

PHARMACOGNOSTICAL STANDARDIZATION AND HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY FINGERPRINT OF ROOTS OF *ANTHOCEPHALUS CADAMBA*

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ABSTRACT

Objective: *Anthocephalus cadamba* is a traditional medicinal plant which is valued for its benefits in the management of various ailments. The current study was aimed to provide requisite pharmacognostic details and high-performance thin-layer chromatography (HPTLC) fingerprint profile for the roots of *A. cadamba*.

Methods: Pharmacognostic evaluation included examination of morphological and microscopic characters, physicochemical analysis, fluorescence study, preliminary phytochemical analysis, and HPTLC fingerprint.

Results: The microscopic studies showed the presence of thick periderm, cortical zone, secondary phloem, and secondary xylem. Microscopic examination of root powder showed abundant fibers and vessel elements. The physicochemical analysis of root powder helped in the assessment of quality control parameters. Fluorescence analysis of root powder imparted characteristic colors on the treatment of the powder with different reagents. Preliminary phytochemical studies reported the presence of alkaloids, saponins, flavonoids, steroids, terpenoids, glycosides, tannins, and other phenolic compounds. The developed qualitative densitometric HPTLC fingerprint can be used as an identifying biochemical marker for ethanol extract of the roots of *A. cadamba*.

Conclusion: The present study provides the information with respect to identification and authentication of crude drug and serves as a reference point for the proper identification of roots of this medicinal plant, thereby contributing to the scientific world of research.

Keywords: *Anthocephalus cadamba*, Pharmacognostic, High-performance thin-layer chromatography, Fluorescence.

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INTRODUCTION

Medicinal plants play an active role in traditional medicines for the treatment of various diseases. However, the key obstacle, which hindered the promotion in the use of traditional medicines in the modern society, was no evidence of documentation and absence of stringent quality control measures [1]. Hence, there is dire need to make sure about the standardization of the plant and parts of the plant to be used as a medicine. From the past few years, implementation of new good manufacturing practices in quality control of raw materials, intermediates, and finished products of botanical origin was taken place [2]. Correct characterization and quality assurance of starting material are an essential step to ensure reproducible quality of herbal medicine which will help us to justify its safety and efficacy [3].

Anthocephalus cadamba (F: Rubiaceae) is a traditional medicinal plant which is valued for its benefits in the management of various ailments. It is native to South Asia which was used traditionally to treat various ailments such as ophthalmic infections, cutaneous diseases, dyspepsia, gum related problems, stomatitis, cough, fever, hematological, and urinary disorders [4]. As the plant shows different pharmacological activities, but till date, there were no reports on pharmacognostic studies and high-performance thin-layer chromatography (HPTLC), this study is inevitable. Hence, the current study is focused to study the pharmacognostic parameters such as microscopic, physicochemical, fluorescence studies, preliminary phytochemical screening, and HPTLC analysis of the roots of *A. cadamba*.

MATERIALS AND METHODS

Collection and authentication of plant material

The roots of *A. cadamba* were collected from Tirumala hills and authenticated by Botanist Dr. Madhavachetty. A specimen (No. 1856)

was deposited in herbarium at Sri Venkateswara University, Tirupati, India. Roots were initially washed, shade dried, and ground in Wiley mill.

Macroscopic studies

The roots were subjected to macroscopic studies which comprised organoleptic characteristics such as color, odor, appearance, and texture of the drug. These parameters were evaluated as per the standard WHO guidelines [5].

Microscopic studies

Microscopic studies were carried as per the methods of Sass, 1940, Brain *et al.*, 1975, Johansen, 1940, and Easu, 1964 [6-9]. The required samples were cut and removed from the plant and fixed in FAA (Formalin – 5 ml + Acetic acid – 5 ml + 70% Ethyl alcohol – 90 ml). After 24 h of fixing, the specimens were dehydrated with graded series of tertiary-Butyl alcohol [6]. Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58–60°C) until TBA solution attained supersaturation. The specimens were cast into paraffin blocks [7].

Sectioning and staining

The paraffin-embedded specimens were sectioned with the thickness of the sections 10–12 µm by the help of rotary microtome. The dewaxing of the sections was done by customary procedure [7]. The sections were stained with Toluidine blue [10]. Wherever necessary, sections were also stained with safranin, fast green, and iodine-potassium iodide.

Photomicrographs

All permanent slides after staining were dehydrated using graded series of ethanol + xylol and mounted in DPX. Photographs of different magnifications were taken with Nikon Labophot 2 microscopic unit. For

normal observations, bright field was used. For the study of crystals, starch grains, and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized light, they appear bright against dark background. Magnifications of the figures are indicated by the scale bars. Descriptive terms of the anatomical features are as given in the standard Anatomy books [9].

Powder microscopy

To study the presence or absence of various types of tissues or structures, the dried root is powdered using electric grinder, passed through sieve No. 60, and then subjected for microscopic studies [11,12].

Physicochemical analysis

Physicochemical parameters such as total ash value, acid-insoluble ash value, water-soluble ash value, pH, moisture content, and extractive values were determined using crude powdered drug of roots as per the WHO guidelines [13].

Fluorescence analysis

Small amount of powdered drug about 1 g is taken in a Petri dish and treated with different chemical reagents. The fluorescence character of the drug was studied by observing under daylight and ultraviolet (UV) light [14].

Preparation of extract

The root powder was defatted with petroleum ether (60–80°C). The defatted marc was air-dried and macerated with ethanol for 24 h. Macerated material was refluxed for 3 h and then filtered. The procedure was repeated twice and obtained filtrate was combined and subjected to distillation under reduced pressure.

Preliminary phytochemical studies

Petroleum ether and ethanol extract of *A. cadamba* was subjected to preliminary phytochemical studies by employing standard methods to detect the presence of various phytoconstituents [15].

HPTLC

A densitometric HPTLC analysis was carried for the development of characteristic HPTLC fingerprinting profile. Ethanol extract of roots was dissolved in methanol and used as a test solution for HPTLC analysis. A 4 µL aliquot of test solution was loaded on 5 cm × 10 cm. Aluminum packed TLC plate coated with 0.2 mm layer of silica gel 60 F₂₅₄ (E. Merck Ltd., Darmstadt, Germany) stored in a desiccator. Hamilton microsyringe (Switzerland) mounted on a Linomat V applicator was used for application. Spotting was done on the TLC plate and the plate was kept in TLC twin trough developing chamber allowing ascending development of the plate with the migration distance 80 mm (distance to the lower edge was 10 mm) which was carried out at 22°C in a Camag chamber previously saturated for 30 min. The mobile phase used is toluene:ethyl acetate:methanol (1:8:1). After development, the plate was subjected to dry by hot air oven for 5 min. Then, densitometric scanning was done with a Camag TLC Scanner 3 equipped with win CATS Software and the chromatograms were recorded.

RESULTS

Macroscopic studies

The root was outer dark brown and inner creamy in color. They were characteristic in odor and slightly bitter in taste. Outer surface of the root is firm, rough, and striated and inner side is soft.

Microscopic studies

The thin root is about 1.3 mm and the thick one is about 3 mm in diameter. Root consists of thick periderm, cortical zone, secondary phloem, and secondary xylem. The root has uniformly thick continuous periderm cylinder extending all around the circumference. The periderm consists of homogeneous squarish thin-walled cells, the cells are in regular radial rows. In the middle part of periderm, there present two layers of lignified cells called phelloids. The remaining periderm tissue includes about many layers of thin-walled suberized cells called phellem cells.

Inner to the periderm is the cortical zone comprising about six layers of cells. The cortical cells include parenchyma cells and fibers. The parenchyma cells are elliptical, compact, and thin walled. Some of them have mucilage inclusions. The fibers are solitary or in small cluster of two or three cells which are scattered in the cortical zone.

Secondary phloem occurs as a continuous cylinder along the outer part of the secondary xylem. The phloem parenchyma elements are squarish in shape and occur in compact radial rows. The sieve elements are in small circular clusters. Secondary xylem cylinder is wide, dense, and solid, measuring 3.4 mm in diameter. It includes vessel elements, xylem fibers, and dilated xylem rays. The vessels are long radial, angular in outline and are in multiples ranges from four to eight with diameter 30–70 µm. Xylem fibers are narrow in sectional outline, polygonal and have thick lignified walls. Xylem rays are distinct, radially oblong, darkly stained, and thin-walled cells (Fig. 1).

Powder microscopy

Microscopic examination of root powder evidenced abundant fibers and vessel elements. Two types of fibers, namely wide fibers (up to 30 µm wide and 750 µm long) and narrow fibers (about 10 µm thick and 450 µm long) were observed. Wide fibers have thin walls, side lumen, and tertiary spiral thickenings. Most of these fibers have multiseriate slit like simple pits. Narrow fibers are but less abundant compared to wide fibers. Their walls are thick and slit-like pits are seen on the lateral walls of the fibers. Vessel elements are long, narrow, and cylindrical and are common. Some of them have long, narrow, tapering tails at the ends. At the end walls, wide circular perforations occurred and the lateral walls are dense and multisclerotic (Fig. 2).

Physicochemical analysis

The physicochemical analysis helped in the assessment of quality control parameters. Various physicochemical parameters were assessed and the identified results are mentioned in Table 1.

Fluorescence analysis

Fluorescence analysis of root powder imparted characteristic colors on the treatment of the powder with various reagents when observed under visible and UV light (Table 2).

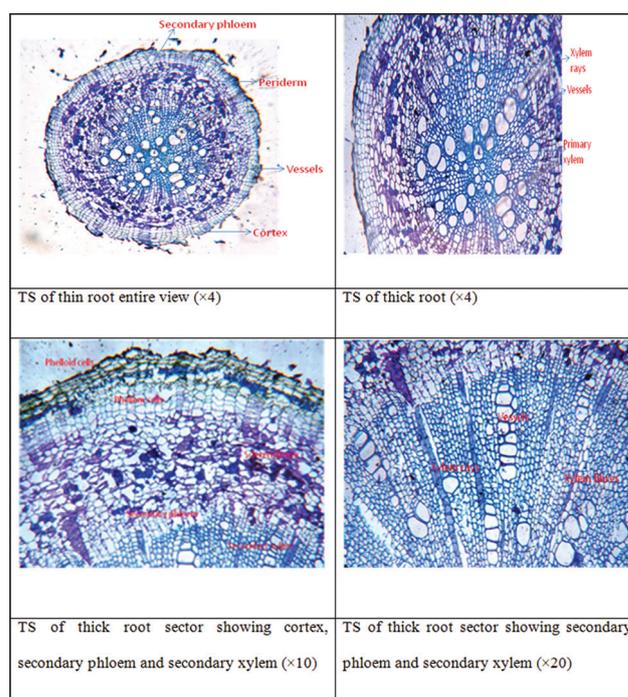


Fig. 1: Microscopic examination of the roots of *Anthocephalus cadamba*

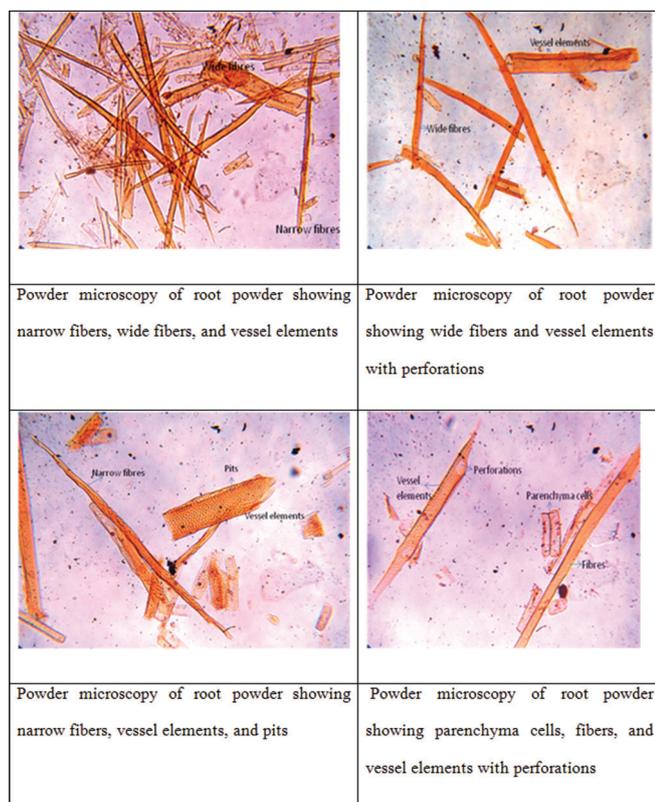


Fig. 2: Powder microscopy of the roots of *A. cadamba*

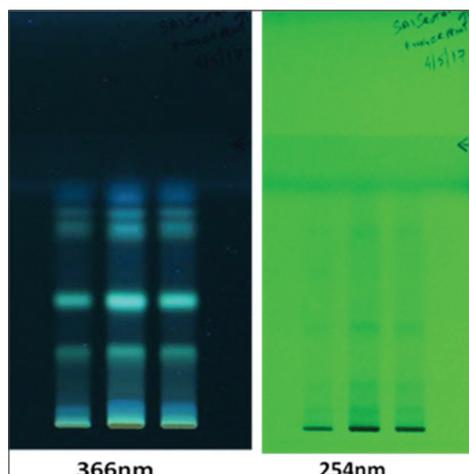


Fig. 3: High-performance thin-layer chromatography fingerprint analysis of ethanol extract of the roots of *A. cadamba*

Preliminary phytochemical screening

The petroleum ether and ethanol extract of the roots of *A. cadamba* showed in Table 3.

HPTLC analysis of the roots of *A. cadamba*

HPTLC analysis of ethanol extract showed the presence of seven phytoconstituents. The corresponding ascending order of R_f values starts from -0.03 to 0.70, in which the highest concentration of phytoconstituents was found to be 50.58% and its corresponding R_f value was found to be 0.35, respectively (Table 4 and Figs. 3, 4).

DISCUSSION

Standardization of the herbal medicine is necessary to assure the quality, efficacy, and safety of the drugs [16]. The primary step in

Table 1: Physicochemical parameters of roots of *Anthocephalus cadamba*

S. No.	Parameters	Results (%w/w)
1	Total ash value	7.94
2	Acid-insoluble ash	2.48
3	Water-soluble ash	3.96
4	Moisture content	7.7
5	Alcohol-soluble extractive	10.75
6	Water-soluble extractive	9.5
7	pH for 1% solution	6.89

Table 2: Fluorescence analysis of root powder of *Anthocephalus cadamba*

S. No.	Reagent	Daylight	UV light
1	Drug powder as such	Pale brown	Bright yellow
2	Conc. H_2SO_4	Blackish-brown	Blackish-brown
3	Dil. H_2SO_4	Pale yellow	Yellow
4	Conc. HCl	Dark brown	Dark brown
5	Dil. HCl	Orangish-brown	Orangish-brown
6	Conc. HNO_3	Muddy brown	Light brown
7	Dil. HNO_3	Pale brown	Yellow
8	Methanol	Pale brown	Bright yellow
9	Chloroform	Cream color	Yellow
10	Petroleum ether	Cream color	Yellow
11	10% Ferric chloride	Light brown	Greenish-yellow
12	10% NaOH	Orangish-brown	Brown
13	Ammonia solution	Pale brown	Yellow
14	CH_3COOH	Light brown	Bright brown
15	Distilled water	Pale brown	Yellow

UV: Ultraviolet

Table 3: Preliminary phytochemical screening of petroleum ether and ethanol extracts of roots of *Anthocephalus cadamba*

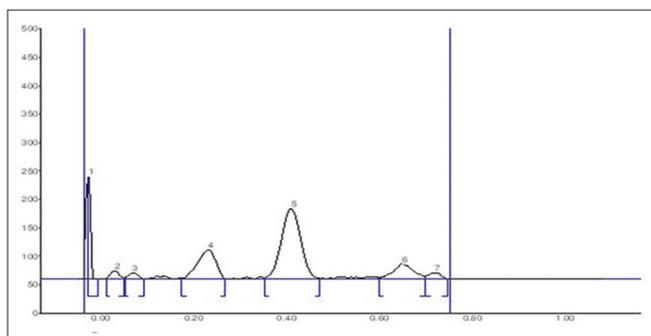
S. No.	Phytoconstituents	Extracts of the root of <i>Anthocephalus cadamba</i>	
		Petroleum ether extract	Ethanol extract
1	Alkaloids	-ve	+ve
2	Proteins and amino acids	-ve	-ve
3	Saponins	-ve	+ve
4	Phenols	-ve	+ve
5	Flavonoids	-ve	+ve
6	Glycosides	-ve	+ve
7	Steroids	+ve	+ve
8	Tannins	-ve	+ve
9	Terpenoids	-ve	+ve
10	Fats and oils	+ve	-ve

+ve - indicates the presence of phytoconstituents, -ve - indicates the absence of phytoconstituents

quality assessment of medicinal plants is ensuring the authenticity of the desired species for intended use [17]. It is conducted by various techniques such as macro- and micro-scopic identification, physicochemical analysis, and fluorescence studies. Observation of morphology and microscopic features is a major aid in the identification of raw materials. The observation of cellular level morphology of these roots helps in quality control profiling. These characteristic profiling mainly play a key role in identification of powdered drugs, since in these cases, many of the diagnostic characters are lost. Physicochemical evaluation serves as valuable source of information and helps in judging the purity and quality of drugs [18]. Ash values determined in our study were especially helpful to find out the existence of any foreign inorganic matter such as metallic salts and silica [19]. Extraction values are primarily

Table 4: High-performance thin-layer chromatography profile of the ethanol extract of roots of *Anthocephalus cadamba*

Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum (%)	End Rf	End height	Area	Area (%)
1	-0.03	179.6	-0.03	180.7	43.19	-0.01	0.0	782.8	10.68
2	0.01	0.3	0.03	13.9	3.33	0.05	2.3	193.7	2.64
3	0.05	2.5	0.07	10.7	2.55	0.09	0.8	157.4	2.15
4	0.17	1.3	0.23	51.5	12.32	0.27	0.2	1439.4	19.64
5	0.35	2.2	0.41	123.7	29.57	0.47	0.9	3706.6	50.58
6	0.60	2.5	0.65	26.9	6.42	0.70	5.0	835.6	11.40
7	0.70	5.1	0.72	11.0	2.63	0.75	0.5	212.0	2.89

Fig. 4: HPTLC chromatograph of the ethanol extract of roots of *A. cadamba*

useful for the determination of exhausted or adulterated drug and it provides an idea about the estimation of specific constituents soluble in that particular solvent used for the extraction [20]. In our current findings, the alcohol extractive value found to be higher than water. This inference that phytoconstituents present in the extracts may be more soluble in alcohol than water.

The moisture content determination indicates the storage capacity of crude drug as the moisture is responsible for its decomposition due to microbial attack or chemical changes [18]. In the present study, the moisture content of our roots is low; therefore, they can be stored without microbial attack. Fluorescence study is another essential parameter for the first-line standardization of crude drug and in identification of adulterants [21]. Phytochemical screening of the plant material is essential to predict the pharmacological activities of the plant materials [22]. The preliminary phytochemical screening revealed the presence of various bioactive phytoconstituents which were immensely beneficial for assessing the pharmacological activity of these roots.

HPTLC is a sophisticated, conventional analytical approach for standardization [23]. "HPTLC fingerprint profile is an important powerful procedure for the determination of bioactive compounds in herbal medicine and found to be a linear, precise, and accurate method for herbal identification and can be used further for authentication and standardization of the medicinally important plants" [24]. The present HPTLC fingerprint analysis showed that there was more number of peaks when the solvent extract was scanned at 366 nm and 254 nm. The variations in number of peaks and R_f values evidence qualitative variation of the different phytochemical constituents present in the root extract. The developed fingerprint aids in the differentiation of these roots from the adulterant and acts as biochemical marker in systematic studies of these roots.

CONCLUSION

The present findings revealed various characteristic features of the roots of *A. cadamba* which aids in differentiation of genuine drug from its adulterants. Thus, the current study provided the information with respect to identification and authentication of crude drug and

served as a reference point for the proper identification of roots of this medicinal plant, thereby contributing to the scientific world of research.

AUTHORS' CONTRIBUTIONS

The experimental part of work and preparation of manuscript was done by the first author (Dr. K. Sai Sruthi). The guidance for experimental design and correction of manuscript was done by the second author (Prof. A. Sreedevi).

CONFLICTS OF INTEREST

No conflicts of interest.

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