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“Integrative Description of *Calotropis* sp. nov. (Apocynaceae) from Karnataka Based on Morphological, Molecular, and Biochemical Data”

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Abstract: During extensive field surveys in the Kalaburagi region of Karnataka, India, a *Calotropis* population exhibiting a consistent combination of morphological features differing from those typically reported for *Calotropis procera* and *Calotropis gigantea* was documented. This population is provisionally referred to here as *Calotropis prefina*. The present study evaluates its taxonomic position using an integrative framework combining detailed morphological assessment, leaf and stem anatomical analysis, biochemical characterization of latex (milk coagulation activity and temperature-dependent pH profiling), chloroplast DNA barcoding (*rbcL*), and herbarium-based authentication. Comparative analyses revealed a stable and diagnostic suite of characters involving plant stature, stem pubescence, latex viscosity, leaf morphology, floral architecture, pollinia structure, vascular organization, and laticifer distribution. *Calotropis prefina* exhibited markedly reduced pollen viability (57.5%) compared with *C. procera* (82.5%) and *C. gigantea* (80%), indicating reproductive differentiation. Biochemical assays showed reduced milk clotting activity and a consistently alkaline, thermally stable latex pH profile in *C. prefina* relative to *C. procera* and *C. gigantea*, indicating functional divergence. Molecular characterization using the chloroplast *rbcL* barcode confirmed the placement of the investigated taxon within the genus *Calotropis*. NCBI BLAST analysis revealed the highest sequence similarity with *Calotropis procera* (98.04% - 97.86%), while *C. gigantea* was also retrieved among the closest matches with comparable identity values. Independent identification through the BOLD Systems database similarly failed to achieve unambiguous species-level discrimination, returning both *C. procera* (97.83%) and *C. gigantea* as closest reference taxon with closely comparable percentage similarity. These results indicate strong genetic affinity within *Calotropis* while highlighting the Phylogenetic interpretation placed *C. prefina* within the *Calotropis* clade, closely allied to *C. procera* and *C. gigantea*, yet forming a distinct lineage. Taken together, the convergence of morphological, anatomical, biochemical, and molecular evidence supports the interpretation of *Calotropis prefina* as a distinct taxonomic entity within the genus *Calotropis*. The provisional designation is applied pending formal nomenclatural validation in accordance with the International Code of Nomenclature, with herbarium authentication currently underway at the Botanical Survey of India.

Keywords: *Calotropis*, morphology, anatomy, pollinia, DNA barcoding, *rbcL*, latex enzymes, milk-clotting activity.

INTRODUCTION

The genus *Calotropis* R.Br. (Apocynaceae, subfamily Asclepiadoideae) comprises perennial shrubs adapted to arid and semi-arid habitats across Africa, the Middle East and South Asia. Members of the genus are readily recognized by their milky latex, opposite thick leaves, complex gynostegial flowers, and pollinia-based reproductive system. Among the currently accepted species, *Calotropis procera* and *Calotropis gigantea* are the most widespread and have been extensively studied with respect to morphology, anatomy, phytochemistry and pharmacological properties (Rahman & Wilcock 1991; Joshi et al. 2010; Kadiyala et al. 2013; Abeysinghe & Scharaschkin 2022).

Despite their apparent distinctiveness, species delimitation within *Calotropis* remains challenging due to overlapping vegetative traits, phenotypic plasticity, and the conservative nature of commonly used chloroplast DNA barcodes. Several authors have emphasized the taxonomic value of floral characters, pollinia morphology, and anatomical features such as trichome distribution, stomatal type and vascular organization in resolving species boundaries within Asclepiadoideae (Metcalf & Chalk 1957; Swarupnandan & Sreedevi 1996; Abeysinghe & Scharaschkin 2022).

Traditional plant taxonomy has relied heavily on morphological and anatomical evidence; however, such characters alone may be insufficient when dealing with cryptic variation or closely allied species complexes. In recent years, integrative taxonomic approaches incorporating biochemical markers and molecular DNA barcoding have proven valuable for species delimitation and authentication (Joshi et al. 2010; Sidhu et al. 2023). Chloroplast DNA markers such as *rbcL* are widely accepted as reliable barcodes for plant identification due to their universality, amplification success, and discriminatory capacity at generic and species levels.

Latex-producing plants such as *Calotropis* also offer a unique opportunity for biochemical characterization. Latex proteases involved in milk clotting have been used as functional markers reflecting enzymatic diversity (Seiber et al. 1982; Rajagopalan and Sivasubramanian 2019; Shrestha and Shrestha 2023). Likewise, latex pH and its thermal stability may indicate underlying biochemical differences among taxon.

During botanical explorations in northern Karnataka, a population of *Calotropis* was repeatedly observed that exhibited a unique combination of characters are clearly distinct from, *C. procera* and *C. gigantea*. Preliminary herbarium submission to the Botanical Survey of India (BSI) indicated that formal taxonomic recognition would require publication of a peer-reviewed scientific study demonstrating consistent diagnostic differences.

In response, the present work aims to

- (i) document and compare the morphology and anatomy of *C. procera*, *C. gigantea*, and the Kalaburagi population;
- (ii) assess molecular affinity using *rbcL* DNA barcoding;
- (iii) evaluate selected biochemical properties of latex; and
- (iv) critically discuss the taxonomic implications of the combined evidence.

MATERIALS AND METHODS

Plant material and study area

The study was conducted in Kalaburagi district, Karnataka, India. *Calotropis procera* and *C. gigantea* were examined along Kusnoor Road, where both species occur sympatric ally. *Calotropis prefina* was studied along Rajapur Road, growing in open wasteland and roadside habitats.

Fresh plant material of novel *Calotropis* sp. provisionally referred to here as “*Calotropis prefina*” was collected from naturally occurring populations in the Kalaburagi region of Karnataka, India. Comparative data for *Calotropis procera* and *Calotropis gigantea* were obtained from co-occurring populations within the same geographical region. Latex samples were collected from healthy, mature plants by making shallow incisions on the stem and immediately processed for biochemical assays.

Material examined

Calotropis procera — INDIA, Karnataka, Kalaburagi district, Kusnoor Road, 17.3185°N, 76.8593°E, roadside scrub, flowering and fruiting (Figure 1,2), coll. Afina Afseen & Preeti Thakur, 2024-25; specimens examined and compared with authenticated herbarium material (BSI; NBU Herbarium; Atlas of Florida, etc.).

Calotropis gigantea — INDIA, Karnataka, Kalaburagi district, Kusnoor Road, 17.3180°N, 76.8590°E, roadside wasteland, flowering and fruiting (Figure 9,10), coll. Afina Afseen & Preeti Thakur, 2024-25; identity verified with herbarium records and published descriptions (BSI; NBU Herbarium; Atlas of Florida, etc.).

Calotropis prefina — INDIA, Karnataka, Kalaburagi district, Rajapur Road, 17.1705°N, 72.6893°E, open wasteland, flowering and fruiting (Figure 17,18), coll. Afina Afseen & Preeti Thakur, 2024-25; voucher specimen submitted to the Botanical Survey of India (BSI) for evaluation.

Morphological observations

Vegetative and reproductive characters were recorded from mature, healthy individuals. Measurements were taken using Vernier callipers. Characters assessed included plant height, branching pattern, stem surface and pubescence, latex appearance, leaf dimensions and texture, floral size and colour, corona and calyx morphology, pedicel length, pollinia structure, gynoeceum dimensions, and follicle length.

Anatomical procedures

Transverse sections of fresh leaves and young stems were stained using toluidine blue O and safranin–fast green differential staining procedure. Sections were stained with 0.05% (w/v) toluidine blue O in phosphate buffer (pH 6.8) for 1–2 min, rinsed briefly in distilled water, and examined immediately under a light microscope. For differential visualization of lignified and non-lignified tissues, selected sections were stained sequentially with safranin O (1% w/v in 50% ethanol) for 2–3 min and counterstained with fast green (0.5% w/v in 95% ethanol) for 30–60 s, followed by brief rinsing in distilled water. Staining procedures followed standard methods after O’Brien et al. (1964) and Ruzin

(1999). Epidermal preparations were examined for stomatal characters and stained using Safranin stain. Observations and photomicrographs were obtained using a compound light microscope under bright-field illumination. Anatomical terminology follows Metcalfe and Chalk (1957).

Pollen Viability Assessment

Pollen viability was evaluated using the **acetocarmine staining technique**, which is widely employed in recent pollen biology and reproductive studies as an indicator of cytoplasmic integrity. Fresh flowers of *Calotropis* spp. were collected at anthesis during early morning hours to ensure maximum pollen freshness (e.g., Obin et al., 2020; Kumar et al., 2022).

Mature anthers were excised and gently crushed on clean glass slides to release pollen grains. A drop of **1–2% acetocarmine stain** was added to the pollen, followed by placement of a coverslip. The slides were allowed to stand for **5–10 minutes** to facilitate uniform staining, as described in recent pollen viability assessments (e.g., Singh et al., 2021; López et al., 2023).

Observations were made under a **light microscope** at 10x and 40x magnification. Pollen grains showing **deep red or dark pink staining with uniform cytoplasm** were considered viable, whereas **faintly stained or unstained grains** were scored as non-viable, following criteria used in recent angiosperm pollen studies (e.g., Chen et al., 2020; Rahman et al., 2024).

To obtain reliable estimates, pollen grains were counted from **multiple microscopic fields and slides**, and counts were pooled until a **minimum of 300 pollen grains per sample** was achieved, in line with current methodological recommendations (e.g., Patel et al., 2021). Three independent samples were analysed.

Pollen viability was calculated using the formula:

$$\text{Pollen viability (\%)} = \frac{\text{Number of viable pollen grains}}{\text{Total pollen grains counted}} \times 100$$

Imaging

A high-definition digital camera was used to document the gross morphology of habitat, stems, leaves, inflorescences, and fruits. The measurements were taken with Vernier callipers. Microscopic images were captured via compound microscopy at 40x – 100x magnification using a digital camera adapter (Ansari and Chauhan 2020).

Plant identification and authentication

Preliminary identification of *C. prefinia* was based on distinct morphological features including leaf arrangement, floral characters, latex exudation, and fruit morphology, following standard taxonomic descriptions (Rahman and Wilcock 1991; Kadiyala et al. 2013). Identification was carried out with the help of Department of Botany, Gulbarga University, Kalaburagi. Owing to the unique combination of morphological traits observed, further authentication was undertaken in consultation with a qualified taxonomist, and comparative analyses were conducted with authenticated specimens

deposited at the Botanical Survey of India (BSI), Pune, and cross verified with online herbaria including the Royal Botanic Garden Edinburgh and the North Bengal University Herbarium.

Chemical screening

Milk coagulation assay or Milk clotting assay (MCA)

Latex protease activity was assessed following established protocols for *Calotropis* species (Rajagopalan and Sivasubramanian 2019; Shrestha and Shrestha 2023), with methodological adaptations based on Arima et al. (1970) and Choedon et al. (2006). Fresh latex (1 mL) was mixed with 0.05 M sodium acetate buffer (pH 5.5), centrifuged to remove debris, and diluted (1:9 v/v). The enzyme extract (1 mL) was added to 5 mL of pre-boiled and cooled milk substrate. The time required for visible coagulation was recorded. Milk coagulation activity was calculated using the formula:

$$\text{MCA (U mL}^{-1}\text{)} = \frac{2400}{T} \times \frac{S}{E}$$

where *T* is coagulation time (s), *S* is milk volume (mL), and *E* is enzyme volume (mL).

Latex pH analysis

Fresh latex was diluted with deionized water (1:9 v/v). pH measurements were conducted using a calibrated digital pH meter across a temperature range of 30–100 °C, maintained using a controlled water bath, following Joshi et al. (2010) and Kadiyala et al. (2013).

Molecular study (DNA barcoding)

Genomic DNA was isolated from fresh leaf tissue of *C. prefina* using Qiagen DNeasy Blood & Tissue kit, as per manufacturer's instructions. The chloroplast *rbcL* region was amplified and sequenced using Applied Biosystems Big Dye Terminator V3.1 Cycle sequencing kit. The obtained sequence was assembled using ChromasProV3.1 sequence assembly software, and further used for BLAST analysed using NCBI BLAST for similarity search and BOLD Systems for species-level identification. Percentage identity values, E-scores, and similarity ranges were recorded. Phylogenetic interpretation was carried out based on clustering patterns (neighbor-joining method) using CLUSTALW and MEGAX software's within Apocynaceae.

Results

1. *Calotropis procera* (Aiton) Dryand.

Habit and stem:

Shrub up to ca. 4 ft tall, erect, sparsely branched (Figure 2); stems smooth, grey-green, glabrous to very sparsely pubescent. Latex copious, thick, milky white (Figure 3).

Leaves:

Lamina ovate, 9–11 cm long, thick and leathery, densely velvety due to abundant trichomes; apex rounded to slightly pointed; base cordate; margin entire; venation pinnate with prominent secondary veins; petiole extremely short (Figure 8(h, i)).

Flowers:

Small, ca. 2.4 cm in diameter (Figure 8(a, b),1); corolla purple with darker margins (Figure 8(c)); lobes narrow and pointed; corona compact, deep purple, five-lobed (Figure 8(d, e)); calyx small, green with purplish mid-region (Figure 8(g)); pedicels ca. 3.0 cm (Figure 8(g)); buds rounded (Figure 8(j, k)).

Pollinia:

Smallest among the three taxon, compact and rounded, opaque, firmly attached to the style (Figure 25).

Pollen grain morphology:

Pollen grains observed in stained preparations are uniform in size and shape, predominantly spheroidal to sub-spheroidal. The pollen wall appears thick, with well-defined outlines and dense cytoplasmic staining, indicating intact cellular contents (Figure 7). Most grains show homogeneous staining, and aborted or collapsed pollen grains are infrequent.

Gynoecium and fruit:

Carpels (bicarpellary) ca. 0.8 cm (Figure 8(f)); follicles shortest, 5.0–5.9 cm long (Figure 8(l, m)), fruiting from March to May.

Leaf anatomy:

Leaf transverse section dorsiventral; epidermis single-layered with thick cuticle. Trichomes dense on both adaxial and abaxial surfaces (Figure 4). Stomata anisocytic, predominantly adaxial (Figure 6). Palisade tissue is compact, consisting of one to two layers; spongy parenchyma reduced. Vascular bundles collateral with fewer xylem elements showing weak to moderate lignification; phloem compact (Figure 4). Laticifers present but sparsely distributed.

Stem anatomy:

Stem transverse section circular; periderm thick and well developed with moderate trichome presence. Cortex narrow. Vascular cylinder compact; xylem vessels fewer and moderately lignified; phloem compact. Laticifers sparse (Figure 5).



Figure 1: *Calotropis procera*



Figure 2: *Calotropis procera* plant



Figure 3: Latex oozing out of *Calotropis procera* stem

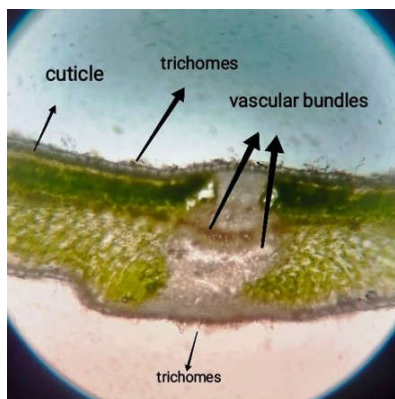


Figure 4: TS of leaf of *Calotropis procera*



Figure 5: TS of stem of *Calotropis procera*

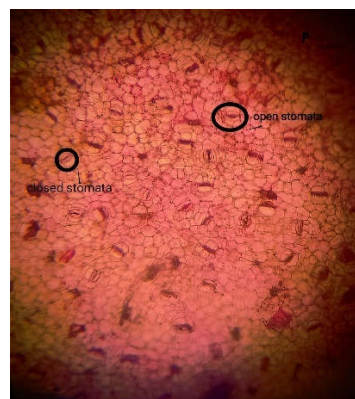


Figure 6: Stomata of *Calotropis procera* leaf

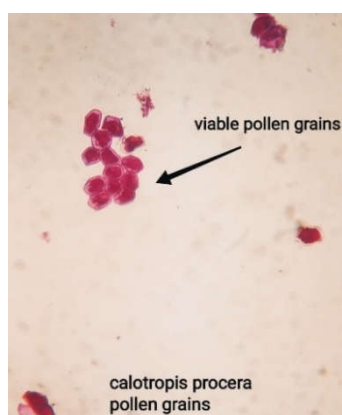


Figure 7: Pollen grains of *Calotropis procera*

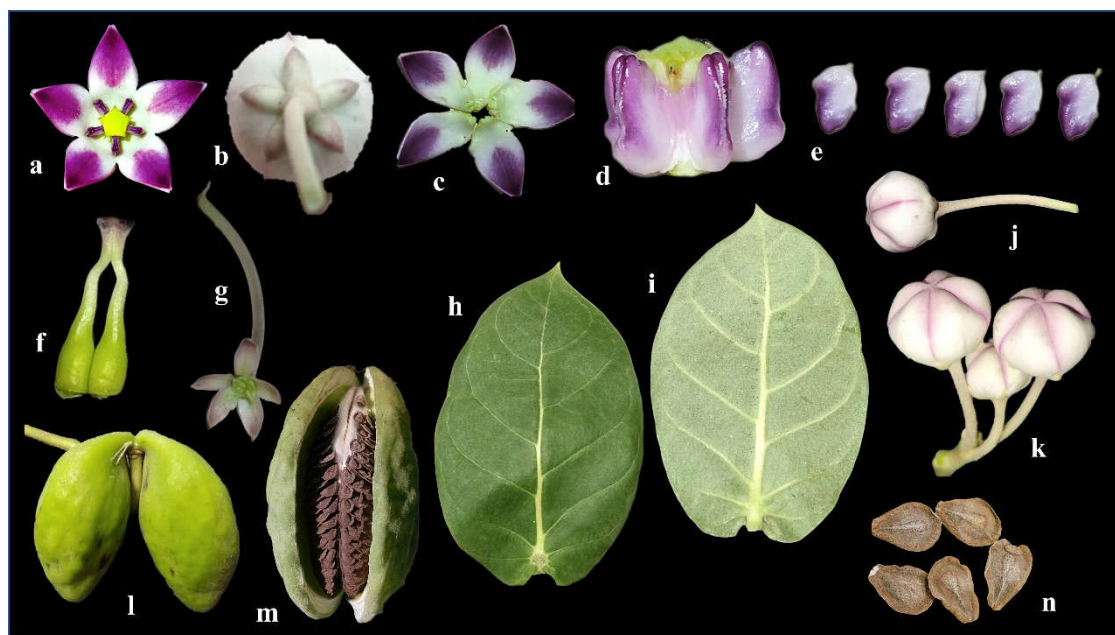


Figure 8: *Calotropis procera* – (a) ventral view of flower; (b) dorsal view of flower; (c) corolla; (d, e) corona; (f) carpel; (g) calyx with pedicel; (h) ventral view of leaf; (i) dorsal view of leaf; (j, k) flower buds; (l) fruit; (m) dispersed follicle; (n) seeds.

2. *Calotropis gigantea* (L.) Dryand.

Habit and stem:

Shrub 6–8 ft tall, profusely branched; stems green to light brown, slightly hairy (Figure 10). Latex white, watery (Figure 11).

Leaves:

Lamina elliptic to obovate, 16–17 cm long, thick and fleshy; surface smooth with waxy coating; apex blunt to slightly pointed; margin entire; petiole short but distinct (Figure 16(d, e)).

Flowers:

Larger, 3.3–3.6 cm in diameter (Figure 16(a, b),9); corolla pale greenish-white (Figure 16(c)); lobes broad and rounded; corona white (Figure 16(f, g); calyx larger than in *C. procera*; pedicels ca. 3.4–3.5 cm (Figure 16(k); buds elongated (Figure 16(l)).

Pollinia:

Larger than *C. procera*, elongated, semi-transparent, moderately attached (Figure 25).

Pollen grain morphology:

Pollen grains are comparatively larger than those of *C. procera*, mostly spheroidal to slightly ellipsoidal. Stained preparations show moderately thick pollen walls and clear cytoplasmic staining, although a small proportion of pollen grains exhibit uneven or weaker staining (Figure 15). The overall pollen population appears relatively uniform, with occasional shrivelled grains.

Gynoecium and fruit:

Carpels (bicarpellary) ca. 1.0–1.2 cm ((Figure 16(j)); follicles 6.0–9.0 cm long (Figure 16(h, i), fruiting November to April.

Leaf anatomy:

Leaf transverse section dorsiventral; epidermis smooth with reduced trichome density. Stomata anisocytic, predominantly abaxial (Figure 14). Palisade tissue well developed; spongy parenchyma broad. Vascular bundles large and numerous; xylem elements strongly lignified; phloem loosely arranged (Figure 12). Laticifers frequent and conspicuous.

Stem anatomy:

Stem transverse section circular; periderm comparatively thinner than in *C. procera*, with higher trichome density. Cortex broader. Vascular tissue robust; xylem vessels well developed and heavily lignified; phloem loosely organized (Figure 13). Laticifers abundant.



Figure 9: *Calotropis gigantea*

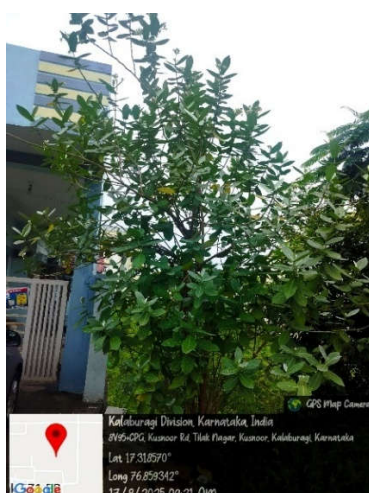


Figure 10: *Calotropis gigantea* plant

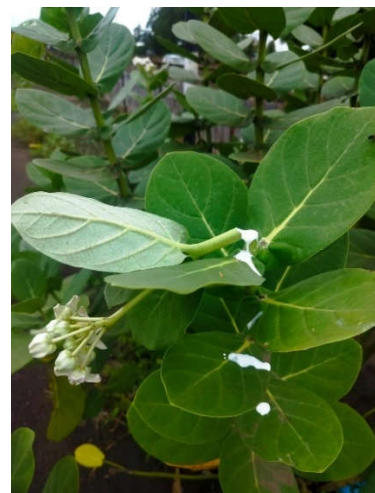


Figure 11: Latex oozing out of *Calotropis gigantea* stem

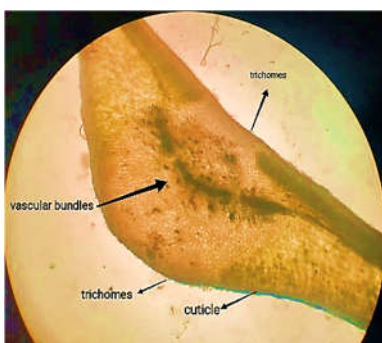


Figure 12: TS of leaf of *Calotropis gigantea*

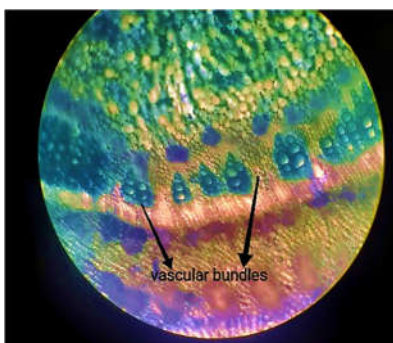


Figure 13: TS of stem of *Calotropis gigantea*

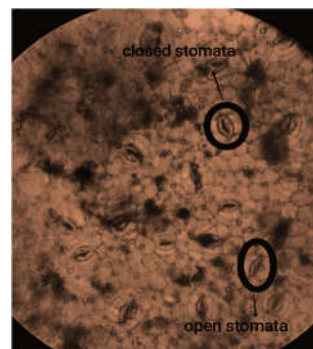


Figure 14: Stomata of *Calotropis gigantea* leaf

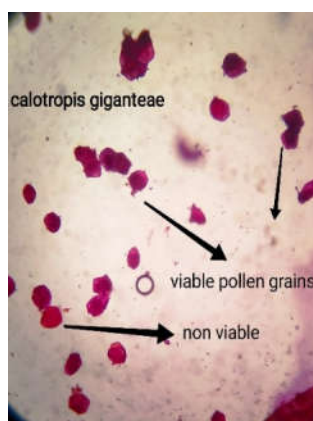


Figure 15: Pollen grains of *Calotropis gigantea*

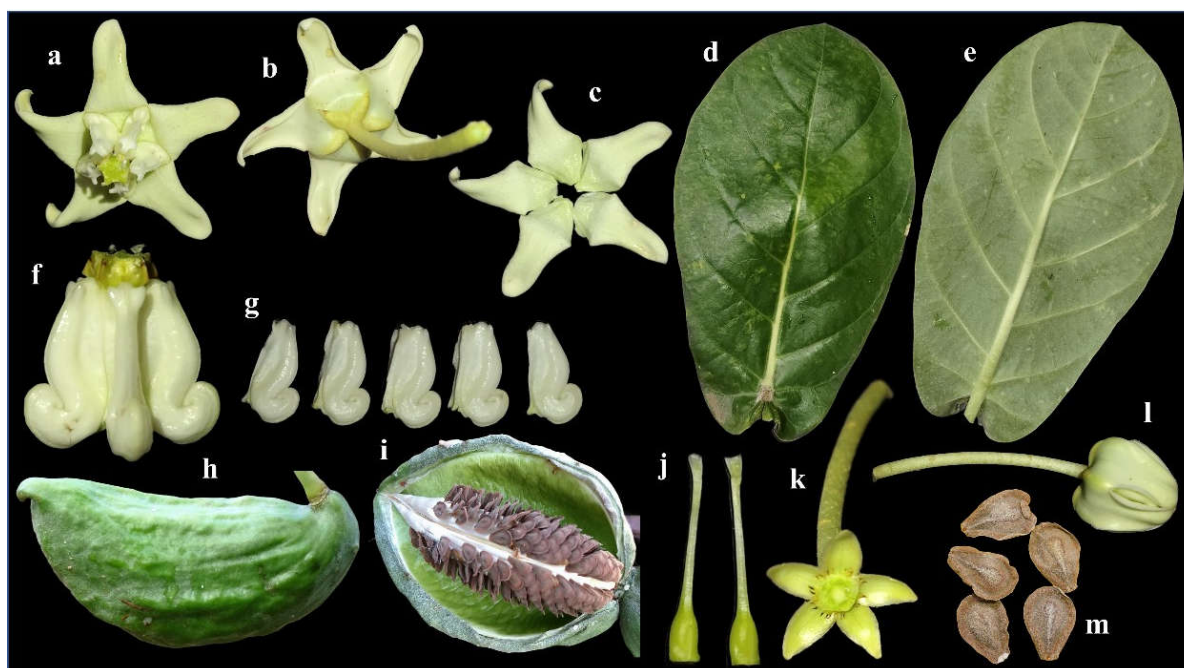


Fig. 16: *Calotropis gigantea* – (a) ventral view of flower; (b) dorsal view of flower; (c) corolla; (d) ventral view of leaf, (e) ventral view of leaf; (f, g) corona; (h) fruit; (i) dispersed follicle; (j) carpel; (k) calyx with

3. *Calotropis prefina* (provisional)

Habit and stem:

Tall shrub up to ca. 8 ft, branched; stems green to light brown, slightly pubescent (Figure 18). Latex white, intermediate in viscosity (Figure 19).

Leaves:

Lamina 11–14 cm long, elongated-elliptic, thick but softer than *C. procera*; surface slightly velvety; apex acute; margin entire; petiole extremely short (Figure 24(t, u)).

Flowers:

Largest among the three taxon, 3.5–3.8 cm in diameter; flowers uniformly purple (Figure 24(a, b),17) or uniformly pink (Figure 24(d, e),17) within the same population; corolla lobes broad; corona large, purple or pink (Figure 24(g, h, j, k)); calyx milky white to light green (Figure 24(m, o)), conspicuously large; pedicels 3.6–4.0 cm; buds elongated (Figure 24(v, w)).

Pollinia:

Largest, elongated and curved, highly transparent, loosely attached; internal pollen grains clearly visible (Figure 25).

Pollen grain morphology:

Pollen grains are the largest among the three taxon and display greater variation in size and shape, ranging from spheroidal to irregularly ellipsoidal. In stained preparations, a substantial proportion of pollen grains show weak, patchy, or absent staining, with visibly reduced cytoplasmic density (Figure 23). Collapsed and malformed pollen grains are comparatively frequent, indicating reduced pollen integrity.

Gynoecium and fruit:

Carpels (bicarpellary) ca. 1.0–1.2 cm (Figure 24(i, l)); follicles variable, 5–10 cm long (Figure 24(q, r)), fruiting from May to February.

Leaf anatomy:

Leaf transverse section dorsiventral; epidermis with dense trichomes predominantly on the abaxial surface, adaxial surface comparatively less pubescent (Figure 20). Stomata anisocytic, mainly adaxial (Figure 22). Palisade tissue moderately compact; spongy parenchyma relatively expanded. Vascular bundles collateral with numerous xylem elements showing moderate lignification; phloem dense with slight dispersion. Laticifers consistently present at moderate frequency.

Stem anatomy:

Stem transverse section circular; periderm thin with sparse trichomes. Cortex of intermediate width. Vascular tissue moderately developed; xylem vessels numerous with moderate lignification; phloem is compact to slightly dispersed. Laticifers present at moderate density (Figure 21).



Figure 17: *Calotropis prefina*



Figure 18: *Calotropis prefina* plant

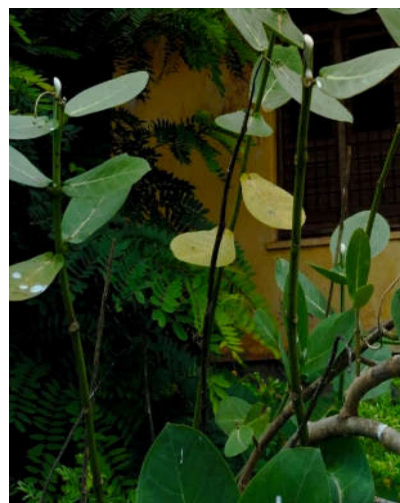


Figure 19: Latex oozing out of *Calotropis prefina* stem

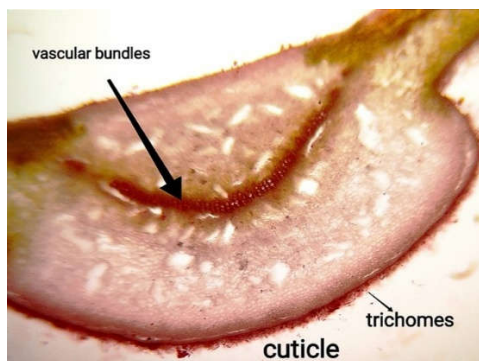


Figure 20: TS of leaf of *Calotropis prefina*

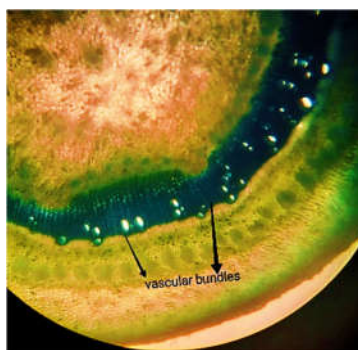


Figure 21: TS of stem of *Calotropis prefina*



Figure 22: Stomata of *Calotropis prefina* leaf

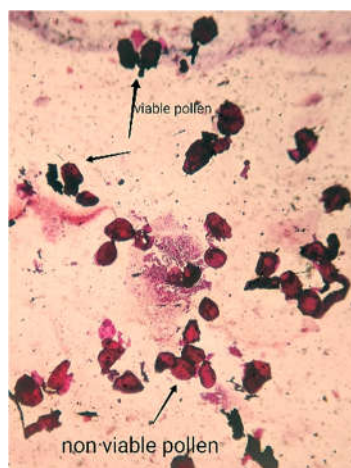


Figure 23: Pollen grains of *Calotropis prefina*

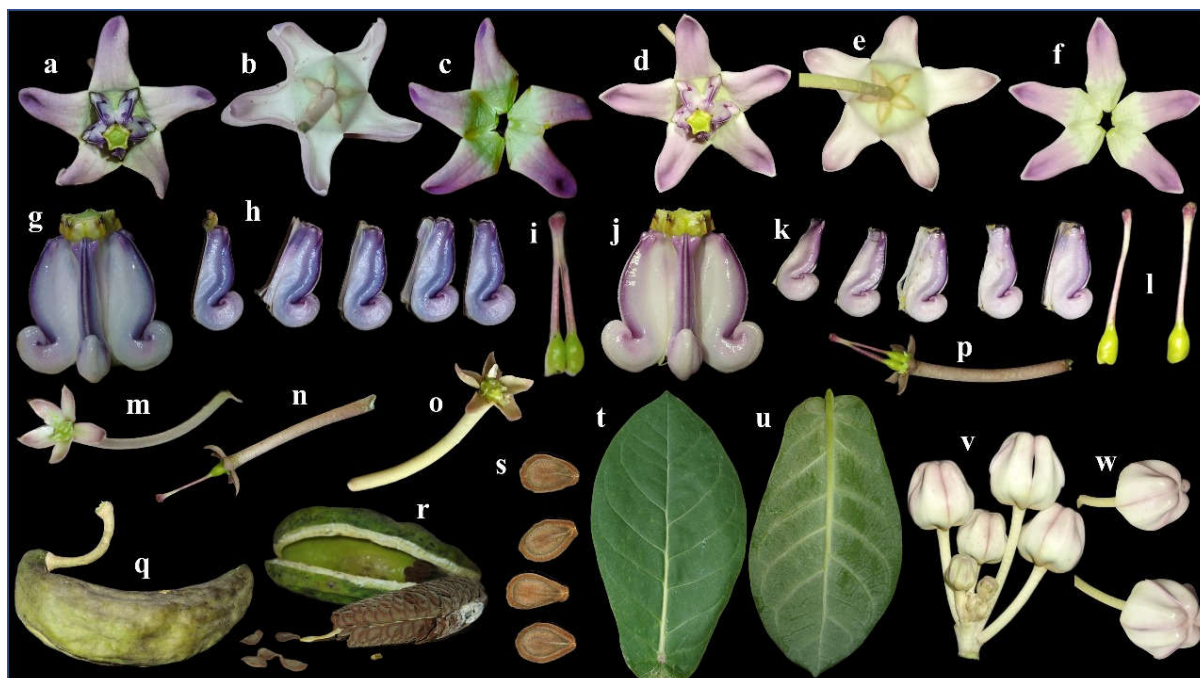


Fig. 24: *Calotropis Prefina* – (a, d) ventral view of flower; (b, e) dorsal view of flower; (c, f) corolla; (g, h, j, k) corona; (i, l) carpel; (m, o) calyx with pedicel; (n, p) carpel with flower stalk; (q) fruit; (r) dispersed follicle; (s) seeds; (t) ventral view of leaf; (u) dorsal view of leaf; (v, w) flower buds.

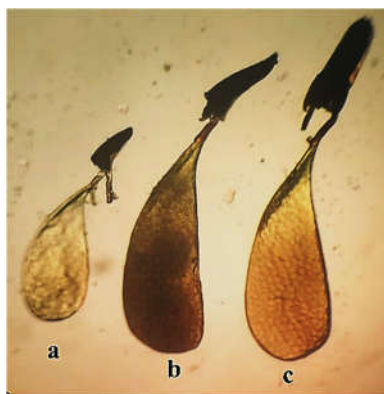


Figure 25: Microscopic view of pollinia of *Calotropis* species a) *Calotropis procera*, b) *Calotropis gigantea*, c) *Calotropis prefina*

Differential diagnosis (provisional)

The third taxon (*C. prefina*) differs from *Calotropis procera* in its taller and more branched habit, intermediate latex viscosity, larger flowers, longer pedicels, elongated and highly transparent pollinia, acute leaf apex, abaxially concentrated trichomes, and more numerous xylem elements. It differs from *Calotropis gigantea* in bearing larger flowers, uniformly purple or pink floral morphs, a conspicuously larger calyx, longer pedicels, loosely attached and highly transparent pollinia, predominantly adaxial stomatal distribution, and a distinct combination of trichome density and vascular anatomy.

Taxonomic notes

The consistent expression of diagnostic vegetative, floral, pollinia, and anatomical characters across examined individuals indicates that *C. Prefina* represents a distinct morpho-anatomical entity within *Calotropis*.

Pollen viability assessment:

***Calotropis procera*:** Pollen viability was recorded at **82.5%**, with the majority of pollen grains exhibiting strong uptake of stain, reflecting high cellular integrity.

***Calotropis gigantea*:** Pollen viability was 80%, marginally lower than in *C. procera*, with a small increase in partially stained or non-viable pollen grains.

***Calotropis prefina*:** Pollen viability was markedly lower at 57.5%, with a high proportion of pollen grains remaining unstained or poorly stained.

Chemical Screening

Milk coagulation assay

The MCA results revealed notable interspecific variation among the three taxa. *Calotropis gigantea* exhibited the strongest coagulating ability (5.714 U mL⁻¹, 35 min), forming a smooth rennet-like curd (Figure 27), followed by *C. procera* (5 U mL⁻¹, 40 min) with thick curd formation (Figure 26). *Calotropis prefina* displayed the lowest MCA (3.571 U mL⁻¹, 56 min), producing a granular curd

(Figure 28). These results indicate reduced milk-clotting enzyme activity in *C. prefina* relative to the other taxon (Table 1).

TABLE 1: Interpretation of MCA assay

Plant species.	Time taken for milk coagulation	MCA value (U/ml)	Remarks	Curd consistency
<i>Calotropis procera</i>	40 mins	5	Moderate coagulation ability	Thick curd
<i>Calotropis gigantea</i>	35 mins	5.714	Strongest coagulating ability	Smooth rennet like curd
<i>Calotropis prefina</i>	56 mins	3.5714	Lowest coagulating ability	Granular curd like



Figure 26: Coagulated milk by *Calotropis procera* latex



Figure 27: Coagulated milk by *Calotropis gigantea* latex



Figure 28: Coagulated milk by *Calotropis prefina* latex

Latex pH profile

Latex pH analysis demonstrated clear differences among species. *Calotropis prefina* showed consistently higher pH values (7.55–8.10) across temperatures when compared with *C. procera* and *C. gigantea* (Table 2). The alkaline nature and relative thermal stability of *C. prefina* latex suggest distinct biochemical behaviour.

TABLE 2: Interpretation of latex pH testing

Temperature (°C)	pH		
	<i>Calotropis procera</i>	<i>Calotropis gigantea</i>	<i>Calotropis prefina</i>
30	7.28	7.11	8.06
40	7.40	7.32	8.10
50	7.33	7.20	8.07
60	7.44	7.29	8.10

70	7.44	7.30	8.02
80	7.10	6.93	7.94
90	7.00	7.04	7.72
100	6.82	6.15	7.55

DNA Barcoding:

The first lane in the gel picture is DNA marker 100-1000 bp size standard. Remaining wells are PCR products loaded for the samples amplified using RBCL primers. Size of the amplicons generated by this primer pair is 568 bp and is also evident from the gel picture below (Figure 29).

The *rbcL* sequence generated from the investigated taxon was analysed using both NCBI BLAST and the BOLD Systems identification platform. BLAST analysis (Figure 30) retrieved *Calotropis procera* as the closest match with 98.04% - 97.86% sequence identity. Other *Calotropis* species, including *C. gigantea*, were also present among the top matches, sharing identical E-values and only marginally lower identity scores, reflecting the high level of sequence conservation within the genus which is statistically significant and not attributable to random sequence matches.

Analysis through the BOLD Systems database produced concordant results (Figure 31), returning both *C. procera* and *C. gigantea* as closest reference taxon with closely comparable percentage similarity values (97.83%) and without clear species-level discrimination. However, none of the retrieved sequences showed complete (100%) identity with *C. prefina*. This absence of perfect sequence matches is noteworthy and suggests that *C. prefina* possesses distinct nucleotide substitutions within the *rbcL* locus that differentiate it from its closest congeners. Given the conserved nature of the *rbcL* gene, even small sequence divergences are taxonomically meaningful and often reflect species-level differentiation rather than intraspecific variation. Thus, the observed divergence of approximately 2–2.2% from *C. procera* and *C. gigantea* supports the genetic distinctiveness of *C. prefina* while still confirming its close evolutionary affinity to these taxa.

The concordance between NCBI BLAST and BOLD Systems results strengthens the reliability of the molecular identification and reduces the likelihood of database-specific bias. Importantly, BOLD Systems—designed specifically for DNA barcode-based species discrimination—also failed to assign *C. prefina* unequivocally to any existing reference sequence, further reinforcing the interpretation that *C. prefina* represents a genetically distinct lineage within the genus.

Phylogenetic reconstruction based on *rbcL* sequences provides additional and compelling support for this conclusion. In the generated phylogeny, *C. prefina* clusters within the *Calotropis* clade, forming a well-supported grouping in close proximity to *C. procera* and *C. gigantea*. Nevertheless, *C. prefina* consistently occupies a distinct, intermediate branch rather than being nested within either of these species clades. This phylogenetic positioning indicates shared ancestry with *C. procera* and *C.*

gigantea while simultaneously demonstrating evolutionary divergence sufficient to justify its separation as an independent taxon.

The intermediate phylogenetic placement of *C. prefina* is particularly significant, as it suggests that the species may represent a transitional or previously under-characterized lineage within *Calotropis*. Such a pattern is consistent with scenarios of recent divergence, incomplete lineage sorting, or historical hybridization events, all of which are common within plant groups characterized by wide geographic distributions and ecological plasticity. While the conserved nature of *rbcL* limits its resolution at lower taxonomic levels, the combined evidence from sequence similarity, barcode identification, and phylogenetic topology collectively supports the recognition of *C. prefina* as a distinct species closely allied to *C. procera* and *C. gigantea*.

Overall, the molecular data convincingly demonstrate that *C. prefina* is not merely a variant of *C. procera* or *C. gigantea*, but rather a genetically distinguishable entity within the genus. These findings underscore the value of integrating DNA barcoding with phylogenetic analysis for resolving taxonomic ambiguities in morphologically similar taxon and provide a robust molecular foundation for the taxonomic recognition and further evolutionary study of *C. prefina*.

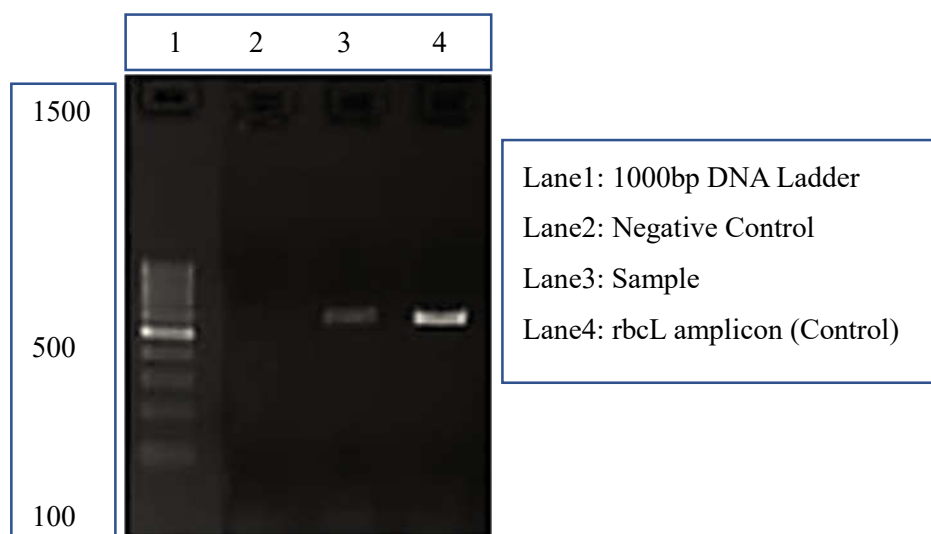


Figure 29: Gel electrophoresis of PCR product

select all 100 sequences selected

GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Calotropis procera ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chl...	Calotropis procera	976	976	99%	0.0	98.04%	651	MN229381.1
Calotropis procera chloroplast	Calotropis procera	976	976	99%	0.0	98.04%	132193	MG678914.1
Calotropis gigantea chloroplast, complete genome	Calotropis gigant...	976	976	99%	0.0	98.04%	165928	NC_041431.1
Calotropis procera chloroplast partial rbcL gene for ribulose-1,5-bisphosphate carboxylase/oxygenase large su...	Calotropis procera	976	976	99%	0.0	98.04%	1437	AJ419736.1
Balanites aegyptiaca voucher R49 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene...	Balanites aegypt...	976	976	99%	0.0	98.04%	715	KX288999.1
Calotropis procera voucher VU/AYAN/016.3 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (r...	Calotropis procera	976	976	99%	0.0	98.04%	665	OR900759.1
Calotropis procera ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chl...	Calotropis procera	976	976	99%	0.0	98.04%	701	MZ291553.1
Calotropis procera chloroplast, complete genome	Calotropis procera	976	976	99%	0.0	98.04%	166010	NC_041440.1
Calotropis procera chloroplast, complete genome	Calotropis procera	976	976	99%	0.0	98.04%	166660	PV296132.1
Calotropis procera voucher VU/AYAN/016.1 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (r...	Calotropis procera	976	976	99%	0.0	98.04%	648	OR859992.1
Calotropis procera voucher VU/AYAN/016.2 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (r...	Calotropis procera	972	972	99%	0.0	97.86%	649	OR859989.1

Figure 30: BLAST analysis result

	Phylum	Class	Order	Family	Genus	Species	Subspecies	Score	Similarity	E-Value	Status
1	Tracheophyta	Magnoliopsida	Gentianales	Apocynaceae	Calotropis	procera		483	97.83	0	Published
2	Tracheophyta	Magnoliopsida	Gentianales	Apocynaceae	Calotropis	gigantea		483	97.83	0	Private
3	Tracheophyta	Magnoliopsida	Gentianales	Apocynaceae	Calotropis	gigantea		483	97.83	0	Private
4	Tracheophyta	Magnoliopsida	Gentianales	Apocynaceae	Calotropis	gigantea		483	97.83	0	Private
5	Tracheophyta	Magnoliopsida	Gentianales	Apocynaceae	Calotropis	procera		483	97.83	0	Published
6	Tracheophyta	Magnoliopsida	Gentianales	Apocynaceae	Calotropis	procera		483	97.83	0	Published
7	Tracheophyta	Magnoliopsida	Gentianales	Apocynaceae	Calotropis	gigantea		483	97.83	0	Published
8	Tracheophyta	Magnoliopsida	Gentianales	Apocynaceae	Asclepias	incarnata		477	97.24	0	Published
9	Tracheophyta	Magnoliopsida	Gentianales	Apocynaceae	Pergularia	daemia		477	97.24	0	Published
10	Tracheophyta	Magnoliopsida	Gentianales	Apocynaceae	Cynanchum	acidum		477	97.24	0	Published
11	Tracheophyta	Magnoliopsida	Gentianales	Apocynaceae	Pergularia	daemia		477	97.24	0	Published
12	Tracheophyta	Magnoliopsida	Gentianales	Apocynaceae	Pergularia	daemia		477	97.24	0	Published

Figure 31: BOLD analysis result

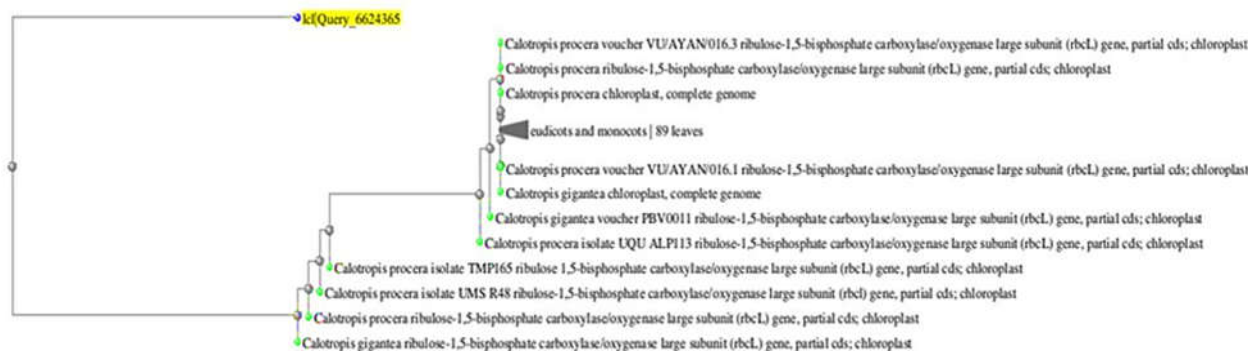


Figure 32: Phylogenetic tree from Consensus sequence of *C. prefina*

Discussion

The present study integrates morpho-anatomical, biochemical, molecular, and herbarium-based evidence to evaluate the taxonomic position of *Calotropis prefina* within the genus *Calotropis*. The convergence of multiple, independent character systems provides a robust and internally consistent framework for interpreting the distinctiveness of this taxon relative to the well-recognized species *C. procera* and *C. gigantea*.

Morpho-anatomical differentiation within *Calotropis*

Morphological and anatomical analyses demonstrate that *C. procera*, *C. gigantea*, and *C. Prefina* presents three clearly differentiated entities. The stability and congruence of diagnostic characters across vegetative, reproductive, and anatomical traits suggest that the observed variation is not environmentally induced plasticity but reflects underlying taxonomic differentiation.

Vegetative traits—including plant stature, branching architecture, stem pubescence, and latex viscosity—have long been recognized as informative characters in distinguishing *C. procera* and *C. gigantea* (Rahman & Wilcock 1991; Joshi et al. 2010). *C. Prefina* exhibits a consistent variable expression of several of these characters, particularly plant height and latex viscosity, yet does not fully correspond to either species. Importantly, these traits were stable across examined individuals, indicating a coherent phenotypic pattern rather than transitional or aberrant forms.

Floral morphology and pollinia characteristics provide especially strong taxonomic signals within Asclepiadoideae (Swarupanandan & Sreedevi 1996; Maity et al. 2019). The markedly larger flowers, longer pedicels, and distinct pollinia morphology—characterized by increased length and pronounced transparency—clearly differentiate *C. Prefina* from both *C. procera* and *C. gigantea*. Pollinia traits are generally conservative at the species level, and differences in transparency and attachment structures are unlikely to arise from environmental variation alone, thereby providing compelling evidence of taxonomic separation.

Reduced pollen viability in the investigated population relative to both congeners provides additional evidence of reproductive divergence, which is particularly significant given the generally conserved reproductive structures within Asclepiadoideae

Anatomical observations from leaf and stem transverse sections further reinforce the morphological findings. Variations in trichome distribution, stomatal positioning, xylem lignification, and phloem organization have been widely employed in comparative anatomical studies of *Calotropis* and related genera (Metcalf & Chalk 1957; Abeyasinghe & Scharaschkin 2022). The anatomical profile of *C. Prefina*—particularly the predominance of adaxial stomata combined with moderately lignified but numerically abundant xylem elements—does not fall within the documented range of either *C. procera* or *C. gigantea*, supporting its structural distinctiveness.

Biochemical evidence of functional divergence

Biochemical analyses provide an additional and independent line of evidence. The reduced milk coagulation activity observed in *C. prefina* suggests functional divergence in latex protease activity, consistent with earlier reports demonstrating interspecific variation in proteolytic enzyme profiles among *Calotropis* taxon (Seiber et al. 1982; Rajagopalan & Sivasubramanian 2019). Furthermore, the consistently alkaline and thermally stable latex pH recorded for *C. prefina* represents a reproducible biochemical trait distinguishing it from *C. procera* and *C. gigantea*. Latex chemistry is known to be under genetic control and has been shown to carry taxonomic signal in laticiferous plant groups, lending additional weight to these observations.

Molecular affinity and genetic differentiation

Molecular analysis using the chloroplast *rbcL* barcode region revealed close affinity between *C. prefina* and other members of *Calotropis*, particularly *C. procera* and *C. gigantea*. However, the observed 2–3% sequence divergence, coupled with the absence of complete sequence identity in both NCBI BLAST and BOLD Systems databases, suggests genetic differentiation rather than simple intraspecific variation. Given the conserved nature of *rbcL*, such levels of divergence are notable and have been reported in barcoding studies resolving closely allied plant taxon (Amin et al. 2020; Sidhu et al. 2023).

Phylogenetic reconstruction further supports this interpretation, with *C. prefina* clustering within the *Calotropis* clade while occupying a distinct and intermediate branch relative to *C. procera* and *C. gigantea*. Although *rbcL* alone does not provide fine-scale resolution, the congruence between sequence divergence, barcode identification, and phylogenetic topology reinforces the inference of a genetically differentiated lineage.

Synthesis and limitations

Taken together, the morpho-anatomical, biochemical, molecular, and herbarium-based evidence presents a coherent and convergent picture of differentiation within the *Calotropis* complex. Rather than relying on a single dataset, the present study demonstrates that multiple independent characters consistently distinguish *C. prefina* from *C. procera* and *C. gigantea*, supporting its treatment as a distinct and well-defined taxonomic entity.

It is acknowledged that the present investigation is primarily descriptive and does not incorporate multi-locus molecular phylogenetics, population-level analyses, or ecological experimentation. Nevertheless, classical morphology, anatomy, and biochemical profiling remain foundational components of plant systematics, particularly when supported by molecular barcoding and herbarium authentication. The integrative approach adopted here provides a strong framework for future studies employing additional molecular markers and broader sampling to further refine evolutionary relationships within *Calotropis*.

Conclusion

This study represents a comprehensive and integrative taxonomic assessment of *Calotropis* populations from northern Karnataka, India, employing coordinated morpho-anatomical, biochemical, molecular, and herbarium-based approaches. While *Calotropis procera* and *C. gigantea* confirm to their established taxonomic circumscription, a third population, provisionally referred to as *Calotropis prefina*, consistently exhibits a distinct and stable combination of vegetative, reproductive, anatomical, biochemical, and molecular traits that sets it apart from both recognized species.

Morpho-anatomical differentiation—particularly in floral architecture, pollinia morphology, pollen viability, and internal stem and leaf anatomy—reveals patterns of character stability that are unlikely to be attributable to phenotypic plasticity. These structural distinctions are complemented by reproducible biochemical differences in latex properties, indicating underlying functional divergence.

Molecular evidence from *rbcL* DNA barcoding further supports this pattern by demonstrating close phylogenetic affinity to *C. procera* and *C. gigantea* while simultaneously revealing consistent sequence divergence and an independent phylogenetic placement.

Importantly, the strength of the present study lies in the convergence of multiple, independent datasets, each of which alone provides partial resolution, but together establish a coherent and robust signal of taxonomic differentiation within the *Calotropis* complex. By integrating classical systematic approaches with molecular and biochemical data and anchoring observations to authenticated herbarium material, this work substantially advances the understanding of species boundaries in a morphologically challenging genus.

Although no formal taxonomic act is proposed here, the evidence presented provides a compelling and well-substantiated foundation for future nomenclatural resolution. The study underscores the continued relevance of integrative taxonomy and highlights the importance of regional-scale systematic investigations in revealing previously under-characterized diversity within widely distributed plant genera.

Supplementary information: about herbarium

<http://flora-peninsula-indica.ces.iisc.ac.in/karnataka/>.

<http://florida.plantatlas.usf.edu/>

<https://www.nbuherbarium.in/xmlrpc.php>

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Author Contributions

Afina Afseen performed, Conceptualization, Methodology, Experimental Work, Data Collection, Data Analysis, Imaging, Manuscript Drafting. Preeti Thakur performed, Experimental Work, Data Collection, Literature Review, Data Curation, Assistance in Manuscript Preparation. Dr. Prithviraj B (Co-guide) performed Supervision, Validation, Technical Guidance, Critical Review and Editing of Manuscript. Dr. Tathagat W (Co-guide) performed Supervision, Resources, Validation, Support in Experimental Design, Critical Review of Manuscript. Prof. Chandrakant K (Guide) performed Conceptualization, Project Administration, Overall Supervision, Final Review and Approval of Manuscript.

Data availability

All data supporting the findings of this study are already included within the article and its supplementary materials.

Declarations

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Conflicts of interest

The authors declare no conflict of interest.

Declaration of generative AI

AI-assisted technologies in the writing process for language improvement only.

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