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Quality control and TLC profile data on selected plant species commonly found in the Brazilian market

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Abstract: The use of thin-layer chromatography (TLC) is a commonplace practice and can be of significant help to different laboratories with quality control, especially those that work with plant extracts and phytotherapeutics. This study evaluated ten species of plants (*Schinus terebinthifolius, Arctium lappa, Trichilia catigua, Camellia sinensis, Mikania glomerata, Croton moritibensis, Achyrocline satureioides, Heteropterys aphrodisiaca, Plantago major, Arctostaphylos uva-ursi*) that are commonly sold by compounding pharmacies, using TLC with reference substances and pharmacopoeic physical and chemical tests (loss on drying, level of extractives, and total ash content). The results showed that the ten species showed losses on drying consonant with the literature. The level of extractives for two species and total ash for five species were also consonant with the literature, and those of the other species were established in this study. The semipurified extracts of the ten species were assayed by TLC, and the analysis with the use of reference substances proved to be effective, in addition to being practical, simple, versatile, and economically viable.

Article

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Introduction

Medicinal plants, herbal drugs, and individual natural products comprise a market worth billions of dollars, in both developed and developing countries. The field of phytotherapy is growing remarkably, with a present worldwide market value of approximately 22 billion dollars, of which Brazil accounts for about 23% (Calixto, 2000). It is estimated that 82% of the Brazilian population uses herb-based products, and the herbal market sector includes about 200 companies with a total output of one billion Brazilian reals and more than a hundred thousand employees within the country (Abifito, 2006).

Phytotherapeutics are marketed through establishments such as the drugstores, where the plant preparations are sold as pharmaceutical specialties and are industrially produced; and the compounding pharmacies, where the medicines are compounded from plant extracts or sold as plant drugs *in natura*. Access to phytotherapeutics made by a compounding pharmacy is guaranteed by the provisions of Law No. 5991/1973 (Brasil, 1973) and Resolution No. 10/2010 (Anvisa, 2010). The increasing public demand for natural medicines has resulted in increased commercial activity and production of these medicines. This has led to growing concern in regard to ensuring the quality and safety of medicinal plants and herbal drugs.

Several national and international agencies have prioritized the issue of ensuring the quality of plant drugs. The effort of the World Health Organization is outstanding: over 20 years ago, it first published quality-control methods for medicinal plant materials, which have been regularly updated. These published methods were followed by a series of monographs on globally important medicinal plants (WHO, 2000). However, for medicinal plants that do not appear in these official codes, studies must be conducted to establish parameters to ensure their quality, according to technical and scientific principles (Farias, 2004).

In order to effectively coordinate the quality of raw materials, processing of materials, and the final products, it has become essential to develop reliable, specific, and sensitive quality-control methods using a combination of classical and modern instrumental methods of analysis. In this context, thin-layer chromatography (TLC), also called planar chromatography, is a widely accepted and extensively used separation technique that is over 65 years old (Poole, 1999). The technique is simple, cost-effective, versatile, and usable in all laboratories worldwide. It can be easily adapted to any given situation of qualitative

or quantitative separation (Ferenczi-Fodor et al., 2006). The uses of TLC in quality control of plant materials include fingerprint profiling for the assessment of chemical constituents of an extract, and quantitative analysis of markers in plant drugs (Mohammad et al., 2010).

Such a chromatographic profile should feature the fundamental attributes of "integrity" and "fuzziness" so as to chemically represent the herbal medicines investigated. This suggests that the chromatographic fingerprint can also successfully demonstrate both "differences" and "uniformity" between various samples, and the authentication and identification of herbal medicines can be accurately conducted even if the number and/or concentrations of chemically characteristic constituents vary in different samples (Liang et al., 2004). Therefore, the chromatographic fingerprint should be considered in evaluating the overall quality of herbal medicines, considering the multiple constituents that are present in medicinal plants and herbal drugs. Thus, TLC remains the method of choice to obtain the first characteristic fingerprint profile of a medicinal plant and herbal drug.

Compounding pharmacies encounter considerable difficulty in establishing parameters for the analysis of plant and herbal drugs. TLC can and should be employed to aid these compounding pharmacies in controlling the quality of their plant and herbal drugs, so as to guarantee the chemical quality of the extracts. Therefore, the present contribution lists several plant species that are commonly used in phytotherapy, and provides data on their physical, chemical, and chromatographic attributes, in order to facilitate quality control.

Material and Methods

Plant material

The plants were selected according to surveys carried out in compounding pharmacies in the city of Maringá, state of Paraná, Brazil, and are listed in Table 1. The species were identified by Prof. Dr. Cassia Mônica Sakuragui. Voucher specimens are deposited in the herbarium of the Department of Biology of the State University of Maringá.

Reagents, standard solutions and materials

All solvents and reagents used were analytical grade from Synth, and diphenylboric acid 2-amino-ethyl ester from Sigma. The reagents used were: ferric chloride at 1% in methanol, vanillin perchloric (solution A: vanillin at 1% in ethanol, and solution B: perchloric acid at 3% in water); natural reagent (diphenylboric acid 2-amino-ethyl ester in methanol at 1% and then spraying polyethyleneglycol 400 at 5% in ethanol); potassium hydroxide at 10% in water; anisaldehyde sulfuric (anisaldehyde 1 mL, sulfuric acid 2 mL, and completed with acetic acid to 100 mL). Deionized water was prepared with a Millipore Milli-Q Plus system (Millipore, Bedford, MA, USA). Hydroquinone, epicatechin, catechin, caffeic acid, chlorogenic acid, gallic acid, *o*-coumaric acid, quercetin, coumarin, lupeol, and rutin (Sigma, USA), isoastilbin and cinchonain Ib (isolated and certified by spectroscopic methods at the Pharmaceutical Biology Laboratory of the Universidade Estadual de Maringá; Marques et al., 2007; Resende et al., 2011), and epicatechin-3-*O*-gallate (kindly provided by Dr. Frank Petereit, Institute for Pharmaceutical Biology and Phytochemistry of the Universität Münster, Germany; Danne et al., 1994) of the highest grade (purity >99.0%) were used as external standards.

Stock solutions (1 mg/mL) of the standards were prepared on the day of analysis in methanol, immediately before use.

Table 1. List of plant species assayed, with their Brazilian common names and botanical identification.

Common name *Species name*	Family	Voucher Code	Part
Aroeira *Schinus terebinthifolius* Raddi	Anacardiaceae	HUEM 12677	Bark
Bardana *Arctium lappa* L.	Asteraceae	RFA 35.777	Leaves
Catuaba *Trichilia catigua* A. Juss.	Meliaceae	MBM 65 901	Bark
Chá-verde *Camellia sinensis* (L.) Kuntze	Theaceae	Opção Fênix, Lot: 065F/05	Leaves
Guaco *Mikania glomerata* Spreng.	Asteraceae	HUEM 8420	Leaves
Marapuama *Croton moritibensis* Baill.	Euphorbiaceae	Ambrosifarma, Lot: 2261998	Bark
Marcela *Achyrocline satureioides* (Lam.) DC.	Asteraceae	HUEM 5270	Inflorescences
Nó-de-cachorro *Heteropterys aphrodisiaca* A. Juss.	Malpighiaceae	HUFMT 22.181	Roots
Tansagem *Plantago major* L.	Plantaginaceae	HUEM 8427	Leaves
Uva-ursi *Arctostaphylos uva-ursi* (L.) *Spreng.*	Ericaceae	Natural Pharma, Lot: 1611R/015	Leaves

Instrumentation

Tigre ASN5 hammer mill; Ika® MS1 Eppendorf shaker; Fisatom® 712 mechanical shaker; AND® HR-200 analytical balance; Gahaka® BG2000 semi-analytical balance; Water bath; Vacuubrand® MZ 2C vacuum pump; Ultraviolet chamber with 254 nm and 365 nm

lamps; Fanen® 315SE hot air oven; Christ® Alpha 1-4 LD lyophilizer; and Büchi® B-480 rotary evaporator.

Preparation of plant extracts

The plant material was used with no separation of particle sizes. For all plant drugs, extracts were prepared at room temperature in a mechanical shaker with helices at 500 rpm, in the proportion of 10% (w/v) of the drug in 50% ethanol (v/v). The extractive solutions were filtered and used in the preliminary analysis. For chromatographic analysis, the extracts obtained were concentrated in a rotary evaporator under reduced pressure (t<40 ºC). After elimination of the organic phase, the extracts (CE) were freeze-dried and stored in amber bottles protected from light, heat, and moisture.

Preparation of final solutions for chromatographic analysis

The amount of CE needed for each plant was established by prior testing. Thus, accurately weighed 200 mg portions of each CE of '*aroeira*', '*bardana*', '*catuaba*', '*chá-verde*', '*marapuama*', '*nó-de-cachorro*', '*tansagem*', '*marcela*'; 100 mg CE of 'guaco', and 50 mg CE of 'uva-ursi' were dissolved in 500 µL water. All extracts were mixed in a tube shaker, and extracted with 500 µL of ethyl acetate (except for *n*-hexane in the case of '*marapuam*a'; Barbosa et al., 2003) in a microtiter shaker at 1400 rpm (IKA, MS1 Minishaker) for 5 min. Then, the tubes were placed in a refrigerated microcentrifuge (Eppendorf, Centrifuge 5415R), at 4000 x *g* for 4 min at 5 ºC, for the total separation of the phases. The organic phase was collected for analysis. After evaporation of solvents, and drying under air flow, the residue was reconstituted to 1 mL with methanol (Test Solution - TS) and applied on chromatography plates (see conditions below) with the aid of volumetric micropipettes, which always dispensed 5 µL or a multiple of this volume.

Phytochemical screening and physical and chemical analyses

For identification of flavonoids, coumarins, iridoids, tannins, simple phenolics, methylxanthines, alkaloids and saponins, tests were conducted according to the respective techniques described by Harborne (1998) and the foaming index test for saponins as described by Schenkel et al. (2003). The loss on drying and total ash content were determined according to the Farmacopeia Brasileira (2010). The methodology used to determine the level of extractives was carried out as described by Deutsches (1986). All tests were conducted in triplicate with three replicates.

Chromatographic analysis

This assay was conducted by analytical thin-layer chromatography (ATLC), using aluminum sheets of silica gel F_{254} (Merck®). All chromatograms were developed in a saturated chamber. Mobile phases employed in this study were selected or developed according to the reference substance for each drug, and are listed in Table 2. Aliquots of the standards and TS (Table 2) were spotted onto a plate with volumetric micropipettes, along a virtual line situated 10 mm from the bottom edge of the plate (100 x 50 mm). The spots were applied at 13-mm intervals. During application, care was taken to dry the spots with a stream of cold air so that they never exceeded a diameter of ca. 5 mm. The plate was developed to a distance of 80 mm, at room temperature. After the chromatogram was developed, the plates were dried and the spots were visualized sequentially under UV light at 254 and 365 nm, and then sprayed with specific chromogenic agents according to the chemical substances analyzed (Table 2).

Results and Discussion

Although the use of herbal drugs is increasing throughout the world, reports on their side effects and instances of adulteration have raised concerns regarding their wide use, and are affecting their marketing (Cianchino et al., 2008). Correct identification and quality assurance of the starting material is an essential prerequisite to ensure reproducible quality of an herbal medicine, which contributes to its safety and efficacy. It is essential to establish a system of standardization for every plant medicine on the market, because of the enormous scope for variation in different batches. Plant material may vary in its chemical content and therefore in its therapeutic effect according to the collection location; the time of year when the material was collected; among different years of collection, even at the same time of year and location; and with differences in environmental factors during the cultivation of the plant (Sahoo et al., 2010). This means that all raw materials should undergo a quality-control test, to ensure the quality of the product.

A general screening was conducted to characterize the chemical composition of the plant drugs under study. The results revealed the presence of secondary metabolites including flavonoids, coumarins, tannins, simple phenolics, methylxanthines, alkaloids, and saponins. No iridoids were detected in any of the extracts. Phytochemicals such as flavonoids, phenolic compounds, and tannins were present in all the crude extracts analyzed, except for the '*marapuama*' extract.

Methylxanthines were present only in '*chá-verde*', alkaloids only in '*aroeira*' and '*marapuama*', saponins only in '*marapuama*' and '*nó-de-cachorro*', and coumarin only in '*guaco*'. From the preliminary results of the chemical analyses, it was possible to determine the chemical marker for each plant drug. These data were corroborated from literature sources (Oliveira et al., 1984 apud Osório & Martins, 2004; Kawashty et al., 1994; Müller & Kasper, 1996; Veneziani & Oliveira, 1999; Pereira et al., 2000; De Souza, 2002; Pizzolatti et al., 2002; Yang & Lambert, 2003; Araújo-Junior et al., 2004; Polydoro et al., 2004; Chan et al., 2007; Marques et al., 2007; Resende et al., 2011; Manian et al., 2008; Silva et al., 2010).

Chemotaxonomic markers are sometimes used as indicators of botanical identity or as tools in manufacturing to help ensure the consistency of products. Therefore, the selection of chemical markers is crucial for the quality control of plant drugs, including authentication of genuine species, harvesting the best-quality raw materials, evaluation of post-harvest handling, and assessment of intermediates and finished products (Ahmad et al., 2010). Table 3 shows the Rf values of the standards used in the development of TLC, and their respective colors.

Thin-layer chromatography (TLC) is a popular

Table 2. Chemical standards, eluent systems and visualizing agents used in the TLC.

Plant drugs Standards	Aliquots (µL)	Eluent systems (v/v)	Chromogenic agent
Aroeira Gallic acid Catechin	25.0 5.0 25.0	1. Toluene: ethyl acetate: methanol: formic acid (75:25:10:6) 2. Ethyl acetate: toluene: formic acid: water (80:10:5:5)	A. Ferric chloride 1% in methanol B. Vanillin perchloric + 105 ºC/5 min
Bardana Chlorogenic acid Rutin	25.0 15.0 5.0	3. Ethyl acetate: water: formic acid: acetic acid (100:27:11:11)	C. NR + PEG 400 + UV 365 nm
Catuaba Cinchonain Ib	15.0 5.0	4. Chloroform: acetic acid: methanol: water (32:16:6:4)	B. Vanillin perchloric + 105 ºC/5 min
Chá-verde Epicatechin Epicatechin-3-*O*-gallate	5.0 10.0 5.0	2. Ethyl acetate: toluene: formic acid: water (80:10:5:5)	B. Vanillin perchloric +105 ºC/5 min
Guaco *o*-Coumaric acid Coumarin	15.0 5.0 5.0	5. Toluene: dichloromethane: acetone (45:25:30)	D. Potassium hydroxide 10% + UV 365 nm
Marapuama Lupeol	10.0 10.0	6. Toluene: dichloromethane: acetone (45:30:25)	E. Anisaldehyde + 105 ºC/5 min
Marcela Caffeic acid Quercetin	10.0 15.0 5.0	1. Toluene: ethyl acetate: methanol: formic acid (75:25:10:6)	C. NR + PEG 400 + UV 365 nm
Nó-de-cachorro Isoastilbin	10.0 5.0	7. Ethyl acetate: formic acid: water (90:5:5)	A. Ferric chloride 1% in methanol
Tansagem Caffeic acid Chlorogenic acid	35.0 15.0 15.0	1. Toluene: ethyl acetate: methanol: formic acid (75:25:10:6)	C. NR + PEG 400 + UV 365 nm
Uva-ursi Gallic acid Hydroquinone	15.0 5.0 5.0	1. Toluene: ethyl acetate: methanol: formic acid (75:25:10:6)	A. Ferric chloride 1% in methanol

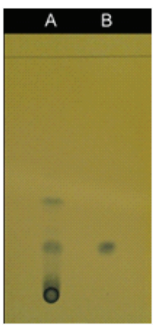
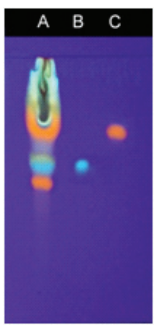
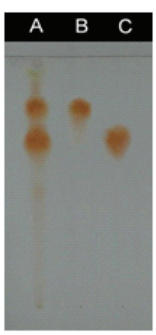
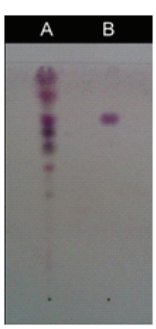
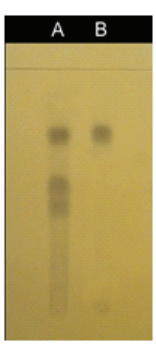
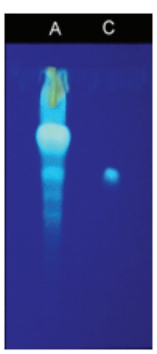
NR: diphenylboric acid 2-amino-ethyl ester; PEG 400: polyethylene glycol 400; UV 365 nm= UV light at 365 nm in the dark.

Table 3. R_f values of the standards used in TLC and their respective colors.

Standard	Plant drugs	Color	R_f
Caffeic acid	*Marcel*a and *Tansagem*	Blue	0.35
Chlorogenic acid	*Bardana* and *Tansagem*	Blue	0.58
Gallic acid	*Aroeira* and *Uva-ursi*	Brownish-blue	0.20
o-Coumaric acid	*Guaco*	Light blue	0.39
Catechin	*Aroeira*	Orange	0.81
Cinchonain Ib	*Catuaba*	Yellow	0.58
Coumarin	*Guaco*	Light blue	0.79
Epicatechin	*Chá-verde*	Red or orange	0.80
Epicatechin-3-*O*-gallate	*Chá-verde*	Red or orange	0.66

Hydroquinone	*Uva-ursi*	Brown	0.49
Isoastilbin	*Nó-de-cachorro*	Brown	0.73
Lupeol	*Marapuama*	Violet	0.76
Quercetin	*Marcela*	Orange	0.40
Rutin	*Bardana*	Orange	0.67

Table 4. Results of chromatographic analysis, extracts, standards, eluent systems, and chromogenic agents as listed in Tables 2 and 3.

	(A) extract of '*aroeira*' (B) catechin (R_f=0.81) Eluent system: 2 Chromogenic Agent: B	
(A) extract of '*aroeira*' (B) gallic acid (R_f=0.20) Eluent system: 1 Chromogenic Agent: A		(A) extract of '*bardana*' (B) chlorogenic acid (R_f=0.58) (C) rutin (R_f=0.67) Eluent system: 3 Chromogenic Agent: C
	(A) extract of '*catuaba*' (B) cinchonain Ib (R_f=0.58) Eluent system: 4 Chromogenic Agent: B	
(A) extract of '*chá verde*' (B) epicatechin (R_f=0.80) (C) epicatechin-3-*O*-gallate (R_f=0.66) Eluent system: 2 Chromogenic Agent: B		(A) coumarin (R_f=0.79) (B) extract of '*guaco*' (C) *o*-coumaric acid (R_f=0.39) Eluent system: 5 Chromogenic Agent: D
	(A) extract of '*marapuama*' (B) lupeol (R_f=0.76 Eluent system: 6 Chromogenic Agent: E	
(A) caffeic acid (R_f=0.35) (B) extract of '*marcela*' (C) quercetin (R_f=0.40 Eluent system: 1 Chromogenic Agent: C		(A) extract of '*nó-de-cachorro*' (B) isoastilbin (R_f=0.73) Eluent system: 7 Chromogenic Agent: C
	(A) extract of '*tansagem*' (B) caffeic acid (R_f=0.35) Eluent system: 1 Chromogenic Agent: C	
(A) extract of '*tansagem*' (C) chlorogenic acid (R_f=0.58) Eluent system: 1 Chromogenic Agent: C		(A) hydroquinone (R_f=0.49) (B) extract of '*uva ursi*' (C) gallic acid (R_f=0.20) Eluent system: 1 Chromogenic Agent: A

method to analyze plant drugs (Pittet & Royer, 2002; Głowniak et al., 2005; Zhang et al., 2008; Barbosa et al., 2009), and has been used for a wide variety of applications. TLC is an important tool, not only for the quality control of medicinal plants, but also for the analysis of herbal drugs (Durón et al., 2009). In several pharmacopoeias, a great variety of plants are identified by use of TLC methods (Farmacopeia Portuguesa, 2002; Farmacopeia Espanhola, 2005; Farmacopeia Brasileira, 2010). TLC has the advantages of low cost, ease of maintenance, and good selectivity of detection. In addition, TLC can analyze several parallel samples in a single run. It also facilitates repeated detection (scanning) of the chromatogram with the same or different parameters. Therefore, the simultaneous assay of several components in a multicomponent formulation is possible. TLC was chosen because this separation system enables simultaneous evaluation of standards and samples, thus matching the working conditions and environment for both, and reducing systematic errors (Kaiser, 2005). The appearance of compounds in common and different bands can be useful for identification and authentication of plant drugs. The TLC profiles of the plant drugs studied are shown in Table 4.

All identifications in the TLC are based on comparison of the migration distances (R_f values), and of the color of the spots between the sample and a standard when the TLC plate is sprayed with a specific chromogenic reagent. The quality of the analysis depends on the correct positioning of the sample and on the reference substances used in the TLC. Accordingly, we opted to apply the samples in spots, to improve the visual impression of the chromatogram. Another fundamental parameter in evaluating samples in TLC is the volume of the sample and reference substances applied, which must be well defined and reproducible because the quality of separation depends on the size, shape, and homogeneity of the application zones (Reich & Blatter, 2003).

In all cases, the criterion for acceptance of the TLC procedure was adequate detection of the specific marker, with respect to the color and relative position of the band. The band of the specific marker should always be present in the chromatogram obtained from the samples.

The physical and chemical tests carried out in this study, including loss on drying, content in extracts, and total ash content, showed that the herbal drugs analyzed were within the technical specifications proposed by the appropriate literature, when available. Data are shown in Table 5.

In conclusion, based on the results obtained, we can suggest that the tests performed can aid in the quality control of the herbal drugs analyzed, contributing significantly to the quality-control protocol. These tests are also useful to indicate the existence of fraud, a common occurrence in the medicinal-plants market, which may consist of adulteration or even falsification of raw plant materials (Liang et al., 2004; Barbosa et al., 2009). However, it should also be borne in mind that TLC plates from different manufacturers, with different thicknesses and particle sizes, can also give variable results for a particular assay method.

Table 5. Results from physico-chemical analyses.

Common name Species name	Physico-chemical analyses					
	Loss on drying		Level of extractives		Total ash	
	[$\bar{x} \pm dp$ (CV%)]	Reference	[$\bar{x} \pm dp$ (CV%)]	Reference	[$\bar{x} \pm dp$ (CV%)]	Reference
Aroeira *Schinus terebinthifolius*	12.93±0.48 (3.55)	8-14%[1]	25.86 ± 0.24 (0.93)	-	4.21 ± 0.11(2.52)	-
Bardana *Arctium lappa*	11.45 ± 0.37 (3.27)	8-14%[1]	33.94 ± 1.32 (3.89)	Min. 20%[3]	3.42 ± 0.10 (2.95)	Max. 15%[3]
Catuaba *Trichilia catigua*	10.42 ± 0.08 (0.77)	8-14%[1]	24.62 ± 0.74 (3.00)	-	6.90 ± 0.06 (0.87)	-
Chá-verde *Camellia sinensis*	8.21 ± 0.21 (2.50)	8-14%[1]	42.39 ± 0.75 (1.79)	-	3.12 ± 0.14 (4.40)	-
Guaco *Mikania glomerata*	8.74 ± 0.20 (2.29)	10%[2]	24.43 ± 0.60 (2.46)	-	5.40 ± 0.11 (2.04)	15%[2]
Marapuama *Croton moritibensis*	9.13 ± 0.07 (0.77)	14%	2.29 ± 0.08 (3.50)	-	2.09 ± 0.07 (3.35)	-
Marcela *Achyrocline satureioides*	6.29 ± 0.16 (2.54)	10%[4]	14.16 ± 0.25 (1.75)	-	3.17 ± 0.08 (2.52)	8%[4]
Nó-de-cachorro *Heteropterys aphrodisiaca*	11.95 ± 0.35 (2.90)	7.5-15.8%[5]	33.41 ± 1.05 (3.14)	31-50%[5]	4.73 ± 0.07 (1.41)	3.5-5.3%[5]
Tansagem *Plantago major*	9.87 ± 0.19 (1.93)	8-14%[1]	42.70 ± 0.77 (1.80)	-	13.70 ± 0.37 (2.70)	-
Uva-ursi *Arctostaphylos uva-ursi*	8.52 ± 0.24 (2.82)	10%[4]	38.35 ± 0.78 (2.03)	-	3.23 ± 0.09 (2.78)	5%[4]

[1]Bacchi (1996); [2]Farmacopeia Brasileira (2002); [3]British Herbal Pharmacopoeia (1996); [4]Farmacopoeia Portuguesa (2002); [5]Marques et al. (2007).

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