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Research Article

Authentication of *Harpullia arborea* (Blanco) Radlk. - a traditional medicinal plant from India using microscopic techniques

Vigi George M*
Department of Botany, C M S College Kottayam (Autonomous), Kottayam - 686001 (Kerala), India
Hari N
Department of Botany, C M S College Kottayam (Autonomous), Kottayam - 686001 (Kerala), India

*Corresponding author. Email: vigi@cmscollege.ac.in

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Abstract
Harpullia arborea (Blanco) Radlk., Sapindaceae, known as “black doll’s eyes” or “tulip tree” is a rainforest tree distributed in the tropical forests of peninsular India. Out of the 26 species of the genus, *H. arborea* is therapeutically more studied and widely used in folk and ayurvedic medicine. In this work, anatomical study of the leaves and stem of *H. arborea* was performed to provide a microscopical diagnosis for its characterization due to its increasing use in folk and ayurvedic medicine and its differentiation from other species with the same popular name. Standard light and scanning electron microscopy procedures were carried out using paradermal sections of leaf surfaces and transverse sections of lamina, midrib, and petiole. Stem preparations include longitudinal, radial and transverse sections of tertiary branches. The leaf epidermis with stellate non-glandular trichomes, cyclocytic stomata, mucilaginous cells and mesophyll without secretory sclereids, and the stem with confluent axial parenchyma, biseriate xylem rays, and the presence of rhomboidal prismatic crystals in the axial cells are the diagnostic microscopic features of the species. This structural characterization of the stem and leaf allows the identification of *H.arborea*, allowing certification of the authenticity of raw material.

Keywords*:* Anatomy, Cyclocytic stomata, Confluent parenchyma, *Harpullia arborea*, Sapindaceae

INTRODUCTION

The genus, *Harpullia* comprises tropical evergreen to semi-evergreen forest trees belonging to the Sapindaceae or Soapberry family. Sapindaceae is comparatively a large family under eudicots, including 144 genera and 1900 species (Buerki *et al*., 2021). The majority of the family members are mainly distributed in the tropical parts of the world and a few genera were exhibiting temperate distribution (Areces-Berazain *et al*., 2021). The leaf and fruit morphology of Sapindaceae members are highly diverse and act as the basis for the infrafamilial groupings in the traditional classifications of the family (Radlkofer, 1933).The genus *Harpullia* is globally distributed in Australia, India, Malaysia, New Guinea and Sri Lanka (Buijsen *et al*., 2003). The genus is represented by 26 species and all are tropical trees (Leenhouts, 1985). Among these, most of the species are reported endemic in the distribution. In India, the genus is represented by *Harpullia arborea* (Blanco) Radlk. and *H.cupanioides* Roxb, and the latter is reported only from Assam (Bora and Kumar, 2003). So, in India, the genus is widely represented by a single species, *H.arborea*, showing its center of distribution in Peninsular India, i.e. Assam, Maharashtra, Karnataka, Tamil Nadu, and Kerala. The genera could be easily distinguished from other forest trees during the fruiting season by their striking scarlet-colored pericarp, exposing black shining seeds when it split open (Adema *et al*., 1994; Acevedo-Rodríguez *et al*., 2010). This striking appearance of their fruits gave the name doll’s eyes to all *Harpullia* species. All the *Harpullia* members were similar in their tree habit, phyllotaxy and leaf, stem and fruit morphology, making the identification and collection of the specific species based on these external morphologies rather difficult.

Out of the 26 species of the genus, *H. arborea* is therapeutically more studied and widely used in folk and ayurvedic medicine (Lipipun *et al*., 2003; Udayan *et al*., 2005). More than 150 compounds have been isolated

and identified from leaf, stem, fruits and seeds. These compounds include diterpenoids, triterpenoids, flavonoids, coumarins and essential oils (Poovapatthanachart, 2003) and are responsible for the antimicrobial and antiradical properties of the various plant parts (Tumewu Lidya *et al*., 2014; Raghavendra *et al*., 2017). From this perspective, since there have been very few microscopical studies of *H.arborea* and its morphological resemblance to other *Harpullia* species, the present study undertook anatomical analysis of the leaves and stem of *H.arborea* to assist in the identification of this medicinal plant.

MATERIALS AND METHODS

Collection and identification of the material

Fresh leaf and branch samples were collected from the plant with nearly 17 cm in diameter at breast height (DBH). The samples were collected in February 2020 from Kakkayam biosphere reserve in Kerala, a part of peninsular India. *H. arborea,* voucher specimens were deposited at the Plant Systematics & Evolutionary science Division of Jawaharlal Nehru Tropical Botanic Garden And Research Institute, Thiruvananthapuram, Kerala, with Voucher Number- 39346. The phyllotaxy and the leaf and branch morphologic characters, including size, shape, texture, color, coating, margin, venation and petiole size, were visually analyzed (Hickey, 1973).

Methodology

To prepare paradermal sections of leaf, a 3 cm^2 sized portion from the middle part of the mature lamina, including leaf margin, was soaked in concentrated nitric acid for two days (Dilcher, 1974). Samples were then washed 2-3 times in distilled water. Peeled-off leaf surfaces were stained with safranin O in 50% alcohol for about 2 minutes before mounting. Scanning Electron Microscope (SEM) images of the leaf surface were taken using JSM-6390, a model Scanning electron microscope from Sophisticated Test and Instrumentation Centre (STIC), Cochin University of Science and Technology (CUSAT), Kerala. For the transverse sections, leaf lamia, midrib and petiole, sections from the middle part were preferred (Cutler et al., 2008). Handmade sections of leaf lamina, midrib, petiole and stem were treated in 30% sodium hypochlorite solution (NaOCl) for 3-5 minutes. Stem preparations include longitudinal (TLS), radial (RLS) and transverse sections (TS) of tertiary branches of 2.5-3 cm in diameter. Stem specimens were boiled in fresh water for 10- 15 minutes in order to soften the material. They were washed repeatedly in distilled water to remove the traces of the bleaching agent. Sections were stained in 1% Toluidine Blue (TBO) and washed with 50% alcohol to remove

Fig. 1. *Harpullia arborea-Habit and Morphology, (a) Tree in its natural habitat. (b) Flowering branch. (c) Pinnately arranged leaflets in a petiole. (d) Portion of main trunk. (e) Ripened scarlet colored split opened fruits. (e) Seeds having black shiny outer covering*

the excess stain. Sections were mounted in 50% glycerin. A total of 5 samples each and 20 microscope fields were examined for the constancy of character. All the samples were analyzed using a Magnus MLXi Plus microscope equipped with Magnus camera adapter. The terminology followed by IAWA Committee (1989) and Metcalfe and Chalk (1950).

RESULTS

Macroscopic features

H.arborea is an evergreen to semi-evergreen forest tree. The younger parts were rather hirsute and the leaves were pinnately compound and without a distal leaflet. There are alternately arranged 4-8 leaflets in each petiole. Each leaflet is elliptic, sparsely hairy and with an acuminate apex. The inflorescence is axillary panicle with yellowish-green, actinomorphic, polygamous flowers and with a prominent floral disc under the ovary. The scarlet colored fruits were very attractive in appearance (Fig. 1). The woody loculisidal capsule, when splits opened, exposes two black shining seeds with orange colored aril.

Microscopic features

Anatomical description of the leaf-blade

The leaf-blade, in front view, expresses the epidermis with indumentum consisting of non-glandular stellate

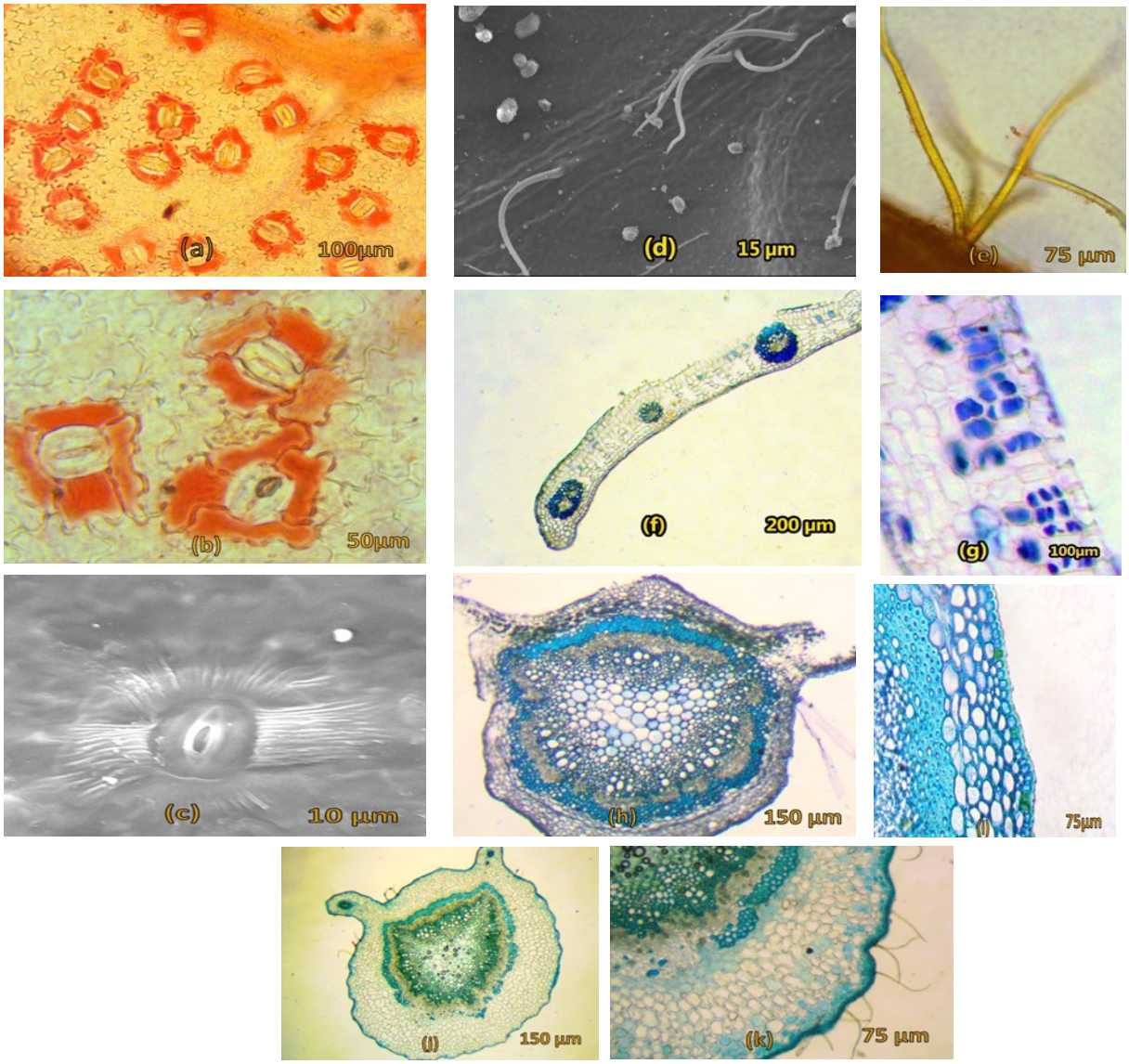

Fig. 2. *Harpullia arborea- Anatomy of Leaf and Petiole:(a) Hypostomatous lamina (b) Cyclocytic stomata and undulating anticlinal walls of lower epidermal cells (c) SEM of stomata showing cuticular striations in subsidiary cells. (d) SEM of non-glandular trichomes on lamina. (e) Stellate unicellular hair tufts (f) T.S of leaf lamina (g) Mucilaginous epidermal cells on upper leaf surface (h) T.S of midrib (i) Secretory idioblasts in epidermal cells of midrib (j) T.S of petiole (k) Enlarged view of petiole T.S showing stellate hairs tufts and prismatic crystals Scale bars: a,g = 100µm; b = 50 µm; c = 10 µm; d = 15 µm; e,i,k = 75 µm; f = 200 µm; h,,j = 150 µm.*

tufts of 2-7 unicellular hairs intermingled with solitary hairs (Fig. 2e). Hairs were more concentrated on the lower surface, especially on the veins and sparsely present on the upper surface. Cuticle on both surfaces was smooth and without papillae or striations. The cuticle on the lower surface form thin loops in the undulations of the epidermal anticlinal wall (Fig. 2b). The guard cells usually lie parallel to the long axis of the subsidiary cells. The leaves were hypostomatic and stomata are predominantly cyclocytic, small, up to 20µm long and subsidiary cells with radiating cuticular striations (Fig. 2c). But above the veins, epidermal cells are rectangular in rows parallel to the veins. The subsidiary cells in *H.arborea* stained more deeply with safranin than the cuticle of unspecialized cells (Fig. 2a).

Lamina is dorsiventral and the adaxial epidermal cells were larger than abaxial cells. Hypodermis is locally developed as an interrupted layer on the adaxial side and is parenchymatous. Mesophyll tissue is with one or two layers of square and erect palisade cells and four to five layers of spongy cells. Spongy tissue forms mesh-like appearance in the studied samples and forms compact continuous uni or biseriate layers just below the abaxial epidermis. Larger veins were slightly raised abaxially. Vascular tissue is embedded in the mesophyll and is not vertically transcurrent or without bundle sheath extensions. Associated with the vein bundles single rhomboidal crystals were noticed in all the studied samples.

Mucilage cells and secretory idioblasts were present in the adaxial epidermal cells (Fig. 2g). Idioblastic sclereids and sclerenchymatous fibers were not present in the mesophyll of the studied samples. Leaf margin has blunt end and marginal vein supported by collenchymatous tissue (Fig. 2f).

The midrib is slightly raised abaxially and with U shaped outline. Single and tufted non glandular hairs were present on the surface. Secretory idioblasts were present in the epidermis and throughout the ground and vascular tissue. Collenchymatous hypodermis present and continues as ground tissue. Vascular system was collateral and surrounded by a prominent sheath of sclerenchyma. Cortical and medullary bundles were absent (Fig. 2h).

Anatomical description of the petiole

Petiolule from the distal end had a cordate outline with an arc-shaped closed vascular system similar to that of midrib, but the sclerenchymatous sheath around the vasculature is less prominent. The two laterally extended leaf trace bundles act as an identification tool for the petiolule (Fig. 2j). Tufts or singular, unbranched non-glandular, unicellular trichomes were present throughout the leaf and petiole surface (Fig. 2k).

Anatomical description of the stem

In cross section, the stem in secondary growth had practically circular contour (Fig. 3a) with non-glandular unicellular hairs and lenticels. Growth boundaries were distinct and marked by flattened fibers. Periderm was pigmented and superficially formed with multilayered phellogen. A prominent wavy continuous ring of pericyclic sclerenchyma was present. Comparatively large pith made of isodiametric parenchymatic cells without intercellular spaces and with cell size increasing towards the center, scleriefied cells, starch containing cells and rhomboid prismatic crystals were also present in the pith tissue (Fig. 3c). More than 50% of xylem vessels are grouped into multiplies of 2 or 4. Paratracheal axial parenchyma shows confluent- aliform distribution around the vessels (Fig. 3b). The major part of the vascular tissue was occupied with xylem fibers. Rhomboidal prismatic crystals were present in the axial parenchyma cells. Vessels were showing the presence of

Table 1. Anatomical markers for the microscopical diagnosis of leaf and stem of *Harpullia arborea*

Sl. No.	Anatomy markers	Plant part
1.	Stellate and non-glandular trichomes	Abaxial leaf surface
2.	Cyclocytic stomata	Abaxial leaf surface
3.	Undulated anticlinal wall	Abaxial leaf surface
4.	Cuticular striations on the subsidiary cell wall	Abaxial leaf surface
5.	Mucilaginous epidermis	Adaxial lamina
6.	Hypodermis absent or poorly developed	Adaxial lamina
7.	Cordate outline with two lateral leaf traces	Petiolule
8.	Diffuse porous stem	Stem
9.	Simple perforation plate and alternate inter vessel pits	Stem
10.	Libriform fibers	Stem
12.	Prismatic crystals in the chambered axial parenchyma	Stem
13.	Confluent axial parenchyma	Stem
14.	Dilating pith	Stem
15.	Biseriate xylem rays	Stem

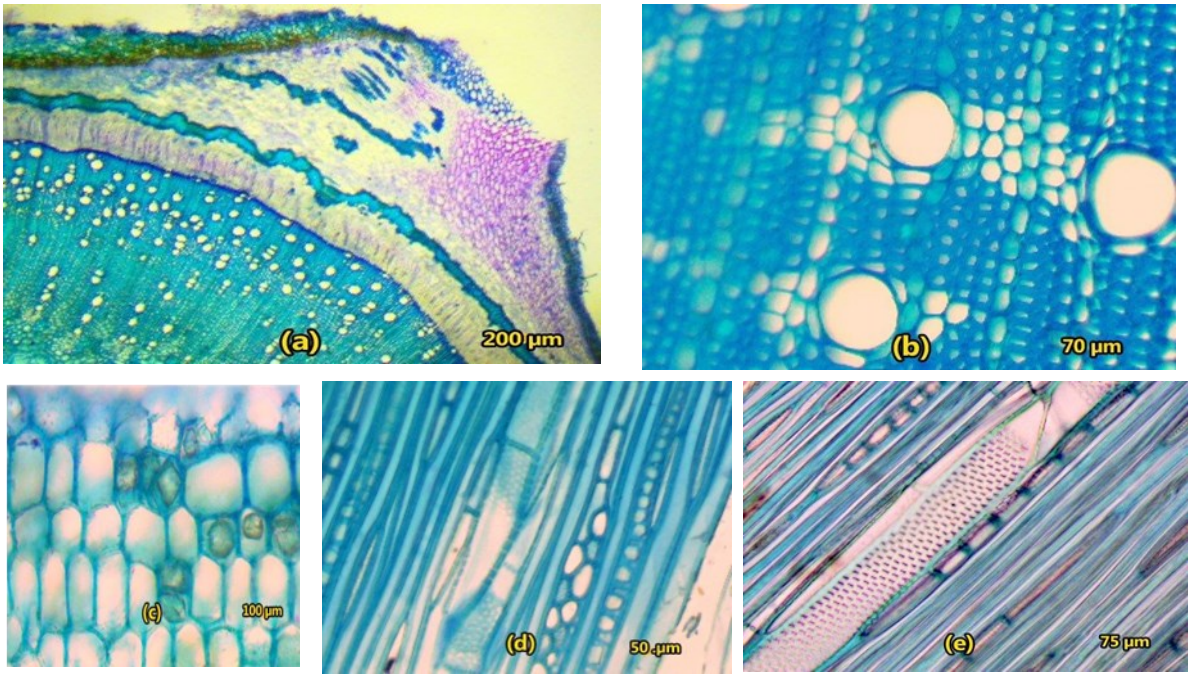

Fig. 3. *Harpullia arborea- Anatomy of Stem: (a)T.S of Stem with secondary thickening (b) Confluent axial parenchyma in stem TS (c) Prismatic crystals in the pith tissue in stem RLS (d) Biseriate xylem rays and prismatic crystals in the axial parenchyma in stem TLS (e) Xylem vessels with alternate inter vessel pits and simple perforation plate in stem TLS. Scale bars: a = 200 µm; b = 70 µm; c = 100 µm; d = 50 µm; e = 75 µm.*

brown colored depositions. The tangential section reveals alternately arranged round shaped intervessel pits with slit like aperture, simple perforation pate (Fig. 3e), prismatic crystals in chambered axial parenchyma and the non septate libriform fibers. Xylem rays had a biseriate pattern and the ray parenchyma was composed of procumbent and sometimes square cells, infrequently with a marginal row of square cells (Fig. 3d). A brief outline of the important leaf and stem anatomical markers that could be used for microscopical diagnosis of *H.arborea* is provided in Table 1.

DISCUSSION

Only a few species of *Harpullia,* such as *H.arborea, H.cupanioides, H.pendula*, *H. petiolaris* and *H.ramiflora,* show pharmaceutical importance (Khan *et al*., 2001; Voutquenne *et al*., 2005; Abdelkader *et al*., 2016; Nabil *et al*., 2019), But among all these species, *H.arborea* had maximum medicinal and therapeutic potentials (Gowri and Vasantha, 2010; Prashith and Vinayaka, 2017). As all the species of *Harpullia* are tropical trees and show similar leaf and fruit morphology, they all are known by the same common name. Due to the similarities among the different species in their external leaf, stem and fruit morphology, the anatomical description of *H. arborea* allows to distinguish it from other species. The presence of trichomes, dorsiventral mesophyll, the mucilaginous epidermis (Fig. 2g), and prismatic crystals (Fig. 2k) were referenced to the Sapindaceae family of the genus by Solereder (1908). The presence of stellate non-glandular trichomes (Fig. 2e), stomata predominantly on lower surface and undulating anticlinal walls of abaxial epidermal cells (Fig. 2b) were very common leaf anatomy features possessed by all the *Harpullia* members (Buijsen, 1995).

The stomatal type is the only one of the important leaf epidermal characters constant within the genus and therefore has a high diagnostic value (Metcalfe and Chalk, 1950). Considering the type of stomata found in the *H.arborea*, it is predominantly cyclocytic (Fig. 2b). The only species of *Harpullia* possessing cyclocytic stomatal type other than the studied species is *H.pendula* and in the remaining 24 species of this genus, paracytic to anaomocytic stomatal types are present (Buijsen, 1995). Subsidiary cells with radiating cuticular striations (Fig. 2c) were present in *H.arborea*, *H.longifolia, H.pendula*, and the subsidiary cells of *H.arborea* were more deeply stained with safranin than the cuticle of unspecialized epidermal cells.

The presence of secretory idioblasts and mucilaginous epidermal cells (Fig. 2i) were indicative of some chemical production by the plant (Evert, 2006), and in the case of *H.arborea*, it was associated with the production of saponins and phenolic compounds, attributing to its phytochemical and bio repellent properties (Raghavendra *et al*., 2017). Secretory idioblasts were present throughout the leaf, petiolule and stem parts. The combination of leaf micro morphological features such as cuticle on the lower surface forming thin loops in the undulations of the epidermal anticlinal wall, cy-

clocytic stomata, subsidiary cells with cuticular striations, stellate tufts of non-glandular hairs intermingled with solitary hairs are useful for anatomical diagnosis of *H. arborea* for its pharmacognostical purposes.

According to Metcalfe and Chalk (1950), the presence of hypodermis and vertically transcurrent vein bundles are taxonomically significant characters. The presence of adaxial hypodermis and vertically transcurrent bundles were common features of *Harpullia.* But, in the case of the *H.arborea*, hypodermis was absent or represented by a single interrupted layer above the veins, and a bundle sheath surrounded the leaf bundles without any lateral extensions (Fig. 2f). In comparison with other species of *Harpullia*, such as *H.camptonuera, H.cauliflora, H. longipetala* and *H.ramiflora*, the mesophyll tissue found in *H.arborea* is distinctive in the absence of idiobalstic sclereids and brachysclereids (Buijsen, 1995). Petiole anatomy is considered a systematically important characteristic as it is less liable to environmental variation (Dickison, 2000; Talip *et al*., 2017). Petiolule with a cordate outline, arc-shaped closed vascular system surrounded by a sclerenchymatous sheath and two laterally extended leaf trace bundles (Fig. 2j) act as an identification tool for *H. arborea* (Solereder, 1908).

The presence of externally originated multi-layered periderm, pericyclic sclerenchyma (Fig. 3a), vessels with simple perforation plate (Fig.3e), paratracheal axial parenchyma, libriform fibers, simple and alternately arranged inter vessel pits (Fig. 3e) are anatomically homogenous features possessed by the stem of all Sapindaceae genera (Klaassen, 1999; Pace *et al*., 2022). The *Harpullia* genera also possess all these above mentioned features and are enough to trace its taxonomic position as a member of Sapindaceae. *Harpullia* belongs to the tribe Dodonaeoideae of Sapindaceae. The presence of growth boundaries marked by marginal parenchyma, diffuse porous vessel distribution, non septate libriform fibers and prismatic crystals in the chambered axial parenchyma (Fig. 3d) in *Harpullia* were showing its affinity to Dodonaeoideae tribe.. (Klaassen, 1999). In addition to the typical Sapindaceae anatomical features, the stem of *H.arborea* showed some specific combined anatomical features like the lenticulated stem surface, confluent axial parenchyma (Fig. 3b), and biseriate rays composed of procumbent cells (Fig. 3d) , large parenchymatous pith with increasing dilation towards the center and prismatic crystals in the pith tissue (Fig. 3c).

Conclusion

The presence of cyclocytic stomata, biseriate xylem rays and stellate non- glandular hair tufts are uncommon features in the Sapindaceae family and are observed in the case of *Harpullia.* Considering the structural aspects of *H.arborea,* the taxa could be distinguished from the other related species by the combination of the following anatomical characteristics like cyclocytic stomata, subsidiary cells with radiating cuticular striations, stellate tufts of non-glandular trichomes, poorly developed hypodermis, cordate shaped petiolule with two laterally extended leaf trace bundles, confluent axial parenchyma in the stem and the absence of idioblasic sclereids and vertically transcurrent vein bundles in the leaf. These structural characteristics of the leaf and stem allow the identification of *H.arborea*, allowing certification of the authenticity of raw material.

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Conflict of interest

The authors declare that they have no conflict of interest.

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