Biotechnological Tools for Extraction, Identification, and Detection of Bioactive Compounds

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Ayushi Varshney, Praveen Dahiya, and Sumedha Mohan

Abstract

Plants are used as medicinal agents due to their wide range of structural diversity and pharmacological activities. The biologically active compounds that are present in plants are referred to as phytochemicals. These phytochemicals are derived from different parts of plants such as leaves, barks, seed, seed coat, flowers, roots, and pulps. The plants are the natural reservoirs of structurally diverse secondary metabolites. The extraction of bioactive compounds from the plants and their quantitative and qualitative estimation is important for the exploration of new biomolecules, which can be used in various industrial applications directly or can be used as a lead molecule to synthesize more potent compounds. This chapter highlights various methodologies used for the analysis of bioactive compounds present in the plant extracts involving the applications of chromatographic techniques such as high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), gas chromatography (GC), and high-performance thin-layer chromatography (HPTLC) and its detection through Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR), and mass spectrometry (MS). The chapter also covers the conventional techniques (Soxhlet method, cold maceration method, hydro-distillation method) for extraction of phytochemicals that generally require large amounts of organic solvents, are high energy expenditure, and are time-consuming. Hence, the new technologies of extraction viz. supercritical fluid extraction (SFC), pressurized liquid extraction (PLE), ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE) that are referred to as clean or green technologies are also discussed here. These recent techniques used to extract bioactive compounds

A. Varshney · P. Dahiya · S. Mohan (✉)
Amity Institute of Biotechnology, Amity University, Noida, Uttar Pradesh, India
e-mail: smehta1@amity.edu

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from natural sources can reduce or eliminate the use of toxic solvents and thus preserve the natural environment and its resources.

Keywords

Phytochemicals · Green technologies · Organic solvents · Industrial applications

23.1 Introduction

The extraction of new bioactive components from natural plants has now become an important aspect for the use in traditional medicines (Yuan et al. 2016). Traditional medicine and medicinal plants are widely used as a fundamental framework for maintaining good health in most developing countries, with around 80% of the world's population relying on herbal remedies (Ekor 2013). Plants possess a variety of chemical components that are utilized for the treatments of both chronic and infectious disorders (Sofowora et al. 2013). Microbial resistance to chemical compounds has special importance, which leads us toward the study of ethnopharmacognosy. Thousands of phytochemicals were discovered to be useful and possess biological activity including anticancer, antibacterial, antioxidant, antidiarrheal, analgesic, and wound healing (Sasidharan et al. 2011). Plant components, particularly phenolic compounds, carotenoids, and vitamins, which are results of plant secondary metabolism, provide resistance against several diseases (Sricharoen et al. 2016). Approximately 20% of known plant species have been used in pharmacological investigations, having a favorable impact on the healthcare system by treating cancer and other disorders (Atanasov et al. 2015). Bioactive chemicals originated from both natural and synthetic have unique metabolic or physiological effects, if their safety have been investigated. Generally, the number of bioactive compounds present in plants is affected by various factors such as the plant variety used, growing conditions, storage, and transport conditions and many more such factors.

Plants that contain important phytochemicals may act as natural antioxidants, supplementing the human body's demands (Zhang et al. 2015). Several studies have shown that antioxidants are abundant in many plants. For example, vitamins A, C, and E, as well as phenolic chemicals found in plants contain mainly flavonoids, tannins, and lignins, all behave as antioxidants (Salehi et al. 2020). Beta carotene, ascorbic acid, and various phenolic compounds play important roles in antiaging, anti-inflammation, and cancer prevention (Zhang et al. 2015). Many institutions and healthcare systems have advocated increasing the consumption of herbal/medicinal plants all around the world (Altemimi et al. 2017). Bioactive compounds have also been utilized in food additives due to their strong antioxidant properties. Antioxidants are compounds that prevent oxidation and minimize oxidative damage in plants by delaying or suppressing oxidation caused by reactive oxygen species (ROS), extending their shelf life, and improving their quality (Tan et al. 2018). Synthetic antioxidants are widely employed due to their stability and widespread

availability, but they have been linked to the consequences of mutagenesis and carcinogenesis, prompting researchers to look for antioxidants isolated from natural plant species (Soquetta et al. 2018).

The extraction of bioactive substances is influenced by several parameters, including the extraction process, raw materials, and extraction solvent (Tiwari 2015). There are two types of techniques: conventional and nonconventional. Organic solvents, heating, and agitation are all required in traditional techniques. Examples of this type of technique include Soxhlet, maceration, and hydrodistillation. Modern techniques, also known as non-conventional techniques, are green or clean procedures since they require less energy and employ organic solvents, both of which are good for the environment (Chel and Kaushik 2018). Because of the variation in the polarity of compounds, it is difficult to come up with a single strategy for extracting all of them efficiently. Generally, a good solvent possesses low toxicity, low boiling point, efficient mass transfer, preservation action, and difficulty dissociating the complex extract. The extract yield obtained also depends upon additional factors including the type of extract being used, the temperature, and the time of extraction (Silva et al. 2016). Many researchers have already looked into the use of green technology in the processing of food (Barba et al. 2016; Boussetta and Vorobiev 2014; Chemat et al. 2017; Mustafa and Turner 2011). This chapter examines the extraction of bioactive chemicals from natural plant species utilizing conventional and nonconventional energy sources because different polarity solvents are required for the identification and isolation of individual compounds. However, the focus of this chapter was on analytical procedures, which included extraction methods and the analysis and identification of bioactive chemicals present in plant extracts using a variety of techniques that included chromatographic techniques and several detection methods.

23.2 Extraction Methods of Phytochemicals

Bioactive compound extraction from plants is a realistic technique since various solvents are utilized at varying temperate conditions. Different bioactive compounds present in plants can dissolve in a particular solvent. Not all compounds can dissolve in a single solvent. Therefore, for the extraction, various solvents are required for the appropriate isolation of all active compounds. A bioactive compound extracted from plants needs further separation from its co-extractive components. Therefore, different solvents are used based on their acidity, polarity, and molecular size.

There are two types of extraction procedures used to separate distinct bioactive chemicals from plant parts:

- conventional and
- nonconventional techniques.

Conventional techniques (Soxhlet extraction, maceration method, and hydrodistillation) use organic solvents in large volumes for extraction and require

Table 23.1 Comparison of different extraction methods

Method	Solvent used	Organic solvent required	Running time	Temperature used
Conventional techniques				
Cold maceration	Aqueous and nonaqueous solvents	Large volume	Lengthy	Room temperature
Soxhlet extraction	Organic solvents	Moderate volume	Lengthy	High temperature
Hydro-distillation	Water	None	Lengthy	High temperature
Percolation	Aqueous and nonaqueous solvents	Large volume	Lengthy	Room temperature but sometimes required high heat
Decoction	Water	None	Moderate	High temperature
Non-conventional techniques				
Pressurized liquid extraction	Aqueous and nonaqueous solvents	Small volume	Short	High temperature
Supercritical fluid extraction	Supercritical solvent (mainly S-CO_2), sometimes with modifier	Very little or none	Short	Room temperature
Ultrasound-assisted extraction	Aqueous and nonaqueous solvents	Very little or none	Short	Room and sometimes high temperature
Microwave-assisted extraction	Aqueous and nonaqueous solvents	Moderate or none	Short	Room temperature

additional time for the process to run, whereas non-conventional extraction techniques (supercritical fluid extraction, extraction with pressurized liquid, ultrasound-assisted extraction, and microwave-assisted extraction) offer some advantages in the practice of less organic solvent, shorter duration of extraction time, and higher selectivity. A summary of the various extraction method used for natural bioactive compounds is listed in Table 23.1.

23.2.1 Conventional Extraction Techniques

23.2.1.1 Soxhlet Extraction

The word Soxhlet was named after "Franz Ritter von Soxhlet," a German agricultural chemist. This is the most suitable method for the continuous extraction of solid–liquid solvent by using high temperatures (Rasul 2018). Soxhlet apparatus is a specific glass-designed refluxing unit, particularly used for the extraction of organic solvents. In the Soxhlet apparatus, the dried and powdered plant material is placed in a thimble constructed of filter paper. The Soxhlet apparatus was fitted into a round bottom flask containing some extract volume and a reflux condenser.

The solvent in the round bottom flask was heated and boiled upon requirement, and the vapors flow up through the side of the tubes, then condense by the condenser, and finally drop into the thimble containing plant material. When the solvent reaches the highest of the tube, it drains out into the flask, thus taking away the portion of the compounds that have been extracted. This whole process is repeated for around 10 cycles for each solvent until the plant material becomes colorless. The resulting solvent extract is filtered, concentrated in a vacuum evaporator, and preserved in vials at 4 °C for further experimentation (Ingle et al. 2017). This technique is a well-established, continuous, highly efficient extraction process that requires less time and solvent than other conventional techniques. The high temperature and longer time duration in Soxhlet extraction will increase the chance of chemical decomposition (Zhang et al. 2018).

23.2.1.2 Cold Maceration Method

Maceration is the process of grinding a sample to improve its surface area for optimum solvent mixing. It is one of the ancient and extensively used techniques used for herbal preparation. Maceration involves solid–liquid extraction of plant material. This method is used to extract essential oils and bioactive substances from various plant sections (Azmir et al. 2013). In this method, the powdered plant material is placed in a closed flask containing solvent. The flask is allowed to stand for 2–3 days with periodic shaking in an incubator shaker. The solvent is diffused into the cell wall to dissolve the chemical components present in plant material during this time. This process is called molecular diffusion. After a few days, the liquid is filtered and evaporated to get the solid residue from the solvent. If water is taken as a solvent for extraction, a slight amount of alcohol needs to be added to prevent the growth of the microorganisms (Pandey and Tripathi 2014). The maceration process involves three basic principles:

1. Plant material was initially powdered with the help of a grinder
2. This increases the surface area of the plant material to allow proper contact between the solvent and the plant material
3. Further, the liquid is drained off but the solid residue was concentrated and collected as an extract.

During the maceration process, occasional shaking is an important step as it facilitates proper extraction by increasing diffusion of the powdered material with the solvent to remove chemical components from the surface for more extraction yield (Devgun et al. 2010).

23.2.1.3 Hydro-Distillation

Hydro-distillation is the most common and old conventional technique that does not require organic solvents to extract plant materials. In this technique, plant material is seal packed in a steel compartment containing water in significant volume and then allowed to boil. On the other hand, fumes are directly injected into the plant samples. Hot water and fumes are significant contributors to the isolation of free bioactive

components from plant tissue. The vapor mixture of water and oil is condensed by indirect cooling through the water. It is considered the most effective method to extract the essential oil from different parts of the medicinal and aromatic plants. The yield of the extract through this method usually relies upon the weight, size, and nature of raw material and volume of water (Parikh and Desai 2011). This process involves three chief physicochemical characteristics: hydro-diffusion, hydrolysis, and heat decomposition. Due to high temperature during extraction, some volatile compounds may vanish, restricting their use for the extraction of thermolabile compounds. The principle behind this technique involves isotropic distillation at atmospheric pressure and heating of water, oil molecules, and other solvents during the extraction process. The advantages and disadvantages of conventional techniques are presented in Table 23.2.

Table 23.2 Advantages and disadvantages of conventional techniques

Extraction method	Advantages	Disadvantages
Soxhlet extraction	• At a time, a large quantity of plant material can be extracted	• Samples were continuously exposed to high temperature for a longer time; hence, the risk of thermal destruction of certain compounds cannot be counterbalanced
	• Solvent can be reused again for the extraction	• It takes a longer time for the extraction process
	• Sometimes, it does not require filtration after the extraction process	• Labor-intensive
	• Does not rely upon the matrix type	• It allows manipulations of restricted variables
	• Simple technique	
Cold maceration	• Simple technique	• Long extraction time usually takes up to 2 weeks
	• No utensils or equipment required	• Pure extraction is not possible
	• No need for a skilled operator	• Very slow and time-consuming process
	• Energy-saving process	• Large volume of solvent is required for extraction
	• Suitable for less potent and inexpensive drugs	
Hydro-distillation	• Higher yield of oil content	• Complete extraction is not done
	• Volatile oil compounds are less prone to hydrolysis and polymerization	• Impart an unpleasant odor to the essential oil
	• Loss of polar compounds can be minimized if refluxing can be controlled	• The continuous exposure to high temperatures can cause hydrolysis of some important components of the essential oil, for example, esters.
	• Oil quality can be improved by steam and water distillation	• Temperature control is problematic as it causes variation in the rates of distillation
	• Cheap and environment-friendly technique	• More space and more fuel are required
	• No organic solvent is required	• Uneconomical process

23.2.2 Nonconventional Extraction Techniques

23.2.2.1 Supercritical Fluid Extraction (SFC)

Supercritical fluid extraction is categorized by the transformation of gas in the supercritical fluid by the change in temperature and pressure. The critical temperature is the highest temperature at which a gas can be converted to a liquid by raising the pressure, while the critical pressure is the highest pressure at which a liquid can be converted to a gas by increasing the temperature (Soquetta et al. 2018). The main transport mechanism in the supercritical solvent phase is convection as a mass transfer operation (Silva et al. 2016). This extraction is generally used to isolate nonpolar bioactive constituents including carotenoids and lipids. This process of extraction is fast, selective, and can be utilized for a small number of samples (Oroian and Escriche 2015). With the use of analytical chromatographic techniques such as gas chromatography (GC) and supercritical fluid chromatography, the main benefit of this extraction process is the ability to detect unknown components contained in the sample (SFC) (Silva et al. 2016). This technique involves two important steps:

- Chemical components first solubilize in the solid matrix and then separate in the supercritical solvent.
- The solvent penetrates through the packed bed and dissolves the solid matrix's components.

The solvent then exits the extractor with a decrease in pressure and increase in temperature, and extracted compound becomes solvent-free (Silva et al. 2016). Supercritical fluids possess low surface tension, low viscosity, disperse easily within the solid matrix, and increased extraction efficiency compared to the liquid solvent used in conventional extraction processes (Pouliot et al. 2014).

23.2.2.2 Extraction with Pressurized Liquid

This method includes transferring solutes from a solid matrix through a separation technique. Liquid solvents are utilized at high pressure and temperature, resulting in a lowering in the solvent's surface tension. As a result, the solvent is capable of penetrating deeper into the cell. Matrix pores are indeed a type of pore found in the matrix. The process causes the matrix to be disrupted. As a result, the mass transfer of the analyte from the sample including solvent rises (Garcia-Castello et al. 2015). The solvents are chosen based on the solubility characteristics of the necessary solute. The physicochemical features of pressurized solvents, such as density, diffusivity, viscosity, and dielectric constant, which may be changed by adjusting the temperature and pressure of the extraction system, make them extremely versatile (Pronyk and Mazza 2009). Extraction using pressurized liquid is appealing because it provides quick extraction with less solvent usage. This method has been used to extract anthocyanins from a variety of plants with great success (Santos et al. 2012).

23.2.2.3 Ultrasound-Assisted Extraction (UAE)

Ultrasound is a type of sound wave that has a frequency range of 20 kHz to 100 MHz. Cavitation is a phenomenon caused by ultrasound-assisted extraction, which requires generation, bubbles' expansion, and deflation (Azmir et al. 2013). UAE is a versatile extraction method that has been used for a long time and may be used on a variety of materials and analytes derived from different forms of samples. Ultrasounds can speed up heat and mass transfer by disrupting plant cell walls, resulting in a better release of target substances from a variety of natural sources (Roselló-Soto et al. 2015).

Ultrasound extraction involves two primary physical phenomena:

1. Diffusion through the cell wall
2. Rinsing the cell content after the walls have been disrupted.

The activity of ultrasound is regulated by temperature, pressure, frequency, and sonication time (Rajha et al. 2015).

Ultrasound is a relatively simple extraction procedure when compared to other extraction techniques; it is versatile, flexible, and requires a low initial investment. Among other molecules and biomaterials, ultrasound has been used to extract polysaccharides, essential oils, proteins, peptides, dyes, pigments, and bioactive substances (Briones-Labarca et al. 2015; Tiwari 2015). This phenomenon can occur in two ways: indirectly or directly. When ultrasound is delivered directly to the medium without the use of a barrier, such as a probe device, the intensity increases 100-fold. The waves must travel through the water until they reach the sample when using an ultrasonic water bath for indirect sonication (Kek et al. 2013). The use of ultrasonic energy has been contemplated to be a promising method for extracting bioactive components from plant samples. It boosts the mass transfer coefficient, speeds the kinetics, and raises the final concentration of bioactive compounds (Zhang 2014).

23.2.2.4 Microwave-Assisted Extraction

Microwave-assisted extraction is a term that encompasses both microwave and classical solvent extraction. It increases the kinetics of extraction by boiling the solvents and plant tissue with a microwave (Delazar et al. 2012). The minute microscopic residues of moisture that occurs in plant cells are the focus for heating in dried plant material. Evaporation occurs as a result of the microwave effect heating the moisture inside the plant cell, putting great pressure on the cell wall. Due to the pressure, the cell wall is forced from within, and the cell wall ruptures. Exudation of active ingredients from burst cells happens as a result of enhancing phytoconstituent production.

MAE has grabbed researchers' interest as a method for extracting bioactive chemicals from a wide range of plants and natural remnants (Anokwuru et al. 2011). Microwaves emit electromagnetic radiation with frequencies ranging from 300 MHz to 300 GHz and wavelengths ranging from 1 cm to 1 m. An electric field and a magnetic field are both present in electromagnetic waves. These are referred to as two fields that are perpendicular to each other. Microwaves were first used to heat

items that could absorb heat transform a portion of the electromagnetic energy into heat. Commercial microwaves generally use 2450 MHz frequency, which equates to an energy output of 600–700 W (Ballard et al. 2010).

Advanced approaches have recently become popular to limit bioactive compound loss without increasing extraction time. As a result, microwave-assisted extraction is a useful technology in a variety of sectors, particularly in the medicinal plant field. Furthermore, this method reduced the number of biological components lost during extraction (Suzara et al. 2013). Because of its potential to minimize both time and extraction solvent volume, microwave-assisted extraction (MAE) has been employed as an alternative to traditional procedures for the extraction of antioxidants (Suzara et al. 2013). The primary goal of MAE is to heat the solvent and extract antioxidants from plants using a smaller amount of these solvents (Altemimi et al. 2017).

23.3 Identification Tools for Phytochemicals

Identification of bioactive compounds and their characterization from plant extracts are still challenging as plant extracts contain a mixture of compounds possessing different polarities. These compounds are isolated using different chromatographic techniques including TLC, HPTLC, paper chromatography, column chromatography, gas chromatography, and HPLC. Chromatographic techniques help in obtaining pure compounds and thus are among the various identification tools for the phytochemical analysis. Chromatography occupies a leading position as it contributes to the highly accurate analysis of organic compounds. It is majorly based on the interaction between the mobile phase, stationary phases, and the mixture components (Sasidharan et al. 2011). The mixture components are separated in the two different phases in chromatography. The pure compound isolated is further utilized for the analysis of the structure and its biological activities. In chromatography, the molecules are separated based on size, shape, and charge (Heftmann 1992).

23.3.1 Thin-Layer Chromatography (TLC)

TLC is considered to be the latest version of paper chromatography and is a widely used laboratory technique. TLC includes adsorbent materials such as alumina, silica, and cellulose on inert materials like glass, plastic, or aluminum foil (Kumar et al. 2013). In TLC, a small amount of sample is applied to a starting point on the chromatography plate, which is allowed to dry. Further, the prepared plate is placed in the developing chamber with a small amount of solvent. The level of solvent should be such that it is not touching the level at which the sample was applied. Along with the solvents, the sample components will move at different speeds on the plate, and thus, the mixture is separated. Highly soluble components will travel farthest on the plate when compared to the less soluble ones (Singhal et al. 2009). R_f (retention factor) can be analyzed by dividing the distance traveled by an individual

compound from its original position by the overall distance traveled by the solvent. TLC is mainly employed for the separation of various components such as amino acids, alkaloids, phenols, steroids, and proteins, utilizing different solvent systems and adsorbents. Adsorbents like silica gel are mainly utilized for the separation of amino acids, alkaloids, sugars, lipids, etc. Adsorbents such as cellulose powder, starch, and Sephadex are used for the separation of mainly amino acids and proteins. Similarly, alkaloids, steroids, phenols, vitamins, and carotenoids are separated using adsorbents aluminum and celite. Once the components are separated, the chromatographic plate is sprayed using different spray reagents like iodine vapors and potassium dichromate, which will confirm the compounds present based on the color development after the spray reagent (Chauhan and Dahiya 2016).

TLC also serves as a tool for screening antimicrobial agents via bioautography. Bioautographic techniques include contact bioautography, agar overlay bioautography, and direct TLC bioautographic detection (Wagman and Bailey 1969).

23.3.2 Contact Bioautography

Agar diffusion or contact bioautography includes the diffusion of antimicrobial agents (Sherman 2008). Developed chromatogram on TLC plate is then placed face down on agar for a certain duration to have proper diffusion. Further, the agar layer is incubated and checked for zones of clearance corresponding to the color spots on the chromatographic plates. The time of incubation for the growth is in between 16 and 24 h, and it can be reduced to approximately 5 h by spraying it with reagent 3,5-tetrazolium chloride. Contact bioautography is a familiar technique for the microbiologist to screen the antimicrobial agents. Diffusion of individual components from chromatogram to agar plate and establishing a closed contact in between the plate and the agar are some of the disadvantages associated with the technique. Various polyether antibiotics, bromoditerpene antibiotic, and several antifungal agents were isolated using contact bioautography (Jayasinghe et al. 2003). The disadvantages such as sensitivity and low resolution can be overcome by using advanced chromatographic tools like HPTLC (high-performance thin-layer chromatography). HPTLC use also reduces the time required and solvent used. Ramirez et al. (2003) observed multiple antibiotic residues in cow's milk by HPTLC contact bioautography.

23.3.3 Direct TLC Bioautography

Direct TLC bioautography includes the spraying/dipping of the bacterial or fungal suspension on the developed TLC plate at a specific concentration (10^6 CFU/mL). The prepared bioautogram is further incubated in a dark and humid chamber for 24–48 h at room temperature. The bioautogram was further sprayed with 2,3,5-triphenyl tetrazolium chloride (TTC) and incubated at room temperature for 4 h. Microbial growth inhibition appeared in the form of the zone of clearance against a

pink backdrop. The R_f values corresponding to the spots possessing clearance zone were determined (Dahiya and Manglik 2013). Direct bioautography is found suitable for both spores forming fungal cultures like *Aspergillus* and *Penicillium* sp. and for bacteria such as *Bacillus* sp., *Staphylococcus* sp., and *E. coli*. TLC bioautography suggests that aloe vera extracts possess anti-MRSA potential, which is maybe because of the presence of tannins in the extracts (Dahiya and Purkayastha 2012a). The bioactive components of juniper essential oil were evaluated against potential inhibitors, *E. coli* and *Staphylococcus aureus* 2, via TLC bioautography, which confirmed the bioactive compound tannin responsible for the antibacterial activity when sprayed with 10% $FeCl_3$ spray reagent (Purkayastha et al. 2012). Similar results were reported by Dahiya and Purkayastha (2012b) for *Psoralea corylifolia* essential oil tested against Enterococcus sp. and Klebsiella pneumonia. TLC bioautography revealed the presence of more than one bioactive component at different R_f values (0.10–0.15 and 0.70–0.83) on plate B against *Enterococcus* sp. and R_f value (0.12–0.15) against *Klebsiella pneumonia*. The significant antimicrobial activity was found to be due to tannins when sprayed with 2% $FeCl_3$ spray solution.

23.3.4 Agar Overlay Bioautography

This technique combines the features of direct and contact bioautography. This technique involves the covering of chromatogram with molten, seeded agar medium. Once the agar solidifies, the bioautogram is sprayed with tetrazolium dye and incubated, which will allow the visualization of inhibition/growth bands. The microorganism acts on the tetrazolium salt and converts it to intensely colored formazan (Saxena et al. 1995). This technique is widely used for bacterial (*E. coli*, *Pseudomonas aeruginosa*, *S. aureus*, etc.) and yeast cultures like *Candida albicans*. Using this technique, the antibacterial activity of isoflavonoid, carotenoids, alkaloids, and several antimicrobial compounds was isolated and characterized (Dewanjee et al. 2015; Zaidi and Dahiya 2015). Manhas and Dahiya (2017) reported hexane extract of *Michelia champaca* leaf, which showed significant antibacterial activity against *S. aureus* 1. Bioautography confirmed three active compounds at different Rf values. The observed inhibition was possibly due to more than one active compound, which is overlapping possibly due to the solvent system used.

23.3.5 High-Performance Liquid Chromatography (HPLC)

HPLC is an analytical technique that is highly flexible as the mobile and stationary phase, and the elution technique can be modified depending on the analysis. It is also known as high-pressure liquid chromatography and can separate the compounds based on their interaction with the column and the solvent phase. There are two types of HPLC that are normal and reverse-phase types. In the normal phase, the solid phase is more polar when compared with the mobile phase, whereas the reverse phase typically includes a more polar mobile phase compared to the stationary phase.

HPLC helps identify and separate organic/inorganic solutes from the sample and phytochemical analysis of plant extracts. Quantification of berberine, an alkaloid obtained from *Tinospora cordifolia*, was studied by HPLC using acetonitrile and water in the ratio of 60:40. Comparative studies related to berberine content obtained from the wild type and micropropagation were studied by Sivakumar et al. (2014) and observed that methanolic extract of micropropagated one gave a higher quantity of berberine (1.2%) as compared to only 0.2% in the wild type.

23.3.6 Gas Chromatography (GC)

Gas chromatography (GC), commonly known as gas–liquid chromatography (GLC), is a technique for separating mixtures into components based on component redistribution over a stationary phase or support material in the form of a liquid, solid, or a combination of both, and a gaseous mobile phase. Many pharmacologically active ingredients of herbal remedies are known to be volatile chemical molecules. As a result, the examination of volatile chemicals in gas chromatography is critical in the analysis of herbal medicines. Because measurements of the area under the peaks revealed on the GC trace are directly related to the quantities of the individual components of the original mixture, GC offers both qualitative and quantitative data on plant compounds. The GC equipment can be set up so that the separated components are subjected to spectral or other analysis after separation. GC is routinely connected to mass spectrometry (MS), and the combined GC-MS equipment has emerged as one of the most important techniques for phytochemical analysis in recent years (Garud et al. 2017). Not only is a chromatographic fingerprint available with the GC-MS, but also information about qualitative and quantitative compositions. This will be very helpful in determining the link between various elements and their pharmacology. As a result, GC-MS is the preferred method for analyzing volatile chemical components in herbal medicines nowadays (Revathy et al. 2011).

23.3.7 High-Pressure Liquid Chromatography (HPLC)

Liquid chromatography is chromatography that uses a liquid as the mobile phase. The "eluent" is the liquid employed as the mobile phase, and the stationary phase is usually a solid or a liquid. The sample solution is supplied to a porous stationary phase, and the mobile phase is delivered at a greater pressure via the column, causing separation depending on the solute's affinity for the stationary phase. The development of HPLC is aided by the need for a higher degree of separation and faster analysis, which is met by refining the stationary phase packing material to a size of 3–10 m and eluent delivery via a high-pressure pump. HPLC instruments comprise mobile phase reservoir, a pump, an injector, a separation column, and a detector as defined by Gupta and Shanker (2008).

The mobile solvent is delivered by the solvent delivery pump, and it is introduced into the mobile phase or onto the chromatographic bed by the injector. The column is the most significant part of the HPLC system because it separates the sample components as it goes through them. The column is a stainless-steel tube with a diameter of 3–5 mm that is filled with silica gel and measures 10–30 cm in length. The separated components in the column will be quantified and recorded in the computer system using a detector. The phytochemical components will be provided as a fingerprint with peaks by the system.

HPLC is the newest chromatographic technology to join the repertoire of phytochemists. This method is mostly utilized for nonvolatile chemicals, such as higher terpenoids, phenolics of all types, alkaloids, lipids, and sugars. It is best for substances that can be identified in the ultraviolet or visible spectrum. It is best for substances that can be identified in the ultraviolet or visible spectrum. As a result, HPLC has seen the most widespread use in the analysis of herbal medicines in recent decades. The most common column used in the analytical separation of herbal medicine is reversed-phase (RP) columns.

HPLC is a very adaptable technology since its mobile phase, stationary phase, and elution process may all be changed to satisfy a variety of analysis needs (IUPAC 2006). The two forms of HPLC are the normal phase and reverse phase, which are distinguished by the stationary phase being more polar than the mobile phase in the normal phase and vice versa in the reverse phase. Normal phase chromatography is used to separate lipophilic compounds such as oils, fats, and lipids. Reverse-phase chromatography is extensively used for phytochemical fingerprinting of medicinal plants since most plant extracts are polar compounds.

23.3.8 High-Performance Thin-Layer Chromatography (HPTLC)

HPTLC is planar chromatography, which is highly sophisticated with advanced features of detection and separation. In this technique, the separation is because of partition/adsorption or both and majorly depends on the solvent and adsorbents used. Using a sample applicator, a sample (0.1–0.5 μL) is applied on a TLC plate designed for HPTLC on the silica gel for the normal phase and C8 and C18 for the reverse phase. The chromatogram is developed, which can be viewed at different wavelengths. In HPTLC, the analysis time is greatly reduced, and efficiency is high due to the smaller particle size generated. It is controlled by software that can develop a peak corresponding to the active compound, and the result can be analyzed (Ingle et al. 2017). Various advancements like the use of densitometers, high-resolution sorbents, UV/Visible/fluorescence scanners, and the use of new software with advanced features make the analysis and detection process more efficient and sensitive resulting in the replacement of HPLC and GC by HPTLC technique. This tool is mainly utilized for the separation and detection of bioactive compounds from medicinal plants and the standardization of herbal drugs. The bulky size, large space needs, technical operator requirement, cost factor, etc., are some of the disadvantages associated with its use.

23.4 Detection of Bioactive Compounds-Fourier Transform Infrared Spectroscopy (FTIR)

Infrared spectroscopy using Fourier transforms is a useful method for identifying functional groups in plant extracts. It aids in molecular identification and structural determination (Ingle et al. 2017). FTIR samples can be made in a variety of ways. Placing one drop of the sample between two plates of sodium chloride is the simplest method for liquid samples. Between the plates, the drop produces a thin film. Solid materials can be milled with potassium bromide (KBr) to form a thin pellet that can be examined. Solid samples can also be dissolved in a solvent such as methylene chloride, and then, a few drops of the solution are dropped onto a single high attenuated total reflectance (HATR) plate and the spectra are recorded in percentage transmittance.

23.4.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

Physical, chemical, and biological aspects of the matter are determined via nuclear magnetic resonance spectroscopy. The one-dimensional approach is commonly employed; however two-dimensional NMR techniques could be applied to achieve the intricate structure of the molecules. The state of being solid, the molecular structure of solids is determined via NMR spectroscopy. Radiolabelled C NMR is used to determine which carbon types are present in a compound. H-NMR is utilized to determine the types of hydrogen contained in a compound and the connections between the hydrogen atoms (Ingle et al. 2017).

The magnetic properties of particular atomic nuclei, such as the nucleus of the hydrogen atom, the proton, carbon, and a carbon isotope, are the focus of NMR. Many researchers have been able to examine molecules using NMR spectroscopy, which records the differences between the various magnetic nuclei and so provides a clear picture of where these nuclei are in the molecule. Furthermore, it will show which atoms are present in adjacent groups. It will eventually be able to determine how many atoms are present in each of these situations. Several attempts have been undertaken in the past to isolate individual phenols using preparative or semi-preparative thin-layer chromatography, liquid chromatography, and column chromatography, with the structures identified afterward by NMR offline (Kemp 1991).

23.4.2 Mass Spectrometry (MS)

In mass spectrometry, organic molecules are bombarded with electrons or lasers and transformed to charged ions, which are highly energetic. The relative abundance of a fragmented ion is plotted against the mass/charge ratio of these ions in a mass spectrum. Relative molecular mass (molecular weight) may be estimated with high accuracy using mass spectrometry, and an exact molecular formula can be derived by knowing where the molecule has been fragmented (Christophoridou et al. 2005).

Mass spectrometry is a strong analytical technique for determining the structure and chemical characteristics of molecules, as well as identifying novel chemicals and quantifying known substances. The molecular weight of a sample can be determined using the MS spectrum.

This method is commonly used for structural elucidation of organic compounds, peptide, or oligonucleotide sequencing and monitoring the presence of previously identified compounds in complex mixtures with high specificity by simultaneously defining the molecular weight and a diagnostic fragment of the molecule.

23.5 Conclusion

The increasing demand for the extraction of plant bioactive components involves a never-ending search for efficient extraction methods. Since bioactive chemicals found in plant material are multi-component combinations, their extraction, identification, and determination still remain a challenge. The extraction of bioactive components is a difficult process that can be achieved using a variety of methods. The traditional approaches are based on the solubility of the solute in the solvent from plant materials. As a result, it frequently uses a substantial amount of solvent to extract the target chemical, even though sometimes aided with increased temperature and mechanical stirring or shaking. It has been proved that replacing traditional procedures with green technology can improve extraction yields, minimize processing time, and prevent the environmental damage caused by toxic solvents. Various studies suggested that a mixture of strategies might help in the optimization of these processes. Bioactive substances such as phenolics, flavonoids, lignins, and anthocyanins are implicated in the protection of various diseases such as cancer, neurological problems, cardiovascular disease, and hypertension. Extracting and optimizing these chemical compounds from plant material are critical. The traditional or conventional method involves hazardous organic solvents that cause a threat to the environment. Each process has its own advantages and disadvantages, and extraction technique selection might be depending on the plant sample employed as a source of bioactive compound. It may be concluded that no single extraction method is appropriate, and each extraction approach is specific to the plants. The measurement of extraction efficiency is also influenced by the use of standard procedures. On the other hand, the growing economic importance of bioactive compounds and bioactive compound-rich commodities may lead to the development of more complex extraction technologies in the future.

References

Altemimi A, Lakhssassi N, Baharlouei A, Watson DG, Lightfoot DA (2017) Phytochemicals: extraction, isolation, and identification of bioactive compounds from plant extracts. Plants 6(42):1–23. https://doi.org/10.3390/PLANTS6040042

Anokwuru CP, Anyasor GN, Ajibaye O, Fakoya O, Okebugwu P (2011) Effect of extraction solvents on phenolic, flavonoid, and antioxidant activities of three Nigerian medicinal plants. Nat Sci 9:53–61

Atanasov AG, Waltenberger B, Pferschy-Wenzig EM, Linder T, Wawrosch C, Uhrin P, Temml V, Wang L, Schwaiger S, Heiss EH, Rollinger JM, Schuster D, Breuss JM, Bochkov V, Mihovilovic MD, Kopp B, Bauer R, Dirsch VM, Stuppner H (2015) Discovery and resupply of pharmacologically active plant-derived natural products: a review. Biotechnol Adv 33(8): 1582–1614. https://doi.org/10.1016/J.BIOTECHADV.2015.08.001

Azmir J, Zaidul ISM, Rahman MM, Sharif KM, Mohamed A, Sahena F, Jahurul MHA, Ghafoor K, Norulaini NAN, Omar AKM (2013) Techniques for extraction of bioactive compounds from plant materials: a review. J Food Eng 117(4):426–436. https://doi.org/10.1016/j.jfoodeng.2013.01.014

Ballard TS, Mallikarjunan P, Zhou K, O'Keefe S (2010) Microwave-assisted extraction of phenolic antioxidant compounds from peanut skins. Food Chem 120:1185–1192

Barba FJ, Zhu Z, Koubaa M, Sant'Ana AS, Orlien V (2016) Green alternative methods for the extraction of antioxidant bioactive compounds from winery wastes and by-products: a review. Trends Food Sci Technol 49:96–109. https://doi.org/10.1016/J.TIFS.2016.01.006

Boussetta N, Vorobiev E (2014) Extraction of valuable biocompounds assisted by high voltage electrical discharges: a review. Comptes Rendus Chimie 17(3):197–203. https://doi.org/10.1016/J.CRCI.2013.11.011

Briones-Labarca V, Plaza-Morales M, Giovagnoli-Vicuña C, Jamett F (2015) High hydrostatic pressure and ultrasound extractions of antioxidant compounds, sulforaphane, and fatty acids from Chilean papaya (*Vasconcellea pubescens*) seeds: effects of extraction conditions and methods. LWT Food Sci Technol 60(1):525–534

Chauhan BS, Dahiya P (2016) Evaluation of *In-vitro* antimicrobial potential and phytochemical analysis of Spruce, Cajeput and Jamrosa essential oil against clinical isolates. Int J Green Pharm 10(1):27–32

Chel A, Kaushik G (2018) Renewable energy technologies for sustainable development of energy efficient building. Alexandria Eng J 57(2):655–669. https://doi.org/10.1016/J.AEJ.2017.02.027

Chemat F, Rombaut N, Meullemiestre A, Turk M, Perino S, Fabiano-Tixier AS, Abert-Vian M (2017) Review of green food processing techniques. Preservation, transformation, and extraction. Innov Food Sci Emerg Technol 41:357–377

Christophoridou S, Dais P, Tseng LH, Spraul M (2005) Separation and identification of phenolic compounds in olive oil by coupling high-performance liquid chromatography with post-column solid-phase extraction to nuclear magnetic resonance spectroscopy. J Agric Food Chem 53: 4667–4679

Dahiya P, Manglik A (2013) Evaluation of the antibacterial, antifungal, and antioxidant potential of essential oil from *Amyris balsamifera* against multi-drug resistant clinical isolates. Asian J Pharmaceut Clin Res 6(5):57–60

Dahiya P, Purkayastha S (2012a) Phytochemical screening and antimicrobial activity of some medicinal plants against multi-drug resistant bacteria from clinical isolates. Indian J Pharm Sci 74(5):443–450

Dahiya P, Purkayastha S (2012b) Phytochemical screening and antibacterial potentiality of essential oil from *Psoralea corylifolia* Linn. Int J Biosci Biochem Bioinformatics 2(3):188–191

Delazar A, Nahar L, Hamedeyazdan S, Sarker SD (2012) Microwave-assisted extraction in natural products isolation. Methods Mol Biol 864:89–115

Devgun M, Nanda A, Ansari SH, Swamy SK (2010) Recent techniques for extraction of natural products. Res J Pharma Tech 3(3):644–649

Dewanjee S, Gangopadhyay M, Bhattacharya N, Khanra R, Dua TK (2015) Bioautography and its scope in the field of natural product chemistry. J Pharmaceut Anal 5(2):75–84

Ekor M (2013) The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. Front Pharmacol 4:177. https://doi.org/10.3389/FPHAR.2013.00177

Garcia-Castello EM, Rodriguez-Lopez AD, Mayor L, Ballesteros R, Conidi C, Cassano A (2015) Optimization of conventional and ultrasound-assisted extraction of flavonoids from grapefruit (*Citrus paradisi* L.) solid wastes. LWT Food Sci Technol 64(2):1114–1122

Garud AM, Jadhav VM, Kadam VJ (2017) Chromatographic analysis of phytopharmaceuticals - a review. Int J Pharm Sci Rev Res 44(1):174–178

Gupta MM, Shanker K (2008) Process-scale high-performance liquid chromatography for medicinal and aromatic plants. In: Khanda SS, Khanuja SP, Longo G, Rakesh DD (eds) Extraction technologies for medicinal and aromatic plants. International Center for Science and High Technology, Trieste

Heftmann F (1992) Chromatography: fundamentals and application of chromatographic and electrophoretic techniques, 5th edn. Elsevier, Amsterdam

Ingle KP, Deshmukh AG, Padole DA, Dudhare MS, Moharil MP, Khelurkal VC (2017) Phytochemicals: extraction methods, identification, detection of bioactive compounds from plant extracts. J Pharmacogn Phytochem 6(1):32–36

Jayasinghe L, Kumarihamy BMM, Jayarathna KHRN et al (2003) Antifungal constituents of the stem bark of *Bridelia retusa*. J Phytochem 62:637–647

Kek SP, Chin NL, Yusof YA (2013) Direct and indirect power Ultrasound-assisted pre-osmotic treatments in convective drying of guava slices. Food Bioprod Process 91(4):495–506

Kemp W (1991) Energy and the electromagnetic spectrum. In: Organic spectroscopy. Macmillan Press, London, pp 1–7

Kumar S, Jyothirmayee K, Sarangi M (2013) Thin-layer chromatography: a tool of biotechnology for isolation of bioactive compounds from medicinal plants. Int J Pharmaceut Sci Rev Res 18(1): 126–132

Manhas N, Dahiya P (2017) *In vitro* antimicrobial activity and phytochemical screening of leaf and stem extracts of *Michelia champaca* Linn. Int Food Res J 24(6):2672–2676

McNaught AD, Wilkinson A (eds) (2006) Compendium of chemical terminology - the gold book. Blackwell Scientific Publications, Oxford. IUPAC (International Union of Pure and Applied Chemistry). http://goldbook.iupac.org

Mustafa A, Turner C (2011) Pressurized liquid extraction as a green approach in food and herbal plants extraction: a review. Anal Chim Acta 703(1):8–18. https://doi.org/10.1016/J.ACA.2011.07.018

Oroian M, Escriche I (2015) Antioxidants: characterization, natural sources, extraction, and analysis. Food Res Int 74:10–36

Pandey A, Tripathi S (2014) Concept of standardization, extraction, and pre-phytochemical screening strategies for the herbal drugs. J Pharmacogn Phytochem 2(5):115–119

Parikh JK, Desai MA (2011) Hydro-distillation of essential oil from Cymbopogon flexuosus. Int J Food Eng 7(1):1–11

Pouliot Y, Conway V, Leclerc PL (2014) Separation and concentration technologies in food processing. In: Food processing: principles and applications, 2nd edn. Wiley, New York, NY, pp 33–60. https://doi.org/10.1002/9781118846315.ch3

Pronyk C, Mazza G (2009) Design and scale-up of pressurized fluid extractors for food and bioproducts. J Food Eng 95(2):215–226

Purkayastha S, Narain R, Dahiya P (2012) Evaluation of antimicrobial and phytochemical screening of Fennel, Juniper and Kalonji essential oils against multi-drug resistant clinical isolates. Asian Pac J Trop Biomed 2(3):S1625–S1629

Rajha HN, Boussetta N, Louka N, Maroun RG, Vorobiev E (2015) Effect of alternative physical pre-treatments (pulsed electric field, high voltage electrical discharges, and ultrasound) on the dead-end ultrafiltration of vine-shoot extracts. Sep Purif Technol 146:243–251

Ramirez A, Gutiérrez R, Diaz G et al (2003) High-performance thin-layer chromatography-bioautography for multiple antibiotic residues in cow's milk. J Chromatogr B Anal Technol Biomed Life Sci 784(2003):315–322

Rasul MG (2018) Conventional extraction methods use in medicinal plants, their advantages, and disadvantages. Int J Basic Sci Appl Comput 2(6):10–14

Revathy S, Elumalai S, Benny M, Antony B (2011) Isolation purification and identification of curcuminoids from turmeric (*Curcuma longa*) by column chromatography. J Exp Sci 2(7): 21–25

Roselló-Soto E, Koubaa M, Moubarik A, Lopes RP, Saraiva JA, Boussetta N et al (2015) Emerging opportunities for the effective valorization of wastes and by-products generated during the olive oil production process: non-conventional methods for the recovery of high-added value compounds. Trends Food Sci Technol 45(2):296–310

Salehi B, Machin L, Monzote L, Sharifi-Rad J, Ezzat SM, Salem MA, Merghany RM, El Mahdy NM, Kılıç CS, Sytar O, Sharifi-Rad M, Sharopov F, Martins N, Martorell M, Cho WC (2020) Therapeutic potential of quercetin: new insights and perspectives for human health. ACS Omega 5(20):11849–11872. https://doi.org/10.1021/ACSOMEGA.0C01818

Santos DT, Veggi PC, Meireles MAA (2012) Optimization and economic evaluation of pressurized liquid extraction of phenolic compounds from Jabuticaba skins. J Food Eng 108(3):444–452

Sasidharan S, Chen Y, Saravanan D, Sundram KM, Yoga Latha L (2011) Extraction, isolation and characterization of bioactive compounds from plants' extracts. Afr J Tradit Complem Altern Med 8(1):1–10

Saxena G, Farmer S, Towers GHN, Hancock REW (1995) Use of specific dyes in the detection of antimicrobial compounds from crude plant extracts using a thin layer chromatography agar overlay technique. Phytochem Anal 6(3):125–129

Sherman J (2008) Planar chromatography. Anal Chem 80:4253–4267

Silva RPFF, Rocha-Santos TAP, Duarte AC (2016) Supercritical fluid extraction of bioactive compounds. TrAC Trends Anal Chem 76:40–51

Singhal S, Singhal N, Agarwal S (2009) Thin layer chromatography. In: Kasture AV, Mahadik KR, Wadodkar SG, More HN (eds) Pharmaceutical analysis – instrumental methods, vol II, 1st edn. Nirali Prakashan, Pune, p 270

Sivakumar V, Sadiq AM, Danarajan MS, Jayanthi M (2014) HPLC method for quantification of berberine in wild and micro propagated *Tinospora cordifolia* – an important ayurvedic medicinal plant. Int J Pharm Res Sch 3(2):432–439

Sofowora A, Ogunbodede E, Onayade A (2013) The role and place of medicinal plants in the strategies for disease prevention. Afr J Tradit Complement Altern Med 10(5):210. https://doi.org/10.4314/AJTCAM.V10I5.2

Soquetta MB, Terra LDM, Bastos CP (2018) Green technologies for the extraction of bioactive compounds in fruits and vegetables. CyTA J Food 16(1):400–412. https://doi.org/10.1080/19476337.2017.1411978

Sricharoen P, Limchoowong N, Techawongstien S, Chanthai S (2016) A novel extraction method for β-carotene and other carotenoids in fruit juices using air-assisted, low-density solvent-based liquid–liquid microextraction and solidified floating organic droplets. Food Chem 203:386–393. https://doi.org/10.1016/J.FOODCHEM.2016.02.093

Suzara S, Costa DA, Gariepyb Y, Rochaa SCS, Raghavanb V (2013) Spilanthol extraction using a microwave: calibration curve for gas chromatography. Chem Eng Trans 32:1783–1788

Tan BL, Norhaizan ME, Liew WPP, Rahman HS (2018) Antioxidant and oxidative stress: a mutual interplay in age-related diseases. Front Pharmacol 9:1162. https://doi.org/10.3389/FPHAR.2018.01162

Tiwari BK (2015) Ultrasound: a clean, green extraction technology. TrAC Trends Anal Chem 71: 100–109

Wagman GH, Bailey JV (1969) Use of silicic acid–glass fiber sheets for bioautography of antimicrobial substances. J Chromatogr 41:263–264

Yuan H, Ma Q, Ye L, Piao G (2016) The traditional medicine and modern medicine from natural products. Molecules 21(5):559. https://doi.org/10.3390/MOLECULES21050559

Zaidi S, Dahiya P (2015) *In vitro* antimicrobial activity, phytochemical analysis and total phenolic content of essential oil from *Mentha spicata and Mentha piperita*. Int Food Res J 22(6): 2440–2445

Zhang XN (2014) Comparative studies on the chemical components and pharmacological effects of *Lonicera macranthoides* Hand-Mazz. and *Lonicera japonica* Thunb. Master's thesis, Southwest University

Zhang YJ, Gan RY, Li S, Zhou Y, Li AN, Xu DP, Bin LH, Kitts DD (2015) Antioxidant phytochemicals for the prevention and treatment of chronic diseases. Molecules 20(12): 21138. https://doi.org/10.3390/MOLECULES201219753

Zhang QW, Lin LG, Ye WC (2018) Techniques for extraction and isolation of natural products: a complete comprehensive review. Chin Med 13(20):1–12. https://doi.org/10.1186/s13020-018-0177-x