Anais da Academia Brasileira de Ciências (2019) 91(1): e20170922
(Annals of the Brazilian Academy of Sciences)
Printed version ISSN 0001-3765 / Online version ISSN 1678-2690
http://dx.doi.org/10.1590/0001-3765201820170922
www.scielo.br/aabc | www.fb.com/aabcjournal

Comparative anatomy and histochemistry of the leaf blade of two species of *Artocarpus*

RAFAELA D. SÁ[1], MARÍLIA B. CADENA[1], RAFAEL J.R. PADILHA[2], LUIZ CARLOS ALVES[2] and KARINA P. RANDAU[1]

[1]Laboratório de Farmacognosia, Departamento de Ciências Farmacêuticas, Universidade Federal de Pernambuco, Av. Prof. Arthur de Sá, s/n, Cidade Universitária, 50740-521 Recife, PE, Brazil
[2]Setor de Microscopia Eletrônica, Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Av. Professor Moraes Rego, s/n, Cidade Universitária, 50670-901 Recife, PE, Brazil

Manuscript received on November 13, 2017; accepted for publication on April 30, 2018

How to cite: SÁ RD, CADENA MB, PADILHA RJR, ALVES LC AND RANDAU KP. 2019. Comparative anatomy and histochemistry of the leaf blade of two species of Artocarpus. An Acad Bras Cienc 91: e20170922. DOI 10.1590/0001-3765201820170922.

Abstract: In Brazil, there are two species of Artocarpus that were introduced: Artocarpus altilis (Parkinson) Fosberg, known as fruta-pão, and Artocarpus heterophyllus Lam., known as jaca. Both are used as food and medicine. The objective of this work was to conduct a comparative anatomical and histochemical study between A. altilis and A. heterophyllus. Techniques of optical, polarized and scanning electron coupled to energy dispersive spectroscopy. The anatomical characterization showed the characters of general occurrence in the family Moraceae and of those that allow the differentiation of A. altilis and A. heterophyllus. The histochemistry revealed the sites of synthesis and/or storage of the metabolites. The chemical microanalyses brought new information about the chemical composition of crystals. The study provides pharmacobotanical data for the quality control of the species.

Key words: *Artocarpus altilis*, *Artocarpus heterophyllus*, crystals, microscopy, Moraceae.

INTRODUCTION

The Moraceae family comprises 40 genera and about 1.217 species of occurrence in the tropical regions of the planet (The Plant List 2013), being that about 50% of the genera are distributed between the region of Mexico to Argentina (Berg 2001). In Brazil, the family is represented by 19 genera and 208 species (Romaniuc Neto et al. 2015), which have an important medicinal and economic value, known to present bioactive secondary metabolites and to be exploited by the wood industry (Royer et al. 2010, Lima et al. 2011).

The genus *Artocarpus* comprises 61 species native to Asia and India, known for their much appreciated fruits (Ragone 2011, Stevens 2012). In Brazil, two species were introduced: *Artocarpus altilis* (Parkinson) Fosberg, known as fruta-pão, and *Artocarpus heterophyllus* Lam., known as jaca (Falcão et al. 2001, Pereira and Kaplan 2013). Worldwide, these two species are known, respectively, as breadfruit and jackfruit (Pereira and Kaplan 2013).

Both are perennial trees that produce latex, very cultivated in domestic orchards in the tropical regions

Correspondence to: Karina Perrelli Randau
E-mail: krandau@hotmail.com
ORCid: http://orcid.org/0000-0002-4486-4420

of Brazil. The fruits are consumed *in natura*, baked or cooked, or in the form of candies and compotes (Lorenzi et al. 2015). Different parts of the plants are also used in traditional medicine to treat injuries, furuncles, back pain, rheumatisms and respiratory problems (Rodrigues 2006, Albuquerque et al. 2007, Agra et al. 2008, Aguiar and Barros 2012).

Studies with extracts, fractions and isolated components of the leaves of the two species demonstrate cardio-protective functions (Nwokocha et al. 2017), dendrite elongation inhibition (Rao et al. 2013), prevent atherosclerosis (Mozef et al. 2015) and have antiviral (Hafid et al. 2017), hypoglycemic and hypolipidemic effects (Chandrika et al. 2006, Chackrewarthy et al. 2010).

In addition to being widely used by the population as a therapeutic resource, these plant drugs can constitute raw materials used in the manufacture of phytotherapic. In some cases, medicinal plants may be erroneously used for presenting morphological similarities. Anatomical and histochemical studies contribute to the correct identification of these species (Brasil 2010). Thus, the objective of this work was to conduct a comparative study between *A. altilis* and *A. heterophyllus* to determine the authenticity parameters for these species.

MATERIALS AND METHODS

PLANT MATERIAL

Adult leaves of specimens of *Artocarpus altilis* and *Artocarpus heterophyllus* were collected in the neighborhood of Aldeia, Camaragibe, Pernambuco, Brazil. A voucher specimen was deposited in the Herbarium Dárdano de Andrade Lima of the Instituto Agronômico de Pernambuco (IPA), under registration number 91180 for *A. altilis* and 91181 for *A. heterophyllus*.

ANATOMICAL CHARACTERIZATION – OPTICAL MICROSCOPY

Various cross-sections were obtained by hand, using a common razor blade, in the middle region of leaf blades fixed in FAA 50% (Johansen 1940). Paradermal sections were also performed on the adaxial and abaxial faces. All sections were subjected to decolorization with sodium hypochlorite solution (50%) (Kraus and Arduin 1997), followed by washing with distilled water. Subsequently, the cross-sections were stained with safranin and astra blue (Bukatsch 1972) and paradermal sections were stained with methylene blue (1%) (Krauter 1985). Then, semipermanent histological slides were prepared to contain the sections of botanical material, following common plant anatomy procedures (Johansen 1940, Sass 1951). The analysis of the semipermanent histological slides were conducted on images in software (LAS EZ), obtained by a digital camera (LEICA ICC50 W) coupled to an optical microscope (Leica DM750M).

ANATOMICAL CHARACTERIZATION – POLARIZED LIGHT MICROSCOPY

Semipermanent histological slides were prepared with cross-sections of leaf blades obtained by the same method used for the analysis in optical microscopy. For the analysis of the slides was used a polarized microscope (Leica DM750M) coupled with a digital camera (LEICA ICC50 W). The images were processed in software (LAS EZ).

HISTOCHEMICAL CHARACTERIZATION

Histochemical tests were made on cross-sections of fresh leaf blades obtained by hand, using a common razor blade (Johansen 1940). The specific reagents used were: potassium dichromate (10%) for phenolic compounds (Gabe 1968); vanillin chloridric for tannins (Mace and Howell 1974); antimony trichloride for triterpenes and steroids

(Mace et al. 1974); Dragendorff's reagent for detecting alkaloids (Yoder and Mahlberg 1976); Sudan III for lipophilic substances (Sass 1951); phloroglucinol for lignin (Johansen 1940); Lugol for starch (Johansen 1940) and hydrochloric acid (10%) to establish the nature of the crystals (Jensen 1962). Controls were performed in parallel with the tests. Semipermanent histological slides were prepared to contain the cross-sections and were analyzed with an optical microscope (Leica DM750M) (Johansen 1940, Sass 1951).

ANALYSIS OF THE ELEMENTAL COMPOSITION OF CRYSTALS

Samples of fresh leaf blades were fixed in 2.5% glutaraldehyde (buffered with 4% formaldehyde). After dehydration in ethanol series, the material was submitted to critical point drying (Hitachi HCP-2) and mounted onto stubs, using double-sided adhesive tape and sputter-coated with gold (Q150T) (Haddad et al. 1998). Finally, the samples were examined with a Scanning Electron Microscope (SEM) (Zeiss EVO LS15). The chemical microanalyses by Energy Dispersive Spectroscopy (EDS) were done with an X-ray detector attached to the scanning electron microscope.

RESULTS

The leaf blade of *A. altilis*, in frontal view in optical microscopy, has cells with straight or slightly sinuous walls on both sides (Figure 1a, b). The leaf blade is hypoestomatic, with anomocytic and actinocytic stomata on the abaxial face (Figure 1b). The presence of druses is observed under optical and polarized microscopy on both faces of the leaf blade (Figure 1a-d). There are non-glandular trichomes and glandular trichomes. The non-glandular trichomes can be of three types: simple (Figure 1e), hooked (Figure 1f, g) and conical (Figure 1h, j). The glandular trichomes are multicellular and have eight or more cells (Figure 1i, j). All types of trichomes described are found on both sides of the leaf blade, except for the simple non-glandular trichome, which is present only on the adaxial side.

The leaf blade of *A. heterophyllus*, in frontal view in optical microscopy, has cells of sinuous walls on both sides (Figure 2a, b). As it was visualized in *A. altilis*, the leaf blade of *A. heterophyllus* is hypoestomatic, with anomocytic and actinocytic stomata on the abaxial face (Figure 2b), and also presents druses on both faces, observed under optical and polarized microscopy (Figure 2a-d). In *A. heterophyllus* there is only one type of non-glandular trichome, the unicellular conical, and it is restricted to the abaxial face (Figure 2e). The glandular trichomes are multicellular, found on both sides of the leaf blade and have 6 or more cells (Figure 2f, g).

In cross-section, analyzed by scanning electron microscopy, the midrib of *A. altilis* shows a biconvex shape (Figure 3a) and *A. heterophyllus* shows midrib ranging from plane-convex to slightly biconvex (Figure 3b). Under optical microscopy, the epidermis on both species is uniseriate, covered with thin cuticle (Figure 3c, d). In both species there are lithocysts mainly in the regions of ribs (Figure 3e, f). Two to four layers of collenchyma are situated below the epidermis in *A. altilis* (Figure 3c), while in *A. heterophyllus* the collenchyma is composed of three to seven layers of cells (Figure 3d).

The two species studied have a collateral vascular bundle in the midrib, associated with sclerenchyma (Figure 3c, d). *Artocarpus heterophyllus* presents a more developed medullar region of parenchyma than *A. altilis* (Figure 3c, d) and has idioblasts in the parenchyma (Figure 3d). Druses are observed in optical and polarized microscopy in the parenchyma and phloem of the two species (Figure 3c, d, g, h).

The mesophyll of both species, in cross-section visualized in optical microscopy, is dorsiventral, consisting of one to two layers of

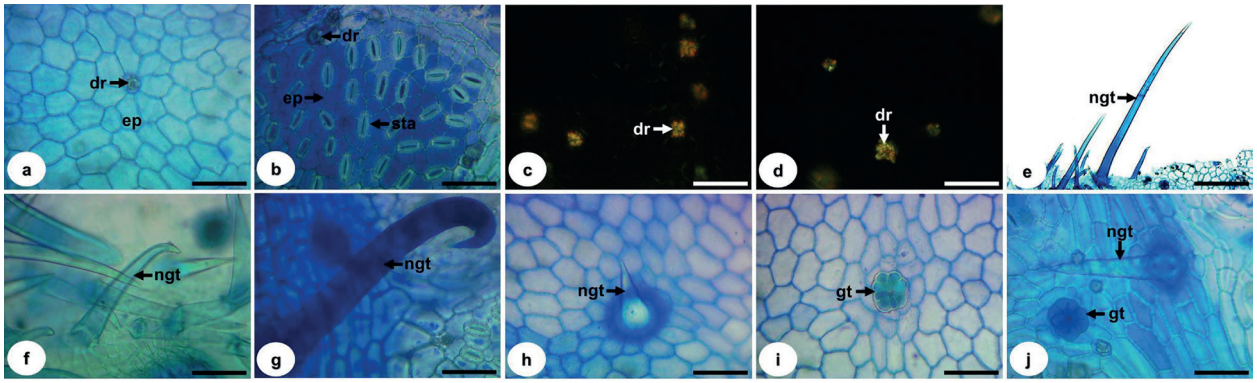

Figure 1 - Paradermal sections of the leaf blade of *Artocarpus altilis*. **a**, **b**, **e**, **f**, **g**, **h**, **i** and **j**: optical microscopy; **c** and **d**: polarized light microscopy; **a**, **c**, **e**, **f**, **h** and **i**: adaxial face; **b**, **d**, **g** and **j**: abaxial face. Abbreviations: dr = druse; ep = epidermis; gt = glandular trichome; ngt = non-glandular trichome; sta = stomata. Bars: **a**, **b**, **e**, **f**, **g**, **h**, **i** and **j** = 50 µm; **c** and **d** = 20 µm.

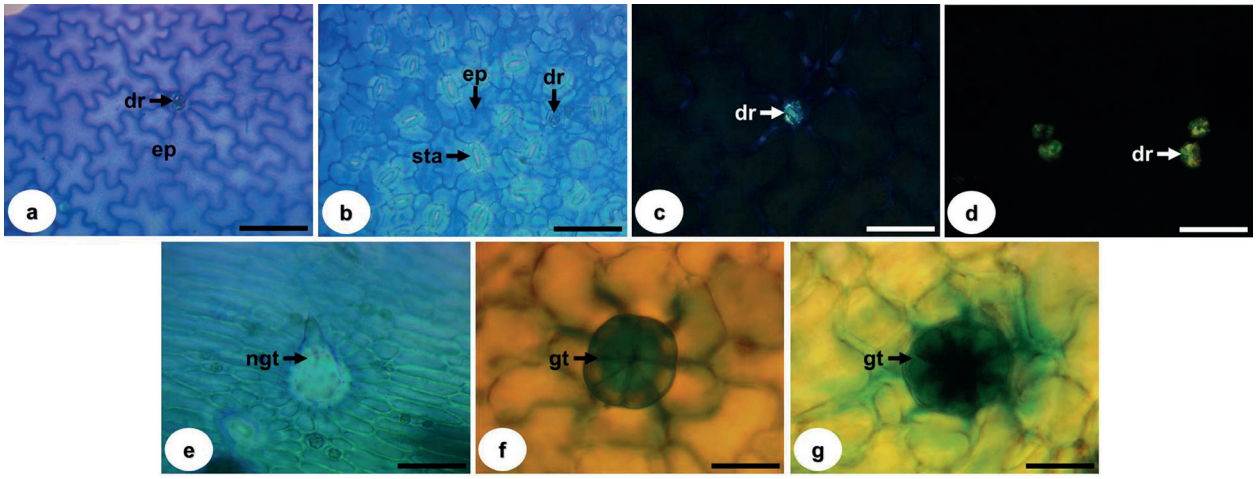

Figure 2 - Paradermal sections of the leaf blade of *Artocarpus heterophyllus*. **a**, **b**, **e**, **f** and **g**: optical microscopy; **c** and **d**: polarized light microscopy; **a** and **c**: adaxial face; **b**, **d**, **e**, **f**, and **g**: abaxial face. Abbreviations: dr = druse; ep = epidermis; gt = glandular trichome; ngt = non-glandular trichome; sta = stomata. Bars: **a**, **b** and **e** = 50 µm; **c**, **d**, **f** and **g** = 20 µm.

palisade parenchyma in *A. altilis* (Figure 3i) and of about three layers of palisade parenchyma in *A. heterophyllus* (Figure 3j). The spongy parenchyma consists of several layers of cells loosely arranged (Figure 3i, j). Small vascular bundles are distributed throughout the mesophyll and are protected by an irregular bundle sheath of parenchyma cells. This bundle sheath has extensions that always connect the two epidermal faces in *A. altilis*, which does not occur in *A. heterophyllus* (Figure 3i, j). Druses are observed in optical and polarized microscopy in the palisade parenchyma and in the bundle sheath (Figure 3k, l).

Table I shows the main anatomical characters of the two species.

Figure 4a and b shows cross-section of the leaf blade of *A. altilis* and Figure 4c and d shows cross-section of the leaf blade of *A. heterophyllus* without the addition of reagent. Phenolic compounds were found in the epidermis of *A. altilis* (Figure 4e) and in epidermis, idioblasts, palisade and spongy parenchyma of *A. heterophyllus* (Figure 4f-h). The presence of tannins was observed in the epidermis of *A. altilis* (Figure 4i) and in the idioblasts and palisade and spongy parenchyma of *A. heterophyllus* (Figure 4j, k).

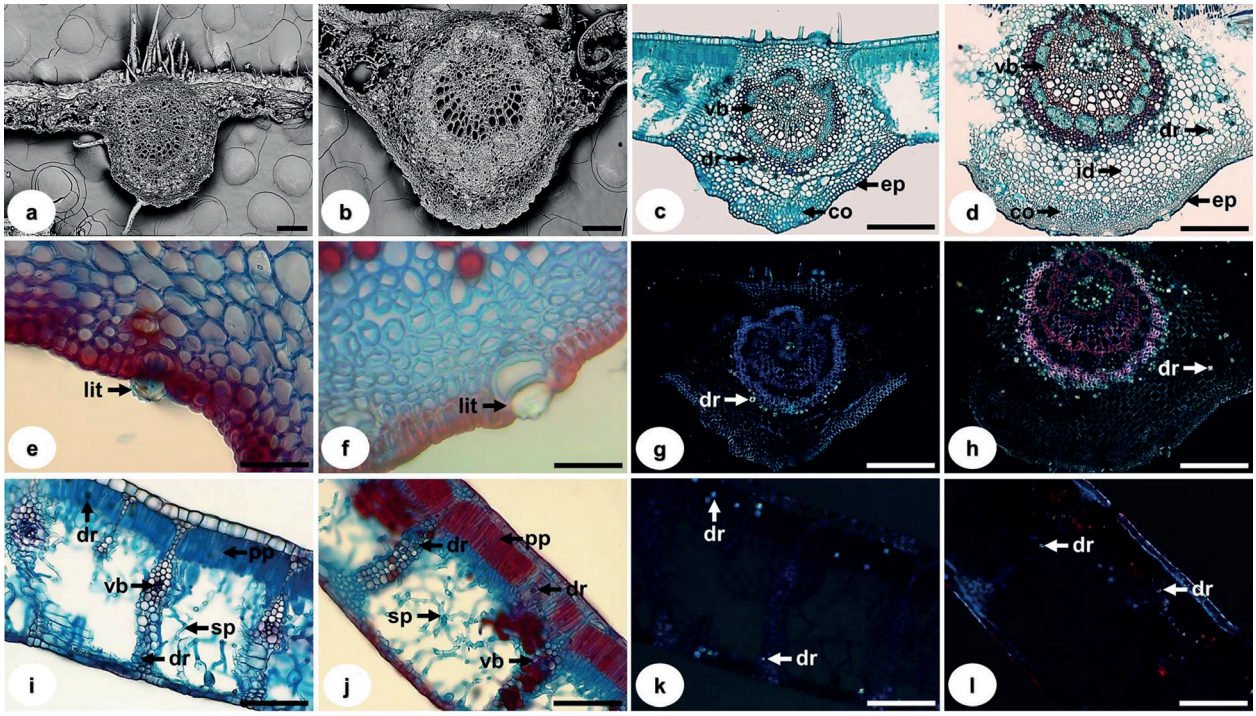

Figure 3 - Cross-sections of the leaf blades of *Artocarpus altilis* and *Artocapus heterophyllus*. **a**, **c**, **e**, **g**, **i** and **k**: *A. altilis*; **b**, **d**, **f**, **h**, **j** and **l**: *A. heterophyllus*; **a** and **b**: scanning electron microscopy; **c**, **d**, **e**, **f**, **i** and **j**: optical microscopy; **g**, **h**, **k** and **l** = polarized light microscopy. Abbreviations: co = collenchyma; dr = druse; ep = epidermis; id = idioblast; lit = lithocyst; pp = palisade parenchyma; sp = spongy parenchyma; vb = vascular bundle. Bars: **c**, **g**, **i**, **j**, **k** and **l** = 100 µm; **a**, **b**, **d** and **h** = 200 µm; **e** and **f** = 50 µm.

TABLE I
Summary of the major anatomical characters of *Artocarpus altilis* and *Artocarpus heterophyllus*.

	Artocarpus altilis	*Artocarpus heterophyllus*
Epidermis	Uniseriate, cells with straight or slightly sinuous walls on both sides.	Uniseriate, cells with sinuous walls on both sides.
Stomata	Anomocytic and actinocytic on the abaxial face.	Anomocytic and actinocytic on the abaxial face.
Trichomes	Adaxial face - non-glandular: simple, hooked and conical; glandular: multicellular, with eight or more cells. Abaxial face - non-glandular: hooked and conical; glandular: multicellular, with eight or more cells.	Adaxial face - glandular: multicellular, with six or more cells. Abaxial face - non-glandular: conical; glandular: multicellular, with six or more cells.
Druses	Epidermis, parenchyma of the midrib, phloem, palisade parenchyma and bundle sheath.	Epidermis, parenchyma of the midrib, phloem, palisade parenchyma and bundle sheath.
Lithocysts	Mainly in the regions of ribs.	Mainly in the regions of ribs.
Midrib	Biconvex shape.	Plane-convex to slightly biconvex shape.
Collenchyma	Two to four layers.	Three to seven layers.
Vascular bundle	Collateral associated with sclerenchyma.	Collateral associated with sclerenchyma.
Mesophyll	Dorsiventral, with one to two layers of palisade parenchyma and several layers of spongy parenchyma.	Dorsiventral, with three layers of palisade parenchyma and several layers of spongy parenchyma.

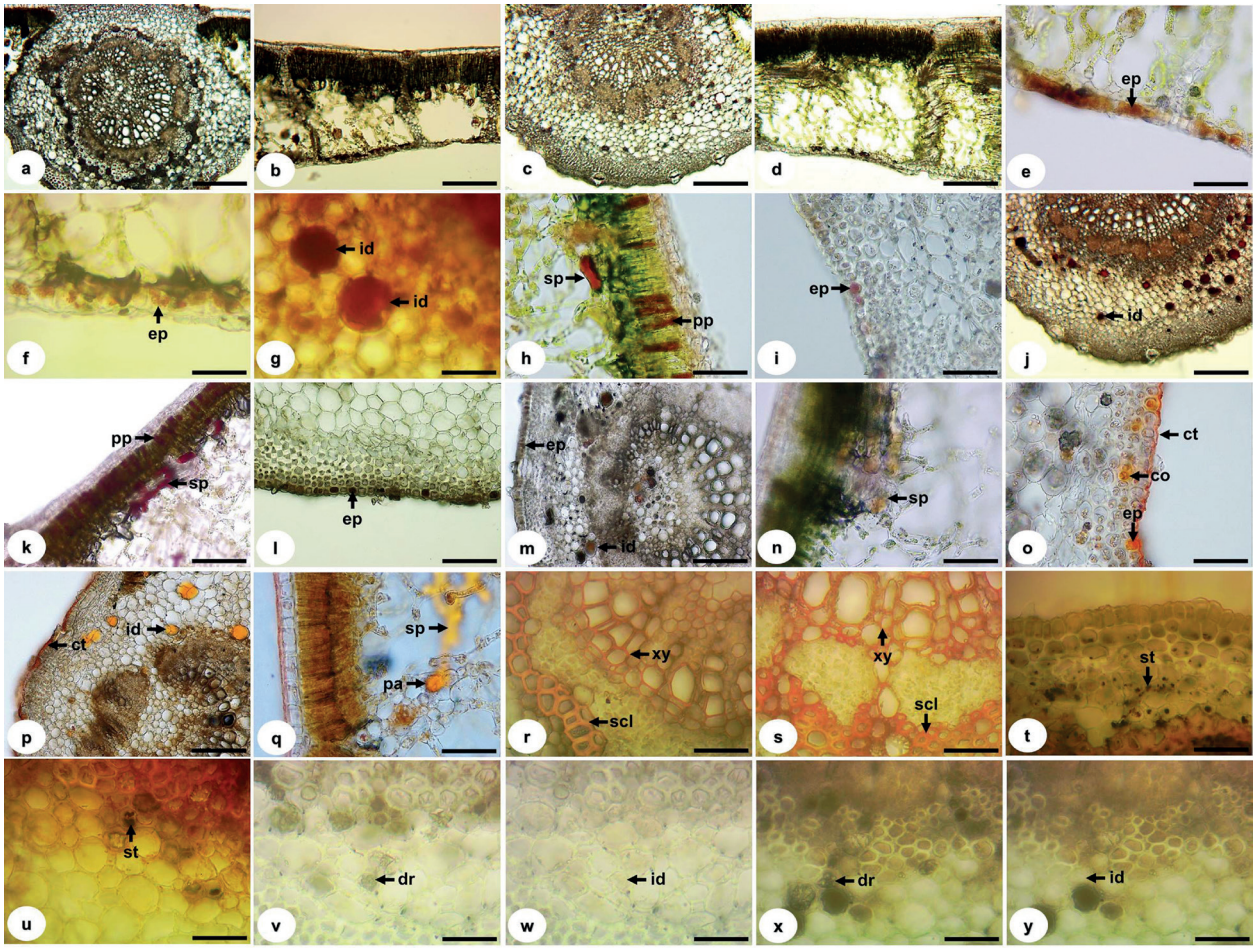

Figure 4 - Histochemistry of the leaf blades of *Artocarpus altilis* and *Artocapus heterophyllus*. **a**, **b**, **e**, **i**, **l**, **o**, **r**, **t**, **v** and **w**: *A. altilis*; **c**, **d**, **f**, **g**, **h**, **j**, **k**, **m**, **n**, **p**, **q**, **s**, **u**, **x** and **y**: *A. heterophyllus*; **a**, **b**, **c** and **d**: control; **e**, **f**, **g** and **h**: potassium dichromate (10%); **i**, **j** and **k**: vanillin chloridric; **l**, **m** and **n**: antimony trichloride; **o**, **p** and **q**: Sudan III; **r** and **s**: phloroglucinol; **t** and **u**: Lugol; **v**, **w**, **x** and **y**: hydrochloric acid (10 %). Abbreviations: co = collenchyma; ct = cuticle; dr = druse; ep = epidermis; id = idioblast; pa = parenchyma; pp = palisade parenchyma; scl = sclerenchyma; sp = spongy parenchyma; st = starch; xy = xylem. Bars: **a**, **b**, **c**, **d** and **j** = 200 µm; **i**, **k**, **l**, **m** and **p** = 100 µm; **e**, **f**, **g**, **h**, **n**, **o**, **q**, **r**, **s**, **t**, **u**, **v**, **w**, **x** and **y** = 50 µm.

Triterpenes and steroids were located in the epidermis of *A. altilis* (Figure 4l) and in the epidermis, idioblasts and spongy parenchyma of *A. heterophyllus* (Figure 4m, n). Lipophilic substances were revealed in the cuticle, epidermis and collenchyma of *A. altilis* (Figure 4o) and in cuticle, idioblasts, spongy parenchyma and bundle sheath in the mesophyll of *A. heterophyllus* (Figure 4p, q). In the two species were found lignin in the xylem and sclerenchyma (Figure 4r, s) and starch in the parenchyma (Figure 4t, u). Figure 4v and x shows the presence of druses in idioblasts and Figure 4w and y shows the dissolution of the druses with the test of hydrochloric acid (10%), indicating that they are of calcium oxalate. Tests with Dragendorff's reagent were negative.

Table II shows a summary of the histochemical characterization of the two species.

The chemical microanalyses performed by SEM-EDS in the druses present in the leaf blades of *A. altilis* (Figure 5a-c) and *A. heterophyllus* (Figure 5d-f) revealed peaks of absorbance for calcium, carbon and oxygen, confirming that they are formed of calcium oxalate.

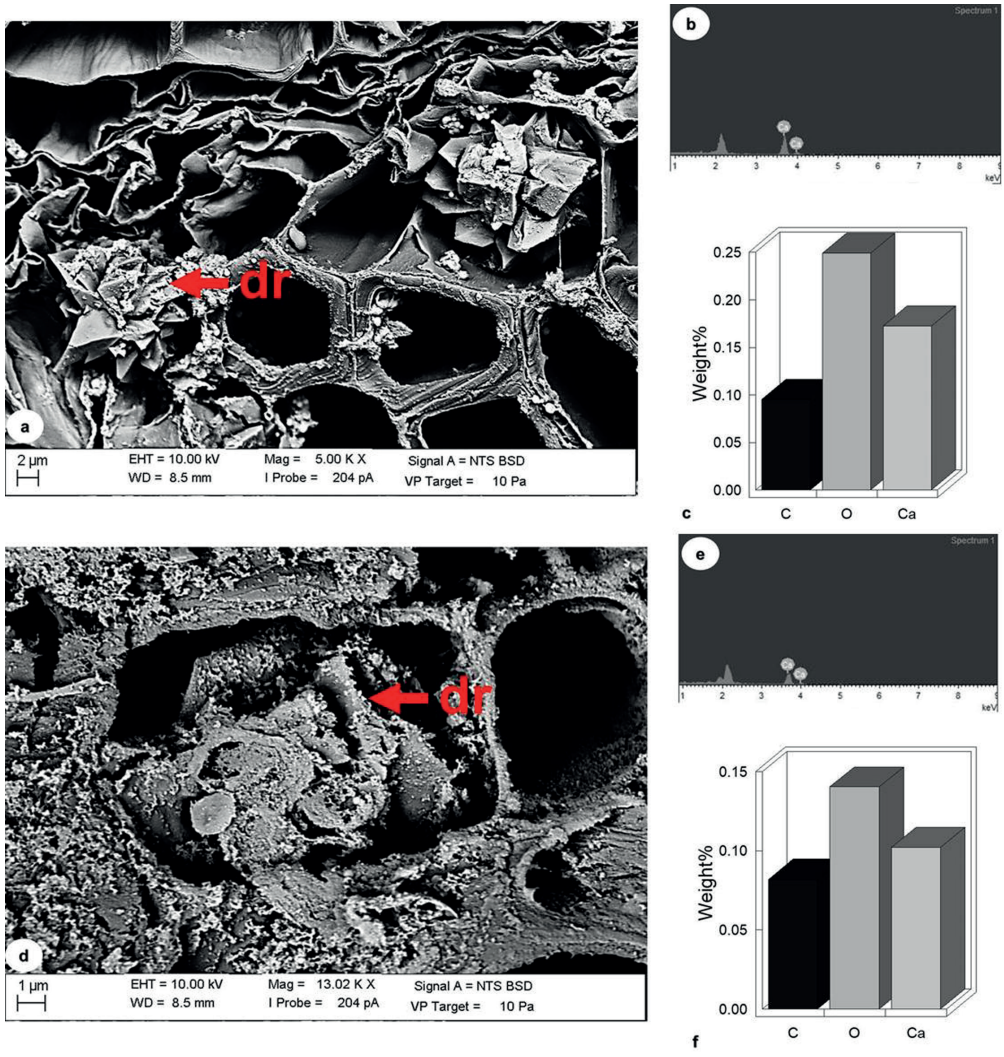

Figure 5 - Scanning electron micrograph and elemental composition of the druses observed in *Artocarpus altilis* and *Artocarpus heterophyllus*. **a**, **b** and **c**: *A. altilis*; **d**, **e** and **f**: *A. heterophyllus*; **a** and **d**: druses; **b** and **e**: analysis of elemental composition of the druses; **c** and **f**: percentage of the chemical constituents of the druses. Abbreviations: dr = druses.

TABLE II
Summary of the histochemical characterization of *Artocarpus altilis* and *Artocarpus heterophyllus*.

	Artocarpus altilis	*Artocarpus heterophyllus*
Phenolic compounds	Epidermis.	Epidermis, idioblasts, palisade and spongy parenchyma.
Tannins	Epidermis.	Idioblasts, palisade and spongy parenchyma.
Triterpenes and steroids	Epidermis.	Epidermis, idioblasts and spongy parenchyma.
Lipophilic substances	Cuticle, epidermis, and collenchyma.	Cuticle, idioblasts, spongy parenchyma and bundle sheath in the mesophyll.
Lignin	Xylem and sclerenchyma.	Xylem and sclerenchyma.
Starch	Parenchyma.	Parenchyma.
Druses	Calcium oxalate.	Calcium oxalate.
Alkaloids	Negative.	Negative.

DISCUSSION

According to Metcalfe and Chalk (1950), in the family Moraceae can be found anomocytic and anisocytic stomata. Gangadhara and Inamdar (1977) found anomocytic stomata in both species and actinocytic stomata only in *A. altilis*.

There are controversies in the literature regarding the types of trichomes found in the two species studied. Martínez (2008) stated that the leaf blade of *A. heterophyllus* is glabrous on the adaxial side. However, in the present study it was found glandular trichome in this face of the leaf blade of *A. heterophyllus*, as was also found by Gangadhara and Inamdar (1977) and Schnetzler et al. (2017). The types of non-glandular trichomes simple, hooked and conical were also described by Gangadhara and Inamdar (1977) for species of *Artocarpus*. Studying trichomes of 25 taxa of Urticales, these authors emphasized that the presence of glandular trichomes located in depressions is a diagnostic feature for *Artocarpus*.

The family Moraceae is known to have cystoliths. Metcalfe and Chalk (1950) stated that true cystoliths occur in *Broussonetia*, *Chlorophora*, *Conocephalus*, *Dammaropsis*, *Fatoua*, *Ficus*, *Malaisia*, *Morus*, *Poulsenia* and *Sparattosyce*, especially in the epidermis of the leaf. In species of *Antiaris*, *Artocarpus*, *Broussonetia*, *Cecropia* and *Parartocarpus* occur structures resembling cystoliths in the hairs.

Periyanayagam and Karthikeyan (2013) and Akinloye et al. (2015) observed druses in the same tissues found in the present study. Wu and Kuo-Huang (1997) described the presence of prismatic crystals in the bundle sheath of *A. altilis*, which was not observed in this study. These previous studies did not perform histochemical tests and chemical microanalyses to determine the chemical composition of the crystals.

The epidermis of Moraceae species usually consists of a single layer of cells, but may have two to three layers, as in *Ficus* species (Sonibare et al. 2006). According to Metcalfe and Chalk (1950), there are idioblasts of tannins in the leaf of *Artocarpus*. However, the present study has demonstrated the presence of other metabolites in idioblasts through histochemistry, such as phenolic compounds triterpenes, steroids and lipophilic substances. Histochemistry was also important to reveal that the two species of *Artocarpus* present the same types of metabolites, however, located in different tissues. Identification of the chemical composition of the crystals by histochemical test and by SEM-EDS is an important diagnostic feature for genus and species of Moraceae.

Thus, the new information of the chemical microanalyses and the histochemistry, allied to the anatomical description, show the characters of general occurrence in the family Moraceae and of those that allow the differentiation of *A. altilis* and *A. heterophyllus*.

ACKNOWLEDGMENTS

The authors are grateful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and to Programa Institucional de Bolsas de Iniciação Científica (PIBIC-UFPE)/Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support in the form of fellowship awards. They also thank CNPq for research funding (409452/016-5).

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