and non-aqueous solvents	Room temperature	Atmospheric	Short	None or moderate	Dependent on extracting solvent
Pulsed electric field extraction	Water, aqueous and non-aqueous solvents	Room temperature, or under heat	Atmospheric	Short	Moderate	Dependent on extracting solvent
Enzyme assisted extraction	Water, aqueous and non-aqueous solvents	Room temperature, or heated after enzyme treatment	Atmospheric	Moderate	Moderate	Dependent on extracting solvent
Hydro distillation and steam distillation	Water	Under heat	Atmospheric	Long	None	Essential oil (usually non-polar)

**Table 4. A summary of phytochemical screening of secondary metabolites**

Secondary Metabolites	Name of test	Methodology	Result(s)	Reference(s)
	Mayer's test	Separately dissolve 0.335 g mercuric chloride in 60 ml of H <sub>2</sub> O and 5 g KI dissolved in 20 ml H <sub>2</sub> O. Mix the 2 solutions and make up the volume to 1000 ml with distilled H <sub>2</sub> O Identification: Dissolved 1 ml of extracts (0.5 g) individually in about 1 ml of 1% HCl, filter and treat with potassium mercuric iodide (Mayers reagent) Or A 1 ml of extract is mixed with (0.5 g) with about 1 ml of 1% HCl, then warm and filter followed by treatment with 2 ml of the filtrate with Mayer's reagent. Observation will indicate the presence of alkaloids	Yellow coloured Precipitate Or turbidity or precipitation, green colour	[86,87]
1)Alkaloid	Dragendorff's test	-Treat the filtrate with solution of potassium bismuth iodide (Dragendorff's reagent) that will give a red precipitate -Spot a drop of extract on a small piece of precoated TLC plate. Spray the plate with Dragendorff's reagent.	Orange spot	[27]
	Hager's test	Treat the filtrates with a solution of saturated picric acid (hager's reagent)	Yellow coloured precipitate	[86]
	Wagner test	2 ml filtrate is added with 1% HCl + steam. add 1ml of the solution with 6 drops of Wagner's reagent.	Brownish-red precipitate	[51]
	TLC method 1	Solvent system: Chloroform: methanol: 25% ammonia (8:2:0:5). Spots can be detected after spraying with Dragendorff's reagent	Orange spot	[88,89]
	TLC method 2	Wet the powdered test sample with a half diluted NH <sub>4</sub> OH and lixivated with EtOAc for 24hrs at room temperature. Separate the organic phase from the acidified filtrate and basify with NH <sub>4</sub> OH (pH 11-12). Then extract it with chloroform (3X), condense by evaporation and use for chromatography. Separate the alkaloid spots using the solvent mixture chloroform and methanol (15:1). Spray the spots with Dragendorff's reagent.	Orange spot	[65,90]
2)Anthraquinone	Borntrager's test	Heat about 50mg of extract with 1ml 10% ferric chloride solution and 1ml of concentrated hydrochloric acid. Cool the extract and filter. Shake the filtrate with equal amount od diethyl ether. Further extract the extract with strong ammonia.	Pink or dee red coloration of aqueous layer	[27]
	Borntrager's test	Add 1 ml of dilute (10%) ammonia to 2 ml of chloroform extract.	A pink-red colour in the ammoniacal (lower) layer	[55,91]
3)Cardiac glycosides	Kellar-Kiliani test	Add 2 ml filtrate with 1ml of glacial acetic acid, 1 ml ferric chloride and 1ml concentrated sulphuric acid.	Green-blue coloration of solution	[69,70]

Secondary Metabolites	Name of test	Methodology	Result(s)	Reference(s)
	Kellar-Kiliani test	Dissolve 50 mg of methanolic extract in 2ml of chloroform. Add H <sub>2</sub> SO <sub>4</sub> to form a layer	Brown ring at interphase	[68]
	TLC method	Extract the powdered sample with 70% EtOH on rotary shaker (180 thaws/min). Further centrifuge the supernatant by adding 6.3% Na <sub>2</sub> CO <sub>3</sub> AT 5000RPM/10MIN. Dry the retained supernatant and re-dissolved in chloroform and use for chromatography. Separate the glycosides using EtOAc-MeOH-H <sub>2</sub> O (80:10:10) solvent mixture	The color and hR <sub>f</sub> values of these spots can be recorded under ultraviolet (UV254nm) light	[65]
4)Flavonoid	Shinoda test	To 2-3 ml of methanolic extract, add a piece of magnesium ribbon and 1ml of concentrated hydrochloric acid.	Pink red or red coloration of solution	[65]
	TLC method	Extract 1g powdered test samples with 10ml methanol on water bath (60oc/5min) Condense the filtrate by evaporation and add a mixture of water and EtOAc (10:1ml), and mix thoroughly. Retain the EtOAc phase and use for chromatography. Separate flavonoid spot using chloroform and methanol (19:1) solvent mixture.	The color and values of these spots can be recorded under ultraviolet (UV254nm)light	[65,91]
	Alkaline reagent test	Treat the extract with dilute NaOH, followed by addition of dilute HCl.	A yellow solution with NaOH, turns colourless with dilute HCl	[88,91]
	Lead acetate test	Treat extract with few drops of lead acetate solution	Yellow colour precipitate	[86]
5)Phenol	Phenol Test	Spot the extract on the filter paper. Add a drop of phoshomolybdic acid reagent and expose to ammonia vapours.	Blue coloration of spot	[26]
	Ferric Chloride test	Treat extracts with 3-4 drops of ferric chloride solution.	Bluish colour formation	[86]
	Folin Ciocalteau's test	To 1 ml of the extract, add 2 ml of distilled water followed by 0.5 ml of sodium carbonate and 0.5 ml Folin Ciocalteau's reagent.	Blue/green colour	[87]
6)Phlobatannin	-	2ml extract was boiled with 2 ml of 1% hydrochloric acid HCl	Formation of red precipitates	[55]
7)Pyrrolizidine alkaloid	-	Prepare 1ml of oxidizing agent, consisting of 0.01ml hydrogen peroxide (30% w/v) stabilized with tetrasodium pyrophosphate (20mg/ml) and made up to 20ml with isoamyl acetate, and add to 1ml of plant extract. Vortex the sample and add 0.25ml acetic anhydride before heating the sample at 60oc for 50-70s. Cool the samples to room temperature. Add 1ml of Ehrlich reagent and place the test tubes in water bath (60oc) for 5 min. Measure the absorbance at 562nm. The method of	Peak were compared with the GC-MS library	[61,92,88]

Secondary Metabolites	Name of test	Methodology	Result(s)	Reference(s)
		Holstege et al. (1995) should be used to confirm results of the screening method		
8)Reducing sugar	Fehling test	25ml of diluted sulphuric acid (H <sub>2</sub> SO <sub>4</sub> ) is added to 5ml of water extract in a test tube and boil for 15mins. Then cool it and neutralize with 10% sodium hydroxide at pH 7 and 5 ml of Fehling solution.	Brick red precipitate	[49]
9)Saponin	Froth test	Extracts is diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 mins. Add 0.5 ml of filtrate with 5 ml of diluted water and shake well.	Formation of a 1 cm layer foam	[70,91]
	Foam test	Shake 0.5 g extract with 2 ml of water and observe.	Production of foam	[86]
	TLC method	Extract two gram of powdered test sample with 10 ml 70% EtOH by refluxing for 10 min. Condense the filtrate, enrich with saturated n-BuOH, and mix thoroughly. Retain the butanol, condense and use for chromatography. Separate the saponins using chloroform, glacial acetic acid, methanol and water (64:34:12:8) solvent mixture. Expose the chromatogram to the iodine vapours.	The colour (yellow) and hRf values of these spots were recorded by exposing chromatogram to the iodine vapours	[65]
10)Steroid	Liebermann-Burchardt test	To 1 ml of methanolic extract, add 1 ml of chloroform, 2-3ml of acetic anhydride, 1 or 2 drops of concentrated sulphuric acid H <sub>2</sub> SO <sub>4</sub>	Dark green colouration	[27]
	-	To 1ml of extract, add 2 ml acetic anhydride and 2 ml concentrated sulphuric acid	Colour change to blue or green	[55]
	TLC method	2 g of powdered extract test samples is added with 10 ml methanol in water bath (80C/15 min) The condensed filtrate is used for chromatography. The sterols can be separated using chloroform, glacial acetic acid, methanol and water (64:34:12:8) solvent mixture. The colour and hRf values of these spots can be recorded under visible light after spraying the plates with an aldehyde sulphuric acid reagent and heating at(100°C/6min)	The colour (Greenish black to Pinkish black) and hRf values of these spots can be recorded under visible light	[65]
11)Tannin	Braemer's test	10% alcoholic ferric chloride will be added to 2-3ml of methanolic extract on (1:1)	Dark blue or greenish grey coloration of the solution	[27,69]
	Ferric chloride test	About 0.5 g of the extract is boiled in 20 ml of distilled water in a test tube, followed by filtration and addition of 1 ml of the filtrate with 1 ml of 0.5% FeCl <sub>3</sub> .	Brownish green colouration	[87]
	Gelatin test	Add 1% gelatin solution containing sodium chloride to the extract.	Formation of white precipitate	[86,93]
12) Terpenoid-Phytosterols	Liebermann-Burchardt test	To 1ml of methanolic extract, is added 1ml of chloroform, 2-3ml of acetic anhydride, then 1 to 2 drops of concentrated sulphuric acid.	Pink or red colouration	[26]
	Salkowski test	5ml extract is added with 2ml of chloroform and 3ml of concentrated sulphuric acid	Reddish brown colour of interface	[56]
13) Volatile oil	-	Add 2ml extract with 0.1ml dilute NaOH and small quantity of diluted HCL shake	Formation of white	[53]

Secondary Metabolites	Name of test	Methodology	Result(s)	Reference(s)
		the solution.	precipitates	
14) Carbohydrates	Molisch's test	Treat filtrates with 2 drops of alcoholic alpha-naphthol solution in a test tube and the production of a violet ring at the junction will indicate the presence of carbohydrates	Violet ring junction	[86]
	Benedict's test	The filtrates is treated with a solution of a mixture of copper sulphate pentahydrate, sodium carbamate, sodium citrate and distilled water (Benedict's reagent) and heat gently.	Orange red precipitate Indicate presence of reducing sugar	[94]
	Fehling's test	Hydrolyze the filtrates with diluted HCl and neutralize with alkali and heat with aqueous copper sulphate and alkaline sodium potassium tartrate (Rochelle salt) Fehling's A and B) respectively.	A red precipitate indicate presence of reducing sugar.	[94]
Protein and Amino acid	Xanthoproteic test	Heat extracts with few drops of concentrated nitric acid	Yellow colour	[93]
	Biuret test	Add 2 ml of mixture of 2 ml of 10% NaOH and 2-3 drops of Cu SO <sub>4</sub> (Biuret Reagent) to crude extract and heat	Purple/blue colour	[93]
	Millon's test	5 ml of distilled water is added into the extract and allowed standing for 3 hrs and then filter. To 2 ml portion of filtrate is added 0.1 ml Million's reagents. Shake and keep for observation	Yellow precipitate	[95]
Anthocyanin and Betacyanin		Add 1 ml of 2N NaOH to 2 ml of the extract and heat for 5 min at 100°C .	Bluish/green colour indicates anthocyanin and yellow colour indicates betacyanin	[87]
Coumarins		1 ml of extract (0.5 g) is covered in test tubes with filter paper. 1 ml of the plant extract is taken in small test tube and covered with filter paper and moistened with 1N NaOH then place the tubes for few minutes in boiling water. The filter paper is removed and examine in UV light.	Yellow fluorescence	[87]
Quinones		Add 1 ml of conc H <sub>2</sub> SO <sub>4</sub> to 1 ml of the extract.	Visualise a Red colour	[87]
Steroids	Sulphuric acid test	Add 2 ml of chloroform to the extract followed by 1 ml of H <sub>2</sub> SO <sub>4</sub> by the side of the mixture.	Reddish brown ring at lower chloroform layer of the interface.	[87]
	Acetic anhydride test	A minimum quantity of chloroform is added and 3-4 drops of acetic anhydride and one drop of conc H <sub>2</sub> SO <sub>4</sub> to 2 ml of the extract	The formation of a purple colour which changes into green	[94]
Lignins		Mix phloroglucinol and HCl with the extract.	Pink colour	[94]

### 3.3.13 Hydro distillation (HD) and steam distillation (SD)

The HD and SD are widely used methods for the extraction of volatile oil. Some natural compounds are prone to decomposition in HD and SD. The chemical composition and antibacterial activity of the primary essential oil and secondary essential oil from *Mentha citrata* have been shown to be significantly affected by the distillation methods. Both primary and secondary essential oil yields by HD are higher than those by SD [77,96]. For phytochemical screening of secondary metabolites, it is important to note that the screening is important as bioactive metabolites give an indication of the type of biological activities to be studied. A summary of phytochemical screening of secondary metabolites is illustrated in Table 4.

## 4. IDENTIFICATION AND CHARACTERIZATION OF HERBAL PLANT PRODUCTS

Plant extracts generally occur as a combination of various types of bioactive compounds with different polarities, and thus their separation remains a big challenge for the process of identification and characterization of phytochemicals. Generally, the isolation of these bioactive compounds applies several separation techniques such as TLC, column chromatography, flash chromatography, Sephadex chromatography and HPLC, that can be used to obtain pure compounds [76,90]. The pure compounds are then used for the determination of structure and biological activity. Apart from that, non-chromatographic techniques such as immunoassay, which use monoclonal antibodies (MAbs), phytochemical screening assay, Fourier-transform infrared spectroscopy (FTIR), can also be used to obtain and facilitate the identification of the bioactive compounds [10].

### 4.1 Thin-layer Chromatography (TLC) and Bio-autographic Methods

TLC is a simple, quick, and low-cost procedure that gives a quick answer on how many components there are in a mixture. TLC is also used to identify compounds in a mixture by comparing the resolution front ( $R_f$ ) of the compound in the sample with the  $R_f$  of a known compound [89,34]. Additional tests involve the spraying of phytochemical screening reagents, which cause colour changes according to the bioactive metabolites existing in a plants extract;

or by viewing the plate under the UV light. This has also been used for confirmation of the purity and identity of isolated compounds [90].

Bio-autography is a technique used for determining bioactive compounds with antimicrobial activity in plant extracts. TLC and bioautographic methods combine chromatographic separation and *in situ* activity determination to facilitate the localization and target-directed isolation of phytochemicals in a mixture. Traditionally, the bioautographic technique uses the growth inhibition of microorganisms to detect anti-microbial components of extracts chromatographed on a TLC layer [61,73]. 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 metabolites are transferred from the TLC plate to an inoculated agar plate by direct contact and the;
- (iii) agar overlay bio-autography, where a seeded agar medium is applied directly onto the TLC plate [62,71]

The inhibition zones produced on TLC plates by one of the above bioautographic technique can be used to visualize the position of the bioactive compound with antimicrobial activity in the TLC fingerprint with reference to  $R_f$  values [62].

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 substances were eluted from the silica with ethanol or methanol [62,90]. Eluted samples were further purified using the above preparative chromatographic method. Finally, the components are identified by HPLC, LCMS and GCMS. Although it has high sensitivity, its applicability is limited to micro-organisms that grow easily on TLC plates. Other problems include the need for complete removal of residual low volatile solvents, such as *n*-BuOH, trifluoroacetic acid and ammonia and the transfer of the phytochemicals from the stationary phase into the agar layer by diffusion [73]. Since bio-autography allows localizing antimicrobial activities of an extract on the chromatogram, it enhances a quick search for new antimicrobial

agents through bioassay-guided isolation [73]. The bioautography agar overlay method is advantageous in that, it uses a small amount of sample when compared to the normal disc diffusion method and thus, it can be applicable for bioassay-guided isolation of compounds. This technique also simplifies the process of identification and isolation of the bioactive compounds [44,71].

## 4.2 The High-performance Liquid Chromatography (HPLC)

HPLC is a reliable, robust, and widely used technique for the isolation of natural products [44,90]. Currently, this technique is gaining popularity among various analytical techniques as the main choice for fingerprinting study for the quality control of herbal plants [44,57]. Natural products are frequently isolated following the evaluation of a relatively crude extract in a biological assay to fully characterize the active entity. The biologically active compound is often present only as a minor component in the extract and the resolving power of HPLC is ideally suited to the rapid processing of such multicomponent samples on both an analytical and preparative scale [44]. Many bench top HPLC instruments now are modular in design and comprise of a solvent delivery pump, a sample introduction device such as an auto-sampler or manual injection valve, an analytical column, a guard column, detector and a recorder or a printer.

Chemical separation is accomplished using HPLC by utilizing the fact that certain compounds have different migration rates given a particular column and mobile phase. The extent or degree of separation is determined by the stationary and mobile phase. Generally, the identification and separation of phytochemicals can be accomplished using an isocratic system (using a single unchanging mobile phase system) [44,73]. Gradient elution in which the proportion of organic solvent to water is altered with time may be necessary if more than one sample component is being studied and differ from each other significantly in retention under the conditions employed.

Purification of the compound of interest using HPLC is the process of separating or extracting the target compound from other (possibly structurally related) compounds or contaminants. Each compound should have a characteristic peak under certain chromatographic conditions depending on what needs to be separated and

how closely related the samples are, the chromatographer may choose the conditions such as:- proper mobile phase, flow rate, suitable detectors and columns to obtain an optimum separation [44].

The identification of compounds by HPLC is an important part of any HPLC assay. To identify any compound by HPLC, a detector must first be selected. Once the detector is selected and is set to optimal detection settings, a separation assay must be developed. The parameters should be such that a clean peak of the known sample is observed from the chromatograph [44,86]. The identifying peak should have a reasonable retention time and should be well separated from extraneous peaks at the detection levels which the assay will be performed. UV detectors are popular among all the detectors because they offer high sensitivity [64] and because the majority of naturally occurring compounds encountered have some UV absorbance at low wavelengths (190-210 nm) [90]. The high sensitivity of UV detection is useful if a compound of interest is only present in small amounts within the sample. Besides UV, other detection methods are being employed to detect phytochemicals among which is the diode array detector (DAD) coupled with mass spectrometer (MS) [75,92].

The Liquid chromatography (LC) coupled with mass spectrometry (MS) (LC/MS), is also a reliable and powerful technique used analyzing complex herbal extracts [50,60]. It provides very useful information for structural elucidation of the compounds when tandem mass spectrometry (MS<sup>n</sup>) is applied. Therefore, the combination of HPLC and MS enhances rapid and accurate identification of phytochemicals in medicinal herbs, especially when a pure standard is not available [76]

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 make a significant contribution on the overall success of natural product isolation. The source material such as dried powdered plant, may initially need to be treated in order to ensure that the compound of interest is efficiently liberated into solution. In the case of dried plant material, an organic solvent like methanol or chloroform, may be used as the initial extractant following the duration of maceration, and the solid material is then removed by decanting of the extract by filtration

[76]. The filtrate is then concentrated and injected into HPLC for separation. The use of guard columns is important in the analysis of crude extract. Many natural product materials possess high levels of strongly binding components, such as chlorophyll and other endogenous materials that may in the long term compromise the performance of analytical columns. The use of guard columns will significantly protect the lifespan of the analytical columns [90].

### 4.3 Non-chromatographic Techniques

#### 4.3.1 Immunoassay

Immunoassays, which use monoclonal antibodies against drugs and low molecular weight natural bioactive compounds, are becoming important tools in bioactive compound analyses. They show high specificity and sensitivity for receptor binding analyses, enzyme assays and qualitative as well as quantitative analytical techniques. Enzyme-linked immunosorbent assay (ELISA) based on MAbs is, in many cases, more sensitive than conventional HPLC methods. Monoclonal antibodies are produced in specialized cells through a technique known as hybridoma technology [13,74,97]. The following steps are involved in the production of monoclonal antibodies via hybridoma technology against plant drugs:

- i. A rabbit is immunized through repeated injection of specific plant drugs to produce a specific antibody, facilitated due to the proliferation of the desired B cells.
- ii. Tumours are produced in a mouse or a rabbit.
- iii. From the above two types of animals, spleen cell (these cells are rich in B cells and T cells) are cultured separately. The separately cultured spleen cells produce specific antibodies against the plant drug, and against myeloma cells that produce tumours.
- iv. The production of hybridoma by fusion of spleen cells to myeloma cells is induced using polyethylene glycol (PEG). The hybrid cells are grown in selective hypoxanthine aminopterin thymidine (HAT) medium.
- v. The desired hybridoma is selected for cloning and antibody production against a plant drug. This process is facilitated by preparing single cell colonies that will grow and can be used for screening of antibody

producing hybridomas.

- vi. The selected hybridoma cells are cultured for the production of monoclonal antibodies in large quantities against the specific plants' drugs.
- vii. The monoclonal antibodies are used to determine similar drugs in the plant extract mixture through enzyme-linked immunosorbent assay (ELISA).

#### 4.3.2 The fourier-transform infrared spectroscopy (FTIR)

FTIR has been shown to be a very useful method for the characterization and identification of compounds or functional groups (chemical bonds) present in an unknown mixture of plants extract [11,55,98]. Also, 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 with a library of known compounds. Samples for FTIR are prepared in several ways. For liquid samples, the easiest is to place one drop of the sample between two sodium chloride plates and the drop forms a thin film between the plates. Solid samples can be milled with potassium bromide (KBr) and 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 [55].

### 5. CONCLUSION

Most stages of the extraction process are very useful and contribute to the study of natural product research. They sample pre-extraction preparation, such as grinding and drying, affects the efficiency and processing of phytochemical of the final extractions; that may eventually affect the final extracts. No single extraction methods are the ideal method, and each extraction procedure is unique to the specified plant or plant parts used. Since bioactive compounds occurring in plant material consist of multi-component mixtures, their separation and determination still creates problems. Practically, most of them have to be purified by a combination of several chromatographic techniques and various other purification methods to isolate bioactive compound(s) of potential pharmaceutical importance in phytomedicine development process.

## DISCLAIMER

The products used for this research are commonly and predominantly used products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

## COMPETING INTERESTS

Authors have declared that no competing interests exist

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