Extraction, Purification, Analysis, and Identification Techniques of Bioactive Phytochemicals

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Abosede O. Fawole, Gbemisola O. Onipede, Olalekan J. Odukoya, and John O. Onuh

4.1 BIOACTIVE PHYTOCHEMICALS EXTRACTION

Extraction is treating plant material with selective solvents to dissolve the bioactive phytochemicals in plant tissues using standard procedures (1). Different kinds of solvents are used to extract bioactive compounds from plants. Among these solvents are ethanol, methanol, petroleum ether, acetone, chloroform, hexane, N,N-dimethylformamide (DMF), water, and ethyl acetate (2; 3). It is expedient to use multiple solvents of differing polarities during the extraction of phytochemicals to achieve a high degree of accuracy. This way, scientists could establish which solvent extracted the highest amount of specific bioactive compounds (4; 5). Also, the form of the plant part to be extracted and the polarity

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of the solvent chosen is critical in the extraction process. For example, a dried form of plant part is better to prevent water interference. At the same time, the solvent must not have similar polarity to the solute of interest to avoid the dissolution of the solute (6). Successful extraction begins with a thorough review of the appropriate literature for suitable protocols that suit a particular plant species or class of compounds. The review is necessary because a high extract yield does not necessarily translate to a high yield of bioactive components in the extract. Thus, plant samples must be carefully selected and prepared, especially those containing free fatty acids and tocopherols that are very sensitive to oxygen and heat (7).

4.1.1 Methods of Extraction

The methods of extraction of bioactive phytochemicals can either be the traditional extraction techniques or exceptional new technologies. The factors that differentiate the extraction methods are the extraction duration, sample particle size, temperature, solvents' pH, and expected compound volatility, among others. The traditional methods include maceration, Soxhlet extraction (SE), decoction, distillation, digestion, tincture, infusion, percolation, conventional reflux extraction (CRE), serial exhaustive extraction (SEE), and aqueous-alcoholic extraction by fermentation (AAE) (8; 9). These conventional methods are easy to operate but take longer to complete. The process is conducted under low or relatively high ambient temperature, with solvents of different polarities. To avoid the destruction of thermosensitive compounds, pure but costly solvents that evaporate quickly are utilized.

The downside of these methods demanded the development of innovative techniques. The exceptional new technologies developed include microwave-assisted extraction (MAE), pulsed-electric field extraction (PEF), ultrasound-assisted extraction (UAE), enzyme-assisted extraction (EAE), pressurized liquid extraction (PLE), high hydrostatic pressure-assisted extraction (HHP), supercritical fluid extraction (SFE), turbo-distillation extraction (TDE), subcritical water extraction (SWE), countercurrent extraction (CCE), high-voltage electric discharge (HVED), and solid-phase extraction (SPE) (8; 7). Many novel sampling strategies involve combining different techniques to extract phytochemicals from plant samples as much as possible. Combining two or more conventional methods, conventional and new technology, or two or more new technologies could provide more representative information of bioactive components in plant material than any single method. It could also provide an effective concept for resolving the issue of information loss during phytochemical sampling tasks.

4.1.2 The Traditional Extraction Techniques

4.1.2.1 Maceration, Digestion, and Infusion

These three methods are closely related. *Maceration* is a cold extraction method conducted at ambient temperature. The procedure works by softening

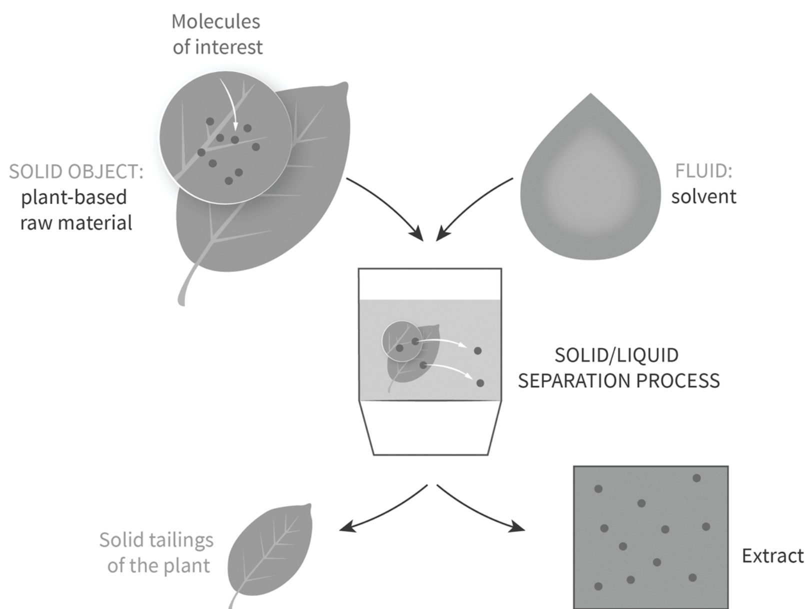

FIGURE 4.1 Plant extraction by maceration (berkem.com).

and breaking the plant's cell wall to release the soluble phytochemicals. Maceration uses the principle of molecular diffusion, ensures the dispersal of the concentrated solution around the sample surface, and adds a new solvent to the menstruum to increase the extraction yield (10). The dried plant part may be ground into powder to increase its surface area before soaking a weighed amount in the appropriate solvent (1). The process is achieved in a closed stoppered vessel where the mixture is left for about 7 days with stirring after each 24 h. Then, the extract is clarified by filtration or using a sealed extractor to prevent solvent evaporation (11). It is a straightforward procedure and is thus used widely (Figure 4.1). However, it is time-consuming and can take weeks for some plant materials to extract. It is thereby recommended for heat-labile phytochemicals.

The digestion method is just a modification made to the maceration technique by applying moderate heat of about 35°C during extraction to increase the efficiency of the solvent(s) used (12). Infusion is maceration done in a short time using either cold or hot water since the bioactive compounds in the plant sample are volatile and extract readily. Then, the plant material is filtered to remove the plant material from the extract. It is the method adopted for preparing tea from plants (13).

4.1.2.2 Soxhlet Extraction

The Soxhlet extractor was invented for lipid extraction from milk solids in 1879 by Franz Ritter von Soxhlet. It is now used for many products whenever total

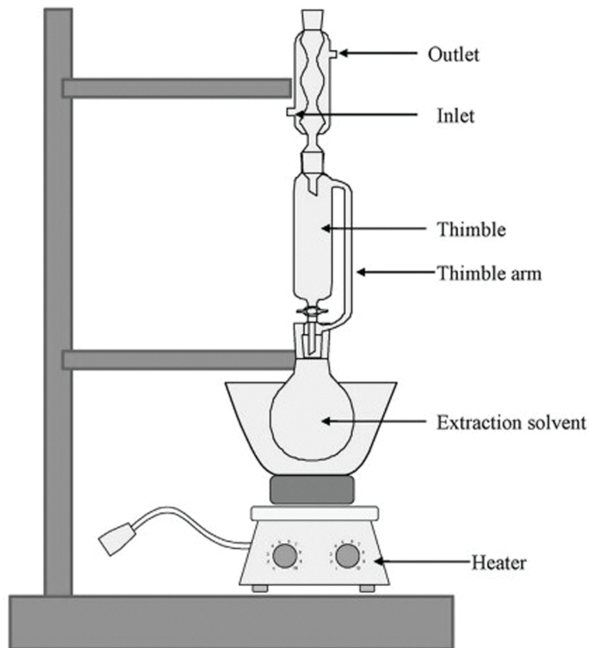

FIGURE 4.2 Experimental Soxhlet extraction apparatus (10).

extractions are needed, including the extraction of phytochemicals (8). The principle here is of a 'greedy' cup (thimble chamber) that empties the liquid inside to the bottom flask once filled past a certain point (siphon arm). The process starts when presoaked ground plant material is placed in a porous bag (cellulose or filter paper) and set in the thimble chamber of the Soxhlet apparatus (Figure 4.2). Then, the vapor of boiling solvent in the bottom flask rises, condenses in the condenser, and drips into the porous cup (thimble), dissolving and extracting the metabolites from the plant sample. A siphoning motion occurs when the smaller side arm of the thimble chamber fills to overflowing, causing the solvent to empty into the bottom flask.

The cycle runs again and continues for a predetermined period (12). This technique is simple to operate and cost-effective and requires a smaller quantity of solvent than maceration as a batch of solvent is recycled. However, the operational risk is exposure to flammable or hazardous organic solvents (14).

4.1.2.3 Decoction

Unlike the maceration technique, decoction requires boiling the plant material in a known volume of water or selected solvent for a specified time to extract the bioactive compounds. Then, it is cooled and filtered. The process combines convection and conduction heat transfer to achieve extraction.

The technique is recommended for more complex plant parts like seeds, roots, and barks (15).

4.1.2.4 Distillation

This extraction procedure is one of the oldest techniques still in use. It mainly extracts many plants' volatile bioactive compounds with essential oils (EOs). It operates the principle that plant pores open when subjected to heat, releasing compounds of interest. It also leverages the principle of separating immiscible liquids at the end. There are two processes: hydrodistillation and steam distillation. The plant materials are boiled in enough water or appropriate solvent at a raised temperature in hydro distillation (16).

In contrast, direct steam is passed through the plant in steam distillation (17). The vapor from either of the procedures is a mixture of oil, bioactive chemicals, and water and condenses by indirect cooling procedure (9). The separator from the condenser automatically separates water from the oil that contains the bioactive compounds (Figure 4.3). This method is easy to set up and useful for heat-sensitive bioactive compounds. Furthermore, it can be done before the dehydration of the plant material. Also, since no organic solvents are required, the procedure is safe 16).

4.1.2.5 Tincture

The technique involves the use of alcohol to extract phytochemicals from plant samples. A fresh plant sample is soaked in ethanol at a ratio of 1:5 w/v. The extract is shelf stable because of the solvent used (7).

4.1.2.6 Percolation

The technique uses a cone-shaped percolator apparatus that is opened at both ends with a drain valve at the outlet. The plant material is placed in the percolator when the valve is closed, covered with enough solvent(s) (menstruum), and left to soak for 24 h.

The valve is opened to drain a few drops of the extract per minute into a container (Figure 4.4). It (the valve) is then closed so that a fresh menstruum is added to repeat the cycle three times or more. Thus, a full extract is recovered from the plant material. The percolation method is very efficient for the extraction of thermolabile compounds, but it is not time efficient and uses a lot of solvents (17).

4.1.2.7 Conventional Reflux Extraction

A conventional reflux extraction system can be adopted for dried and finely ground plant parts using a small- or large-scale reactor. The ratio of plant material to solvent can be 1:5, 1:7, or 1:10 w/v depending on the nature of the experiment at different temperatures between 60°C and 80°C (19). The solvent is usually

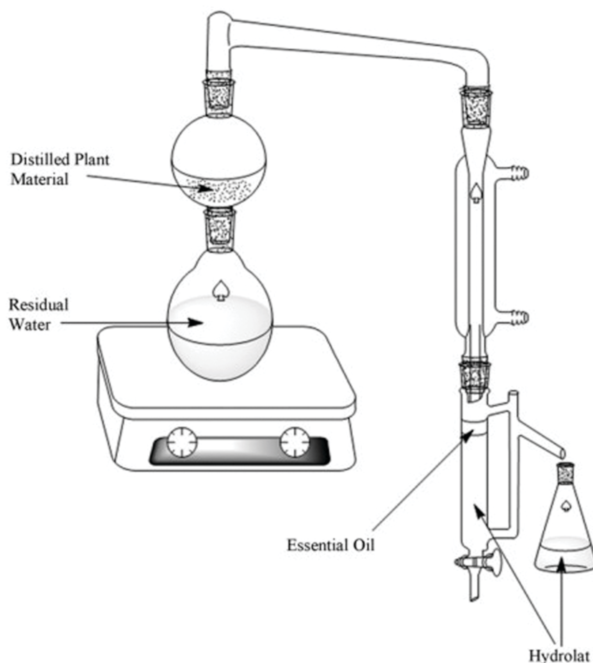

FIGURE 4.3 Schematic diagram of a distillation apparatus (18).

aqueous ethanol, and less quantity is needed than in some traditional methods. The extract can be clarified by filtration. This technique is time efficient as extraction can be achieved within 1 to 3 h (8).

4.1.2.8 Serial Exhaustive Extraction

SEE is a fractionation technique that extracts nonpolar biochemicals with solvents of increasing polarity without introducing chemical changes. Three extraction series could be adopted, starting with a nonpolar solvent, then an intermediate polarity solvent, and ending with a polar solvent. This technique extracts essential biological compounds such as major antioxidants in plants such as *C. woodii*. It is appropriate for thermally labile compounds (20).

4.1.2.9 Aqueous-alcoholic Extraction by Fermentation

This technique uses fermentation to extract active phytochemicals from plants, such as the Ayurveda plants used in alternative medicine. The technique is performed traditionally by submerging the crude plant in water that was initially boiled and cooled in an earthen vessel. The fermentation process takes a length of time, and alcohol is produced. During fermentation, bioactive components of the plant are extracted, and the alcohol produced serves as a preservative. Huge

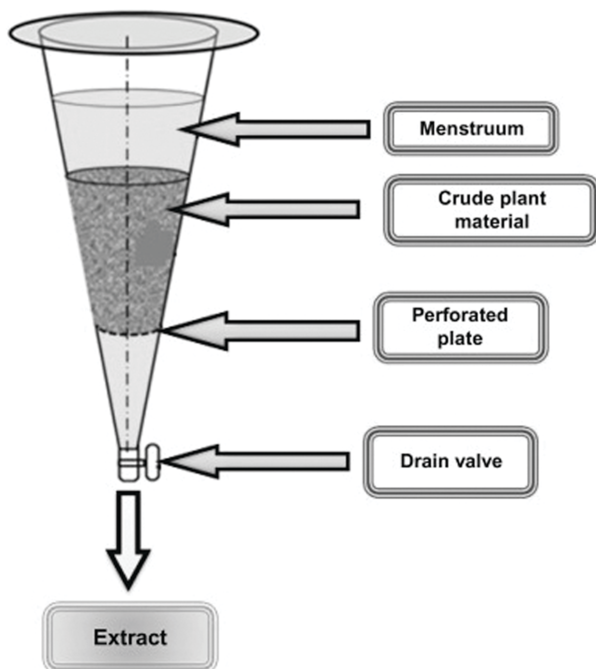

FIGURE 4.4 Schematic diagram of a Percolator. (Adapted from community.freetrade.io.)

wooden vats, large metal vessels, or porcelain jars are used for large-scale production (21).

4.1.3 Exceptional New Technologies

4.1.3.1 Microwave-Assisted Extraction

MAE is an excellent technique to reduce the loss of bioactive constituents of plant materials without necessarily increasing the amount of solvent and extraction period. It uses microwave energy based on a direct impact on the molecules of polar materials through ionic conduction and dipole rotation (10). The principle of heating targets the moisture of fresh or the traces of moisture in dried plant materials. There is evaporation when the moisture heats up, causing the cell wall to be pressured and ruptured. Hence, the exudation of bioactive constituents from the ruptured cells gives superior recovery results (1). It is mainly used to extract phytochemicals, especially the organic and organometallic compounds of various plant sources such as myrtle leaves, tomatoes, grapes, and berries (22). Nguyen et al. (23) successfully performed MAE on *Phyllanthus amarus* at 600 W and 10 s per one-minute irradiation time for 10 min with a total irradiation time of 100 s. MAE improves crude extract purity and marker compound stability.

It also reduces processing costs by reducing the energy and solvent needed for extraction (9).

4.1.3.2 Pulsed Electric Field Extraction

This novel technique is based on cell membrane disruption to increase extraction. An electric current is passed through the membrane of the plant cell. On the dipole nature of membrane molecules, electric potential separates bioactive compounds according to their charges in the cell membrane (24). In the PEF treatment of plants, a simple circuit with exponential decay pulses with a treatment chamber and two electrodes is employed. Plant materials are placed inside the treatment chamber, designed to operate either in a continuous or batch mode (25). It considerably increases the optimum recovery of extracts in a short time. Factors such as pulse number, field strength, specific energy input, nature of the plant material being treated, and treatment temperature can influence the efficacy of PEF extraction (9). It is a very effective technique for extracting thermolabile phytochemicals since it minimizes degradation (26). This method requires low treatment temperature and no solvent; hence it is very economical and gives a good quality product.

4.1.3.3 Ultrasound-Assisted Extraction

It is one of the most accessible extraction techniques. It uses standard laboratory equipment such as ultrasonic bath, ultrasonic probe system, and shaking water bath. The extraction procedure involves smashing the plant material and mixing it with a suitable solvent. Then the mixture is placed into the ultrasonic bath while temperature and extraction time are controlled (6). Ultrasound in the frequency of 20 kHz and above would effectively leach out any plant matrix's organic and inorganic bioactive components. However, factors such as the plant moisture content, particle size, appropriate solvent, frequency, temperature, pressure, and time of sonication must be well figured out. UAE has several advantages over classical methods but could be integrated into conventional systems to improve efficiency (27). The technique is fast as several process steps could be skipped. It is both economical and eco-friendly.

4.1.3.4 Enzyme-Assisted Extraction

EAE is an extraction method that can access phytochemicals locked in the polysaccharide-lignin network by hydrophobic or hydrogen bonds. A solvent does not readily leach out such compounds in a regular extraction procedure. Hence EAE has been used by many scientists as a technique or a pretreatment to recover bonded phytochemicals by adding specific hydrolyzing enzymes. Therefore, its efficiency is based on the hydrolytic action of enzymes, first on the components of the cell wall and cell membrane, then on the macromolecules within the cell that enable the release of the compounds (10). The enzymes mainly used in EAE include cellulase, amylase, and pectinase.

There are two EAE methodologies: enzyme-assisted cold pressing (EACP) and enzyme-assisted aqueous extraction (EAAE). In the latter, the enzymatic action degrades the seed cell wall, thereby rupturing the polysaccharide-protein colloid that could trigger emulsion formation and cause a low yield. Nevertheless, the EACP technique is a nonaqueous system in which the enzymes only hydrolyze the seed cell wall and facilitate maximum product yield (28). As a result, EAE increases oil extractability and gives a higher yield of free fatty acids and phosphorus contents when compared to traditional oil extraction methods (29).

4.1.3.5 Supercritical Fluid Extraction

The physical characteristics of a gas, such as carbon dioxide or propane, change when it is compressed and heated and becomes a supercritical fluid. Supercritical fluid thus possesses gas-like properties (diffusion, viscosity, and surface tension) and liquid-like features (density and solvation power). An SFE system consists of a tank of mobile phase (supercritical fluid), a pump to pressurize the gas with a controller to maintain the high pressure inside the system, a cosolvent vessel and pump, an oven that contains the extraction vessel, and a trapping vessel. In addition, different types of meters could be attached to the system, such as a flow meter and a dry or wet gas meter (9).

The SFE procedure involves compressing a gas (usually CO_2, others include methane, ethylene, xenon, fluorocarbons, and nitrogen) into a dense liquid (supercritical fluid). Then the liquid is pumped through a cylinder containing the plant material to be extracted. The extract is separated from the gas when extract-laden liquid is pumped into a separation chamber. Since it is operated at room temperature, it is an ideal technique for thermolabile phytochemicals. It gives a complete extraction within a reduced time and uses a small amount of solvent. In addition, a supercritical fluid is recyclable, thus reducing waste generation. The extraction process and mechanism can be manipulated and adjusted for optimal yield by increasing the temperature and pressure (30). However, it is a very costly technique compared to conventional liquid extraction. Also, the commonly used solvent, nonpolar carbon dioxide, has limited dissolving power, specifically for polar solutes (31).

4.1.3.6 Pressurized Liquid Extraction

It is an automated technique introduced by Dionex Corporation in 1995 to extract solid samples with a liquid solvent(s), either aqueous or organic. The technique requires small solvents and can be concluded within a short time. This advantage is because high pressure and temperatures are combined to provide faster extraction (32). PLE is known by other names, such as pressurized solvent extraction (PSE), enhanced solvent extraction (ESE), high-pressure solvent extraction (HSPE), pressurized fluid extraction (PFE), and accelerated fluid extraction (ASE) (33). It is considered more effective than MAE, hot-solvent extraction, and UAE and a potential alternative method to SFE (34).

4.1.3.7 High Hydrostatic Pressure-Assisted Extraction

High hydrostatic pressure-assisted extraction (HHPE) is an emerging nonthermal and eco-friendly method used to obtain phytochemicals more efficiently from fruits, vegetables, and other plant tissues. Efficient extraction in terms of the highest yield could be achieved by mixing freeze-dried plant tissue with an appropriate solvent. For example, it could be green solvents like soybean or sunflower oil, with a solid:solvent ratio of 2:10 (g ml^{-1}) (35). The pre-HHPE treatment could be set between temperatures of 20°C and 40°C, pressure of 300 and 500 MPa, and for 2 to 8 min. First, pressurized extracts should be mixed in a vortex for about 1 min and placed in a food-grade bag, vacuumed-sealed and stored at 4°C or used immediately. Next is pressurization in a high hydrostatic pressure (HHP) unit with pressure and temperature not exceeding 900 MPa and 95°C, respectively. Finally, extract-enriched oil should be separated by centrifugation and stored at a temperature of about –20°C until further analysis.

The temperature must be monitored during HHPE and not be kept rigorously constant during pressurization due to adiabatic heat that hikes when pressure increases (36). Conversely, HHPE can operate at refrigerated or room temperatures, thereby preventing the denaturation of bioactive compounds, especially volatile oils. Thus, it enables the extraction of heat-sensitive compounds since it does not require heating, except for minor temperature rises from the compression phase.

This methodology is more effective and faster than conventional extractions and enzyme-assisted extraction. However, Cascaes Teles et al. (37) reported that the combination of both EAE and HHPE technologies gave the best results. They suggested that the combination of the methods is sustainable and able to recover high-value compounds from plant materials. Furthermore, HHPE increases the mass transfer rate, efficiently increases the permeability of the cells, enhances the diffusion of solvent(s), and improves the extraction of compounds. Therefore, HHPE could be a helpful technique to extract and modify compounds' bioaccessibility that help improve health-related conditions (38).

HHPE acts on noncovalent bonds only and not covalent bonds. Thus, it only causes alterations in large molecules of the plant tissues by altering their membranes and secondary, tertiary, and quaternary structures. The larger molecules include lipids and proteins. HHPE operation, therefore, leaves small molecules like bioactive compounds, flavor, pigmentation compounds, vitamins, and peptides intact. The drawback of this technology is that it is costly and so not affordable to some food industries (39).

4.1.3.8 Turbo-distillation Extraction

Turbo-distillation (THD) reactor was developed for the extraction of essential oils from the plant parts like seeds, bark, and wood that are hard. The reactor first grinds the agglomerates and most giant chunks of this hard part to allow

effective contact for water to seep into the solid phase. Then, a steady turbulent flow is kept inside the reactor to maximize the evaporation area. The technique differs from conventional hydrodistillation (HD), only possessing a mechanical stirrer that breaks down piles and chunks of hard plant matrices. The stirrer also homogenizes the medium simultaneously to prevent the powdered matrix from lying at the bottom of the reactor, where it can be burnt and essential oil degraded (40). It saves energy and reduces extraction time. However, it is not a suitable technology for extracting essential oils from soft matrices (41).

4.1.3.9 Subcritical Water Extraction

SWE is a powerful technology in which water is used under high temperatures between 100°C and 374°C and pressure from 10 to 60 bar. The latter is the critical water temperature, and the pressure range is below supercritical conditions but high enough to maintain the liquid state during the extraction process. This type of extraction mechanism follows the 'like dissolves like' rule. A distinctive feature of subcritical water is that its polarity can be considerably decreased by increasing temperature, making it behave similarly to methanol and ethanol (42). SWE should be carried out at the highest permitted temperature that is obtained experimentally for different plant materials. This is because essential oil components can be degraded if the extraction temperature is raised above a specific value. For instance, the best temperature condition for extracting essential oils was found to be between 125°C and 175°C (43).

SWE apparatus can be constructed of stainless steel in the laboratory, and extraction can be performed in a batch or continuous system. The main components of this apparatus include three tanks, an extraction vessel, two pumps (one for water and extract and the other for flushing the tubings), an oven that heats the extraction vessel, a heat exchanger that cools extract, a pressure restrictor, and sample collection system (44). *Subcritical water* is a green extraction fluid used for various nonpolar plant materials. SWE is faster, cleaner, and cheaper than traditional extraction methods. It replaces the use of organic solvents and gives high yields.

4.1.3.10 Countercurrent Extraction

In CCE, a fine slurry is produced from a pulverized wet plant material using a Bonotto extractor. The slurry is moved within the extractor in one direction to come in contact with an extracting solvent. The extract gets more concentrated as the starting plant material moves further. Finally, the extract comes out at the end of the extractor. A significant extract yield is achievable only if there is an optimization of the amount of solvent versus the plant material with their flow rates. This technique is fast, and there is no risk due to high temperature as it is usually performed at ambient temperature. Thus, it is suitable for thermolabile phytochemicals (21).

4.1.3.11 Solid-phase extraction

SPE is a simple liquid-solid phase extraction technology that involves the sorption of solutes from a liquid state sample onto a solid particle or adsorbent like beads or resins. The mechanism is like the one commonly found when molecules are retained on stationary chromatographic phases. The suspended solutes are separated based on their physical and chemical properties. Solid phases extraction media commonly used are reversed-phase material, silica gel, hydrophilic interaction liquid chromatography stationary phases in prepacked glass or plastic columns, normal phase, and ion exchange media or mixed-mode material (45). An SPE procedure involves the analyte of interest is typically adsorbed on a stationary phase, washed, and then evaluated with a different mobile phase. In another procedure, concentrated analytes are eluted from the column. Its benefits include reducing ion suppression, simplifying the complex sample matrix, functionalization of sample matrix to analyze compounds by class, purification of bioactive compounds, enhancement of MS applications, and concentration of very low-level compounds (10).

4.1.3.12 High Voltage Electrical Discharges

This green extraction process is a liquid-phase discharge technology. It is a nonthermal technique suitable for the mass transfer of bioactive compounds from different plant materials. The HVED principle is based on the electrical disruption of cell tissues in water and enhancing the release of valuable intracellular components (46). Three HVED systems with the exact primary mechanism have been developed: batch, continuous, and circulating extraction systems. Factors such as solvent selection, electric field intensity, solvent-solid ratio, flow rate, and treatment time are critical to this technique. The method gave a higher extraction yield than that obtained with pulsed electric fields and ultrasounds-assisted extraction. In addition, it is performed under less processing time and lower power consumption and achieves fewer extract impurities (47).

4.1.3.13 Purge-and-Trap Extraction

The technique can be employed using an automated purge-and-trap system for solid and liquid plant samples. The system comprises a flow meter, splitter, and adsorbent. The flow meter controls a nitrogen source, and the splitter unit divides the flow into several channels when extracting from more than one sample. The plant material must be pretreated by incubating its ground form at optimized purging temperature such as 60°C for about 10 min (48). Small samples between 5 g and 25 g can be extracted using inorganic solvents. Thus, it is a good technique for research or routine work purpose. Extraction takes a short period with low detection limits (49).

4.2 STANDARD PROCEDURES FOR SCREENING BIOACTIVE PHYTOCHEMICALS

Phytochemicals are naturally occurring secondary metabolites of plants. Plants synthesize a large variety of these chemical substances, including steroids, terpenoids, alkaloids, flavonoids, saponins, tannins, and phenolic compounds. Therefore, it is imperative to screen plants for the presence of these phytochemicals because they are the basic constituents of many therapeutic drugs (50). Phytochemical screening is done to qualitatively evaluate the chemical compositions of different plant extracts usually employing precipitation and coloration reactions to identify the major secondary metabolites (51). Many researchers have described these screening methods based on the standard procedures earlier described by (52) with several modifications.

4.2.1 Screening for Tannins

i. Add drops of 1% lead acetate solution to a defined quantity of extract. The formation of a yellow or red precipitate indicates the presence of tannin (51).
ii. Add 1% $FeCl_3$ solution drops to a defined quantity of plant extract. The formation of blue-black coloration indicates the presence of tannin (53).

4.2.2 Screening for Saponins

i. Add 5 ml of distilled water to 5 ml of extract, shake vigorously in a test tube, and apply a little heat. The formation of a stable foam indicates the presence of saponins (50).
ii. Add drops of sodium bicarbonate to 5 ml of extract. Shake the mixture vigorously and leave to stand for 3 minutes. The formation of honeycomb-like froth indicates the presence of saponin (51).
iii. In a boiling water bath, boil 0.5 g of plant sample with 15 ml of double distilled water. The formation of intensive froth indicates the presence of saponin (53).

4.2.3 Screening for Phenols

i. Dissolve 500 mg of plant sample in 5 ml of distilled water. Then, add drops of neutral 5% ferric chloride. The formation of dark green coloration indicates the presence of phenols (50).

ii. Add 3 ml of distilled water to 1 ml of extract, then add a few drops of ferric chloride solution. The presence of phenols is indicated by the formation of blue or green color (51).
iii. Add 4–5 drops of 2% $FeCl_3$ solution to 10 ml of the plant ethanolic extract. The presence of phenols is indicated by a change in color (53).

4.2.4 Screening for Terpenoids

i. Dissolve 2 ml of plant extract in 2 ml of $CHCl_3$, then evaporate to dryness. Add 2 ml of concentrated H_2SO_4 and heat for about 2 minutes. The formation of grey coloration indicates the presence of terpenoids (50).
ii. Add 2 ml chloroform ($CHCl_3$) to 0.5 g of plant extract or 5 ml of plant methanolic extract. Carefully add 3 ml of concentrated H_2SO_4 to form a layer. The formation of reddish-brown coloration indicates the presence of terpenoids (51; 53).

4.2.5 Screening for Flavonoids

i. Add 1 ml of 10% lead acetate solution to 1 ml of extract. A yellow precipitate's formation indicates flavonoids' presence (50).
ii. Add 5–10 drops of dilute HCl and a small piece of ZnCl or Mg to 0.5 ml of extract in a test tube. Boil the solution for a few minutes. The formation of reddish-pink or dirty brown coloration indicates the presence of flavonoids (51).
iii. Heat 2 g of a crude extract with 10 ml of ethyl acetate over a water bath for 5 min. Filter the solution through Whatman paper No 1. Mix 1.4 ml of the filtrate with 10% dilute ammonia solution and shake vigorously. The formation of yellow coloration indicates the presence of flavonoids (53).

4.2.6 Screening for Alkaloids

i. Add 3 ml of 1% HCl to 3 ml of extract in a test tube. Stir the mixture in a steam bath. Introduce 1 ml of each of the mixture into two test tubes. To one of the test tubes, add drops of Dragendorff's reagent. The formation of an orange-red precipitate indicates the presence of alkaloids. To the second test tube, add Mayer's reagent. The appearance of a buff-colored precipitate indicates the presence of alkaloids (50).
ii. Dissolve 1.36 g of mercuric chloride in 60 ml of distilled water. Also, dissolve 5 g of potassium iodide in 10 ml distilled water. Mix the two

solutions and make the volume up to 100 ml to form Meyer's solution (potassium mercuric iodide). Add drops of the reagent to 1 ml of extract. The formation of a white or pale precipitate indicates the presence of alkaloids (51).

iii. Add 2 ml of 2N HCl to 2 ml of methanolic extract in a test tube. Shake vigorously to mix and allow to stand for 5 minutes. Decant the aqueous phase formed. Add drops of Mayer's reagent ($HgCl_2$ + KI in water) and shake. The formation of a creamy-colored precipitate indicates the formation of alkaloids (53).

4.2.7 Screening for Glycosides

i. Add drops of dilute HCl to 2 ml of extract. Add 2 ml of sodium nitroprusside in pyridine and sodium hydroxide solution. The formation of pink to blood-red coloration indicates the presence of cardiac glycosides (50).
ii. Add 5 ml of 5% $FeCl_3$ to 5 ml of extract. Heat for 5 minutes in a boiling water bath. Allow it to cool. Add drops of benzene or any other organic solvent, then shake well. Decant the organic layer and add an equal volume of dilute ammonia. The pinkish-red formation in the ammonia layer indicates the presence of glycosides (51).
iii. Add 2 ml of glacial acetic acid containing 2% $FeCl_3$ to 5 ml of the plant methanolic extract. Add 1 ml of concentrated H_2SO_4 slowly along the wall of the test tube. The formation of a brown ring at the interphase of the two liquids indicates the presence of glycosides (53).

4.2.8 Screening for Steroids

i. Salkowski's test: Add 1 ml of concentrated sulfuric acid carefully along the side of a test tube containing a mixture of 2 ml of extract and 2 ml of chloroform. Production of red coloration in the chloroform layer indicates the presence of steroids (50; 51).
ii. Liebermann Burchard test: Dissolve 2 ml of organic plant extract in 2 ml chloroform. Treat the mixture with concentrated sulfuric acid and acetic acid. The formation of greenish coloration indicates the presence of steroids (50).
iii. Treat 5 ml of methanolic plant extract in a test tube with 0.5 ml of anhydrous acetic acid. Cool on an ice bath for 15 minutes. Add 0.5 ml of chloroform to the solution. Carefully add 1 ml of concentrated sulfuric acid (H_2SO_4) along the wall of the test tube. The formation of a reddish-brown coloration at the interphase of the two liquids indicates the presence of steroids (53).

4.3 TECHNIQUES FOR PURIFICATION OF BIOACTIVE PHYTOCHEMICALS

The modern techniques used to purify bioactive compounds from plants offer the benefits of developing many bioassays and providing clear-cut techniques for isolating and separating these bioactive compounds from various sources. Several bioactive molecules have been isolated and purified using the thin-layer chromatographic and column chromatographic methods. These methods offer convenience and are economical. The stationary phases are also readily available (6).

4.3.1 Thin Layer Chromatography

Thin layer chromatography is a technique used to separate nonvolatile mixtures such as bioactive molecules extracted from plants, animals, and microorganisms. It is performed on a sheet of an inert substrate such as plastics, glass, or aluminum foil coated with a thin layer of adsorbent materials. These adsorbent materials, referred to as the stationary phase, usually include silica gel, alumina (aluminum oxide), or cellulose. The sample to be analyzed is applied to the adsorbent plate, and a solvent or mixture of solvents (called the mobile phase) is drawn up the plate through capillary action. The different components of the sample ascend the TLC plate at different rates; thus, separation is achieved (54).

Depending on the adsorbent used in the TLC, the adsorption of the bioactive compounds is based on the principle of adsorption chromatography, or partition chromatography, or both. The components with more affinity for the stationary phase migrate slower than the other components with lesser affinity. Once the components separate, individual spots can then be visualized, and identification made using a wide range of spectroscopic techniques, including UV-visible, mass spectroscopy (MS), infrared (IR), and nuclear magnetic resonance (NMR) (6).

The TLC system consists of the TLC plates, the TLC chamber, the mobile phase, and a filter paper. The TLC plates are usually ready-made with a thin layer of the stationary phase (made of uniform thickness and fine particles) applied on the surface layer with the plates. The TLC chamber helps to provide a uniform environment for the development of spots, keeps away dust, and prevents evaporation of the solvent(s). The mobile phase comprises a solvent or a mixture of solvents. The last component of the TLC system is the filter paper placed in the chamber and moistened in the mobile phase. The filter paper helps to develop an even rise in the mobile phase over the stretch of the stationary phase (54).

The performance of a compound on a TLC is usually described in terms of its R*f* value (i.e., its relative mobility). The R*f* value, also described as the retention factor, is a unique value constant from one experiment to another for each compound under constant chromatographic conditions. The chromatographic conditions include the solvent(s) system, amount of sample spotted, temperature, the adsorbent, and thickness of the absorbent. However, these factors are often

difficult to keep constant from one experiment to the next. In order to overcome this pitfall, relative Rf is usually considered. Relative R*f* describes the R*f* value of a compound to a standard (54).

Researchers have used thin layer chromatography to purify bioactive compounds from various plant materials. Altemimi et al. (55) combined densitometry with TLC to isolate and purify flavonoids and polyphenols from peach and pumpkin extracts. The combination of densitometry and image analysis has earlier been reported to show the ability to measure components of medicinal plants (56). Three solvents mixtures were tested to determine the best separation solvents: ethyl acetate and acetone (5:4 – v/v); hexane and chloroform (10:10 – v/v); and ethyl acetate, formic acid, and water (10:2:3 – v/v). The best separation solvent mixture was found to be the combination of ethyl acetate, formic acid, and water (10:2:3 – v/v), corresponding to the earlier reports of Dmitrienko et al. (57). In the TLC analysis of an extract from the bark of *Jatropha podagrica*, silica gel 60 was used as the solid phase, while the mobile phase was a mixture of hexane and ethyl acetate in the ratio 8:2 (v/v). The purity of the fractions confirmed with GC-MS was 97.67% (58).

The advantages of TLC as a method for the purification of bioactive compounds include rapid isolation and higher selectivity of compounds with minute differences in chemistry. Also, the standard of purity of the sample can be easily assessed (54). Despite these advantages, TLC has the shortfall of not being able to differentiate between enantiomers and some isomers. In order to overcome this, the R*f* values for the compound of interest must be known earlier. In addition, the length of separation for TLC is usually shorter than that of other chromatographic techniques because of the short stationary phase (54).

Aside from the ability of TLC to separate phytochemicals with ease and rapidly, it has also been used to detect the biological activity of the separated constituents, a method described as TLC bioassay (59) or TLC bioautography (60). This method is widely used to measure the antimicrobial activity of the separated constituents against fungi and bacteria. The TLC bioautography combines separation using chromatography with the determination of the antimicrobial activity of a target bioactive component of an extract by growth inhibition of microorganisms (60), and this has been considered the most efficient assay for detecting antimicrobials (61).

There are three ways through which TLC bioautography can be done:

i. Direct bioautography (TLC-DB) in which the microorganism is grown directly on the TLC plate (60; 62).
ii. Contact bioautography in which the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact (60; 63).
iii. Agar overlay bioautography, in which a seeded agar medium is applied directly onto the TLC plate (60; 63), is considered a hybrid of direct and contact bioautography (64).

The TLC bioautography techniques are great tools for probing and studying the antimicrobial properties of extracted phytochemicals (65). In TLC-DB, analytical

methods earlier described by researchers such as Choma (66) and Morlock and Schwack (67) are combined with antimicrobial assays (62). All the procedures involved in the analysis, including TLC separation, antimicrobial activity detection, and visualization, are done directly on the TLC plate (62).

Despite the high sensitivity of TLC bioautography, its application is limited to microorganisms that can grow on the TLC plates (60). There is also the concern about the need for the removal of residual low-volatile solvents, such as ammonia, trifluoroacetic acid, and n-BuOH, coupled with the transfer of the active biomolecules from the stationary phase by diffusion into the agar layer (68). It is also essential to understand that bioautography is not a quantitative measure of the antimicrobial activity of phytochemicals. However, only an indication of the number of compounds separated that have antimicrobial activity (69). The absence of bioactivity in some constituents of the extract is not an indication that they are inactivity as many may have additive or synergistic interaction with other constituents of the plant extract (70).

4.3.2 Column Chromatography

Column chromatography is a method in which the substances to be separated are introduced into a column packed with an adsorbent material. The constituents of the substance pass through the column at different rates based on their affinity for the adsorbent and the solvent or solvent mixture, allowing them to be separated into fractions. The constituents are collected in solution as they pass from the column at different intervals. The mobile phase in column chromatography can be liquid or gas (54). In column chromatography, the stationary phase can either be coated on a matrix and packed into the column or applied as a thin film to the inside wall of the column. The substance to be separated (usually referred to as analyte) is then introduced into the column, and the mobile phase (referred to as eluent) is passed through the column by use of a pumping system or is applied as gas pressure (58).

Two methods are employed in the preparation of columns in column chromatography: the dry and the wet column preparation. In the dry column preparation, dry stationary phase powder is introduced into the column before adding the mobile phase. The mobile phase is allowed to run through the dry stationary phase until it becomes completely wet and remains so throughout the process (71; 72). Dry column preparation or dry packing often results in air bubble formation, which can be challenging to remove. When this occurs, the column has to be removed and repacked (72). In the wet column preparation, the mobile phase (eluent) is used to make a slurry of the stationary phase powder before introducing it into the column. Wet column preparation (wet packing) can be muddling, but it works well and is preferred for silica gel columns, while dry packing works better for alumina (72). After the column has been packed, the mobile phase is passed through the column to elute the various components of the sample to be analyzed. The components are retained by the stationary phase and separated from each other while running at different rates through the column with the mobile phase (71).

The adsorbent or stationary phase in column chromatography is usually solid. Previously, cellulose powder was commonly used. This has been replaced with silica gel and alumina. The stationary phases are usually finely ground powders of gels which are microporous for increased surface area. A significant ratio exists between the stationary phase's weight and the analyte mixture's dry weight that can be loaded into the column. For columns with silica gel, the ratio is between 20:1 and 100:1, depending on how close the analyte components to be eluted are to one another (71). The mobile phase is a solvent or solvent mixture that moves the compounds to be separated through the column. The retention factor informs the choice of the mobile phase of the compound of interest, which usually is around 0.2–0.3. This helps to minimize the time and amount of eluent needed to run the chromatography. A thin layer chromatography pretest is used to optimize the eluent (71).

The analyte (sample to be analyzed) is usually a mixture of components. This is dissolved in a small amount of the mobile phase before being introduced into the column. Then, the analyte gets adsorbed on the upper part of the column from where the individual components are eluted with the gradual introduction of the mobile phase (73). Elution of the components of the analyte can be achieved through two methods: the isocratic elution method or the gradient elution method. In the isocratic elution method, a solvent with the same polarity is used all through the process of separation, while in the gradient elution method, solvents with gradually increasing polarity or increasing elution strength are used for the separation (73).

Monitoring the progress of separation can be visually monitored if the components to be separated are colored; however, if the components are colorless, a small portion of the eluent should be collected sequentially in tubes and the composition of each collection analyzed by TLC chromatography (73). Devika and Koilpillai (74) obtained a colorless compound from the column chromatographic analysis of *Tagetes erecta* Linn using silica gel 60 F254 precoated aluminum plate of 0.2 mm thickness and ethyl acetate:methanol (1:1) as the developing solvent. The colorless eluent was visualized by dipping the plate in 1% vanillin sulfuric acid and heating it at 105°C to obtain distinct spots (74).

4.4 IDENTIFICATION TECHNIQUES

The extracts from a plant contain a cross-section of bioactive compounds with changing polarity. Thus, segmenting them has proven to be a significant struggle in identifying and classifying a bioactive element. Therefore, a diverse technique which includes paper chromatography, column chromatography, gas chromatography, TLC, OPLC, HPLC, and HPTLC, ought to be used to identify pure substances. The regular activity and the organization of the unadulterated elements are then decided (1; 75). After being separated and purified, phytochemicals are often identified by checking several classes of compounds and then comparing

these data with those in the literature. Parameters such as homogeneity are checked, which is determined if it travels in a single spot in thin layer chromatography or paper chromatography. Other parameters are optical rotation, boiling points, melting points, and R_f value which is the distance a compound moves in chromatography relative to the solvent font (60).

Also, identification can be achieved by measuring the spectral characteristics of the plant substance used, such as UV, IR, NMR, and MS. Different identification techniques have been adopted to classify the various phytochemicals in plant extracts, which are explained in detail below. These detection techniques are a vital mechanism in a bioactive phytochemical study (60; 75).

4.4.1 Chromatography Technique

In a qualitative and quantitative study, chromatography has been proven to be a significant biophysical technique for separating, purifying, and identifying mixture components. Chromatography works on the attitude of molecules that are contained in a mix that is being applied to a surface and the unchanging phase of the fluid splitting from one another while moving with the aid of a mobile state. Some molecular attributes connected to partition (liquid-solid), adsorption (liquid-solid), affinity, or differences among their molecular masses affect this separation procedure. Chromatography separates molecules based on size, shape, and charge (76).

In the chromatography process, analytes are first dissolved in solvents, after which they are taken through a solid phase which serves as a sieving medium. The molecule is subjected to separation as it moves through the molecular sieve. When identifying and isolating on an industrial scale, column chromatography is usually advised. The column chromatography is used with automatic fraction collecting, as this gives a more purified component in better amounts. The major chromatic procedures that offer qualitative information while permitting quantitative data to be obtained are paper chromatography, thin layer chromatography, and gas chromatography. This may be done by using one or combining two or the three chromatographic techniques (77).

4.4.1.1 Paper Chromatography

In paper chromatography, the chemical substances are separated by taking advantage of their various migration rates across sheets of filter paper. One significant benefit of paper chromatography is that separation is done on sheets of filter paper, which serves as both a support and a medium of separation (78). Another benefit is the repetition ability of the retention factor (R_f) values calculated on a paper. The inactive phase in this type of chromatography is the paper sheet. A sample is inserted very close to the filter paper's bottom. The filter paper is then put in a chromatographic chamber that contains solvent. The solvent moves ahead through a passage, bringing soluble molecules with it. A paper with a low porosity level has a sluggish rate of solvent transport, but thick sheets offer a larger

sample capacity (79). Compounds are often seen as colored or UV-fluorescent spots, after it has reacted with the chromogenic reagents. The reaction can be done in two ways which are either by dipping or spraying. Dipping is usually done for large sheets because it is easier; hence the solvent is modified to allow easy drying; after it is dried the paper may then be heated to develop the colors.

4.4.1.2 Thin Layer Chromatography

According to Stahl (80), thin layer chromatography (TLC) separates a mixture of chemicals into their constituents by using a glass plate covered with a very thin layer of adsorbent, such as silica gel or alumina. The chrome plate is the type of plate used in this operation. First, a little area of the mixture to be separated is put 2 cm above one end of the plate with the solution. After which, the plate is put in a sealed jar containing an eluant, which raises the plate, taking various components of the mixture to various heights. TLC has more benefits than paper chromatography, including adaptability, speed, and sensitivity. TLC has a benefit of adaptability because different absorbents aside from cellulose can be spread on a glass plate or any other support for chromatography. Hahn-Deinstrop (81) described TLC as an adsorption chromatography technique in which the separation of materials occurs due to the interaction between tiny layers of adsorbent on a plate. The usual method of identification of phytochemicals using TLC is normally done by spraying the plate with concentrated H_2SO_4 as a simpler procedure, usually for the detection of steroids and lipids (82). Hence compounds that absorb at 254 nm are identified by adding a fluorescent dye to the slurry during the preparation of the plate or by spraying the plate with a similar dye.

4.4.1.3 Gas Chromatography

GC is another type of separation technique used to separate unstable substances. The components in this process are distributed between a gas and a liquid phase. The liquid phase is in the state of inertia, while the gas phase is active. Therefore, the component of the chemical rate of migration is determined by its dispersion in the gas phase. Since the gas phase is in the active state, a component that distributes itself entirely into the gas phase will flow at the same pace as the flowing gas. In contrast, a component that distributes itself in the liquid state will remain stationary (75).

In GC, a sample is vaporized and injected into the chromatographic column's head. A movement of the inactive gaseous mobile phase moves the sample through the column. A liquid stationary phase is contained in the column itself, which is adsorbed onto the surface of an inert solid. The major parameters of GC are the nature of the stationary phase and the temperature of the operation, and these are dependent on the polarity and volatility of the compounds being identified. Most recently, GC apparatus are now set up to subject the compounds to further analysis, such as automatically linking the GC apparatus to mass spectrometry, thereby combining the GC-MS apparatus, which has emerged in recent years for phytochemical analysis (83).

4.4.1.4 Adsorption Chromatography

In this chromatography procedure, various chemicals are adsorbed on the adsorbent to changing degrees, which depends on the component's absorptivity in the adsorption chromatography procedure. The mobile phase, in this case, is moved across the stationary (waiting) phase, bringing the component with more excellent absorptivity to a closer distance than those with lower absorptivity. Adsorption chromatography, similarly known as liquid/solid chromatography or displacement chromatography, is centered solely on the interaction between the solute and the inactive phase fixed active sites (10). Noncovalent bonds, nonpolar contacts, van der Waals forces, and hydrophobic interactions communicate with the useful groups of molecules to be separated at the active spots of the inert phase. The mobile phase starts by dissolving the loosely bound compound, which then allows for the separation of various groups of the compound.

4.4.1.5 High-Performance Liquid Chromatography

High-performance liquid chromatography is another type of separation technique that determines and separates organic and inorganic solutes in various plant samples (84). Generally, the identification and separation of phytochemicals can be accomplished using a nonchanging mobile phase. HPLC separates substances based on their relationship with solid particles in a densely packed column and the solvent in the active phase. In modern HPLC, a nonpolar solid phase, a polar liquid phase, usually C_{18} as an example of the nonpolar solid phase, and a combination of water and another solvent as the polar liquid phase are used. The analyte should be dissolved down a column at high pressure of up to 400 bar before passing through a diode array detector (DAD). Identification of compounds by HPLC is a crucial part of any HPLC assay. To identify any compound by HPLC, a detector must first be chosen; the detector chosen is then set to an optimal detection setting. In identifying analytes, a diode array detector is used to inspect the absorption spectra of the analytes (60). One benefit of HPLC is its ability to identify substances that cannot be vaporized or broken down when exposed to high temperatures. Another advantage is that it can replace gas chromatography in usage.

4.4.1.6 Partition Chromatography

Partitioning the components into two phases is a process to isolate the components from the sample mixture. The two phases are in the liquid state. In this process, the mobile phase is the immiscible solid surface covered in the liquid surface on the stationary phase. The stationary phase takes out the liquid surface, resulting in the formation of a stationary phase. In the process of the mobile phase moving away from the stationary phase, separation occurs between the components. The partition coefficient determines the separation. Liquid/liquid chromatography: In this procedure, the molecules to be separated communicate between two immiscible liquid phases based on their solubility in partition chromatography.

4.4.1.7 Ion-Exchange Chromatography

Ion-exchange chromatography, along with other ion chromatography like ion-partition/interaction and ion-exclusion chromatography, is an essential analytical technique for separating and identifying ionic substances. Like other types of column-based liquid chromatography techniques, ion exchange is made up of mobile and stationary phases, which are needed to separate differently ionizable molecules (85–87). The mobile phase is made of an aqueous buffer system, where the sample mixture to be determined is placed. Furthermore, the stationary phase is usually a chemically derived inactive organic matrix containing ionizable functional groups loaded with displaceable oppositely charged ions (87). Ion-exchange chromatography is a system for separating ions and polar compounds centered on their electrical characteristics (88).

4.4.1.8 Affinity Chromatography

In affinity chromatography, separations are centered on distinct relationships between pairs of interacting substances, including macromolecules and their substrates, cofactors, allosteric effectors, and inhibitors. The affinity chromatography technique is based on a particular binding relationship between an immobilized ligand and its binding mate. Examples include enzyme-substrate, enzyme-inhibitor, and antibody-antigen communications. The degree of the purification can be equitably high depending on the specificity of the interaction, which usually results in it being the first, if not the only, stage in the purification approach. During this chromatography, an assortment of chemicals will be placed in the columns. In order to allow the desired chemical to attach to the ligand, the buffer is used to wash away substances that have no affinity for the ligand. Thus, a buffer with different pH or greater ionic strength is used to elute the analyte (89).

4.4.1.9 Size Exclusion Chromatography

Size exclusion chromatography, also known as molecular sieve chromatography, is a technology-based chromatography that does molecule separation in a solution based on its size and, in some cases, mass (90). This technique commonly uses macromolecular complexes, such as industrial polymers. Some other terms for it include gel filtration and gel permeation chromatography. This chromatography has zero contact or chemical attraction between the stationary phase and the solutes.

4.4.1.10 Column Chromatography

Column chromatography is another procedure for separating the components of a mixture. However, it uses a column of suitable adsorbents packed in a glass tube. The combination is placed over the column, and a suitable eluent is allowed to travel down the column gently. The separation of the mixture's components occurs based on the degree of adsorption of the components on the

wall adsorbent column. The component with the highest absorptivity is placed on top, while the other components flow down to changing heights. Molecular sieves, ion exchange, and adsorption phenomena are used in column chromatography (19).

4.4.1.11 High-performance Thin-layer Chromatography

HPTLC is an improved version of thin-layer chromatography. Here, the components of a mixture are separated with the aid of a high-performance layer and data is collected. The high-performance layer is created with precoated plates with a sorbent particle size of 5–7 microns and a layer thickness of 150–200 microns. The separation type and the plate effectiveness improve when the layer's thickness and particle size are reduced. In HPTLC, the separated materials after chromatography can only be seen with the naked eye using HPTLC (75).

4.4.1.12 Optimum Performance Laminar Chromatography

OPLC acts as a bridge connecting TLC and HPTLC and their benefits. This type of chromatography is a dynamic, analytical, and preparative mechanism that may be utilized in research and control labs. OPLC combines HPLC's user-friendly interface and flash chromatography's capacity with the multidimensionality of TLC for separation. Like another chromatographic process, OPLC works on the same principle: a pump forces a liquid active phase through a phase of inert, such as a bonded phase medium or silica (91).

4.4.2 Nonchromatographic Techniques

There are other nonchromatographic techniques that can also be employed for the identification of bioactive compounds. These techniques are immunoassay, phytochemical screening assay, and FTIR (Fourier-transform infrared spectroscopy) (60).

4.4.2.1 Immunoassay

Immunoassays are used in bioactive compound analyses. Immunoassays involving monoclonal antibodies (MAbs) with low-molecular-weight natural bioactive compounds are often used. Immunoassays are often used for receptor binding, enzyme assays, and qualitative and quantitative analysis because they possess high specificity and sensitivity. Therefore, in most cases, immunoassays may be adopted to identify phytochemicals because they are more sensitive than the HPLC methods (60).

4.4.2.2 Phytochemical Screening Assay

Phytochemical screening assay is another method that can be used to identify phytochemicals for several reasons, some of which are because it is simple, quick, and not expensive and gives a quick answer to the various types of phytochemicals in a mixture. The assay includes tests such as Dragendorff's test, Wagner test, Borntrager's test, Keller–Kiliani test, Shinoda test, NaOH test, phenol test, Fehling test, Salkowski test, Braemer's test, Liebermann–Burchardt test, and frothing test or foam test (92–95).

4.4.2.3 Fourier-Transform Infrared Spectroscopy

This is a proven tool for the characterization and identification of compounds or functional groups in an unknown plant extract mixture. In FTIR, the spectrum of the unknown compound is usually identified by comparing it to the data of the known compounds. The samples used for FTIR are prepared by placing one drop of a sample between two plates of sodium chloride, forming a thin film between the plates. This method is done for liquid samples. However, solid samples are prepared by milling the sample with potassium bromide (KBr) and then reduced into a thin particle which is then used for the analysis (60; 96).

4.5 CONCLUSION

Extraction, purification, and identification of bioactive phytochemicals are very complicated processes due to their enclosure in vacuoles of plant cells or lipoprotein bilayers. Extraction involves the use of several techniques involving conventional, nonconventional, or a combination of both techniques to recover the bioactive phytochemicals in plant matrices. Extraction depends on the choice of technique(s), cost of extraction, organic solvent consumption, extraction times and efficiency, and temperature. Following extraction, the bioactive phytochemicals are subjected to purification to clean up and separate fractions of plant constituents. Analytical methods adopted for the purification often require optimization and validation before being employed to effectively remove undesired components and recover bioactive phytochemicals with intact biological features. Purified bioactive food phytochemicals are subsequently identified to determine the class of compounds. These identification procedures measure certain parameters and compare them with data in the literature.

REFERENCES

1. Ingle KP, Deshmukh AG, Padole DA, Dudhare MS, Moharil MP, Khelurkar VC. Phytochemicals: Extraction methods, identification and detection of bioactive compounds from plant extracts. J Pharmacogn Phytochem. 2017;6(1):32–6.
2. Senguttuvan J, Paulsamy S, Karthika K. Phytochemical analysis and evaluation of leaf and root parts of the medicinal herb, *Hypochaeris radicata* L. for in vitro antioxidant activities. Asian Pac J Trop Biomed. 2014;4(Suppl 1):S359–67.
3. Zhi PR, Liang LZ, Yi ML. Evaluation of the antioxidant activity of *Syzygium cumini* leaves. Molecules. 2008;13(10):2545–56.
4. Anokwuru CP, Anyasor GN, Ajibaye O, Fakoya O, Okebugwu P. Effect of extraction solvents on phenolic, flavonoid and antioxidant activities of three Nigerian medicinal plants. Nat Sci. 2011;9(7):53–61.
5. Koffi E, Sea T, Dodehe Y, Soro S. Effect of solvent type on extraction of polyphenols from twenty three Ivorian plants. J Anim Plant Sci. 2010;5(3):550–8.
6. Altemimi A, Lakhssassi N, Baharlouei A, Watson DG, Lightfoot DA. Phytochemicals: Extraction, isolation, and identification of bioactive compounds from plant extracts. Plants. 2017;6(4):1–23. doi:103390/plants6040042
7. Fongang FYS, Bankeu KJJ, Gaber EB, Iftikhar A and, Lenta NB. Extraction of bioactive compounds from medicinal plants and herbs. In: IntechOpen; 2021. pp. 1–39.
8. Jha AK, Sit N. Extraction of bioactive compounds from plant materials using combination of various novel methods: A review. Trends Food Sci Technol [Internet]. 2022;119(October 2021):579–91. Available from: https://doi.org/10.1016/j.tifs.2021.11.019
9. Azmir J, Zaidul ISM, Rahman MM, et al. Techniques for extraction of bioactive compounds from plant materials: A review. J Food Eng [Internet]. 2013;117(4):426–36. Available from: http://dx.doi.org/10.1016/j.jfoodeng.2013.01.014
10. Srivastava N, Singh A, Kumari P, et al. Advances in extraction technologies: isolation and purification of bioactive compounds from biological materials [Internet]. Natural Bioactive Compounds, Sinha RP and Hader DP (Eds). Elsevier Inc.; 2021. 409–433 p. Available from: http://dx.doi.org/10.1016/B978-0-12-820655-3.00021-5
11. Azwanida NN. A review on the extraction methods use in medicinal plants, principle, strength and limitation. Med Aromat Plants. 2015;04(03):3–8.
12. Hussain MK, Khan MS, Khan MF. Techniques for Extraction, Isolation, and Standardization of Bio-active Compounds from Medicinal Plants. Springer Nature Singapore Pte Ltd. 2019. pp. 179–97.

13. Harbourne N, Marete E, Christophe J, Riordan DO. Conventional extraction techniques for phytochemicals. Handbook of Plant Food Phytochemicals: Sources, Stability and Extraction, Tiwari BK, Brunton NP and Brennan CS (Eds). 2013. pp. 400–10.
14. Luque de Castro MD, García-Ayuso LE. Soxhlet extraction of solid materials: An outdated technique with a promising innovative future. Anal Chim Acta. 1998;369(1–2):1–10.
15. Nagalingam A. Drug delivery aspects of herbal medicines. In: Japanese Kampo Medicines for the Treatment of Common Diseases: Focus on Inflammation, Arumugam S and Watanabe K (Eds). 2017. pp. 47–57.
16. Silva L V., Nelson DL, Drummond MFB, Dufossé L, Glória MBA. Comparison of hydrodistillation methods for the deodorization of turmeric. Food Res Int. 2005;38(8–9):1087–96.
17. Rasul MG. Conventional extraction methods use in medicinal plants, their advantages and disadvantages. Int J Basic Sci Appl Comput. 2018;(6):10–4.
18. Gawde AJ, Cantrell CL, Zheljazkov VD. Dual extraction of essential oil and podophyllotoxin from Juniperus virginiana. Ind Crops Prod. 2009;30(2):276–80.
19. Chua LS, Latiff NA, Mohamad M. Reflux extraction and cleanup process by column chromatography for high yield of andrographolide enriched extract. J Appl Res Med Aromat Plants [Internet]. 2016;3(2):64–70. Available from: http://dx.doi.org/10.1016/j.jarmap.2016.01.004
20. Ngouana V, Zeuko'O Menkem E, Youmbi DY, Yimgang LV, Toghueo RMK, Boyom FF. Serial exhaustive extraction revealed antimicrobial and antioxidant properties of *Platycerium stemaria* (Beauv) Desv. Biomed Res Int. 2021;2021; 4141–50.
21. Handa SS. An overview of extraction techniques for medicinal and aromatic plants. In: Extraction Technologies for Medicinal and Aromatic Plants, Handa SS, Khanuja SPS, Longo G and Rakesh DD (Eds). 2008. pp. 21–8.
22. González de Peredo AV., Vázquez-Espinosa M, Espada-Bellido E, et al. Development of new analytical microwave-assisted extraction methods for bioactive compounds from myrtle (*Myrtus communis* L.). Molecules. 2018;23(11).
23. Nguyen VT, Pham HNT, Bowyer MC, Van Altena IA, Scarlett CJ. Influence of solvents and novel extraction methods on bioactive compounds and antioxidant capacity of Phyllanthus amarus. Chem Pap. 2016;70(5):556–66.
24. Bryant G, Wolfe J. Electromechanical stresses produced in the plasma membranes of suspended cells by applied electric fields. J Membr Biol. 1987;96(2):129–39.
25. Puértolas E, López N, Condón S, Álvarez I, Raso J. Potential applications of PEF to improve red wine quality. Trends Food Sci Technol. 2010;21(5):247–55.
26. Ade-Omowaye BIO, Angersbach A, Taiwo KA, Knorr D. Use of pulsed electric field pre-treatment to improve dehydration characteristics of plant based foods. Trends Food Sci Technol. 2001;12(8):285–95.

27. Vinatoru M, Bartha E, Badea F, Luche JL. Sonochemical and thermal redox reactions of triphenylmethane and triphenylmethyl carbinol in nitrobenzene. Ultrason Sonochem. 1998;5(1):27–31.
28. Latif S, Anwar F. Physicochemical studies of hemp (Cannabis sativa) seed oil using enzyme-assisted cold-pressing. Eur J Lipid Sci Technol. 2009;111(10):1042–8.
29. Domínguez H, Núñez MJ, Lema JM. Enzyme-assisted hexane extraction of soya bean oil. Food Chem. 1995;54(2):223–31.
30. Lang Q, Wai CM. Supercritical fluid extraction in herbal and natural product studies – A practical review. Talanta. 2001;53(4):771–82.
31. Zhang Z, Li G. A review of advances and new developments in the analysis of biological volatile organic compounds. Microchem J [Internet]. 2010;95(2):127–39. Available from: http://dx.doi.org/10.1016/j.microc.2009.12.017
32. Richter BE, Jones BA, Ezzell JL, Porter NL, Avdalovic N, Pohl C. Accelerated solvent extraction: A technique for sample preparation. Anal Chem. 1996;68(6):1033–9.
33. Nieto A, Borrull F, Pocurull E, Marcé RM. Pressurized liquid extraction: A useful technique to extract pharmaceuticals and personal-care products from sewage sludge. TrAC – Trends Anal Chem. 2010;29(7):752–64.
34. Kaufmann B, Christen P. Recent extraction techniques for natural products: Microwave-assisted extraction and pressurised solvent extraction. Phytochem Anal. 2002;13(2):105–13.
35. Lara-Abia S, Gomez-Maqueo A, Welti-Chanes J, Cano MP. High hydrostatic pressure-assisted extraction of carotenoids from papaya (*Carica papaya* L. cv. Maradol) tissues using soybean and sunflower oil as potential green solvents. Food Eng Rev [Internet]. 2021;13(3):660–75. Available from: https://doi.org/10.1007/s12393-021-09289-6
36. Otero L, Molina-García AD, Sanz PD. Thermal effect in foods during quasi-adiabatic pressure treatments. Innov Food Sci Emerg Technol. 2000;1(2):119–26.
37. Cascaes Teles AS, Hidalgo Chávez DW, Zarur Coelho MA, Rosenthal A, Fortes Gottschalk LM, Tonon RV. Combination of enzyme-assisted extraction and high hydrostatic pressure for phenolic compounds recovery from grape pomace. J Food Eng. 2020;288(October 2019).
38. Briones-Labarca V, Giovagnoli-Vicuña C, Cañas-Sarazúa R. Optimization of extraction yield, flavonoids and lycopene from tomato pulp by high hydrostatic pressure-assisted extraction. Food Chem [Internet]. 2019;278(November 2018):751–9. Available from: https://doi.org/10.1016/j.foodchem.2018.11.106
39. Moreira SA, Pintado M, Saraiva JA. High hydrostatic pressure-assisted extraction: A review on its effects on bioactive profile and biological activities of extracts [Internet]. Present and Future of High Pressure Processing: A Tool for Developing Innovative, Sustainable, Safe and Healthy Foods, Barba SJ, Tonelle-Sampson C, Puertolas E and Lavilla M (Eds). Elsevier

Inc.; 2020. 317–28 p. Available from: https://doi.org/10.1016/B978-0-12-816405-1.00014-5

40. Périno S, Chemat-Djenni Z, Petitcolas E, Giniès C, Chemat F. Downscaling of industrial turbo-distillation to laboratory turbo-Clevenger for extraction of essential oils. Application of concepts of green analytical chemistry. Molecules. 2019;24(15).
41. Périno-Issartier S, Ginies C, Cravotto G, Chemat F. A comparison of essential oils obtained from lavandin via different extraction processes: Ultrasound, microwave, turbohydrodistillation, steam and hydrodistillation. J Chromatogr A. 2013;1305:41–7.
42. Cheng Y, Xue F, Yu S, Du S, Yang Y. Subcritical water extraction of natural products. Molecules. 2021;26(13):1–38.
43. Khajenoori M, Asl AH, Hormozi F, Eikani MH, Bidgoli HN. Subcritical water extraction of essential oils from Zataria multiflora Boiss. J Food Process Eng. 2009;32(6):804–16.
44. Asl AH, Khajenoori M. Subcritical Water Extraction. In: IntechOpen [Internet]. 2013. pp. 459–87. Available from: http://dx.doi.org/10.1039/C7RA00172J%0Ahttps://www.intechopen.com/books/advanced-biometric-technologies/liveness-detection-in-biometrics%0Ahttp://dx.doi.org/10.1016/j.colsurfa.2011.12.014
45. Hamuel JD. Phytochemicals: Extraction Methods, Basic Structures and Mode of Action as Potential Chemotherapeutic Agents. In: Phytochemicals – A Global Perspective of Their Role in Nutrition and Health. 2012. pp. 1–32.
46. Roselló-Soto E, Barba FJ, Parniakov O, et al. High voltage electrical discharges, pulsed electric field, and ultrasound assisted extraction of protein and phenolic compounds from olive kernel. Food Bioprocess Technol. 2015;8(4):885–94.
47. Li Z, Fan Y, Xi J. Recent advances in high voltage electric discharge extraction of bioactive ingredients from plant materials. Food Chem [Internet]. 2019;277(October 2018):246–60. Available from: https://doi.org/10.1016/j.foodchem.2018.10.119
48. Sonmezdag AS, Kelebek H, Selli S. Identification of aroma compounds of lamiaceae species in turkey using the purge and trap technique. Foods. 2017;6(2):1–9.
49. Izco JM, Torre P. Characterisation of volatile flavour compounds in Roncal cheese extracted by the ‘purge and trap’ method and analysed by GC-MS. Food Chem. 2000;70(3):409–17.
50. Bargah RK. Preliminary test of phytochemical screening of crude ethanolic and aqueous extract of *Moringa pterygosperma Gaertn*. J. Pharmacogn. Phytochem. 2015; 4(1):07–09.
51. Senthilkumar M. Phytochemical screening of *Gloriosa superba* L. from different geographical positions. Int. J. Sci. Res. Publ. 2013; 3(1):1–5. ISSN 2250-3153.
52. Harborne JB. ‘*Phytochemical Methods*’, London: Chapman and Hall Ltd.; 1973. pp. 49–188.

53. Bhattacharyya S, Roy S. Qualitative and quantitative assessment of bioactive phytochemicals in Gobindobhog and black rice, cultivated in West Bengal, India. Int. J. Pharm. Sci. Res. 2018; 9(9):3845–51.
54. Aryal S. Thin Layer Chromatography – Definition, Principle, Parts, Steps, Uses. In: Microbe Notes. 2022. https://microbenotes.com/thin-layer-chromatography
55. Altemimi AW, Watson DG, Kinsel M, Lightfoot DA. Simultaneous extraction, optimization, and analysis of flavonoids and polyphenols from peach and pumpkin extracts using a TLC-densitometric method. Chem. Cent. J. 2015; 9(1):1–15.
56. James J. Dubery I. Identification and quantification of triterpenoid centelloids in *Centella asiatica* (L.) urban by densitometric TLC. J Planar Chromatogr – Mod TLC. 2011;24(1):82–87.
57. Dmitrienko SG, Kudrinskaya VA, Apyari VV. Methods of extraction, pre-concentration, and determination of quercetin. J Anal Chem. 2012; 67(4):299–311.
58. Minh TN, Xuan TD, Tran H, Van TM, Andriana Y. Isolation and purification of bioactive compounds from the stem bark of *Jatropha podagrica*. Molecules 2019; 24:889. doi:10.3390/molecules24050889
59. Panda S. Rath CC. Phytochemicals as Natural Antimicrobials: Prospects and Challenges. In: Bioactive Phytochemicals: Perspectives for Modern Medicine, Gupta VK (Ed), New Delhi, India: Astral International PVT Ltd.; 2012; 1: 329–78.
60. Sasidharan S, Chen Y, Saravanan D, Sundram KM, Latha LY. Extraction, isolation and characterization of bioactive compounds from plants' extracts. Afr. J. Tradit Complement Altern Med. 2011; 8(1):1–10.
61. Shahverdi AR, Abdolpour F, Monsef-Esfahani HR, Farsam H. TLC Bioautographic assay for the detection of nitrofurantoin resistance reversal compound. J. Chromatogr. B. 2007. 850:528–30.
62. Grzelak EM, Majer-Dziedzic B, Choma IM, Pilorz KM. Development of a novel direct bioautography–thin-layer chromatography test: Optimization of growth conditions for gram-positive bacteria, *Bacillus subtilis*. J. AOAC Int. 2013; 96(2):386–91.
63. Favre-Godal Q, Queiroz EF, Wolfender J. Latest developments in assessing antifungal activity using TLC-bioautography: A review. J. AOAC Int. 2013; 96(6):1175–88.
64. Islam N, Parveen SA, Nakazawa N, Marston A, Hostettmann K. Bioautography with the fungus, *Valsa ceratosperma* in the search for antimycotic agents. Pharm Biol. 2003;41:637–40.
65. Choma IM, Grzelak EM. Bioautography in thin layer chromatography. J. Chromatogr A. 2011;1218:2684–91.
66. Choma IM. The use of thin-layer chromatography with direct bioautography for antimicrobial analysis. LCGC Eur. 2005;18:482–88.
67. Morlock G, Schwack W. Hyphenation in planar chromatography. J. Chromatogr A. 2010; 1217:6600–609.

68. Cosa P, Vlietinck AJ, Berghe DV, Maes L. Anti-infective potential of natural products: How to develop a strong in vitro 'proof-of-concept'. J. Ethnopharmacol. 2006;106:290–302.
69. Suleiman MM, McGaw LJ, Naidoo V, Eloff JN. Detection of antimicrobial compounds by bioautography of different extracts of leaves of selected South African tree species. Afr. J. Trad. CAM. 2010; 7(1):64–78.
70. Ahmad I, Aquil F. *In vitro* efficacy of bioactive extracts of 15 medicinal plants against ESβL-producing multidrug-resistant enteric bacteria. Microbiol. Res. 2007;162:264–75.
71. Shusterman AJ, McDougal PG, Glasfeld A. 'Dry- column flash chromatography'. J. Chem. Educ. 1997;74(10):1222. doi:10.1021/ed074p1222
72. Meyers CLF. Column Chromatography. In: Synthesis of Unmodified Oligonucleotides. Cur. Prot. Nucl. Acid. 2000; A.3E.1–A.3E.7.
73. Aryal, S. Column Chromatography – Definition, Principle, Parts, Steps, Uses. In: Microbe Notes. 2022. https://microbenotes.com/column-chromatography/
74. Devika R, Koilpillai J. Column chromatographic separation of bioactive compounds from *Tagetes erecta* Linn. Int. J. Pharm. Sci. Res. 2015;6(2):762–66.
75. Banu KS, Cathrine L. General techniques involved in phytochemical analysis. Int J Adv Res Chem Sci [Internet]. 2015;2(4):25–32. Available from: www.arcjournals.org
76. Heftmann F. Chromatography: Fundamentals and Application of Chromatographic and Electrophoretic Techniques. 5th edn. Amsterdam, The Netherlands: Elsevier; 1992.
77. Coskun O. Separation techniques: Chromatography. North Clin Istanb. 2016;3(2):156–60. doi: 10.14744/nci.2016.32757. PMID: 28058406; PMCID: PMC5206469.
78. Harborne JB. Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis. 2nd edn. Chapman and Hall Publishers, 3, Springer. Germany. 1998.
79. Sherma J, Zweig G. Paper Chromatography. New York: Academic Press; 1971.
80. Stahl E. Thin Layer Chromatography. Springer-Verlag, Berlin. 1965.
81. Hahn-Deinstrop E. Applied Thin Layer Chromatography: Best Practice and Avoidance of Mistakes. Weinheim, Germany: Wiley-VCH; 2000.
82. Sasidharan S, Chen Y, Saravanan D, Sundram KM, Latha LY. Extraction, isolation and characterization of bioactive compounds from plants' extracts. Afr J Tradit Complement Altern Med. 2011;8(1):1–10.
83. Burchfield AP, Storrs EE. Biochemical Applications of Gas chromatography. New York: Academic Press; 1962.
84. Hancock WS. High Performance Liquid Chromatography in Biotechnology. New Jersey: Wiley-Interscience; 1990.
85. Wisel A, Schmidt-Traub H, Lenz J, Strube J. Modelling gradient elution of bioactive multicomponent systems in non-linear ion-exchange chromatography. J Chromatogr A. 2003;1006 101–20.

86. Fritz JS, Gjerde DT. Ion Chromatography (Fourth Completely Revised and Enlarged Edition). Weinhein: Wiley-VCH Verlag GmbH; KGoA Weinhein; 2009.
87. Cummins PM, Dowling O, O'Connor BF. Ion-Exchange Chromatography: Basic Principles and Application to the Partial Purification of Soluble Mammalian Prolyl Oligopeptides. In: Walls D, Loughran ST (Eds) Protein Chromatography Methods and Protocols. New York: Springer; 2011. pp. 215–28.
88. Weiss J, Wesiss T. Handbook of Ion Chromatography. Weinheim, Germany: Wiley-VCH; 2004.
89. Draczkowski P, DM, KJ. Affinity Chromatography as a Tool for Quantification of Interactions Between Drug Molecules and Their Protein Targets. In: Dr., Magdeldin S, editors. Affinity Chromatography. InTech; 2012. pp. 275–304.
90. Paul-Dauphin S, Karaca F, Morgan TJ. Probing Size Exclusion Mechanisms of Complex Hydrocarbon Mixtures: The Effect of Altering Eluent Compositions. Energy Fuels. 2007;21(6):3484–89. doi:10.1021/ef700410e.
91. Nyiredy S. The bridge between TLC and HPLC: Overpressured layer chromatography (OPLC). TrAC Trends in Analytical Chemistry. 2001;20(2):91–101.
92. Kumar GS, Jayaveera KN, Kumar CK, Sanjay UP, Swamy BM, Kumar DV. Antimicrobial effects of Indian medicinal plants against acne-inducing bacteria. Tropical Journal of Pharmaceutical Research. 2007;6(2):717–23.
93. Parekh J, Karathia N, Chanda S. Evaluation of antibacterial activity and phytochemical analysis of *Bauhinia variegata* L. bark. African Journal of Biomedical Research. 2006;9(1).
94. Onwukaeme DN, Ikuegbvweha TB, Asonye CC. Evaluation of phytochemical constituents, antibacterial activities and effect of exudate of Pycanthus Angolensis Weld Warb (Myristicaceae) on corneal ulcers in rabbits. Tropical Journal of Pharmaceutical Research. 2007;6(2):725–30.
95. Akinyemi KO, Oladapo O, Okwara CE, Ibe CC, Fasure KA. Screening of crude extracts of six medicinal plants used in South-West Nigerian unorthodox medicine for anti-methicillin resistant Staphylococcus aureus activity. BMC Complementary and Alternative Medicine. 2005;5(1):1–7.
96. Eberhardt TL, Li X, Shupe TF, Hse CY. Chinese Tallow Tree (*Sapium sebiferum*) utilization: Characterization of extractives and cell-wall chemistry. Wood and Fiber Science. 2007 Sep 27:319–24.