ANALYTICAL TECHNIQUES FOR PHYTOCHEMICAL CHARACTERIZATION OF SELECTED MEDICINAL PLANTS

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ABSTRACT

Phytochemical study helps to identify bioactive compounds in medicinal plants, mainly alkaloids, flavonoids, tannins, terpenoids, and phenolics, which are known for their therapeutic potential. Traditional extraction methods such as maceration and Soxhlet remain useful, but modern green approaches like supercritical fluid extraction and ultrasound-assisted extraction offer higher efficiency and sustainability. For compound identification, classical methods including Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), Gas Chromatography-Mass Spectrometry (GC-MS), and Fourier Transform Infrared Spectroscopy (FTIR) remain reliable. Latestadvance, such as Nuclear Magnetic Resonance (NMR), Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS), metabolomics, and machine learning–assisted spectral analysis, have further enhanced accuracy in profile complex plant metabolites. Together, these integrated methods strengthen quality control, ensure standardization, and accelerate natural product–based drug discovery. Phytochemical analysis thus continues to bridge traditional plant knowledge with modern pharmaceutical applications.

Keywords: - Phytochemical analysis, bioactive compounds, Chromatography, Spectroscopy technique, medicinal plant.

INTRODUCTION

The term phytochemical is derived from the Greek word "*phyton"* (meaning plant) and "*chemical"* (a chemical substance).So, the phytochemical meaning to naturally occurring compounds in plants that often have biological significance, such as antioxidants, flavonoids, and carotenoids. These compounds play a vital role in human health, both as nutrients as well as medicinal agents (Rabizadeh et al., 2022). Plants are one of the most important sources of medicine, having been utilized in health care for millennia. Ayurveda, originating in India 5,000–10,000 years ago, is widely regarded as one of the oldest medical systems in the world and has significantly influenced other traditional systems, such as Traditional Chinese Medicine. For this reason, Ayurveda is often called the “Mother of all healing” (Wangchuk, 2015).

Despite remarkable progress in modern medicine, millions of people continue to suffer and die annually from communicable and non-communicable diseases. This highlights the continuing importance of medicinal plants in global health. The World Health Organization (WHO) has recognized medicinal plants as one of the greatest sources of drug discovery, underscoring the need for detailed investigation into their phytochemistry, pharmacological properties, and safety (Wangchuk, 2015).

Globally, the Food and Agriculture Organization (FAO) reported in 2002 that over 50,000 plant species were used medicinally. More recently, the Royal Botanic Gardens, Kew, through its Medicinal Plant Names Services (MPNS), recorded 39,112 plant species cited in medicinal, pharmaceutical, or herbal sources (Royal Botanic Gardens, Kew, 2024), demonstrating a significant update over earlier estimates. In Nepal, ethnobotanical surveys reflect extraordinary diversity: a 2022 study documented 1,762 medicinal plant species used across 77 districts (Ghimire et al., 2022). These plants are applied for a wide variety of ailments, including anxiety, asthma, arthritis, digestive problems, hypertension, and high cholesterol, using plant parts such as stems, leaves, fruits, flowers, bark, bulbs, or the entire plant.

Phytochemicals are broadly classified into primary metabolites, such as sugars, amino acids, proteins, nucleic acids, and chlorophyll, which are essential for growth and survival, and secondary metabolites, such as alkaloids, terpenoids, flavonoids, lignans, steroids, curcuminoids, saponins, and phenolics, which mediate ecological interactions and often account for the therapeutic activities of medicinal plants (Rabizadeh et al., 2022; Elshafie et al., 2023; Simşek et al., 2024). These compounds exhibit antioxidant, anti-inflammatory, antimicrobial, and anticancer properties, making them central to drug discovery and modern pharmacology.

Nepal's unique geography and diverse climatic zones foster a rich repository of medicinal plants. The Karnali Province, in particular, harbors numerous medicinal and aromatic plants used traditionally by local ethnic groups such as the Gurung, Newar, Majhi, Raute, Tamang, and Tharu (Aryal et al., 2023). Previous studies in Nepal have identified phytochemicals including alkaloids, tannins, flavonoids, saponins, and steroids (Shrestha & Adhikari, 2001), while antioxidant and phenolic-rich extracts from species like *Spondias pinnata* and *Bauhinia asiatica* have been reported (Sai et al., 2019). Nevertheless, phytochemical investigations focusing specifically on indigenous species of Karnali Province remain scarce.

Given the ecological significance and ethnobotanical richness of Karnali Province, comprehensive phytochemical studies are urgently needed. Such research will (a) validate traditional medicinal claims, (b) identify novel bioactive compounds for therapeutic development, and (c) promote sustainable use and conservation of these valuable plant resources (Aryal et al., 2023; Rabizadeh et al., 2022; Elshafie et al., 2023).

LITERATURE REVIEW

Phytochemical characterization of medicinal plants is essential for drug discovery, quality control, and validation of ethnomedicinal practices, as plants produce diverse bioactive secondary metabolites including alkaloids, flavonoids, terpenoids, and phenolics (Plumb et al., 2023). Extraction remains the first critical step, where traditional methods such as maceration and Soxhlet are widely applied, but greener technologies like ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) are increasingly used due to their efficiency and preservation of thermolabile compounds (Shehu et al., 2025). Supercritical fluid extraction (SFE), particularly using CO_2, has also gained importance for

volatile and non-polar constituents with minimal solvent residues (Aati et al., 2024). For separation and fingerprinting, thin-layer chromatography (TLC) and high-performance TLC (HPTLC) are cost-effective tools for preliminary screening, while HPLC and its advanced forms (UHPLC, UPLC) remain the gold standard for quantification of phenolics, flavonoids, and glycosides with high sensitivity (Kowalska et al., 2022). Gas chromatography–mass spectrometry (GC–MS) is particularly suited for profiling volatile oils and terpenoids, as shown in recent work on Artemisia essential oils analyzed via HS-SPME/GC-MS coupled with antioxidant assays (Aati et al., 2024). In terms of structural elucidation, hyphenated mass spectrometry methods such as LC-HRMS and LC–MS/MS enable untargeted metabolite profiling and fragmentation-based identification (Plumb et al., 2023), while nuclear magnetic resonance (NMR) spectroscopy remains indispensable for full structural confirmation, especially when combined with MS data (Kılınç et al., 2023). Quantitative NMR is emerging as a powerful approach for absolute quantification without external standards, though its adoption in phytochemical studies remains limited (Ravaglia et al., 2024). Untargeted metabolomics integrating LC–MS, NMR, and multivariate statistics has become central for chemotaxonomy and biomarker discovery in medicinal plants (Kılınç et al., 2023), while curated resources such as the Natural Products Magnetic Resonance Database (NP-MRD) facilitate dereplication and annotation of plant metabolites (Wishart et al., 2022).

RESEARCH GAPS

Despite these advances, several gaps persist. First, many phytochemical studies rely on qualitative screening or unvalidated protocols, which limits reproducibility across laboratories (Shehu et al., 2025). Second, access to advanced tools such as LC-HRMS and high-field NMR is limited in developing regions, including Nepal, creating geographic disparities in phytochemical characterization (Kılınç et al., 2023). Third, although NMR offers absolute quantification without standards, its application in medicinal plant research remains rare (Ravaglia et al., 2024). Fourth, untargeted metabolomics is hampered by incomplete spectral databases, especially for endemic and under-studied species, leading to low annotation confidence (Wishart et al., 2022). Finally, chemical profiling is often disconnected from pharmacological validation, with few studies linking identified metabolites to mechanisms of action or in-vivo safety (Aati et al., 2024).

METHODS AND METHODOLOGY

Collection of Plants

Medicinal plants were collected from various forest areas. The collection process was challenging due to difficult region and the possibility of misidentifying species. However, collecting plants from the wild confirms that they are free from pesticides (WHO, 1998). After collection, plants were cleaned to remove dust, soil, and other attached particles.

Cleaning of Plants

Collected plants were first washed with distilled water to remove dust and mud. To eliminate fungi, bacteria, and other microorganisms, absolute alcohol was used (Mrozek-Szetela, Rejda, & Wińska, 2020).

Drying

The primary purpose of drying medicinal plants is to remove water content and prevent the degradation of plant materials. Drying can be performed using either natural or artificial methods (Sahira Banu & Cathrine, 2015).

Natural Drying

In natural drying, plants are exposed to sunlight or air-dried on drying frames, barns, or sheds. Complete drying may take several weeks depending on temperature and humidity (Sahira Banu & Cathrine, 2015; Müller & Heindl, 2006).

Artificial Drying

Artificial drying uses warm-air driers, reducing drying time from days to hours or even minutes. Plants are placed on plates inside the drier with warm air blown over them. This method is particularly useful for sensitive flowers and leaves but requires manual labor for loading and unloading (Sahira Banu & Cathrine, 2015; Eapen et al., 2025).

Powdering

The medicinal plants powdered after drying with the help of grinder(WHO, 1998).

METHODS OF EXTRACTION

Plant Tissue Homogenization

The powdered medicinal plants were mixed with a specified quantity of solvent and shaken vigorously for 5–10 minutes or left for 24 hours. The extract was then filtered, and the filtrate could be dried under reduced pressure and re-dissolved in the solvent to determine concentration. Some researchers centrifuge the filtrate to clarify the extract (Sahira Banu & Cathrine, 2015).

Serial Exhaustive Extraction

This method involves successive extraction with solvents of increasing polarity, from non-polar (hexane) to polar solvents (methanol), ensuring a wide range of compounds is extracted. Soxhlet extraction is commonly used but is unsuitable for thermolabile compounds due to prolonged heating (Kallassy, 2017).

Soxhlet Extraction

Soxhlet extraction is used when the desired compound has limited solubility in a solvent, while impurities are insoluble. The soluble compound can then be separated by simple filtration (Sahira Banu & Cathrine, 2015).

Maceration

In maceration, whole or coarsely powdered plant material is kept in contact with a solvent in a stoppered container, with frequent agitation until the soluble matter dissolves. This method is suitable for thermolabile drugs (Pandey & Tripathi, 2014).

Decoction

Decoction involves boiling crude plant material in water for 15 minutes, followed by cooling, straining, and adjusting the volume with cold water to obtain the desired extract (Sivanandham, 2015).

Infusion

Infusion is a dilute solution prepared by macerating plant material for a short time with cold or boiling water (Sahira Banu & Cathrine, 2015).

Digestion

Digestion is a type of maceration with gentle heating, increasing the solvent efficiency when moderate temperature is acceptable (Sahira Banu & Cathrine, 2015).

Percolation

Percolation uses a percolator to extract active ingredients. The plant material is moistened with solvent, allowed to stand, packed in the percolator, and solvent is added to extract compounds gradually. The percolate is then clarified by filtration or decanting (Sahira Banu & Cathrine, 2015).

Sonication

Sonication uses ultrasound (20–2000 kHz) to increase cell wall permeability and produce cavitation. Although effective for certain roots like *Rauwolfia*, large-scale use is limited due to cost and potential degradation of compounds (Sahira Banu & Cathrine, 2015).

QUALITATIVE AND QUANTITATIVE ANALYSIS OF PHYTOCHEMICALS

Preliminary Qualitative Analysis

Alkaloids

- **Mayer's test:** White creamy precipitate indicates alkaloids (Sahira Banu & Cathrine, 2015).
- **Wagner's test:** Reddish-brown precipitate confirms alkaloids (Sahira Banu & Cathrine, 2015).

Amino Acids

- **Ninhydrin test:** Purple color indicates presence of amino acids (Maroyi, 2019).

Carbohydrates

- **Molish's test:** Violet ring indicates carbohydrates (Sahira Banu & Cathrine, 2015).
- **Benedict's test:** Colored precipitate confirms sugars (Sharma et al., 2020).

Fixed Oils and Fats

- **Spot test:** Oil stain on paper indicates fixed oils.
- **Saponification test:** Soap formation indicates fixed oils/fats (Arabic, 2015).

Phenolic Compounds

- **Ferric chloride test:** Dark green color indicates phenolics.
- **Gelatin test:** White precipitate indicates phenolics.
- **Lead acetate test:** White precipitate confirms phenolics (Sahira Banu & Cathrine, 2015).

Flavonoids

Yellow coloration with ammonia or aluminum chloride confirms flavonoids (Sivanandham, 2015).

Test for Catechins:- 3 ml of test solution in alcohol was added with Ehrlich reagent and a few drops of concentrated HCl to form pink color indicates the presence of catechins.

Test for flavonols and flavones:- 3 ml of the filtrate was mixed with 4 ml of 1% aluminium chloride in methanol in a test tube, yellow colour observed indicated the presence of flavonols and flavones (Sivanandham, 2015).

Test for phytosterols

a. Libermann-Burchard's test:- In 2 ml acetic anhydride 50 mg extract dissolved and 1 or 2 drops of concentrated sulphuric acid are added slowly along the sides of the test tube. Group of colour change shows the presence of phytosterols (Sivanandham, 2015).

Test for Proteins:-

100 mg sample dissolve in 10 ml of distilled water and filtered with the help of Whatmann No.1 filter paper. The filtrate is used for protein test.

a. Millon's test:-

2 ml of filtrate is taken in a testube and few drops of Millon‟s reagent are added to form white precipitate indicates the presence of proteins.

b. Biuret test:-

2 ml of filtrate is taken in a test-tube. Added 1 drop of 2% copper sulphate solution and 1 ml of ethanol (95%), followed by excess of potassium hydroxide pellets. The Pink color layer indicates the presence of protein (Tiwari et al., 2011).

Test for Saponins:-

The 50 mg extract is dissolved with distilled water and solution made up to 20 ml. The suspension is shaken in a graduated cylinder for 15 minutes. A two cm layer of foam indicates the presence of saponins (Balamurugan et al., 2019).

Test for gum and Mucilages:-

The extract (100 mg) is dissolved in 10 ml of distilled water and to this 2 ml of absolute alcohol is added with constant stirring. White or cloudy precipitate indicates the presence of Gums and Mucilages (T et al., 2015)

Test for volatile oil:- 50 mg of powdered material (crude drug) is taken and subjected to hydro-distillation. The distillate is collected in graduate tube of the assembly, where in the aqueous portion automatically separated out from the volatile oil (Altemimi et al., 2017).

Qualitative and quantitative Analysis

This analysis can be done by using Gas Chromatography Mass Spectroscopy (GCMS). GCMS can be applied to solid, liquid and gaseous samples. First the samples are converted into gaseous state then analysis is carried out on the basis of mass to charge ratio. High Performance Liquid Chromatography is applicable for compounds soluble in solvents. High performance thin layer chromatography is applicable for the separation, detection, qualitative and quantitative analysis of phytochemicals.

Gas Chromatography

Volatile compounds are analysed by using gas chromatography. In this method, there is a gas and a liquid phase. The liquid phase is stationary where the gas phase is a mobile phase. These compounds to be analysed are also in the mobile phase with a carrier gas which is usually helium, hydrogen or argon. The chemicals are separated depending on the migration rate into the liquid phase. Higher percentage of the chemical will lead to faster migration in the liquid phase. This is mostly used in qualitative and quantitative phytochemical analysis (Altemimi et al., 2017).

High Performance Liquid Chromatography: (HPLC)

HPLC is also known as High- Pressure Liquid Chromatography. This method involves the interaction of liquid solvent in the tightly packed solid column or a liquid column. These acts as the stationary phase while the liquid (solvent) acts as the mobile phase, high pressure enables the compounds to pass to the detector. As HPLC compounds are analysed after vaporisation, thermolabile compounds cannot be analysed with this technique (Altemimi et al., 2017).

High Performance Thin Layer Chromatography: (HPTLC)

High Performance Thin layer Chromatography is a modified version of thin layer chromatography. High Performance Thin layer Chromatography is planer chromatography where separation of sample components is done on high performance layers with detection and acquisition using an advanced work station. These high performance layers are pre-coated with a sorbent of particle size 5-7 microns and a layer thickness of 150-200 microns. The reduction in the thickness of the layer and the particle size results in increasing the plate efficiency along with nature of separation. HPTLC is suitable for qualitative, quantitative and micro-preparative chromatography (Balamurugan, et. al., 2019).

Optimum Performance Laminar Chromatography: (OPLC)

OPLC combines the advantages of TLC and HPLC. The system separates about 10-15 mg samples, with simultaneous processing of up to 4 or 8 samples at a time depending on the model. In OPLC a pump is used to force a liquid mobile phase through a stationary phase, such as silica or a bonded-phase medium (Balamurugan et al., 2019).

METHODS OF DETECTION

Spectroscopy is used in the detection of phytochemicals. The following are frequently used in the study of phytochemicals.

UV spectroscopy- UV spectroscopyuseful for chromophore identification, also for general classification of compounds, for structural identification and also for quantitative estimation.

IR spectroscopy- Useful for identify the functional groups present in a compound, for purity checking and structural identification by co-IR and finger print region comparison and quantitative analysis.

Mass spectroscopy- To determine the molecular weight of the compound and structural moiety identification.

C-NMR spectroscopy- 13C-NMR spectra gives structural information about organic compounds with the number, types, and chemical environment of carbon atoms.

1H-NMR - To find out how many types of hydrogen atoms are present in the compound and to find out the connection of hydrogen atoms.

X-Ray Crystallography:- X-ray crystallography is an experimental technique that exploits the fact that X- ray are diffracted by crystals. X- rays have the proper wavelength (in the Angstrom range 10-8) to be scattered by the electron cloud of an atom of comparable size. Based on the diffraction pattern obtained from

X- Ray scattering off the periodic assembly of molecules or atoms in the crystal, the electron density can be reconstructed. Additional phase information can be extracted either from the diffraction data or from supplementing diffraction experiment to complete the reconstruction. A model is then progressively built into the experimental electron density, refined against the data and the result is a quite accurate molecular structure (Sahira Banu & Cathrine, 2015).

CONCLUSION

Phytochemical study of medicinal plants is very important to identified the natural compounds that gives the medicinal value. These compounds include alkaloids, flavonoids, phenolics, terpenoids, saponins, and steroids, which have health benefits such as antioxidant, anti-inflammatory, and antimicrobial effects.

Collecting, cleaning, drying, and powdering the plants properly is important to preserve their active compounds. Extraction methods like maceration, Soxhlet, and modern techniques like ultrasound or supercritical fluid extraction help to get these compounds efficiently.

For analysis, methods such as TLC, HPTLC, HPLC, GC-MS, and OPLC, along with spectroscopy techniques like UV, IR, NMR, and mass spectrometry, are used to identify and measure these compounds. Modern tools like LC-MS/MS and metabolomics provide even more accurate results.

Using these methods together helps ensure the quality and consistency of medicinal plant products. It also supports traditional medicine, helps discover new drugs, and promotes sustainable use of plant resources, especially in rich areas like Karnali Province, Nepal. Overall, combining traditional knowledge with modern techniques gives a clear understanding of the medicinal value of plants.

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