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Original Article

Microscopic and UV/Vis spectrophotometric characterization of *Cissampelos pareira* of Brazil and Africa

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ABSTRACT

Cissampelos pareira L., belonging to Menispermaceae family, has worldwide distribution, occurring in tropical and subtropical regions of the Americas, Africa and Asia. It is the most popular species of *Cissampelos*, known for its medicinal uses of leaves and roots. The study aims to find distinctive leaf anatomical characters, and also demonstrate the importance of spectral data to identify *C. pareira* samples, in order to contribute to its taxonomy and quality control of its drugs. Anatomical leaf analyses were performed by optical and scanning electron microscopy. The spectral profile was obtained from methanolic extracts of *C. pareira* samples from Brazil and Africa, with application of UV–vis spectrophotometry data, which were analyzed by principal component analysis (PCA). Some anatomical characters such as leaf epidermal cells walls, stomata, trichomes, mesophyll, features of midrib and petiole, and the spectral profile within the wavelength ranging between 770 and 240 nm (eight bands) differs between Brazilian and African samples. The results represent an additional support to the taxonomy of *C. pareira*, and the quality control of their leaf drugs, mainly in relation to misidentified samples.

Introduction

Cissampelos pareira L., belonging to Menispermaceae family, has worldwide distribution, occurring in tropical and subtropical regions of the Americas, Africa and Asia (Ortiz, 2001). In Brazil, it is encountered in different types of vegetation, from Caatinga, Atlantic Forest and Amazon forest (Braga, 2015). According to Schmelzer and Gurib-Fakim (2008), in Africa this species occurs in subtropical forest, savannah, deciduous shrubs, often persisting in cleared land and plantations, also in secondary vegetation and near rock outcrops.

It is the most popular species of *Cissampelos* not only for its wide distribution, but mainly because its leaves and roots are widely used as medicinal. According to Napralert (2013), *C. pareira* has more than eighty folk names. In Brazil, it is known as "parreira", "abuta", and "parreira-brava" (Lewis and Elvin-Lewis, 1977; Rury, 1983); in Africa, it is called in folk medicine as "chegonde" and "karigi-munana" (Hedberg et al., 1983; Rukunga et al., 2009); and in India, it is known as "ambastha", "patha" and "laghupatha" (Vaidya, 1988).

In many ethnobotanical reports, the leaves of *C. pareira* are recognized as a natural medicine for various purposes. The leaf juice is used as antiseptic, anthelmintic, insecticidal and parasiticidal, and against dermatitis (Singh and Ali, 1992), asthmas (Singh and Maheshwari, 1994), genitourinary disorders (Sanchez Medina et al., 2001), diarrhea, dysenteries and gastrointestinal disorders (Kumar et al., 2006; Kamble et al., 2008), antifertility (Ganguly et al., 2007; Priya et al., 2012), and antidiabetic (Yadav et al., 2013). The topical use of leaves is indicated to treat hemorrhages from cuts, burns and wounds (Ramasubramaniaraja and Babu, 2010; Shukla et al., 2012), and also to treat abscesses (Abbasi et al., 2010; Haque et al., 2011). In addition, in India, the leaves are also used as cattle feed to increase milk production, and also in some food systems as thickeners, gelling agents, texture modifiers and stabilizers (Vardhanabhuti and Ikeda, 2006; Priya et al., 2012), *inter alia.*

The leaves of *C. pareira* have been reported to be a rich source of isoquinoline and bisbenzylisoquinoline alkaloids (Shukla et al., 2012), such as berberine (Kupchan et al., 1960a), curine (Chowdhury, 1972), hayatine (Sharma, 1987) and magnoflorine (Ahmad et al., 1992). In addition, have also been isolated essential oil (Kupchan et al., 1960b), flavonoids (Ramirez et al., 2003; Amresh et al., 2007a), polysaccharides (Vardhanabhuti and Ikeda, 2006), and pectin (Singthong et al., 2004; Arkarapanthu et al., 2005) have also been isolated.

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Biological and pharmacological activities of leaves and aerial parts (leaves and branches) of *C. pareira* were demonstrated in several studies. The cissampeloflavone, isolated from leaves, showed activity against *Trypanosoma cruzi* and *T. brucei rhodesiense* (Ramirez et al., 2003). The plant extract exhibited antifungal activity against *Aspergillus niger* and *Saccharomyces cerevisiae* (Kumar et al., 2006). The ethanol extract of the aerial parts showed anti-inflammatory and analgesic activities (Amresh et al., 2007b). The contraceptive and cytotoxic effects were demonstrated by Priya et al. (2012) and Ganguly et al. (2007), respectively. The anti-diabetic activity was confirmed by Jannu et al. (2011) and Yadav et al. (2013). In addition, a preliminary study carried out by Thakur and Rana (2013) confirmed the anxiolytic effect of *C. pareira* leaves.

According to Rhodes (1975) and Hoot et al. (2009), *C. pareira* has problems in its interspecific delimitation with imprecise limits, mainly caused by its wide distribution and great plasticity of their vegetative forms. On the other hand, the leaf anatomical studies have shown to be an additionall support to the plant taxonomy, as already done in *Solanum* (Nurit-Silva et al., 2007; Nurit-Silva and Agra, 2011; Sampaio et al., 2014), and also to the Menispermaceae family, including *Cissampelos* by De Wet et al. (2002), Porto et al. (2008, 2011, 2012), for example.

The spectroscopic chemical techniques have emerged and contributed as an additional tool to contribute to plant taxonomy, and also as a support to the quality control of herbal drugs, allowing information to be obtained without the need for previous isolation of chemical constituents, as demonstrated before for *Baccharis* (Lonni et al., 2005) and *Solanum* (Basílio et al., 2012).

Although the leaves of *C. pareira* are commonly used in traditional medicine, and there is evidence of many activities of their compounds, a literature survey showed a lack of studies of the leaf comparative anatomy, as well as spectroscopic analysis of UV–visible of the leaf extracts. In this way, this study aimed to find leaf anatomical characters, distinctive to *C. pareira*, on samples of plants from Brazil and Africa, revealing the importance of anatomical studies combined with spectral data, would be useful to the quality control of its drugs, as well as to the taxonomy of *C. pareira*.

Materials and methods

Plant material

Botanical expeditions and field observations were carried out by N.M. Porto, in areas of Atlantic Forest and Rain Forest, for sample collection of Menispermaceae, including leaves of *Cissampelos pareira* L. in the following Brazilian States: Alagoas, Pará Maranhão, Paraíba, Pernambuco and Sergipe (Table 1). For each individual, an average of three leaf samples were taken from the second to the fifth nodes of the leaf blades and the proximal, median and distal portions, and petiole were fixed in FAA (50%) for 24 h (Johansen, 1940), and preserved in ethanol 70 GL. The other part of fertile material was pressed and dried for herbaria, according to Bridson and Forman (1999). The voucher specimens were deposited at the Herbarium Prof. Lauro Pires Xavier (JPB), of the Universidade Federal da Paraíba.

In addition, leaf samples from herbarium specimens identified as *C. pareira* were also analyzed from the following herbaria, acronyms by Thiers (2015): Herbarium of Centro de Pesquisas do Cacau (CEPEC), Herbarium Prof. Jayme Coelho de Morais (EAN), Herbarium of Embrapa Amazônia Oriental (IAN), Herbário Prof. Lauro Pires Xavier (JPB), Herbário Museu Paraense Emílio Goeldi (MG), Herbarium Jardim Botânico do Rio de Janeiro (RB),

Table 1
Selected voucher specimens of *Cissampelos pareira* and species of outgroup.

Species	Specimen code	Country, State and Municipality	Voucher specimen	Herbarium
Anomospermum chloranthum	AC	Brazil, Pará, Santarém	M Silva 2619	MG
Anomospermum steyermarkii	AS	Brazil, Roraima, Uaicá	GT Prance s/n	MG
Cissampelos andromorpha	CA1	Brazil, Paraíba, Conde	NM Porto 30	RB
	CA2	Brazil, Pará, Belém	NM Porto 45	JPB
	CA3	Brazil, Pernambuco, Catende	NM Porto 07	JPB
	CA4	Brazil, Espírito Santo, Santa Teresa	W Pizziolo 329	RB
Cissampelos pareira	CP1	Brazil, Rondônia, Porto Velho	JA Silva 39	IAN
	CP2	Brazil, Pará, Monte Alegre	RL Fróes 30443	IAN
	CP3	Brazil, Distrito Federal, Brasília	HS Irwin s/n	IAN
	CP4	Brazil, Santa Catarina, Ipumirim	AL Gasper 2020	RB
	CP5	Brazil, Goiás, Pirenópolis	HS Irwin s/n	RB
	CP6	Brazil, Mato Grosso do Sul, Corumbá	A C. Cervi 3276	RB
	CP7	Africa, Ethiopia, Ghion	JW Ash 655	MO
	CP8	Africa, Tanzania, Tanga district	H Faulkner 5631	MO
	CP9	Africa, Uganda, Kyadondo	PK Rwaburindore 205	MO
	CPa	Brazil, Bahia, Filadélfia	AM Giulietti 1886	CEPEC
	CPb	Brazil, Mato Grosso do Sul, Corumbá	A Pott 3158	RB
	CPc	Brazil, Bahia, Coribe	MM Lopes 1374	CEPEC
	CPd	Brazil, Paraíba, Maturéia	MF Agra 5061	JPB
	CPe	Brazil, Santa Catarina, Capão Alto	M Verdi 1156	RB
Cissampelos sympodialis	CS1	Brazil, Paraíba, João Pessoa	MF Agra 7133	JPB
	CS2	Brazil, Ceará, Fortaleza	Celismar s/n	JPB
	CS3	Brazil, Bahia, Juazeiro	Zehntren 211	RB
Cissampelos tropaeolifolia	CT1	Brazil, Sergipe, Capela	NM Porto 19	JPB
	CT2	Brazil, Alagoas, Coruripe	NM Porto 47	JPB
	CT3	Brazil, Maranhão, Ribeirãozinho	NM Porto 48	JPB
	CT4	Brazil, Pará, Conceição do Araguaia	T Plowman 8755	IAN
Hyperbaena domingensis	HD	Brazil, Pernambuco, Sirinhaém	M Oliveira 1553	UFP
Orthomene hirsuta	OH	Brazil, Amazonas, São Gabriel	GA Black 48-2473	IAN
Orthomene schomburgkii	OS	Brazil, Pernambuco, Igarassu	BS Amorim 1668	UFP
Sciadotenia brachypoda	SB	Brazil, Amazonas, São Paulo Olivença	NT Silva 4146	IAN

Herbarium Prof. Geraldo Mariz (UFP) of the Universidade Federal de Pernambuco (UFPE), and Herbarium of Missouri Botanical Garden (MO). A list of voucher specimens used in this study is given in Table 1.

Anatomical and histochemical analysis

The plant material was divided in two portions, one for analysis by optical microscopy and the other by scanning electron microscopy (SEM). Transverse sections were performed on leaves of *C. pareira* by free hand using commercial razor blades. Subsequently, the sections were cleared by sodium hypochlorite (20%) until complete clarification, neutralized with acetic acid (0.2%), washed in distilled water, and stained with a solution of Astra blue and Safranin, modified by Bukatsch (1972). Leaf epidermis was separated from the mesophyll by dissociation in Jeffrey solution (Johansen, 1940), and then stained with Safranin with 1% solution in 50% alcohol, according to Franklin (1945).

For alkaloid detection, transverse sections were treated with Dittman's and Wagner reagents (Furr and Mahlberg, 1981). All leaf sections were mounted in glycerinated gelatin (50%). The observations and microphotographs were performed by a photomicroscope (Leica DM750), with image processing software (Qwin System) coupled to a video camera (Leica ICC50 HD) for image capture.

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) of leaf epidermis was performed in dry material to optimize the observation of waxes and epidermal appendages. Leaf fragments of 1 cm^2 were fixed in a solution of 4% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.0) for 24 h, at 48 °C, then washed in 0.1 M sodium cacodylate buffer (pH 7.0), followed by post fixation in 1% OsO_4, in 0.1 M Na-cacodylate buffer (pH 7.0) for 1 h, at room temperature. Subsequently, the fragments were dehydrated in a crescent ethylic series, and dried at the critical point, placed on aluminum stubs with double-sided tape, air-dried and, finally, coated with gold. Finally, photomicrographs and microscopic analysis were performed by scanning electron microscopy (JEOL JSM-5600), on leaf epidermis, at an accelerating voltage of 15 KV. Micromorphological characterization was complemented by the analysis of epicuticular waxes of the leaf epidermis that were classified here according to Barthlott et al. (1998).

Chemical analysis

Samples of dried leaves of *C. pareira* (from Brazil and Africa) and of nine species used as an outgroup of Menispermaceae family were investigated: *Cissampelos andromorpha* DC., *Cissampelos sympodialis* Eichl., *Cissampelos tropaeolifolia* DC., *Anomospermum chloranthum* Diels, *Anomospermum steyermarkii* Krukoff & Barneby, *Hyperbaena domingensis* (DC.) Benth., *Orthomene hirsuta* (Krukoff & Moldenke) Barneby & Krukoff, *Orthomene schomburgkii* (Miers) Barneby & Krukoff, *Sciadotenia brachypoda* Diels. All UV spectrophotometric analysis were performed using a modified version of a previously published method (Basílio et al., 2012). Briefly, methanol extracts of dried leaves were prepared by ultrasound extraction for 20 min at room temperature, and then filtered through membranes with a pore size of 0.45 mm, modified from Basílio et al. (2012).

The UV/Vis spectra were recorded with a spectrophotometer (UV-1650PC, Shimadzu, Kyoto, Japan). For all absorbance measurements Quartz cells (1 cm) were used. The spectra were recorded in triplicate from 770 to 200 nm. The standardized procedure was repeated for all species and the data was automatically reduced to an ASCII file.

The spectra were normalized by setting the absorbance at 770 nm equal to zero and subsequently mean centered. The data matrix was processed by FITOPAC v.2.1.2 software. Finally, a principal component analysis (PCA) was performed using the methods

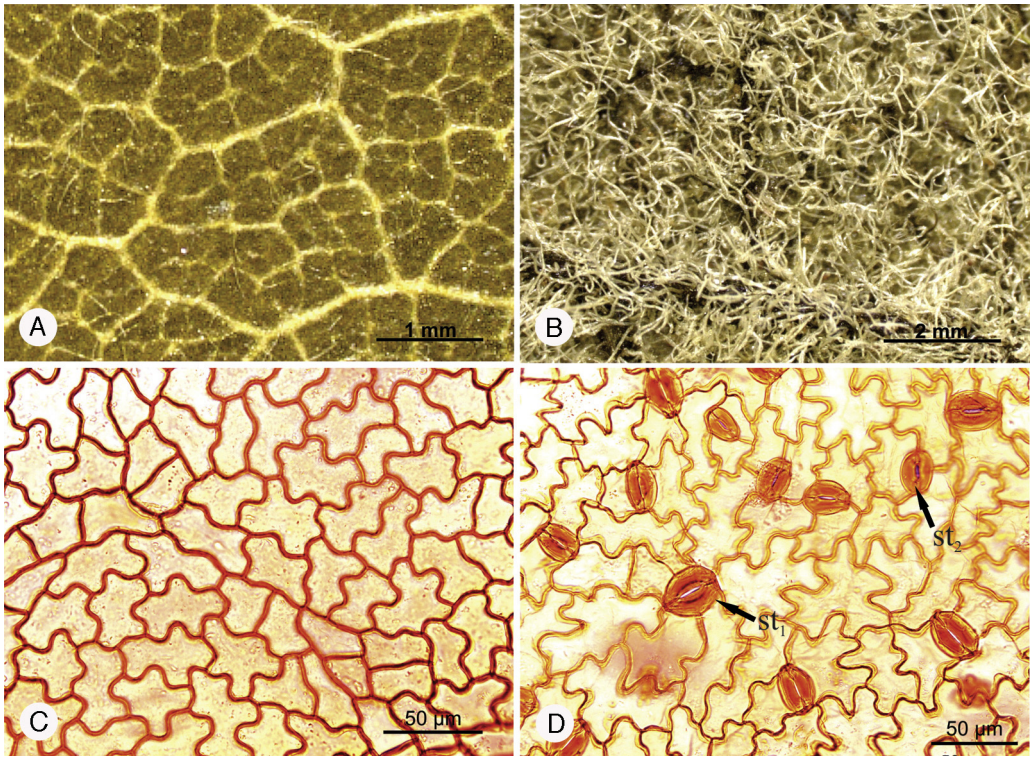

Fig. 1. *Cissampelos pareira* (*MF Agra 5061*), front view: (A) adaxial surface laxe-pilose; (B) abaxial surface pubescent; (C) leaf epidermis with waved anticlinal walls cells on the adaxial surface by light microscopy; (D) Leaf epidermis with stomata (st_1, anomocytic and st_2, anisocytic) on the abaxial surface by light microscopy.

variance-covariance between the groups with Past software, version 2.15 (Hammer et al., 2001).

Results and discussion

Anatomic study

The leaf epidermis of *C. pareira*, in front view, presented cells with curve to waved anticlinal walls on the glabrescent adaxial surface (Fig. 1A and C), and waved and hairy on the abaxial surface (Table 2, Fig. 1B and D). In transverse section, the epidermis of *C. pareira* was uniseriate with tabular cells, and a thin and smooth cuticle (Fig. 3A). Metcalfe and Chalk (1950), Porto et al. (2011) and Sudhakaran (2012) have reported this pattern of cell walls to *C. pareira*. It was also recorded in other species of *Cissampelos* (Hong et al., 2001; De Wet et al., 2002; Porto et al., 2011), and other genera of Menispermaceae as *Cocculus* and *Stephania* (Metcalfe and Chalk, 1950). Only two samples from Brazil (CP6 and CPb), and two from Africa (CP8 and CP9) showed thickened cuticle (Figs. 4E and 5C, E) that differed from the *C. pareira* pattern (Table 2). To Wilkinson (1979), in general the presence and thickness of the cuticle is determined by environmental factor and does not have taxonomic importance.

Eight specimens identified as *C. pareira* displayed the leaf epidermis pattern different from those known for this species. Two samples from Brazil (CP6 and CPb) showed straight anticlinal walls on the adaxial, and curve on the abaxial (Table 2, Fig. 4C and D). According to Stace (1965), the features of anticlinal walls are a mesomorphic character, and environmental conditions such as humidity play a significant role in determining the pattern of anticlinal cell walls. These features also have been reported to vary in response to changes in light regimes by Rôças et al. (2001) and Mantuano et al. (2006).

Four samples from Brazil, CP3, CP5, CPa and CPc (Table. 2, Fig. 4G), and three from Africa (CP7, CP8 and CP9) showed an epidermal papillae (Table. 2, Fig. 5A). The presence, number and distribution of papillae constitute an important feature that has been used to define boundaries at specific and generic level in many taxonomic groups (Hong et al., 2001). According to Bone et al. (1985), the cell walls of the leaf epidermis with convex curvature would be advantageous to increase the energy capture efficiency, which is important for plants that must survive at extremely low light levels. Furthermore, papillae in the leaf epidermis minimize the area of contact causing a very low adhesion of water on leaf (Ensikat et al., 2011).

Epicuticular waxes as tubules and clusters of tubules were confirmed in SEM, in both surfaces of *C. pareira* (Fig. 2C and D), as previously referred by Porto et al. (2011). The epicuticular waxes also have taxonomic value in the characterization of the leaf epidermis, according to Barthlott et al. (1998). Moreover, the presence of wax tubules in leaf epidermis could be related to the higher water repellency (Ensikat et al., 2011).

The leaves were hypostomatic with anomocytic and anisocytic stomata occurring simultaneously, both at the epidermal level (Fig. 3A), however, the anomocytic type was predominant (Fig. 1D), which corroborate with the pattern previously described to *C. pareira* by De Wet et al. (2002) and Porto et al. (2011). Simple bicelled to many-celled trichomes were present on both surfaces of the leaf epidermis (Figs. 1A, B and 2A, B), which are common on the leaf epidermis of some Menispermaceae species, according to Wilkinson (1978).

Dorsiventral leaves with adaxial mesophyll and a single layer of palisade was the pattern for *C. pareira* (Table 2, Fig. 3A). However, bisseriate palisade was observed in five samples, being two from Brazil (CP6 and CPb), and three from Africa (CP7, CP8, CP9)

Table 2
Selected leaf anatomical characters of *Cissampelos pareira* L. and related, and their states.

Species	Country	Specimen code	Characters															
			Anticlinal walls of epidermal cells		Epidermal cuticle		Epidermal papillae	Stomata level	Layers of palisade parenchyma	Crystals	Midrib	Vascular bundles	Alkaloid	Secretory cavities				
			AD	AB	AD	AB	AB	AB	AD	ME	BL	PT	ME	AP	MI	PT		
Cissampelos pareira and related	Africa	CP1	wv	wv	–	–	–	el	1	st	bc	6–8	+	–	–	–		
		CP2	wv	wv	–	–	–	el	1	st	bc	6–8	+	–	–	–		
		CP3	wv	wv	–	–	+	el	1	st	bc	9–12	–	–	+	+		
		CP4	wv	wv	–	–	–	el	1	–	bc	6–8	+	–	–	–		
		CP5	wv	wv	–	–	+	el	1	st	bc	9–12	–	–	+	+		
		CP6	st	st	+	+	–	ae	2	st	pc	9–12	–	–	+	+		
		CPa	wv	wv	–	–	+	el	1	st	bc	9–12	–	–	+	+		
		CPb	st	st	+	+	–	ae	2	st	pc	9–12	–	–	+	+		
		CPc	wv	wv	–	–	+	el	1	st	bc	9–12	–	–	+	+		
	Brazil	CPd	wv	wv	–	–	–	el	1	st	bc	6–8	+	–	–	–		
		CPe	wv	wv	–	–	–	el	1	st	bc	6–8	+	–	–	–		
		CP7	wv	wv	–	–	+	el	2	st	pc	6–8	+	–	+	–		
		CP8	wv	wv	+	–	+	ae	2	dr, st	pc	6–8	–	+	+	+		
		CP9	wv	wv	+	–	+	ae	2	dr, st,	pc	6–8	–	+	+	+		

Legends: ae, above the epidermal level; AB, abaxial surface; AD, adaxial surface; AP, apex leaf; BL, blade leaf; bc, biconvex; dr, druses; el, at epidermal level ME, Mesophyll; MI, Midrib; PT, petiole; pc, plane-convex; st, stiloyds; st, straight to curve; wv, waved; –, absent; +, present; 1, uniseriate 2, bisseriate.

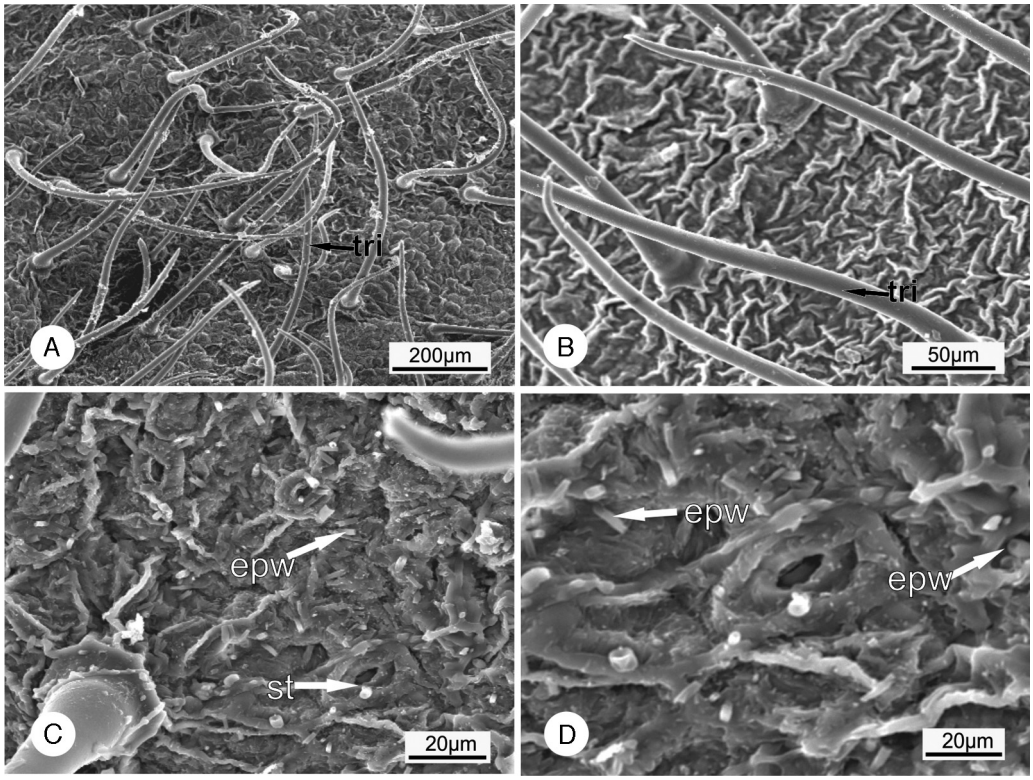

Fig. 2. *Cissampelos pareira* (*RL Fróes 30443*), leaf epidermis in front view by SEM: (A) adaxial surface with long finger hairs; (B) detail of the wrinkled epidermis and trichomes on the abaxial surface; (C) detail of the abaxial epidermis with epicuticular waxes, as tubule; (D) stoma in detail and epicuticular waxes on the abaxial surface (*Legends*: epw, epicuticular wax; st, stomata; tri, trichome).

(Table 2, Fig. 5C) According to Esau (1972), Levitt (1980) and Rozema et al. (1997), palisade and spongy parenchyma are tissues known to reveal responses related to light and soil water variations. All samples showed 3–5-layered spongy parenchyma with large intercellular spaces (Fig. 3A), and several collateral vascular bundles distributed throughout the mesophyll.

The leaf margin was slightly curved toward the abaxial surface with a many-layered collenchyma and a single vascular bundle. The dorsiventral organization of the leaf mesophyll is characteristic to *Cissampelos*, according to Metcalfe and Chalk (1979) and De Wet et al. (2002), and does not constitute an exclusive character of *C. pareira*.

The midrib was biconvex, in transverse section, more prominent and rounded to the abaxial surface with a sub-epidermal 3–5-layered lacunar collenchyma, followed by the fundamental parenchyma. The vascular system has a single vascular bundle with a sclerenchymatous ring at the middle portion, and 1–2 smaller vascular bundles at the base and apex (Table 2, Fig. 3B). Five samples, three from Africa (CP7, CP8 and CP9) and two from Brazil (CP6 and CPb) showed midrib with plane-convex shape, differing from the more common biconvex pattern characteristic of *C. pareira* (Table 2, Figs. 4D and 5D). In our studies, the morphology of the midrib in *Cissampelos* and other genera of Menispermaceae (in prep.), constitutes an important feature to separate taxa at specific level, which is also corroborated by studies of De Wet et al. (2002).

In transverse section, the petiole of *C. pareira* showed circular contour with uniseriate epidermis and simple long trichomes; the cortex under the epidermis has continuous collenchyma similar to the midrib; the vascular system is formed by 6–8 collateral vascular bundles (Fig. 3C–E), which are surrounded by a sclerenchymatic ring at the middle portion (Table 2, Fig. 3D). Six samples (CP3, CP5, CP6, CPa, CPb and CPc) showed 9–12 collateral vascular bundles (Table 2, Fig. 4E), thus differing from the pattern observed for *C. pareira* and therefore suggesting these samples belong to different taxa.

According to Sinnot (1914), Metcalfe and Chalk (1950) and Howard (1979), the characteristics of the angiosperms have great taxonomic significance because the environment does not influence it. Moreover, the petiole vascularization is diagnostic to distinguish some genera and species and also has taxonomic importance (Wilkinson, 1978, 1986, 1989). On the other hand, the sclerenchymatic sheath was also referred to *C. sympodialis* by Porto et al. (2008), and to other South African *Cissampelos* species by De Wet et al. (2002). It is common in vegetative organs of Menispermaceae, especially in lianas (Carlquist, 1996).

Idioblasts were observed in the vascular tissue of the cortical parenchyma of the midrib and petiole and showed a positive reaction to alkaloids in all samples of *C. pareira* (Table 2, Fig. 3F). Differently, the samples CP3, CP5, CP6, CP8, CP9, CPa, CPb and CP5 showed no positive reaction to alkaloids (Fig. 5G and H). These results corroborate with existing chemical studies of *Cissampelos* (Semwal et al., 2014), as well as confirm the localization of the production and/or accumulation of these compounds in the leaf (Chowdhury, 1972; Cavalcanti et al., 2014). According to Menachery (1996) and Barbosa-Filho et al. (1997), the occurrence of alkaloids can be a chemotaxonomic character of Menispermaceae.

Secretory cavities were observed at the leaf apex of two samples from Africa (CP8 and CP9), and also in the midrib and petiole of eight samples (Table 2, Figs. 4H and 5E, F), three from Africa (CP7, CP8 and CP9), and six from Brazil (CP3, CP5, CP6, CPa, CPb and CPc). Although this character has been reported for some species of *Cissampelos* and other genera of Menispermaceae (Metcalfe and Chalk, 1950, 1979; Wilkinson, 1989; De Wet et al., 2002), it was never mentioned before to *C. pareira*, and provides further evidence that these samples with secretory cavities belong to different taxa. According

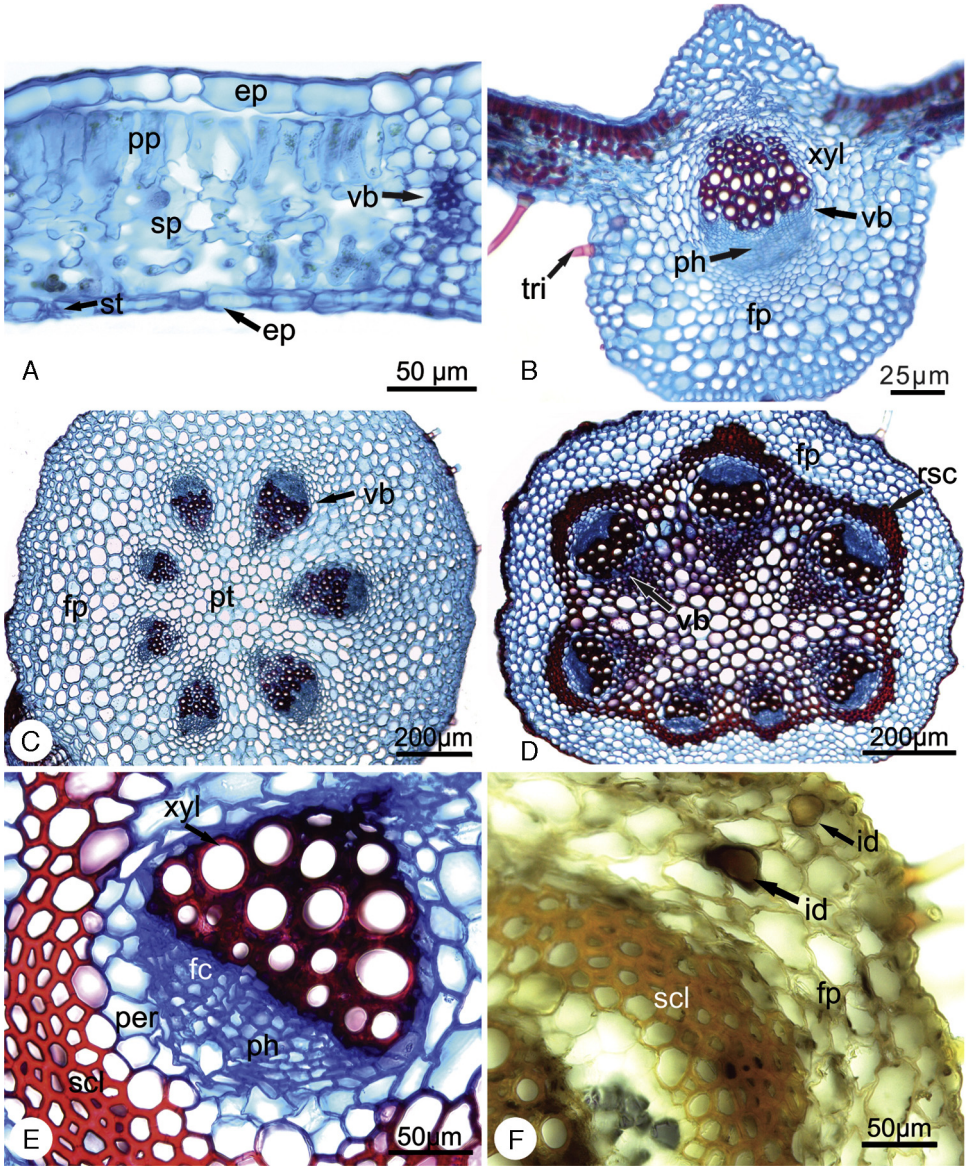

Fig. 3. *Cissampelos pareira* (*AL Gasper 2020*) in transverse sections: (A) dorsiventral mesophyll; (B) midrib with collateral vascular bundle; (C) vascular bundles of petiole in the basal portion; (D) vascular bundles of petiole in the median portion with a sclerenchymatous ring; (E) detail of collateral vascular bundle; (F) stained idioblasts showing positive reaction for alkaloids (*Legends*: col, collenchyma; ef, exchange fascicular; ep, epidermis; fc, fascicular cambium; fp, fundamental parenchyma; id, idioblast; per, pericycle; ph, phloem; pp, palisade parenchyma; pt, pith; rsc, sclerenchymatous ring; scl, sclerenchyma; sp, spongy parenchyma; st, stomata; tri, trichome; vb, vascular bundle; xyl, xylem).

to Fahn (1988), secretory cavities are distinctive for angiosperms and have important taxonomic value.

The results of the leaf anatomy of thirteen samples analyzed identified as *C. pareira*, revealed that only five of these samples (CP1, CP2, CP4, CPd and CPe), all from Brazil, showed a set of characters that match the pattern described for *C. pareira* by Porto et al. (2011) and Sudhakaran (2012), which are summarized here: hypostomatic leaf blades; anomocytic and anisocytic stomata with predominance of the first type at the epidermis level; epidermal anticlinal walls curves on the adaxial surface, and wavy on the abaxial surface; epidermis with thin cuticle and simple uniseriate trichomes; dorsiventral mesophyll with uniseriate palisade parenchyma, with few and rare styloid cristals (Table 2, Fig. 4H); biconvex midrib; collateral vascular system; petiole with 6–7 vascular bundles; idioblasts positive for alkaloids; and no secretory cavities.

Nine samples (CP3, CP5, CP6, CP7, CP8, CP9, CPa, CPb and CPc) were mistakenly identified as *C. pareira*, as they have a set of characters different of the pattern of *C. pareira*. These set of different characters displayed by these samples allow to separate them into three groups, which could belong to distinct and unidentified taxa (Table 2): *Cissampelos* sp1, CP3, CP5, CPa and CPc (Fig. 4A and B); *Cissampelos* sp2 (Fig. 4C–H), CP6 and CPb; and *Cissampelos* sp3, CP7, CP8 and CP9 (Fig. 5A–H).

Cissampelos sp1 and *Cissampelos* sp3 have secretory cavities, biconvex midrib, and epidermal papillae on the abaxial surface. However, they present distinctive characters: *Cissampelos* sp1 has uniseriate palisade parenchyma, and the petiole with 9–12 vascular bundles (Table 2); while in *Cissampelos* sp3 the palisade parenchyma is biseriate and the petiole has 6–8 vascular bundles.

On the other hand, samples of *Cissampelos* sp2 differs from *Cissampelos* sp1 and *Cissampelos* sp3 mainly by the anticlinal walls of epidermal cells that are straight and smooth with thin cuticle (Fig. 4C and D), without papillae. However, it shows a biseriate mesophyll and plane-convex midrib, similar to *Cissampelos* sp3, but different from *Cissampelos* sp1 that has an uniseriate palisade, and biconvex midrib. The number of petiole vascular bundles of

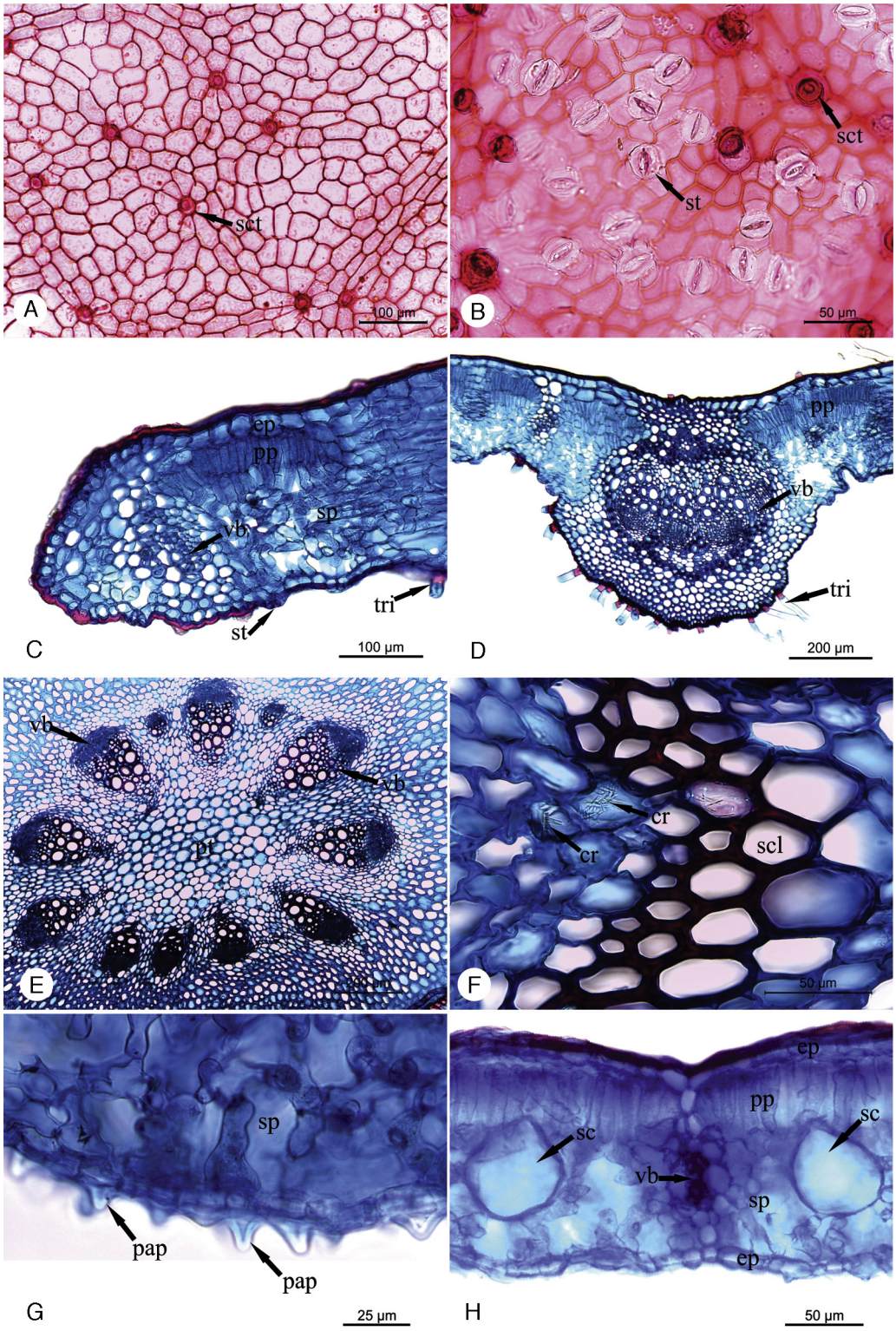

Fig. 4. (A, B) *Cissampelos* sp1 (HS Irwin s/n – IAN), leaf epidermis in front view: (A) Leaf epidermis with straight anticlinal cells walls on the adaxial surface; (B) Leaf epidermis with curve anticlinal cells walls on the abaxial surface; (C–H) *Cissampelos* sp2 (AC Cervi 3276), transverse sections: (C) leaf margin somewat rounded; (D) Plane convex midrib with collateral vascular bundle; (E) Petiole of 12 collateral vascular bundles in the basal portion; (F) detail of parenchyma with styloids cristals, (G) detail of epidermal papillae on the abaxial surface; (H) detail of mesophyll with secretory cavities; (*Legends*: cr, crystals; ep, epidermis; pap, papillae; pp, palisade parenchyma; pt, pith; sc, secretory cavity; scl, sclerenchyma; sct, scar of trichome; sp, spongy parenchyma; st, stomata; tri, trichome; vb, vascular bundle).

Cissampelos sp2 is similar to that showed by *Cissampelos* sp1 (9–12), but different from *Cissampelos* sp3, with 6–8 vascular bundles.

The evidences found in this work suggest that nine samples were mistakenly identified as *C. pareira*, and probably belong to three different taxa, as they have a distinctive set of characters from those observed *C. pareira*. With regard to identification of these indeterminate taxa, further investigations are required with a higher sampling allowing for the identification of the taxa involved.

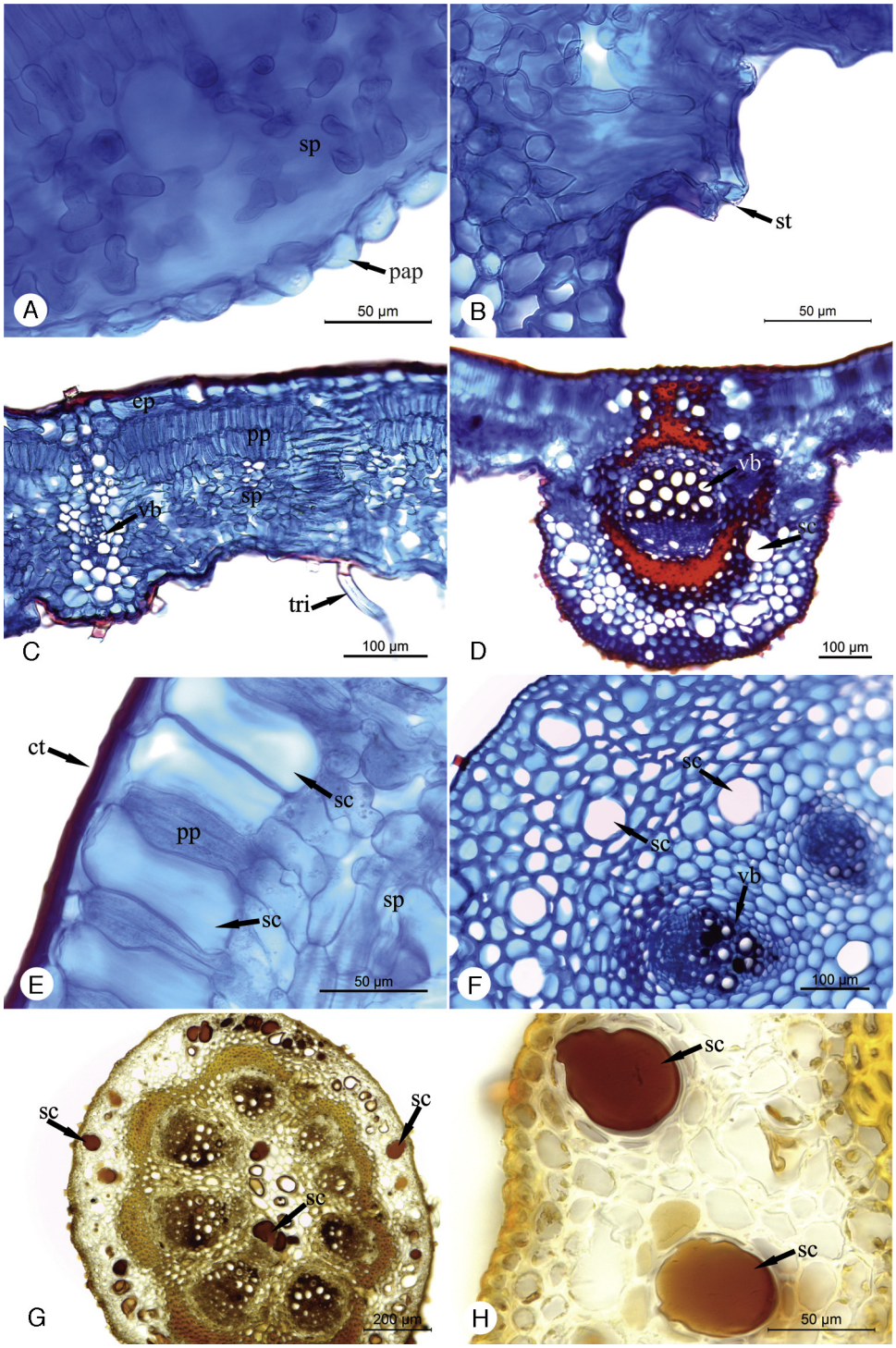

Fig. 5. *Cissampelos* sp3, transverse sections of leaf blades. (A) (*JW Ash 655*): detail of epidermal papillae; (B–D) (*PK Rwaburindore 205*): (B) detail of a stoma on the abaxial surface; (C) dorsiventral mesophyll with bisseriate palisade; (D) plane-convex midrib with collateral vascular bundle; (E) (*H Faulkner 5631*): detail of mesophyll with secretory cavities; (F, H) (*PK Rwaburindore 205*): (F) Palisade with secretory cavities; (G, H) stained secretory cavities showing positive reaction for alkaloids (*Legends*: ct, cuticle; ep, epidermis; pap: papillae; pp, palisade parenchyma; sc, secretory cavity; sp, spongy parenchyma; st, stomata; tri, trichome; vb, vascular bundle).

Spectroscopy analysis and principal component analysis (PCA)

The spectral profile showed saturated absorbance in the characteristic region of conjugated enones (240–190 nm) while the region above 550 nm showed no relevant absorbance, only bands of possible interferents, related to chlorophyll and/or other substances (Shipman et al., 1976). These results led us to select the spectral range from 550 to 250 nm (600 variables) for chemometric studies (Fig. 6).

The characterization of spectra was carried out by the analysis of methanol extracts of specimens. Four distinct absorption bands were observed (Table 3): Band I (λ_{max} 500–504.5 nm); Band II (λ_{max} 391.5–468 nm); Band III (λ_{max} 312.5–368.5 nm); Band IV (λ_{max} 223–291 nm).

In the interspecific analysis all analyzed species showed similar absorption for the bands I and III, which may be related to the overlap of absorption in this region. However, in the region of band II (λ_{max} at 403.5–410.0 nm), *Cissampelos tropaeolifolia* showed

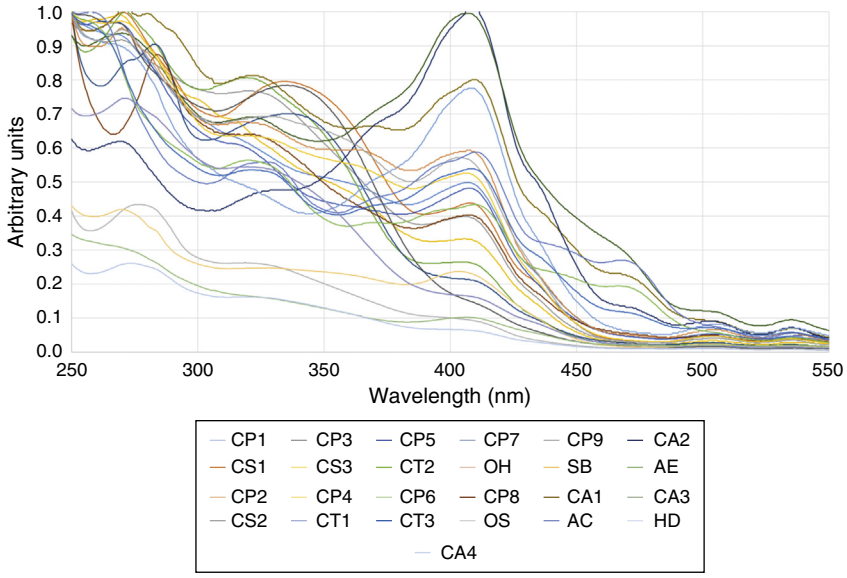

Fig. 6. *Cissampelos* species: UV–vis spectra of in the spectral region selected for chemometric analysis (550–250 nm). For the numerical code of the species see Table 1.

significantly different absorption values at λ_{max} 466.5 nm, when compared with other species.

The intraspecific analysis of samples of *C. pareira* revealed shifts in the wavelength of maximum absorption in the band IV between Brazilian (λ_{max} at 261.5–272.0 nm) and African (λ_{max} at 282.0–284.0 nm) specimens, except for the sample CP9 that showed no band. The spectral analysis of *C. pareira* samples from Brazil revealed absorption maxima at 261.5 and 272 nm, which are characteristic of the C=N chromophore (Nagarajan et al., 2011). Based on the data available, such absorbance can be related to the presence of tropoisoquinoline alkaloids, which were already isolated from *C. pareira* (Morita et al., 1993), and is also common in other species of *Cissampelos* (Menachery, 1996).

The spectral data of the outgroup samples, *A. chloranthum*, *A. steyermarkii*, *H. domingensis*, *O. hirsuta*, *O. schomburgkii*, *S. brachypoda* were different, as expected, from *C. pareira* and other species of the genus, especially *C. andromorpha*, *C. sympodialis* and *C. tropaeolifolia* in the PC1 factor (Fig. 7A).

Table 3
Maximum absorption values (λ_{max}) for *Cissampelos* species and out-group.

Species (Country)	Collector (Herbarium)	Code	λ_{max} (nm)[a]			
			Band IV	Band III	Band II	Band I
Anomospermum chloranthum	M Silva 2619 (MG)	AC	271.5	319.5	–	501.5
Anomospermum steyermarkii	GT Prance s/n (MG 42173)	AS	–	–	405.5	501.5
Cissampelos andromorpha	NM Porto 30 (JPB)	CA1	270.5 and 279.5	323 and 366.5	409.5	–
	NM Porto 45 (JPB)	CA2	269	–	409	503
	NM Porto 07 (JPB)	CA3	270	320.5	406.5	–
	W Pizziolo 329 (RB)	CA4	–	–	408	504.5
Cissampelos pareira (Brazil)	JA Silva 39 (IAN)	CP1	267.5	–	407	504.5
	RL Fróes 30443 (IAN)	CP2	270	334	407.5	504.5
	HS Irwin s/n (IAN 129416)	CP3	269.5	320.5	405.5	504.5
	AL Gasper 2020 (RB)	CP4	272	312.5	408	504.5
	HS Irwin s/n (RB 144809)	CP5	261.5	–	407.5	504.5
	A Pott 3158 (RB)	CP6	271.5	319.5	404.5	503.5
Cissampelos pareira (Africa)	JW Ash 655 (MO)	CP7	283	336.5	–	–
	H Faulkner 5631 (MO)	CP8	284	–	407	–
	PK Rwaburindore 205 (MO)	CP9	–	335.5	–	–
Cissampelos sympodialis	Agra 7133 (JPB)	CS1	270	318.5 and 361.5	407	503.5
	Celismar s/n (JPB)	CS2	270	326	403.5	503.5
	Zehntren 211 (RB)	CS3	269.5	317.5	406	504
Cissampelos tropaeolifolia	NM Porto 19 (JPB)	CT1	257	326	410 and 468	–
	NM Porto 47 (JPB)	CT2	–	320.5 and 368.5	409.5 and 466.5	–
	T Plowman 8755 (IAN)	CT4	278 and 282.5	–	–	–
Hyperbaena domingensis	M Oliveira 1553 (UFP)	HD	273	318	–	504.5
Orthomene hirsuta	GA Black 48-2473 (IAN)	OH	–	–	406.5	–
Orthomene schomburgkii	BS Amorim 1668 (UFP)	OS	276	318.5	–	503
Sciadotenia brachypoda	NT Silva 4146 (IAN)	SB	269.5	328	403	503

[a] Wavelengths of maximum UV/Vis absorbance (≥0.01 nm) between 260 and 505 nm.

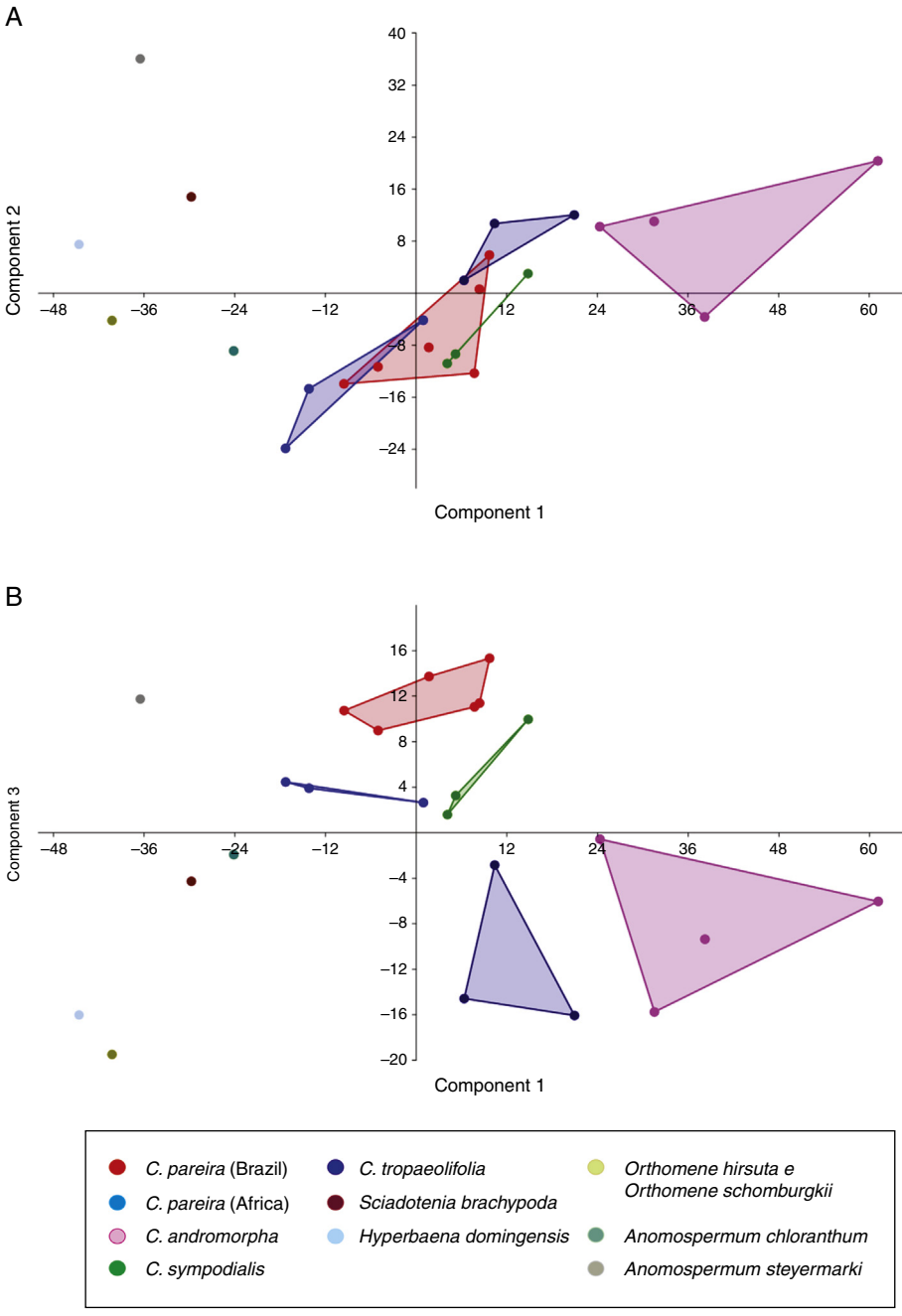

Fig. 7. *Cissampelos* species (A) PC1 × PC2; (B) PC1 × PC3: PCA spectral profile (770–250 nm).

The principal component analysis of spectral data allowed the differentiation of samples of *C. pareira* of African and Brazilian origin mainly based on their score values at PC3. The spectra of Brazilian samples CP4 and CP6 showed proximity with the samples of the African group, CP7, CP8 and CP9 (Fig. 8). The samples of *C. pareira* specimens were close to *C. tropaeolifolia* and *C. sympodialis* with positive score values in the PC3 (Fig. 7B). It is worth noting a group of African (CP7, CP8 and CP9) and Brazilian samples (CP4 and CP6) that, although similar, has spread throughout the dispersion diagram (Fig. 8). Based on the results, in general, the PCA analysis was able to delimit the species of *Cissampelos* analyzed.

The results showed that application of UV/Vis spectrophotometry is a valid technique for identifying *Cissampelos* samples, confirming the observations already made for other angiosperms groups such as Asteraceae by Lonni et al. (2005) and *Solanum* by Basílio et al. (2012).

The chemical data corroborates with the anatomical features of *C. pareira* samples, which differentiates them from samples of other species with the following characters: biseriate parenchyma, stomata above the level of the epidermis, presence of epidermal papillae as well as secretory cavities in the mesophyll.

The principal components analysis (PCA) provided important spectral information, which allowed the separation of African-tropical and Neotropical samples. These differences may be related to geographical distribution, considered by Griffin and Lin (2000) as an aspect that influences the chemical composition of plants. However, when analyzing chemical data together with anatomical characters, usually neglected in taxonomic studies, these differences suggest that the studied samples belong to different taxa, probably in speciation, after being subjected to different selection processes, with the development of adaptive characters.

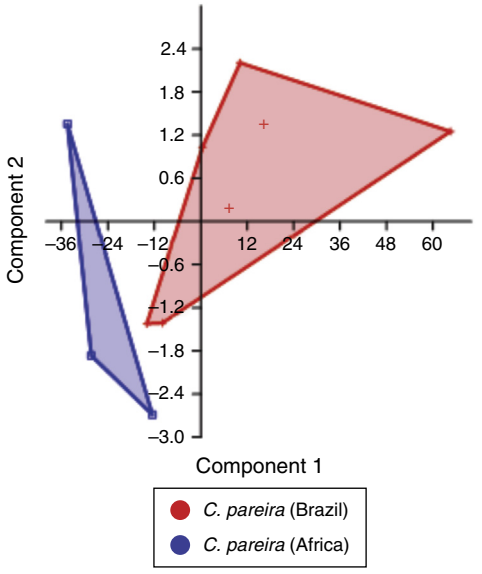

Fig. 8. *Cissampelos pareira* and relatives from Brazil and Africa (PC1 × PC2): PCA spectral profile (770–250 nm).

Conclusions

Characters of epidermal cells, mesophyll (palisade parenchyma layers), midrib shape, number of vascular bundles in petiole, secretory cavities, and the chemical results proved to be useful and distinctive to separate *C. pareira* from related species, providing an additional tool for its taxonomy, and quality control of their drugs composed by leaves. Further chemical and taxonomic studies are needed to enable the identification of all taxa, as well as to explain some variations in spectral and anatomical data to *C. pareira* and its related species.

Authors' contributions

NMP (PhD student) contributed in collecting plant samples and identification, and running the laboratory work (preparing herbaria samples, chemical extraction, obtaining of spectra and plant anatomy studies), analysis of the data and preparation of the paper. YLB contributed to UV analysis, supervised by IJLDB. MFA designed the study with *Cisampelos pareira*, and supervised all laboratory and field work, as well as contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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