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Original Article

Anatomy, histochemistry and oxalic acid content of the leaflets of *Averrhoa bilimbi* and *Averrhoa carambola*

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ABSTRACT

Averrhoa bilimbi L. and *A. carambola* L., Oxalidaceae, are the only two species of the genus *Averrhoa* L. Their leaves are widely used in folk medicine as an adjuvant in the treatment of diabetes. Some species may contain, for example, calcium oxalate crystals, which may lead to risk in its use when there is predisposition of individuals with reduced renal activity. Therefore, there are still few studies on the content of oxalic acid present in them, highlighting the importance of this investigation. The objective of this work was to conduct a comparative anatomical and histochemical study between the species and determining its content of oxalic acid. Semipermanent histological slides were prepared, following common plant anatomy procedures, for analysis of the leaflets in optical microscopy, polarization and scanning electron microscopy coupled with energy-dispersive X-ray spectrometry. To determine the total, soluble and insoluble oxalate content was used titration with potassium permanganate. The anatomical characterization allowed identifying the characters useful in the differentiation of the species. The histochemistry revealed the location of the metabolites. Chemical microanalyses demonstrated that the crystals are of calcium oxalate. *A. carambola* presented the highest levels of total oxalate and soluble oxalate. The study assists in the identification and quality control of *A. bilimbi* and *A. carambola* and brings new data on its oxalic acid content, which are important, in view of the medicinal use of the species.

Introduction

The Oxalidaceae R. Br. family comprises eight genera and about 601 species (The Plant List, 2013), distributed in the tropical and subtropical regions of the world (Souza and Lorenzi, 2012). In Brazil, the family is represented by the genera *Averrhoa* L., *Biophytum* DC. and *Oxalis* L., comprising 103 species found in all regions of the country. The genus *Oxalis* is the most numerous, while the genus *Averrhoa* has only two species in Brazil flora, *A. bilimbi* L. and *A. carambola* L. (Abreu and Fiaschi, 2015). *A. bilimbi* is a perennial tree of 5–9 m in height, with little dense and low crown. In Brazil, it is popularly known as "azedinho", "bilimbi", "biri-biri" and "limão-caiena". *A. carambola* is also a perennial tree, but its crown is elongated and dense, with 5–10 m in height. It is popularly known as carambola and "fruta-estrela" (Lorenzi et al., 2015).

The leaves of these species are widely used in folk medicine for diabetes (Messias et al., 2015), disorders of the nervous system, colic (Rodrigues and Andrade, 2014), against urinary, kidney and liver diseases (Albuquerque et al., 2007; Agra et al., 2008). Scientific studies have proven the antidiabetic potential of leaf extracts from these plants (Tan et al., 2005; Ferreira et al., 2008; Daud et al., 2013; Putra et al., 2017).

The use of medicinal plants is commonly reported in the literature as an adjuvant in the treatment of diabetes (Santos et al., 2012). However, its popular use is not always done correctly in relation to the quality of the vegetable raw material and may still pose a serious risk to the health of the population, because they are composed of complex mixtures of substances (Leal and Tellis, 2015). Some species may contain, for example, calcium oxalate crystals (Franceschi and Horner, 1980) and there are still few studies on the content of oxalic acid present in them, which may lead to risk in its use when there is predisposition of individuals with reduced renal activity (Nakata, 2012). Some studies have shown that the absorption and excretion of a rich diet in oxalate can be considered as an

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important factor in the development of kidney stones (Siener et al., 2003; Holmes and Assimos, 2004).

Therefore, for the determination of the quality parameters related to these species and to increase the knowledge about the chemical compounds present in them, the objective of the study was to perform an anatomical and histochemical study of the leaflets of *A. bilimbi* and *A. carambola*, besides determining its oxalic acid content.

Materials and methods

Plant material

The botanical material of *Averrhoa bilimbi* L., and *A. carambola* L., Oxalidaceae, was collected in February 2017, by Rafaela Damasceno Sá and Alex Lucena de Vasconcelos, in the metropolitan region of Recife, Pernambuco, Brazil (8°04′03″S, 34°55′00″W). After drying according to standard herbarium techniques (Bridson and Forman, 1999), vouchers were deposited in the Herbarium Dárdano de Andrade Lima of the Instituto Agronômico de Pernambuco, under registration number 89750 for *A. bilimbi* and 89332 for *A. carambola*.

Anatomical characterization

For the anatomical characterization in optical microscopy and polarized light microscopy were used mature leaflets from the third to five node from three specimens of each species, fixed in FAA (formaldehyde, acetic acid and ethyl alcohol 50%) (Johansen, 1940). Cross-sections were obtained by hand, using a common razor blade, at the middle region of the leaflets. Paradermal sections were also performed on the adaxial and abaxial faces. The sections were subjected to decolorization with sodium hypochlorite solution 50% (Kraus and Arduin, 1997). Cross-sections were stained with safranin and astra blue (Bukatsch, 1972) and paradermal sections were stained with methylene blue 1% (Krauter, 1985). Semipermanent histological slides were prepared containing the sections, 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 digital camera (Leica ICC50W) coupled to an optical and polarized microscope (Leica DM750M).

For the anatomical characterization in Scanning Electron Microscopy (SEM), samples of fresh leaflets were fixed in 2.5% glutaraldehyde (buffered with 0.1 M sodium cacodylate) and post-fixed using 2% osmium tetroxide solution (buffered with 0.1 M sodium cacodylate). The material was submitted to dehydration in ethanol series and to critical point drying (Bal-Tec CPD 030), mounted onto SEM stubs, using double-sided adhesive tape and sputter-coated with gold (Leica EM SCD 500) (Haddad et al., 1998). The samples were examined with a scanning electron microscope (Quanta 200 FEG) in the Centro de Tecnologias Estratégicas do Nordeste (CETENE).

Histochemical characterization

Histochemical tests were made on cross-sections of fresh leaflets 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's iodine reagent 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 containing the cross-sections and were analyzed in optical microscope (Alltion microscope).

Analysis of the elemental composition of crystals

Cross-sections of leaflets were processed following the same methodology described for the analysis in SEM (Haddad et al., 1998). The chemical microanalyses by Energy Dispersive Spectroscopy (EDS) were done with X-ray detector attached to the Zeiss-EVO-LS15 scanning electron microscope.

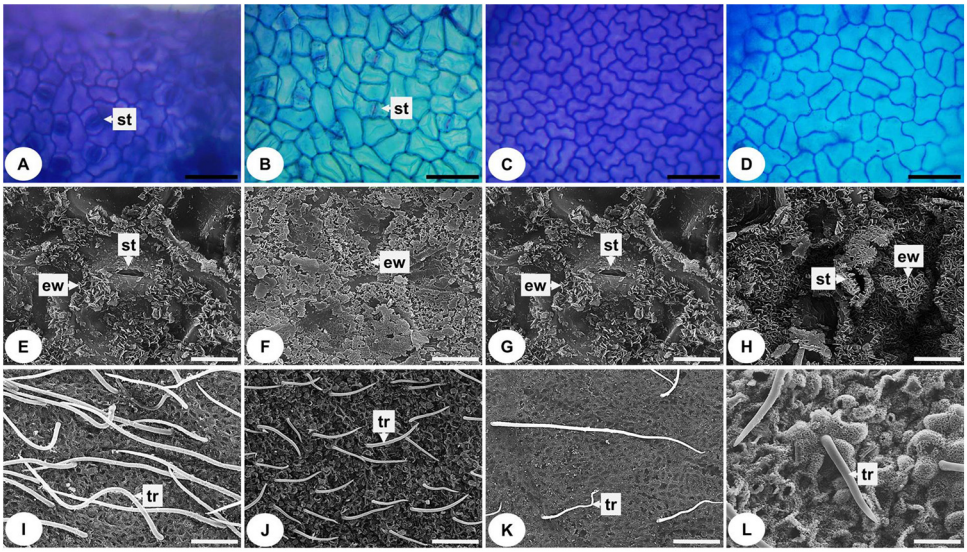

Fig. 1. Photomicrographs of the leaflets surface view of *Averrhoa bilimbi* and *A. carambola*. A, C, E, G, I, K: *Averrhoa bilimbi*. B, D, F, H, J, L: *Averrhoa carambola*. A–D: optical microscopy. E–L: scanning electron microscopy. A,B,E,F,I,J: surface view of the abaxial face. C, D, G, H, K, L: surface view of the adaxial side. ew: epicuticular wax; st: stomata; tr: trichome. Bars: A, B, C, D: 50 μm; E, F, G, H: 10 μm; I, J, K: 100 μm; L: 25 μm.

Determination of oxalic acid

Leaflets were oven dried at 60 °C for 48 h and powdered in a blender. For the determination of total oxalate and soluble oxalate, 0.5 g of the powder of the leaflets were weighed into 100 ml erlenmeyer flasks and 50 ml of 2 N hydrochloric acid (total oxalate) or distilled water (soluble oxalate) were added. The flasks were placed in a shaking water bath at 80 °C for 30 min. After filtration, the extracts were diluted with 50 ml of distilled water (Al-Wahsh et al., 2012). From each extract, 25 ml aliquots were taken for titrations with standard 0.02 mol l^{-1} potassium permanganate solution standardized against sodium oxalate. To acidify the medium of the extracts prepared with distilled water (soluble oxalate) were used 20 ml of 0.02 mol l^{-1} sulfuric acid. Titrations (in triplicate) were performed under heating at 50 °C and the turning was colorless to persistent pink for more than 30 s. The insoluble oxalate content was calculated by the difference between the total oxalate and the soluble oxalate. Oxalate concentrations were expressed as g/100 g dry matter.

Results

In surface view, under optical microscopy, the two species present hypoestomatic leaflets with paracytic stomata (Fig. 1A and B). The leaflet of *A. bilimbi* has epidermal cells with sinuous walls on both faces (Fig. 1A and C), while the leaflet of *A. carambola* has epidermal cells with walls that are straight to slightly sinuous in the abaxial face (Fig. 1B) and with slightly sinuous walls in the adaxial face (Fig. 1D). In SEM, it can be seen in detail that the epicuticular wax, which covers the epidermal cells in the two species, is of the squamous type, with crystalloids arranged in rosettes (Fig. 1E–H). It is also observed, in both species, the presence of simple filiform non-glandular trichomes on both faces (Fig. 1I–L), but, more abundant in the abaxial face (Fig. 1 I and J).

In cross-section, under SEM, the midrib of *A. bilimbi* shows a plane-convex shape (Fig. 2A) and the midrib of *A. carambola* shows a concave–convex shape (Fig. 2B). Under optical microscopy, the epidermis of both species is uniseriate, covered with thick cuticle (Fig. 2C and D). In *A. bilimbi*, below the epidermis of the adaxial face is about two layers of collenchyma, followed by a collateral vascular bundle in the shape of an arch, surrounded by sclerenchyma (Fig. 2C). In *A. carambola*, below the epidermis of the adaxial face is also about two layers of collenchyma, however, they are followed by three layers of palisade parenchyma (Fig. 2D). After the palisade parenchyma there is a collateral vascular bundle in the shape of an arch (Fig. 2D), but, as opposed to *A. bilimbi*, the vascular bundle in *A. carambola* is not surrounded by sclerenchyma. There are only a few isolated fibers close to the vascular bundle (Fig. 2D).

In the phloem of the two species, prismatic crystals are visualized under optical microscopy and polarized microscopy (Fig. 2C–F). The abaxial region of the midrib of the two species is composed of parenchyma (Fig. 2C and D), however, only in the parenchyma of *A. bilimbi* are visualized prismatic crystals (Fig. 2C and E).

The mesophyll of the species, in cross-section, under optical microscopy, is dorsiventral (Fig. 2G and H). In *A. bilimbi*, the palisade parenchyma is formed by two layers of cells (Fig. 2G) and in *A. carambola* the palisade parenchyma consists of three layers of cells (Fig. 2H). Prismatic crystals are found in the mesophyll of *A. bilimbi* in both palisade and spongy parenchyma (Fig. 2G and I), while in *A. carambola* the prismatic crystals predominate in the palisade parenchyma (Fig. 2H and J).

Fig. 3A and B shows cross-sections of the leaflets of *A. bilimbi* and the Fig. 3C and D shows cross-sections of the leaflets of *A. carambola* without addition of reagent. Phenolic compounds were found in the palisade parenchyma and spongy parenchyma of the two species (Fig. 3E and F). Tannins were evidenced in the phloem and in the parenchyma of the midrib in *A. bilimbi* (Fig. 3G) and in the palisade parenchyma and spongy parenchyma of *A. carambola* (Fig. 3H).

The presence of lipophilic compounds was observed in the cuticle of the two species (Fig. 3I and J), as well as the presence of lignin in the xylem (Fig. 3K and L) and starch in the parenchyma of the midrib (Fig. 3M and N). Fig. 3O and Q shows the presence of prismatic crystals in the leaflets of *A. bilimbi* and *A. carambola*, respectively, and the Fig. 3P and R shows the dissolution of the prismatic crystals with the test of hydrochloric acid 10%, indicating that

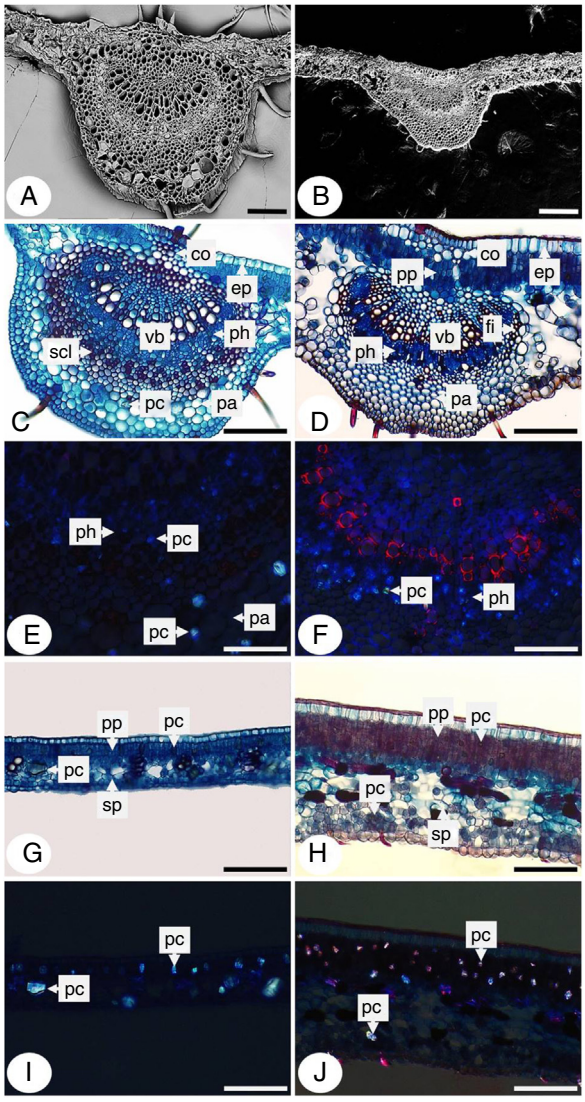

Fig. 2. Photomicrographs of the leaflets cross-sections of *Averrhoa bilimbi* and *A. carambola*. A, C, E, G, I: *Averrhoa bilimbi*. B, D, F, H, J: *Averrhoa carambola*. A and B: scanning electron microscopy. C, D, G, H: optical microscopy. E, F, I, J: polarized microscopy. A–F: midrib area. G–J: mesophyll area. co: collenchyma; ep: epidermis; fi: fiber; pa: parenchyma; pc: prismatic crystal; ph: phloem; pp: palisade parenchyma; scl: sclerenchyma; sp: spongy parenchyma; vb: vascular bundle. Bars: A: 100 μm; B: 300 μm; C, D, E, F, G, H, I, J: 100 μm; E, F: 50 μm.

Table 1
Average concentrations (g/100 g dry matter) of oxalates in leaflets of *Averrhoa bilimbi* and *A. carambola*.

Species	Total oxalate	Soluble oxalate	Insoluble oxalate
Averrhoa bilimbi	5.45 ± 0.28	3.38 ± 0.11	2.07
Averrhoa carambola	5.92 ± 0.47	4.87 ± 0.11	1.05

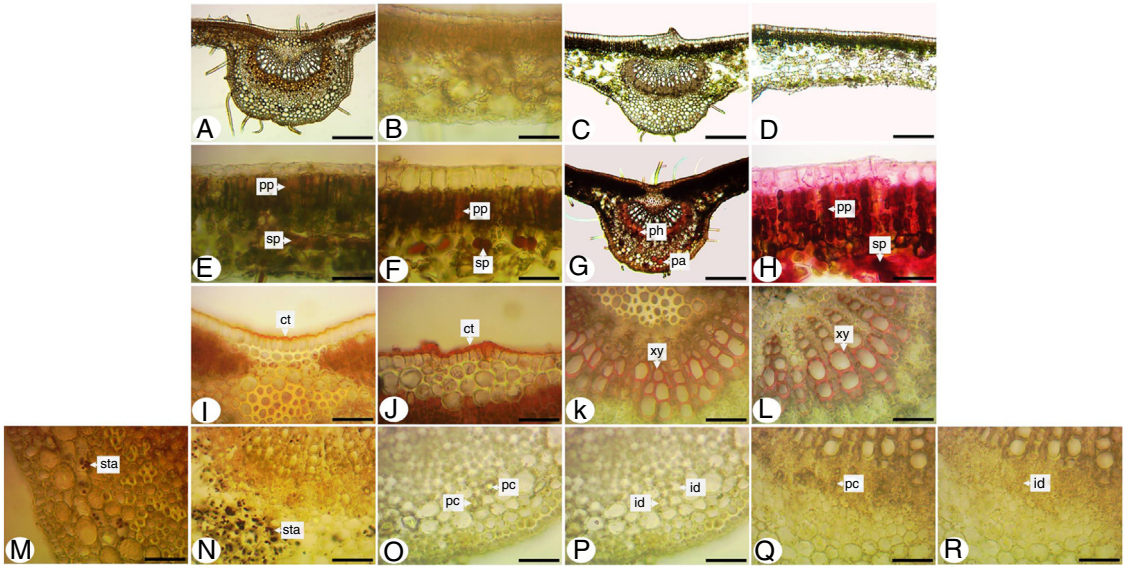

Fig. 3. Photomicrographs of the leaflets cross-sections of *Averrhoa bilimbi* and *A. carambola* and their histochemistry. A, B, E, G, I, K, M, O, P: *Averrhoa bilimbi*. C, D, F, H, J, L, N, Q, R: *Averrhoa carambola*. A–D: controls. E and F: potassium dichromate 10%. G and H: vanillin chloridric. I and J: Sudan III. K and L: phloroglucinol; M and N: Lugol's iodine reagent. O–R: hydrochloric acid 10%. ct: cuticle; id: idioblasts; pa: parenchyma; pc: prismatic crystal; ph: phloem; pp: palisade parenchyma; sp: spongy parenchyma; sta: starch; xy: xylem. Bars: A, C, D, G: 200 μm; B, E, F, H, I, J, K, L, M, N, O, P, Q, R: 50 μm.

they are of calcium oxalate. The tests for alkaloids, triterpenes and steroids were negative for both species.

The chemical microanalyses performed by SEM-EDS in the prismatic crystals present in the leaflets of *A. bilimbi* (Fig. 4A–C) and *A. carambola* (Fig. 4D–F) revealed peaks of absorbance for calcium, carbon and oxygen, confirming that they are formed of calcium oxalate.

The mean concentrations of oxalate determined in the leaflets of the species studied are shown in Table 1. The highest values of total oxalate and soluble oxalate were found in *A. carambola*, 5.92 ± 0.47 g/100 g dry matter and 4.87 ± 0.11 g/100 g dry matter, respectively.

Discussion

The two species presented, as common characters, the type of stomata and its position in the leaflet, the type of epicuticular wax, trichome, vascular bundle and the amount of collenchyma layers. According to Metcalfe and Chalk (1950), paracytic stomata are common in the genera *Averrhoa*, *Biophytum* and *Eichleria*. In the genus *Oxalis*, Jooste et al. (2016) found four types of stomata: anomocytic, anisocytic, actinocytic and an unusual 4-celled anisocytic stomatal type. The type of hypoestomatic leaflet was reported by Sunarti and Tihurua (2008) in species of *Averrhoa*. In *Oxalis* there are species with epiestomatic, hypoestomatic and amphistomatic leaflets (Jooste et al., 2016).

Epicuticular wax with crystalloids arranged in rosettes is also found in the families Fabaceae, Connaraceae, Malpighiaceae, Erythroxylaceae and Asteraceae (Ditsch and Barthlott, 1997). Second Barthlott et al. (1998), the types of epicuticular waxes are of great systematic significance. Regarding the type of trichomes, Sunarti and Tihurua (2008) observed non-glandular trichomes on both faces of the leaflet of *A. bilimbi* and only on the adaxial face of the leaflet of *A. carambola*, diverging from the result described in this study for *A. carambola*. Jorge et al. (2005) also determined that in the leaflet of *A. carambola* the non-glandular trichomes are present with more frequency in the abaxial face. Collateral vascular bundles are common in the Oxalidaceae family (Metcalfe and Chalk, 1950). Valsan and Raphael (2016) reported that the collenchyma in *A. bilimbi* is composed of five to six layers of cells.

The leaflet anatomy also revealed a distinct set of characters among the species, such as the epidermal pavement cell types, the midrib shape, the presence of sclerenchyma around the vascular bundle and the amount of layers of palisade parenchyma.

Reis and Alvim (2013) and Jooste et al. (2016) found that the epidermal pavement cell types and the amount of layers of palisade parenchyma are useful characters for the differentiation of *Oxalis* species. According to Jooste et al. (2016), the variability of the epidermal pavement cell types might be explained as a response to environmental factors. Guedes (2009) also observed a plane-convex shape in the midrib of *A. bilimbi*.

The presence of prismatic crystals is common in the Oxalidaceae family and, according to Metcalfe and Chalk (1950), they are generally cubic and solitary. Sunarti and Tihurua (2008) observed prismatic crystals in *A. bilimbi*, *A. carambola*, *A. dolichocarpa* Rugayah & Sunarti and *A. leucopetala* Rugayah & Sunarti. Guedes (2009) mentioned the presence of druses in the leaflet of *A. bilimbi*, which was not evidenced in the present study.

Guedes (2009) performed histochemical tests in the leaflets of *A. bilimbi* for starch and lipophilic compounds and their results were similar to ours. Positive phytochemical tests for phenolic compounds and terpenes corroborate the work of Azeem and Vrushabendraswami (2015) and Mewara et al. (2017). Valsan and Raphael (2016) detected phenolic compounds in the leaves of *A. bilimbi*, but did not observe terpenes. These authors and Mewara et al. (2017) obtained positive tests for alkaloids in the leaves of *A. bilimbi* and *A. carambola*, respectively. However, these two studies used different tests of which was used by us to detect alkaloids, which may explain the divergence of results.

No histochemical or chemical microanalyses performed by SEM-EDS were found in the literature to demonstrate the chemical nature of the crystals of the species studied as being of calcium oxalate. Biomineralization is a common process in plants and calcium minerals comprising about 50% of the known biominerals. Functions of cellular ion balance, osmotic regulation, vegetable defense against herbivory, tissue mechanic support, metal detoxification, capture and reflection of solar energy are attributed to calcium oxalate crystals (Franceschi and Horner, 1980; Franceschi and Nakata, 2005).

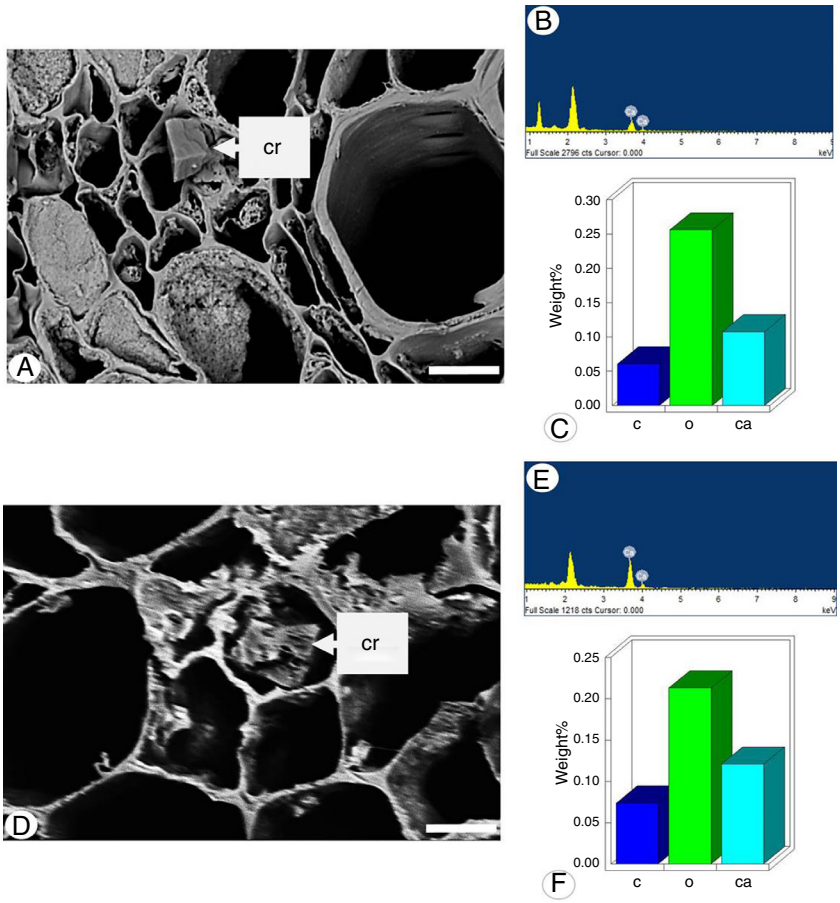

Fig. 4. Scanning electron micrograph and elemental composition of the crystals of the leaflets of *Averrhoa bilimbi*. and *A. carambola*. A, B, C: *Averrhoa bilimbi*. D, E, F: *Averrhoa carambola*. A, D: Crystal in the midrib. B,E: Analysis of elemental composition of the crystal. C, F: Percentage of the chemical constituents of the crystal. cr: crystal. Bars: A: 6 μm; D: 8 μm.

Oxalates can be found in amounts ranging from 3 to 80% of the dry weight of the plants (Nguyen and Savage, 2013). No data were found on the oxalate content in the leaves of the species studied. Wagner et al. (1975) determined the oxalate content in fruits of *A. carambola* of eighteen cultivars of the United States and found average levels (mg/100 g of fresh fruit) ranging from 39 mg to 679 mg. Joseph and Mendonça (1989) investigated the oxalate content in the green and ripe fruits of the sweet and sour of *A. carambola* and in the green and ripe fruits of *A. bilimbi* collected in Guyana. The highest levels of oxalate were found in the green fruits of both species. In the sour *A. carambola*, the average content (mg/100 g of fresh fruit) ranged from 3.79 to 5.9, In the sweet *A. carambola* ranged from 0.18 to 1.4 and in *A. bilimbi* ranged from 8.45 to 11.20.

According to Nakata (2012) the ingestion of juices and foods rich in oxalates may be risky in their use in individuals with reduced renal activity. There are reports in the literature of cases of renal lesions in patients caused by the ingestion of fruit juices from *A. bilimbi* and *A. carambola* and the recommendation is to avoid the consumption of these fruits (Nair et al., 2014; Paschoalin et al., 2014; Scaranello et al., 2014; Vanelli et al., 2014; Oliveira and Aguiar, 2015).

Studies have shown that oxalate contents vary in different parts of the plant, but that, generally, the highest concentrations are found in leaves (Siener et al., 2006; Huang et al., 2015). Thus, in view of the high concentrations of oxalate found, the present study also warns the use of leaves of *A. bilimbi* and *A. carambola* by patients with renal impairment.

Conclusion

The results obtained are of great importance, since these species are medicinally used by people with renal impairment and, in a pioneer way, this study brings data referring to the quantitative of oxalic acid in the leaves, besides contributing with the anatomy and histochemistry differential of the two single species of the genus *Averrhoa*, which may aid in their identification.

Authors' contributions

RDS and ALV contributed in collecting plant sample, confection of a voucher specimen, running the laboratory work, analysis of the data and drafted the paper. AVS, RJRP, LCA and LALS contributed in analysis of the data. KPR designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

Authors declare no conflicts of interest.

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