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Microscopic and Microchemical Characterization of Leaves and Stems of *Acmella bellidioides*

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HIGHLIGHTS

- This is the first anatomical investigation of *Acmella bellidioides*.
- The common name “arnica-do-campo” is often applied to multiple species.
- Microscopy techniques were used to characterize the species’ structures.
- Peltate glandular trichome is an anatomical marker for *Acmella bellidioides*.

Abstract: *Acmella bellidioides* (Asteraceae), commonly known as "arnica-do-campo", is a South American native medicinal plant mainly found in Brazil, Argentina, Uruguay and Paraguay. The indigenous people in these regions use the flowers of this plant to treat diseases of the digestive, musculoskeletal and sensory systems. Many *Acmella* species are morphologically similar, and there are a few anatomical studies available in the literature that can be used to identify and distinguish them. Several other members of Asteraceae, such as *Calea uniflora, Chaptalia nutans, Porophyllum ruderale, Pseudobrickellia brasiliensis* and *Solidago chilensis*, are also called "arnica-do-campo" or "arnica". Applying the same common name to several other species makes it difficult to identify the plant correctly and allows it to be more easily adulterated. The present study characterizes *A. bellidioides* using microscopy and microchemical techniques to provide pharmacobotanical data to support the authentication of the species. The notable anatomical markers identified in *A. bellidioides* are hypostomatic leaves, anomocytic stomata, peltate glandular trichomes on the leaf abaxial surface, midrib vascular system with three collateral bundles in an open arch, and the presence of prismatic crystals in the leaves and stems. These characteristics can help species identification and differentiation of *A. bellidioides* from other *Acmella* species and Asteraceae species known as arnica-do-campo.

Keywords: Anatomy; Asteraceae; histochemistry; microscopy; quality control.

INTRODUCTION

Acmella bellidioides (Sm.) R.K.Jansen (syn. *Spilanthes arnicoides* DC.), popularly known as arnica-do-campo, is a small perennial herb found in Brazil, Argentina, Paraguay and Uruguay. It is also cultivated as a medicinal plant [1]. *Acmella bellidioides* is traditionally used by the indigenous people in the form of chewing, gargling or as a poultice for the treatment of diseases affecting the digestive, musculoskeletal and sensory systems [2]. Several plants growing in Brazil are commonly called “arnica-do-campo” or “arnica” in association with the pharmacopeia herb *Arnica montana* L. (Asteraceae), including *Chaptalia nutans* (L.) Pol., *Solidago chilensis* Meyen, *Porophyllum ruderale* (Jacq.) Cass., and *Pseudobrickellia brasiliensis* (Spreng.) R.M.King & H.Rob [3-5].

Application of the same common names to multiple species and the morphological similarities among different plants are some of the main elements that promote adulterations of the plant material, damaging the therapeutic efficacy and promoting the risk of intoxication [6]. To address this problem, microscopy analyses of the botanicals can be used for accurate identification and authentication of the plant materials [7-10]. Correct species identification is crucial for the safety and efficacy of botanicals [6].

Athayde and coauthors [11] studied and compared the anatomical characteristics of ten species in the arnica-do-campo complex, such as *Calea uniflora* Less., *Chaptalia nutans* and *Lychnophora ericoides* Mart., all belonging to Asteraceae family. These authors stated that several species could be designated as "arnica," making identification difficult. Ramachandran and Radhakrishnan [12] observed that *Acmella* species have similar morphology and evaluated ten "arnica" species microscopically. However, no anatomical study has so far been carried out for *A. bellidioides*. To bridge this gap, the present work aimed to provide detailed microscopic and microchemical analyses of the leaves and stems of *A. bellidioides* to support authentication and quality control of this herb.

MATERIAL AND METHODS

Botanical material

Leaves and stems of *A. bellidioides* were collected in April 2018 from the State University of Ponta Grossa campus (latitude 25º 5' 11" S; longitude 50º 9' 39" W) in Paraná, Brazil. The plant material was identified by the taxonomist O. S. Ribas and the herbarium specimen (MBM 191067) was deposited in the Curitiba Botanical Garden herbarium in Paraná, Brazil. Access to the botanical material was authorized by the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (CGEN/SISGEN - AF2FB38).

Microscopical analyses

Leaves and stems of *A. bellidioides* were fixed in FAA 70 (formaldehyde 37%, glacial acetic acid, and alcohol 70%) [13] and then preserved in 70% alcohol [14]. Hand-cut cross-sections were prepared with a razor blade and double-stained with basic fuchsin and astra blue [15]. Semi-permanent slides were prepared with glycerin 50% and closed with colorless nail polish [16]. To analyze epidermal surfaces, small sections of the leaves were washed and then treated with hypochlorite solution (5%) until translucent. The materials were then washed with distilled water and neutralized in an acetic acid solution (5%). The sections were rewashed in distilled water, stained and mounted as described above [16]. The slides were analyzed and imaged using an Olympus CX31 optical microscope equipped with a C7070 digital camera at UEPG pharmacognosy laboratory.

Scanning Electron Microscopy (SEM) was performed on fragments of fixed leaves and stems. The tissues were gradually dehydrated in ethanol solutions of increasing concentrations and dried in a Balzers CPD 030 critical point dryer (BAL-TEC AG, Balzers, Liechtenstein) and then coated with gold using a SC7620 Quorum sputter coater. Electron micrographs were taken using a Mira 3 field-emission SEM (Tescan, Brno-Kohoutovice, Czech Republic). During the SEM procedure, energy dispersive x-ray spectroscopy (EDS) was performed to verify the elemental chemical composition of the crystals. This analysis was randomly made for the crystals, and the cells devoid of crystals as control, at an acceleration voltage of 15 kV using an EDS detector attached to the SEM [17]. These analyses were performed at the Multiuser Laboratory Complex (C-Labmu) at the State University of Ponta Grossa.

Histochemical tests

The following reagents were used in the histochemical tests: Sudan III to detect lipophilic compounds [18]; ferric chloride 2% [13] and potassium dichromate 20% for phenolic compounds [19]; phloroglucinol/HCl for the identification of lignified structures [20]; methylene blue (0.1%) to reveal mucilaginous cells [13] and Lugol solution for starch [14].

RESULTS AND DISCUSSION

Acmella bellidioides leaves (Figure 1A, B, C), in frontal view, show wavy anticlinal epidermal cell walls on adaxial face (Figure 1D) and sinuous anticlinal epidermal cell walls on adaxial face (Figure 1H); cells near the venation area assume more elongated walls. Anomocytic stomata are observed only on the abaxial side, characterizing the leaves as hypostomatic (Figure 1F, H, I). Ramachandran and Radhakrishnan [12] have found anomocytic, anisocytic and diacytic types of stomata on both sides of leaves in ten *Acmella* species. Moreover, amphistomatic leaves were observed in *Acmella calva* (DC.) R.K.Jansen, *Acmella ciliata* (Kunth) Cass., *Acmella ghoshinis* (Sheela) Reshmi & Rajalakshmi ex Kottaim., *Acmella paniculata* (Wall. ex DC.) R.K. Jansen, *A. radicans* (Jacq.) R.K. Jansen, *Acmella uliginosa* (Sw.) Cass., *Acmella uliginosa* (Sw) *var. pentamera* Reshmi & Rajalakshmi and *Acmella vazhachalensis* (Sheela) Reshmi & Rajalakshmi [21]. Also, another arnica plant, *Sphagneticola trilobata* (L.) Pruski, showed stomata on both surfaces [11].

Three types of trichomes are observed: i) 2-3-celled simple non-glandular trichomes with rough cuticle surfaces (Figure 1E, F); ii) about 6-celled uniseriate non-glandular trichomes with obtuse apex and smooth cuticle (Figure 1G); and iii) peltate glandular trichomes (Figure 1I). The glandular trichomes are only found on the abaxial leaf surface. Simple and multicellular non-glandular trichomes have also been identified in nine other species of *Acmella* [21]. However, the peltate glandular trichome was not previously described for any *Acmella* species. This feature is a good anatomical marker for the identification of *A. bellidioides*.

In cross-section, the leaf is dorsiventral with an unilayered epidermis covered by a striated cuticle (Figure 2A, B). Dorsiventral leaves were also previously observed in nine species of *Acmella* [12]. The mesophyll consists of a 2-layered palisade and about 5-layered spongy parenchyma. Secretory ducts were observed in the palisade parenchyma near the minor collateral vascular bundles. The midrib, in cross-section, has a biconvex shape (Figure 2C), with a slight prominence on the adaxial surface. Subjacent to the epidermis, 1-2 layers of angular collenchyma are observed (Figure 2D); however, more layers can be seen in the prominence of the adaxial surface. Simple non-glandular trichomes are also observed in the midrib (Figure 2C, E).

The vascular system is represented by three collateral vascular bundles in an open arc (Figure 2C), with the central one having the largest diameter. In contrast, only a central collateral vascular bundle was described for *Acmella* species in the study of Ramachandran and Radhakrishnan [12]. Secretory ducts (Figure 2F) containing essential oil are observed close to the vascular bundles. Druses of calcium oxalate (Figure 2G) are dispersed throughout the ground parenchyma (Figure 2G).

Calcium oxalate crystals are common in plants and can be formed in any organ or tissue. They occur in different morphotypes, such as druses, prisms, sand crystals, styloids, and raphides, each with several different shapes and sizes [22]. The occurrence and morphotypes of crystals in plant tissues are useful in species identification [7, 17, 23, 24]. EDS spectra of the crystals in *A. bellidioides* showed prominent peaks of calcium, carbon, and oxygen (Figure 3), suggesting that these crystals are composed of calcium oxalate.

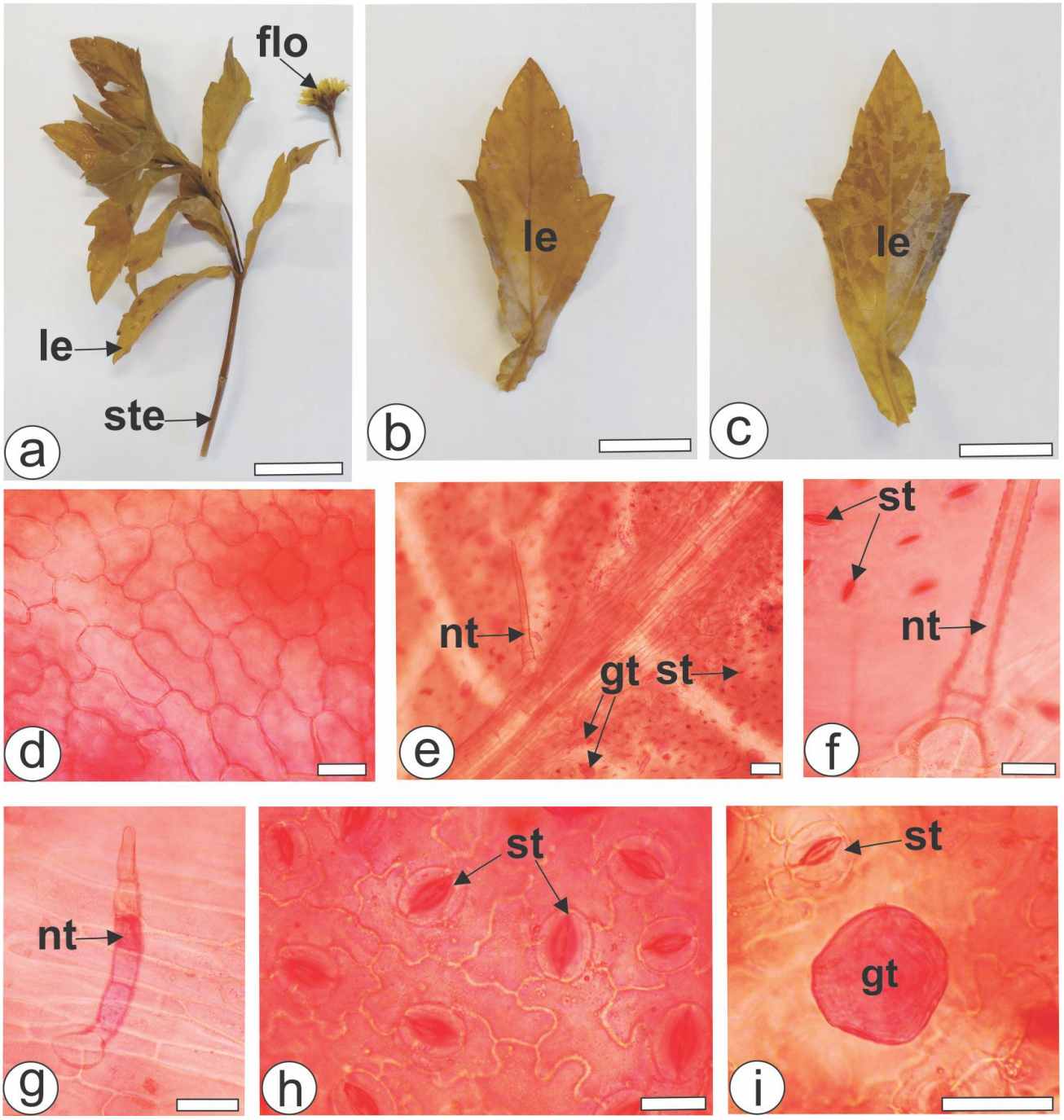

Figure 1. Morpho-anatomy of leaves of *Acmella bellidioides.* a. leaves and flower of *A. bellidioides*; b. adaxial surface; c. abaxial surface; d-I. leaf epidermis in surface view (d. adaxial surface; e-i. abaxial surface). [le: leaf; flo: flower; ste: stem; nt: non-glandular trichome; gt: glandular trichome; st: stomata]. Scale bars: a = 19 cm, b = 7 cm, c = 7 cm, d = 50 µm, e = 100 µm, f-i = 50 µm.

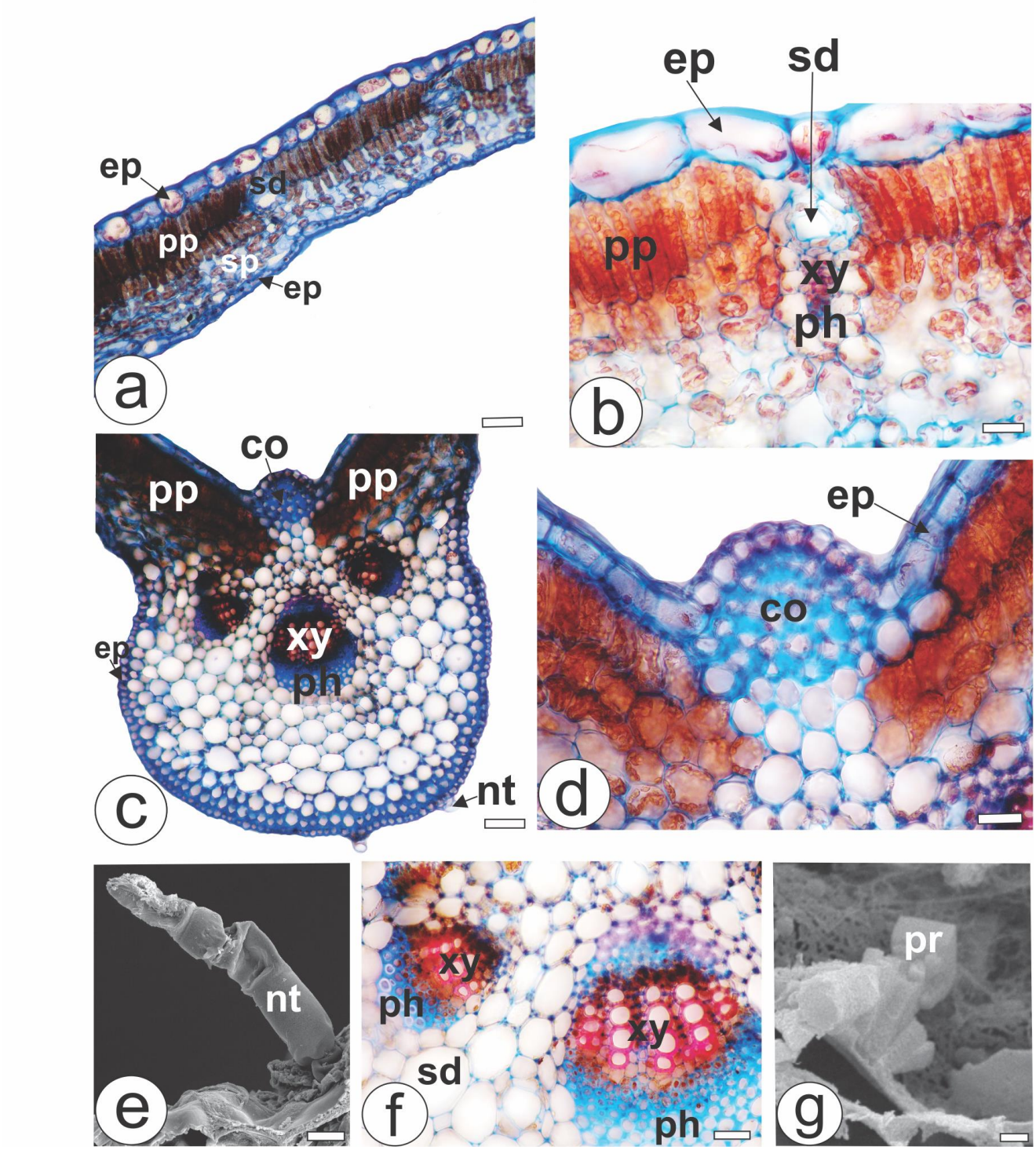

Figure 2. Leaf anatomy of *Acmella bellidioides.* Cross-sections of leaf blade (a-b, e) and midrib (c, d, f, g). [a-d, f: stained in astra blue/basic fuchsin; e, g: SEM). [ep: epidermis; pp: palisade parenchyma; sp: spongy parenchyma; sd: secretory duct; xy: xylem; ph: phloem; co: collenchyma; nt: non-glandular trichome; pr: prismatic crystal]. Scale bars: a, c = 100 µm, b,d,f = 50 µm, e = 20 µm, g = 10 µm.

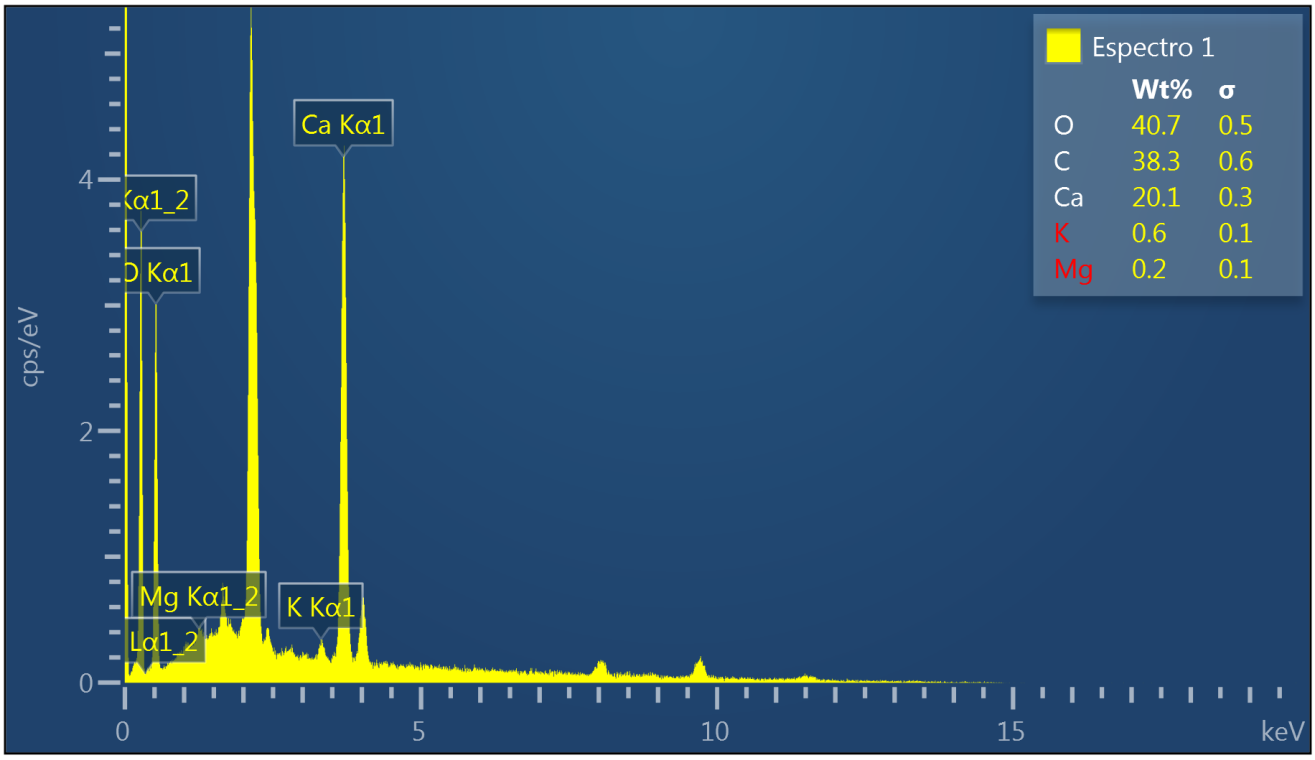

Figure 3. Energy-dispersive X-ray Spectroscopy (EDS) spectrum of prismatic crystal from *Acmella bellidioides.*

The stem is circular in cross-section (Figure 4A). The epidermis is unilayered and covered externally by a slightly thick cuticle (Figure 4C, D). Non-glandular trichomes are observed (Figure 4F, G). Beneath the epidermis, 2-3 layers of laminar collenchyma are present (Figure 4C, D, E, H). The cortex is formed by layers of angular collenchyma (Figure 4C, E) filled with phenolic compounds (Figure 6E). About 17 secretory cavities are arranged in a circle in the stem cortex. The vascular cylinder has phloem on the outside and xylem on the inside (Figure 4I, J, K). A layer of amiliferous endoderm surrounds the vascular system, which is represented by a collateral vascular cylinder. Perivascular fibers are found attached to the phloem (Figure 4I, L). The pith comprises thin-walled cells and contains numerous crystals and secretory ducts (Figure 4M). Ramachandran and Radhakrishnan [12] also found secretory ducts in nine species of *Acmella.* Druses and prisms of calcium oxalate (Figure 5) are found (Figure 4M, N, O). As per the literature, druses are absent in *A. paniculata* and *A. uliginosa* var. *pentamera*, while styloid crystals were found in *Acmella tetralobata* (Reshmi & Rajalakshmi) and *A. vazhachalensis* [12]. The prismatic crystals observed in this study can help the differentiation of *A. bellidioides* from other *Acmella* species.

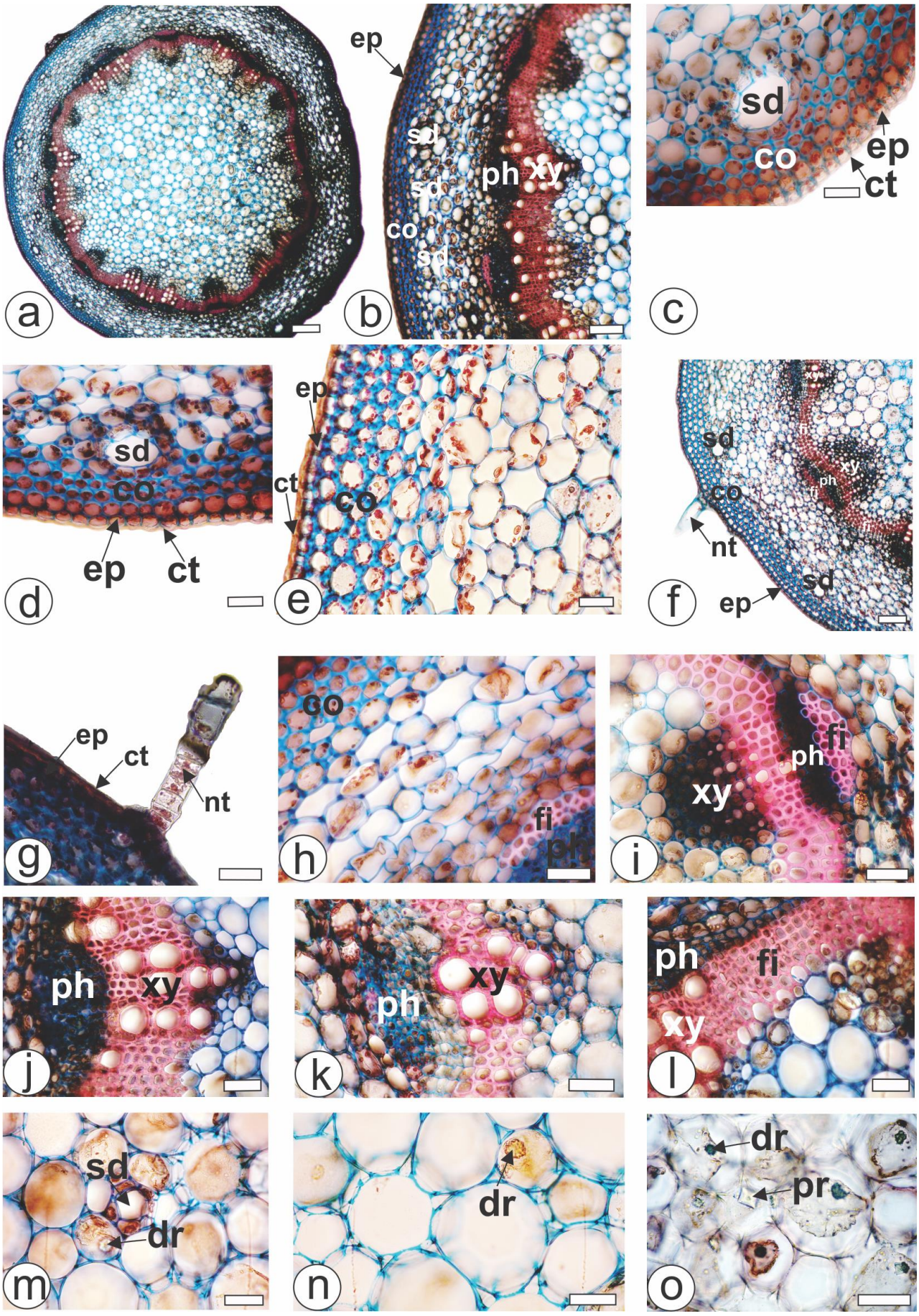

Figure 4. Stem anatomy of *A. bellidioides* – Cross-sections of the stem (a-o). [a-o: stained in astra blue/basic fuchsin]. [ep: epidermis; sd: secretory duct; co: collenchyma; ph: phloem; xy: xylem; ct: cuticle; nt: non-glandular trichome; fi: fiber; dr: druse; pr: prismatic crystal]. Scale bars: a, f = 200 µm, b = 100 µm, c-e, g-o = 50 µm.

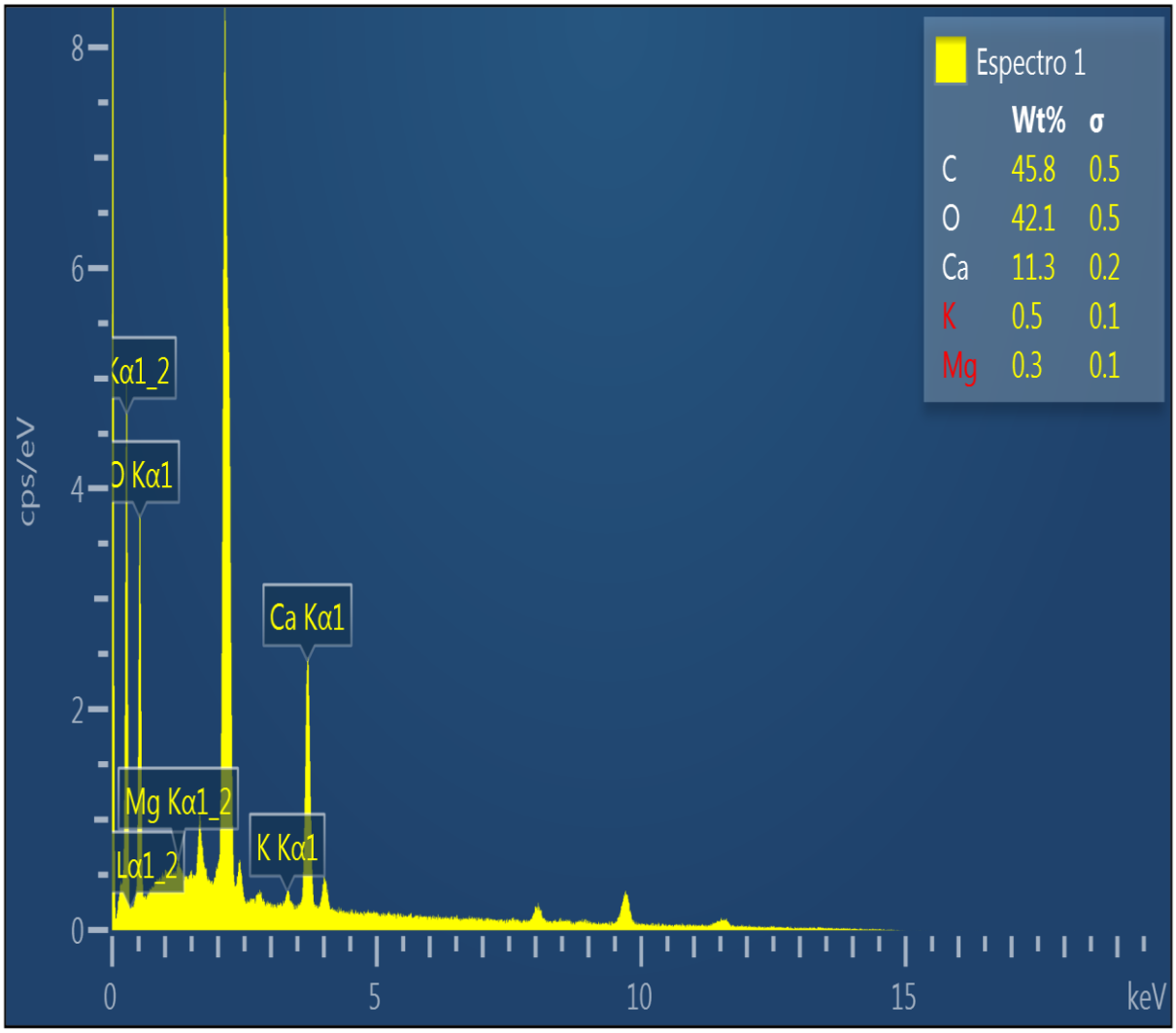

Figure 5. Energy-dispersive X-ray spectroscopy (EDS) spectra of druses in *Acmella bellidioides* stems.

Based on the histochemical tests, lipophilic compounds were found in the cuticle (Figure 6A), secretory ducts (Figure 6D) in the leaves and stems, and phenolic compounds in the leaf mesophyll (Figure 6B) and in the cortex and vascular bundles of the stems (Figure 6E). Palisade parenchyma has a greater amount of phenolic compounds compared to the spongy parenchyma. Lignified structures (Figure 6G) were detected in the fibers and xylem of the stems. Aggregated or isolated starch grains were visualized in the leaves (Figure 6C) and stems (Figure 6F).

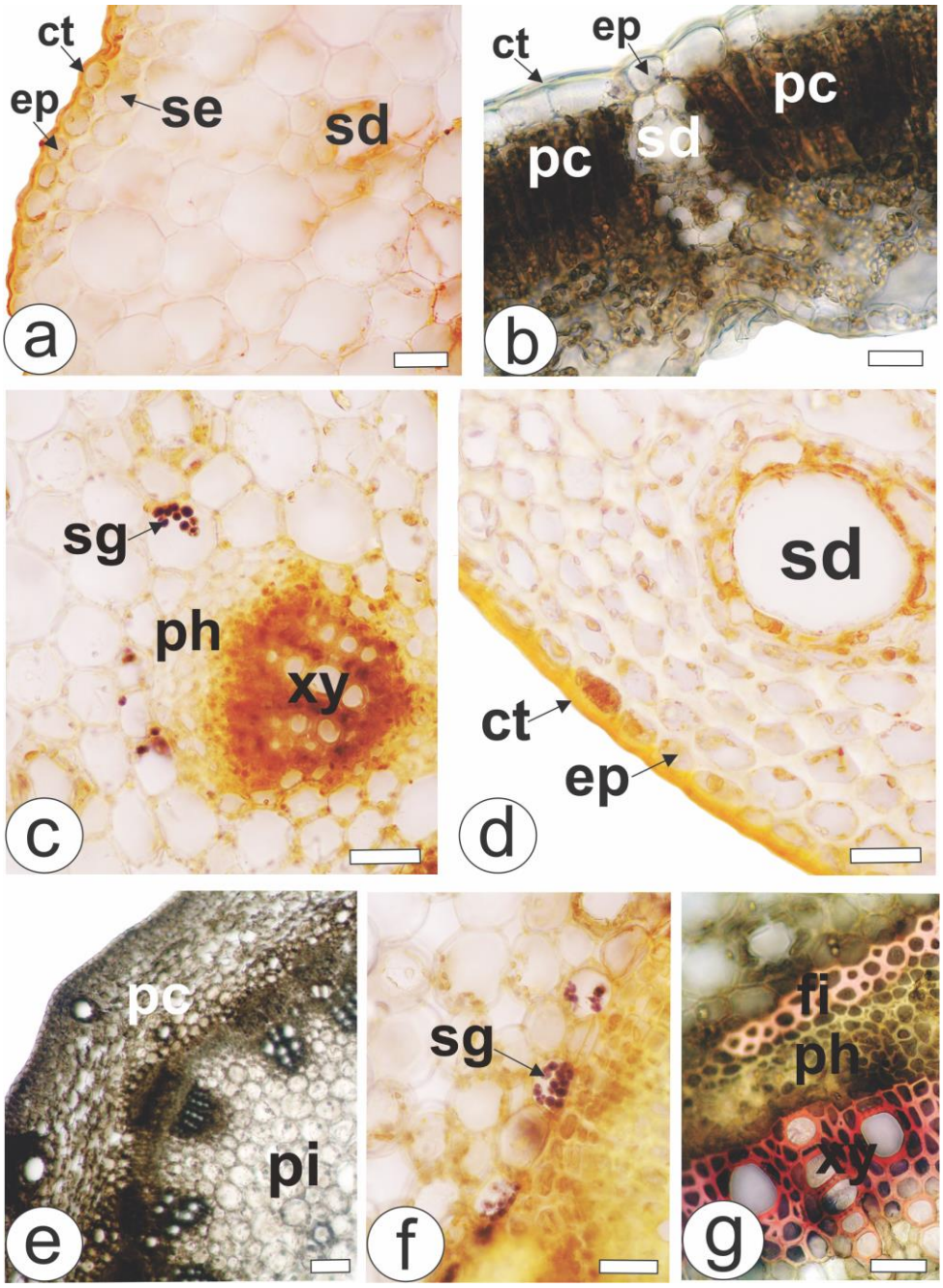

Figure 6. Histochemical tests. Cross-sections of leaves (a-c) and stems (d-g). [a, d: Sudan III; b, e: ferric chloride; c, f: iodine solution; g: phloroglucinol/HCl]. [ep: epidermis; ct: cuticle; se: subepidermal layer; sd: secretory duct; pc: phenolic compounds; xy: xylem; ph: phloem; sg: starch grains; pi: pith; fi: fiber]. Scale bars: a, d, e = 100 µm; b, c, f, g = 50 µm.

CONCLUSION

Microscopy and histochemical analyses of plant tissues play vital role in the taxonomy and characterization of morphologically similar plant species and semi-processed botanical raw materials. The present study provides a detailed anatomical report of *Acmella bellidioides* illustrated with light and scanning electron micrographs. Noteworthy anatomical characteristics observed in this study are hypostomatic leaves, anomocytic stomata, peltate glandular trichomes, midrib with three collateral vascular bundles in an open arch, and prismatic crystals in leaves and stems. These findings would support the identification and differentiation of *A. bellidioides* from other species of the arnica-do-campo complex and form a basis for future studies of other taxa in the genus *Acmella*.

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Conflicts of Interest: The authors declare no conflict of interest.

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