PHYTOCHEMICAL CHARACTERIZATION OF PLANTS

Abstract

Historically and today, phytochemicals have played a pivotal role in benefiting humanity. Their significance spans various sectors, such as pharmaceuticals, nutraceuticals, dietary supplements, and the food and beverage industries. This wide array of applications includes diverse phytoconstituents like alkaloids, phenols, tannins, saponins, terpenoids, and more. These compounds exhibit various beneficial properties, including anti-oxidative, anti-inflammatory, antiviral, anticancer, antimicrobial, and others. As a result, the screening of these phytoconstituents holds immense importance in the realms of drug discovery and development. This chapter delves into the categorization, therapeutic uses of phytochemicals, and their identification using traditional and modern techniques in comprehensive detail.

Keywords: phytochemicals, alkaloids, tannins, drug discovery

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I. INTRODUCTION

Medicinal plants stand as an abundant source of diverse phytochemicals and have served as medicinal remedies since ancient eras. The therapeutic effectiveness of these plants arises from the presence of phytoconstituents, synthesized through the plant's primary and secondary metabolism. These phytochemicals play crucial roles in the growth of plants and serve as defence mechanisms against pests, insects, and diseases. Alkaloids, phenolic, steroids, glycosides, terpenes, and other significant phytochemicals exist in various plant parts, correlating with a range of beneficial properties such as anti-oxidative, antiviral, anticancer, antimicrobial, and anti-inflammatory effects. [1-4]

Modern medicine heavily relies on synthetic or semi-synthetic antibiotics to combat microbial diseases, but many microbes have developed resistance to these medications. Consequently, there's an increasing global demand for herbal drugs. According to the World Health Organization (WHO), roughly a quarter of current medications derive from plant-based components, while 80% of the world's population uses herbal medicines as their primary healthcare solution. Integrating these medicinal plants in synthesizing modern medicine is vital, creating a crucial link known as 'Phyto-medicines' between traditional and contemporary medical practices.

Understanding the relationship between plant-based compounds and their biological activities is paramount in developing new compounds tailored to address various health issues and chronic diseases. Exploring phytochemicals is pivotal in innovating novel plant-based natural products like dietary supplements, cosmeceuticals, textiles, food, and more that hold significant commercial value across diverse industries. This emphasis on phytochemical study greatly contributes to developing compounds with targeted activities, facilitating advancements in treating various health conditions. [5-7].

Phytochemical extraction from plants encompasses both traditional and innovative methods. Conventional approaches such as percolation, maceration, decoction, digestion, infusion, hot continuous extraction, and serial exhaustive extraction have long been employed. Meanwhile, contemporary techniques, including supercritical CO_2 extraction, microwave-assisted extraction, enzyme-assisted extraction, ultrasound-assisted extraction, and pressure fluid extraction, are increasingly utilized to extract bioactive compounds from plants.

Throughout the extraction process, a diverse array of solvents, such as chloroform, ether, water, acetone, and ethanol, are chosen based on the specific nature of the phytochemicals being targeted for extraction [8-10].

Utilizing modern methods like Gas Chromatography (GC), Liquid Chromatography (LC), High-Performance Liquid Chromatography (HPLC), and High-Performance Thin Layer Chromatography (HPTLC) offers significant advantages in identifying phytochemicals, supplementing conventional techniques. Conventional phytochemical tests remain a straightforward and cost-effective option for initial phytochemical characterisation, and evaluating plant materials through these methods can substantiate their therapeutic potential against various ailments. Hence, the current chapter focuses on categorising, extracting, and detecting phytochemicals using conventional and modern methodologies.

II. CLASSIFICATION OF PHYTOCHEMICALS

Typically, phytochemical components fall into two categories: primary and secondary metabolites. Primary metabolites, crucial for plant sustenance, encompass sugars, amino acids, proteins, and lipids. On the other hand, secondary metabolites can be categorized into several main groups: nitrogen-containing compounds such as alkaloids, glucosinolates, etc.; hydroxyl-containing compounds like phenols, flavonoids, tannins, etc.; terpenes, terpenoids, sulphur-containing organic compounds [11-12] are presented in Table 1.

These compounds are produced within the primary metabolic pathway and are not actively involved in generating new cells. They exhibit antiviral, antifungal, and antibiotic properties, serving as the plant's defence against pathogens. Additionally, they function as UV-absorbing agents, shielding leaves from light-induced damage. These biological activities render them valuable in medicinal applications for human diseases. Figure 1 illustrates the diverse applications of these phytochemicals.

Among others, phenolic compounds, comprising flavonoids (a diverse group of polyphenolic compounds), phenolic acids, and tocopherol, constitute one of the extensive categories of plant metabolites characterized by an aromatic ring featuring one or more hydroxyl groups. These compounds exhibit various biological properties, including anti-ageing, anti-inflammatory, anti-carcinogenic, antioxidant effects, and cardiovascular protection. Moreover, they demonstrate anticancer activities. Tannins, sizable polyphenolic biomolecules containing hydroxyl and other groups like carboxyl, serve as therapeutic agents in inflammation, burns, and related conditions.

Terpenoids, commonly synthesized in flowers, vegetative tissues, and roots, reduce total cholesterol and triglycerides, regulate blood pressure, and display antibacterial and anti-inflammatory properties. Alkaloids, conversely, encompass a range of applications such as cytotoxicity, analgesic effects, and antibacterial activity [13,14]. The imperative applications of herbal drugs and their major phytoconstituents are presented in Table 2.

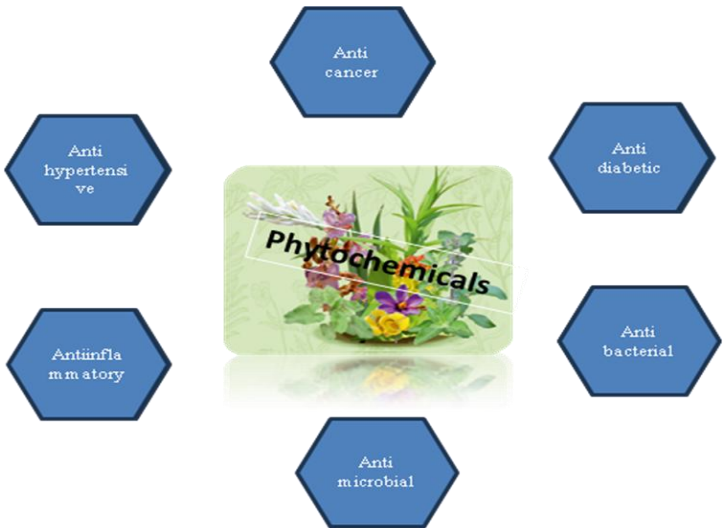

Figure 1: Various applications of phytochemicals

Table 1: Major Classification of Phytochemicals

S.No	Major classes	Subclasses	Representatives
1	Phenolics	Simple phenols	a) Phenolic acids (Caffeic acid, Ferulic acid, Gallic acid, Sinapic acid, etc.) b) Coumarins (Psoralene, Coumarin, etc.)
		Polyphenols	a) Flavonoids (Quercetin, Kaempferol, Curcumin, Catechin, etc.) b) Non- Flavonoids Tannins (Gallotannins, Ellagitannins, etc.)), Lignins (Pinoresinol),etc.
2	Terpenes	Monoterpenes (C10)	Geraniol, (+)-Limonene, pyrethroids, etc.
		Triterpenes (C30), etc.	Azadirachtin, phytoecdysones
3	Terpenoids	Monoterpenoids (C10)	Thymol, Cymenol, etc.
		Diterpenoids (C20)	Phytol, α-tocopherol, etc.
		Triterpenoid (C30), etc.	β-Sitosterol, Erythrodiol, Uvaol, etc.
4	Nitrogen containing compounds (organonitrides)	Alkaloids	Atropine, Taxol, Aconitine, Papaverine, etc.
5	Sulphur containing organic compounds (organosulfides)	Indoles, methiin, propiin, alliin, Glutathione, phytoalexins	Allicin, piperine, etc.

Table 2: Some of the important Herbal Drugs and its Applications

S.No.	Herbal material	Application	Important compound	References
1	Garlic	Antibacterial, anticancer, antifungal, antiinflammatory, chemopreventive, hepatoprotective, neuroprotective, etc	diallyl thiosulfonate (allicin), diallyl sulfide, dimethyl disulfide, etc	[15-17]
2	Ginger	Helps to treat arthritis, Diarrhea, improves immunity, etc.	[6]-gingerol, [14]-shogaol, hexahydrocurcumin, tetrahydrocurcumin, gingerenone A, etc.	[18]

3	Cinnamon	Improves the health of colon, reduce the risk of diabetes Anti-inflammatory, antimicrobial, etc.	polyphenols and cinnamaldehyde	[19]
4	Turmeric	Antioxidant, antimicrobial, anti-inflammatory, anticancer, hypoglycemia and anticoagulant.	curcumin and its derivatives	[20]
5	Artichoke, pear, and basil	Antiinflammatory, antifatigue, and antistress properties	Caffeic acid	[21]
6	Carrots, leafy greens and red orange	Anticarcinogenic, cornea protection, stimulates DNA repair enzymes, etc.	Carotene	[22]
7	Clove	Boosts immunity, improve digestion, anti allergic, anti cancer, etc.	Eugenol, eugenyl acetate, α-humulene, 2-heptanone, and β-caryophyllene	[23,24]
8	Fennel	Purify the blood, treat asthama, boosts metabolism, etc.	Phenolic compounds	[25]

Extraction of Phytochemicals

Phytochemicals are abundantly present in all parts of plants, including leaves, flowers, fruits, roots, stems, and bark. Extracting these compounds can be influenced by various factors such as the choice of solvents, temperature, and extraction method. Solvents, in particular, play a pivotal role in this extraction process.

An ideal extraction solvent should possess low toxicity, easy evaporation, and solubility. Given that plants contain diverse bioactive compounds with differing polarities selecting solvents becomes crucial. More polar bioactive compounds tend to be extracted efficiently using polar solvents such as water, ethanol, methanol, and acetone. Conversely, less polar compounds are better extracted using solvents like chloroform or ether due to their lower polarity [26-29] presented in Table 3

Table 3: Selection of Solvents for Extraction/Identification of Phytochemicals

S.No.	Solvent	Identification/extraction of phytochemical
1	Water	Anthocyanins, polypeptides, tannins, terpenoids, saponins, starches
2	Ethanol	Polyacetylenes, flavonol, sterols, tannins, terpenoids, saponins, polyphenols, Alkaloids
3	Methanol	Anthocyanins, tannins, terpenoids, saponins, polyphenols, totarol, lactones, flavones, xanthoxylins
4	Acetone	Phenols, flavanols, tannins
5	Chloroform	Terpenoids, flavonoids
6	Ether	Alkaloids, terpenoids, coumarins, fatty acids

III. DETECTION OF PHYTOCHEMICALS

The essential role of qualitative phytochemical screening lies in identifying diverse biochemical compounds within plants. This process involves employing various phytochemical tests that trigger chemical reactions, leading to observable color changes or the formation of distinct colored precipitates for identification purposes. [30-36].

1. Identification Tests for Phytochemicals

- **Test for carbohydrates:** Three commonly utilized tests—Benedict's, Molisch, and Fehling's—identify carbohydrates within plant extracts.

  - **Benedict's test:** Begin by dissolving a small quantity of the plant extract in 5ml of distilled water and then filter the solution. Afterwards, introduce Benedict's reagent. The presence of sugars is indicated by a color change to green, yellow, or red.
  - **Molisch test:** Following filtration, combine the filtrate with 2 or 3 drops of alcoholic alpha-naphthol and carefully add a few drops of sulfuric acid along the sides of the test tube. The presence of sugars is denoted by the appearance of a violet color.

  - **Fehling's test:** Mix 1ml of the filtrate with equal amounts of Fehling's solutions A and B, then heat the mixture in water. The formation of a red precipitate serves as an indicator of the presence of sugars.

- **Test for Amino acids & proteins :** Performing the Biuret test involves taking a small filtrate volume and introducing two drops of a 2% $CuSO_4$ solution. Following this, add 1 ml of a 95% ethanol solution and subsequently, an excess of KOH. The presence of a pink colouration confirms the presence of amino acids.

- **Test for Oils:** Take a small quantity of extract and press between the two filter papers. The appearance of spots indicates the presence of oils

- **Test for Alkaloids:** Dissolve the plant extracts in diluted HCl and subsequently filter the solution. The resulting filtrate is utilized for testing the presence of alkaloids.

- **Mayer's test:** Introduce a few drops of Mayer's reagent into the filtrate. Confirm the presence of alkaloids if a creamy white or yellow precipitate forms.
- **Wagner's test:** Add a few drops of Wagner's reagent to the filtrate. A reddish-brown precipitate serves as an indicator of the presence of alkaloids.
- **Hager's reagent**: Incorporate 1-2 ml of Hager's reagent into the filtrate. The confirmation of alkaloids is indicated by a creamy white precipitate.
- **Dragondroff test:** Utilize a portion of the filtrate and add 1-2 ml of the reagent. The formation of an orange or red precipitate signifies a positive test for alkaloids

- **Test for Phenolic compounds**

- **Gelatin test:** Combine 5 ml of the plant extract (filtrate) with 2 ml of 1% gelatin solution and 10% NaCl solution. The emergence of a white precipitate indicates the presence of phenols.
- **Ferric chloride test:** Introduce a few drops of a 5% ferric chloride solution into the filtrate. Confirmation of phenols is observed through the appearance of a dark green or bluish-black color.
- **Lead acetate test:** Mix 5 ml of the plant extract (filtrate) with 3 ml of a 10% lead acetate solution. A positive indication for phenols is demonstrated by the formation of a milky white precipitate.

- **Test for Tannins**

- **Ferric chloride test:** To the filtrate, add few drops of ferric chloride (5%) solution gives a dark green indicates the presence of tannins

- **Test for Flavonoids**

- **Lead acetate test:** Utilize 1 ml of the filtrate and introduce a few drops of a 10% lead acetate solution. A yellow precipitate formation indicates the presence of flavonoids.
- **Shinoda test:** Mix the plant extract with 5 ml of alcohol and magnesium ribbons, followed by the addition of a few drops of concentrated HCl. Confirmation of flavonoids is affirmed if the solution turns a crimson red color.

- **Test for Saponins**

- **Foam test:** Mix few mg of the plant extract with 5 ml of distilled water and shake vigorously. The formation of frothing indicates the presence of saponins

- **Test for Steroids:** To few ml of extract, add 2 ml of $CHCl_3$ and 2 ml of conc. H_2SO_4, the appearance of red color/yellowish green confirms the presence of steroids

- **Test for Terpenoids:** To the $CHCl_3$ filtrate, add few drops of Conc. H_2SO_4 shake well and allow to stand few minutes, the appearance of golden yellow layer at the bottom confirms the presence of terpenoids

- **Salkowski's test:** Add 2 ml $CHCl_3$ and 3 ml Conc. H_2SO_4 to the 5mg of plant extract. Formation of reddish brown colour layer indicates the presence of terpenoids

- **Test for Anthraquinones:** Dissolve 10 mg of extract in isopropyl alcohol, to this add few drops of Conc. Ammonium hydroxide solution, formation of red colour after few minutes shows the presence of anthraquinones

- **Test for Glycoside**

  - **Borntager's test:** Add 3 ml of $CHCl_3$ to 2 ml of filtrate and shake well. Formation of $CHCl_3$ layer will be observed. Separate the $CHCl_3$ layer and add (10%) Ammonia solution. The appearance of pink colour indicates the presence of glycosides

- **Test for phytosterols**

  - **Libermann-Burchard's test:** Dissolve 2 mg of dry extract in acetic anhydride, heat to boil, cool it, and add 1 ml of Conc. H_2SO_4 along the sides of the test tube. The appearance of green color indicates the presence of steroids.

2. Modern phytochemical Characterization Techniques

Chromatographic and spectroscopic methods are valuable for identifying, quantifying, and determining the structure of phytochemicals. This involves various chromatography techniques such as thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), liquid chromatography (LC), gas chromatography (GC), as well as combined techniques such as chromatography coupled with mass spectrometry (MS). Additionally, spectroscopy methods including UV-Vis, IR, NMR, among others, are also explored for their efficacy in analyzing phytochemicals. [37-42].

3. Chromatographic Techniques

Detecting secondary metabolites in herbal products often proves to be a time-intensive endeavour. Nonetheless, specific chromatography-based methodologies streamline the identification of these metabolites, simplifying the process of characterizing various bioactive chemicals.

4. Thin-Layer Chromatography (TLC) and High-Performance Thin-Layer Chromatography (HPTLC)

Thin-layer chromatography (TLC) stands as one of the most prevalent and uncomplicated methods for substance separation. Operating based on the principle of adsorption, this technique separates biomolecules on a TLC plate, relying on their affinity toward both the mobile phase (liquid) and stationary phase (solid). TLC offers advantages like rapid analysis and minimal sample preparation, making it extensively used as a qualitative method in herbal drug analysis.

High-Performance Thin-Layer Chromatography (HPTLC) finds frequent application in quantifying active components and detecting adulterants, pesticides, mycotoxins, and other substances present in herbs and related herbal products. Recently, Tirupataiah et al[43], identified the embelin in *embelia ribes* fruits in $CHCl_3$ extract using Chloroform: Ethyl

acetate: Formic acid (5:4:0.5 v/v/v) as a mobile phase at different wavelength 254 and 366 nm.

5. High-Performance Liquid Chromatography (HPLC)

In recent decades, HPLC has emerged as the predominant analytical technique for evaluating herbal medicines. Operating on a similar principle to TLC, it typically employs reversed-phase (RP) columns. HPLC finds extensive use in the pharmaceutical industry, employed for identifying and purifying herbal constituents through both analytical and preparative methods. Additionally, it serves a crucial role in pesticide and aflatoxin analysis.

Narasimhaji et al., estimated asiaticoside content in *Centella asiatica* (L.) using HPLC and recommended that the best procurement time for the whole plant is in Grishma ritu (May and June) [44]. Govindrajan et al., used the HPLC–photodiode array method to estimate furocoumarins for quality control and standardization of *Heracleum candicans* [45]. Sujit et al. have done phytochemical analysis for *S. indica, S. declinata, S. thaipingensis and S. asoca* flowers using RP-HPLC and confirmed the presence of total 121, 110, 111, and 121 types of phytochemicals, respectively [46].

6. Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS technology has significantly progressed and enables the analysis of naturally occurring metabolites like carbohydrates, DNA, peptides, and proteins. A study conducted by A. K. Meena et al. utilized LC-MS to investigate the *Cassia fistula* stem bark and its smaller branches. The report highlighted the presence of active compounds in both the stem bark and small branches of the plant extracts [47].

7. Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS)

Gas chromatography (GC) stands as a widely recognized analytical method renowned for its exceptional sensitivity and resolution. Primarily, it proves valuable in the identification and quantification of volatile organic compounds (VOCs), essential oils, and fatty acids. Its utility extends to the detection of environmental toxins, pesticides, and similar substances, finding applications across various industries such as cosmetics, herbal products, and pharmaceuticals.

Gas chromatography–Mass spectrometry (GC-MS), a hyphenated analytical technique merging separation and mass detection methods, has emerged from this coupling. This technique is highly effective in detecting a diverse range of compounds like volatile organic compounds (VOCs), medicated oils, and other substances present in test samples. Using, GC-MS, Olivia, et al, [48] identified 23 compounds in the aqueous methanol fraction of *Hibiscus asper* leaves. Narasimhamurthy et al., tested the leaf and the rhizome of *Amomum nilgiricum* using GC-MS and identified variety of bioactive compounds [49].

8. Spectroscopic Techniques

Spectroscopy involves studying the interaction between matter and electromagnetic radiation. This technique gauges the energy absorbed or emitted by a sample across different

wavelengths. Its applications include identifying a sample's composition, assessing the physicochemical properties, and determining the molecular structure. Diverse spectroscopic techniques like UV-Vis, FT-IR, NMR, and others are available and discussed extensively for their varied applications and functionalities.

9. Ultraviolet-Visible Spectroscopy

This operates based on the Beer-Lambert principle and primarily concerns the absorption of ultraviolet (UV) and visible light by a sample. It finds extensive application across biology, materials science, analytical chemistry, and various other fields. UV-Vis spectroscopy aids in estimating sample concentration, identifying functional groups within a molecule, and facilitates the examination of a molecule's geometric and electronic structure. This technique spans wavelengths from approximately 200 nm to 800 nm.

Chromophores, present in most plant metabolites, are light-absorbing functional groups. When these chromophores absorb UV/Vis light, they transition from a ground state to a higher energy state, generating distinctive spectra that contribute significantly to molecule identification. [50]. It is frequently used in the detection and quantification of several phytoconstituents like alkaloids, phenols, tannins, flavonoids, and others in various parts of the plants by observing λ_{max} at a particular wavelength.

10. Fourier Transform-Infrared Spectroscopy [FT-IR]

Most molecules exhibit light absorption within the infrared region (IR) of the electromagnetic spectrum, which spans from 4000 to 400 cm^{-1}, between visible and microwave wavelengths. Unlike other methods, IR spectroscopy is a non-destructive analytical technique commonly utilized for identifying functional groups within various compounds. It finds extensive application in quality assessment within food, beverages, pharmaceuticals, herbal products, and others [51]. Recently, Wongsa et al. showed the phenolic profiles of 25 herbal infusions using FT-IR spectroscopy [52].

11. Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is a prominent analytical technique extensively employed for elucidating the structure of diverse compounds. Various NMR techniques, including solid-state NMR, ^{13}C-NMR, ^{1}H-NMR, among others, are available for this purpose. Solid-state NMR spectroscopy is specifically utilized for determining the molecular structure of solids.

The 13C isotope is employed in 13C-NMR spectroscopy, with typical delta (δ) values ranging between 0 and 220 ppm for discerning the carbon type within a compound. Meanwhile, 1H-NMR spectroscopy is utilized to identify the type of hydrogen and its connectivity to carbon. Tetramethylsilane commonly serves as the internal reference standard, and samples are typically prepared in d6-DMSO for analysis. [38,39].

IV. CONCLUSION

Naturally, various plant parts encompass many phytochemicals, many possessing therapeutic properties. Analyzing these phytochemicals is pivotal in evaluating the medicinal potential of plants and serves as a foundation for isolating specific compounds fostering new drug discoveries. Several extraction methods and solvents are available for extracting these phytochemicals. Polar solvents prove effective for extracting polar compounds, while non-polar solvents are suitable for non-polar compounds.

Qualitative analysis of phytochemicals provides an initial understanding regarding the presence of alkaloids, phenols, saponins, terpenes, and other compounds. A comprehensive investigation into plant metabolites using advanced instruments like HPTLC, GC-MS, LC-MS, UV-Vis, IR, among others, aids in both quantification and structural determination of biomolecules. This detailed analysis lays the groundwork for developing novel drugs derived from various plant components

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