Original Research Article
ISSN: **3041-5357**
Journal homepage: https://diversity.researchfloor.org/

Journal of Diversity Studies

Phytochemical Profiling Integrated with HPTLC Fingerprinting for Standardization of *Phyllanthus pinnatus* (Wight) G. L. Webster

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ABSTRACT

Phyllanthus pinnatus, a medicinal plant belonging to the family Phyllanthaceae, has been widely utilized in traditional medicine for its diverse therapeutic properties. The present study was undertaken to evaluate the phytochemical composition and to develop a High-Performance Thin-Layer Chromatography (HPTLC) fingerprint profile for the standardization of P. pinnatus leaves. Sequential Soxhlet extraction was performed using solvents of increasing polarity, namely petroleum ether, chloroform, ethyl acetate, and methanol. Preliminary qualitative phytochemical screening revealed the presence of a wide range of bioactive constituents, including alkaloids, flavonoids, phenolic compounds, tannins, glycosides, and terpenoids. Quantitative analysis demonstrated that the methanolic extract contained the highest concentration of phytochemicals, with alkaloids (524 mg/g), flavonoids (536 mg/g), phenols (385 mg/g), and tannins (330 mg/g). The chloroform extract exhibited moderate levels of alkaloids, phenols, and tannins, while the ethyl acetate extract was particularly rich in flavonoids. HPTLC fingerprint profiling of the methanolic extract revealed distinct and well-resolved chromatographic bands at wavelengths of 254 nm and 366 nm, with characteristic Rf values indicating the presence of multiple phytoconstituents. These findings confirm that P. pinnatus is a rich source of secondary metabolites, particularly flavonoids, phenolics, and alkaloids, which are commonly associated with the genus Phyllanthus. The established phytochemical profile and HPTLC fingerprint provide a reliable and reproducible reference for the authentication, quality control, and standardization of this medicinal plant.

Keywords: *Phytochemicals, Soxhlet extraction, HPTLC, Phyllanthus pinnatus and Phyllanthaceae*

Citation: Shaheen and P. Vijaya [2026]. Phytochemical Profiling Integrated with HPTLC Fingerprinting for Standardization of *Phyllanthus pinnatus* (Wight) G. L. Webster. *Journal of Diversity Studies.* **DOI: https://doi.org/10.51470/JOD.2026.5.1.39**

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Article History: *Received 03 November 2025 | Revised 01 December 2025 | Accepted 04 January 2026 | Available Online February 02, 2026*

Introduction

The global human population in 2025 is estimated to range between 8.1 and 8.2 billion, reflecting the continued pattern of steady demographic growth observed over recent decades. Projections from leading international organizations indicate an annual population growth rate of approximately 0.85% to 0.9%, highlighting ongoing demographic transitions across both developed and developing regions. Real-time population estimates from platforms such as Worldometer further suggest that the global population has reached nearly 8.2 billion in 2025 [1]. These trends underscore the increasing pressure on natural resources, infrastructure, and socio-economic systems, emphasizing the need for sustainable development strategies to address the challenges associated with rapid population growth. Around 80% of the global population utilizes traditional medicine for at least some component of their healthcare, with particularly high reliance reported across many Asian and African countries. Major traditional medical systems contributing to this global usage include Traditional Chinese Medicine, Ayurveda from the Indian subcontinent, and diverse African traditional medicine frameworks, each of which incorporates extensive herbal pharmacopoeias and complex therapeutic doctrines developed over centuries.

This substantial dependence on plant-based and other indigenous therapeutic modalities is shaped by the historical consolidation of local medical knowledge, its deep cultural embeddedness, and persistent structural barriers to accessing conventional biomedical services, especially in low- and middle-income settings with constrained health infrastructure. These traditional systems collectively represent millennia of empirically accumulated and increasingly systematized information on the pharmacological properties, safety profiles, and clinical applications of botanical and other natural products, some of which have informed the discovery and development of contemporary pharmaceuticals. The continued prominence of traditional herbal medicine in primary health care highlights both its perceived clinical utility in the management of acute and chronic conditions and the enduring inequities in the global distribution of biomedical technologies, regulated pharmaceuticals, and formally trained healthcare providers, particularly in resource-limited regions where traditional medicine often constitutes the most accessible and economically viable care [2-3]. Herbal medicines are gaining global prominence as plant-derived bioactive compounds increasingly serve as therapeutic agents for diverse pathologies, including bacterial infections and cancer.

Despite their pharmacological potential, the clinical utility of these natural products remains constrained by suboptimal bioavailability and stability issues. Nano vesicular delivery platforms such as liposomes, niosomes, and solid lipid nanoparticles offer innovative strategies to overcome these limitations by enhancing targeted delivery, solubility, and pharmacokinetic profiles of herbal actives. Nevertheless, translation of these herbal-nano formulations into clinical practice faces persistent challenges in large-scale manufacturing, long-term biocompatibility, and regulatory pathways for approval 3-4].

The growing global reliance on medicinal plants has raised serious concerns regarding their long-term sustainability and conservation. Increasing demand, driven by expanding use in traditional and modern healthcare systems, has intensified pressure on natural plant populations. In many regions, medicinal plants are still primarily collected from the wild rather than cultivated systematically, leading to unsustainable harvesting practices [5]. This is particularly problematic for species in which therapeutically active compounds are concentrated in roots, bark, or other vital tissues, as their removal often results in the destruction of the entire plant. Consequently, a significant number of medicinal plant species are now considered threatened, posing risks not only to biodiversity but also to the future availability of plant-based therapeutics.

Phytochemicals, which are naturally occurring bioactive compounds synthesized by plants, play a crucial role in their defense mechanisms against environmental stress and biological threats. These compounds are widely distributed across various plant-based foods and medicinal sources, including fruits, vegetables, grains, herbs, and nuts. A vast diversity of phytochemicals has been identified, encompassing major groups such as carotenoids, polyphenols, terpenoids, phytosterols, saponins, and complex polysaccharides. Many of these compounds exhibit potent biological activities, including antioxidant, antimicrobial, antiviral, anti-inflammatory, and antiparasitic effects. Their functional versatility makes them valuable not only in traditional medicine but also in modern pharmacological research [6]. The convergence of rising dependence on plant-based remedies, rapid loss of plant biodiversity, and increasing demand for safer therapeutic alternatives highlights the urgent need to reassess the role of medicinal plants within contemporary healthcare frameworks. Ensuring sustainable utilization while maintaining ecological balance has become a critical challenge for researchers, policymakers, and healthcare practitioners alike.

Plants have long served as a rich reservoir of bioactive molecules with significant pharmacological potential, and their use in disease management dates back to ancient civilizations. Over time, numerous plant-derived compounds have contributed to the development of modern medicines, demonstrating the enduring importance of natural products in drug discovery. In recent years, phytochemicals have gained renewed attention as promising agents in the prevention and treatment of chronic diseases. Their structural diversity enables them to interact with multiple biological pathways, making them particularly effective against complex conditions such as cancer, cardiovascular disorders, inflammatory diseases, autoimmune conditions, and neurological disorders. As a result, plant-based compounds continue to serve as an important source of lead molecules for the development of new therapeutic agents [7].

the exploration of phytochemicals for managing chronic inflammation has opened new avenues in biomedical research. Given their natural origin, broad-spectrum activity, and relatively low toxicity, these compounds are increasingly being investigated as alternatives or complements to synthetic drugs. Harnessing the therapeutic potential of phytochemicals, while ensuring sustainable sourcing of medicinal plants, represents a promising strategy for addressing current and future healthcare challenges.

Plants are naturally enriched with free radical scavenging compounds such as vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and various secondary metabolites, all of which contribute significantly to antioxidant activity ([8]. Numerous investigations have demonstrated that these antioxidant constituents exhibit multiple biological properties, including anti-inflammatory, anti-atherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, and antiviral effects [9-10]. The consumption of natural antioxidants has been correlated with a lower incidence of chronic diseases such as cancer, cardiovascular disorders, diabetes, and other age-related ailments [11]. Consequently, there has been growing global interest in phytochemicals derived from berries, tea, herbs, oilseeds, legumes, fruits, and vegetables [12]. Medicinal plants have long been utilized in traditional healing systems, and their therapeutic applications have expanded considerably in recent decades [13].

The effectiveness of herbal medicines largely depends on the authenticity and quality of the raw plant materials used. Ensuring the safety, quality, and therapeutic reliability of medicinal plants and herbal formulations has therefore become a critical concern, highlighting the need for proper standardization. Recent developments in chromatographic and spectroscopic fingerprinting techniques have significantly contributed to the quality assessment of complex herbal preparations. Although many pharmacopoeias provide monographs describing the physicochemical characteristics of plant materials, modern analytical approaches focusing on the identification and quantification of active phytoconstituents are essential for reliable standardization of herbal products [14]. The World Health Organization has also emphasized the necessity of applying advanced analytical methods and appropriate quality standards to ensure the safety and efficacy of medicinal plant products [15]. Among various analytical tools, high-performance thin-layer chromatography (HPTLC) has emerged as a highly efficient and reliable technique for profiling chemical and biochemical markers. HPTLC provides superior resolution of active constituents with satisfactory accuracy within a relatively short analytical time [16]. This technique has become increasingly popular due to its operational advantages, including minimal mobile phase requirement, rapid analysis, and the capability to analyze multiple samples simultaneously on a single plate, unlike high-performance liquid chromatography (HPLC) (Ramya et al., 2010). Additionally, HPTLC allows direct analysis of turbid samples and suspensions, supports automated sample application, and permits repeated scanning of the same plate under different conditions, thereby improving analytical flexibility and cost-effectiveness [17].

The genus *Phyllanthus*, belonging to the family Phyllanthaceae, comprises a large and diverse group of plant species, with more than 1,300 species distributed across tropical and subtropical regions of Asia, Africa, the Americas, and Australia.

Members of this genus have been extensively utilized in traditional medicine systems, particularly in Asian countries, where they play a significant role in ethnopharmacological practices. These plants have been traditionally employed for a wide range of therapeutic purposes, including the management of digestive disorders, genitourinary conditions, respiratory ailments, skin diseases, liver-related disorders such as jaundice, and kidney stones. They are also valued for their astringent, diuretic, febrifuge, and antiseptic properties. In South American traditional medicine, certain *Phyllanthus* species are used to regulate uric acid levels, reflecting their diverse medicinal applications across cultures [18], various species within the genus have been reported to exhibit therapeutic potential in the treatment of conditions such as hypertension, fever, inflammation, diarrhea, urinary tract infections, diabetes, wounds, rheumatism, and arthritis. The wide spectrum of biological activities associated with *Phyllanthus* species is largely attributed to their rich phytochemical composition. These plants are known to contain a variety of bioactive compounds, including alkaloids, terpenoids, and an array of polyphenolic constituents such as flavonoids, phenolic acids, stilbenes, anthocyanins, coumarins, and lignans. Such compounds are recognized for their potent antioxidant and pharmacological properties, contributing to the therapeutic efficacy of these plants.

Notably, plant-derived polyphenols have gained considerable attention as safer alternatives to synthetic drugs, particularly in the management of metabolic disorders such as hyperuricemia. Unlike conventional antihyperuricemic medications, which may be associated with adverse side effects, natural polyphenols obtained from dietary and medicinal plant sources are generally considered to be less toxic and more biocompatible, making them promising candidates for alternative therapeutic strategies.

The genus *Phyllanthus* encompasses a wide range of growth forms, including herbs, shrubs, and trees, many of which possess significant pharmacological importance. Extensive phytochemical investigations have revealed that species within this genus contain hundreds of distinct chemical constituents, highlighting their potential as a valuable source of bioactive molecules. Furthermore, extracts derived from certain *Phyllanthus* species have demonstrated antiviral activity, particularly against the hepatitis B virus, underscoring their importance in medicinal research. While several species such as *Phyllanthus amarus*, *Phyllanthus emblica*, and *Phyllanthus niruri* have been widely studied for their biological activities, comparatively fewer studies have focused on other species within the genus [19]. Recent scientific interest has therefore shifted toward less-explored species, with an emphasis on validating their traditional uses through phytochemical and pharmacological investigations. In this context, *Phyllanthus pinnatus* represents an important yet underexplored member of the genus. This species, belonging to the family Phyllanthaceae, is part of a globally distributed group of plants known for their medicinal value. Further research into its phytochemical profile and biological activities is essential to fully understand its therapeutic potential and to support its use in evidence-based medicine.

Materials and Methods

Plant Material

Fresh leaves of *Phyllanthus pinnatus* (Wight) G.L. Webster (family Phyllanthaceae), weighing approximately 1 kg, were collected from the Lingala Nallamalla Forest located in Achampet Mandal, Nagar Kurnool District, Telangana State, India (16°38′60.00″ N, 80°07′59.88″ E). The collection was carried out during the months of August to September 2023, a period considered suitable for obtaining optimal phytochemical content.

Authentication of Plant Material

The collected plant material was taxonomically authenticated by the Department of Botany, Osmania University, Hyderabad, Telangana, India. A voucher specimen was prepared and deposited in the Herbarium Hyderbadensis, Department of Botany, Osmania University, under the voucher number OUAS-217 for future reference and verification.

Drying and Powder Preparation

The freshly collected leaves were thoroughly washed with double-distilled water to remove adhering dust and other extraneous matter. The cleaned plant material was then shade-dried at room temperature (25 ± 2 °C) for approximately three weeks until a constant weight was achieved. Shade drying was preferred to preserve thermolabile bioactive compounds. The dried leaves were subsequently ground into a fine powder using a mechanical grinder. The powdered material was sieved to obtain uniform particle size and stored in airtight containers under dry conditions for further experimental analysis.

Extraction Using Soxhlet Apparatus

The extraction of phytoconstituents from *Phyllanthus pinnatus* leaves was carried out using a Soxhlet apparatus following a sequential solvent extraction method. Initially, fresh leaves were thoroughly washed under running water to remove dust, debris, and other contaminants, followed by rinsing with distilled water to ensure complete cleanliness. The cleaned leaves were shade-dried and subsequently ground into a fine powder using a mechanical grinder. The powdered material was sieved to obtain uniform particle size, ensuring consistency during extraction. A measured quantity of the dried leaf powder was subjected to successive extraction using solvents of increasing polarity, namely petroleum ether, chloroform, ethyl acetate, and methanol. Each extraction was performed in a Soxhlet apparatus for approximately five hours, allowing continuous hot percolation and efficient recovery of phytochemicals. The extraction temperature was maintained according to the respective boiling point of each solvent to facilitate optimal solvent cycling and effective extraction. Following extraction, the obtained solvent extracts were concentrated by evaporating the solvents in a hot air oven maintained at 45 °C until dryness. The dried extracts were collected, weighed, and stored in airtight containers for subsequent phytochemical analysis.

Calculation of Percentage Yield

The percentage yield of each extract was determined by weighing the dried extracts obtained after complete solvent evaporation. The yield was calculated based on the initial weight of the plant material used for extraction using the following formula:

$$\text{Percentage of yield } (\%) = \left(\frac{\text{Dry weight of extract}}{\text{Dry weight of a plant}} \right) \times 100$$

Qualitative Screening of Phytochemicals in *Phyllanthus pinnatus* Leaves

Preliminary phytochemical screening of *Phyllanthus pinnatus* leaf extracts was performed to identify the presence of various secondary metabolites.

Standard qualitative methods were employed to detect major classes of bioactive compounds, including alkaloids, flavonoids, saponins, steroids, terpenoids, phenolic compounds, tannins, glycosides, cardiac glycosides, coumarins, phytosterols, leucoanthocyanins, resins, fixed oils, anthraquinones, and quinones. These tests were conducted to determine the presence or absence of specific phytoconstituents based on characteristic color changes or precipitate formation.

Test for Alkaloids

Approximately 50 mg of solvent-free extract was mixed with 5 mL of dilute hydrochloric acid and filtered. The filtrate was subjected to alkaloid detection using Mayer's reagent.

Mayer's Test

To 3 mL of the filtrate, a few drops of Mayer's reagent were added along the side of the test tube. The formation of a white or creamy precipitate indicated the presence of alkaloids.

Test for Flavonoids

Shinoda Test

A small quantity of the extract was treated with a pinch of zinc dust followed by the addition of concentrated hydrochloric acid. The development of a pink to magenta coloration after a few minutes confirmed the presence of flavonoids.

Test for Steroids

To 1 mL of the extract, 1 mL of chloroform and 2–3 mL of acetic anhydride was added, followed by the careful addition of 1–2 drops of concentrated sulfuric acid. The appearance of a dark green coloration indicated the presence of steroidal compounds.

Test for Saponins

About 0.5 g of powdered plant material was boiled gently with 20 mL of distilled water for 2 minutes and then filtered while hot. After cooling, 5 mL of the filtrate was diluted with distilled water and shaken vigorously. The formation of stable froth indicated the presence of saponins.

Detection of Steroids and Terpenoids

Salkowski's Test

The extract was dissolved in chloroform, and a few drops of concentrated sulfuric acid were carefully added along the side of the test tube to form a lower layer. The appearance of a reddish-brown coloration at the interface indicated the presence of steroids, while a yellow to reddish coloration suggested the presence of terpenoids.

Detection of Phenolic Compounds

Ferric Chloride Test

Approximately 50 mg of the extract was dissolved in 5 mL of distilled water. To this solution, a few drops of neutral 5% ferric chloride solution were added. The development of a dark green or bluish-black coloration confirmed the presence of phenolic compounds.

Detection of Tannins

Potassium Dichromate Test

To the test solution, 2% potassium dichromate solution was added. The formation of a yellow precipitate indicated the presence of tannins.

Detection of Glycosides

The extract was dissolved in alcohol or prepared using a hydroalcoholic solution and subjected to the following tests:

a) Baljet's Test

The test solution was treated with 2% sodium picrate solution. The appearance of a yellow to orange coloration indicated the presence of glycosides.

b) Legal's Test

The extract was treated with pyridine and made alkaline, followed by the addition of 2% sodium nitroprusside solution. The formation of a pink to red coloration confirmed the presence of glycosides.

c) Keller–Killiani Test

Approximately 100 mg of the extract was dissolved in 1 mL of glacial acetic acid containing one drop of ferric chloride solution. This was carefully layered with 1 mL of concentrated sulfuric acid. The formation of a brown ring at the interface indicated the presence of glycosides, particularly cardiac glycosides.

Detection of Cardiac Glycosides

A 2 mL portion of the filtrate was treated with 1 mL of glacial acetic acid, followed by the addition of 1 mL ferric chloride solution and 1 mL concentrated sulfuric acid. The appearance of a greenish-blue coloration confirmed the presence of cardiac glycosides.

Detection of Coumarins

One milliliter of the extract was placed in a test tube and covered with filter paper moistened with dilute sodium hydroxide solution. The setup was heated in a water bath for a few minutes. The filter paper was then removed and observed under ultraviolet (UV) light. The presence of green fluorescence indicated the presence of coumarins.

Test for Phytosterols

The extract was dissolved in chloroform, and a few drops of concentrated sulfuric acid were added. The solution was shaken gently and allowed to stand. The appearance of a reddish coloration in the lower chloroform layer indicated the presence of phytosterols.

Detection of Quinones

Approximately 1 mL of the crude extract was treated with a few drops of dilute sodium hydroxide (NaOH) solution. The development of a blue-green or reddish coloration indicated the presence of quinones.

Detection of Resins

To 2 mL of the extract, 5–10 drops of acetic anhydride were added and gently heated to ensure dissolution. Subsequently, 0.5 mL of concentrated sulfuric acid was added carefully. The appearance of a bright purple coloration confirmed the presence of resins.

Detection of Leucoanthocyanins

Equal volumes (1 mL each) of the crude extract and isoamyl alcohol were mixed thoroughly. The formation of a red coloration in the upper layer indicated the presence of leucoanthocyanins.

Detection of Anthraquinones
Approximately 1 g of powdered plant material was treated with chloroform and shaken for about 5 minutes. The mixture was then filtered, and 5 mL of ammonia solution was added to the filtrate and gently shaken. The appearance of a bright pink coloration in the upper aqueous layer confirmed the presence of anthraquinones.

Detection of Fixed Oils
A small quantity of the extract was placed between two sheets of filter paper and pressed gently. The formation of a persistent oily stain on the paper indicated the presence of fixed oils.

Quantification of Total Alkaloid Content
The total alkaloid content of the plant extract was determined using the bromocresol green (BCG) method. Briefly, 1 mg of the extract was dissolved in dimethyl sulfoxide (DMSO), followed by the addition of 1 mL of 2 N hydrochloric acid. The solution was filtered and transferred into a separating funnel. Subsequently, 5 mL of bromocresol green solution and 5 mL of phosphate buffer were added. The mixture was extracted successively with chloroform in volumes of 1, 2, 3, and 4 mL through vigorous shaking. The chloroform extracts were pooled into a 10 mL volumetric flask and the final volume was adjusted with chloroform. A calibration curve was prepared using atropine as the standard (20–100 µg/mL), processed under identical conditions. The absorbance of both standard and sample solutions was measured at 470 nm against a reagent blank using a UV–Visible spectrophotometer. The total alkaloid content was calculated from the calibration curve and expressed as milligrams of atropine equivalents per gram of extract (mg AE/g).

Quantification of Total Flavonoid Content
The total flavonoid content was estimated using the aluminium chloride colorimetric method. In this procedure, 1 mL of the plant extract was mixed with 4 mL of distilled water in a 10 mL volumetric flask. To this, 0.3 mL of 5% sodium nitrite was added and allowed to react for 5 minutes. Subsequently, 0.3 mL of 10% aluminium chloride solution was added, followed by incubation for another 5 minutes. Then, 2 mL of 1 M sodium hydroxide was added, and the final volume was adjusted to 10 mL using distilled water. Standard solutions of quercetin (20–100 µg/mL) were prepared similarly to construct a calibration curve. The absorbance of the reaction mixture was measured at 510 nm using a UV–Visible spectrophotometer against a reagent blank. The total flavonoid content was determined from the calibration curve and expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g).

Quantification of Total Tannin Content
The total tannin content was determined using the Folin–Ciocalteu method. Briefly, 0.1 mL of the plant extract was transferred into a 10 mL volumetric flask containing 7.5 mL of distilled water. To this, 0.5 mL of Folin–Ciocalteu phenol reagent was added, followed by 1 mL of 35% sodium carbonate (Na_2CO_3) solution. The mixture was then diluted to 10 mL with distilled water, mixed thoroughly, and incubated at 30 °C for 30 minutes. A series of gallic acid standard solutions (20–100 µg/mL) were prepared under similar conditions to construct a calibration curve. The absorbance of both standard and sample solutions was measured at 725 nm using a UV–Visible spectrophotometer against a reagent blank.
The total tannin content was calculated from the calibration curve and expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g).

Quantification of Total Phenolic Content
The total phenolic content was estimated using the Folin–Ciocalteu spectrophotometric method. In this procedure, 1 mL of the plant extract was mixed with 9 mL of distilled water. Subsequently, 1 mL of Folin–Ciocalteu reagent was added, and the mixture was shaken thoroughly. After 5 minutes, 10 mL of 7% sodium carbonate (Na_2CO_3) solution was added, and the final volume was adjusted to 25 mL with distilled water. Standard gallic acid solutions (20–100 µg/mL) were prepared to generate a calibration curve. The reaction mixture was incubated at 30 °C for 90 minutes to allow complete color development. The absorbance was measured at 550 nm using a UV–Visible spectrophotometer against a reagent blank. The total phenolic content was calculated and expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g).

HPTLC Analysis
High-Performance Thin-Layer Chromatography (HPTLC) analysis of the methanolic extract of *Phyllanthus pinnatus* leaves was carried out to establish a characteristic fingerprint profile. The concentrated methanolic extract was applied onto pre-coated silica gel 60 F_{254} aluminum plates (10 × 10 cm) using an Automatic TLC Sampler (ATS 4, CAMAG) equipped with a 25 µL Hamilton syringe. Sample application was performed under a controlled nitrogen flow at a constant rate of 15 µL/s, producing bands of 5 mm length with a distance of 10 mm between tracks. Prior to sample application, the plates were pre-washed with methanol and activated. Chromatographic separation was achieved using a mobile phase consisting of chloroform:methanol (8:2, v/v), which provided optimal resolution of phytoconstituents. The development was carried out in a twin-trough glass chamber saturated with 10 mL of the mobile phase for 20 minutes at room temperature. Linear ascending development was performed up to a migration distance of approximately 8.5 cm. After development, the plates were air-dried using a hot air dryer under controlled ventilation. Densitometric scanning was performed using a CAMAG TLC Scanner III in absorbance–reflectance mode at wavelengths of 254 nm and 366 nm. The slit dimensions were maintained at 4 × 0.20 mm, and scanning was conducted at a speed of 20 mm/s. Instrument control and data analysis were carried out using WinCATS software (version 1.4.3, CAMAG). A deuterium lamp, emitting a continuous ultraviolet spectrum in the range of 200–400 nm, was used as the radiation source.
The relative concentration of separated compounds was estimated based on the intensity of the reflected light. Chromatograms were documented digitally using a CAMAG DigiStore 2 documentation system, which included an illuminator, Reprostar 3, and a high-resolution digital camera. The obtained HPTLC fingerprint profile serves as a reliable tool for the identification, authentication, and quality assessment of *P. pinnatus* leaf extract.

Results and Observations
Plant Profile of *Phyllanthus pinnatus*
The taxonomic classification of *Phyllanthus pinnatus* was established based on standard floristic references. The plant belongs to the family Phyllanthaceae and is widely recognized for its medicinal importance. The detailed classification is presented in Table 1.

Table 1: Taxonomic Classification of Phyllanthus pinnatus

Taxonomic Rank	Classification
Kingdom	Plantae
Phylum	Streptophyta
Class	Equisetopsida
Subclass	Magnoliidae
Order	Malpighiales
Family	Phyllanthaceae
Genus	*Phyllanthus*
Species	*Phyllanthus pinnatus*

Morphological Description of *Phyllanthus pinnatus*

Phyllanthus pinnatus, commonly referred to as the "Sand Potato Bush," is a bushy shrub typically attaining a height of 1–1.5 m, although it may occasionally grow into a small tree reaching up to 4 m. The plant is completely glabrous (hairless) in nature. The main branches are rough in texture, marked by stipular scars and reduced scaly leaves. The leaf-bearing branchlets are fascicled, short (4–7 mm long), sparsely foliated, and often deciduous. The leaves exhibit considerable variation in shape, ranging from elliptic and ovate-elliptic to oblong-elliptic or obovate. They are generally small, measuring about 5–23 mm in length and 4–17 mm in width, with a membranous texture and a pale glaucous appearance on the lower surface. The leaf base is typically obliquely wedge-shaped and may be rounded or blunt, while the apex varies from pointed to rounded or slightly apiculate. The petiole is short, usually measuring 1–3 mm in length. Flowers are borne directly on older branches and are unisexual, arranged in dense clusters (glomerules). Male flowers are located in the proximal axils and are borne on slender, thread-like pedicels measuring 3–9 mm in length. Each male flower possesses six sepals arranged in two whorls (3 + 3), which are ovate to obovate in shape. The floral disc is annular and six-lobed, with six prominent stamens having free, thread-like filaments. Female flowers occur in the distal axils and are supported by longer, thread-like pedicels ranging from 8 to 37 mm in length. They consist of six broadly ovate to elliptic sepals with scarious margins. The floral disc is relatively larger, nearly flat, and may be entire or slightly lobed. The fruit is a nearly spherical capsule, measuring approximately 6–8 mm in diameter and 10–11 mm in length. It is characteristically three-lobed, with a hard (crustaceous) surface and a wrinkled texture marked by longitudinal ridges. *Phyllanthus pinnatus* is commonly found in semi-deciduous and scrub forest ecosystems, typically at elevations up to 1000 m. It is widely distributed in regions such as Telangana, Andhra Pradesh, Tamil Nadu, Sri Lanka, and parts of East Africa. The flowering period generally extends from February to October.

Flowering: February, April to July, and September **(Fig; 1A-B).**

Figure 1: Habitat of P.pinnatus

Phytochemical Studies of *P.pinnatus*

Qualitative phytochemical studies

Phytochemical screening of *Phyllanthus pinnatus* leaves was performed using sequential solvent extraction with petroleum ether, chloroform, ethyl acetate, and methanol to investigate the presence of key bioactive compounds. The results demonstrated distinct solubility patterns of secondary metabolites depending on the polarity of the solvent used, indicating the diverse phytochemical composition of the plant (Table 2). Alkaloids were found exclusively in the methanolic extract, exhibiting a strong positive reaction in Mayer's test. This suggests that alkaloids present in *P. pinnatus* are predominantly polar in nature, as non-polar solvents like petroleum ether and moderately polar solvents like chloroform and ethyl acetate failed to extract them. Flavonoids were moderately present in both ethyl acetate and methanol extracts, as confirmed by the lead acetate test. This reflects the medium to high polarity of flavonoids, making them soluble in semi-polar to polar solvents. Their absence in petroleum ether and chloroform further supports their polarity-dependent solubility.

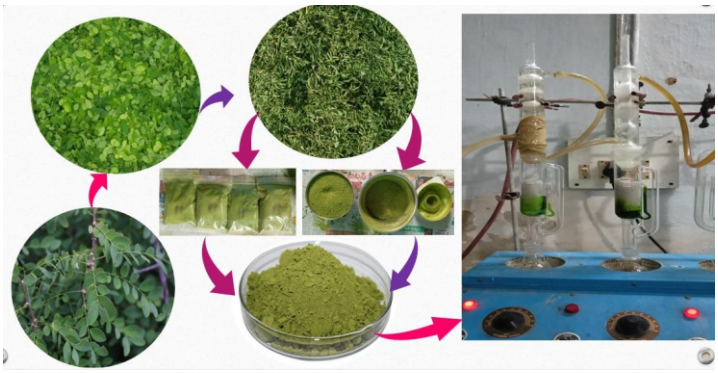


Figure 2: Soxhlet extraction process: infographic representing pre-extraction process which subject to Soxhlet extraction

Figure 3: Extraction P. pinnatus with Soxhlet apparatus

Saponins, known for their surface-active properties, were found only in the methanol extract, with a strong positive result in the foam test. This again highlights the necessity of highly polar solvents for efficient extraction of these glycosidic compounds. Their complete absence in petroleum ether, chloroform, and ethyl acetate indicates low solubility in nonpolar or semipolar environments. Steroids and terpenoids were strongly present in the ethyl acetate extract and moderately present in both chloroform and methanol extracts, based on Salkowski's test. Their absence in petroleum ether suggests that the compounds extracted belong to a class of moderately polar terpenoids or conjugated steroidal compounds rather than non-polar types. Phenolic compounds were moderately present in chloroform and methanol extracts, as shown by the ferric chloride test, while absent in petroleum ether and ethyl acetate extracts. This result suggests the presence of phenolic compounds with moderate polarity, which are not extractable with non-polar solvents like petroleum ether. Tannins followed a similar pattern to phenols, with moderate presence detected in chloroform and methanol extracts through the gelatin test.

The findings further confirm that tannins in *P. pinnatus* are polar to moderately polar in nature and require solvents with higher polarity for effective extraction.

Glycosides, important plant secondary metabolites with sugar moieties, were found in all extracts except petroleum ether. A strong reaction was observed in both chloroform and methanol extracts using Borntrager's test, with moderate presence in the ethyl acetate extract. The results indicate that glycosides are primarily soluble in medium to highly polar solvents. Coumarins were detected in chloroform and ethyl acetate extracts but not in petroleum ether or methanol. The NaOH test indicated a moderate presence in both cases. This pattern suggests coumarins in *P. pinnatus* may exhibit intermediate polarity, being more extractable in solvents like chloroform and ethyl acetate. Phytosterols, which are structurally similar to cholesterol, were highly concentrated in petroleum ether and chloroform extracts, showing strong and moderate positive results, respectively. This indicates their non-polar nature, as they are not detected in polar solvents like ethyl acetate or methanol.

Quinones were found to be weakly present in the chloroform extract and strongly present in the methanol extract. The precipitate test confirmed the presence of these oxidized phenolic derivatives in polar solvents, consistent with their general chemical nature. Resins were only detected in the methanolic extract using the acetic anhydride test, indicating their polar or amphipathic character. Their absence in other solvent extracts reflects poor solubility in non-polar or medium-polarity solvents. Cardiac glycosides, a special class of bioactive glycosides with known pharmacological activity, were moderately present in the ethyl acetate extract and strongly present in the methanol extract, as indicated by the Keller-Killiani test. These findings support the use of polar solvents for their effective isolation. Leucoanthocyanins, precursors to anthocyanins, were moderately present in ethyl acetate and strongly present in methanol, as detected using the isoamyl alcohol test. These water-soluble flavonoid derivatives again confirm the requirement of polar solvents for extraction. Anthraquinones were absent in all solvent extracts, indicating that either these compounds are not present in *P. pinnatus* leaves or are present in very low concentrations below the detection limit of the qualitative test employed. Fixed oils, which are non-volatile lipids, were found abundantly in petroleum ether, chloroform, and ethyl acetate extracts using the spot/stain test. Their absence in the methanol extract aligns with the general insolubility of oils in polar solvents and validates the efficiency of non-polar and semi-polar solvents in lipid extraction.

Table 2: Qualitative analysis of phytochemicals using various chemical tests

S.NO	Phyto. Name	Pet. ether	Chloroform	Ethyl acetate	Methanol
1.	**Alkaloids** Mayer's	-	-	-	+++
2.	**Flavonoids** Lead acetate test	-	-	+	+
3.	**Saponins** Foam test	-	-	-	+++
4.	**Steroids & Terpenoids** Salkowki's test	-	++	+++	++
5.	**Phenols** Ferric chloride test	-	++	-	++
6.	**Tannins** Gelatin test	-	++	-	++
7.	**Glycosides** Borntrager's Test	-	+++	++	+++
8.	**Coumarins** NaOH test	-	++	++	-
9.	**Phytosterols** Salkowski's test	++	+++	-	-
10.	**Quinones** Precipitate test	-	+	-	+++
11.	**Resins** Acetic anhydride test	-	-	-	+++
12.	**Cardiac Glycosides** Kellar - Kiliani	-	-	++	+++
13.	**Leuco anthocyanins** Isoamyl alcohol test	-	-	++	+++
14.	**Anthraquinones** Borntrager's	-	-	-	-
15.	**Fixed oils** Spot test/ Stain test	+++	+++	+++	-

Figure 4: Qualitative phytochemical analysis of P. pinnatus

Table 3: Quantitative analysis of phytochemicals

	Alkaloids (Atropine equivalent) mg/g	Flavonoid (Quercetin equivalent) mg/g	Phenol (Gallic acid equivalent) mg/g	Tannin (Gallic acid equivalent) mg/g
Petroleum ether	0	0	0	0
Ethyl acetate	0	450	0	0
Chloroform	316	0	260	195
Methanol	524	536	385	330

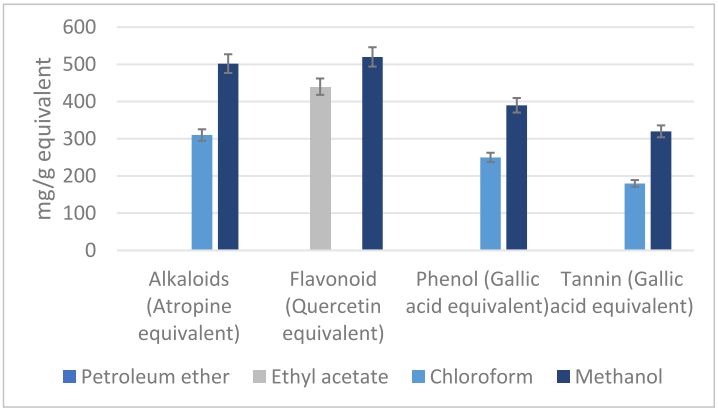


Figure 5: Quantification of phytochemical content from P. pinnatus leaf extract with different solvents

Flavonoids were predominantly extracted in the methanolic extract (536 mg/g, Quercetin equivalent), with a significant amount also present in the ethyl acetate extract (450 mg/g). No flavonoids were detected in the chloroform or petroleum ether extracts, suggesting these solvents are ineffective for extracting flavonoid compounds from *P. pinnatus*. Phenolic content followed a similar trend, being highest in the methanol extract (385 mg/g, Gallic acid equivalent), moderately present in the chloroform extract (260 mg/g), and completely absent in petroleum ether and ethyl acetate extracts.

Methanol proved to be the most effective solvent in extracting a wide range of polar phytochemicals including alkaloids, saponins, phenols, tannins, glycosides, quinones, cardiac glycosides, leucoanthocyanins, and resins. Ethyl acetate and chloroform also showed moderate efficiency in extracting semi-polar compounds like flavonoids, steroids, terpenoids, and coumarins. In contrast, petroleum ether primarily extracted non-polar compounds such as phytosterols and fixed oils. This polarity-based extraction pattern provides a comprehensive understanding of the solvent-specific phytochemical composition of *Plectranthus pinnatus*.

Quantitative Phytochemical analysis of *P. pinnatus*

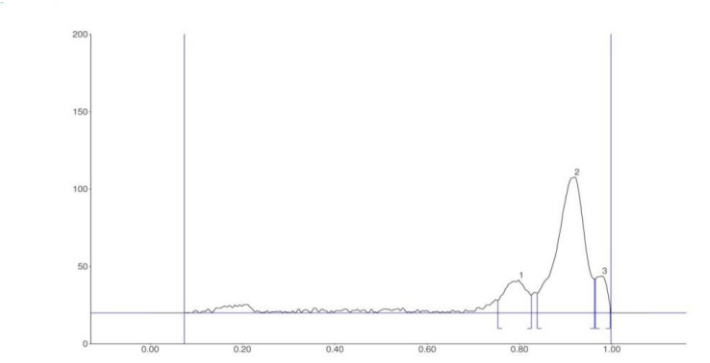
The quantitative estimation of key phytochemical constituents—alkaloids, flavonoids, phenols, and tannins were performed on various *Phyllanthus pinnatus* leaf extracts using respective standards for calibration. The results are expressed in milligrams per gram (mg/g) of dry extract weight, based on equivalent standard compounds: atropine for alkaloids, quercetin for flavonoids, and gallic acid for both phenols and tannins. The data obtained is presented in **Table 5**. Among all extracts, the methanolic extract demonstrated the highest concentration of all four phytochemical classes, indicating its superior ability to extract polar bioactive compounds. Specifically, the alkaloid content was highest in the methanol extract at 524 mg/g (Atropine equivalent), followed by the chloroform extract at 316 mg/g, while both petroleum ether and ethyl acetate extracts showed no detectable levels of alkaloids.

Tannin content was most pronounced in the methanol extract at 330 mg/g (Gallic acid equivalent), followed by the chloroform extract (195 mg/g), with no detectable tannins in petroleum ether or ethyl acetate extracts. These findings confirm that methanol is the most efficient solvent for the extraction of a wide range of polar phytoconstituents, whereas non-polar solvents like petroleum ether showed negligible extraction capacity for these compounds. The chloroform extract exhibited moderate levels of alkaloids, phenols, and tannins, indicating its intermediate polarity is suitable for selective extraction. Ethyl acetate was notably efficient for flavonoids but did not yield other classes of compounds in measurable amounts.

Profile of hptlc finger printing of *p. pinnatus*

The chromatographic profile of the leaf methanol extract, when scanned at 254 nm, demonstrated a well-resolved separation pattern with three distinct spots (Figure 6 & 7). Among these, spot 2 exhibited the highest peak area, indicating maximum composition, with an Rf value of 0.92. The other two spots were observed at Rf values of 0.80 and 0.98. All three spots appeared brown in color under 254 nm, suggesting the presence of UV-absorbing phytoconstituents in varying concentrations. The differential intensities of these spots reflect the relative abundance of the separated compounds in the extract. Further densitometric scanning at 366 nm revealed a more complex phytochemical profile with six clearly resolved spots

(Figure 8&9). Among them, spot 2 again showed the maximum composition with an Rf value of 0.19, indicating that this particular compound is predominant under fluorescence detection as well. The six fluorescent spots were recorded at Rf values of 0.14 (blue), 0.19 (light green), 0.36 (blue), 0.49 (orange), 0.78 (yellow), and 0.95 (red). The variation in fluorescence colors suggests the presence of chemically diverse bioactive constituents, possibly belonging to different classes such as flavonoids, phenolics, or other secondary metabolites. The distinct chromatographic fingerprints obtained at both 254 nm and 366 nm confirm the phytochemical complexity of the studied drug. These characteristic Rf values and densitometric patterns serve as reliable identification markers for authentication and quality control of the plant material. Such reproducible HPTLC fingerprint profiles can be considered valuable reference standards for inclusion in pharmacopoeial monographs, this fingerprint analysis represents a vital quality assurance parameter, ensuring the reliability, consistency, and reproducibility of the herbal drug.



winCATS Planar Chromatography Manager

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.75	8.1	0.80	21.2	15.92	0.83	11.5	743.3	15.25	unknown *
2	0.84	12.7	0.92	87.8	66.09	0.96	21.8	3721.9	76.37	unknown *
3	0.97	22.0	0.98	23.9	17.99	1.00	3.6	408.0	8.37	unknown *

Figure 6: HPTLC densitogram of methanolic extract of P. pinnatus scanned at 254 nm by using chloroform: methanol (8:2 v/v)

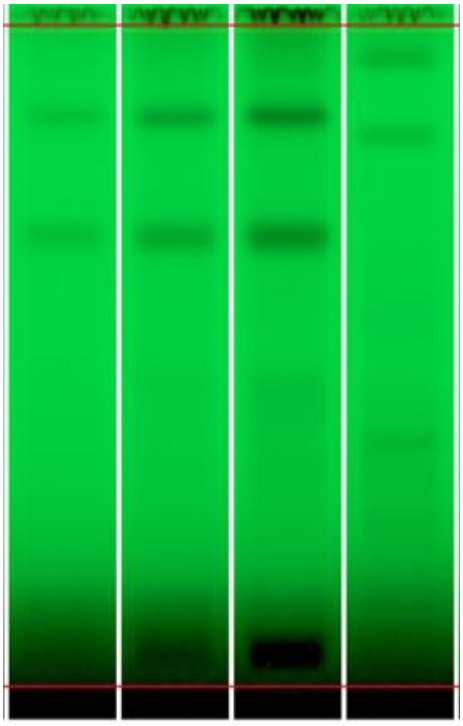
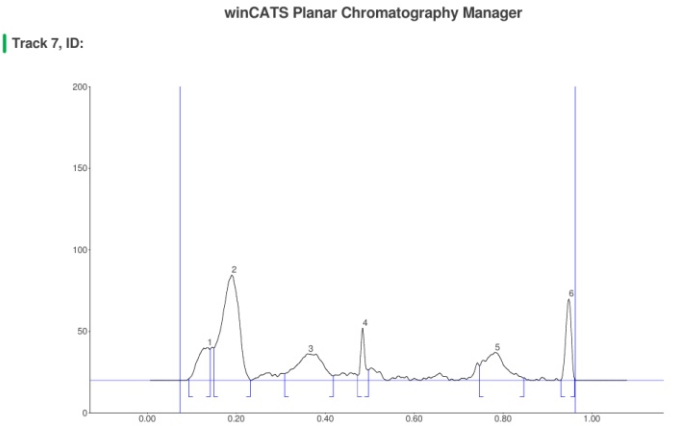


Figure 7: High performance thin layer chromatography image of P. pinnatus at 254 nm in chloroform: methanol (8:2 v/v)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.09	1.1	0.14	20.2	10.09	0.14	19.1	402.7	9.66	unknown *
2	0.15	20.0	0.19	64.6	32.21	0.23	0.3	1797.2	43.13	unknown *
3	0.31	4.2	0.36	16.3	8.15	0.42	2.7	704.3	16.90	unknown *
4	0.47	3.1	0.49	32.3	16.09	0.50	6.5	219.2	5.26	unknown *
5	0.75	8.9	0.78	17.2	8.59	0.85	1.5	597.9	14.35	unknown *
6	0.93	0.1	0.95	49.9	24.88	0.96	0.8	445.5	10.69	unknown *

Figure 8: HPTLC densitogram of methanolic extract of P. pinnatus scanned at 366 nm by using chloroform: methanol (8:2 v/v)

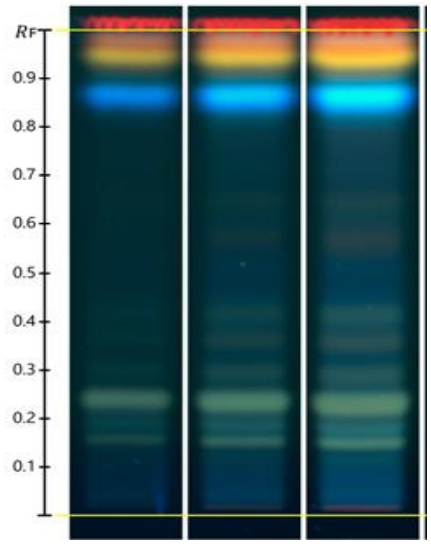


Figure 9: High performance thin layer chromatography image of P. pinnatus at 366 nm in chloroform: methanol (8:2 v/v)

Discussions

Phyllanthus pinnatus belongs to the genus Phyllanthus, which is widely acknowledged in traditional systems of medicine for the management of digestive, hepatic, renal, metabolic, and infectious disorders. Although specific pharmacological investigations on *P. pinnatus* remain limited, its therapeutic potential can reasonably be interpreted in the context of the extensive phytochemical and biological evidence reported for other well-studied Phyllanthus species. Members of this genus are known to contain diverse secondary metabolites such as lignans, flavonoids, tannins, and triterpenoids, many of which exhibit documented antiviral, antioxidant, antidiabetic, anti-inflammatory, and anticancer activities. Ethnomedicinally, *Phyllanthus* species are extensively utilized in regions such as East Africa, India, and Sri Lanka for the treatment of jaundice, digestive ailments, renal calculi, fever, malaria, and various skin diseases. Given the geographical distribution of *P. pinnatus* in these areas, it is plausible that the species contributes to local traditional healthcare practices for similar indications. Such traditional claims provide a strong rationale for scientific validation through phytochemical and pharmacological investigations.

Comprehensive reviews of the genus have reported more than 500 isolated compounds and have substantiated a broad spectrum of bioactivities, including hepatoprotective, nephroprotective, antidiabetic, anti-inflammatory, antimicrobial, and anticancer effects [19-23], these findings position Phyllanthus as a significant phytopharmaceutical resource and a promising reservoir of bioactive lead molecules. Therefore, systematic studies on *P. pinnatus* are warranted to validate its traditional uses, characterize its phytochemical profile, and explore its potential therapeutic applications. Phytochemicals represent a diverse group of compounds synthesized by plants during their growth and metabolic processes. These substances occur abundantly in everyday foods like fruits, vegetables, grains, nuts, cocoa, chocolate, juices, tea, coffee, and wine. Unlike primary metabolites essential for plant growth, development, and reproduction, most phytochemicals serve non-essential roles and earned the label "secondary metabolites" in 1891 [24].

Plants in the *Phyllanthus* genus stand out for their rich variety of secondary metabolites, such as polyphenols, flavonoids, alkaloids, terpenoids, and organic acids. For centuries, various Phyllanthus species have played key roles in traditional medicine, addressing ailments including kidney and urinary issues, gut infections, diabetes, and hepatitis B. Their therapeutic effects stem from specific chemicals that trigger targeted physiological responses in humans. This medicinal potential has sparked extensive research into their chemical makeup. Preclinical and clinical trials using extracts and isolated compounds from these plants validate many traditional uses against diverse health conditions. Notably, *Phyllanthus* species contain terpenes, alkaloids, lignans, flavonoids, and tannins, each exhibiting a range of biological activities [25].

The qualitative phytochemical analysis of *Phyllanthus pinnatus* leaves revealed a diverse array of secondary metabolites with clear polarity-dependent extraction patterns. Methanol extracts exhibited the highest diversity of bioactive compounds, including alkaloids, saponins, glycosides, quinones, and cardiac glycosides, indicating that many phytoconstituents of *P. pinnatus* are predominantly polar. Similar findings have been reported in other *Phyllanthus* species, where polar solvents effectively extract flavonoids, tannins, alkaloids, and phenolic compounds associated with significant biological activities [26]. The flavonoids and phenolics detected mainly in semi-polar to polar fractions corroborate earlier studies demonstrating that these compounds contribute to antioxidant and antimicrobial properties in *Phyllanthus* plants. The presence of terpenoids, sterols, and fixed oils in non-polar and semi-polar extracts further reflects the chemical diversity typical of the genus. Extensive phytochemical investigations have reported more than 500 compounds from *Phyllanthus*, including lignans, flavonoids, tannins, and triterpenoids responsible for its therapeutic potential [26-27]. The quantitative phytochemical analysis of *Phyllanthus pinnatus* leaf extracts revealed significant variations in metabolite content depending on solvent polarity. Among the tested solvents, the methanolic extract exhibited the highest concentrations of alkaloids (524 mg/g atropine equivalent), flavonoids (536 mg/g quercetin equivalent), phenols (385 mg/g gallic acid equivalent), and tannins (330 mg/g gallic acid equivalent), highlighting the efficiency of polar solvents in extracting bioactive phytochemicals. The chloroform fraction showed moderate levels of alkaloids (316 mg/g), phenols (260 mg/g), and tannins (195 mg/g), whereas flavonoids were predominantly detected in the ethyl acetate extract (450 mg/g).

In contrast, petroleum ether extract showed no detectable levels of these compounds, indicating the limited solubility of these metabolites in non-polar solvents.

These findings are consistent with previous studies reporting that species of the genus *Phyllanthus* are rich sources of phenolics, flavonoids, tannins, and alkaloids, which are more efficiently extracted using polar solvents such as methanol. The abundance of phenolic and flavonoid compounds in methanol extracts is often associated with strong antioxidant and pharmacological activities observed in *Phyllanthus* species [28-29]. The HPTLC fingerprint analysis of the methanolic leaf extract of *Phyllanthus pinnatus* revealed a distinct chromatographic pattern, confirming the presence of multiple phytoconstituents. At 254 nm, three well-resolved spots were detected with Rf values of 0.80, 0.92, and 0.98, among which the band at Rf 0.92 showed the highest peak area, indicating its predominance in the extract. Visualization at 366 nm revealed a more complex profile with six fluorescent bands at Rf values of 0.14, 0.19, 0.36, 0.49, 0.78, and 0.95 exhibiting blue, light green, orange, yellow, and red fluorescence. The variation in fluorescence intensity and color suggests the presence of chemically diverse phytochemicals, particularly phenolic and flavonoid compounds commonly detected under UV fluorescence conditions. HPTLC fingerprinting is widely recognized as a reliable analytical technique for the characterization and quality control of herbal drugs, as the number, position (Rf), and color of chromatographic zones serve as unique identification markers for plant materials [30-31]. The reproducible chromatographic profile obtained in this study therefore provides a valuable reference for the authentication, standardization, and pharmacognostic evaluation of *P. pinnatus.*

Conclusions

The present study established a comprehensive phytochemical profile and HPTLC fingerprint for *Phyllanthus pinnatus* leaves, contributing to its scientific standardization. Qualitative and quantitative analyses revealed the presence of diverse secondary metabolites, with the methanolic extract showing the highest levels of alkaloids, flavonoids, phenols, and tannins, indicating the effectiveness of polar solvents for extracting bioactive compounds. The characteristic HPTLC fingerprint with distinct Rf values provides a reproducible chemical marker for authentication and quality control. These findings support previous reports that *Phyllanthus* species are rich in bioactive compounds such as flavonoids, tannins, alkaloids, and terpenoids with important pharmacological potential, the developed phytochemical and chromatographic profile offer a reliable reference for the standardization and future pharmacological investigation of *P. pinnatus*.

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