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Original Article

Leaf histochemistry analysis of four medicinal species from Cerrado

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ABSTRACT

Chemical components act in plant defense and protection, but many of them are extracted and used medicinally. For Cerrado, active chemical components are used in the treatment of diseases, which strengthens the necessity for pharmacological studies of plants of that environment. The objective was to evaluate the histochemistry of the leaf blade of *Byrsonima verbascifolia* (L.) DC., Malpighiaceae, *Campomanesia adamantium* (Cambess.) O.Berg, Myrtaceae, *Roupala montana* Aubl., Proteaceae, and *Solanum lycocarpum* A. St.-Hil., Solanaceae, species that have been reported as producers of secondary metabolites for pharmacological use. The 3rd node leaves (median, intercostal and margin regions) were collected, fixed, included in Paraplast® or 2-hydroxyethyl methacrylate, sectioned in microtome, stained and photographed on microscope. This analysis aimed to find leaf regions which produced chemical compounds. For histochemical tests, intercostal areas were selected from median region leaf of the 3rd node. Samples fresh and newly collected and fixed and embedded in Paraplast® were used. Tests were conducted for lipids, terpenoids, phenolic compounds, alkaloids, sugars and proteins. Alkaloids were observed only in *R. montana*, as well as the results for phenolic compounds. Flavonoids are present in *B. verbascifolia* and *R. montana*. The lipid composition was showed for the chemical compounds of *B. verbascifolia* and *C. adamantium*, which proved to be part of the essential oils or resins oils in *C. adamantium* idioblasts. The chemical compounds of *B. verbascifolia*, *C. adamantium* and *R. montana* are present mainly in idioblasts among the parenchyma and epidermal cells. *C. adamantium* has secretory cavities, but only with lipid content. The identification of chemical compounds has not been possible in mature leaves of *S. lycocarpum*.

Introduction

Plant secondary metabolism produces products that can aid in the defense and protection, besides the attraction of pollinators (Evert, 2006). Those substances have been extracted and utilized for medicine production, vaccines and other forms of treatment (Barbosa-Filho et al., 2008; Souza et al., 2008). In this context, the anatomic study of pharmacologic use plants may contribute to quality assurance and correct identification (Mauro et al., 2008; Carpano et al., 2009; Gomes et al., 2009). Beyond that, it allows to elucidate the aspects referring to secreting structures and consequently to storage and secretion of secondary metabolites, which could lead to the correct localization and extraction of medicinal chemicals.

Secreting structures are frequently reported to the different organs of Cerrado plants (Castro et al., 1997; Rodrigues et al., 2011; Boudouris and Queenborough, 2013), this being Biome rich in medicinal use plants (Silva et al., 2010). Leaves, xylopodia and barks are quoted as producers of active pharmacological substances (Silva et al., 2010). According to Ribeiro and Walter (1998) the Cerrado is the second largest Biome in Brazil, occupying around 23% of national territory (Oliveira and Marquis, 2002). It is considered the floristically richest Savannah in the world with elevated endemism, being one of the prioritized Brazilian areas to conservation (Myers et al., 2000).

Malphigiaceae, Myrtaceae, Proteaceae and Solanaceae are well represented on the Cerrado (Rizzini, 1971; Pereira-Silva et al., 2004) that also contain plant species provided with active substances useful to diseases treatment. *Byrsonima* (Malpighiaceae) presents species with antimalarial activity (Milliken, 1997). *Byrsonima crassifolia* (L.) Kunth and *Byrsonima verbascifolia* (L.) DC., and are used for antifever in different countries in Latin America (Rutter, 1990; Garcia-Barriga, 1992). The leaves of species of *Campomanesia* (Myrtaceae) are useful for treatment of diarrhea and bladder issues (Piva, 2002). Besides the ethyl acetate extract of fruits of *Campomanesia adamantium* (Cambess.) O. Berg has shown an inhibitory effect against *Mycobacterium tuberculosis*, a pathogenic bacterium that causes most cases of tuberculosis (Pavan et al., 2009). The leaves of the *Roupala montana* Aubl. (Proteaceae) are utilized as antipyretics

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and antiseptics for treatment of wounds and ulcers (Butler et al., 2000). Solanaceae is a source of alkaloids provided with pharmacological actions, especially *Solanum crinitum* Lam., *S. lycocarpum* A. St.-Hil. and *S. gomphodes* Dunal, for being used in the treatment of diabetes (Araújo et al., 2010b).

Based on the exposure, this study aimed to evaluate the histochemistry of the leaves of *B. verbascifolia* (L.) DC., Malpighiaceae, *C. adamantium* (Cambess.) O.Berg, Myrtaceae, *R. montana* Aubl., Proteaceae, and *S. lycocarpum* A. St.-Hil., Solanaceae, species reported as producers of secondary metabolites of pharmacological activities. Also, it intends to describe the secretory structures that produce such compounds, and identify the cells and tissues in which they are stored.

Materials and methods

Studied species and deposit at the herbarium

Byrsonima verbascifolia (L.) DC., Malpighiaceae, *Campomanesia adamantium* (Cambess.) O.Berg, Myrtaceae, *Roupala montana* Aubl., Proteaceae, and *Solanum lycocarpum* A. St.-Hil., Solanaceae, were collected at the "Campo sujo" (shrub Savannah) of Cerrado within Serra do Cipó, Minas Gerais, Brazil (19°22′01′′S and 43°37′10′′W). Vouchers were deposited at the Herbarium of Universidade Federal de Minas Gerais (BHCB), under the registration numbers: 161584, 161585, 161586 and 167052.

Light microscopy

Leaves ($n = 5$) of the 3rd node of five individuals were collected, fixed in FAA (formalin, acetic acid, 50% ethanol, 1:1:18 v/v/v) and stored in ethanol 70% (Johansen, 1940).

Inclusions in Paraplast® (Kraus and Arduin, 1997) and/or in 2-hydroxyethyl methacrylate (Leica Instruments, historesin) were done with fragments of the median region. Then, cross sections of 5–10 μm were obtained using a rotary microtome (Leica® Biocut Jung, USA). The material prepared in historesin was stained with 0.05% toluidine blue – pH 4.7 (O'Brian et al., 1964) and Paraplast® with 0.5% safranin and astra blue, 2:8 (Kraus and Arduin, 1997). All leaves were mounted in Entellan® (Kraus and Arduin, 1997) and photographed with use of a light microscope (Primo Star Zeiss®) coupled with digital camera (Canon A650).

Histochemical tests

Transverse sections of the median area of the intercostal region were obtained from fresh and recently collected leaves from the 3rd node ($n = 5$), using table microtome (Rolemberg and Bhering Trade, model LPC). The tested metabolites classes are listed in Box 1. Fresh selections, unfixed and unstained, were utilized as negative control. The positive control was conducted as recommended by the respective authors of histochemical tests. The tests were repeated on material included in Paraplast® in order to obtain thinner sections and thus improve the visualization of the results. In both cases, the slides were mounted in the reagent itself or jelly glycerin. The sections were photographed under a light microscope (Primo Star Zeiss®) coupled with digital camera (Canon A650).

Box 1: Metabolite groups, reagents and authors of the methodologies used in histochemical tests.

Metabolite groups	Reagent	References
Lipids		
Total	Sudan red B	Brundrett et al. (1991)
Terpenoids		
Essential oils and resin-oils	Nadi reagent	David and Carde (1964)
Steroids	Antimony trichloride	Hardman and Sofowora (1972), Mace et al. (1974)
Phenolic compounds		
General	Ferric chloride III	Johansen (1940)
Tannins	Vanillin–hydrochloric acid	Mace and Howell (1974)
Lignin	Phloroglucinol	Johansen (1940)
Flavonoids	DMACA - *p*-Dimethylaminocinnamaldehyde	Feucht and Schmid (1983)
Alkaloids		
General	Dragendorff	Furr and Mahlberg (1981)
Polysaccharides		
General	PAS - Periodic acid–Schiff's reagent	Mcmanus (1948)
Starch	Lugol	Jensen (1962)
Pectins	Ruthenium red	Johansen (1940)
Mucilage	Tannic acid/Ferric chloride III	Pizzolato and Lillie (1973)
Proteins		
Total	Bromophenol blue	Mazia et al. (1953)

Results

There was no occurrence of external secretory structures on the leaf lamina in any species studied, but only internal secretory structures occurred in them (classification of Evert, 2006). These structures occur in the midrib (Fig. 1A, D and G), the intercostal region (Fig. 1B, E, H) and the margin (Fig. 1C, F and I), particularly in idioblasts (Fig. 1A–I), in most species. In these cells, the chemical compounds occupy uniformly all vacuole, in general (Fig. 1A and I). The idioblasts present primary walls when in the epidermis and parenchyma (Fig. 1A–F), or secondary, when attached to the fibers (Fig. 1G–I).

The size and shape vary according to the tissue to which it is associated, in most cases being similar to the neighboring cells of the same meristematic origin (Fig. 1A–I). In such cases, recognition is only possible by the presence of chemical compounds.

Byrsonima verbascifolia

In midrib, idioblasts containing chemical compounds are shown between xylem and phloem cells, and in parenchyma originated from the ground meristem (Fig. 1A). In the intercostal region and in the edge of the leaf, the compounds are found in epidermal cells on both sides, in the mesophyll and in the smaller diameter bundles (Fig. 1B and C). In the negative control it was not possible to identify chemical compounds (Fig. 2A).

The histochemical analysis showed lipids in all idioblasts with content (Fig. 2B; Box 2) and concentrated flavonoids in the cells of the spongy and palisade parenchyma (Fig. 2C; Box 2).

Campomanesia adamantium

In the midrib, chemical compounds are widely found in epidermal cells, in the ordinary parenchyma and in the parenchyma cells and fibers of xylem and phloem (Fig. 1D). The intercostal region contains chemical compounds mainly in the epidermis and bundle sheath cells (Fig. 1E). In the edge of the leaf, the content can be widely viewed in all tissues (Fig. 1F). Secretory cavities are distributed among the mesophyll cells (Fig. 1F).

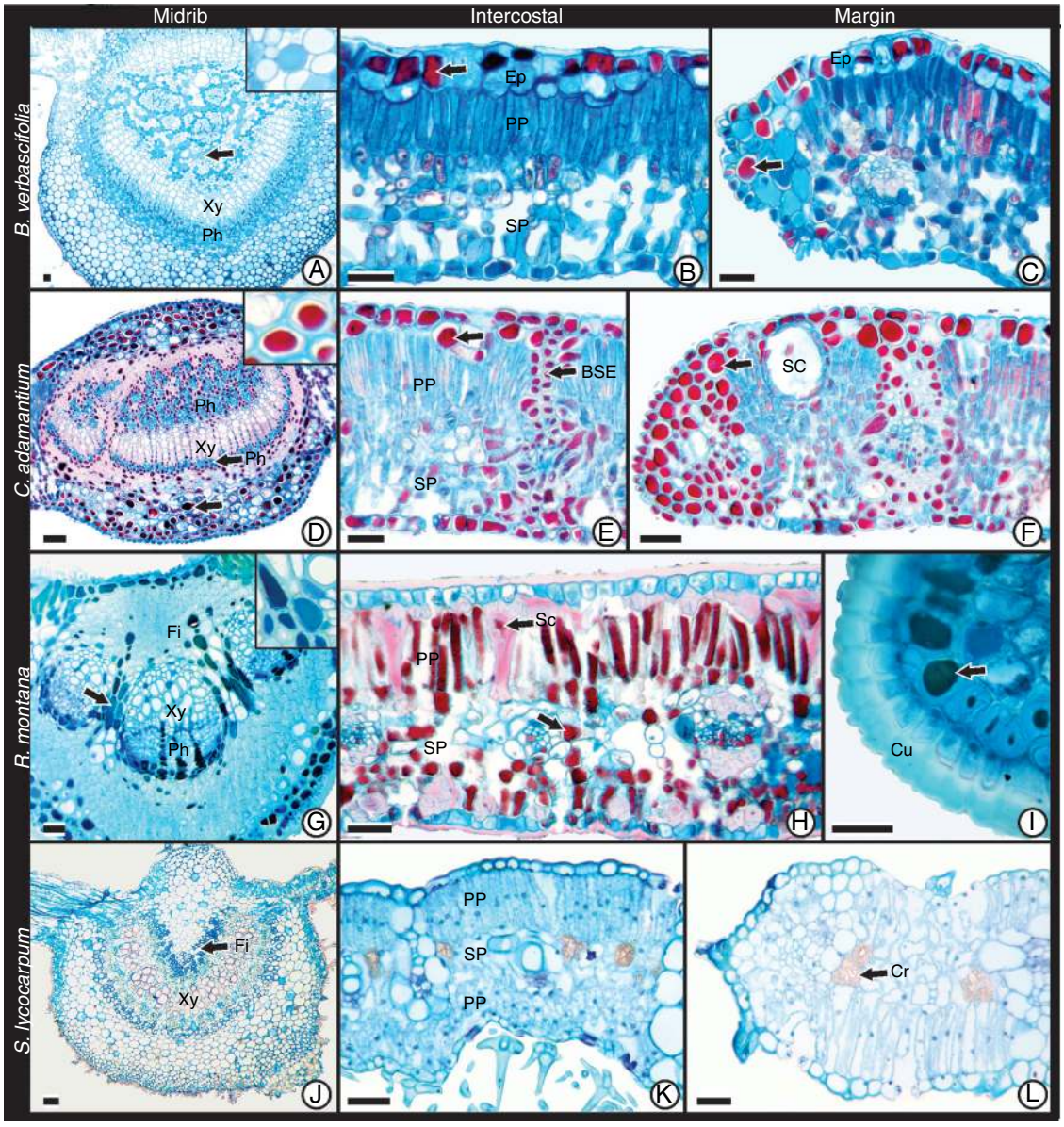

Fig. 1. Cross sections of *Byrsonima verbascifolia* (A–C), *Campomanesia adamantium* (D–F), *Roupala montana* (G–I) and *Solanum lycocarpum* (J–L). (A, D, G, J) Midrib; (B, E, H, K) Intercostal region; (C, F, I, L) Margin. *Abbreviations*: Xy, xylem; Ph, phloem; Fi, fiber; PP, palisade parenchyma; SP, spongy parenchyma; BSE, bundle sheath extension; SC, secretory cavity; Cr, crystal; Sc, sclereid; Cu, cuticle; Ep, epidermis. Arrows indicate idioblasts with chemical compounds. Bars = 50 μm.

In the section without chemical treatment (negative control) it was not possible to visualize any chemical compounds of potentially pharmacological use (Fig. 2D). From the histochemical tests it was noted that essential oils or oil resins (Fig. 2E, Box 2) and lipids (Fig. 2F; Box 2) are widely distributed in the leaf blade. In the secretory cavities positive results for general lipids were found (Fig. 2G; Box 2).

Roupala montana

In midrib, the chemical compounds are present in subepidermal parenchyma cells, in the fibers that surround the vascular system and in the parenchyma and fiber cells of xylem and phloem (Fig. 1G). In the intercostal region, the compounds occur in the epidermis of both leaf surfaces, parenchyma cells in the mesophyll and in the minor vascular bundles (Fig. 1H). In the edge, the compounds are associated with fibers and parenchyma cells (Fig. 1I).

Chemical compounds were not visualized in negative control (Fig. 2H). Positive results were found for phenolic compounds in the epidermal cells of adaxial surface (Fig. 2I, Box 2). Flavonoids (Fig. 2J, Box 2) and alkaloids (Fig. 2J, Box 2) were present in the mesophyll cells.

Solanum lycocarpum

No evidence of chemical compounds was found (Fig. 1J, K, L) and all the histochemical tests had negative results (Box 2).

Discussion

Most chemical compounds found are present in idioblasts. According to Fahn (1979) idioblasts are cells that differ from others by presenting distinctive shape, size or different content from other cells of the tissue. They can accumulate and/or secrete large amounts of compounds such as oil, resin, mucilage and tannin

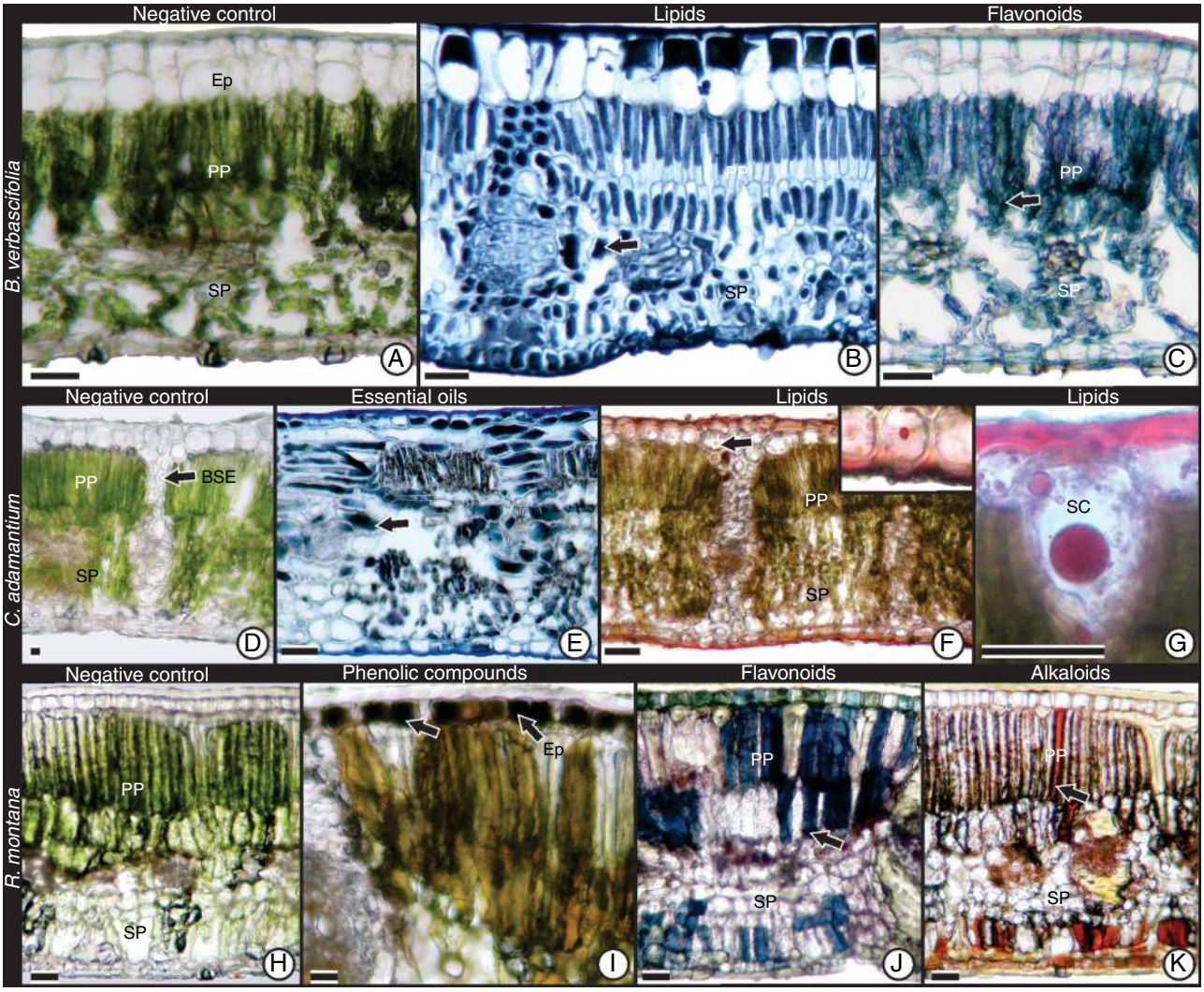

Fig. 2. Cross sections with positive results for histochemical tests. (A–C) *Byrsonima verbascifolia*; (D–G) *Campomanesia adamantium*; (H–K) *Roupala montana.* (A, D, H) Negative control; (B, F, G) lipids; (C, J) flavonoids; (E) essential oils; (I) phenolic compounds; (K) alkaloids. *Abbreviations*: Ep, epidermis; PP, palisade parenchyma; SP, spongy parenchyma; BSE, bundle sheath extension; SC, secretory cavity. Arrows indicate idioblasts with chemical compounds. Bars = 50 μm.

(Fahn, 1979). Those different cells present a large distribution in the leaf, from the midrib until the edge, except for *S. lycocarpum*, that contrary to expectations did not show positive results from the histochemical analysis adopted.

Rinaldo et al. (2010) studied six species of *Bysonima* and found the presence of two flavonoids: catechin and epicatechin, including for *Bysonima verbascifolia.* These compounds present antitumor and antioxidant activity and may act in the treatment of gastric ulcers, inflammation, skin infections and fever (Heinrich et al., 1992; Aguiar et al., 2005). The results indicate that the production site and/or accumulation of flavonoids in *B. verbascifolia* are associated with parenchyma cells, especially in the palisade and spongy parenchyma. Parenchyma mesophyll cells containing chemical compounds have been reported in six species of *Byrsonima* from Brazilian Cerrado (Araújo et al., 2010a), but without histochemical characterization.

In Myrtaceae, secretory cavities producers of lipophilic compounds are widely found in species (Solereder, 1908; Metcalfe and Chalk, 1950), as observed for *Campomanesia adamantium*. The wide production of essential oils or oil resins in *C. adamantium*

Box 2: Positive results of histochemical tests for the secretory structures on the leaf blade of the four species studied. Abbreviations: (+) = positive result.

Metabolite groups		Secretory structures	Species			
			B. verbascifolia	*C. adamantium*	*R. montana*	*S. lycocarpum*
Alkaloids	General	Idioblast			+	
Phenolic compounds	General	Idioblast			+	
	Flavonoids	Idioblast	+		+	
Lipids	Total	Idioblast	+	+		
		Secretory cavity		+		
Terpenoids	Essential oils and resin-oils	Idioblast		+		

leaves corroborates the studies of chemical characterization of other species of *Campomanesia*. Essential oils are present in *C. guazumifolia* (Cambess.) O.Berg, *C. xanthocarpa* (Mart.) O.Berg and *C. rhombea* O.Berg, being rich in sesquiterpenes, while *C. aurea* O.Berg features predominance of monoterpenes (Limberger et al., 2001). For *C. adamantium*, essential oils of the leaves have a low income, with presence of mono and sesquiterpenes (Vallilo et al., 2006; Coutinho et al., 2008), however with the wealth of flavanones and chalcones (Coutinho et al., 2008). Despite these results, the test performed to flavonoids (DMACA) has not detected such a compound in the evaluated leaf areas. Coutinho et al. (2010) reported that the flavonoids content in *C. adamantium* leaves is influenced by seasonal variation, which may explain the fact that this compound was not found in the analysis, especially considering that the environment at the location of collection shows two distinct seasons (Klink and Machado, 2005).

The Proteaceae chemical profile is composed mainly by flavonoids, saponins, polyphenols glycosides, coumarins and alkaloids (Butler et al., 2000). The flavonoids isolated from the aerial part of *R. montana* have been used to reduce the motor activity of adult *Schistosoma mansoni* (Neves et al., 2015). These compounds were identified in the mesophyll cells in the spongy and palisade parenchyma. Furthermore, the alkaloids, typical of Proteaceae (Butler et al., 2000), were also found in leaves of *R. montana*, in the same region where the flavonoids are present.

In *S. lycocarpum*, the pharmacobotany work usually shows the main use of the fruit for the treatment of diseases such as asthma, flu, colds, and also as a tonic (Rodrigues and Carvalho, 2001). The leaves are used as emollient and antirheumatic (Rodrigues and Carvalho, 2001). Despite the use, no evidence of production of potentially active chemical compounds in green and mature leaves was found. Histochemical studies for *S. lycocarpum* were conducted by Araújo et al. (2010b), but only elucidated the chemical characteristics of structural components. Aires et al. (2005) showed allelopathic effect in senescent leaves of this species, which indicates possible production of chemical compounds in that organ. In young leaves glandular trichomes were reported (Elias et al., 2003), which do not remain in the mature leaf (Araújo et al., 2010b). Chemical analysis uncovered the presence of tannins, flavonoids, steroids and triterpenes, coumarins and saponins, for *S. lycocarpum* leaves (Gallon et al., 2015), being assigned antibacterial, antifungal and antiviral for tannins (Carvalho et al., 2007). Thus, it is possible that the chemical compounds from the leaves of *S. lycocarpum* are produced only in young leaves or in senescence, or which are undetectable by histochemical tests in specific months of the year due to low productivity.

In summary, through the adopted histochemical tests it was possible to identify in tissue level, the region of synthesis and/or storage of metabolites of pharmacological use in the leaves of *B. verbascifolia*, *C. adamantium* and *R. montana*. The same was not possible to the mature leaves of *S. lycocarpum*. The potentially active compounds were concentrated on idioblasts present primarily among the parenchyma and epidermal cells, which are differentiated from the others only by the presence of chemical compounds.

Author's contributions

VCK (PhD student), part from his thesis, participated in all stages of the work; FHAV supervised the doctoral student VCK in all stages of the work.

Conflicts of interest

The authors declare no conflicts of interest.

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