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REGULAR ARTICLE

SYSTEMATIC STUDIES IN HERBACEOUS *PHYLLANTHUS* SPP. (REGION: TIRUCHIRAPPALLI DISTRICT IN INDIA) AND A SIMPLE KEY TO AUTHENTICATE 'BHUMYAMALAKI' COMPLEX MEMBERS

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SUMMARY

The taxonomic status of Phyllanthaceae and current systematic position is highlighted. In India, 12 herbaceous species of *Phyllanthus* have been identified and among them herbs referred to as 'Bhumyamalaki' complex has been extensively used as traditional medicine for various ailments. The medicinally important herb in this group is *P. amarus* Schum. & Thonn. and is often adulterated with its allied species and hence a simple key to differentiate them is evolved as confusion exists in identification of these herbaceous species due to their similarity and close proximity. *P. niruri* L. is a native of New World and endemic to America and does not occur in India, although there are many publications in India claiming work on *P. niruri* L. Those reports are actually pertaining to investigations in 'niruri complex' but not on *P. niruri* L. Herbaceous *Phyllanthus* species found in Tiruchirappalli district has been recorded and their morphological and anatomical parameters were assessed and a simple key developed. SCAR Analysis for validating the identity of *P. amarus* is presented for authentication at molecular level. *P. debilis*, a coastal zone species is first reported from an inland area.

Key words: *Phyllanthus amarus*, *Phyllanthus debilis*, *Phyllanthus maderaspatensis*, *Phyllanthus virgatus*, *Phyllanthus fraternus*, *Phyllanthus urinaria*, Phyllanthaceae, Morphology, Taxonomic key, Herbal Authentication

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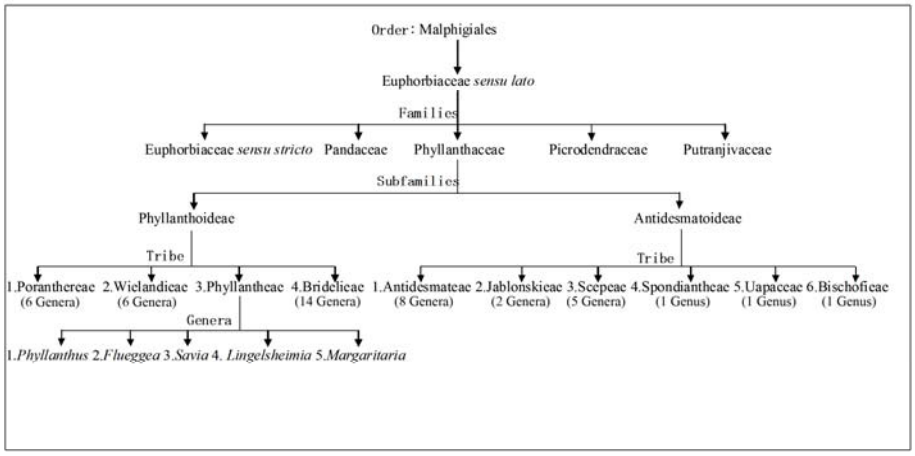
1. Introduction

Systematic studies elucidating deep level angiosperm relationships is leaping fast from the past decade due to rapid advancements in new phylogenetic informations. The eudicots clade includes 75% of all angiosperm species (remaining 25% in monocotyledons) and comprises several major subclades like rosids, asterids, saxifragales, santalales and caryophyllales (Judd and Olmstead, 2004; Soltis *et al*., 2005; APG II, 2003). Rosids contain 140 families (70,000 species) and comprises three important groups: Viz., Vitaceae, Eurosids I and Eurosids II (Cantino, 2007; Endress and Matthews, 2006). Eurosids I is known as Fabidae and includes Rosales, Fabales, Cucurbitales, Fagales, Zygophyllales and COM group (Cantino, 2007). COM group consists of the orders Celastrales, Oxalidales and Malphigiales. Malphigiales is one of the largest orders of flowering plants, containing 39 families, 716 genera and 15935 species which are approximately about 7.8% of the eudicots. The order is very diverse and hard to recognize except with molecular phylogenetic evidence. Molecular clock calculations estimate the origin of stem group Malphigiales at around 100 Mya (million years ago) and the origin of crown group Malphigiales at about 90 Mya

(Wanga *et al*., 2009). Euphorbiaceae *sensu lato* is the largest among Malphigiales and were defined by a few shared morphological characters including unisexual flowers with superior syncarpous ovaries, apical-axile placentation and one or two epitropous ovules per locule. Characters that are usually present include alternate, stipulate leaves, actinomorphic flowers, and presence of a floral disc, pistillode and obturator, as well as a tricarpellate, explosively dehiscing schizocarp leaving a central columella.

The recent classification of angiosperms recognises five lineages of *Euphorbiaceae sensu lato* (Figure 1) at family rank: *Euphorbiaceae sensu stricto*, *Pandaceae* Engl. & Gilg, *Phyllanthaceae* Martynov, *Picrodendraceae* Small and *Putranjivaceae* (APG III, 2009). Phyllanthaceae is the second largest segregate of Euphorbiaceae *sensu lato*, comprising c. 2000 species in 49 genera. Phyllanthaceae includes two subfamilies, Phyllanthoideae and Antidesmatoideae. Phyllanthoideae consists of four tribes and *Phyllanthus* belongs to the tribe Phyllantheae. Members of Phyllanthaceae are pantropical and include trees, shrubs, phyllocladous taxa, semi – succulents, annual herbs, and even a free floating species. Vegetatively, most Phyllanthaceae are uniform with pinnate venation, entire margins and simple indumentums. Flowers are small and actinomorphic but display great diversity in shape, size and number of floral organs (Hoffmann *et al*., 2006; Kathriarachchi *et al*., 2006).

Fig. 1: Phylogenetic classification of Phyllanthaceae (Hoffman P, Kathriarachchi H and Wurdack K J, 2006)



The genus *Phyllanthus* L. was first described by Linnaeus in 1753 and consists of *ca*.833 species (Govaerts *et al*., 2000) in the world and is chiefly distributed in moist humid tropics. In India, it is represented by *ca*. 40 species (Henry & Santapau, 1973), although Hooker f. (1887) has recorded 56 species from the then British India. In total, 12 species of herbaceous *Phyllanthus* have been identified in India. They are used since ancient times in different systems of medicine, particularly for the treatment of liver disorders and urinary infection. The herbs known as 'Bhumyamalaki' in Indian literature refer to a complex group of *Phyllanthus amarus* Schum. & Thonn., *Phyllanthus fraternus* Webster, *Phyllanthus debilis* Klein *ex* Wild and *Phyllanthus urinaria* L. (Chaudhary and Rao, 2002). Although these species closely resemble each other they also show sufficient characters to maintain them as distinct species. However, ethnomedical uses and some aspects of pharmacological activities among these species are different (Theerakulpisut *et al*., 2008). Confusion exists in identification of these herbaceous species mainly due to referring them all with a common vernacular name, their similarity in gross morphology, close proximity in growth habitat and lack of guideline legal system to

check the authenticity and quality of the medicinal plant sold.

Webster (1955, 1956a, b, 1957, 1958, 1967, 1970 and 1994; Webster and AiryShaw, 1971) has worked exhaustively on *Phyllanthus* and has provided detailed taxonomic accounts of West Indian *Phyllanthus*. He observed that true *P. niruri* L. is a native of New World and endemic to America and does not occur in India. But there are many publications published even today from India bearing title on *P. niruri* L. In 1985, Mitra and Jain, after critical examination of Indian materials of *Phyllanthus* revealed that the *Phyllanthus niruri* L. described in Flora of British India (Hooker f, 1887) is actually a mixture of three closely related but distinct species namely *P. amarus* Schum. & Thonn., *P. debilis* Klein *ex* Wild and *P. fraternus* Webster. Those reports done in India on *P. niruri* L. are actually pertaining to investigations in any one member of this 'niruri complex' but not on *P. niruri* L. The present study was conducted to investigate the diversity of *Phyllanthus* spp. found in Tiruchirappalli district of Tamilnadu, India and attempt to resolve the nomenclatural problem persisting in this genus by analysing the morphological and anatomical characters of these plants and evolve a simple key for easy identification of the related species. Through distinct morphological features, the identity of *P. amarus* can be confirmed but as an additional proof the validity of Sequence Characterised Amplified Regions (SCAR) markers developed earlier was assessed (Jain *et al*., 2008). PCR-based methods, including Random Amplified Polymorphic DNA (RAPD), have been used for authentication of medicinal plant materials. However, RAPD markers are difficult to reproduce and are therefore preferentially converted to more specific SCAR markers. For building a SCAR primer, a RAPD gel has to be run first using random primers and then the unique bands have to be isolated, cloned, sequenced and developed as SCAR primers useful for unique identification of that species. Use of SCAR marker for identification of *P. emblica* L. in its commercial samples and in Triphala churna, a multi-component ayurvedic formulation has been done earlier (Dnyaneshwar *et al*., 2006).

2. Materials and Methods

Tiruchirappalli district is centrally located in the state of Tamilnadu, India with a total geographic extent of 4404.12 sq. Km. It lies between 78° 10' to 79°5' east longitude and 10°15' and 11°2' north latitude. It lies at an altitude of 78 m above sea level. It falls under the Cauvery Delta agroclimatic zone. The annual mean maximum temperature is 37.7^{o}C and the annual mean minimum temperature is 18.9°C. The annual total rainfall is 778 mm. The study area was thoroughly surveyed for the presence of *Phyllanthus* spp. throughout the year from time to time to observe the seasonal variation in diversity (if any). They were identified referring to standard floras (Mathew, 1982, 1983a, b and c, 1988; Gamble and Fischer, 1915-1936; Nair and Henry, 1983; Henry *et al*., 1987; Henry *et al*., 1989).

For SCAR analysis fresh leaf samples (young leaves) collected from the study areas in Tiruchirappalli district were used for the isolation of genomic DNA (Sharma *et al*., 2003). Forward and Reverse primers were designed (Jain *et al*., 2008) and were synthesized by Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore. The PCR reaction mix (25 µl) used in the experiment was: DNA template - 1.72 µl (25 ng); dNTP mix - 0.5 µl (200 µM each); Taq DNA polymerase - 0.5 µl (0.2 unit); PCR buffer - 2.5 µl; Forward Primer - 1 µl (10 pmol); Reverse Primer - 1 µl (10 pmol) and Sterile D. H2O - 17.78 µl. The PCR was carried out using a DNA engine thermocycler (Eppendorf Pro-S gradient cyler) programmed for the initial denaturation at 94^{o}C for 5 min and 40 cycles of 94°C for 1 min, 38^{o}C for 1 min and 72^{o}C for 2 min, with a final Extension of 72^{o}C for 5 min. The amplified products were separated on 1.8% agarose gel containing 0.5 µg / ml of ethidium bromide and photographed with Alphainnotech alphaimager (USA). The profile was analysed using the Alphaview for Alphaimager systems (version 1.2.0.1) software.

Voucher specimens were deposited in the Department Herbarium, Department of Biotechnology, Bharathidasan University for future reference. For anatomical details of

the species, Cross Sections (C.S.) were obtained free hand with sectioning blades, then stained in safranine and mounted in glycerine. Semi permanent slides, so prepared, were examined under Nikon PFX microscope. The photography was made with the help of Labomed CXR3, LaboAmerica, Inc., US, Compound microscope with camera. For external features Motic SMZ – 140, China, Stereo Zoom microscope with DM -143 camera was used.

3. Results

Four herbaceous species of *Phyllanthus* were found in the study area and are *P. amarus* Schum. & Thonn., *P. debilis* Klein *ex* Willd., *P. maderaspatensis* L. and *P. virgatus* Forster f (Figure 2). The SCAR profile showed the characteristic 1150 bp band confirming the identity of *P. amarus* and additional bands (475 bp, 400bp, 250 bp, 100bp and 50 bp) were also produced (Figure 3). Anatomical comparison between the species and Morphological distinctions of the species revealed the prominent differences (Figures 2, 4 and 5). The morphological and anatomical distinctions were listed (Table 1).

Table 1: Morphological and Anatomical characters of herbaceous *Phyllanthus* spp. seen in Tiruchirappalli District

Character	*P. amarus* Schum. & Thonn.	*P. debilis* Klein *ex* Wild	*P. maderaspatensis* L.	*P. virgatus* Forster f.
Vegetative Characters				
Stem shape	Terete	Angular	Angular	Angular
Stem surface	Hispidulous	Glabrous	Glabrous	Glabrous
Cataphyll	Three. Triangular-lanceolate; acuminate and turn black at maturity	Three. Narrowly lanceolate and acuminate	Absent	Absent
Branchlet	Present	Present	Absent	Absent
Leaf shape	Oblong	Narrowly elliptic in upper part and cuneate at base	Spathulate	Oblong-elliptic
Leaf tip	Rounded	Acute	Apiculate	Obtuse
Reproductive Characters				
Tepals	Five	Six	Six	Six
Disc segments	Five	Six	Six	Six
Stamens	Three	Three	Three	Three
Filaments	Completely connate	Connate at the base and free at tip	Completely connate	Free
Style	Three. Minutely bifid at tip	Three. Bifid about to the middle. Appressed to the ovary	Three. Minutely bifid at tip. Free	Three. Bilobed at apex. Free
Female Disc	Star shaped	Irregular	Rectangular	Orbicular
Capsule Surface	Slightly warty	Smooth	Smooth	Tubercled
Ovary Chambers	Trigonous	Trigonous	Trigonous	Trigonous
Ovules	Six	Six	Six	Six
Placentation	Axile	Axile	Axile	Axile
Anatomical Characters				
Stem hairs	Present	Absent	Absent	Absent
Shape	Circular	Angular (pentagonal) with protruded bulges at the angles	Angular (pentagonal) with bulges at the angles	Circular with protrusion at the poles
Epidermis	Present	Present	Present	Present
Hypodermis	Outer 2-3 layers of collenchyma and inner 2-3 layers of chlorenchyma	4-5 layers of chlorenchyma	4 – 5 layers of chlorenchyma	3-4 layers of chlorenchyma
Vascular Bundles	Conjoint, Collateral, Open and Endarch. Arranged in a ring	Conjoint, Collateral, Open and Endarch. Arranged in a ring	Conjoint, Collateral, Open and Endarch. Arranged in a ring	Conjoint, Collateral, Open and Endarch. Arranged in a ring
Pith	Large, central and parenchymatous	Large, central and parenchymatous	Large, central and parenchymatous	Small, centered and parenchymatous
Branchlets	Epidermis, 2-3 layers of chlorenchyma and two conjoint, collateral, open vascular bundles	Epidermis, 2-3 layers of chlorenchyma and two conjoint, collateral, open vascular bundles	Absent	Absent

As evident from the table, *P. maderaspatensis* and *P. virgatus* can be easily distinguished from other members of *Phyllanthus* by virtue of their unique characters like leaf shape, absence of cataphylls, absence of branchlets, etc. The capsule of *P. amarus* Schum. & Thonn. has been reported to be smooth hither to (Chaudhary and Rao, 2002) but the observation made in this study has revealed its minutely warty surface nature. *P. debilis* Klein *ex* Wild originally thought to be confined to coastal belts (Chaudhary and Rao, 2002; Mitra and Jain, 1985) are identified in some parts of the study area (inland) in rich distribution. *P. debilis* Klein *ex* Wild collected from the study area showed characters such as leaf margins glabrous, female calyx lobes subequal, staminal filaments connate at base and slightly free at tip, styles distinctly bifid at tip, female disk irregularly lobed, etc. to confirm its identity (Figure 4). Mathew (1983) reported the distribution of *P. debilis* Klein *ex* Wild in hills (900 – 1200 m), fallow fields and river banks. But Chaudhary and Rao (2002) and Mitra and Jain, 1985 in their monographs on taxonomic study of herbaceous *Phyllanthus* species recorded its distribution only in coastal regions of West Bengal, Orissa, Maharashtra, Andhra Pradesh, Karnataka, Kerala, Tamil Nadu and Andaman and Nicobar islands. However, here we report its luxuriant distribution in Sathamangalam Revenue Village of Tiruchirappalli district, which is a plain area and far from sea-shore. Based on the morphological characters observed, a simple and easy to follow dichotomous key is presented below to distinguish *P. amarus* Schum. & Thonn. from *P. debilis* Klein *ex* Wild.

Key for distinguishing *P. amarus* Schum. & Thonn. and *P. Debilis* Klein *ex* Wild

1a. Terete stem with hispidulous surface, Cataphyll turn black at maturity, leaf shape oblong with rounded apex, Five tepals, Stamen completely connate into a column, Style minutely bifid at tip and not appressed to the ovary, star shaped female disc and slightly warty capsule……...*P. amarus* 1.

1b. Angular stem with glabrous surface, Cataphyll remain green, leaf shape narrowly elliptic in upper part and cuneate at base with acute apex, Six tepals, Stamen connate at the based and free at tip, Style bifid about to the middle and appressed to the ovary , irregularly lobed female disc and smooth capsule……..*P. debilis* 2.

In *P. debilis* Klein *ex* Wild the proximal axils bear male flowers and distal axils bear female flowers but in *P. amarus* Schum. & Thonn. both male and female flowers occurring together has been observed. This floral character can also serve as additional parameter for differentiating these two species, but, the male flower distribution along the axils is reported as varying over geographical area (Chaudhary and Rao, 2002) and hence not included in the key. The elaborate characters described in earlier works (Chaudhary and Rao, 2002; Mathew, 1983; Mitra and Jain, 1985; Bagchi *et al.*, 1992) help one to identify the group but characters with sharp variation and distinction aiding easy examination are short-listed and presented as a simple dichotomous key here to correctly distinguish members of the 'Bhumyamalaki' complex:

1. a. Tepals 5, Stem terete throughout……..*P. amarus* 1.

b. Tepals 6, Stem angular throughout or angular above……...2

2. a. Leaves hispidulous along margins, female flower in lower axils and 1-3 male flowers in upper axils ……*P. urinaria* 2.

b. Leaves glabrous along margins, proximal few nodes of branchlets with 2-4 male flowers, succeeding distal nodes with solitary female flower…...…….3

3. a. Leaves with acute tip, Staminal filaments slightly free at tip, styles distinctly bifid at tip, female tepals sub equal and female disk almost rounded or irregularly lobed, styles appressed and spreading ……*P. debilis* 3.

b. Leaves with obtuse tip, staminal filaments completely connate into a column, styles minutely bifid at tip, female tepals unequal in size and female disk deeply lacinate, styles erect and spreading……*P. fraternus* 4.

The characters mentioned in the key are arranged sequentially based on their importance and not like the usual vegetative to reproductive character order.

Fig.2 Habit and Uprooted view

Fig. 3: SCAR profile of *Phyllanthus amarus* Schum. & Thonn. collected from three study areas in Tiruchirappalli district

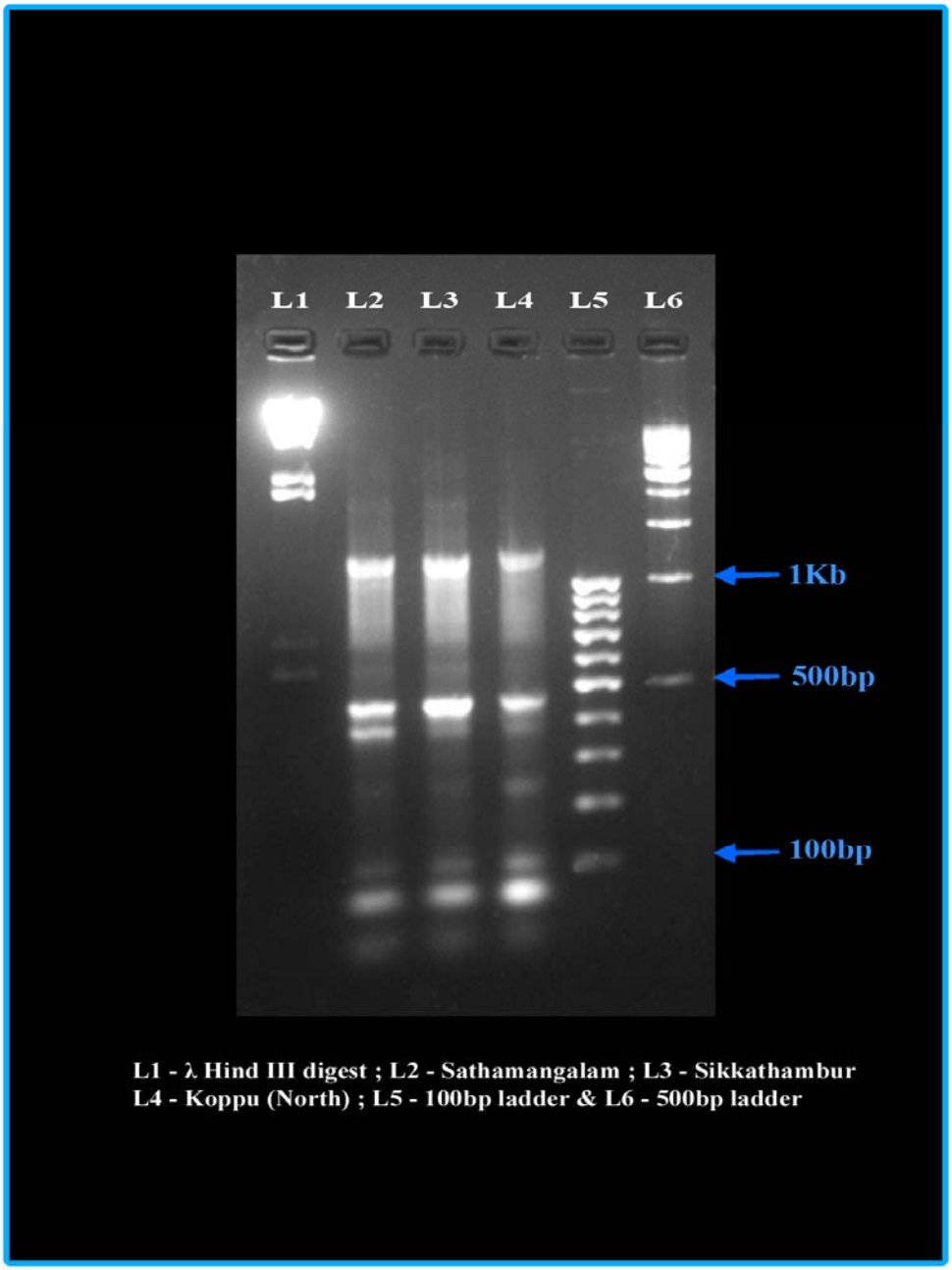


Fig. 4: Stereo zoom macroscopic view of some vegetative and reproductive parts of *Phyllanthu*s spp. Studied

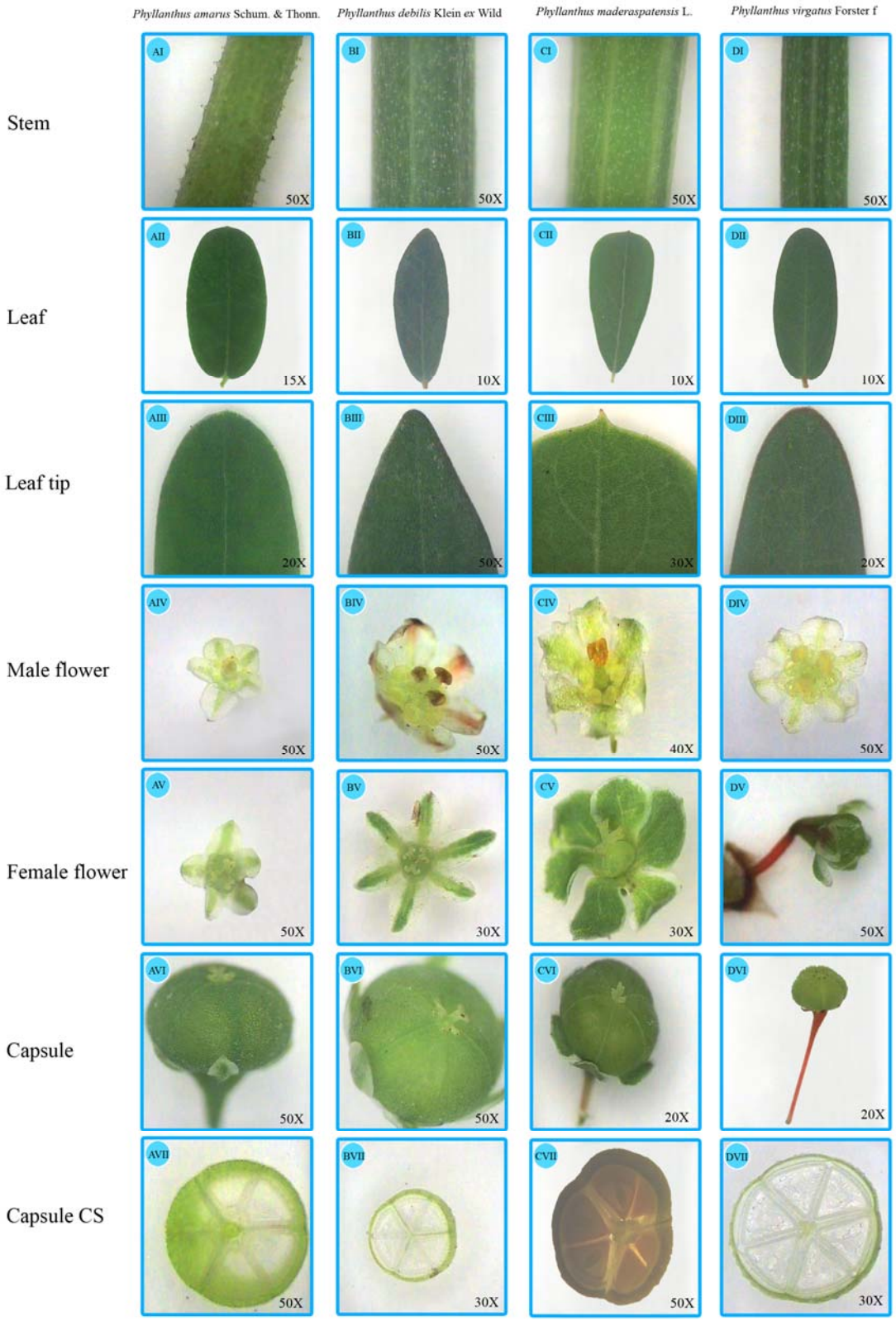
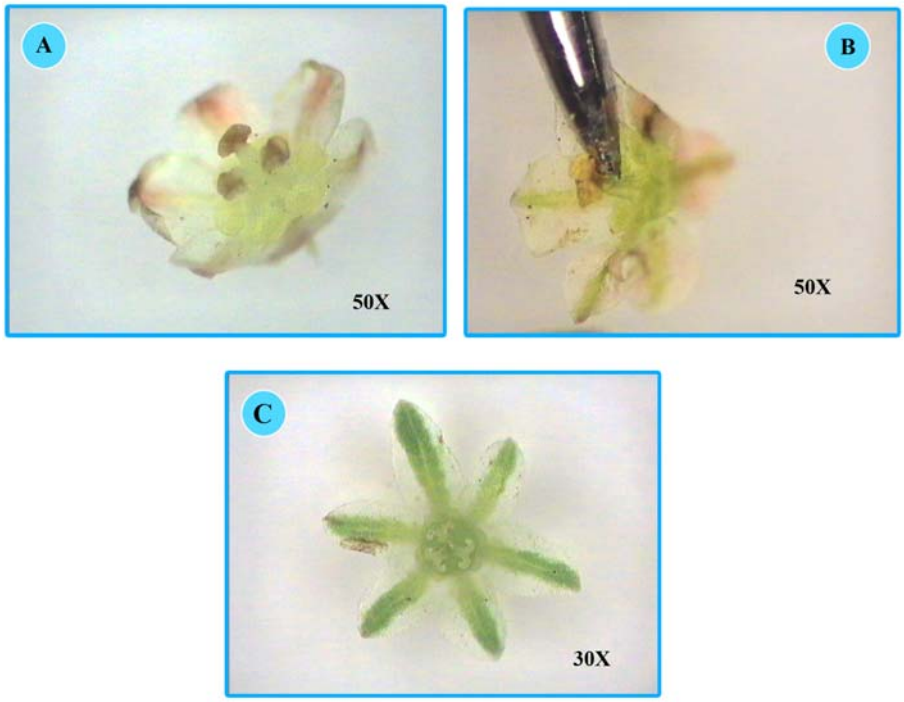


Fig. 5: Distinguishing features of *Phyllanthus debilis* Klein *ex* Wild observed



A – Staminal filaments free at tip
B – Staminal filaments connate at base
C – Subequal tepals, distinctly bifid styles which are appressed and spreading and irregularly lobed female disk are seen

4. Discussion

A study by National Medicinal Plants Board (NMPB), Government of India (Ved, D. K. and G. S. Goraya, 2008) has revealed the total number of medicinal plants traded in India as 960. Among them, 178 species fall under high volume trade or consumption category (>100 Million Tonnes/Year). *P. amarus* Schum. & Thonn. is one among them and a prioritized medicinal plant of National Medicinal Plants Board (NMPB) for high demand in the domestic and international markets for cultivation, conservation and development. It has become common to add admixtures with morphologically allied and geographically co-occurring species to raw drugs (Srirama *et al*., 2010; Bisset, 1984; Khatoon *et al*., 2006; Mitra and Kannan,2007; Nair *et al*., 1983; Sunita, 1992; Ved and Goraya, 2008). The annual volume of *Phyllanthus* trade in India is about 2000–5000 meteric tonnes (Ved and Goraya, 2008). Almost all of this is sourced from the wild or natural populations of the species (Kuipers, 2003; Ved and Goraya,2008). However due to a high level of morphological similarity among the mentioned species (Chaudhary and Rao, 2002; Ganeshaiah *et al*., 1998) raw drug samples often contain species admixtures (Dymock, 1883; Dymock *et al*., 1893; Kirtikar and Basu, 1975; Nadkarni, 1954; van Rhede, 1690). Khatoon *et al. (*2006) showed that the three species of *Phyllanthus* (*P. amarus*, *P. fraternus* and *P. maderaspatensis*) that are often mixed together have significantly different phytochemistry and only *P. amarus* was found to contain phyllanthin and hypophyllanthin, the two major compounds believed to be responsible for the hepato protective activity (Calixto *et al*., 1998). In Tiruchirappalli district *P. debilis* Klein *ex* Wild is often misidentified as *P. amarus*

Schum. & Thonn. Local herbal vendors and collectors often sell this plant in shandies with the Tamil Vernacular name 'keezhanelli'. Plant collectors pay little attention to proper identification of the plants for sale. This lack of accuracy often leads to an inadequate linkage of the trade data with the specific plant species though many of these species may be of high conservation concern. The SCAR profile obtained here is slightly different from the earlier work (Jain *et al*., 2008). The profile obtained by them showed only a single band of 1150 bp length. But, in this investigation along with the characteristic 1150 bp band, additional bands (475 bp, 400bp, 250 bp, 100bp and 50 bp) were produced. This may be due to difference in PCR stringency levels. Eventhough SCAR markers (Jain *et al*., 2008) and DNA barcodes (Srirama *et al*., 2010) are useful for distinguishing the *Phyllanthus* species, they are beyond the reach of a local herb collector. Hence, we believe simple morphological and anatomical character based keys for easy identification of herbs existing along with closely related allied species should be attempted with. Most of the time the taxonomic keys are prepared exhaustively and serves the purpose of scientific purposes and so keys for layman with minimal technical details should either be developed or abridged from elaborate ones. The key presented here to a large extent can be examined with naked eye and does not need any DNA marker or laboratory. *P. amarus* can be easily distinguished from its allies by its unique characteristics of terete stem and five tepals.

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