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Content of phenolic compounds in monofloral aroeira honey and in floral nectary tissue

Abstract – The objective of this work was to quantify the content of total phenolic compounds in monofloral honey from aroeira (*Astronium urundeuva*) trees and to verify, through histochemical tests, if these compounds are present in the floral tissues. The apiary, with *Apis mellifera* bees, was installed among aroeira trees in the semiarid region of the state of Minas Gerais, Brazil. From the anatomy of the flowers and of the inflorescence rachis, an ornamented epidermis, tector and glandular trichomes, idioblasts, and a developed secretory system were observed. Aroeira honey has an average phenolic content of 142.5±22.6 mg 100 g^{-1}, a value considered very high when compared with those of other monofloral honeys from Brazil and around the world. Histochemical tests detected the presence of phenolic substances in the idioblasts and secretory ducts associated with the phloem in the floral tissues, especially in the nectar parenchyma, epidermis, and glandular trichomes. Phenolic compounds are present in the floral tissue of both floral morphs, mainly in the nectary where honeybees collect nectar. The obtained results are the first, in the literature, indicative that the phenolic compounds produced by aroeira trees are transferred through the nectar to the honey. This study contributes to the establishment of quality standards for monofloral aroeira honey and to the identification of its botanical origin.

Index terms: *Apis mellifera*, *Astronium urundeuva*, Africanized honeybee, histochemical tests.

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Conteúdo de compostos fenólicos em mel monofloral de aroeira e no tecido do nectário floral

Resumo – O objetivo deste trabalho foi quantificar os compostos fenólicos totais no mel monofloral de aroeira (*Astronium urundeuva*) e verificar, por meio de testes histoquímicos, se estes compostos estão presentes nos tecidos florais. O apiário, com abelhas *Apis mellifera*, foi instalado em meio a aroeiras, na região do semiárido do estado de Minas Gerais, Brasil. A partir da anatomia das flores e da raque da inflorescência, observou-se epiderme ornamentada, tricomas tectores e glandulares, idioblastos e sistema secretor desenvolvido. O mel de aroeira apresenta, em média, 142.5±22.6 mg 100 g^{-1} de compostos fenólicos totais, valor considerado muito elevado quando comparado aos de outros méis monoflorais provenientes do Brasil e do mundo. Os testes histoquímicos detectaram a presença de substâncias fenólicas nos idioblastos e nos ductos secretores associados ao floema nos tecidos florais, especialmente no parênquima nectarífero, na epiderme e nos tricomas glandulares. Os compostos fenólicos estão presentes no tecido floral de ambos os morfos florais, principalmente no nectário onde as abelhas coletam o néctar. Os resultados obtidos são os primeiros, na literatura, indicativos de que os compostos fenólicos produzidos pelas árvores de aroeira são transferidos através do néctar para o mel. Este estudo contribui para o

estabelecimento de padrões de qualidade do mel de aroeira e para a identificação da sua origem botânica.

Termos para indexação: *Apis mellifera*, *Astronium urundeuva*, abelha africanizada, testes histoquímicos.

Introduction

Honey produced mainly from a single plant species is termed monofloral and is characterized by particular organoleptic, microscopic, and physicochemical properties, as well as by a higher market value than heterofloral honey, obtained from more than one origin (Thrasyvoulou et al., 2018). The honey produced by honeybees from the nectar of flowers of the aroeira [*Astronium urundeuva* Engl. (Syn. *Myracrodruon urundeuva* Allemão)] (Anacardiaceae) tree is considered monofloral (Calaça et al., 2018). This honey has unique microscopic and organoleptic characteristics, such as flavor and aroma, and also a striking amber or dark-amber color and low moisture values (Bastos et al., 2016; Santos et al., 2018; Oliveira & Bendini, 2021).

In 2022, the Brazilian National Institute of Industrial Property granted a geographical indication certification to the honey produced by *Apis mellifera* Linnaeus honeybees visiting flowers of aroeira trees in the northern region of the state of Minas Gerais, Brazil. This honey is produced in the Brazilian seasonally dry tropical forest (SDTF) belt mostly by backyard beekeepers during the dry season of the year (Demier et al., 2020), representing a valuable economic activity in this semiarid region since other livestock and agricultural activities are affected by drought.

The aroeira is a native mass-flowering dioecious tree (Mitchell & Daly, 2017) that occurs in SDTFs, where its natural populations are abundant. In Brazil, the species is found in the Caatinga, Cerrado, and in some sites of the Atlantic Rain Forest (Domingos & Silva, 2020). The aroeira blooms during the dry season, when honeybees intensively visit its flowers to collect nectar (Calaça et al., 2018, 2022).

Aroeira is widely used in folk medicine due to its several compounds, especially tannins and other phenolic compounds (Domingos & Silva, 2020). Different parts of the aroeira plant – wood, bark, and/or leaves – have analgesic, antibacterial, antifungal, antioxidant, anti-inflammatory, anti-ulcer, antiviral, larvicidal, and termite-repellent activities (Domingos & Silva, 2020). Therefore, in some honeys, the phenolic compounds and flavonoids that are part of their composition and that are related to their biological activities might be of plant origin (Consonni & Cagliani, 2015; Machado et al., 2020). Escuredo et al. (2013) highlighted the possible association of phenolic compounds with the nutritional value and therapeutic properties of a honey (Escuredo et al., 2013). Some monofloral honeys, such as those from the aroeira and assa-peixe [*Cyrtocymura scorpioides* (Lam.) H.Rob. (Syn. *Vernonia scorpioides* (Lam) Pers] trees, have shown a remarkable antioxidant activity and an increased amount of phenolic compounds and flavonoids (Pena Júnior et al., 2022).

To date, the histochemical studies of the vegetative parts of the aroeira tree have focused on: leaves, showing the presence of starch granules, calcium oxalate crystals, fatty compounds, resins, phenols, and alkaloids in the tissue (Nascimento-Silva et al., 2011); and bark, characterized by the presence of secretory ducts and dead phloem between periderms, with large amounts of phenols in all tissues (Sousa et al., 2022). However, there are no known works about the presence and location of these substances in the floral tissues of aroeira, particularly in the nectary, where honeybees collect nectar to produce honey.

The objective of this work was to quantify the content of total phenolic compounds in monofloral honey from aroeira trees and to verify, through histochemical tests, if these compounds are present in the floral tissues.

Materials and Methods

The study was carried out in a well-preserved SDTF fragment in the municipality of Janaúba, in the state of Minas Gerais, Brazil (43°31'06"W, 15°40'34"S), covering a total area of 5.9 km^2, with a mean annual precipitation of 730.46 mm and an annual temperature ranging from 15.2 to 40.2°C (INMET, 2018). The experimental apiary was installed in the dense aroeira populations formed in the fragment. To guarantee that only aroeira honey was produced, colonies of *A. mellifera* were monitored throughout the entire flowering period of the tree each year (Calaça et al., 2018). To measure total phenolic compounds, nine monofloral aroeira honey samples were harvested from the apiary – three in July 2016, four in June 2017, one in June 2018, and one in June 2019.

To ensure that all samples were from monofloral aroeira honey, a melissopalynological analysis was performed at Fundação Ezequiel Dias according to Louveaux et al. (1978). The honey samples had pollen spectra with more than 93.2% pollen from the aroeira tree (Table 1) as established in Calaça et al. (2018). For pollen identification, on slides, under the Olympus BX50 light microscope (Olympus Optical, São Paulo, SP, Brazil), at 400–100x magnification, the obtained pollen was compared with the collection of reference pollen grains from plant species of the SDTF fragment where the honey samples were harvested.

Inflorescences were collected from aroeira trees at the same site to determine the presence of phenolic compounds in the floral tissues and to carry out the anatomy and histochemical tests. One inflorescence was collected from every ten male and ten female trees at the study site. From each inflorescence, the rachis and four flower buds – two for the anatomical analysis and two for the histochemical tests – were analyzed. Fertile branches were herborized, identified, and deposited at the herbarium at Fundação Zoobotânica de Belo Horizonte under accession number BHZB11912. Fresh flower material was stored in liquid nitrogen or ethanolic series 70%. As in Calaça et al. (2022), female and male flowers designate pistillate and staminate flowers, respectively.

For the anatomical and histochemical studies, flowers were dehydrated in an alcoholic series 70 to 95%, embedded in Paraplast, and sectioned in transverse and longitudinal sections of 4.0 to 7.0 µm thicknesses using a rotary microtome. Then, the sections were stained with Safranin and Astra Blue in an aqueous solution (Johansen, 1940) and mounted on slides with Entellan. Anatomical cuts were made on the inflorescences and flower buds embedded in Paraplast (Figure 1), and histochemical tests were performed for the detection of phenolic compounds using ferric trichloride (Johansen, 1940) and of tannins and flavonoids using vanillin-hydrochloric acid (Mace & Howell, 1974). Semipermanent slides of freehand sections of fresh material were mounted on glycerinated jelly. Relevant results were documented with the Olympus BX50 microscope (Olympus Optical, São Paulo, SP, Brazil), using the ImagePro Plus, version 10.0, software (Media Cybernetics, Rockville, Maryland, USA). Permissions for the collection of plant and honey samples were obtained from Instituto Chico Mendes de Conservação da Biodiversidade

Table 1. Melissopalynological analysis of nine aroeira (*Astronium urundeuva*) honey samples, identified as monofloral.

Sample ID	Aroeira pollen type (%)	Other pollen types (%)
1	93.3	6.7
2	97.5	2.5
3	98.8	1.2
4	99.4	0.6
5	98.9	1.1
6	95.9	4.1
7	99.0	1.0
8	99.3	0.7
9	99.2	0.8

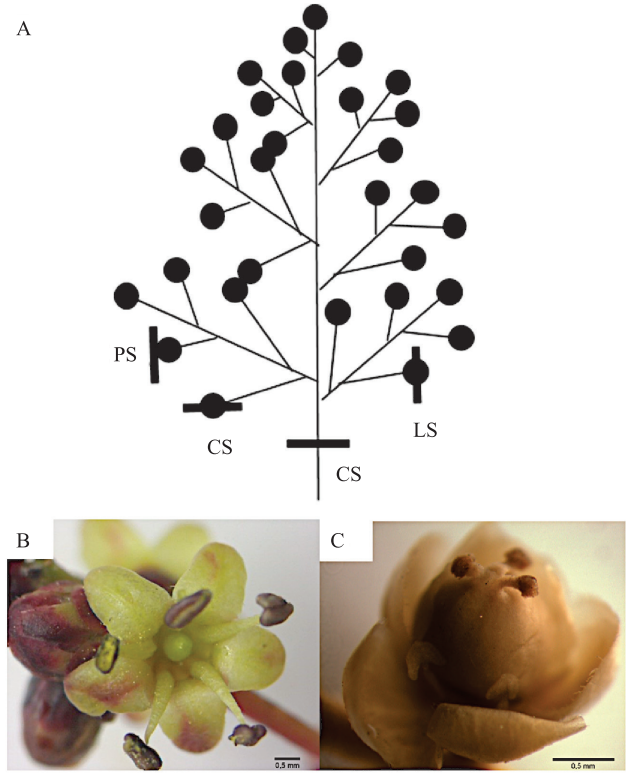

Figure 1. Representation of the inflorescence of the aroeira (*Astronium urundeuva*) tree, showing types and locations of anatomical cuts (A), as well as male (B) and female (C) flowers. PS, paradermic section; CS, cross section; and LS, longitudinal section. Representation (A) and photos (B and C) by Lívia Cristina de Paiva Gardoni and Paula Calaça, respectively.

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The total phenolic compounds of all nine honey samples were quantified using the Folin & Ciocalteu method (Singleton et al., 1999), and the results were compared with those from the literature. A sample of 1.0 g honey was weighed and diluted with 10 mL Milli-Q water in a volumetric flask. Afterwards, the solution was filtered and transferred to test tubes in triplicate. A volume of 2.5 mL of 0.2 N Folin & Ciocalteu phenol reagent (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was added to the tubes, homogenized, and allowed to settle for 5 to 8 min. After this time, 2.0 mL of a 75 g L^{-1} sodium carbonate solution were added to the samples, homogenized again, and allowed to rest for 2 hours. Absorbance was measured using the UV-1650PC spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at 760 nm, with ultrapure water as a blank. A calibration curve was constructed for total phenolic compounds from a 1.0 g L^{-1} standard solution of tannic acid (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). The points on the curve were: 50, 100, 150, 200, and 250 µg mL^{-1}. A volume of 500 µL of each standard solution was pipetted and transferred to test tubes in triplicate, following the same steps as for the evaluated samples. The standard curve had a coefficient of determination of 99.8%. Total phenolic values were expressed in mg 100 g^{-1} honey and then compared with those obtained for other monofloral honeys also quantified by the Folin & Ciocalteu method in different studies (Al-Mamary et al., 2002; Meda et al., 2005; Kaškonienė et al., 2009; Tuberoso et al., 2009; Lianda et al., 2012; Pena Júnior et al., 2022).

Results and Discussion

The average content of total phenolic compounds in aroeira honey was 142.5±22.6 mg 100 g^{-1} honey, with minimum and maximum values of 107.2 and 178.5 mg 100 g^{-1}, respectively. Compared with other monofloral honeys in the literature, except manuka honey [*Leptospermum* sp. (Myrtaceae)] from New Zealand and Salam tree (*Acacia ehrenbergiana* Hayne) honey from Yemen, aroeira honey showed the highest average total phenolic contents (Table 2).

The average total phenolic contents in aroeira honey is, therefore, higher than those of other honeys worldwide, with values of 12.16–117.38 mg 100 g^{-1} in Europe (Kus et al., 2014), 1.33±0.00–155.16±0.98 mg 100 g^{-1} in Asia (Noor et al., 2014), 2.87–114.7 mg 100 g^{-1} in Africa (Idris et al., 2011), and 58.26–152.52 mg 100 g^{-1} in Brazil (Sant'Ana et al., 2014). Moreover, the total phenolic content of the studied aroeira honey is threefold higher than that of orange blossom honey, which is widely consumed in the Brazilian territory (Lianda et al., 2012).

In the anatomical analysis, the aroeira flower showed an ornamented epidermis due to a striated cuticle (Figure 2 A). The epidermis of the rachis region of the inflorescence was formed by tector and glandular trichomes (Figure 2 B and C), followed by collenchyma and parenchyma layers. The presence of idioblasts and of a large number of secretory ducts was also observed next to the phloem in the medulla (Figure 2 B, D, and F). According to Lima et al. (2017), trichomes in the reproductive organs can protect plants from herbivores and pathogens by acting as physical and chemical defenses. The analyzed flower parts also had a significant amount of calcium oxalate in form of druse, as already found for leaves (Nascimento-Silva et al., 2011).

Another detected structure was an evolved secretory system made up of secretory ducts found near or in association with the phloem used to transport compounds, including those from the plant specialized metabolism (Figure 2 D).

The tissues of the floral parts of both floral morphs showed similar anatomical features: striated cuticle, epidermis with tector trichomes at the margins, and idioblasts in the mesophyll (Figure 2 E, F, and G). In male and female flowers, numerous secretory ducts and idioblasts were present mainly in the region of the nectariferous disk (Figure 2 H, J, and K) since idioblasts and secretory ducts are characteristic of the family Anacardiaceae, to which aroeira belongs (Sousa et al., 2022). Furthermore, in species of the genus *Astronium*, schizolysigenous ducts are associated with the phloem, extending to the dead phloem in the outer bark and to leaves, as observed by Sousa et al. (2022) and Nascimento-Silva et al. (2011), respectively, as well as to flowers, as reported, for the first time in the present study, in inflorescences of the aroeira tree.

The nectary disk, in male flowers, is intrastaminal, has five lobes, and covers internally the apex of the receptaculum, except in the central region where a pistillode is located (Figure 1 B and Figure 2 I); in female flowers, however, the disk surrounds the base of the ovary (Figure 2 H). In both flower morphs, the epidermis of the nectary consists of a layer of rectangular and glabrous cells with numerous modified stomata from which secretions are discharged into the lumen. Beneath the epidermis are several layers of nectary parenchyma with typical small cells – with thin walls and a dense protoplast – that were intensely stained with Safranin and Astra Blue, which showed the secretory nature of the tissue (Figure 2 L). The secretory ducts, nectary tissue, idioblasts, epidermis, and glandular trichomes of the floral structures had a positive reaction to ferric chloride for total phenolic compounds (Figure 2 C and D and Figure 3 A, B,

Table 2. Total phenolic compounds in monofloral honeys from Brazil and around the world evaluated in different studies.

Study	Honey name	Honey botanical origin	Country	Mean ± SD[(1)] (mg 100 g^{-1})
Present study	Aroeira	*Astronium urundeuva* (Anacardiaceae)	Brazil	142.5±22.6
Pena Júnior et al. (2022)	Aroeira	*Astronium urundeuva* (Anacardiaceae)[(2)]	Brazil	76.83±15.26
	Assa-peixe	*Cyrtocymura scorpioides* (Syn.: *Vernonia scorpioides*) (Asteraceae)		107.93±1.12
	Betonca	*Hyptis* sp. (Lamiaceae)		62.95±2.11
	Caiaté	*Omphalea diandra* (Euphorbiaceae)		42.93±2.25
	Candeinha	*Eremanthus incanus* (Asteraceae)		51.38±2.26
	Cipó-uva	*Serjania lethalis* (Sapindaceae)		42.93±3.29
	Eucalyptus	*Eucalyptus robusta* (Myrtaceae)[(2)]		68.74±6.74
	Manuka	*Leptospermum* spp. (Myrtaceae)		141.73±2.37
	Pequi	*Caryocar brasiliense* (Caryocaraceae)		48.82±**1.33**
	Velame	*Croton urucurana* (Euphorbiaceae)		45.52±**0.52**
Meda et al. (2005)	Acacia	*Acacia* (Fabaceae)	Burkina Faso	93.4±0.87
		Combretaceae		55.9±0.83
		Lannea (Anacardiaceae)		43.0±0.63
		Vitellaria (Sapotaceae)		86.7±0.70
Al-Mamary et al. (2002)	Acacia	*Acacia edgeworthii* (Fabaceae)	Yemen	100.1±2.80
	Acacia	*Acacia ehrenbergiana* (Fabaceae)		246.2±0.97
	-	*Ziziphus* (Rhamnaceae)[(3)]		131.8±6.20
	-	*Ziziphus* (Rhamnaceae)[(3)]		98.5±2.24
	Orange	*Citrus* spp. (Rutaceae)	USA	61.1±2.60
Tuberoso et al. (2009)	Asphodel	*Asphodelus microcarpus* (Asphodelaceae)	Italy	30.1±1.31
Kaškonienė et al. (2009)	Rape	*Brassica napus* (Brassicaceae)	Poland	7.2±0.10
	Buckwheat	*Fagopyrum esculentum* (Polygonaceae)		20.2±1.70
	Heather	*Calluna vulgaris* (Ericaceae)		20.1±0.60
	Lime tree	*Tilia* sp. (Malvaceae)		15.3±0.60
Venugopal & Devarajan (2011)	Manuka	*Leptospermum* spp (Myrtaceae)	New Zealand	161.3±0.88
Lianda et al. (2012)	Orange	*Citrus* spp. (Rutaceae)	Brazil	40.4±2.72

[(1)]Standard deviation. [(2)]Mean and standard deviation of six aroeira and two eucalyptus honey samples. [(3)]Honeys with the same botanical origin, but from different locations in Yemen.

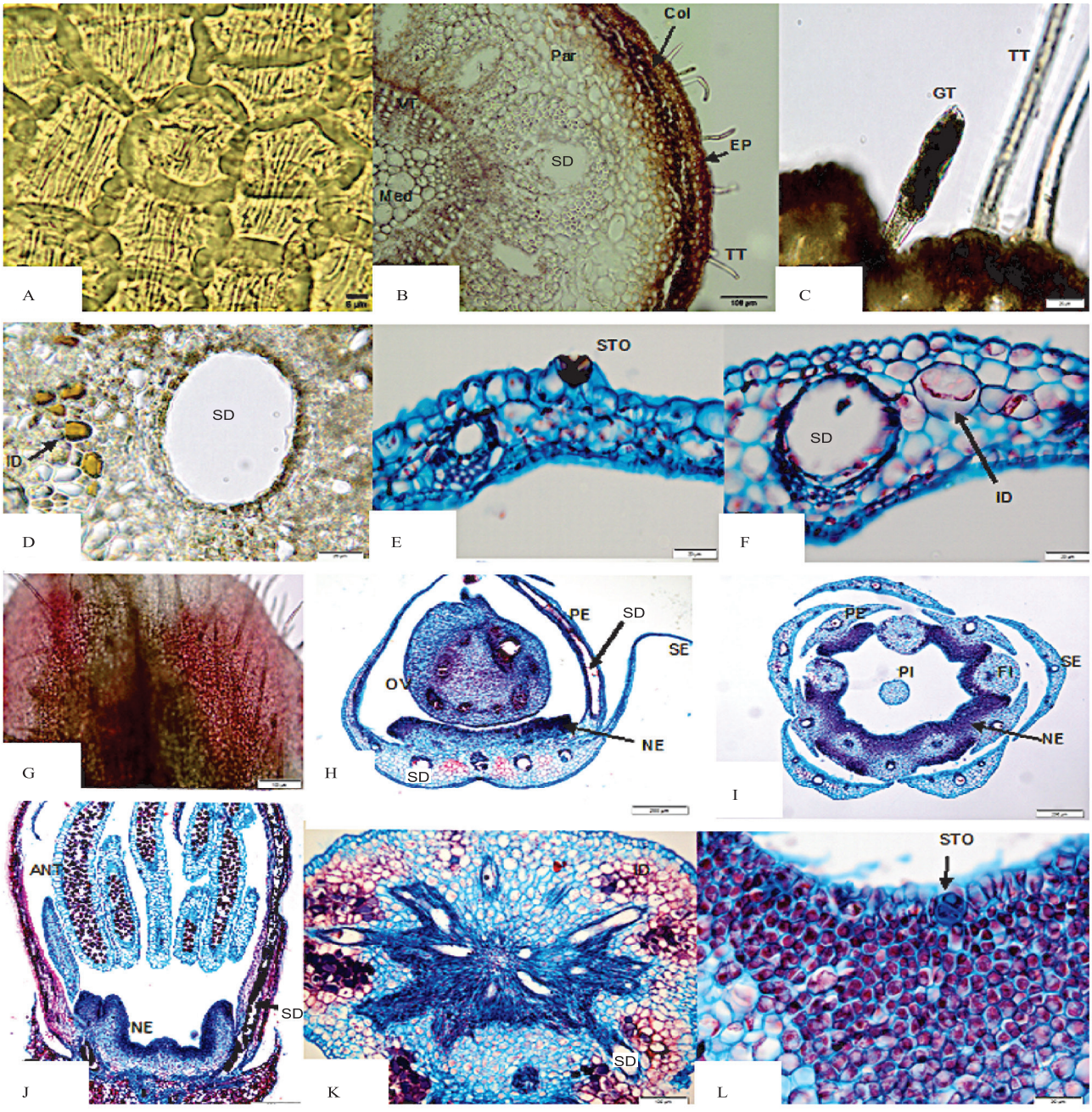

Figure 2. Histochemistry of male and female inflorescences of the aroeira (*Astronium urundeuva*) tree, showing: cuticle striation in a paradermical section of the sepal (A); cross section of the rachis (B); tector and glandular trichomes with a positive result for phenolic compounds in the rachis (C); secretory duct and idioblasts in the rachis, colored by the phenolic test (D); detail of the sepal (E); detail of the petal in cross section (F); positive reaction to vanillin in the sepal (G); longitudinal (H) and cross section (I) of a female bud; longitudinal section of a male bud (J); cross section on the base of a female bud, near the nectary, showing innervation (K); and detail of the nectary tissue (L). Med, medulla; SD, secretory duct; Par, parenchyma; Col, collenchyma; EP, epidermis; TT, tector trichome; GT, glandular trichome; ID, idioblast; STO, stomata; OV, ovary; NE, nectary; SE, sepal; PE, petal; PI, pistillode; FI, fillet; and ANT, anther. Photomicrography obtained by optical microscopy, with 200 to 400x magnification; sections were stained with Safranin and Astra Blue in an aqueous solution (Johansen, 1940).

C, and D). The epidermis of the floral parts and the glandular trichomes also had a positive reaction but to flavonoids (Figure 3 E and F). Tannins were found in the anthers and mesophyll of the sepals and petals (Figure 2 G).

The floral anatomy and histochemical tests evidenced the presence of phenolic compound contents in: floral tissues (nectaries); idioblasts; and secretory ducts associated with the phloem, which irrigates the floral tissue, mainly in regions next to nectary and in the nectary parenchyma itself. Since the studied honey is monofloral, the high phenolic content was likely transferred to the honey via the nectar.

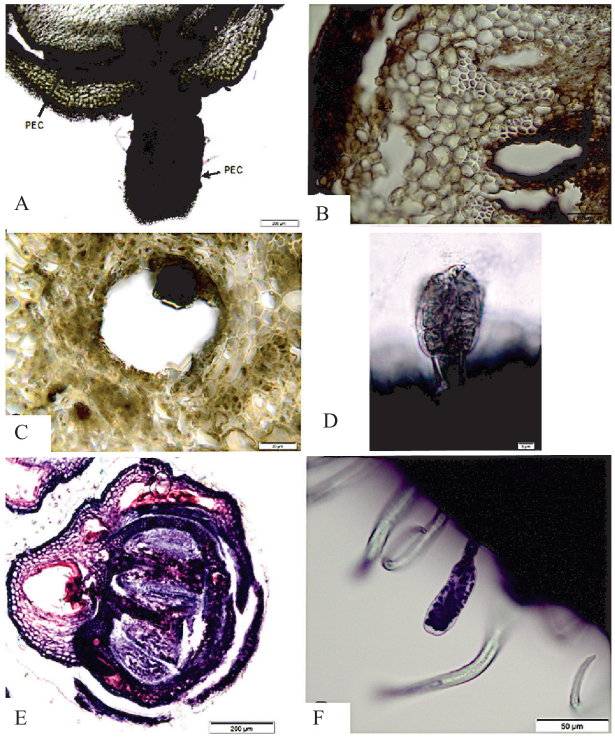

Figure 3. Histochemistry of aroeira (*Astronium urundeuva*) inflorescences, showing a positive reaction to ferric chloride in the following plant structures: epidermis (A), petiole (B), secretory duct (C), glandular trichome (D), male bud through the p-Dimethylaminocinnamaldehyde (DMACA) test (E), and glandular trichome also by the DMACA test (F). PEC, petiole. Photomicrography obtained by optical microscopy, with 200 to 400x magnification; sections were stained with Safranin and Astra Blue (Johansen, 1940).

According to Kumar et al. (2020), widespread secretory ducts, idioblasts, and other parts of the inflorescence might be related to plant defenses against herbivore attack and fungal growth. In addition, phenolic compounds – astringent, toxic, and antioxidant substances – are related to pigmentation and might be associated with pollinator attraction, ultraviolet light protection, structural support, temporary storage of nutrients, and host plant location (Price et al., 1987). In vacuoles and/or cell walls, due to their antioxidant activity, these compounds may absorb radiation and reduce the caused damage by acting as a sunscreen (Kumar et al., 2020). This explains why plants severely exposed to sunlight are rich in phenolic compounds, as is the case of the epidermal tissue of aroeira inflorescences in the xerophytic environment of Brazilian SDTFs.

The present study represents a first step in the chemical characterization of aroeira honey. The histochemical tests detected the presence of phenolic compounds in the secretory ducts associated with the phloem, responsible for irrigating floral tissues, especially in the nectary parenchyma. The obtained results show, for the first time in the literature, that the phenolic compounds produced by aroeira trees are transferred to the honey through the nectar; for this reason, these substances can be used as floral markers for the authentication of the botanical origin of monofloral honeys (Consonni & Cagliani, 2015). Therefore, the present study is a contribution to the establishment of quality standards for aroeira honey, to the identification of the botanical origin of this honey, and to the economic valuation of this genuine bee product from the semiarid region of Brazil, facilitating its commercialization in foreign markets.

Despite these positive results, the present study has limitations because it was not possible to quantify the total phenolic content exclusively from the nectar secretion of the two flower morphs since aroeira is a dioecious species. In future studies, it would be important to identify these phenolic compounds by chromatographic techniques, which could be done using a honey extract, as well as to verify the antioxidant and other biological activities of this particular type of monofloral honey.

Conclusions

1. The monofloral honey of aroeira (*Astronium urundeuva*) has a high total phenolic content of 142.5±22.6 mg 100 g^{-1} honey, which exceeds by far those of several other monofloral honeys in the literature, and the histochemical tests show the presence of these phenolic substances in the idioblasts and secretory ducts associated with the phloem in floral tissues, especially in the nectar parenchyma, epidermis, and glandular trichomes of the floral structures.

2. Phenolic compounds are present in the floral tissue of both floral morphs, mainly in the nectary where honeybees (*Apis mellifera*) collect nectar.

3. The phenolic compounds produced by aroeira trees enter the honey through the nectar, which was observed for the first time in the present study.

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