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Pharmacognostic Survey on *Paeonia Broteri* – An Iberian Endemism

Joel Pereira[1], Generosa Teixeira[2, 3]

[1]Faculdade de Farmácia, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003, Lisboa, Portugal.
[2]cE3c- Centre for Ecology, Evolution and Environmental Changes & CHANGE - Global Change and Sustainability Institute, Faculdade de Ciências, Universidade de Lisboa, C2, 1749-016, Lisboa, Portugal.
[3]DCFM, Faculdade de Farmácia, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003, Lisboa, Portugal.

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*Corresponding Author:
Generosa Teixeira

ABSTRACT

The genus *Paeonia* (Paeoniaceae) includes perennial plants distributed throughout the northern hemisphere and some of these are used in traditional medicine and cultivated as ornamental species. *Paeonia broteri* is endemic to Iberian Peninsula and the current paper is the first report on a pharmacognostic survey on its roots, leaves and corresponding powders. Using light and scanning electron microscopy, simple and compound polyhedral starch grains, with a fissure-type hilum and no striae, are recorded in the root phelloderm cells and the presence of idioblasts with druse-type calcium oxalate crystals, in addition to isolated stone cells, are also common. Irregular and sinuous epidermal cells can be detected on both leaf surfaces and anomocytic stomata, with an elliptical-rounded shape, are only on the abaxial face. In leaves, druse-type calcium oxalate crystals appear along the vascular tissues. The histochemical tests allowed the *in situ* localization of some chemical groups and the most relevant detected were polyphenols and terpenoids.

KEYWORDS: *Paeonia broteri*; micromorphology; anatomy; histochemistry; roots and leaves; powdered analysis

I. INTRODUCTION

The genus *Paeonia* L. was previously classified in the Ranunculaceae but chemotaxonomic studies led to its placement in a monogeneric family, the Paeoniaceae Raf. (1; 2; 3; 4; 5). This comprises 35 species, spread over 3 sections: Moutan, Oneapia, and Paeonia, all living in the temperate areas of North hemisphere. The first includes 8 woody species confined to Eastern Central China and the second incorporates 2 species, limited to Western North America (6; 7). The section Paeonia is the largest, with 25 herbaceous perennial species, widely distributed throughout temperate Eurasia (8) and it includes *Paeonia lactiflora* Pallas (Syn: *P. albiflora* Pallas; *P. edulis* Salisb.; *P. officinalis* Thunb) (4). This *taxon* has been used in Chinese medicine for centuries and is encompassed in the *Chinese Pharmacopoeia* as two different traditional Chinese medicines, *Radix Paeoniae Alba* and *Radix Paeoniae Rubra* (9; 4; 10; 11). It is recommended for the treatment of rheumatoid arthritis, systemic lupus erythematosus, hepatitis, dysmenorrhea, muscle cramping and spasms and other practices are mentioned, such as antimicrobial, antioxidant, antitumor, diuretic, cardiovascular-system and central-nervous-system (12; 13; 4). Phytochemical studies have revealed more than 262 compounds, belonging to different chemical groups, monoterpenoid glucosides, triterpenoids, steroids, stilbenoids, flavonoids, tannins and phenols (4; 14; 11), to whom the aforementioned biological and pharmacological activities are attributed, indicating the huge potential of these plants.

Although some *Paeonia* have been evaluated for their biological and pharmacological activities, the species identification itself and the corresponding pulverized materials becomes difficult, even in the best known *taxa*, as there is not much data on their micromorpho-anatomy, and the distinction between species has raised some doubts, which may put at risk the safety and efficacy of its use (9; 15; 16).

Some species of section Paeonia can be found in Iberian Peninsula: *P. mascula* (L.) Mill., *P. coriacea* Boiss., *P. officinalis* (Boiss. & Reut.) (Syn: *P. humilus* Retz.) and *P. broteri* Boiss. & Reut. (Syn: *P. broteroi* Boiss. & Reut; *P. lusitanica* auct.) which is endemic in Portugal (17). It can be seen in shady locations, nearby temporary water courses, growing in the understory of oak populations. Plants can reach up to 50-70 cm height, with tuberous roots and deeply lobed compound bright green leaves. Exemplars are appreciated and grown as ornamental, as in late spring they show large flowers 10-15 cm in diameter, with 5-10 pinkish-

red petals, numerous bright yellow anthers and woolly carpels. To the best of our knowledge, this study reports for the first time the micromorpho-anatomical characters in *P. broteri* roots and leaves. It is a contribution to improve our knowledge of this *taxon*, as those data provide referential information to determine the identity and quality of the plant material.

II. MATERIALS AND METHODS

Plant Material

Samples of tuberous roots and leaves of *P. broteri* were collected from native populations, in a mountainous area of Southwestern Portugal (38º 8' N – 8º 33'W). Plant material was identified and voucher specimens were deposited in the Lisbon Botanical Garden Herbarium (LISU 221345).

Light microscopy (LM)

The microscopy studies were performed on fresh, fixed, paraffin embedded and powder material. Fresh leaves were hand-sectioned transversely and paradermally and then clarified in chloral hydrate (18). The samples fixation was achieved in a 2.5% glutaraldehyde solution in a 0.1 M sodium phosphate buffer, pH 7.2, for 5 h at 4°C and dehydrated in a graded ethanol series (19). Some fixed material was washed in TBA and embedded in paraffin blocks (20) and cross sectioned at 8-10 µm, using a Leitz 1512 Minot microtome. Roots and leaves powdering followed the *European Pharmacopoeia* (21) instructions to achieve their microscopic characterization. Images were obtained on a Nikon Labophot-2, equipped with an AxioCam color CCD, software AxioVision 4.8.2.

We performed some histochemical tests, according to previous works (22; 23), to detect *in situ* the presence of the main chemical groups. Results were semi-quantitatively analyzed, between negative (-), slight positive (+), moderate positive (++), strongly positive (+++) and inconclusive (inc).

Scanning electron microscopy (SEM)

Some fixed samples were critical point dried with liquid CO_2, on a Critical Point Polaron BioRad E3500, coated with gold on a Jeol JFC-1200 and examined at 20 kV with a Jeol JSM-5220 LV scanning electron microscope. Measurements were done by computer-assisted image analysis, always at the same magnification.

III. RESULTS AND DISCUSSION

Roots morpho-anatomy

P. broteri tuberous roots are hard, light brown, up to 2 cm in diameter. Complete cross-sections were difficult to obtain, but the pieces allowed the understanding of secondary growth: from the outer to the inner layers we find the periderm that comprises the phellem, a few layers of light brown suberized cells, originating from the phellogen (cambium), this one with 2 to 4 layers of slightly yellowish flat cells, followed by the phelloderm (secondary parenchyma cortex), with 8 - 12 rows of circular cells (Fig.1A). From here, the secondary phloem ring and the vascular cambium ring surround the entire secondary xylem ring, where the medullary radial rays can be seen. The primary vascular tissues are still visible, with some pockets of primary phloem scattered by secondary phloem ring, and the primary xylem vessels, cross arranged in the center. In the root sections, the phelloderm cells stand out as they are filled with simple and compound starch grains (Fig.1A, 1B, 1C and 1D). The simple grains are circular to polyhedral, 7.4 ± 4.3 µm in diameter, with a cleft hilum and no striae (Fig.1B); the compound granules have 2-3-4 aggregate elements. The observation of micro morphological and anatomical characteristics are important data for the identification and authentication of plant drugs and which acquire particular emphasis on the powder analysis. WHO considers the monograph of Radix Paeoniae (9) whose definition is the dried root of *P. lactiflora* and about the plant microscopic characteristics it states that the literature description is not available and have to be established in accordance with national requirements. This same monograph remarks the light grayish brown powder, the masses of gelatinized starch granules fairly abundant, 5-25 µm in diameter. It is known that the starch storage in roots occurs in starch grains, inside amyloplasts, located in reserve parenchyma cells. They have characteristics that make it possible to allow the identification and authentic - ation of their vegetable origin: shape, size, type of hilum, presence of striations and existence of occasional compound granules (21). Radix Paeoniae (9) and others (15) do not mention most of these characteristics, as only their diameter is revealed. The lack of the other starch grain identification items of *P. lactiflora* does not allow their complete characterization and distinction. Even so, we notice that *P. lactiflora* has starch grains with very different diameters, from small to large (5 - 25 µm), while the average size of *P. broteri* root starch grains is smaller but more homogeneous (7.4 ± 4.3 µm).

In the vacuoles of some phelloderm cells we can also observe druse calcium oxalate crystals (Fig. 1D), random distributed, with 20.5 ± 9.2 µm in diameter. According to the Radix Paeoniae monograph (9), clusters of calcium oxalate 11-35 µm in diameter, packed in parenchyma cells in rows or singly, are found and their existence can explain the bitter flavor mentioned in the same monograph. Taking into account the deviation values, the size of the crystals is approximate in both species, although there are smaller crystals in *P. lactiflora* roots. Nothing is said as to the crystal type found in the *P. lactiflora* roots, although the mention of their diameter points to a druse configuration. Also throughout the root phelloderm, we can found stone cells with pitted cell walls (Fig.1E and Fig. 1F) positive for lignin. The analysis of the light yellow to light brown colored root powder confirmed all the characters described above and which are condensed in Table 1.

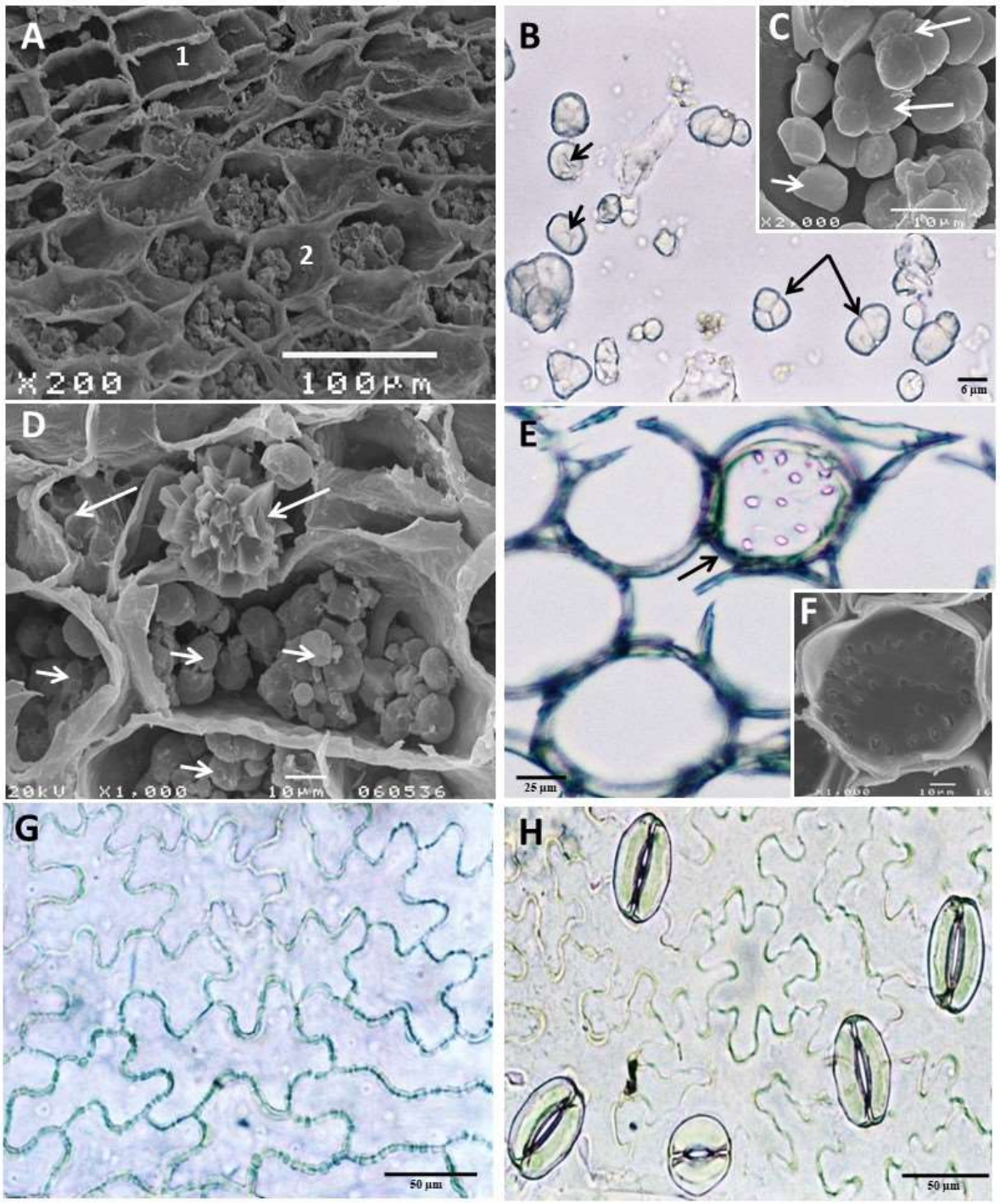

Figure 1. *Paeonia broteri*: **A, C, D, F** – SEM images and **B, E, G, H** – LM images. **A** – Detail of root cross section, with phelogen (1) and phelloderm cells (2); **B** and **C** – Root powder, starch grains, simple (small arrows) and compound (large arrows); **D** – Root phelloderm cells with druse calcium oxalate crystals (large arrows) and starch grains (small arrows); **E** and **F** – Root phelloderm isolated sclereid cell (stone cell, arrow) with simple pits; **G** – Leaf adaxial face epidermal cells; **H** – Anomocytic stomata in the abaxial leaf epidermis.

Table 1. *Paeonia broteri* main characteristics of the roots and of the corresponding powder.

***Paeonia broteri* Roots**
Tuberous Roots colour / Roots powder colour
light brown / light yellow to light brown
Phelloderm Cells: large and filled with starch grains
Starch Grains
- shape: round to polyhedral
- dimension: 7.4 ± 4.3 µm
- hilum type: cleft
- striae: without striae
- isolated / compound: isolated and compound, with 2-4 elements
Calcium oxalate crystals: druse type, 20.5 ± 9.2 µm in diameter
Stone cells: yes

Leaves and petiole anatomy

In the leaf, the upper epidermis has very irregular cells, with sinuous cell walls and no defined orientation (Fig. 1G). The lower surface epidermal cells are similar but have elliptical and circular stomata randomly distributed and without any defined orientation. They belong to the anomocytic type (Fig. 1H) and the stomatal index was estimated at 21.3 ± 0.4. Both leaf epidermises are glabrous, that is, plant hairs are not found. Leaf cross sections, with a total thickness of 208.4 ± 23.1 µm, show a common dicotyledonous anatomy: a dorsiventral blade with an asymmetric parenchyma, 1-2 layers of palisade parenchyma, 4-6 layers of spongy parenchyma, 57.9 ± 7.8 µm and 122.5 ± 15.4 µm thick, respectively. Among the mesophyll cells, druse calcium oxalate crystals are found inside the vacuoles of specialized cells, the crystal idioblasts, and most of these accompany both the secondary and the main vessels. A cuticle, 8.1 ± 1.2 µm thick, similar on both faces, covers equally upper and lower single epidermal layers.

The petiole anatomy also shows a dicotyledonous structure, highlighting the presence of isolated lignified sclereid stone cells in the pith. The powdering of leaves and petioles yields an olive green powder and its microscopic observation confirms the sinuous cell wall of epidermal cells, the anomocytic stomata, the druse crystals loose or still inside the parenchyma cells and the isolated lignified sclereid cells (Table 2). Despite the root being the organ considered as a drug, there are some references regarding the chemical composition of the aerial organs, as well as some *in vitro* biological activities of these organs (4; 24; 25). The epidermis is the organs outermost tissue and in plant leaves it can show a huge amount of information, used as differentiating characters in all Magnoliopsida. Some of these differences constitute a better adaptation to environmental conditions and so they can also sign about the habitat of a plant (26). For example, the existence of a thick cuticle, high cell walls sinuosity of epidermal cells, hypostomatal distribution, high stomatal index and dorsiventral mesophyll, such as in *P. broteri* leaves, indicate adaptations to reduce water loss (27; 28; 29). These are useful in "montado" habitats, which have dry summers and high solar radiation. We also found calcium oxalate crystals in leaves and petioles, whose diameter is similar to that found in root crystals. At the leaves level, these crystals are mainly found along the veins. Various functions have been attributed to the crystals gathering in plant tissues, such as calcium storage, avoidance of toxic accumulation of oxalic acid and provide mechanical support and chemical plant protection against herbivores (30) and other environmental damages (15). The existence of isolated and disperse stone cells in the roots and in the petiole might also be related with the support function of these sclereid cells inside the plant organs.

Table 2. *Paeonia broteri* main characteristics of the leaves and of the corresponding powder.

***Paeonia broteri* Leaves**
Leaves colour / Leaves powder colour
bright green / olive green
Adaxial epidermis cells / Abaxial epidermis cells
- irregular with sinuous cell walls / irregular with sinuous cell walls
- glabrous / glabrous (no plant hairs)
Stomata:
- shape: elliptical and circular (only on abaxial epidermis)
- type: anomocytic
- stomatal index: 21.3 ± 0.4
Leaf cross sections
- total thickness: 208.4 ± 23.1 µm
- cuticle: 8.1 ± 1.2 µm thick
- palisade parenchyma: 1-2 layers; 57.9 ± 7.8 µm thick
- spongy parenchyma: 4-6 layers; 122.5 ± 15.4 µm thick
Calcium oxalate crystals: along the veins
- type: druse
Stone cells: in the petiole pith

Histochemical study

The results of the histochemical tests are reported on Table 3. We noticed some differences both in the detection of chemical groups and in their location in root and leaf tissues. The application of Tannic Acid for mucilages detection, revealed positive in roots parenchyma cells and in leaves epidermal and parenchyma cells. With PAS test for total polysaccharides, pinkness appeared in the roots parenchyma cells and less in leaves tissues. The localization of acidic polysaccharides with Alcian Blue was positive in leaves tissues but inconclusive in roots. Alkaloids were not detected in any organ with the Wagner reagent. When applying Ferric Trichloride, for phenols, parenchyma root and leaf mesophyll cells stained dark green. Acidic Phloroglucin colored lignin in xylem tissue and sclerenchyma cells, including stone cells. The treatment of

leaf sections with Vanillin, for tannins, has just revealed a light orange-brown coloring in the root parenchyma. The application of Sudan Red III, for total lipids detection, revealed inconclusive in roots and a strong orange coloration in the cuticle on the outside of the cell walls of the leaves epidermal cells. The Nile Blue test for neutral lipids was negative but positive for acidic lipids in epidermal cells. All root and leaf structures stained positive with Copper acetate / Rubeanic Acid, for fatty acids. Considering terpenoids detection in roots and leaves, all revealed positive, although in different concentrations. The results of histochemical tests on *P. broteri* roots and leaves point to the differentiated presence of the main chemical groups, with the exception of alkaloids, what is in accordance with a previous phytochemical screening performed on extracts of different polarities (25) and with other phytochemical studies (4).

Table 3. *Paeonia broteri* histochemical test results to characterize *in situ* roots and leaves.

Histochemical Tests	**Chemical Groups**	***In situ* roots**	***In situ* leaves**
Tannic Acid	**Mucilages**	++ pc	+++ ep / pc
	Polysaccharides		
P.A.S.	Total	+++ pc	++ ep / pc
Alcian Blue	Mucopoly - saccharides	inc	++ ep / pc
Wagner reagent	**Alkaloids**	-	-
	Polyphenols		
Ferric Trichlor- ide	Phenols	+++ pc	+++ pc
Acidic Phloroglucin	Lignin	+++ xy /sc	+++ xy /sc
Vanillin	Tanins	+ pc	-
	Lipids		
Sudan Red III	Total	inc	+++ ep
	Neutral	-	-
Nile Blue	Acidic	+ pc	+++ ep
Nile Blue Copper Ac. / Rubeanic Ac.	Fatty acids	++ pc	+ pc
	Terpenoids		
Nadi reagent	Essential oils	+++ pc	+ pc
Antimony trichloride	Steroids	+ pc	+ pc
2,4-dinitro-phenilhidra-zine	Terpenoids with a carbonyl group	++ pc	+ pc

- negative; +++ strongly positive; ++ medium; + low; ep epidermis; inc inconclusive; pc parenchyma; sc sclerenchyma; xy xylem.

In conclusion, our study is the first on the morpho-anatomy of *P. broteri* roots, leaves, petioles and their powdering materials, revealing noteworthy features which can be the main identifying characters: simple and compound starch grains, calcium oxalate druses and stone cells in the roots and epidermal sinuous cell walls with elliptical and circular anomocytic stomata in the leaves and druse cristals and stone cells in petioles. Histochemical tests on the organs were conclusive on mucilages, total polysaccharides, tannins, acidic lipids and terpenoids but no alkaloids. These data, acting as diagnostic tools, make it possible to authenticate and standardize this species as a plant drug, promoting its pharmaceutical and economic importance.

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Competing interests

The authors have no conflicts of interest to declare.

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