Natural Product Extraction Techniques

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Conventional extraction techniques, including Soxhlet extraction, maceration, percolation, and decoction, are known to be time-, solvent-, and energy-intensive. In contrast, various non-conventional techniques for the extraction process, such as supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), and ultrasound-assisted extraction (UAE), have been developed to increase the yield of bioactive compounds and address the aforementioned issues. These contemporary techniques have demonstrated their sustainability compared to conventional ones. Low-temperature and environmentally friendly extraction techniques have gained popularity for producing high-quality bioactive extracts. UAE, in particular, has attracted considerable interest due to its advantages over conventional extraction methods, which include greater extraction efficiency, preservation of bioactive compound stability, shorter extraction time, and industrial application. Thus, non-conventional extraction techniques like SFE, MAE, and UAE offer sustainable and efficient alternatives to conventional methods, allowing for increased bioactive compound yield, shorter extraction times, and improved stability, making them popular choices for high-quality extraction in low-temperature and environmentally friendly approaches.

natural product extraction extracting natural products

1. Introduction

Researchers are increasingly interested in the use of plant-derived, bioactive extracts due to the beneficial effects of their phytochemical constituents on human health. Phytochemical constituents, primarily polyphenols, are synthesized as secondary metabolites by plants through diverse metabolic pathways within plant cells [1]. Additionally, the extraction of bioactive compounds from animal tissues, such as beef fat and deer antler, remains popular, necessitating specialized extraction procedures. Extraction is a technique utilized in natural product research to separate bioactive compounds from various natural materials. Both plant and animal tissues are capable of yielding a variety of bioactive extracts, which can be prepared using a vast array of extraction techniques.

2. Extraction

Extraction is a procedure that uses selective solvents to separate the pharmaceutically active components of plant or animal tissues from the inactive or inert portions. Polar solvents (e.g., water, ethanol, and methanol), intermediate-polar solvents (e.g., acetone, methylene chloride, and ethyl acetate), and nonpolar solvents (e.g., n-hexane, ether, and chloroform) are frequently used in the extraction of natural products [2]. The majority of the

active ingredients in a crude extract tend to act synergistically. Purification of biologically active substances is not always required. To save time and expense on purifying bioactive compounds from crude extracts, the standardized extract can be used as the formula's active ingredient [3]. The quality and quantity of bioactive constituents found in plant and animal materials are largely dependent on the selection of an appropriate extraction technique. During the extraction process, the solvents move into the solid raw materials and solubilize compounds with similar polarity in accordance with the principle of like dissolves like. In addition to employing an appropriate extraction method, the selection of an appropriate solvent is crucial. Moreover, extraction methods are continually modified [4].

There are two broad categories of extraction techniques: conventional and non-conventional (modern technique). Soxhlet, maceration, percolation, decoction, and hydro-distillation are the most frequently employed conventional extraction techniques in small research areas or Small and Medium-Sized Enterprises (SMEs) manufacturing facilities. However, these techniques have a variety of disadvantages, including lengthy extraction times, high costs, low extraction selectivity, poor extraction efficiency, high solvent consumption, and bioactive compound degradation due to prolonged exposure to high extraction temperatures. To overcome the limitations of conventional extraction methods, novel extraction techniques such as SFE, MAE, and UAE have been developed. These techniques can increase separation efficiency, reduce the use of raw materials, solvents, and energy, and have minimal environmental impact. In addition, the use of "green" solvents, which have numerous advantageous properties, such as being completely biodegradable, recyclable, noncorrosive, noncarcinogenic, and nonozone-depleting, enables the production of extracts that are recognized as safe and preferred by consumers [5].

2.1. Conventional Extraction Methods

2.1.1. Soxhlet Extraction

Soxhlet extraction is the most effective technique for the continuous extraction of solid raw materials using a hot solvent. Except for the extraction of thermolabile compounds, Soxhlet extraction is a general and well-established technique with superior performance to other conventional extraction techniques. The powdered solid substances are placed in a thimble within the Soxhlet apparatus. A round-bottomed flask containing the solvent and a reflux condenser is attached to the apparatus. The Soxhlet extraction procedure is as follows: (1) The solvent is heated by reflux. (2) Solvent vapor ascends the side tube, condenses in the condenser, and falls into the thimble. (3) Extraction of the solid matrix with a fresh solvent (4) From the extraction chamber, solutes are transferred to the reservoir. (5) The procedure is repeated until the extraction is complete [6].

2.1.2. Maceration

Maceration is a method of solid–liquid extraction. Traditionally, maceration was used to recover bioactive compounds from plant or animal materials by combining solvents with or without heat and agitation or shaking to enhance mass transfer and the solubility of compounds. The solvent is added to the closed vessel containing the powdered solid matrix. A long time is allowed for extraction (varying from several hours to days), and a sufficient

amount of time is allowed for the solvent to diffuse through the plant cell wall or animal tissue in order to solubilize the chemical constituents present in those materials. The process occurs solely via molecular diffusion [7].

2.1.3. Percolation

The most common technique for extracting active ingredients for the preparation of tinctures and fluid extracts is percolation. Typically, a percolator (a narrow, cone-shaped vessel with open ends) is utilized. The powdered solid materials are moistened with an adequate amount of solvent and allowed to stand for several hours in a tightly sealed container before being packed, and the percolator's top is sealed. An additional solvent is added to form a thin layer above the mass, and the mixture is macerated for an extended period of time in a closed percolator. The percolator's outlet is then opened, and the liquid extract is allowed to slowly drip. Additional solvent is added as necessary until the percolate reaches approximately three-quarters of the required volume. The extract is then pressed, the required volume of solvent is added to the percolate, the combined liquid is clarified by filtration or by standing, and then decanted [3].

2.1.4. Decoction

A decoction is a water-based preparation that can be used to extract water-soluble compounds from medicinal plant material. When dealing with tough, fibrous plants, barks, and roots, decoction is the preferred method. In this procedure, the plant material is typically reduced to small fragments or powder for effective dissolution. The liquid extract is produced by boiling plant material in water. On occasion, aqueous ethanol or glycerol can be substituted for water. The amount of water used in this method is dependent on the plant material's hardness [4].

2.2. Non-Conventional Extraction Methods

2.2.1. Microwave-Assisted Extraction (MAE)

Microwaves are electromagnetic waves with a frequency between 300 MHz and 300 GHz that are non-ionizing [8]. Ionic conduction and dipole rotation, which are most frequently present simultaneously, are two phenomena that govern microwave heating because of their direct impact on polar materials and solvents. Ionic conduction is the electrophoretic migration of ions under the influence of an alternating electric field. The solution's resistance to the migration of ions generates friction, which eventually causes the solution to heat up. Dipole rotation is the realignment of a molecule's dipoles in a rapidly varying electric field. Microwaves produce heat by interacting with polar compounds in the plant and animal matrix, such as water and certain organic molecules, via ionic conduction and dipole rotation [4][9]. Both ethanol and methanol will absorb less microwave energy than water, but their overall heating efficiency will be greater than that of water. On the other hand, hexane, and other less polar solvents (low dielectric constant), such as chloroform, are microwave-transparent and do not generate heat [8]. In MAE, heat and mass transfer occur in the same direction, producing a synergistic effect that accelerates extraction and increases extraction yield. Evaporation of water increases the pressure within plant and animal cells, resulting in swelling and eventual rupture. This facilitates the leaching of constituents from ruptured cells into the surrounding solvent. MAE

can also be carried out without a solvent. In a dry or solvent-free MAE, the sample water acts as a solvent and enables cell lysis.

2.2.2. Supercritical Fluid Extraction (SFE)

A fluid in conditions of pressure and temperature beyond its critical point is known as a “supercritical fluid,” which is a dense fluid with characteristics between those of a gas and a liquid. Its viscosity and diffusivity are similar to those of a gas, while its density is comparable to that of a liquid. Consequently, a supercritical fluid can function as a liquid-like solvent, except with enhanced mass transfer kinetics [10]. SFE relies on the solubilizing properties of supercritical fluid, which can be obtained by applying pressure and temperature above the critical point of the sample mixture. Although carbon dioxide (CO_2) is the favored extraction solvent for nonpolar substances, the polarity of supercritical carbon dioxide (SC-CO_2) can be increased by adding a miscible polar solvent (like hexane, methanol, ethanol, isopropanol, and dichloromethane) as a modifier or co-solvent. Ethanol is recommended as a co-solvent due to its lower toxicity and miscibility with CO_2. Due to the properties of SC-CO_2, which include high selectivity, relative affordability, safety, non-flammability, non-corrosiveness, inertness (least alteration of bioactive substances to maintain their therapeutic or functional properties), and the capability to extract thermally unstable compounds, it is extensively used to extract many natural products, including non-polar compounds such as lipids and volatile oils, by optimizing the extraction pressure and temperature [4].

2.2.3. Ultrasound-Assisted Extraction (UAE)

Ultrasound refers to any sound wave or acoustic energy that exceeds the human hearing threshold of 20 kHz. For the extraction of natural products, ultrasonication treatments typically range from 20 to 100 kHz and 10 to 1000 W/cm^2 in power density. The ultrasonic wave travels through large quantities of materials based on their mechanical and physical characteristics, such as their composition and structure. As a result of the ultrasonic waves’ combination of compression and rarefaction, molecules are displaced from their original position [11]. The UAE principle relies on cavitation effects, which enhance mass transfer, and close interaction between the solvent and plant or animal tissues. The cavitation bubbles are formed when the acoustic pressure produced by rarefaction cycles exceeds the attractive forces of the liquid medium’s molecules. The cavitation bubbles will expand and contract during the rarefaction and compression cycles if the acoustic pressure is totally inadequate. If the bubbles do not attain the critical size for implosion or collapse, the phenomenon is referred to as stable cavitation, which lessens the cavitation effect.

The ultrasonic devices used in the UAE can be briefly categorized into three groups based on their mode of action: ultrasonic bath mode, sonotrode (ultrasonic probe) mode, and a special-design mode derived from the first two modes [12]. The ultrasonic cleaning bath is the most commonly used and readily available method. Typical components include a transducer, a container, a timer, and a heater. The sound energy derived from the electric current and converted by the transducers can be transferred into the bath’s medium. The extraction vessel in small and lab-scale UAE is typically a flask or tube that is partially submerged in water and held in a specific position to maximize cavitation. The ultrasonic bath is considered the extraction vessel at larger scales. A mechanical stirrer is always required in this circumstance to prevent the matrix from floating or sinking and escaping the ultrasonic

irradiation. From the foregoing, it is clear that bath sonication is an indirect contact method employing an ultrasonic bath of high intensity. A water bath receives ultrasonic energy, which is then transferred to a vessel or sample container.

2.3. Parameters Affecting the Quality of Extracts Obtained from UAE

2.3.1. Frequency

It is important to note that ultrasonic frequency is a critical parameter that can regulate the physical effects of burst bubbles [13]. Due to the inverse relationship between ultrasonic frequency and rarefaction phase duration, the implosion of cavitation bubbles is hampered at high frequencies. More mass transfer resistance is provided by the huge number of bubbles generated at high frequency [14].

2.3.2. Power and Amplitude

The rate at which sound energy is emitted per unit of time is called ultrasound power, and it varies depending on the mass of the solvent, the solvent's heat capacity, and the time interval over which the power is measured [15]. The transfer of energy to the extraction liquid during UAE has also been quantified in terms of amplitude percentage. Increases in power and amplitude cause greater vibration in the solvent medium, which in turn increases the solvent's penetration and extraction efficiency [16].

2.3.3. Solvent

The polarity of the solvent is one of the factors influencing the type and quantity of extracted molecules as well as the extraction yield. Various solvents with high and low polarity, such as water, alcohols (ethanol and methanol), acetone, etc., have been used in the UAE method to extract numerous bioactive compounds. The selection of the solvent depends primarily on the solubility of the required substances [17].

2.3.4. Processing Time

Increasing sonication time has an impact similar to increasing power and temperature. Increasing sonication duration initially increases yield, but when time is extended further, yield decreases. In response to an initial increase in sonication time, the cavitation impact of the ultrasound increased the tissue matrix's swelling, breakdown, and pore creation. All of these attributes increase the exposure of the solute and solvent, resulting in a better extraction yield. However, prolonged ultrasound exposure causes structural damage to some solutes and diminishes the extraction yield, especially due to oxidative degradation [18].

2.3.5. Extraction Temperature

Similar to the effect caused by an increase in power and amplitude, an initial increase in temperature increases the extraction yield, but further increases in temperature decrease the yield. Increasing the temperature decreases the solvent's viscosity, which increases the solvent's diffusivity in the tissue matrix, resulting in a greater extraction

yield [14]. The formation of cavitation bubbles is influenced by temperature and solvent type, resulting in cell rupture and subsequent release of bioactive compounds [19].

2.3.6. Solvent-to-Solid Ratio

The solvent-to-solid ratio is the volume of solvent to the amount of sample extracted [14]. The extraction yield increases with increasing solvent-to-solid ratio until a particular limit is reached, after which it decreases. At low solvent-to-solid ratios, the viscosity of the sample-solvent mixture is high, which makes the cavitation effect more difficult because the negative pressure in the rarefaction cycle must achieve a greater cohesive force in the high-viscosity mixture. With an increase in the initial ratio of solvent to solid, the viscosity and concentration of the mixture decrease, resulting in a greater cavitation effect.

3. Techniques for Separation and Chemical Characterization of Extracts

As part of the overall quality and biosafety assessment, chemical characterization of extracts involves the identification and quantification of the extract's chemical components. The study of biological extracts derived from diverse plants, animals, or microorganisms differs from the study of isolated (pure) compounds. The extracts are typically multicomponent mixtures of active, partially active, and inert substances, and their activities are rarely exerted on a single target. It is commonly believed that multiple components in the extract can contribute to a synergistic response and that the therapeutic response is sometimes related to conventional preparation and administration. The composition of the extract varies based on the extraction technique and the raw materials employed (such as growing conditions, development stage, and harvesting protocols) [20].

Thin-layer chromatography (TLC)-densitometry, for instance, is one of the most straightforward chromatographic separation techniques used for qualitative and quantitative analysis. Nevertheless, TLC can only detect substances that fluoresce under long-wavelength ultraviolet light (360 nm) or absorb under short-wavelength ultraviolet light (254 nm). Additionally, certain compounds can be detected by spraying reagents that undergo a chemical reaction with a compound on the TLC plate, transforming it into a colored compound [21]. In order to purify or isolate large quantities of natural products, preparative high-performance liquid chromatography (preparative HPLC) typically requires big columns, substantial sample loading, a high mobile phase volume, and elevated flow rates in an HPLC system. To obtain small amounts of pure compounds, it is sometimes possible to conduct isolation with analytical-scale HPLC systems. In general, bioassay screening requires small quantities of pure substances, whereas structure elucidation requires large quantities. Normal phase (suitable for separation of low-polar or lipophilic natural products), reversed phase (suitable for isolation of high-polar or hydrophilic natural products), size exclusion (suitable for separation of lower molecular weight compounds), and ion exchange (suitable for separation of amines or acids) are among the various HPLC modes currently available for purifying most classes of natural products [22]. Gas chromatography (GC) is one of the most essential and widespread techniques for the separation of natural products, especially terpenes and other high-temperature-stable volatile compounds. If natural products are nonvolatile, they must undergo silylation, acylation, alkylation, or other suitable derivatization reactions to make

them volatile. Chromatographic techniques for GC are categorized as adsorption chromatography (interaction between a solid stationary phase and the gas phase) and partition chromatography (interaction between a liquid stationary phase and the gas phase). To accomplish a good separation in GC, the column must be carefully chosen based on the type of natural products, the number of crude extracts, and their complexity. For instance, polyethylene glycol used as the stationary phase of a GC column is appropriate for separating compounds with polar functional groups, such as organic acids and volatile oils [23].

For structure elucidation or identification, ultraviolet-visible (UV-Vis) spectrophotometry, Fourier transform infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry (MS) has been utilized. The UV-Vis spectrophotometer provides information about the structure of conjugated or aromatic molecules. Different functional groups of natural products can be identified using FTIR spectra. One-dimensional (1D) NMR experiments, such as proton and carbon-13 NMR, convey information regarding the qualitative and quantitative composition of natural products. Initially, they are employed to ascertain the chemical structure of various classes of natural products, including amino acids, organic acids, sugars, phenolic compounds, alcohols, and esters [24]. In addition to 1D-NMR experiments, numerous 2D-NMR experiments have been developed for structure elucidation by providing information on the correlations of nuclei via scalar or dipolar couplings, revealing the connections between proton and proton and proton and carbon-13 or heteroatom within molecular structures [25]. Despite the fact that compounds are absolutely identified through the interpretation of 2D-NMR experiments, 1D-NMR experiments still provide valuable information. For example, the number of carbon-13 signals helps to identify the structures of terpenes made up of isoprenoid units; the increase in area under the proton signal and the decrease in carbon-13 signals help to identify symmetric molecules; and specific proton and carbon-13 chemical shifts help to confirm the presence of aromatic rings or carbonyl groups in molecular structures. In addition, proton NMR spectra disclose the presence of certain functional groups, including hydroxyl and primary and secondary amine groups [25][26]. Therefore, NMR can be used to guide the isolation of natural products. MS can determine the molecular weights and elemental compositions of compounds with diverse chemical and physical properties, making it essential for natural product structure elucidation. If a molecular ion is created with enough excitation energy, it will undergo a sequence of unimolecular processes to produce fragment ions with relative abundances specific to its structure. The mass spectra can reveal the functional groups and how they are linked to generate a unique molecular structure by analyzing fragmentation patterns [27].

Before chromatographic separation, sample preparation is crucial for the elimination of interferences and unintended substances. There are numerous techniques for sample preparation, including solid-phase extraction (SPE) and liquid-liquid extraction (LLE). In general, compounds of interest are extracted using LLE in order to separate compounds based on their relative solubilities in two distinct immiscible liquids, typically aqueous (polar) and an organic solvent (non-polar) [28]. LLE enhances the concentration of the substance of interest, resulting in a proportional increase in the ratio of the area beneath the proton NMR signal (integration value) in the structure of the substance of interest to that of other substances [29].

Liquid or gas chromatography-coupled spectroscopic techniques such as high-performance liquid chromatography-ultraviolet (HPLC-UV), high-performance liquid chromatography-mass spectrometry (HPLC-MS), high-performance

liquid chromatography-nuclear magnetic resonance (HPLC-NMR), gas chromatography-flame ionization detection (GC-FID), and gas chromatography-mass spectrometry (GC-MS) are hyphenated techniques that have been used to simultaneously separation and identification of the constituents of an extract [30][31][32]. These techniques permit the rapid determination of known natural products using a minute amount of sample material. To detect different classes of compounds using HPLC-UV, different detection wavelengths are required. With HPLC-UV analysis, only compounds containing chromophores can be detected [32]. HPLC-NMR is a viable approach for routinely analyzing complex extracts. The combination of carbon-13 NMR and proton NMR provides detailed structural information on all organic compounds. However, the method is costly, maintenance-intensive, and relatively insensitive [32][33].

It is well known that the chemical composition of natural extracts is complex. The structure elucidation and quantification of biologically active compounds in extracts necessitate specialized equipment, particularly the hyphenated technique. As mentioned above, combining a separation technique with an online spectroscopic detection technology produces the hyphenated technique [30][31][32][33][34]. The separation part, typically an HPLC or GC, and detection part, one or more spectroscopic detectors such as UV–vis, MS, and NMR, are coupled for simultaneous separation, structural identification, and quantification of components present in a complex extract [35]. As part of the optimization of extraction, the hyphenated technique was used to prove the chemical structure and analyze the content of active substances in the extract (experimental response). Examples of the implementation of hyphenated techniques to optimize extraction are provided in the subsections that follow.

3.1. High-Performance Liquid Chromatography-Diode Array Detection (HPLC-DAD)

The photodiode array (PDA) detector, also known as the diode array (DAD) detector, is an advanced type of UV-vis spectrophotometer that can be coupled to an HPLC to produce the HPLC-DAD. It is remarkably useful for analyzing natural compounds containing chromophores, such as polyphenols, flavonoids, and aromatic alkaloids. For each peak on an HPLC chromatogram, a PDA detector provides three-dimensional (3D) UV-vis data, typically consisting of UV-vis absorption spectra from 190 to 500 nm. The data are accessible in the domains of time, concentration, and wavelength. Therefore, it is possible to rapidly anticipate unique absorption regions corresponding to particular compounds, which represent the peak's purity [35][36]. Organic solvents used as components of a mobile phase have UV absorption cutoff wavelengths. The solvent itself absorbs all light at wavelengths that are shorter than the cutoff. When selecting a solvent with significant UV absorption at the wavelength at which measurements are performed, complications arise. In such instances, the substance's signal and the solvent's signal will overlap, resulting in an inaccurate determination [35]. The following are examples of optimizing extraction using HPLC-DAD for the determination of bioactive compounds. The developed HPLC-DAD method provided a rapid and dependable method for identifying and quantifying bioactive scopoletin in a noni extract (*Morinda citrifolia* L). The optimal yield of scopoletin was obtained by extracting at an increased temperature of 60 °C for 12 min with ethanol as the extraction solvent and a solid-to-solvent ratio of 1:30 (*w*/*v*) [37].

3.2. High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS)

An HPLC-MS combines the chemical separation capability of an HPLC with the ability of a mass spectrometer to detect and confirm the molecular identity selectively. The sample eluted from an HPLC column is then accelerated through either a quadrupole or an ion trap mass analyzer, and the ions are identified according to their mass-to-charge (*m*/*z*) ratios. MS is one of the most sensitive and selective chemical analysis techniques, and it provides data on the molecular weight and fragmentation pattern of natural products. MS provides invaluable information for confirming the identities of natural products, particularly if they are known compounds [35][38]. In a mass spectrometer, matrix-assisted laser desorption/ionization (MALDI), electrospray ionization (ESI), and atmospheric pressure chemical ionization (APCI) are common ionization techniques. MALDI's usefulness for analyzing heterogeneous samples makes it an attractive technique for the mass analysis of complex extracts. It can measure masses up to 300,000 Da. However, matrix background, which can be a problem for natural products with a mass of less than 700 Da. ESI and APCI are soft ionization techniques that can analyze a practical mass range of up to 70,000 Da and 2000 Da, respectively, with high sensitivity. However, ESI has a relatively low salt tolerance, whereas APCI may decompose samples prior to vaporization through thermal desorption [38][39].

3.3. High-Performance Liquid Chromatography-Diode Array Detection-Mass Spectrometry (HPLC-DAD-MS)

Hyphenated techniques, particularly HPLC coupled to DAD and MS, have proven to be extraordinarily beneficial for optimizing the extraction of natural products [40]. Using a reliable HPLC–DAD–ESI-MS/MS method, 25 compounds in the extract of *Aloe barbadensis* Miller were characterized based on their spectral data or comparison with the reference standards. It was also used to check the quality of *A. barbadensis* extracts by measuring the amount of 8-C-glucosyl-7-O-methyl-(*S*)-aloesol, aloe-emodin, aloin A, and aloenin B [40]. For multi-response optimization of phenolic antioxidants from white tea (*Camellia sinensis* L. Kuntze), the contents of catechin and epicatechin in the extracts were determined by HPLC-DAD, and the phenolic profile of the extracts was identified by HPLC–DAD–Q-TOF–MS/MS in the optimal condition on the basis of their HPLC retention time, detection wavelength, and mass spectra based on the values of mass-to-charge ratio (*m*/*z*) and comparisons with fragmentation patterns. White tea extract is a good source of antioxidants, particularly flavan-3-ols, for dietary supplements, according to the results [41].

3.4. High-Performance Liquid Chromatography-Nuclear Magnetic Resonance (HPLC-NMR)

HPLC-NMR has improved the ability to isolate and interpret the structures of complex natural products. The structural information derived from a hybrid HPLC-NMR technique may be adequate for identifying unidentified extract constituents. In general, reversed-phase HPLC columns with binary or tertiary solvent mixtures yield insufficient NMR spectra due to the presence of strong solvent signals and weak natural product signals on the same spectra. To suppress solvent signals, pre-saturation, soft-pulse multiple irradiations, or water suppression enhancement can be utilized [42]. The development of efficient solvent suppression techniques enables the measurement of reversed-phase HPLC-proton NMR spectra [43]. 2D total correlation spectroscopy (2D-TOCSY) permits the determination of all spin system neighbors simultaneously. So long as there is coupling between each

intervening proton, correlations between distant protons will be observed. It can provide information on four to five bonds if each successive proton is coupled.

Trends and applications of various modes of HPLC-NMR operation, such as online-flow mode, stop-flow mode, and loop-storage mode, were discussed, as well as practical applications in natural product analysis. In online-flow mode, the HPLC is directly coupled to the NMR probe, and spectra are continually obtained while peaks of natural products are eluting. The NMR flow cell's short exposure time for eluted peaks reduces its sensitivity. Depending on solvent properties, solvent composition changes during elution may alter sample and solvent chemical shifts [44]. In stopped-flow mode, the NMR probe's interface is directly connected to the HPLC detector's outflow. It has a higher signal-to-noise ratio and enables the detection of only particular peaks. As soon as the eluted peaks reach the NMR detection device, the HPLC pump must be halted until the NMR signals are gathered. The primary disadvantage of this mode may be its dependence on separations resolved for retention times longer than 2 min [45]. In loop-storage mode, the HPLC detector outlet must be directly connected to the sample storage loops. In these loops, the eluted peak is immediately collected. Upon completion of the separation, the HPLC pump can be used to force the previously collected peaks into the NMR flow cell using a valve.

According to RSM-based extraction optimization for *Azadirachta indica* leaves, proton NMR-based metabolite profiling of the obtained extract revealed the presence of numerous potential bioactive compounds that can be considered a source of α-glucosidase inhibitors and antioxidants; therefore, it can also be used as active constituents of functional foods [46]. Metabolomics techniques rely on identifying as many small molecules as possible and have been used to characterize the relationships between the metabolome (the complete set of small natural molecules) and the corresponding genetic makeup, source, quality, or other biological characteristics. NMR is the primary analytical technique currently employed for metabolomics research. The optimal extraction of anthelmintic metabolites from *Lysiloma latisiliquum* leaves was optimized by comparing eight distinct solvent systems. As an internal standard, trimethylsilylpropanoic acid was used to examine the proton-NMR spectra of tannin-free extracts in methanol-d_4. The principal component analysis (PCA) of the proton NMR data indicated hydrophilic solvents as optimal for the extraction of *L. latisiliquum* leaves with potent anthelmintic activity and revealed that the bioactive metabolites are high-polarity, glycosylated substances. The results of the study support proton-NMR metabolomics as a useful technique for the elucidation of bioactive metabolites in plant extracts in the absence of prior phytochemical studies [47]. *Croton membranaceus* root-bark extracts were analyzed using the high-performance liquid chromatography-solid phase extraction-nuclear magnetic resonance (HPLC-SPE-NMR) technique. After post-column dilution of the eluate with water, the separated peaks were trapped on SPE cartridges and eluted with acetonitrile-d_3 into an NMR probe. Scopoletin, the main extract ingredient, was trapped more efficiently on an SPE phase (polystyrene-type polymer) than on a C18 phase.

3.5. High-Performance Liquid Chromatography-Diode Array Detection-Solid Phase Extraction-Nuclear Magnetic Resonance (HPLC-DAD-SPE-NMR)

For identifying radical-scavenging compounds in *Rhaponticum carthamoides* extracts, a high-performance liquid chromatography apparatus coupled to a solid-phase extraction unit and NMR detector (LC-DAD-SPE-NMR) was

used, along with online radical-scavenging detection. Without the need for offline chromatographic stages, the technique enabled the detection and identification of particular compounds. UV spectra, 1D and 2D proton and carbon-13 NMR spectra, and MS spectra were used to reveal the structures of flavonoid glycosides. The SPE apparatus was adaptable, enabling analyte focusing, and multi-trapping. This performs better than HPLC-NMR with partially deuterated liquids. Future integration of LC-DAD-SPE-NMR with online bioassays and online MS will accelerate high-throughput screening identification and dereplication [48].

3.6. High-Performance Liquid Chromatography-Photodiode Array Detection-Mass Spectrometry-Solid-Phase Extraction-Nuclear Magnetic Resonance (HPLC-PDA-MS-SPE-NMR)

A high-performance liquid chromatography-photodiode array detection-mass spectrometry-solid-phase extraction-nuclear magnetic resonance (HPLC-PDA-MS-SPE-NMR) could be used to directly determine the structures of unrefined extracts containing minute amounts of natural products. The results demonstrated that post-column SPE is an effective method of analyte concentration and accumulation not only for NMR but also for circular dichroism (CD) spectroscopy. Thus, the combination of the hyphenated technique and CD enabled rapid detection of (*R*)-(-)-gossypol in *Thespesia danis* twigs. Consequently, an HPLC-PDA-MS-SPE-NMR-CD system is envisioned as an efficient natural product discovery instrument, as the chirality of the compounds is a crucial property in terms of biological activity [49].

3.7. Gas Chromatography-Flame Ionization Detection (GC-FID)

GC-FID is characterized by its simplicity, dependability, relatively high sensitivity, and excellent linearity for a broad range of natural products. It consists of four main parts: a carrier gas source, a sample introduction system, a GC column, and a flame ionization detector (FID). FID uses the flame that results from the combustion of hydrogen and air. When compounds are introduced to a flame via the FID jet, a large number of ions are produced. A polarizing voltage is applied to the FID collector, which will attract the ions and produce a current proportional to the amount of analyte in the flame. The carrier, hydrogen, and oxygen flows must be properly adjusted for optimal FID operation [50].

GC-FID was utilized for the simultaneous quantitative analysis and chemical characterization of volatile oils extracted from *Alpinia oxyphylla* fruits by hydrodistillation. According to the results, the volatile oils contained *p*-cymene and nootkatone as significant components. A GC-FID fingerprinting method was developed, and chemometrics was used to analyze the profiles. The profiles of the vast majority of samples were uniform and stable. These findings demonstrated that GC-FID determination and fingerprinting analysis are highly effective, organized, and practicable methods for the thorough profiling of volatile oils in *A. oxyphylla* [51]. Using GC-FID, the volatile oils of 12 species of *Eucalyptus* were identified and quantified in terms of their constituents and relative concentrations. Monoterpenes, α-pinene and 1,8-cineol were the most abundant secondary metabolites isolated from *Eucalyptus globulus*. Large quantities of 1,8-cineol are also detected in *Eucalyptus mycrocoris*, *Eucalyptus resinifera*, and *Eucalyptus urophylla*. However, the chromatographic profiles of these species were significantly

distinct. In conclusion, the validated GC-FID method can accurately quantify the 1,8-cineol content in various *E. globulus* matrices and aid in monitoring the entire manufacturing process.\

3.8. Gas Chromatography-Mass Spectrometry (GC-MS)

Due to its simplicity of use, high sensitivity, and ability to effectively separate extracts, GC is one of the most prevalent chromatographic techniques for separating volatile substances. In gas-liquid chromatography (GLC), the mobile phase is a carrier gas, and the stationary phase is a liquid with a high boiling point that is adsorbed on an inert solid support. Since GLC is the most versatile and selective gas chromatography separation technique, the majority of natural product studies will concentrate on GLC. Components of the extract are distributed between a stationary phase and a gaseous mobile phase that transports the components through the stationary phase. The affinity of each compound with the stationary phase varies due to their diverse properties and structures. Consequently, under the same propelling force, the retention duration of various components in the column differs, resulting in their movement out of the column in various orders [23]. GC-MS is a technique that combines the characteristics of GC and MS in order to separate and identify volatile substances within an extract. Instruments such as quadrupole, ion trap, and time-of-flight (TOF) mass analyzers are widely used today. Quadrupoles are the most common mass analyzers because they can accommodate a broad *m*/*z* range and are relatively inexpensive. High-resolution time-of-flight mass spectrometry (HRMS) is exceptional in its capacity for accurate mass measurement of high-resolution fragment ions [52].

Betel leaf (*Piper betle* L.) var. *Bangla* is a volatile, oil-rich plant belonging to the *Piperaceae* family that is traditionally employed as an herbal medication. The optimization of volatile oil extraction from betel leaves and biochemical characterization is of great interest for industrial applications. The GC-MS analysis uncovered numerous volatile compounds with a variety of antimicrobial activities. Different percentages of eugenol, estragole, linalool, α-copaene, anethole, chavicol, and caryophyllene were discovered to be abundant in the betel leaf extract [53]. Using GC/GC-MS, the plant *Aethionema sancakense* was identified as a new species based on its essential oil and fatty acid compositions. Linoleic acid, α-humulene, camphene, and heptanal were identified as the principal essential oil components of the aerial part of *A. sancakense* [54].

4. Quality Control, Standardization and Biological Activity of Extracts

Natural products have distinct characteristics compared to conventional synthetic molecules. They are distinguished by their structural diversity and complexity. In general, natural substances have a larger chemical structure, a greater number of sp^3 carbon and oxygen, fewer nitrogen and halogen, a greater number of hydrogen bond acceptors and donors, higher hydrophilicity (a lower n-octanol-water partition ratio), and greater molecular rigidity than those of synthetic compounds [55][56]. Although the selection of raw materials of plants and animals for study is sometimes based on their traditional medicinal uses, this is not always the case; other characteristics, such as chemical diversity and the absence of previous studies, may also justify further study. Numerous natural

products have been reported to possess a variety of intriguing and important biological activities, including antioxidant, antimicrobial, antiparasitic, anti-inflammatory, anti-diabetic, and antiproliferative [57].

Natural products for human sustenance are typically extracted using aqueous ethanol, aqueous glycerin, or hot water as extracting solvents. Due to their respective boiling points, removing ethanol is difficult, removing water is more difficult, and removing glycerin is the most difficult. In addition, water extracts are inherently unstable and contain polar components that impede chemical analysis [58]. Standardized extracts or products contain specific amounts of chemical constituents. Standardization involving multiple analytical methods, including hyphenated techniques, provides information that could be beneficial for selecting and adjusting dosages [59][60]. Standardized extracts may contain fewer chemical constituents than non-standardized extracts, as the process of increasing concentrations of some constituents may entail extraction techniques that reduce extract complexity.

Natural product extracts must contain various contaminants, including heavy metals, pesticides, residual solvents, fungal spores, toxins, or pathogens, within permissible limits [61]. Certain contaminants can only be detected using specialized analytical procedures. GC-MS, for example, is suitable for the analysis of particular pesticides and residual solvents, but it is insensitive to a large number of non-volatile contaminants [62].

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