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Morpho-anatomy and chemical profile of native species used as substitutes of quina (*Cinchona* spp.) in Brazilian traditional medicine. Part I: *Polyouratea hexasperma*

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Abstract: This research is part of a larger study of the Brazilian species that are commonly referred to as quinas and are usually used as substitute of *Cinchona* species. The purpose of the present study was the botanical characterization of the whole and powdered stem bark of *Polyouratea hexasperma* (A. St.-Hil.) Tiegh., Ochnaceae, by morphological and anatomical description, and the analysis of its chemical profile. The external texture of the bark, the whitened pit in the inner bark and the presence of cristarque cells, as well as the shape and arrangement of other lignified cells, are the most important macroscopic and microscopic features for the characterization of the bark. Chlorogenic and caffeic acids were detected in the chemical analysis and can also be used in the identification of the bark.

Introduction

Quina (or china) is the traditional name originally assigned to *Cinchona* species, specifically *Cinchona calisaya* Wedd. and *C. succirubra* Pav. ex Klotzsch, Rubiaceae. These plants are native to Peru, and their barks are sources of quinine and other alkaloids that have important medical applications. Quinine has been used to treat humans with malaria for over 350 years and is still in use, especially in cases of chloroquine-resistant *Plasmodium falciparum* (Kaur et al. 2009; Dondorp & Nosten, 2009). Quinine is also commercially used as a flavoring agent in tonic water due to its bitter flavor (Brasic, 1999).

Species of the *Cinchona* genus that produce quinine do not naturally grow in Brazil, and the interest in finding natural substitutes for *Cinchona* barks stimulated the search for *Cinchona* species in the Brazilian territories in the 18th and 19th centuries (Dean, 1996; Ribeiro, 2005). A consequence of these searches was the appearance of several substitutes for *Cinchona* plants that also received the name quina. In 1799, for example, the Brazilian naturalist Frei M. C. Vellozo wrote a book entitled *Quinographia Portugueza*, in

which he describes data on almost thirty substitutes for species of *Cinchona* which produces quinine (Vellozo, 1799). The German naturalist K. F. von Martius, in 1854, also registered several substitute species in his book *Systema Materia Medica Vegetal* (Martius, 1854). In 1916, Waldemar Peckolt, publish a monography with the description of fifteen species used as quinas in different regions of Brazil (Peckolt, 1916). In his travel notebook, the French naturalist A. Saint-Hilaire registered several species of quina used by the Brazilian population (Brandão et al., 2012), and in his book, *Usual Plants of Brazilians* (Saint-Hilaire, 2009), he reported the presence of seven substitutes for *Cinchona* spp. Barks from the quina-mineira (*Remijia ferruginea* (A. St.-Hil.) DC.) and quina-do-campo (*Strychnos pseudoquina* A. St.-Hil.) species were already widely used as tonics and febrifuges in conventional medicine. Monographs for these barks were included in the first edition of the Brazilian Pharmacopoeia (Brandão et al., 2009). Despite not being currently used in the preparation of industrialized medicine (Brandão et al., 2010), the barks of these quina plants are still being sold in popular markets all over the country (Brandão et al., 2013).

In recent years, our research group has focused on the restoration of information and samples of the plants used in traditional Brazilian medicine in the past centuries. A database including images and data on such plants is available at www.dataplant.org.br. The present study is part of a larger project in which we are retrieving and evaluating the bitter native species known as quina. Analytical methods for the characterization of vegetal drugs from these species are scarce or do not exist, and this lack of methods may make their identification, as well as their quality control, difficult. The final objective of this project is to gather information to better understand these remedies from native Brazilian species and to promote their better use and conservation. In this study, we show the results from the morpho-anatomic and chemical profile analysis of the bark of the quina species *Polyouratea hexasperma* (A. St.-Hil.) Tiegh., Ochnaceae.

Materials and Methods

Plant material

The samples of *Polyouratea hexasperma* (A. St.-Hil.) Tiegh., Ochnaceae, bark were collected from a small tree on the University of Brasília campus (DF, Brazil), located at 15.46.12.9S, 47.52.06W at an altitude of 1040 m. The voucher was deposited in the University of Brasília herbarium (Fagg, C.W. 2191 UB), and the vegetal drug (dried bark) was deposited in the Dataplant-UFGM (DAT-105).

Morpho-anatomical analysis

Soon after the samples were collected, the samples were fixed in a solution of formalin, acetic acid, and 70% ethanol (FAA), in a proportion of 1:1:18 per volume (Johansen, 1940), rinsed in distilled water, and stored in 70% ethanol. These samples were dehydrated in an ethanol series, embedded in Leica HistoResin, and sectioned using a rotary microtome Leica RM2145 (Leica Microsystems, Wetzlar, Germany). The 3-7 μm sections were stained with 0.05% toluidine blue solution (O'Brien et al., 1964). The slides were mounted using Verniz vitral incolor 500[®] (Paiva et al., 2006). Free-hand sections were obtained with the aid of a Ranvier's microtome and were submitted to various histochemical tests; these included ferric chloride (Johansen, 1940) and potassium dichromate (Gabe, 1968) staining to detect phenolic compounds, acid phoroglucin staining for lignin detection (Sass, 1951), lugol staining for starch (Kraus & Arduin, 1997), Dragendorff's reagent (Yoder & Malhberg, 1976) and Wagner's reagent (Furr & Mahlberg, 1981) tests for alkaloids and Sudan III (Sass, 1951) and Sudan IV staining for lipid detection (Gerlach, 1984). Part of each sample was submitted to the maceration process for dissociation and tissue component analysis. For

this analysis, the samples were placed in Franklin solution and maintained in a kiln at 60 °C for 72 h (Kraus & Arduin, 1997). After this process, the macerate was washed with distilled water to completely remove the Franklin solution and was stored in 50% ethanol. The macerates were stained with 1% ethanolic safranin. The slides were analyzed and described using an Olympus CX31 optical microscope and photographed using a digital Olympus C-7070 camera with wide zoom.

Chromatographic profile for phenolic and alkaloids in TLC

Preparation of fractions enriched in phenolic substances

Briefly, 1 g of dried *P. hexasperma* bark was extracted under reflux conditions with 20 mL 70% ethanol for 30 min. The sample was then filtered through filter paper and concentrated and dried using a rotary evaporator. The resulting residue was diluted with 50 mL water and extracted three times with 30 mL ethyl acetate. The ethyl acetate fraction was concentrated to dryness and dissolved in 1 mL methanol for subsequent analysis.

Preparation of alkaloid fraction

Briefly, 3 g of dried *P. hexasperma* bark was extracted under reflux conditions with 20 mL 0.1 M HCl for 30 min. The solution was alkalized with NH_4OH to pH 9.0 and extracted three times with 20 mL ethyl ether. The organic phase was concentrated to dryness, and the resulting residue was dissolved in 2 mL methanol for subsequent analysis.

Chromatographic analysis

TLC was performed on silica gel plates (Macherey-Nagel Alugram Xtra UV 254) using solvents and reagents specific for the detection of phenolic substances, including flavonoids and alkaloids. For the detection of phenolic substances, an ethyl acetate: acetic acid: formic acid: water (100:26:26:13) mixture was used, followed by spraying with NP/PEG. The rutin hydrate (Sigma Aldrich), chlorogenic acid (Aldrich) and quercetin (Sigma Aldrich) flavonoids were used as reference standards. For the detection of alkaloids, a toluene:methanol:diethylamine (80:10:10) mixture was used, followed by spraying with Dragendorff reagent. Samples of quinine hydrochloride dihydrate (Sigma), quinidine (Sigma), cinchonin (Aldrich), and cinchonidin (Sigma-Aldrich) were used as reference standards.

Results and Discussion

Poliouratea hexasperma (A. St.-Hil.) Tiegh., Ochnaceae, is a small tree that grows between two and three meters in height. This species is characteristically found in the Brazilian Cerrado (Figure 1A), but it also occurs in Bolivia and Paraguay (Killeen et al., 1998). A. de Saint-Hilaire first observed the use of this species as a substitute for *Cinchona* spp. in the 19th century. He first described this species in 1824 and named it with the basionym *Gomphia hexasperma* A. St.-Hil. The bark of this species has been used to heal wounds caused by insect

bites in both animals and humans; the bark has also been used as an astringent (Martius, 1854; Saint-Hilaire, 2009). On the other hand, Peckolt (1916) not cited this species in your study of Brazilian quinas.

Externally, the bark of *P. hexasperma* is corky and ranges in color from grey to yellowish grey. The bark has sparse deep discontinuous fissures, which can be longitudinal or transversal (Figure 1B). The bark can also have grey-green lichens. When cut on the transverse plane, the bark can be divided into an outer bark and an inner bark, according to Trockenbrodt (1990); this definition considers all the tissues outside of the vascular cambium

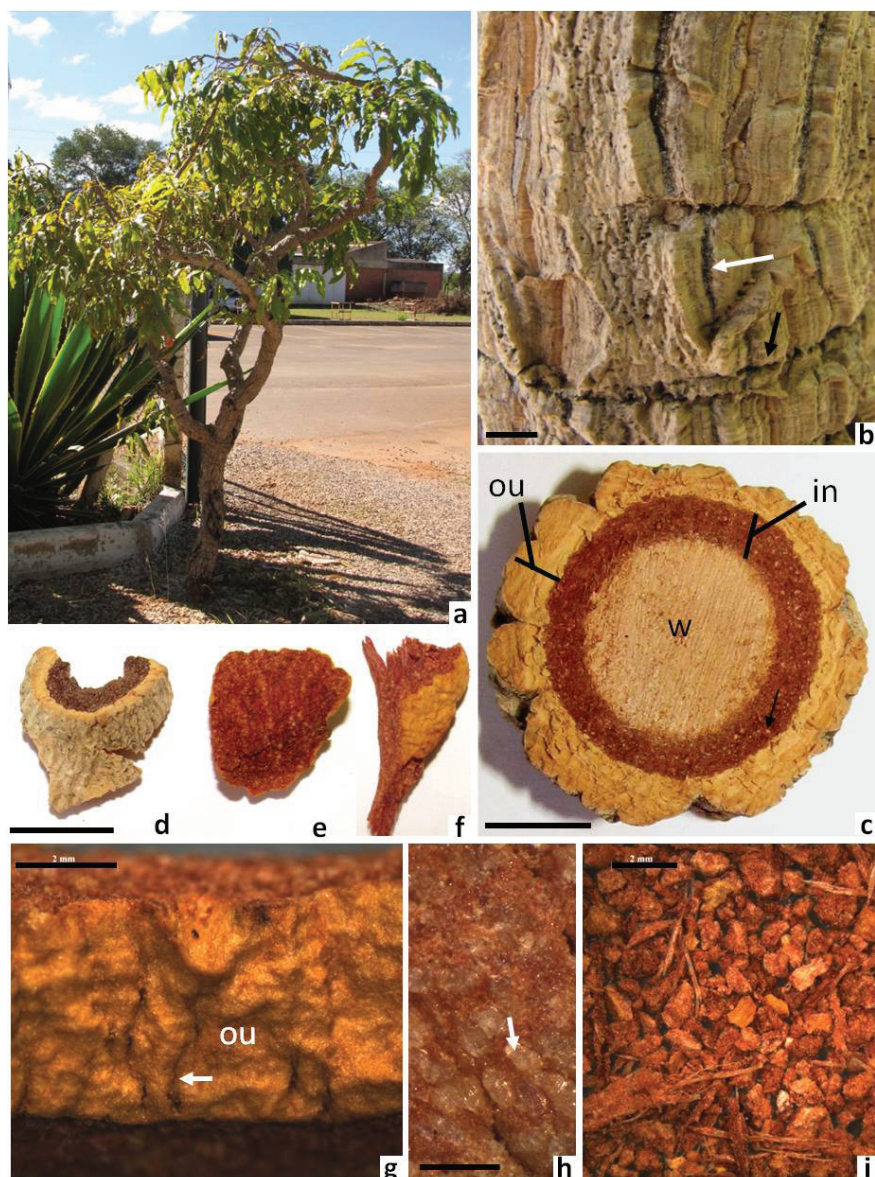


Figure 1. *Polyouratea hexasperma* (A. St.-Hil.) Tiegh. A. Tree. B. Externally aspect of bark showed longitudinal and transversal fissures (arrows white and black, respectively). C. Cross-section showed outer (ou), inner(in) bark and wood (w). Arrow points the whitened dots. D-F. Dry fragments of bark (plant drug). G. Detail of outer bark (ou) without cork. Arrows points the fissure. H. Detail of whitened dots (arrow) of inner bark. I. Powder of the bark with fibrous pieces. Scale bars: 1 cm (B-F), 2 mm (G, I) and 0,5 mm (H).

as bark, regardless of its specific structure. According to this same author, the terms outer bark and inner bark are recommended, and the term bast, which is sometimes used to identify the inner bark, should be avoided. The outer bark is creamy-brown in color, and the inner bark is reddish-brown with white dots (Figure 1C). Dry fragments (plant drug) can be represented by complete bark or parts (Figure 1D-F). When the plant drug is devoid of cork, the outer bark is yellowish in color and appears wrinkled, with small transverse fissures (Figure 1F-G). The white dots observed on the inner bark are translucent when magnified (Figure 1H). The powder is reddish in color, with little yellow pieces and fibrous pieces (Figure 1I).

When cross-sectioned, the outer bark is formed by the periderm with phellem, phellogen and phelloderm cells (Figure 2A,B). The phellogen has a subepidermal origin according to ontogeny analyse in young stem (data not shown). The phellem is very regular and formed by several layers of juxtaposed rectangular cells, with slightly thickened walls, followed by one layer of phellogen (Figure 2C). In the phelloderm, the cells developed thick walls (Figure 2A,B), lignified and formed a continuous ring just below the phellogen (Figure 2B). The deposition of lignin in these cells was thicker in the inner and anticlinal walls, thereby giving this structure the shape of the letter “U” (Figure 2C, arrows). These cells are called cristarque

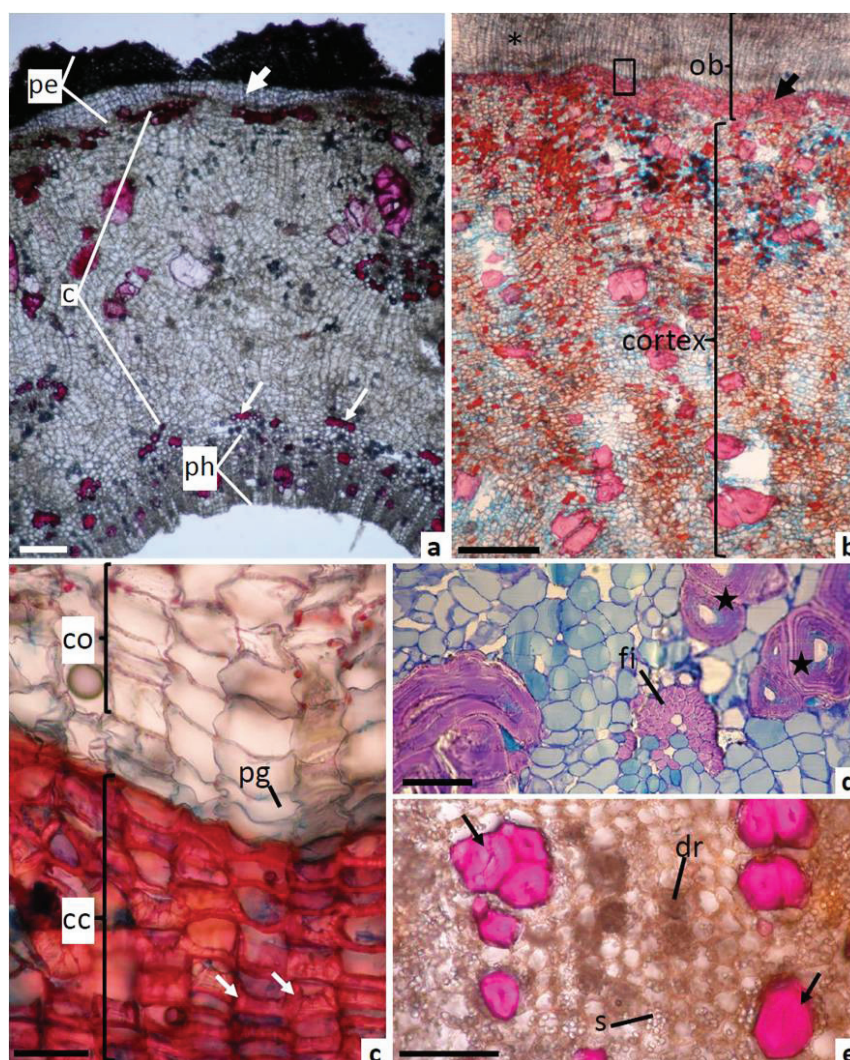


Figure 2. Microscopy characteristics in transversal section of the bark of the *Polyouratea hexasperma* (A. St. Hi.) Tiegh. A. General view of the young bark showed the periderm (pe) with phelloderm without lignification (arrow), cortex (c) and secondary phloem (ph). Thin arrow showed the fibers of the primary phloem. B. Part of the outer bark (ob) showed the phellem (asterisk) and phelloderm with lignified cell wall (arrow) and part of the inner bark showed the cortex (c) with clusters of sclereids. C. Magnification of square in (b). Cork with rectangular cells (co), phellogen (pg) and cristarque cells (cc) of the phelloderm showed thickening on the inner and anticlinal walls (arrows). D. Cortex region with stone cells (stars) and bundle of primary phloem fibers (fi). E. Secondary phloem region with lengthened sclereids isolated or clustered (arrows), druse (dr) and starch grains (s) in the parenchyma cells. Scale bars: 200 µm (A-B), 100 µm (D), 50 µm (C,E).

cells and usually have a crystal inside the lumen (Rao et al., 1967; Dickinson, 2000). Nevertheless, the presence of the crystals was not detected in the lumen of these cells in *P. hexasperma*. The presence of the cristarque cells is a diagnostic characteristic of the Ochnaceae family and a few others families (Dickinson, 2000). Phelloderm cells with thickened walls are common in tropical plants (Roth, 1981), and these cells can provide protection to the inner tissue when the cork is lost (Yamamoto, 1989).

The inner bark is formed by the cortex and the secondary phloem (Figure 2A). In the cortex, there are a large number of parenchyma cells interspersed with clusters of sclereids, as well as single sclereids and groups

of sclerenchyma fibers (Figure 2B and 2D). These fibers are reminiscent of the primary phloem (Figure 2A). In the secondary phloem, lengthened sclereids were found close to the vascular cambium; these sclereids were found in isolation as well as in clusters (Figure 2E). Idioblasts containing druses (Figure 2E and 3B) and lipidic substances in the shape of oil droplets (Figure 3B) or adhered to druses were observed mostly in the parenchyma cells of the secondary phloem. Cells with phenolic compounds and idioblasts containing starch grains (Figure 2E) were distributed throughout the cortex and the secondary phloem, especially in the parenchyma cells of the cortex. The presence of phenolic compounds in these regions of

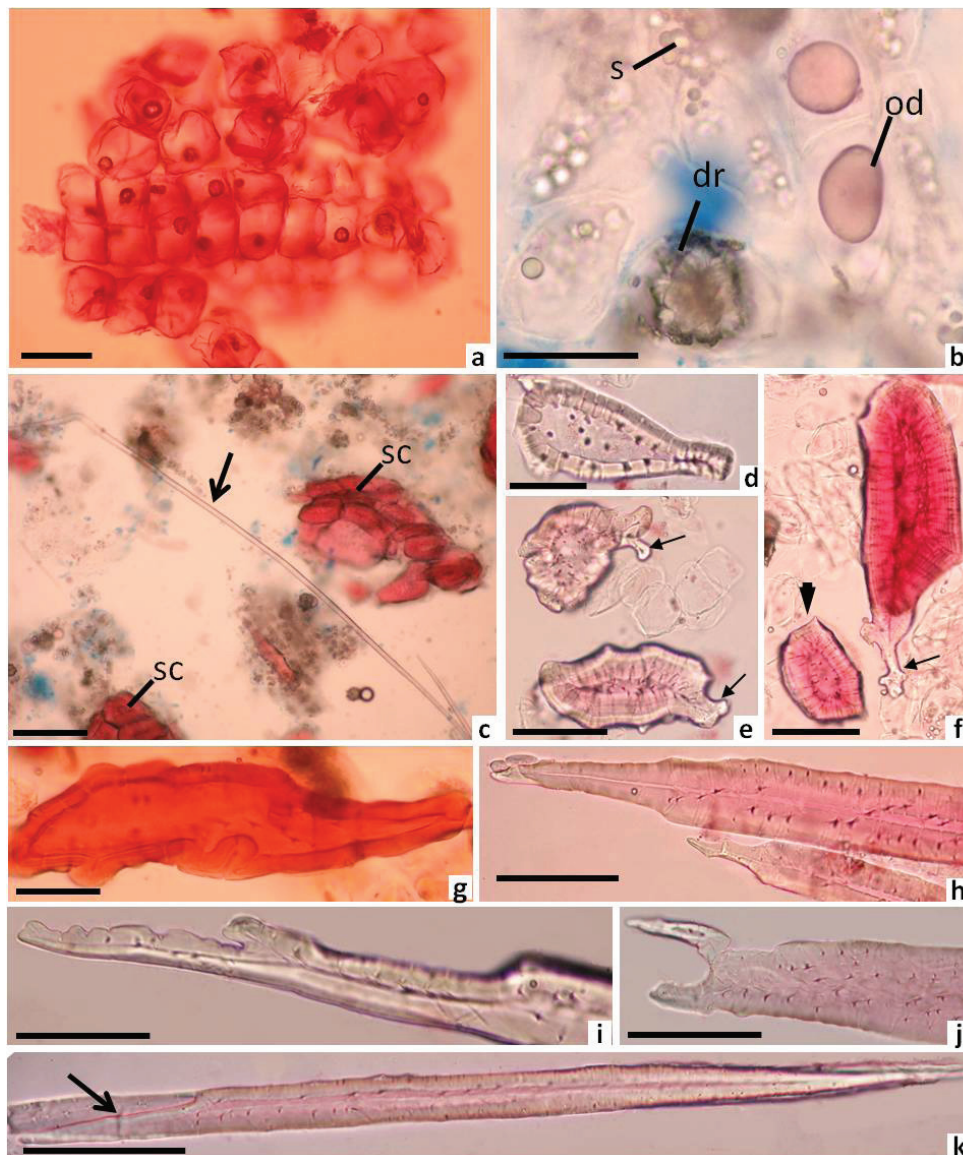


Figure 3. Macerate of *Polyouratea hexasperma* (A. St. Hi.) Tiegh. A. Cork cells. B. Idioblasts with druses (dr), starch grains (s) and oil droplets (od). C. Part of primary phloem fiber (arrow) and cluster of sclereids (sc). D. Stone cell. E. Sclereids with adorned ends (arrows). F. Stone cell (thick arrow) and sclereid with adorned end (arrow). G. Sclereid with irregular form. H-K. Lengthened sclereids. H-J. Details of adorned ends. K. Detail of intrusive growth at the end of the sclereids (arrow). Scale bars: 100 μ m (C) and 50 μ m (A-B, D-K).

the bark is common in plants from the Cerrado (Costa et al., 1997), and these compounds usually help protect the plant from ultraviolet B radiation and insect herbivory in the leaves (Izaguirre et al., 2007). However, the function of these compounds in the bark remains unknown.

After the maceration process, the following cell types were identified: clustered or isolated square-shape cork cells (Figure 3A), idioblasts containing druses and oil droplets (Figure 3B), sieve tube members with sieved areas and sieve plates, parenchyma cells and lignified cells. The lignified cells showed variation in the cells types, such as narrow and elongated primary phloem fibers (Figure 3C), and sclereids of diverse shape; these sclereids included stone cells or brachysclereids (Figure 3D, F), short sclereids with or without adorned ends (Figure 3E,F), sclereids with irregular forms (Figure 3G) and lengthened sclereids with intrusive growth and adornments at the end of the cells (Figure 3H-K). Short sclereids with and without adorned ends had polylamellate walls with branched pits (Figure 3D-G). Phloem fibers and lengthened sclereids (Figure 3H-K) did not possess polylamellate walls, but the lengthened sclereids did possess branched pits. These pits were not visible in the phloem fibers.

Fibers and sclereids in the secondary phloem and cortex are common features and are found in most barks (Esau, 1977; Parameswaran, 1980; Trockenbrodt, 1990; Junikka, 1994). However, the characterization of the cells types, such as their shape, location and source, are important (Parameswaran, 1980; Evert, 2006) and could assist in the diagnosis of vegetal drugs.

Previous phytochemical studies have shown the presence of terpenoids, isoflavonoids, flavonoid glycosides, biisoflavanones and biflavones in the roots, leaves and stem barks of *P. hexasperma*. The biflavone are considered to be chemical markers of the genus *Polyouratea* (Moreira et al., 1994; 1999; Daniel et al., 2005; Carvalho et al., 2008; Fernandes, 2008; Suzart et al., 2007). The biflavone 7-*O*-methyl-agathisflavone has been shown to inhibit DNA topoisomerase and therefore possesses anticancer activity (Grynberg et al., 2002; Daniel et al., 2007). Flavonoids were only weakly observed in our study, being rutin and quercetin absent. Conversely, chlorogenic acids among other caffeic acids were clearly observed in the TLC analysis and the presence of these substances can be useful for characterization of the bark. Alkaloids were also weakly observed in R_f of 0.60, above the bands for quinine, quinidine, cinchonine and cinchonidine, which were used as standards. In spite of TLC analyzes reveal the presence of these substances in the bark, histochemical tests were negative for alkaloids, hindering its localization in tissues.

Conclusion

This study reports the morpho-anatomical and chemical characteristics of the bark of the quina substitute *Polyouratea hexasperma* (A. St.-Hil.) Tiegh. The presence of cristarque cells in the phelloderm and the adorned sclereids are anatomical characters of diagnostic value. Furthermore, the presence of chlorogenic acids may be useful for identification of the extracts of the bark in addition to already existing chemical markers. Wherefore, these characteristics could contribute to the identification of this species and help in quality control analyses.

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Authors' Contribution

NSS contributed in anatomy and histochemical studies. MGLB is the coordinator of the research and GPC has done the chromatographic analyses. CWF contributed in collecting plant material, identification and herbarium confection. All the authors have read the final manuscript and approved the submission.

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