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Research

Pharmacognostical, preliminary phytochemical probe, phenetics and DNA barcoding of *Calotropis gigantea* Linn leaves

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Abstract

Background: *Calotropis gigantea* belongs to Apocynaceae, a large erect lactiferous shrub, native to continental Asia, South East Asia and had been introduced in the pacific islands, Australia, Central and northern South America, South Africa, India, Srilanka, China, Thailand, Philippines, Malaysia, Indonesia and Cambodia. Tribal communities used this plant to treat bronchial asthma, cholera, convulsions, pneumonia, toothache, ringworm and small pox infections, fever, rheumatism, leprosy, constipation, wounds. The phytochemical survey reported the presence of cardenolides, triterpenoids, anthocyanins and hydrocarbons. The plant exhibited antimicrobial, antioxidant, anti asthmatic, anticonvulsant, hepatoprotective, hypoglycemic, procoagulant, abortifacient, cytotoxic, insecticidal and ovicidal effects

Materials and methods: Leaves were collected from Mangalakudi village, Madurai district, Tamil Nadu, in the month of February 2025. It was identified and authenticated by Dr.Stephen. The collected leaves were washed with water; shade dried, powdered and aqueous extract is prepared. The extract was concentrated and stored in air tight container for further use.

Results and discussion: The present study analysed the pharmacognostical parameters such as macroscopical, microscopical evaluation, determination of physicochemical constants including inorganic elements, qualitative and quantitative analysis of phytoconstituents, phenetics and DNA barcoding in leaves of *Calotropis gigantea*.

Conclusion: The present article draws phytochemical analysis of the plant shows presence of beta carboline alkaloid, cardiac glycoside, tannin, saponin, steroid, terpenoid and quantitatively estimates digoxin(0.0003mg/g), gallic acid(0.0005mg/g), tannic acid(0.006 mg/g) and thymol(0.001mg/g) equivalent in *Calotropis gigantea.*

Keywords: Apocynaceae, *Calotropis gigantea* Linn, DNA barcoding, pharmacognostical , phenetics, physico-chemical, qualitative and quantitative analysis of phytoconstituents.

INTRODUCTION

Calotropis gigantea belongs to Apocynaceae, a large erect lactiferous shrub, native to continental Asia & South East Asia and has been introduced in the Pacific islands, Australia, Central, northern South America, South Africa, India, Srilanka, China, Thailand, Philippines, Malaysia, Indonesia and Cambodia[1,2].*Calotropis* genus contains two different species *gigantea* (Purple flowered plant) and *procera* (White flowered plant)[3]. Santals tribe of Jharkhand and West Bengal, India used this plant to treat bronchial asthma, cholera, convulsions, pneumonia, toothache, ringworm and small pox infections. People of Kolayat tehsil, Rajasthan used the plant to treat fever. People of Chitheri Hills, Dharmapuri, Tamilnadu and people of Raipur, Chhatisgarh plains used the plant preparations to treat rheumatism, People of North Kanara, Karnataka used to treat leprosy. People of Kamrup, Assam used to treat constipation, Tribals of eastern Rajasthan used to get rid of wounds[4]. The phytochemical survey reported the presence of cardenolides from the latex and leaves, triterpenoids, anthocyanins from flowers and hydrocarbons [5]. It exhibited antimicrobial[6] antioxidant[7], antiasthmatic[8], anticonvulsant[9], Hepatoprotective[10], Hypoglycemic[11], Procoagulant[12], abortifacient[13], cytotoxic[14], insecticidal[15] and ovicidal effects[16,17]. The present study is to examine *Calotropis gigantea* sourced from a distinct habitat offering fresh insight into the plant's Pharmacognostical, Physico-chemical parameters, Qualitative analysis of phytoconstituents present in *Calotropis gigantea* leaf and quantitative estimation of harmaline, digoxin, thymol was determined along with initiation of phenetic studies.

MATERIALS AND METHODS

Collection of leaf and authentication

Leaves were collected from Mangalakudi village, Madurai district, Tamil Nadu, in the month of February 2025. It was identified and authenticated by Dr. Stephen, Professor in the Department of Botany, American College, Madurai–625 002. A herbarium specimen has been preserved in the department for future reference.

Pharmacognostical evaluation

Morphology

Morphological characters such as size, shape, apex, margin, venation, base, petiole, surface and colour of leaves of *Calotropis gigantea* were studied [18,19].

Microscopical study

Thin fresh and transverse sections of the leaf, including the petiole and midrib, of *Calotropis gigantea* were prepared. The sections were stained using phloroglucinol in combination with concentrated hydrochloric acid, mounted with dilute HCl, and subsequently observed under the microscope [20].

Quantitative microscopy

It was performed using square pieces of leaf taken from the region between the midrib and the margin. These samples were decolourised and examined under a microscope to assess vein islet number, vein termination, and palisade ratio. For stomatal index analysis, the upper and lower epidermal layers were carefully peeled from separate leaf pieces, mounted in glycerin water on a slide, and observed microscopically to determine stomatal index and stomatal type [21].

Preparation of leaves Powder

The fresh matured leaves of *Calotropis gigantea* were collected, washed with water and dried. The dried leaves were made into a fine powder.

Powder microscopy

The coarse powder was treated with routine reagent to identify the diagnostic features of the plant [22].

Behaviour of Powder with various chemical reagents

A small quantity of leaf powder was transferred into a test tube, to which 1–2 drops of freshly prepared reagents were added. The resulting colour changes were observed under visible light as well as under UV at 254 nm and 365 nm, following standard procedure[23].

Physicochemical parameter

The powder was subjected to physiochemical parameters such as loss on drying, ash value, and extractive value with different solvents in increasing order of polarity as per standard procedures recommended by Ayurvedic pharmacopoeia of India[24]. The ash was subjected to inorganic elements identification as per Atherden[25] and heavy metals analysis of lead were performed as per I.P 1985[26].

Preparation of fresh juice of *Calotropis gigantea* (*C.gigantea*$_{frj}$)

Fresh leaves of 25g were weighed. It was washed with water and 30 ml of water is added and made into a fresh juice of 25.5ml.

Preliminary phytochemical screening

Preliminary phytochemical screening were carried out by using different reagents for identification the presence of phytoconstituents as per standard procedures[27].

Quantitative estimation of phytoconstituents

Limit of Detection (LOD)

LOD of an individual analytical procedure is the lowest amount of an analyte in a sample that can be detected but not necessarily quantified

Limit of Quantitation (LOQ) of *C.gigantea*$_{frj}$

LOQ of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected and quantified

Procedure

C.gigantea$_{frj}$ were prepared and scanned under UV range of (200-700nm). *C.gigantea*$_{frj}$ were analyzed to identify the presence of peak and the corresponding wavelength was recorded. Subsequently, *C.gigantea*$_{frj}$ at concentrations of (0 to 10 µg/ml) were prepared and examined under UV spectroscopy at the wavelength identified from the *C.gigantea*$_{frj}$. The LOD and LOQ values were calculated based on the standard deviation of the response and the slope derived from the *C.gigantea*$_{frj}$[28,29].

Determination of digoxin equivalent in *C.gigantea*$_{frj}$

Procedure

The method was adopted as per I.P 1985, Various concentrations of (0 to 0 to 25 µg/ml) of *C.gigantea*$_{frj}$ were prepared by dissolving 0.5ml of *C.gigantea*$_{frj}$ and add 5ml of a mixture of 65 volumes of chloroform and 35 volumes of methanol. Add 20ml of glacial acetic acid and shake continuously for one hour. Allow to stand and filter the supernatant liquid through a sintered glass filter (grade 4, maximum pore size, 5 to 15µ), rejecting the first few ml of filterate. Dilute 5 ml of the filterate to 25ml with glacial acetic acid containing 0.005%w/v of ferric chloride and 2%v/v of sulphuric acid and allow to stand for one and half an hour. From this 5,10,15,20 and 25µg/ml is prepared and compared with Digoxin as standard under UV at 590nm [26].

Determination of gallic acid equivalent in *C.gigantea*$_{frj}$

Procedure

The method was adopted as per Singleton V.L, *C.gigantea*$_{frj}$ of 5,10,15,20 and 25µg/ml is taken. To each of this solution add 5ml of distilled water and 0.5ml of folin ciocalteu's reagent is added, mixed and shaken. After 5 minutes, 1ml of 10% sodium carbonate solution is added and the volume is made upto 10ml with distilled water. It is allowed to incubate for 2 hours at room temperature. Intense blue colour is developed. The reaction mixture without sample is used as blank. After incubation, absorbance was recorded at 725nm and compared with gallic acid as standard[30].

Determination of tannic acid equivalent in *C.gigantea*$_{frj}$

Procedure

The method was adopted as per Boham AB, Various concentrations of (0 to 25µg/ml) *C.gigantea*$_{frj}$ were prepared by dissolving 0.5ml of *C.gigantea*$_{frj}$ and add 0.5ml of Folin-Denis reagent and 0.8mL of distilled water was added. The tubes were kept aside for 15min. To this, 1mL of sodium carbonate solution was added and the remaining volume was made up with distilled water. From this 5,10,15,20 and 25µg/ml is prepared and compared with Tannic acid as standard. Then the tubes were shaken and the absorbance was recorded at 700nm after 30min[31].

Determination of thymol equivalent in *C.gigantea*$_{frj}$

Procedure

The method adopted by Gurupriya S, Various concentrations of (0 to10µg/ml) *C.gigantea*$_{frj}$ were prepared by taking 0.5ml *C.gigantea*$_{frj}$ and 10ml methanol was poured into it. The blend was shaken well and filtered to taken 5ml methanolic juice of the plant sample. Then 2ml Chloroform was blended in the extract of the plant sample and 3 ml sulphuric acid was added in plant juice. From this 2,4,6,8 and 10µg/ml is prepared and compared with thymol as standard under UV at 538nm[32].

Phenetics

Numerical taxonomy of plant derived by using five characters such as leaf blade, venation, apex base and margin[33].

DNA Barcoding

Genomic DNA Isolation

About 100 mg of plant tissue was ground with liquid nitrogen to make fine powder using mortar and pestle. Added 1 ml of preheated CTAB extraction buffer with 20µl of β-mercaptoethanol to the mortar and finely ground. The contents were transferred into a 2 ml centrifuge tube and incubated for 20 to 30 min at 65°C on a

water bath. After centrifuging using Refrigerated Centrifuge (Eppendrof, 5418R) the tube at 12,000 rpm for 10 min the supernatant was transferred to a fresh centrifuge tube and added equal volumes of chloroform: isoamyl alcohol (24:1) mixture and mixed gently by inverting tubes till an emulsion was formed. The tubes were centrifuged at 13,000 rpm for 12 min. The clear aqueous phase was transferred to fresh centrifuge tubes and equal volumes of ice-cold isopropanol was added. The sample was incubated overnight at -20° C and centrifuged at 12000 rpm for 3 min. The supernatant was discarded and the pellet was washed with 70% ethanol. DNA pellets were then air-dried at room temperature by allowing evaporation of uncovered centrifuge tubes. The pellets were suspended in an appropriate volume (20-30 µl) of $T_{10}E_1$ buffer.

Qualitative and Quantitative estimation of DNA

The quality and concentration of genomic DNA were checked by running the DNA sample on 1% agarose gel. The DNA concentrations were rechecked by visual assessment of band intensity under UV-trans-illuminator (Biorad, GelDoc Go, USA). The quantity of 1 µl of isolated DNA was checked using Nanodrop (Thermoscientific, Nanodrop One, USA).

PCR Amplification

The DNA barcode candidate ITS was used for PCR amplification as the same resulted in amplification. The isolated DNA was used as a template for PCR reaction and carried out in a thermocycler (Applied Biosystem,Veriti™, USA). The PCR products were then loaded onto 1% agarose gel and the amplification was confirmed.

Sequence analysis

FASTA format of the nucleotides were obtained using Finch TV from the chromatogram. The FASTA was fed into Basic Local Alignment Search Tool (BLAST) algorithm of NCBI to identify the closest matching sequence in the nucleotide database of GenBank. The sequence was converted to Barcode using the software BioRad barcode generator.

NCBI submission

After confirmation of the species, the sequence was submitted to NCBI with the necessary details to obtain a GenBank ID[34].

RESULTS AND DISCUSSION

Morphological study

Calotropis gigantea leaves were simple, oblong, and arranged in the opposite direction. They were dark green in ventral and light green in dorsal view, with a bitter taste and a fragrant odour. It has a subsessile petiole, a cordate base, and an acute apex. Its entire edge features parallel venation and a smooth texture. Because of the latex, it has white cottony tomentosum.(Tab.1& Fig.1)

Table1: Morphological characters of leaves of *Calotropis gigantea*

S.No.	Parameters	Observation
1.	Colour	Ventral: Dark green Dorsal: Light green
2.	Odour	Aromatic
3.	Taste	Bitter
4.	Leaf type	Simple
5.	Shape	Oblong
6.	Arrangement	Opposite
7.	Apex	Acute
8.	Base	Cordate base
9.	Petiole	Sub sessile
10.	Margin	Entire
11.	Venation	Parallel
12.	Texture	Smooth
13.	Length	12cm
14.	Width	6.2cm
15.	Petiole length	2.54cm

Fig 1: Habitat

Fig 1.1: Dorsal view of leaf

Fig 1.2: Ventral View of leaf

Microscopical study

Microscopic examination of *Calotropis gigantea* leaf showed the following characterisitics using safranin as dye and glycerin as humectant and viewed under microscope of lens 45x

Epidermis

Three rows of long, closely spaced parenchyma palisade cells made comprised the top epidermis and stomata encircled by many guard cells were visible in the lower epidermis.

Stomata

The lower epidermis contains stomata has two semicircular guard cells which are parallel and bigger subsidiary cells surrounds stomata and it is known as paracytic stomata.

Vascular bundles

Bicollateral hollow vascular cylinder made up of long, tubular cells. The cells are round, with a broad lumen and thick, lignified walls. Short radial lines are used to arrange the elements.

Fibres

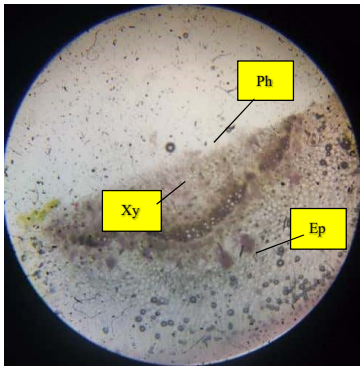
Fibers are cells of the sclerenchyma there is a elongated cell is present that is brachysclereid.

Laticifers

These are specific tissues or cells that are present in some plants and that generate the milky substance known as latex and that was very noticeable in *Calotropis gigantea* because the plant easily releases latex when it is injured. long, narrow tubular laticifers are commonly observed. They are anastomosing (non-septate and branching) and non-articulate. Producing and storing the latex is the main responsibility of laticifers.

T.S of midrib

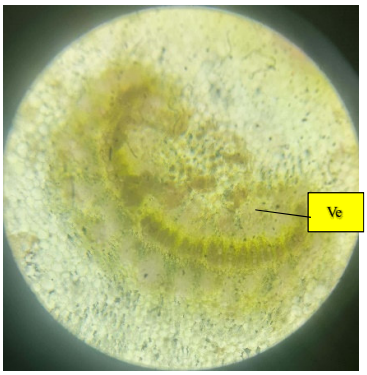
The proximal side of midrib is flat and distal side of midrib is convex. It contains parenchymatous cells and layer of vascular bundles in the proximal side of midrib. It shows oil globules at the distal side.



Ep- Epidermis; Ph- Pholem; Xy-Xylem

Fig 2: T.S of midrib (10x)

T.S of petiole



Ve-Vessel walls

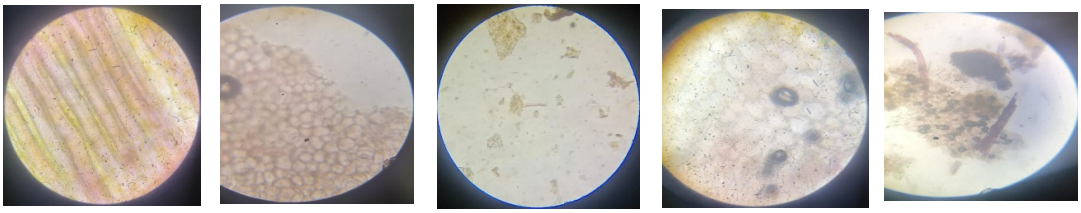
Fig 3: T.S of Petiole (10X)

Histochemical studies

The section of the leaves of *Calotropis gigantea* Linn., were stained by using specific reagents such as N/50 Iodine, sulphuric acid, Phloroglucinol and Conc. Hydrochloric acid, KOH to observe and locate lignin,cellulose, suberin, calcium oxalate respectively as per the protocol.

Table 2: Histochemical studies of *Calotropis gigantea* leaf

S.No.	Reagent	Test for	Observation	Identification
1.	Phloroglucinol + Hcl	Lignin	Pink	Vessels
2.	Iodine solution followed by Sulphuric acid	Cellulose	Yellow	Parenchyma cells
3.	Heating with KOH	Suberin	Yellow	Trichomes, stomata
4.	Picric acid	Alkaloids	Red	Fibres



Vessels **Parenchyma cells** **Trichomes** **Stomata** **Fibres**

Fig 4: Histochemical studies of *Calotropis gigantea*

Quantitative microscopy of *Calotropis gigantea*

Stomata: Paracytic Stomata is present on both surface.

Vein islet number and Veinlet termination number

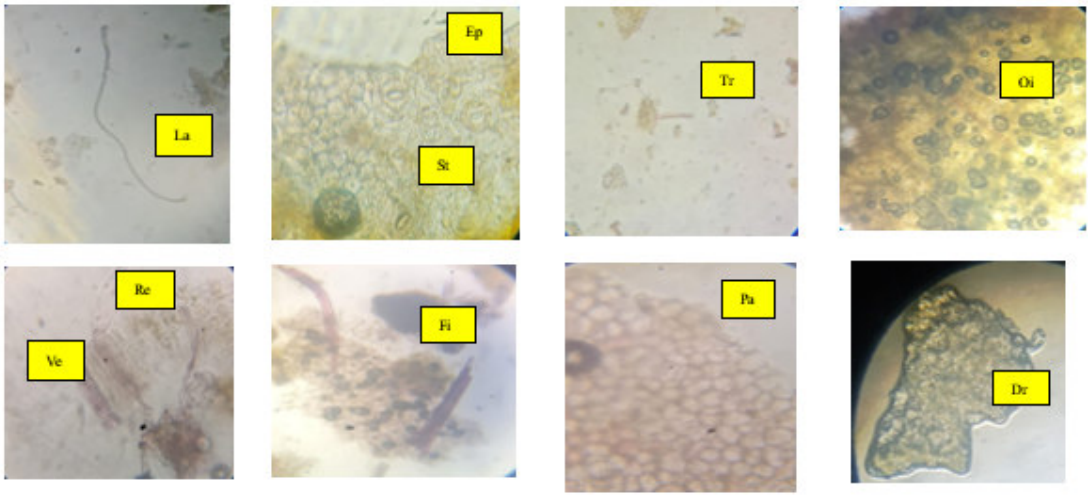
Leaf surface showed the presence of veins and vein islets and vein termination.

Table 3: Quantitative microscopy of *Calotropis gigantea*

S.No.	Parameters	Cells /mm2
1.	Epidermal number (Upper epidermis)	104
2.	Vein islets number	5
3.	Vein termination	3
4.	Stomatal number	52
5.	Stomatal Index	33
6.	Palisade ratio	4-6

Powder microscopy

The shade dried, powdered plant material was used for powder microscopic analysis. The organoleptic characters were observed and to identify the different characteristic features various staining reagents are used. It showed epidermal cells, stomata of paracytic type, parenchyma cells.



La- Laticifer; Ep- Epidermis; St- Stomata; Tr- Trichomes; Oi- Oil globules; Ve- Vessels; Re- Reticulate thickenings; Fi- Fibres; Pa- Parenchyma cells; Dr- Druse crystal

Fig 5: Powder microscopy of *Calotropis gigantea* Linn leaves

Behaviour of powder with various chemical reagents

Powdered drug of plant gave different fluorescence under visible and ultraviolet (UV)radiation (254nm& 365nm) when treated with various reagents. The colour observed in different radiations were recorded. It is used for the identification of plant and powdered drug.

Table 4: Behaviour of powder with various chemical reagents of *Calotropis gigantea* leaves

		Observation		
S.No.	**Reagent**	**Visible (<400 nm)**	**UV (254nm)**	**UV (365 nm)**
1.	Powder+ Hcl	Green	Red	Brown
2.	Powder+ Hcl+ H_2O	Green	Orange	Brown
3.	Powder+ HNO_3	Yellow	Pink	Grey
4.	Powder+ HNO_3+H_2O	Yellow	Pink	Green
5.	Powder+ H_2SO_4	Black	Brick red	Pale green
6.	Powder+ H_2SO_4+ H_2O	Black	Red	Pale Green
7.	Powder+ Acetic acid	Green	Brown	Red
8.	Powder+ NaOH	Green	Orange	Pale green
9.	Powder+ Alcoholic NaOH	Green	Orange	Pale green
10.	Powder+ Picric acid	Yellow	Orange	Brown
11.	Powder+ Fe_3Cl_4	Dark green	Brick red	Brown
12.	Powder+ Iodine	Green	Orange	Dark brown
13.	Powder+ Ammonia	Green	Orange	Brown

Determination of physicochemical parameters & inorganic element analysis

The physicochemical parameters of the plant drug were estimated using standard procedures which showed the loss on drying and total solid content was found to be 14±6.68% w/w and 86%w/w & total ash of 17.3±8.26% w/w, water soluble ash 8.3±3.92%w/w, acid insoluble ash 10.3±7.87%w/w .The percentage extractive value of Petroleum ether, Ethyl acetate, Ethanol, Aqueous was found to be 4.07±2.52%w/w, 4.86±1.22%w/w,

14.34±12.56%w/w, 13.73±9.14%w/w respectively. To the ash of the leaves was treated with 50%v/v hydrochloric acid and kept for 1 hr.It was filtered ,filtrate was used for inorganic analysis.

Table 5: Physiochemical parameters of *Calotropis gigantea*

S.No.	Physiochemical Parameters	Results
1.	Foreign matter	Nil
2.	Loss on drying	14±6.68%w/w
3.	Total solid	86%w/w
4.	Petroleum ether extractive	4.07±2.52%w/w
5.	Ethyl acetate extractive	4.86±1.22%w/w
6.	Ethanol extractive	14.34±12.56%w/w
7.	Aqueous extractive	13.73±9.14%w/w
8.	Total ash	17.3±8.26%w/w
9.	Water soluble ash	8.3±3.92%w/w
10.	Acid insoluble ash	10.3±7.87%w/w
11.	Presence of inorganic elements	Sulphates
12.	Heavy metals – Lead	Absent

Qualitative phytochemical analysis

The extract were subjected to preliminary phytochemical screening to determine the presence of various phytoconstituents. Its showed the presence of alkaloid, carbohydrates, glycosides, phytosterol, phenol, tannins, saponins, flavonoid and proteins.

Table 6: Qualitative phytochemical analysis of *Calotropis gigantea*

S.No.	Phytochemical analysis	Observation
1.	Test for Alkaloids	+
2.	Test for Beta carboline alkaloid	+
3.	Test for Cardiac glycosides	+
4.	Test for Phenolic compounds	+
5.	Test for Tannins	+
6.	Test for Saponins	+
7.	Test for Phytosterols	+
8.	Test for Steroids	+
9.	Test for Terpenoids	Trace
10.	Test for Proteins	-
11.	Test for Amino acids	-
12.	Test for Flavonoids	-
13.	Test for Anthroquinone glycosides	-
14.	Test for Coumarin	-
15.	Test for Quinone	-
16.	Test for Anthocyanin	-
17.	Test for Gums & mucilage	-

+ Presence, - absence

Quantitative estimation of phytoconstituents

Quantitative analysis such as total alkaloid equivalent, total cardiac glycoside equivalent, total phenolic equivalent, total tannin equivalent, total terpenoid equivalent were estimated for the *C.gigantea*$_{frj}$

Limit of Detection (LOD) and Limit of Quantitation (LOQ) of *C.gigantea*$_{frj}$

Various concentrations (0 to 10 µg/ml) of *C.gigantea*$_{frj}$ leaf were prepared as blank, observed under UV at 235nm. Absorbance was observed and displayed in table 7

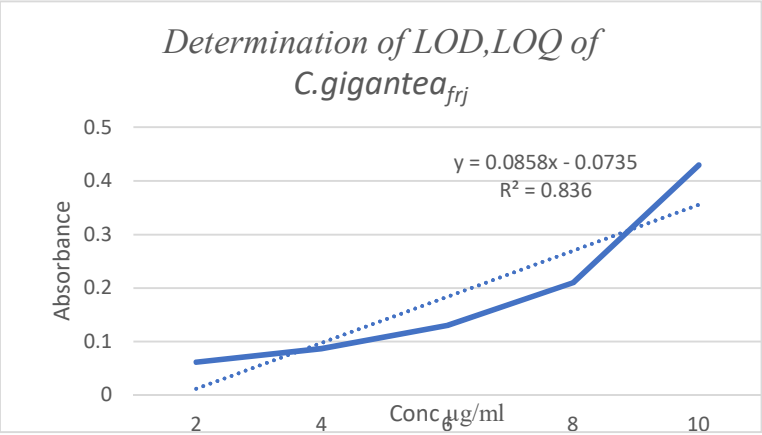


Fig 6: LOD, LOQ of *C.gigantea*$_{frj}$

Table 7: Determination of LOD, LOQ in *C.gigantea*$_{frj}$

S.No.	Concentration (µg/ml)	Absorbance of *C.gigantea*$_{frj}$
1.	2	0.0626
2.	4	0.0873
3.	6	0.1305
4.	8	0.2103
5.	10	0.4295
	LOD	0.094
	LOQ	0.27

The quantitative estimation LOD and LOQ of *C.gigantea*$_{frj}$ was found to be 0.094 and 0.27.

Determination of digoxin equivalent in *C.gigantea*$_{frj}$

Various concentrations (0 to 25 µg/ml) of *C.gigantea*$_{frj}$ leaf were treated by colorimetric method, Ferric chloride produce reddish brown colour and the absosrbance was observed and displayed in table 8

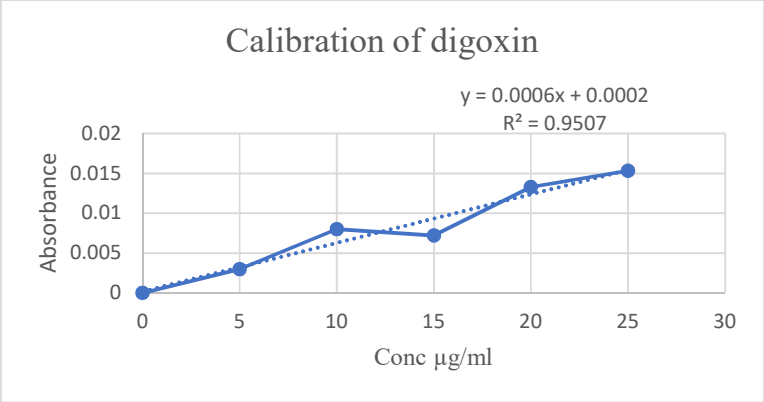


Fig 7: Calibration of digoxin

Table 8: Determination of digoxin equivalent in *C.gigantea*$_{frj}$

S.No.	Concentration (µg/ml)	Absorbance of digoxin	Absorbance of *C.gigantea*$_{frj}$
1.	0	0	0
2.	5	0.0004	0.014

3.	10	0.008	0.017
4.	15	0.0072±0.000125	0.019±0.000272
5.	20	0.0133±0.000272	0.033±0.000272
6.	25	0.01533±0.000272	0.035
		DE	**0.0003mg/g**

Quantitative estimation of cardiac glycoside was done by colorimetric method using digoxin as standard. The total digoxin equivalent in *C.gigantea*$_{frj}$ was found to be 0.0003 DE/g (Fig 7&Tab 8).

Determination of gallic acid equivalent in *C.gigantea*$_{frj}$

Various concentrations (0 to 25 µg/ml) of *C.gigantea*$_{frj}$ leaf were treated with Folin- cio calteu, which produce blue colour and the absosrbance was observed and displayed in table 9.

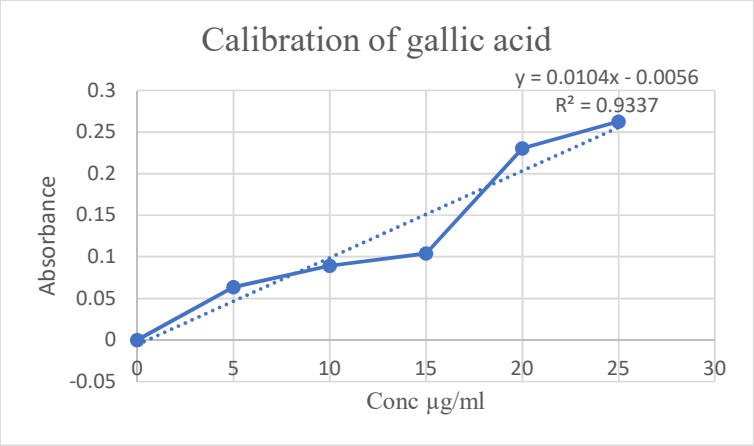


Fig 8: Calibration of gallic acid

Table 9: Determination of gallic acid equivalent in *C.gigantea*$_{frj}$

S.No.	Concentration (µg/ml)	Absorbance of gallic acid	Absorbance of *C.gigantea*$_{frj}$
1.	0	0	0
2.	5	0.063±0.0002	0.01±0.0002
3.	10	0.089±0.0010	0.010±0.0002
4.	15	0.154±0.0006	0.014±0.0051
5.	20	0.230±0.0001	0.060±0.0004
6.	25	0.262±0.0002	0.053±0.0002
		GAE	**0.0005mg/g**

Quantitative estimation of phenolic was done by Folin- cio calteu method using gallic acid as standard. The total gallic acid equivalent in *C.gigantea*$_{frj}$ was found to be 0.0005 GE/g (Fig 8 &Tab 9).

Determination of tannic acid equivalent in *C.gigantea*$_{frj}$

Various concentrations (0 to 20 µg/ml) of *C.gigantea*$_{frj}$ leaf were treated with Folin Denis, which produce blue colour and the absosrbance was observed and displayed in table 10

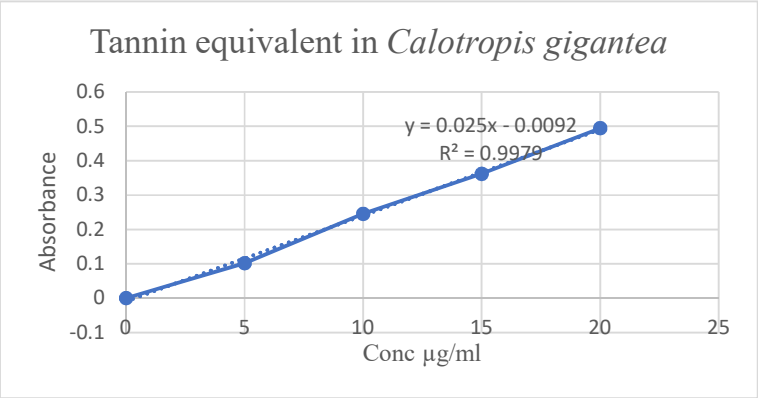


Fig 9: Calibration of tannic acid

Table 10: Determination of tannic acid equivalent in *C.gigantea*$_{frj}$

S.No.	Concentration (µg/ml)	Absorbance of tannic acid	Absorbance of *C.gigantea*$_{frj}$
1.	0	0	0
2.	5	0.1018±0.0001	0.077±0.0002
3.	10	0.2454±0.0006	0.098±0.0006
4.	15	0.3619±0.0002	0.101±0.0001
5.	20	0.4948±0.0007	0.214±0.0002
		TAE	**0.006 mg/g**

Quantitative estimation of tannin was done by Folin denis assay using tannic acid as standard. The total tannic acid equivalent in *C.gigantea*$_{frj}$ was found to be 0.006 TAE/g (Fig 9 &Tab 10).

Determination of thymol equivalent in *C.gigantea*$_{frj}$

Various concentrations of (0 to 10 µg/ml) *C.gigantea*$_{frj}$ leaf were treated by colorimetric method, which produce reddish brown colour and the absosrbance was observed and displayed in table 11.

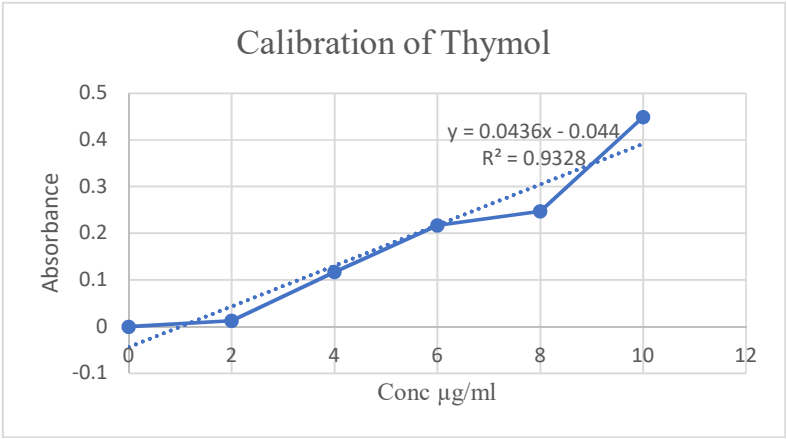


Fig 10: Calibration of thymol

Table 11: Determination of thymol equivalent in *C.gigantea*$_{frj}$

S.No.	Concentration (µg/ml)	Absorbance of thymol	Absorbance of *C.gigantea*$_{frj}$
1.	0	0	0
2.	2	0.0005±0.0001	0.0224±0.0001

3.	4	0.0117±0.0003	0.0246±0.0001
4.	6	0.2171±0.0030	0.1037±0.0138
5.	8	0.2476±0.0026	0.1758±0.0141
6.	10	0.4492±0.0011	1.1267±0.0007
		TE	**0.001mg/g**

Quantitative estimation of thymol was done by colorimetric method using thymol as standard. The total thymol equivalent in *C.gigantea*$_{frj}$ was found to be 0.001 TE/g (Fig 10 &Tab 11).

Phenetics

There are two species of *Calotropis*. These are taken as the botanical sources of Erukkam in many texts of Dravyaguna and medicinal plants.

- *Calotropis gigantea L.*
- *Calotropis procera* Ait.

Review from 11 floras shows that *Calotropis procera* and *Calotropis gigantea* are maximum mentioned botanical sources. Still, an additional third variety has been mentioned by some floras.

- *Calotropis acia* (Buch-Ham.)
  Other variety of *Calotropis* includes
- *Calotropis wallichii*
- *Calotropis spicata*

Table 12: Table of similar *Calotropis gigantea* characters with other four species of *Calotropis*

S.No.	Species	Blade	Venation	Apex	Base	Margin
1.	*Calotropis gigantea*	1	1	1	1	1
2.	*Calotropis procera*	1	1	1	1	1
3.	*Calotropis acia*	1	0	1	0	0
4.	*Calotropis Wallichii*	1	1	1	1	1
5.	*Calotropis spicata*	1	0	1	0	1

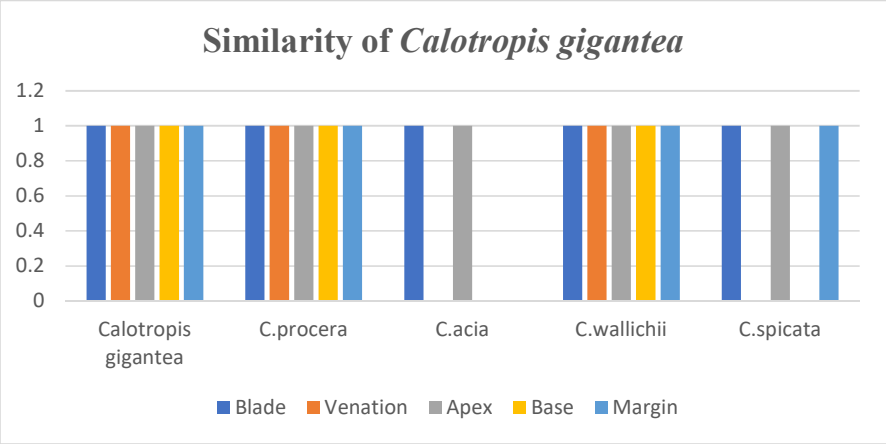


Fig 11: Chart of similarity characters of *Calotropis gigantea* Linn leaves

Table 13: Table of dissimilar *Calotropis gigantea* characters with other four species of *Calotropis*

S.No.	Species	*Calotropis gigantea*	*Calotropis procera*	*Calotropis acia*	*Calotropis wallichii*	*Calotropis spicata*
1.	*Calotropis gigantea*	0	0	0.4	0	0.6
2.	*Calotropis Procera*	0	0	0.6	0	0.4
3.	*Calotropis Acia*	0.4	0.6	0	0.6	0.2

4.	*Calotropis wallichii*	0	0	0.6	0	0.4
5.	*Calotropis spicata*	0.6	0.4	0.2	0.4	0

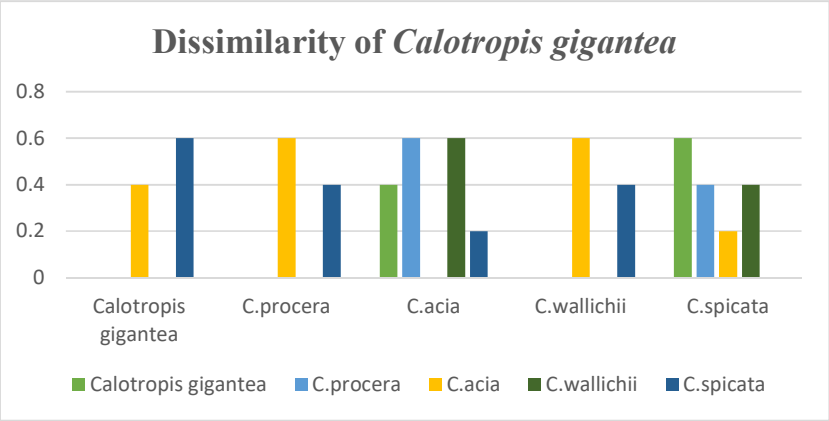


Fig 12: Chart of Dissimilarity characters of *Calotropis gigantea* Linn leaves

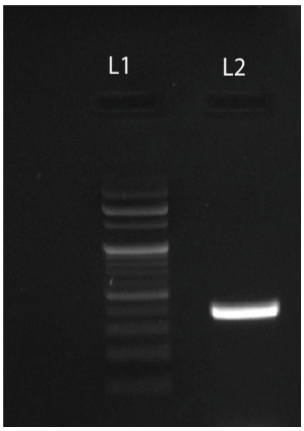
Sample matching coefficient
SSM=NS/NS+ND*100
Where,
NS= Number of similarity characters
ND= Number of dissimilarity characters
NS=15; ND=5
The matching coefficient of *Calotropis gigantea* with respect to other species was found to be 75%

DNA Barcoding

Genomic DNA was successfully isolated from the authenticated *Calotropis gigantea* sample, exhibiting high purity as indicated by spectrophotometric analysis (A260/A280 = 2.04) and confirming integrity through agarose gel electrophoresis. PCR amplification using ITS primers produced clear, specific amplicons, which were verified via gel electrophoresis. Sequencing of the PCR products provided high-quality nucleotide data, which matched *Calotropis gigantea* in BLAST analysis. The obtained sequences were converted into a DNA barcode and submitted to GenBank (Accession No. PX121659), providing a reliable molecular identification for this species. These results validate the use of ITS-based DNA barcoding for the authentication and genetic characterization of *Calotropis gigantea*.

Table 14: Quality check and quantification of DNA

Sample code	Concentration	in ng/µl	$A_{260/280}$	$A_{230/260}$
C14052501H	248.75		2.04	1.78



L1: Ladder; L2: Sample
Fig 13: Gel image of PCR amplified product

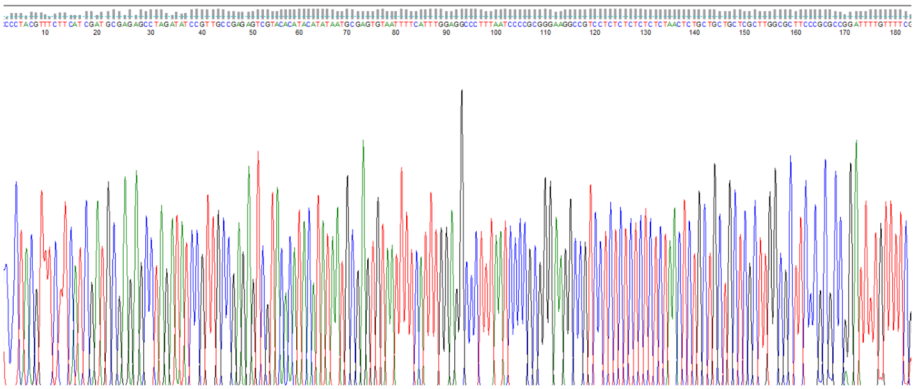


Fig 14: Chromatograms of *Calotropis gigantea*

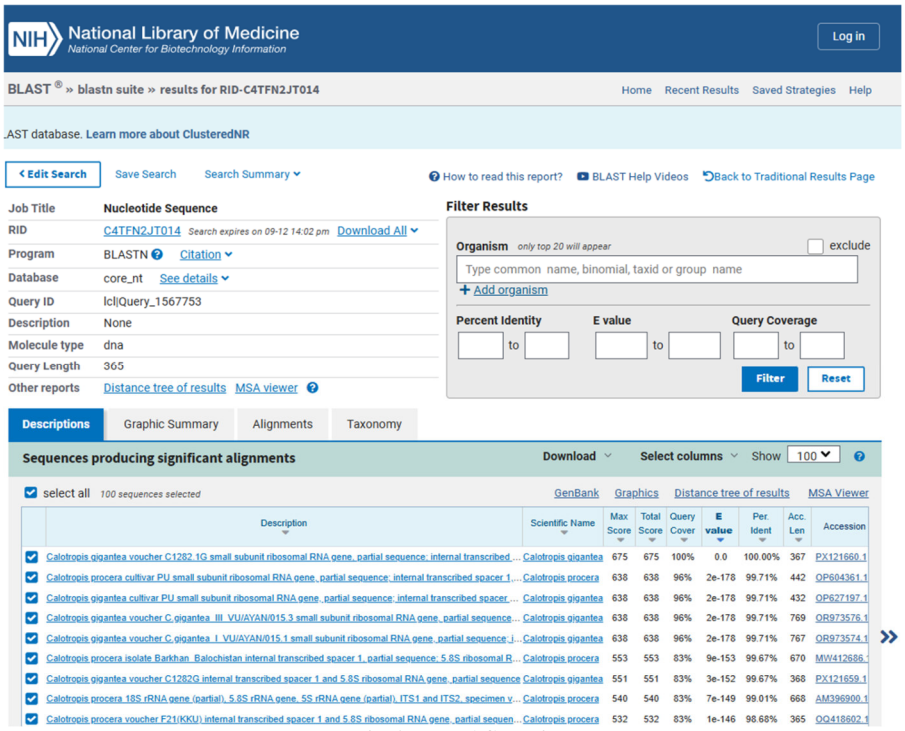
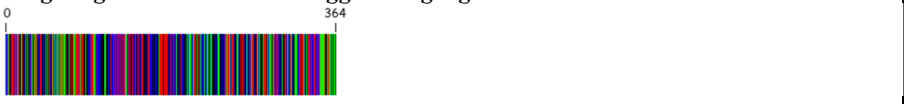


Fig 15: BLAST Hit

Table 15: Sequence obtained

Sample code	Sequence with Barcode
1282.C06052504G	**cctacgtttcttcatcgatgcgagagcctagatatccgttgccgagagtcgtacacatacatataatgcgagtgtaatttt catttggaggccctttaatccccgcgggaaggccgtcctctctctctctctaactctgctgctgctcgcttggcgcttcccg cgccggattttgttttcgctgctcgacgcgtccggcgacaaaccattgagtcaccgaccaagcaagggcgcaacggcg cttattactttcggaacgtgttcgcacgtcattcggtacagggatttgacaatgatccttccgcaggttcacctacggaaa ccttgttacgacttctccttcctctaaaaatggataacagaag** 

CONCLUSION

This study is the first to report phenetics and DNA barcoding for *Calotropis gigantea* collected in Mangalakudi village, Madurai district, Tamil Nadu. Pharmacognostical and phytochemical evaluations have established definitive parameters useful for the accurate identification and standardization. These findings can serve as a valuable reference for future research. Additionally, the phenetic analysis provides important taxonomic insights that help distinguish *C.gigantea* from other species within the *Calotropis* genus and DNA barcoding performed for the authentication and genetic characterization of *Calotropis gigantea*.

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