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Full Length Research Paper

Anatomical description, alkaloid content and quality control of the bark of Pau-pereira (*Geissospermum laeve*, Apocynaceae)

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Fifteen samples commercialized as Pau-pereira have been purchased from open-air fairs, herbal stores, newsstands, and Internet sites, and their anatomical features were compared with two reference samples of *Geissospermum laeve*, Apocynaceae. In addition, an analysis was carried out to track the presence of flavopereirine, an alkaloid described in *G. laeve* and other species of the genus, in both ethanolic extract and infusions of the reference samples, as well as the 15 samples acquired from commercial sources.

Key words: Bark anatomy, Brazilian Pharmacopeia, *Geissospermum*, medicinal plant, natural product.

INTRODUCTION

The bark of *Geissospermum laeve* (Vell.) Miers (synonym *Geissospermum vellosii* Allemão, nom. illeg.), either in powder or splinter form, is sold in open-air markets, newsstands and herbal stores as febrifuge to relieve indigestion and stimulate appetite (Pio Corrêa, 1984). This species is commonly known as Pau-pereira ("pereira bark"), and it is an endemic large tree of the Atlantic Forest of the Brazilian east coast (Lorenzi, 2000). Pau-pereira was listed in the first edition of the Brazilian Pharmacopoeia (Albino, 1926), but it was removed in the second edition (Pharmacopeia dos Estados Unidos do Brasil, 1959) and, also, not included in the National Formulary (Brasil, 2005). This plant is part of Brazil's history of natural products because the first alkaloid in the country was isolated from its bark by Ezequiel Corrêa dos Santos, the patron of Brazilian pharmacists (Almeida et al., 2009). It was also studied by Rodolpho Albino, the developer of the Brazilian Pharmacopeia (Albino, 1926). Considered one of the 10 most useful Brazilian trees in phytomedicine (Peckolt, 1942), the isolated alkaloids of *G*. *laeve* present an array of pharmacological activities (Araújo et al., 2010; Lima et al., 2009; Hall and Beljanski, 2005; Gouvêa, 1964; Aurosseau, 1961a, b; Raymond-Hamet, 1954).

The purpose of this study was to comparatively assess the anatomic features of the barks and alkaloid contents of the 15 samples freely commercialized as Pau-pereira with 2 reference samples of *Geissospermum laeve* to determine the quality and authenticity of Pau-pereira sold over the counter.

MATERIALS AND METHODS

Reference sampling

Two individuals of *G. laeve* were collected, one from the arboretum of the Instituto de Pesquisas Jardim Botânico of Rio de Janeiro and another in the city of Nova Iguaçu, both in the State of Rio de Janeiro, Brazil. Portions of the bark were deposited in the wood collection of the Instituto de Pesquisas Jardim Botânico of Rio de Janeiro under numbers RBw 9572 and RBw 9573, and in this work, they are denoted as "reference samples" (Table 1). These reference samples were collected at approximately 1.3 m above ground with the help of a chisel. Afterwards, they were fixed in FAA(acetic acid, formaldehyde and ethanol 70%), according to Johansen (1940), for three days, followed by immediate storage in

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Table 1. Suppliers and origin of the samples commercialized as Pau-pereira and reference samples.

Origin of the sample / purchase site and origin	Type of tissue
1 – Herbal store Rio de Janeiro, RJ	Phloem, periderm and wood
2 – Herbal store Rio de Janeiro, RJ	Phloem and periderm
3 – Open-air market Nova Iguaçu, RJ	Phloem and periderm
4 - Newsstand Rio de Janeiro, RJ	Phloem and periderm
5 – Open-air market Niterói, RJ	Phloem and periderm
6 – Open-air market Rio de Janeiro, RJ	Phloem
7 – Open-air market Niterói, RJ	Phloem and periderm
8 – Open-air market Niterói, RJ	Phloem and periderm
9 – Open-air market Rio de Janeiro, RJ	Phloem
10 – Open-air market Teresópolis, RJ	Periderm
11 – Herbal store Rio de Janeiro, RJ	Phloem and periderm
12 – Herbal store Bahia, BA	phloem and periderm
13 – Internet site	Phloem and periderm
14 – Open-air market Rio de Janeiro, RJ	Phloem and periderm
15 - Newsstand Pará, PA	Phloem and periderm
RBw 9572 – reference sample Rio de Janeiro, RJ	Phloem, periderm and wood
RBw 9573 – reference sample Nova Iguaçu, RJ	Phloem, periderm and wood

ethanol 70%.

Samples of commercially sold material

Specimens commercially sold under the common name Pau-pereira were acquired through open-air markets, Internet sites, herbal stores and newsstands, totaling 15 samples, mainly originating from the State of Rio de Janeiro, Brazil (Table 1). From the 15 samples analyzed, 27% were acquired in herbal stores and/or stores that sell natural products, 53% in open-air markets, and 20% at newsstands and Internet sites.

Anatomical analyses

The macroscopic study was carried out using dry material, both by naked eye observation and by hand lens (10× magnification). Obtaining integral bark sections of good quality for anatomical analysis is extremely difficult, especially in the case of dehydrated material, which is nearly always the case with commercially sold Pau-pereira. After softening in boiling water, the material was embedded by sequential immersion in a graded series of aqueous solutions of polyethylene glycol 1500 (Rupp, 1964). Samples were subsequently sectioned with the help of an anti-tearing resin made from expanded polystyrene dissolved in butyl acetate brushed on the samples and an adhesive tape attached before sections 15 to 25 µm thick were cut (Barbosa et al., 2010) with a sliding microtome. The sections were double stained in astra blue and safranin 9:1 (Bukatsch, 1972) and were mounted in Permount resin. The anatomical description of the bark followed the method of Richter et al. (1996).

Obtaining ethanolic extracts and teas

To obtain ethanolic extracts, 20 g of the commercially sold Pau-pereira, previously milled, was submitted to maceration with 100 ml of ethanol P.A. for 7 days at room temperature, according to the method of Rapoport et al. (1958). The extract was filtered and concentrated in a rotary evaporator to obtain crude extract. To obtain the infusion, teas were obtained according to the instructions found on the packaging. Thus, ten grams (1 tablespoon) of milled Pau-pereira was added to 500 ml of warm distilled water. The infusion lasted around 30 min per sample. Afterwards, the infusion was filtered and dried at reduced pressure. For both ethanolic extracts and infusions, three replicates were prepared (Table 2).

Analysis by thin layer chromatography (TLC)

The 60 F_{254} silica gel chromatoplates (E. Merck, Darmstadt) were used with a dichloromethane: methanol 15% elution system. Alkaloids were analyzed after treatment with Dragendorff reagent, as previously described by Stahl (1969). The chemical profiles of both ethanolic extracts and infusions were compared between reference and commercial samples. In addition, through TLC, we verified the presence of flavopereirine in the different extracts and infusions obtained. Flavopereirine perchlorate (Chromadex®) was used as a reference substance, and cinchonine was used to detect alkaloids.

Assessment of total alkaloid contents

To determine total alkaloid contents, 500 mg of dry ethanolic extract was used. The sample was initially solubilized in 5 ml of methanol P.A. and 30 ml of HCl 1% solution and heated for 10 min. Acid extraction with dichloromethane was performed until the organic phase gave a negative alkaloid test, as verified by precipitation reaction with Dragendorff reagent. After the acid extraction (pH 1), a solution of NH_4OH 10% was added to the aqueous phase until pH 10 was obtained. Afterwards, repeated extractions were carried out with dichloromethane until no alkaloid was detected in the organic phase. The acid and alkaline fractions obtained from the 15 commercial samples were analyzed separately by TLC to evaluate the efficiency of the extraction method used to track flavopereirine alkaloid. The mass percentages of the fraction obtained are given in Table 2.

Table 2. Extraction yield and percentage of alkaloid fractions obtained.

Sample	Extraction yield (%)		Ethanolic extract		Infusion	
	Ethanol	Infusion	pH 1 (% w/w)	pH 10(% w/w)	pH 1 (% w/w)	pH 10 (% w/w)
1	5	17	18	2	4	1
2*	5	16	4	4	4	1
3	4	14	14	32	7	18
4	5	25	22	5	8	1
5	2	7	25	23	10	18
6	5	18	12	28	5	13
7	2	6	27	51	11	13
8	2	5	23	50	9	11
9	4	15	10	22	3	9
10	2	6	26	50	8	12
11	5	21	10	27	5	19
12	3	4	17	5	8	5
13*	6	31	7	2	4	2
14	3	18	6	3	3	2
15	6	12	18	2	5	2
RBw 9572	9	23	10	4	3	1
RBw 9573	5	30	19	36	4	9

* Samples that did not present flavopereirine by TLC analysis.

Table 3. Main characteristics of the bark of the material analyzed.

Pattern	1	2	3	4
Bark color	Yellow	Yellow	Reddish-brown	Yellow
Detaches easily?	Yes	Yes	Yes	No
Constitution of the bark	Rhytidome and secondary phloem	Rhytidome and secondary phloem	Rhytidome and secondary phloem	Periderm, cortex and secondary phloem
Diagnostic characteristic of the bark	Lines of fibrosclereids	Lines of fibrosclereids	Less conspicuous lines of fibrosclereids (larger cells) and dilated parenchyma cells	Fibrosclereids in isolated groups, secretory cells and dilated parenchyma cells

Pattern 1: reference samples (*G. laeve*); pattern 2: commercial material that can be identified as *G. laeve*; pattern 3: commercial material that shares some similarities with *G. laeve* and thus rises to Patterns 1 and 2; pattern 4: commercial material that shares no structural similarities with *G. laeve*.

RESULTS

Bark anatomy

To perform the anatomical comparison between commercial samples and reference samples, four anatomical bark patterns were established, and the main structural features are summarized in Table 3. These four patterns are as follows: pattern 1: reference samples (true *G. laeve*); pattern 2: commercial material that can be identified as *G. laeve*; pattern 3: commercial material that shares some similarities with *G. laeve* and thus rises to patterns 1 and 2; pattern 4: commercial material that shares no structural similarities with *G. laeve*.

Samples representing patterns 1 and 2 (Figures 1A, B and 2A, D to F) have yellowish bark and two types of tissue: one with papyraceous detachment in plates (rhytidome) and another that is more rigid (secondary phloem). The rhytidome is a tissue comprising of several periderms, which, in their turn, are composed of suber and phelloderm, produced by the phellogen, a lateral meristem. In this standard, each periderm of this rhytidome (Figure 2A) has a suber that is comprises of 5 to 10 layers of cells without intercellular spaces, except in regions that produce lenticels, and the phelloderm has 2 to 3 layers of thin cells, as seen in transversal section (Figure 2E). The secondary phloem is comprised of sieve tube elements with simple sieve plates, axial parenchyma

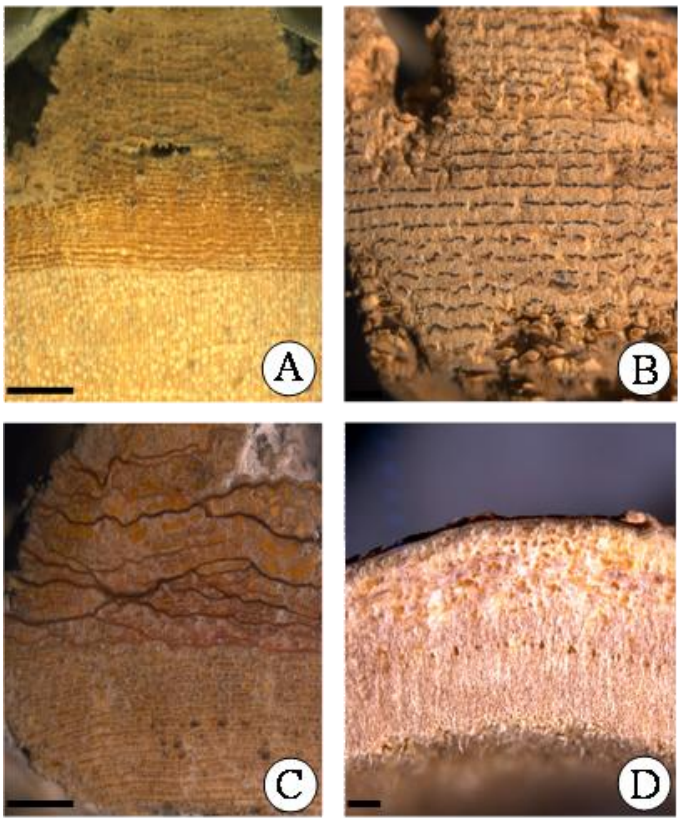

Figure 1. Bark, macroscopy, transversal section. (A) Pattern 1 (*G. laeve*). (B to D) Commercial materials: (B) Pattern 2 (sample 10, open-air market, Teresópolis); (C) Pattern 3 (sample 15, newsstand, Pará) (D) Pattern 4 (sample 15, newsstand, Pará). Bar = 50 mm.

cells, and rays 3 to 4 cells in width (Figure 2A and F). In the macroscopic analysis, sclerenchyma layers are observed, while in the microscopic analysis, these layers are classified as phloem fibers (Figures 1A and 2F). The functional periderm originates in the most internal layers of the secondary phloem, in general forming narrow undulations, leading outside portions of non-conducting phloem, as seen in Figures 1A and 2E.

Samples representing pattern 3 (Figures 1C and 2B) have brown bark that does not detach easily. In macroscopic view, we observe that the rhytidome is reddish brown, which differs from *G. laeve* (patterns 1 and 2). The sclerenchyma is orange in the lines of the secondary phloem. The bark of this type is also comprised of the secondary phloem and rhytidome. The suber is comprised of 2 to 6 layers of radially oblong cells, and the phelloderm has 1 to 2 layers of thin cells. The secondary phloem is comprised of sieve tube elements with simple sieve plates, parenchyma cells, uniseriate rays, but fewer in number than pattern 1, and fibrosclereids that form tangential ranges. The cells of the secondary non-conducting phloem are dilated in the tangential direction, which differs from pattern 1. The range of fibrosclereids differs from patterns 1 and 2 (*G. laeve*), as samples in this pattern are conspicuously larger.

Samples representing pattern 4 (Figures 1D and 2C) have yellow bark that does not detach easily. There is no rhytidome. Pattern 4 samples differ from other specimens in that they do not exhibit sclerenchyma lines. Small ruptures corresponding to fibrosclereids, as confirmed by internal morphology, stand out in the phloem and are arranged in isolated groups. The bark is comprised of a periderm, cortex and secondary phloem. Small groups of dispersed fibrosclereids stand out in the secondary phloem, as well as the presence of secretory cavity. The phloem rays are uniseriate and less numerous than found in samples representing pattern 1.

From 15 commercial samples analyzed, 6 presented the pattern 4 profile (Figure 2C), thus failing to match the internal morphology type of Pau-pereira. Particularly, sample 15 had a mixture of bark matching both patterns 3 (Figure 2B) and 4. Nine samples presented the same morphologic pattern of Pau-pereira (*G. laeve*).

Chemical composition

According to the results given in Table 2, we verified that the best extraction yield (%) was through the preparation of infusions (4 to 31%) when compared to ethanolic extracts (2 to 9%) obtained for the same samples. However, the %w/w of the fractions obtained in pH 1 and 10 of the ethanolic extracts was higher. Similar values were observed in the extraction process for acid and alkaline pH, especially for the samples purchased in open-air markets from different cities of Rio de Janeiro. In addition, the best extraction yield was obtained in pH 1 and 10, which varied from 5 to 51% for ethanolic extracts and 4 to 19% for the infusions.

The analysis by thin layer chromatography (TLC) showed that all alcoholic extracts and 13 of the teas obtained from the commercial samples revealed the presence of alkaloids through the orange color by positive reaction with Dragendorff's reagent. The chromatographic profile of the commercial samples was similar to the reference samples of Pau-pereira used, and the alkaloid flavopereirine (Rf = 0.47, CH_2Cl_2: MeOH 15%) was found in 13 commercial samples analyzed (Table 2).

DISCUSSION

Bark anatomy

A single description of *G. laeve* bark is found in the first edition of the Brazilian Pharmacopeia by Albino (1926). The structural pattern described here differs from that observed by Albino by the presence of sclerenchyma

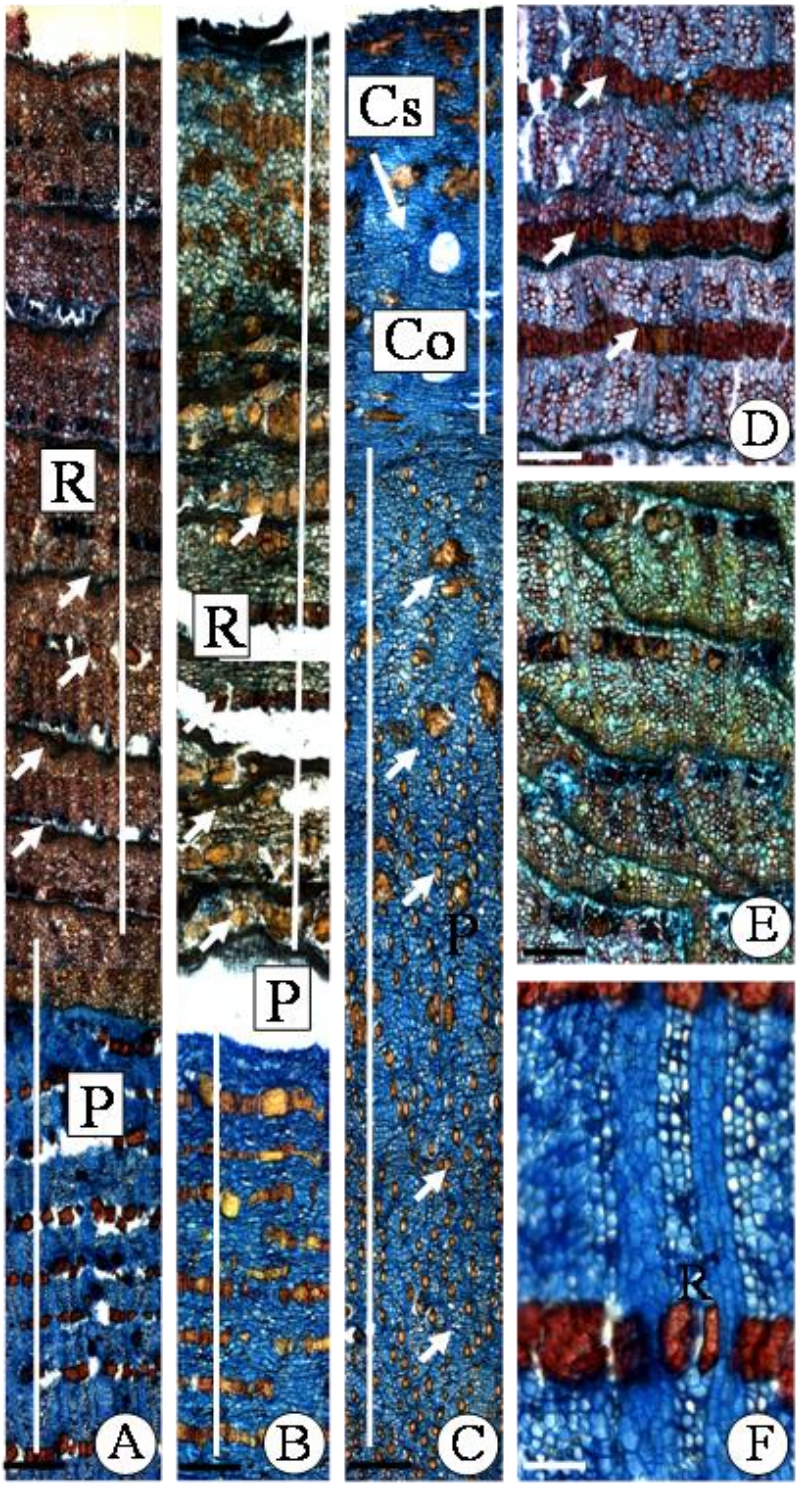

Figure 2. Bark, microscopy, transversal section. (A) Reference sample (pattern 1, *G. laeve*): Observed rhytidome (R), periderm (P) and sclereid lines (arrows). (B) Pattern 3: Commercial material (sample 15). Observed sclereids (arrows) are larger; rhytidome (R), periderm (P) and parenchyma dilatation. (C) Pattern 4: Commercial material (sample 15) - Cortex (Co), secretory cell (Cs), sclereids in small groups (arrows) and parenchyma dilatation. (D) Pattern 2: Commercial material (sample 10) - Schereid lines (arrows). (E) Pattern 2: Commercial material (sample 10). Rhytidome. (F) Detail of Figure 2A – secondary phloem. Ray (R). Bars A, B, C = 120 µm; D, E = 60 µm; F = 45 µm.

cells arranged in lines in the secondary phloem and absence of laticifers. According to our results, Pau-pereira is characterized by lines of fibrosclereids in the secondary phloem.

Pickard (2007) considers the laticifers and secretory ducts as two different tube systems in plants, in addition to conduction elements (vessel elements and sieve tube elements). A laticifer is a cell or a row of cells producing latex (Fahn, 1979). General consensus holds that latex consists of a variant chemical composition that is not necessarily milky in appearance and that may present as colorless or as a yellow, orange, red, or brown color (Fahn, 1979; Pickard, 2007).

We did not observe latex in Pau-pereira. It is difficult to distinguish laticifers because they do not exhibit a shape that is different from their adjacent cells (Monacelli et al., 2005), thus often requiring analyses in longitudinal sections. In this work, we observed longitudinal sections of Pau-pereira, but it was still not possible to distinguish laticifers in the secondary phloem. However, we cannot rule out the possibility that laticifers are present in xylematic rays or pith because, according to Metcalfe and Chalk (1950), the presence of laticifers is universal in the family.

In commercially sold samples, we found pattern 3 to be similar, but not equal, to *G*. *laeve*. This standard also has a range of fibrosclereids with distribution similar to Pau-pereira. However, the fibrosclereids in these samples were longer, most likely indicating that these species come from the same family Apocynaceae. In future work, we intend to analyze the structural standard of the bark of *Geissospermum sericeum* Miers, a species that can be found in the same locations as *G*. *laeve*. Standard 4 is clearly distinguishable from *G*. *laeve*. These samples exhibit no rhytidome or sclereid lines, and secretory cells are present in the cortical region. The bark is not easily detached in these samples, indicating that their external morphology is very different from pattern 1. This standard was found in samples from different suppliers. Although, suppliers most likely know that patterns 4 properties are similar to *G. leave*, they are, nonetheless, falsely selling this material as “true” Pau-pereira.

The structural standard analysis of Pau-pereira carried out in the present work was sufficient to characterize the samples sold commercially. The present work clearly identifies the standard(s) of commercially sold *G. laeve* and demonstrates that other taxa are available on the open market, but that these species do not correspond to the same standard and thus may be harmful.

Chemical composition

Even though some commercial samples analyzed release a sweet aroma, all presented bitter taste by the presence of alkaloids, as confirmed by TLC. According to the botanical analysis, only 9 of the commercial samples presented characteristics similar to reference samples, and according to the literature, most of the species of the genus *Geissospermum* produce alkaloids, such as geissospermine and flavopereirine. Thus, some of the materials commercialized may be other species of the genus *Geissospermum*, as also suggested by the bark anatomy comparisons.

Alkaloid assessment revealed that extraction in alkaline pH is the best method to obtain flavopereirine. However, extraction in acid pH must also be carried out to isolate other alkaloids produced by the species because the presence of alkaloids by TLC analysis has also been confirmed. According to the results shown in Table 2, we can observe that the samples presenting features similar to the botanical description of Pau-pereira were also the samples that presented a higher percentage in mass in the fractions obtained in pH 1 and 10.

Since the consumption of Pau-pereira in traditional medicine is through teas or tincture, two extraction methods, aqueous and alcoholic, were carried out to evaluate the efficiency of each extraction, and the best extraction yield was aqueous.

Conclusion

In this study, we were able to distinguish between false and true Pau-pereira barks based on anatomical analyses and the total alkaloid content of the barks.

Accordingly, we have demonstrated that at least 6 of the 15 samples sold as Pau-pereira barks in the City of Rio de Janeiro are fraudulent.

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