PHARMACOGNOSTICAL STUDIES AND DNA BAR CODE SCREENING FOR LEAVES OF *CLINACANTHUS NUTANS* (BURM.F.) LINDAU

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Abstract:
Pharmacognostical standardization of crude drugs is essential before carrying out its scientific validation as it furnishes the identity and authenticity of the plant. The present study is concerned with the macro-microscopical evaluation & DNA bar code screening of the leaves of *Clinacanthus nutans* (Burm.f.) Lindau. It is a perennial herb of the Acanthaceae family, widespread in Tamilnadu & Kerala and locally known as Visha pacchelai is a folk medicine. Macroscopic characteristics viz. size, shape, colour, odour and taste were determined. Microscopic evaluations were performed by using a high-resolution microscope. The anatomy of the leaves was examined by following the standard method of section cutting. Species identification using DNA barcoding was done based on the pattern of nucleotide arrangement in a fragment of DNA of a particular species and its maximum percentage of identity. The macroscopic assessment showed that the plant leaves are dark green in colour, simple, opposite, narrowly elliptic-oblong or lanceolate having slightly characteristic odour with salt, astringent and sweet taste. Dorsiventral lamina, irregular polygonal cells, uniseriate epidermis-bearing trichomes, V-shaped petiole, horseshoe shape vascular bundles, thin xylem fibres, pitted tracheids, anisocytic stomata, unicellular warty, septate trichome were all revealed in detailed microscopy. DNA quantification by spectrophotometer showed an A260/280 ratio of 1.8, indicating the purity of DNA. The macro-microscopic findings and DNA barcode analysis of the *Clinacanthus nutans* (Burm.f.) Lindau leaf are applicable for standardization and authentication of the plant.

Keywords: *Clinacanthus nutans* (Burm.f.) Lindau, Morphology, Pharmacognosy, Macro-microscopy, DNA barcoding, Identity, Quality.

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INTRODUCTION

Clinacanthus nutans (Burm.f.) Lindau belongs to the family of Acanthaceae is a folk medicine specially used to treat snake bite poisoning. In Tamil Nadu, it is known as vishapachalai and used to treat kidney diseases by Irula tribal of Thandarai, Chengalpattu, Kancheepuram district of Tamilnadu, India. The plant was identified as an exotic medicinal species during an ethno-medicinal survey in 2014-2016 [1]. The synonyms of *C. nutans* are *Clinacanthus burmanni Nees, Justicia nutans (Burm. f),* and *C. nutans var. robinsonii Benoist.* It is prevalent in several Asian countries, including Indonesia, Malaysia, Thailand, Vietnam, and China. It is also known as "Sabah Snake Grass" and "Saled Pangpon Tua Mea" (Saliva of the Female Mongoose) in Thailand [2].

The preliminary phytochemicals found in leaves include steroids, triterpenoids, amino acids, alkaloids, flavonoids, isoflavonoids, saponins, tannins, glycosides, quinones, phenolic compounds, carbohydrates, amino acids, and proteins [3]. Traditional herbal medicine in Malaysia, Indonesia, Thailand, and China employs this plant to treat diabetes, gout, herpes simplex lesions, skin rashes, bug and snake bites, and other ailments [4]. Pharmacological screening has further confirmed a variety of traditional uses such as antivenom, analgesic, anti-inflammatory, immunomodulating, neuroprotective, and neuromodulating functions, antidiabetic, antioxidant, antiviral, antibacterial, antifungal, anticancer, wound healing, plasmid DNA protective, lipid elevated inhibition, and oral mucositis and stomatitis activities[5].

According to the World Health Organization (WHO), more than 80% of the world's population prefers traditional medicine as their first choice of medical treatment [6, 7]. Although modern drugs are available, herbal medicine retained its image for historical and cultural reasons. Many people in developing nations rely on traditional healers and medicinal herbs to address their basic medical needs. Since the usage of herbal medicines has increased, it is difficult to evaluate the authenticity, safety, and efficacy in developing countries. There has been a rapid increase in the standardization of selected medicinal plants of potential therapeutic significance [8]. The use of plant drugs is subject to their correct identification. Generally, the potent drugs are either adulterated or substituted depending upon morphological characters or biological activity [9]. Despite advancements in research methods, pharmacognostical investigations are still the most accurate way to identify plant-based medications. DNA barcoding also serves as a tool for confirming the identity of specific plant species.

Through molecular species identification and DNA barcoding has become a robust and adaptable tool for authenticating plants and herbal medicines. DNA barcoding employs markers that are conserved within species but diverge between species such that species-specific sequences are derived with a single set of universal primers. In taxonomy, biodiversity evaluation, conservation, and environmental protection, DNA barcoding are employed for species delimitation, identification of cryptic species, and understanding species composition in biodiversity hotspots. Several research studies has used DNA barcoding techniques to screen for contamination, mislabelling, adulteration, and product substitution in herbal goods and raw pharmaceuticals [10].

To date, *Clinacanthus nutans* (Burm.f.) Lindau is not an official herbal medicine and there is no full-scale pharmacognostic studies so far except for the few attempts on biochemical evaluation of cultivated samples [12, 13, 14]. Hence the present study was undertaken for pharmacognostic standardization of *Clinacanthus nutans* (Burm.f.) Lindau leaves with an emphasis on macro and microscopic characters and DNA barcoding evaluation, which helps in the scientific identification and authentification of *Clinacanthus nutans* (Burm.f.) Lindau.

Material and Methods:

Sample collection and Authentication:

Plant leaves of *Clinacanthus nutans* (Burm.f.) Lindau were collected from the Thandarai village of Chengalpattu district of Tamilnadu, India. The sample was authenticated by Dr. K. N. Sunil Kumar; HOD of the Department of Pharmacognosy, Siddha Central Research Institute, Chennai. Herbarium specimen voucher No. 548.15062304 was deposited there for future reference. To remove foreign matter and contaminants, the leaves were cleaned, washed with distilled water and dried under the shade.

Chemicals and instruments:

Microscopical equipment, such as a compound microscope and a trinocular microscope, as well as glass slide covers, watch glass, and other conventional glassware, were employed in the investigation. Microphotographs were obtained using a Nikon Lab Photo 2 microscope connected to an Olympus Bx43 with a DSLR digital camera. Formalin, acetic acid, and ethanol (95%) were employed as common solvents, whereas glycerine, toluidine blue, iodine solution, phloroglycerinol,

hydrochloric acid, chloral hydrate, and sodium hydroxide were utilised as reagents.

Macroscopical studies:

According to the protocols established by Brain and Turner, the macroscopical characteristics of the leaves of *Clinacanthus nutans* (Burm.f.) Lindau, including shape, size, taste, fracture, colour, and aroma, were elucidated [15].

Microscopic studies and powder analysis:

The leaves were immersed in water for two days before being fixed for 48–72 hours in FAE (1:1:18) (formalin-5 ml + acetic acid-5 ml + 70% ethyl alcohol–90 ml). After the dehydration, cleaning, and infiltration procedures, the specimen was prepared on microtome slides (transverse slices), stained and examined under a light microscope. Rotating microtomes were used to segment the paraffin-embedded specimens. Sections ranged in thickness from 10 to 12 µm. The portions underwent the standard dewaxing process. Toluidine blue was used to stain the sections. Some cytochemical reactions were also obtained. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc., wherever necessary sections were also stained with safranin and fast-green and iodine (for starch). Images were captured using a Nikon Lab Photo 2 Microscopic Unit at various magnifications [16–18].

Powder microscopy:

The mature leaves were shade-dried and ground into a fine powder using an electric grinder for powder microscopy. A glass slide containing the stained powder sample was examined under the microscope. The powdered medication was assessed individually by treating it with various reagents such as phloroglucinol, glycerine, iodine solution, safranin, water, Sudan red, and toluidine blue to identify several microscopic features. A quadrangular compound microscope (with a camera attachment) was used to observe all the preparations. Digital camera Olympus Bx43 with DSLR was used in both instances to take the photomicrographs [19].

Photomicrographs:

A professional camera unit was used to take photomicrographs at various magnifications. Bright light was employed for routine observations and polarised light was used for the investigation of crystals, starch grains, and lignified cells because these structures exhibit the birefringent property under polarised light, which makes them stand out against a dark backdrop. The scale-bars are used as an indication that the figures were significantly magnified [20].

DNA Barcoding

Approximately 100 mg of fresh leaf sample has been used to isolate genomic DNA by following cetyltrimethylammonium bromide (CTAB) method with minor modifications. The pellet of isolated DNA has been dissolved in 100µl TE buffer. The isolated DNA has been checked on 0.8% agarose gel. DNA quality and quantity were estimated by agarose gel electrophoresis and spectrophotometer. Universal rbcL primers set (forward 5′-ATGTCACCACAAACAGAGACTAAAGC - 3′ and reverse 5′- GTAAAATCAAGTCCAC CRCG - 3′) has been used for amplification of DNA barcoding marker (Fazekas et al. 2003). The PCR reactions mixture has been constituted contained genomic DNA (50 ng), 2.5 µM dNTPs, 1.0 µM of each primer 1X *Taq* buffer, and 1-unit *Taq* polymerase enzyme (NEB, MA, USA) [14]. PCR amplification steps include initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 40 sec, final extension at 72°C for 5 min, and hold at 16°C. The PCR amplified products were checked on 1.5% agarose gel and compared with 100 bp molecular weight ladder (NEB, MA, USA) [21,22].

DNA sequencing and analysis

The PCR amplified products were purified using EZ-10 Spin Column (Bio Basic Inc. Ontario, Canada) and sequenced using a Big-dye terminator V3.1 Cycle sequencing kit following the manufacturer's protocol (Applied Biosystem, USA). The quality of the DNA barcode sequences was checked using Sequence Scanner Software v1.0 (Applied Biosystems, CA, USA). Sequence chromatograms were viewed and manually corrected using Chromas software. Regions of overlapping, poor or overcrowded peaks at the start and end of the sequence reads were removed. A similarity search was done using the Basic Local Alignment Search Tool (BLAST) algorithm against the non-redundant nucleotide database at National Center for Biotechnology Information NCBI [23]. Species identification was carried out based on the maximum percentage of identity [24].

Phylogenetic Analysis

A Neighbour Joining tree of molecular distance analysis was performed using 1000 bootstrap replicates with the Kimura 2-parameter model from

the alignments created using the ClustalW program in MEGA version 11.0.13 [25].

Results and Discussion:

Clinacanthus nutans (Burm.f.) Lindau is a perennial herb which can grow up to 1 m tall with pubescent branches and cylindrical, striate, and glabrescent stems. The morphological and organoleptic characters of *Clinacanthus Nutans* (Burm.f.) Lindau are shown in Table 1 & Fig 1.

Table1. Morphological and organoleptic characteristics of *Clinacanthus nutans* (Burm.f.) Lindau

Parameter	Leaf
Color	Dark green
Odour	Characteristic
Taste	astringent, salt and taste
Size	2.5–13.0 cm long × 0.5–1.5 cm wide
Shape	simple, opposite, narrowly elliptic-oblong or lanceolate

Microscopic evaluation of leaves of *Clinacanthus nutans* (Burm.f.) Lindau:

Microscopy is one of the easiest and cost-effective method for the precise identification of plants. There is no previous microscopic studies done for *Clinacanthus nutans* (Burm.f.) Lindau. So an attempt has been made to analyze the raw plant material of *Clinacanthus nutans* (Burm.f.) Lindau by microscopical studies. The results showed that the lamina of the leaf was of dorsiventral type and the upper and lower epidermis are covered with thick cuticle, both epidermal cells embedded with cystolith. The upper epidermis is followed by a single row of palisade parenchyma and spongy parenchyma; mesophyll is differentiated into outer single palisade cells extending up to the vascular region, followed by 2 to 3 layers of spongy mesophyll containing irregular polygonal cells.

The transverse section of the midrib shows uniseriate upper and lower epidermis covered by thick cuticle and bearing trichomes; epidermal cells embedded with cystolith; a patch or 5-7 layers of collenchymatous tissue is present under the raised adaxial portion of the rib and beneath the upper epidermis; also lining much larger above the abaxial epidermis; rest of the midrib is parenchymatous ground tissue; a centrally placed vascular bundles are arranged in a form of arc shape which are conjoint and bicollateral in nature; xylem vessels are present as uniseriate rows.

TS of the petiole is V-shaped in outline with a slightly wavy margin; shows a single layer of epidermis covered with thick cuticle and bearing few simple multicellular covering trichomes, followed by cortical region composed of 4 to 5 rows of collenchymatous cells followed by parenchymatous ground tissue embedded with, conjoint bicollateral vascular bundles arranged in a horseshoe shape, meristele is encircled by an endodermis layer (Fig.2, 3, 4).

Leaf powder microscopy

The results of powder microscopy of leaf showed fragment epidermal cells in sectional and the surface view from the stem region; xylem fibres are thin-walled with wide lumen and sharp end; followed by vessels with pitted, and spiral thickenings, radially and tangentially cut medullary rays crossing fibres; vessels with tailed projections; pitted tracheids from stem; followed by a fragment of the upper epidermis in surface view with an anticlinal wall, a fragment of the lower epidermis in surface view with anisocytic stomata and wavy anticlinal walls; unicellular warty, septate trichome and brownish mass (Fig. 5).

Fig. 1. Habitat photo of *Clinacanthus nutans*

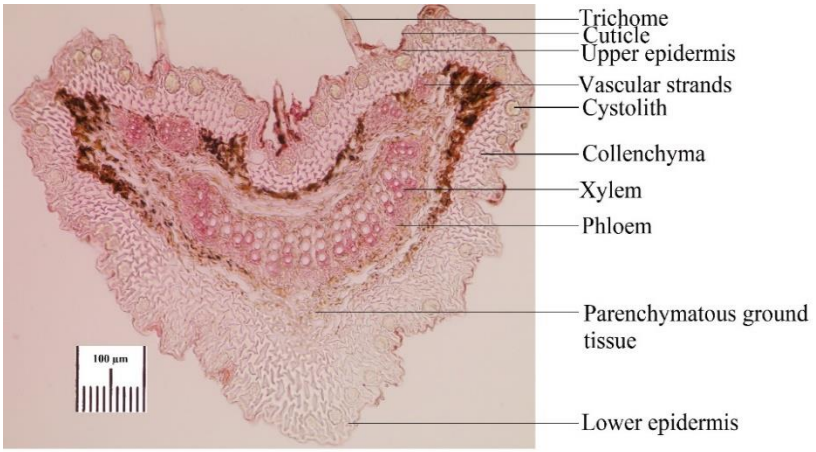


Fig. 2. TS of petiole of *Clinacanthus nutans*

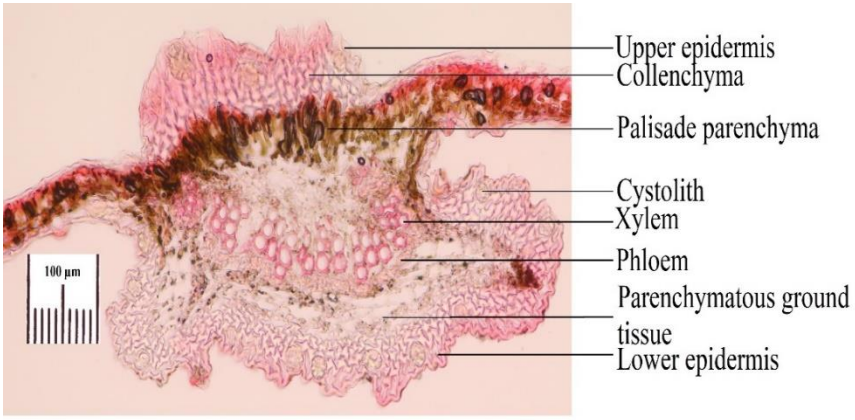


Fig. 3. TS of lamina passing through midrib of *Clinacanthus nutans*

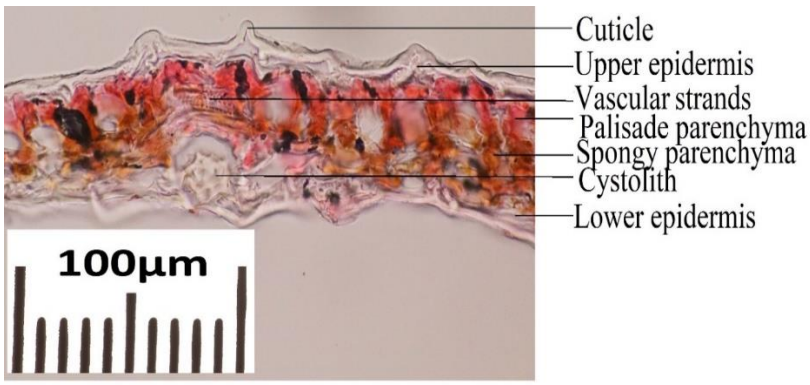
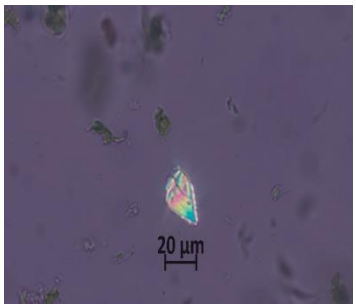
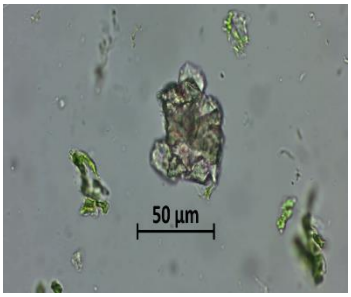
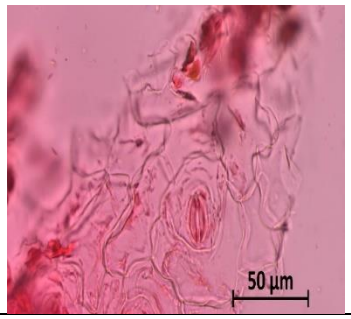
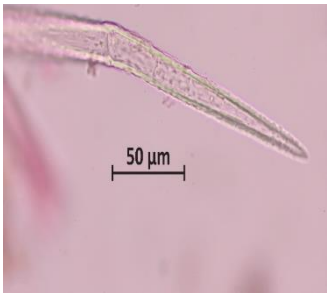


Fig. 4. TS of Lamina

Powder microscopy of Clinacanthus nutans

	
Annular vessels	Fragment of trichome
	
Crystal structure	Crystals
	
Diacytic Stomata	Trichome

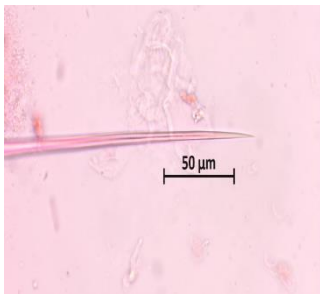
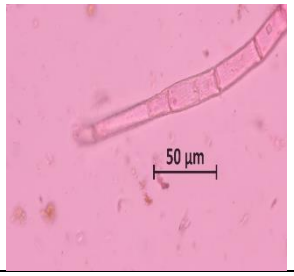
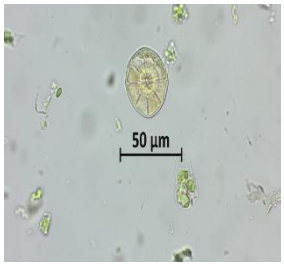
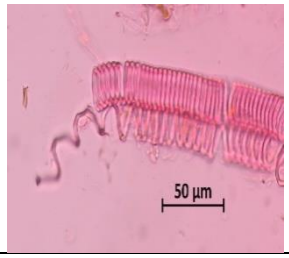
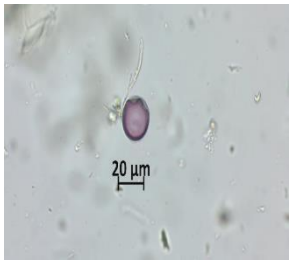
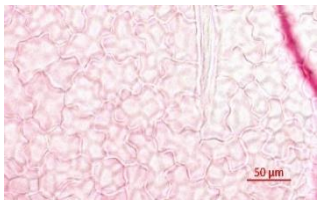
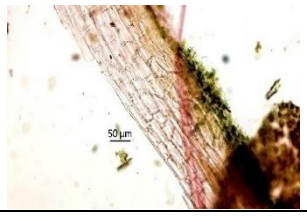
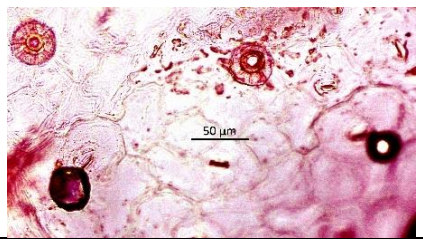
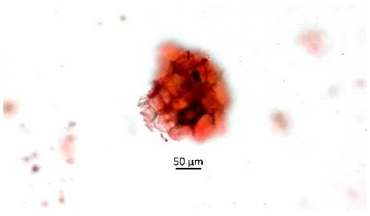
	
Fibres with narrow lumen	Fibres with sharp end
	
Uniseriate multicelular Trichome	Separated cicatrix
	
Spiral vessel	Starch grains
	
Upper epidermis of leaf in surface view	Epidermal cells from midrib region
	
Hooked warty trichome	Unicellular septate and warty trichome
	
Fragment of lower epidermis with cicatrix	Brownish mass

Fig. 5. Powder microscopy of *Clinacanthus nutans* – Leaf part

DNA barcoding:

The DNA extracted from the leaves of *C. nutans* showed slightly degraded DNA bands without any RNA contamination in agarose gel electrophoresis. DNA quantification by spectrophotometer showed an A260/280 ratio of 1.8, indicating that the DNA found in the leaves of *C. nutans* is pure. The size of polymerase chain reaction (PCR) amplified product of ribulose-bisphosphate carboxylase gene (rbcL) barcode was observed at approximately 550 bp. Agarose gel electrophoresis of DNA and *rbcL* marker amplified from the leaf of *C. nutans* has been shown in Fig 6a & 6b respectively. Sequencing with forward and reverse primers generated 711 bp and 558 bp, respectively. After manual correction of sequencing and aligning, both read contig has generated a 595 bp long sequence. A BLAST search of this nucleotide stretch against NCBI nucleotide databases showed 99% identity with 100% query coverage to the chloroplast genome of *C. nutans* (NC_042162.1), rbcL gene sequences of *C. nutans* voucher PS0749MT01 (GQ436501.1), and rbcL gene sequences of *C. nutans* (MH069767.1).The rbcL sequence from this study has been submitted to GenBank accession number: OR129836. (Fig. 6, 7).

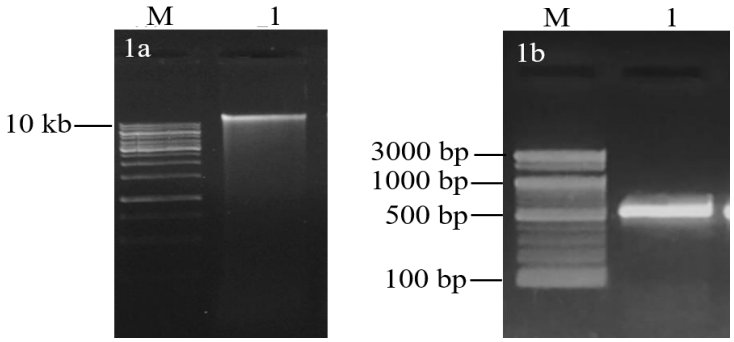


Fig.6a. 0.8 % Agarose gel electrophoresis: Lane represents **M**: 1kb DNA marker; **1**: DNA of C. *nutans*). **6b**. 1.5% Agarose gel electrophoresis: Lane represents **M**: 100bp DNA marker; **1**: PCR amplified product of *rbcL* DNA barcode marker from C. *nutans*.

Phylogenetic analysis

To confirm the phylogenetic positions of sequenced *C. nutans* (this study), rbcL sequences of *Clinacanthus nutans* (GQ436501.1), *Clinacanthus nutans* (MH069767.1), Ecbolium amplexicaule (JQ933315.1), *Cosmianthemum viriduliflorum* (KX527392.1), *Justicia adhatoda* (MG946890.1), *Justicia adhatoda* (MG946843.1), *Pseuderanthemum malaccense* (KR529941.1), *Pseuderanthemum malaccense* (KR529941.1), *Ruttya fruticose* (L02434.2), *Codonacanthus pauciflorus* (KX527299.1) from the Acanthanceae family were downloaded from the NCBI database. The *rbcL sequences of Nicotiana tabacum (OP711257.1)* from the Solanaceae family were used as outgroups. The phylogenetic analysis of *C. nutans* in a clade with closely related to *other C. nutans* rbcL sequences (Fig 7).

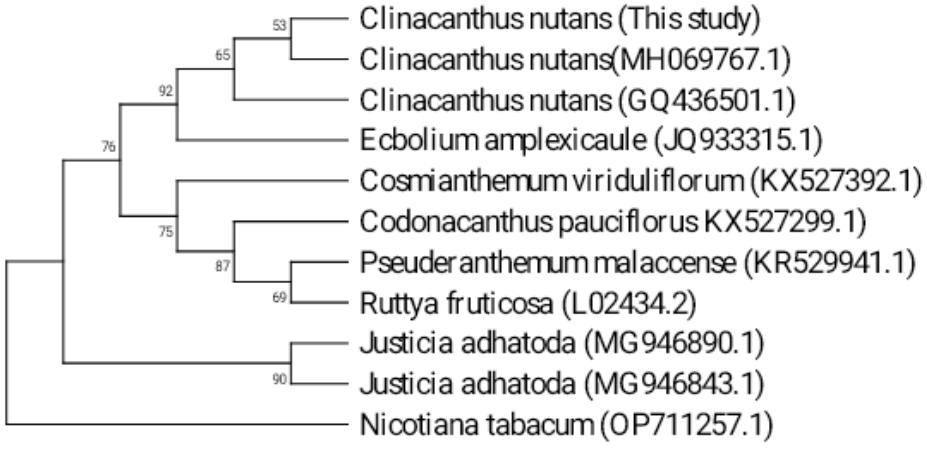


Fig.7. Phylogenetic tree analysis based on rbcL sequences and from other species of the *Acanthaceae* family and *Nicotiana tabacum* from the *Solanacea*e family as outgroup.

Conclusion:

The current investigation supports the applicability of macroscopic, microscopic, and DNA barcoding studies of *Clinacanthus nutans* leaves, which have been carried out with the aim of identifying diagnostic characters that will significantly contribute to the preliminary identification and accurate authentication of the botanical species of the plant drug. The safety and effectiveness of herbal medication depend on accurate identification and quality control, which is an essential prerequisite. In the current study, an effort has been made to describe the morphological traits and DNA profile of *Clinacanthus nutans*, which can be used as a crucial diagnostic tool to assess herbal medicine as an indigenous resource.

In conclusion, the parameters which are reported here can be considered as distinctive enough to identify and decide the authenticity of this drug in herbal industry/trade and this can be used as microscopic standards in future.

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CONFLICT OF INTEREST: The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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