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Histological Assessment of *Acalypha Paniculata* and Its Phytoconstituent Systemisation

A. Elumalai[1], N. Irfan[1], M. Vijaya Vara Prasad[1], Ismail Y.[1], Haja Nazeer Ahamed[1], D. Prabu[2] and P. Ashok Kumar[3]

[1] Crescent School of Pharmacy, B.S. Abdur Rahman Crescent Institute of Science and Technology, Vandalur, Chennai, Tamil Nadu, India 600 048
[2] Department of Microbiology, G.S.T. Road, Dr. ALM PG Institute of Basic Medical Sciences, University of Madras, Taramani, Chennai 113, Tamil Nadu, India
[3] School of Life Sciences, B. S. Abdur Rahman Crescent Institute of Science and Technology, Vandalur, Chennai, Tamil Nadu, India 600 048

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ABSTRACT This study explores the pharmacognostic characterisation of *Acalypha paniculata*, a plant species within the Euphorbiaceae family known for its traditional medicinal uses. The investigation focuses on the leaf histology and phytoconstituent profile of *Acalypha paniculata*. Microscopic analysis reveals distinct features, including rectangular epidermal cells, multicellular covering trichomes, caryophyllaceous stomata, and cortex-heteromorphic structures. Phytochemical examination confirms the presence of alkaloids, terpenoids, carbohydrates, steroids, flavonoids, and saponins. Physicochemical parameters such as moisture content, total ash value, and extractive values are measured, with the soxhlet ethanolic extract. HPTLC densitometric analysis identifies four distinct spots with Rf values of 0.2, 0.4, 0.7 and 0.9, respectively. ATR-FTIR spectroscopy reveals nine functional group peaks between 3350 cm^{-1} to 881 cm^{-1} and GC-MS analysis detects 11 bioactive compounds. The comprehensive findings contribute to the understanding of *Acalypha paniculata*'s characteristics, facilitating future identification and authentication. The phytoconstituent profile suggests potential applications in new drug development, emphasising the plant's pharmacological significance.

INTRODUCTION

According to the World Health Organisation (WHO), traditional herbal medicine is relied upon by more than 80 percent of the country's population for their healthcare requirements. Particularly, India has a rich biodiversity and a vast knowledge of using medicinal plants to treat various diseases, including infectious diseases (WHO 2022). Medicinal plant usage is limited due to a lack of ethnomedical studies, and the descriptions of the plants listed are incomplete (Silveira and Boylan 2023). In the Ayurvedic tradition, roughly, 20,000 plant species are recognised for their medicinal properties, and many of these continue to be utilised in conventional medical practice (Singh et al. 2023). These natural remedies are currently receiving increased attention due to their potential to offer numerous benefits to society, particularly in the realms of medicine and pharmacology.

The precise identification and quality control of plant materials is crucial in ensuring the reproducibility of herbal medicine quality, which is vital for both efficacy and safety. This field is closely related to other allied fields, including biosynthesis, chemotaxonomy, phytochemistry, biotransformation, microbial chemistry, and toxicity screenings of natural drugs (Pandey et al. 2008). Inanimate plants are important API (Active Pharmaceutical Ingredient) resources for local communities and medicinal plant businesses. Raw materials are frequently subject to adulteration when brought to the market, natural businesses and local groups often face the problem of adulteration and the transfer of the world's raw materials.

In this analysis, the researchers decided to use *A.paniculata* for research to find a way to identify it according to taxonomic and pharmacognostic analysis. *A.paniculata* is a member of the Euphorbiaceae family and is predominantly found in the

Address for correspondence:
Irfan Navabshan
Assistant Professor,
Crescent School of Pharmacy,
BS Abdur Rahman Crescent Institute of Science and Technology,
Vandalur, Chennai, Tamil Nadu, India 600 048
E-mail: irfan@crescent.education

Parvathamalai hills of the Eastern Ghats in Tamil Nadu (Balakrishnan and Chakraborty 2007). It is an herbaceous plant that can grow to be 0.5 to 1 metre tall at various altitudes, adapted to low nutrient availability in soil. Locally, the plant is called kothukuppameni and the different parts have been traditionally used to treat many diseases, such as bacterial and fungal infections, gastric ulcer and arthritis. In folklore medicine, the powdered leaves are applied in dressing wounds and decoctions made from the plant aerial parts were commonly utilised to ease pain arising from inflammation caused by fractures and arthritis. The juice of fresh leaves was used in itchy skin infections (Seebaluck et al. 2015; Venkatachalapathi et al. 2018; Matthew 1983).

The medicinal benefits of *A.paniculata* are noteworthy, however, there is currently insufficient information on its standardisation parameters and phytoconstituent data. The objective of this study is to offer a comprehensive analysis of the standardisation parameters of *A.paniculata* to establish the pharmacognostic authentication by using various methods, including microscopy and macroscopy, physicochemical parameters, and extractive values. In addition, the phytoconstituents profile was catalogued using various spectral and chromatographic methods. The utilisation of these parameters can ensure the drug's quality and facilitate the development of a suitable monograph for its accurate identification and phytoconstituent may be used as a lead in new targets for infectious diseases.

MATERIAL AND METHODS

Plant Materials

The collection of *A.paniculata*'s aerial parts was done in November 2022 from the Parvathamalai hills located in the Indian state of Tamil Nadu. The plant's authentication and identification were performed in the Department of Pharmacognosy, Central Siddha Research Institute (CCRS), The Indian Government's Ministry of Ayush, in Chennai 600106 (Ref: H12092201S).

Pharmacognostical Standardisation

Macroscopic and Microscopic Evaluation

For organoleptic evaluation, fresh leaf pieces of *A.paniculata* were washed and air-dried in the shade. This evaluation included observation of visual attributes like size, shape, colour and odour. The leaf type, petiole, arrangement pattern, and external characteristics of the lamina, including the shape of the leaf, apex, venation, base, and margin, as well as the average length and width, were documented. Furthermore, the morphology and arrangement of the leaf were examined (Nafees et al. 2022; Kuster et al. 2016; WHO 2007).

The arrangement of tissues, the type of stomata, trichomes, vascular bundles, crystalline structures, and other leaf constants are revealed by the microscopic characteristics in the transverse section of the leaf. The cross-sections were made using a free-hand sectioning technique, treated with chloral hydrate solution for clarification, and tinted with freshly prepared safranin and fast green dyes. Different alcohol grades were utilised to improve visibility. The images of the transverse sections were captured using a Nikon ECLIPSE E200 trinocular microscope under bright field illumination (Wan 1972; Bokhari et al. 2022; Nafees et al. 2022).

Physicochemical Evaluation

Evaluating the quality and purity of herbal medicines is crucial, and it is achieved by the physicochemical analysis method. Importantly following key indices such as moisture content, ash value (total ash, acid insoluble, water soluble), and extractive value (water soluble and alcohol soluble) are typically assessed. The moisture content was determined by measuring the weight of the crude drug before and after removing water by parching it in a hot air oven. To determine the total ash value, the powdered specimen is heated gradually between 600 °C and 700 °C until it turns white, and the resulting residue is weighed. The determination of acid-insoluble ash content involves dissolving a portion of the total ash in concentrated hydrochloric acid, boiling it, collecting the residue in a filter paper, washing, drying, and weighing it. The determination of water-soluble ash content is done similarly. Loss on drying was determined by placing 2-5 grams of air-dried material in an oven set at 100-105°C and measuring the weight difference before and after drying. The determination of extractive values involves macerating finely powdered samples (2 g) in solvents with different polarities (for example, petroleum ether, ethanol and water) and stirring on a shaker for an hour. The

resulting extracts are filtered, evaporated to desiccation, and weighed. All of these procedures were completed according to standard operating procedures (SOPs) in compliance with WHO standards (Raman 2006; Mukharjee 2019; García-Bores et al. 2020; Khandelwal 2022).

Phytochemical Evaluation

The standard methods for phytochemical screening (Harborne 1973) were used to identify the existence of different compounds in *A.paniculata*. Alkaloids were detected using Dragendroff's test, Wagner's test, Hager's test, and Mayer's test. Glycosides were detected using the Keller-Killani test, Legal's test, Baljet test, and Modified Borntrager's test. Steroids were detected using the Libermann-Burchard test and Salkowski test. Flavonoids were detected using the Shinoda test and Lead Acetate test. Amino acids were detected using the Xanthoproteic test, Ninhydrin test, Biuret test, and Millon's test. Carbohydrates were detected using the Molish's test, Barfoed test, Seliwanoff test, Fehlings test, and Benedict's test. Phenolic compounds were detected using the Gelatin test and Iodine test, while Saponins were detected using the Foam test and Phenazone test (Idu et al. 2022).

Thin Layer Chromatography

Ethanolic extract (10 mg) was prepared by dissolving it in 1 ml of ethanol. Using a micropipette, samples of about 5-10 ìl volume were applied to silica gel G60 F 254 TLC plates, which were precoated and placed 2 cm above the bottom. The ascending mobile phase was permitted to travel up to 3/4th of the adsorbent phase. Different solvent systems were used to develop the TLC plate, which was then derivatised with anisaldehyde sulphuric acid and Dragendroff's reagent. After spraying with different derivatising reagents, the plates were put in a hot air oven for a minute to develop colours and then examined under UV light (Stahl 1969; Wagner 2007; Pandiyan and Ilango 2022). The observed spots' Rf value was calculated using the formula below:

$$R_f = \frac{\textit{Distance travelled by solute}}{\textit{Distance travelled by solvent}}$$

HPTLC

HPTLC Fingerprint analysis was performed by using the methods of Harborne. The ethanolic extract of 100mg was dissolved in 1m HPTLC grade methanol to prepare the test sample, which was then centrifuged at 2000 rpm for 5 minutes. The analysis was conducted using the CAMAG HPTLC system, which included an automatic applicator called Linomat 5 equipped with a 100 µm syringe. A sample of 2µl was spread on an HPTLC 60 F254 plate (4 × 10 cm, thickness 250 µm) coated with silica gel in smears, with a 5 mm bandwidth from the bottom and 15 mm from the sides. The twin-groove glass chamber was impregnated with a suitable mobile phase for the detection of alkaloids, terpenoids, flavonoids, saponins, and the plate was allowed to develop in it. After development, the plates were parched in a hot air oven at 60°C for 10 minutes to remove the mobile phase completely. The densitometry was carried out in a TLC scanner equipped with WINCATS 1.4.2 software, scanned at 254 nm to identify peak densitogram, peak display, and peak numbers, as well as their heights and areas (Shaikh and Patil 2020; Nonglang et al. 2022).

Spectroscopic Method

The Fourier Transform Infrared Spectrophotometer (FTIR) is an extremely effective instrument for detecting and identifying the various functional groups that are present in compounds (Shaffai et al. 2023). For FTIR analysis, 5mg of the dried ethanolic extract was used and encapsulated with a 100mg KBr pellet, and the powdered sample was loaded into the FTIR Spectroscope (SHIMADZU, IRTRACER 100) according to the instrument manual. The scan range was set to 400-4000 cm^1.

GC-MS investigation of *A.paniculata* was done using Shimadzu, QP2010 Plus from Japan with an auto-sampler. The GC-MS/HP column was utilised with measurements of 0.25 mm in diameter, 30 m in length, and 0.25 µm in particle size. Ultra-pure helium was employed as the carrier gas. The flow rate of helium gas was maintained at 1.12 mL/min, with a linear velocity of 39 cm/s. The oven temperature was programmed to increase at a frequency of 10°C per minute, ranging from 110°C to 280°C, and remained constant thereafter. The sample injection was performed in splitless mode with a split

ratio of 10:1, and a volume of 0.5 µL was used. The injector temperature was set at 250 °C. The MS transfer line was maintained at a constant temperature of 250 °C, while the ion source was held at a temperature of 200 °C. The detector voltage for the mass spectra was set to 0.94 kV. Full-scan mass spectra were recorded by scanning the mass range (m/z) of 85-500 at a rate of 10,000 u/s. Chemical constituents and peaks were identified through direct searching in the NIST library (Tulukcu et al. 2019; Olivia et al. 2021; Ajilore et al. 2021; Singh et al. 2022).

RESULTS

Macroscopy

A.paniculata is an erect herb that can grow a maximum of up to 1 metre in height. Broadly ovate leaves, measuring 4-8 x 2-5 cm, with an acuminate apex and a rounded to cordate base. The margins are serrated, and the apex is acuminate. The flowers are monoecious, with male flowers in axillary spikes that are slender and elongated, measuring 6-10 cm in length. Female flowers are found in terminal panicles, with stigmas that are laciniate 5-6 fid. The schizocarp is glandular-hairy, and the seeds are reticulatedly pitted.

Microscopy

Transverse Section of Stem

The circular transverse section of *A.paniculata* stem exhibits a revealing outermost layer of thick-walled, rectangular epidermal cells. The cortex exhibits heteromorphism, consisting of 2-3 layers of outer collenchyma cells pursued by 4-5 stratum of parenchymatous cells. The vascular bundle, encompassing a wide xylem encircled by phloem, is enclosed by a continuous, single-layered endodermis. The phloem is composed of parenchyma and phloem fibres, while the xylem consists of fibres, vessels, and tracheids. Additionally, centrally located parenchymatous pith can be observed. The petiole of *A.paniculata* displays an oval transverse section, exhibiting an outermost layer of single-layered epidermis with a few trichomes. The cortex is composed of 2 to 3 outer layers of collenchyma cells followed by 4 to 5 layers of parenchyma cells, exhibiting heteromorphism. A continuous, single-layered endodermis surrounds the vascular bundle, which is enclosed by phloem and central xylem.

Transverse Section of A.paniculata Leaf

Midrib: The transverse section of the midrib of *A.paniculata* leaf reveals a single-layered epidermis with a distinct cuticle and a few multicellular covering trichomes. The cortex is composed of 3 to 4 stratums of collenchyma cells tailed by 5 to 6 parenchymal layers. The vascular region is located in the centre of the section and is surrounded by a distinct pericycle.

Lamina: The transverse section of *A. paniculata* lamina reveals that it is dorsiventral and hypostomatic. The single-layered epidermis is covered by a thick cuticle and a few covering and trichomes in the lower epidermis. The mesophyll is composed of outer single-layered, radially elongated palisade parenchyma cells and trailed by 3 to 4 layers of spongy parenchyma. A few sandy crystals are randomly distributed throughout the spongy parenchyma. Veins can be seen traversing through the mesophyll tissue.

Quantitative Microscopy

The quantitative microscopic observation of epidermal peelings from *A.paniculata* leaves reveals the presence of diacytic stomata on both the upper and lower epidermis, with a stomatal number ranging between 45 and 50 and a stomatal index falling within the range of 43 to 46. The palisade ratio, which signifies the proportion of palisade cells in the leaf, is noted to be in the range of 20 to 28. Additionally, the vein islet count is reported to be between 5 and 8, and the vein termination count ranges from 15 to 18. These findings provide valuable insights into the microstructural characteristics of *A.paniculata* leaves. Notably, numerous sandy crystals are randomly distributed throughout the leaf.

Powder Microscopy

A.paniculata leaf powder has a distinctive odour and taste, with a greyish colour. Microscopic examination revealed the presence of multicellular covering trichomes, warty trichomes, epidermal fragments with diacytic stomata, epidermal frag-

ments with paracytic stomata, mesophyll cells, pitted parenchyma, pitted vessels, annular vessels, fibre bundles, and sandy crystals.

Physicochemical Analysis

Based on the physiochemical analysis, the fresh leaf was found to have a low moisture content of 3.5 percent. Additionally, the sample had a relatively low total ash value, indicating that it is not highly mineralised. The acid-insoluble ash value was slightly elevated in comparison to the water-soluble ash value, indicating a higher content of insoluble minerals present in the sample. The high value of alcohol soluble extractives indicates that significant amounts of compounds can be extracted with alcohol. The relatively high value of water-soluble extractives, on the other hand, indicates the existence of water-soluble compounds. Overall, the sample's composition suggests that alcohol extraction would be suitable for isolating the active compounds.

Preliminary Phytochemical Screening

Various chemical tests were performed on ethanolic extract, and the results revealed the existence of alkaloids, carbohydrates, flavonoids, phenolic compounds, steroids, amino acids, and saponins.

Thin Layer Chromatography

In this study, reformed solvent systems were used in TLC profiling of *A.paniculata* ethanolic extract. For the detection of alkaloids, Toluene: Formic acid: Ethyl acetate (7:1:2) was used as a solvent system along with Dragendroff's reagent as the derivatising agent. The results revealed the greenish, purple, red and light yellowish orange spots having different Rf values of 0.3, 0.7, and 0.8 under short wavelength UV254 nm. Similarly, the Chloroform: Acetic acid: Methanol: Water (6:3:0.5:0.5) solvent system was used for saponins detection with Anisaldehyde Sulphuric acid as the derivatising agent. The sample showed only one red coloured spot with an Rf value of 0.7 under short wavelength UV254 nm. Lastly, for terpenoids detection, the Ethyl acetate: Glacial acetic acid: Water: Formic acid (8:1:1:1) solvent system was used along with Natural Product Reagent as the derivatising agent. Two spots, pink and yellowish orange were observed with different Rf values of 0.4 and 0.8 respectively. The majority of the detected phytochemicals were non-polar compounds, as their Rf values were lower than 0.7.

HPTLC Densitogram

According to the FTIR densitogram results, the use of Toluene: Formic acid: Ethyl Acetate (7:2:1) solvent system was found to be optimal for alkaloid extraction from *A.paniculata* leaves. After applying Dragendroff's reagent, the plates were pictured in absorbance mode at 254 nm, 366 nm, and visible light (400-600 nm), with the most promising outcomes recorded at 400 nm, displayed clear separation of all chemical constituents without any tailing or diffuseness. The Rf values ranged from 0.01 to 0.99, and out of the eight components, the Rf values of 0.2, 0.7, 0.8, and 0.9 were predominant, with percentage areas of 16.81 percent, 38.24 percent, 46.87 percent and 11.25 percent, respectively. The bands indicated the existence of alkaloids, saponins, and terpenoids, with the observation of one greenish orange, two purple, and one pink band.

FTIR Analysis

The ethanolic extract of *A.paniculata* leaf was subjected to FTIR analysis, revealing peaks at 2974.23, 2889.37, 1637.56, 1386.82, 1328.95, 1083.99, 1043.49, 877.61 and 433.98. The peak observed at 2974.23 indicates C-H asymmetric stretching, while the peak at 2889.37 indicates C-H symmetric stretching, indicating the presence of aliphatic compounds. The peak observed at 1637.56 shows C=O stretching, confirming the presence of ketone compounds. Peaks observed at 1386.82 and 1328.95 indicate O-H bending and C-C stretching respectively, indicating the presence of nitro compounds. Peaks observed at 1083.99 and 1043.49 indicate C-O stretching of carbohydrates. Peaks below 1000 correspond to C-H stretching and correspond to terpenoids compounds.

GCMS Analysis

The GC-MS analysis of *A.paniculata* leaf's ethanolic extract was piloted to identify the phytoconstituents. The gas chromatography fractions

were analysed using mass spectrometry, and the results revealed the presence of 11 different compounds, which were identified based on their mass spectra. The compounds were N-cyclohexyl-2-pyridin-3-ylpiperidine-1-carbothioamide, 4,6-Dimethyl-2-[5-(4-Methyl-1,2,5-Oxadiazol-3-Yl)-2h-1,2,4-Triazol-3-Yl], Thieno[2,3-B]Pyridin-3-Amine, Alloaromadendrene, 11,21-Dihydroxypregn-4-ene-3,20-dione, 1,2-Benzenedicarboxylic Acid, Dodecahydropyrido[1,2-B]Isoquinolin-6-One, Geraniol, benzyl N [4 amino 6 (dimethylamino) 1,3,5 triazin 2 yl] N (1H indol 3 ylmethyl) carbamate, Trans-3,4-Dihydro-2,2-Dimethyl-6-Fluoro-4-(Piperidin-1-Yl)-2h-1-Benzopyran-3-Ol, Carbamic Acid, and N,N-Dimethylglycine.

DISCUSSION

Pharmacognostical standardization is key for the development of herbal monographs and pharmacopoeial standards, aligning with the recommendations of the World Health Organization (WHO). Given that safety and efficacy are paramount considerations, ensuring the consistent quality of herbal drugs necessitates precise identification and quality assurance of the raw materials. Employing pharmacognostic protocols such as macromorphology, micromorphology, chemical tests, and advanced analytical techniques like HPTLC and GC-MS analysis is pivotal. These methodologies contribute to the identification of authentic herbal drugs by yielding specific results characteristic of particular plant species (Smeriglio et al. 2024; Hameed et al. 2024).

This research aims to establish the distinctive characteristics of *Acalypha paniculata*, which can serve as effective quality control measures to ensure the safety, efficacy, and consistency of this herbal medicine. The parameters investigated in this study are valuable for accurately identifying and authenticating *Acalypha paniculata*, a plant of significant traditional medicinal importance. The identification of medicinal plants, particularly *Acalypha paniculata*, is challenged by the morphological similarities within the genus Acalypha.

Macroscopic examination reveals distinct features such as oval-shaped leaves with a cordate base and an acuminate apex, along with monoecious flowers with serrated borders. Microscopy of the stem shows unique characteristics, including thick-walled, rectangular epidermal cells with diacytic stomata. Similar studies reported the importance of stomata in the proper identification of medicinal plants (Dubale et al. 2023; Sweta et al. 2023). From the results obtained, the extractive values are comparably higher in alcohol soluble extractives than water soluble extractive, indicating that significant amounts of compounds can be extracted with alcohol.

Identification of the different classes of phytochemical constituents of the plant is an important parameter, which gives an indication of the pharmacologically active metabolites present in the plant (Sonibare et al. 2023). In this study, preliminary chemical tests confirm the presence of alkaloids, flavonoids, saponins, and carbohydrates. HPTLC analysis aids drug standardization by providing chromatographic insights into complex mixtures. It offers Rf values for phytocompounds, revealing their characteristics like polarity and separation. In this study, the solvent system Toluene: Formic acid: Ethyl Acetate (7:2:1) showed effective separation, confirming ethanolic solvent as efficient for extraction. Finally, GC-MS study explored the plant's active biological compounds; the chromatogram showed 11 different compounds belong to different classes of alkaloids, flavonoids, and saponins. Further experiments are required to validate the observed biological potential and isolate the responsible compounds in their pure form.

CONCLUSION

To identify and confirm the purity of herbal materials, pharmacognostic studies are conducted. In the case of *Acalypha paniculata*, this study has yielded valuable information on various parameters including macroscopy, microscopy, physicochemical evaluation, phytochemical screening, HPTLC, ATR-FTIR, and GC-MS analysis. These findings can be utilised for the identification and authentication of this plant. However, further research through in-vitro and in-vivo screening on animals is necessary to explore the pharmacology of this unique indigenous species in various investigations.

RECOMMENDATIONS

Further studies are recommended to explore the specific therapeutic activities of the identified phytoconstituents of *Acalypha paniculata* through

in-depth pharmacological investigations. Investigation into formulation strategies is necessary to enhance the bioavailability and stability of active compounds, thereby ensuring the creation of standardised herbal products. Bioavailability studies, ecological impact assessments, and pharmacovigilance studies will address practical considerations in the application of *Acalypha paniculata* in medicine. Additionally, collaboration between traditional healers and modern scientists can integrate traditional knowledge with contemporary research, fostering a holistic approach to the plant's medicinal properties.

DISCLOSURE

All authors reported that there are no conflicts of interest.

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