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# Pharmacognostic evaluation, and development and validation of a HPLC-DAD technique for gallocatechin and epigallocatechin in rhizomes from *Limonium brasiliense*

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ABSTRACT

*Limonium brasiliense* (Boiss.) Kuntze, Plumbaginaceae, is a plant from the southern coast of Brazilian that is used for the treatment of premenstrual syndrome, menstrual disorders and genito-urinary infections. The aim of the present study was to determine the quality control parameters for rhizomes collected during different periods by pharmacopoeial and non-pharmacopoeial methods, and to develop and validate a HPLC-DAD method for quantitative control of marker substances. The measured parameters were: granulometric analysis ($d_{50}$ = 0.21–0.48 mm), loss on drying (11.1–12.4%), total ash (4.9–5.7%), dry residue by extraction with acetone:water (7:3, v/v) (30.6–39.5%), total polyphenol content (8.5–15.8%), and chromatographic fingerprint by HPLC and TLC. Besides, the acetone:water (7:3, v/v) extraction solvent in combination with a turbo-extractor, yielded the crude extract with a significant increase in tannins ($F_{4,20}$ = 37.0, $p$ < 0.001). The antioxidant potential of the crude acetone:water (7:3, v/v) extract, as well as the ethyl acetate and water fractions obtained after the partition process was evaluated by DPPH and the results were, respectively: $IC_{50}$ 6.87, 5.91, and 6.92 μg/ml. The validation parameters for the HPLC-DAD method showed adequate specificity, precision and accuracy. The gallo- and epigallocatechin contents were, respectively, 0.8–2.7% and 1.2–2.2%. These data contribute to analysis of the pharmacognostic quality control of the commonly used part from this species.

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## Introduction

*Limonium brasiliense* (Boiss.) Kuntze, Plumbaginaceae, is a perennial herb that grows in Argentina, Uruguay and the southern coast of Brazil. It is popularly known as "baicuru" or "guaicuru" (Murray et al., 2004). Preparations of their dried underground parts have been used in the treatment of premenstrual syndrome, menstrual disorders and genito-urinary infections (Fenner et al., 2006). Because of its traditional use, a clinical study was conducted to test the association of a liquid extract from *L. brasiliense* and the analgesic paracetamol in 36 women with symptoms related to menstrual disorders including dysmenorrhea, leucorrhea or amenorrhea. The improvement was excellent in 83.3% and good in 13.9% of the women, only 2.8% of them considered a regular improvement for this treatment. The treatment time varied between 11 and 50 days (Janhs and Crescente, 1976). Studies also report that these extracts have biological activities such as bacteriostatic, anti-inflammatory and antioxidant effects, and a toxicological evaluation of the extract demonstrated a low toxicity (Antonelli-Ushirobira et al., 2015a,b). The presence of hydrolysable and condensed tannins, leucoanthocyanins, flavonoids, β-sitosterol, saponins, and coumarin has been reported in *L. brasiliense* (Murray et al., 2004). A recent morpho-anatomical study resolved some doubts in the literature as to the nature of the part used for medicinal purposes and defined its structure as the rhizome (Antonelli-Ushirobira et al., 2015a,b).

One of the main complications of phytomedicine studies is the complexity of the process to evaluate natural products, because their quality and compounds characterization is difficult and differences may be induced in the pharmacokinetic, pharmacodynamic,

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and safety profiles (Nisbet and Moore, 1997). In addition, the chemical constituents of medicinal plants may vary according to genetic factors, weather, soil quality and other external factors (Gobbo-Neto and Lopes, 2007). This way, without suitable quality control parameters for the production of medicinal plants in order to obtain herbal drugs, it is impossible to ensure the reproducibility between different batches, or to assay for the absence of contaminants. Therefore, chemical, physical and physicochemical methods, as well as modern analytical methods, should be used to help define the features of natural drugs, in order to recognize and understand their possible variations (Gobbo-Neto and Lopes, 2007; Schulz et al., 2004). Tannins seem to be a featured chemical class present in *L. brasiliense*, however they are often present as complexes with other structures and the analytical data depends on several factors such as sample preparation, storage and extraction technique (Mueller-Harvey, 2001; Schofield et al., 2001).

In order to guarantee the safety and effectiveness of the phytotherapies, analytical methods for content determination are an integral part of the quality control of the source material. The determination of quality control parameters for plant drugs and extracts with high tannin content is complicated due to the structural complexity of this class. Frequently the biological, biochemical and chemical procedures must be conducted in concert. Although high-performance liquid chromatography methods are usually conducted according to the most common laboratorial routine, its reliability requires an efficient process of development and validation (Mueller-Harvey, 2001; Lopes et al., 2009).

The purpose of the present study was to evaluate pharmacopoeial and non-pharmacopoeial parameters, and to develop and validate a high-performance liquid chromatography method for the determination of gallocatechin and epigallocatechin from *L. brasiliense* rhizomes.

## Materials and methods

### *Plant material*

Rhizomes of *Limonium brasiliense* (Boiss.) Kuntze, Plumbaginaceae, were collected in Rio Grande, in the state of Rio Grande do Sul, Brazil (S 32°09′22″/W 52°06′01″) in May 2006 (sample A), (S 32°00′00″/W 52°09′00″) May 2010 (sample B), and (S 31°59′33″/W 52°10′43″) February 2013 (sample C). Voucher specimens were deposited at the Herbarium of the State University of Maringá under numbers HUEM-12151, HUEM-21152, and HUEM-27725, respectively. The plant material was collected under a permit from IBAMA-SISBIO, No. 11995-2, July 5, 2007, and 11995-3, November 2, 2010, authentication code 46367613, under the responsibility of J.C.P. Mello. Access to the botanical material was authorized and licensed by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), registered under No. 010252/2015-0. The plant materials were cleaned with water to remove soil, dried in a circulating-air oven (37 ± 2 °C), and powdered in a hammer mill (Tigre ASN-5).

### *Extract preparation*

The crude extract (CE) was prepared from the dried rhizomes (samples: A – 0.63 kg; B – 5.6 kg; C – 5.0 kg) using an acetone:water solution (7:3, v/v, 10%, w/v) and an Ultra-Turrax (UTC115KT, Wilmington, NC, USA; 3000 rpm, 4× 5 min, $t$ < 40 °C). Next, the extract was filtered, the organic phase was removed in a rotavapor (Büchi R-135) and the residue was lyophilized (Christ Alpha 1–4) to yield the CE (A: 43.3%; B: 28.3%; C: 27.6%). The CE was solubilized in water in the proportion of 10% (w/v) and partitioned with ethyl acetate (10 times with the volume of water). The ethyl-acetate (FAE) and aqueous (FAQ) fractions were concentrated and lyophilized as previously described to yield, respectively, FAE (A: 9.2%; B: 11.6%; C: 10.4%) and FAQ (A: 83%; B: 82.4%; C: 72.4%).

### *HPLC method*

High-performance liquid chromatography (HPLC) was conducted on an Agilent Model 1290 Infinity instrument with an Agilent Zorbax C-18 (250 mm × 4.6 mm) 5 μm column. The mobile phase was composed of water:concentrated phosphoric acid (100:0.2, v/v, Solvent A) and acetonitrile:concentrated phosphoric acid (100:0.2, v/v, Solvent B) and used in the following elution gradient: 0 min 10% B; 15 min 15% B; 35 min 19% B; 36 min 80% B; 44 min 80% B; 45 min 10% B; 52 min 10% B. The oven temperature was controlled at 24 °C (0–18 min), 35 °C (18–45 min), and 24 °C (45–52 min). The flow rate was 0.4 ml/min, and the injection volume was 10 μl. The detection was by UV in 210 nm and DAD (200–400 nm) was employed for determination of peak purity. The FAE samples were prepared in methanol:water (1:1, v/v) at 200.0 μg/ml with the help of an ultrasonic bath for 10 min and filtered (Millipore Milex-HV 0.45 μm) prior to injection. Reference substances (gallic acid, gallocatechin, catechin, epigallocatechin, epicatechin, and ellagic acid, Sigma) were prepared in methanol:water (1:1, v/v) and injected into the HPLC instrument to identify the possible substances present in FAE samples by the comparison of retention times (RT), absorbance spectrum and increase to peak area with the addition of reference solutions in sample solution (spiked sample), as well as the concentrations of these present substances in the FAE for extern standard.

### *Validation of the HPLC method*

For validation of the analytical method, the guidelines established by the International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH, 2005) and by Brazilian regulation RE No. 899/2003 of the National Health Surveillance Agency (Anvisa, 2003) were employed. The FAE from sample C was used to test the validation parameters.

Linearity between the peak area and concentration was analyzed using three calibration curves obtained from reference solutions of gallocatechin (GC) and epigallocatechin (EGC) at five different concentrations in the range of 3.4–5.1 μg/ml and 3.2–4.8 μg/ml, respectively.

Specificity was determined by peak purity (≥0.99) of GC and EGC peaks from the sample solution. The DAD detector was employed. In addition, the sample solution was stored under relevant stress conditions (acid/basic hydrolysis, heat, oxidation and light) to promote the partial degradation of the sample and to show by the peak purity if the analyzed chromatographic peak could be attributable to only one component. For each condition, *ca.* of 2 mg of FAE was weighed into a 10 ml volumetric flask. For acid stress, 1 ml of 2 M HCl was added, and after 4 h at room temperature it was neutralized with 1 ml of 2 M NaOH. For basic stress, 1 ml of 0.1 M NaOH was added, and after 3 h at room temperature it was neutralized with 1 ml of 0.1 M HCl. For heat stress, the FAE was stored in an oven at 60 °C for 40 h. For oxidation stress, 1 ml of 30% $H_2O_2$ was added and stored for 40 h at room temperature. For light stress, two samples, one in amber flask and other in transparent flask, were for 14 h in photostability chambers (Weiss, model Pharma 500-L). After all above procedure, the samples were diluted with methanol:water (1:1; v/v) to the concentration of 200 μg/ml and submitted to HPLC conditions.

The limits of detection (LOD) and quantification (LOQ) were determined from the calibration curves of the GC and EGC. The LOD was established by using the expression $3\sigma/S$ and LOQ by

expression $10\sigma/S$, where $\sigma$ is the standard deviation of the response and $S$ is the slope of the calibration curve.

Precision was evaluated on two levels: repeatability (intra-day) and intermediate (inter-day). The test of repeatability was carried out using three samples of the FAE at 80% (*ca.* 1.60 mg), at 100% (*ca.* 2 mg) and 120% (*ca.* 2.2 mg) in a 10 ml volumetric flask. For intermediate precision, the same procedure was performed on at least two different days on another HPLC instrument of the same model. Repeatability and intermediate precision were expressed as the residual standard deviation (RSD%) of the concentrations of GC and EGC in the dried FAE. A RSD% over 10% was considered unacceptable for the complex matrix.

The accuracy of the method was established based on the GC and EGC recovery tests using the addition of standard. The sample solutions were prepared from 1 mg of FAE in a 10 ml volumetric flask (50% of the normal preparation). The accuracy was assessed at three different levels in three replicates. A defined volume of reference solution equivalent at 12.72 μg of GC and 12.03 μg of EGC was added for the lower concentration (LC); at 21.22 μg of GC and 20.08 μg of EGC for the intermediate concentration (IC), and at 29.66 μg of GC and 28.06 μg of EGC for the higher concentration (HC). The known concentration was determined (based on assay of FAE by precision parameter) and the measured value was compared with the theoretical value. The accuracy was assessed as the recovery percentage and the method was considered accurate if the recovery percentages were between 85 and 115%.

Robustness was determined by comparing sample solutions that were analyzed under the established conditions and by changing the following parameters: from oven temperature gradient to a constant temperature at 24 °C and 35 °C, and eluent flow from 0.4 ml/min to 0.6 ml/min. In addition, the stability of the sample solution was assessed after 30 h at room temperature and in the injector at 10 °C.

*Pharmacognostic quality control assays*

The granulometry of the powder was measured by sieves of mesh sizes 150, 180, 212, 300, 600, and 850 μm (Bertel AGT-01) according to the method described by Brazilian Pharmacopeia (2010) and the median diameter ($d_{50}$) could be determined by the point of intersection of the curve of the percentage of residue fraction and percentage of passage fraction. The levels of total ash and loss on drying were determined according to methods described by the Brazilian Pharmacopeia (2010). The total polyphenol (TP) content was determined for milled rhizomes according to the method described by Glasl (1983). The milled rhizomes (20 g) from samples A, B, and C were extracted with acetone:water (7:3, v/v) by turbo-extraction (Ultra-Turrax® UTC IKA-125, 4× 5 min, $t < 40$ °C) in the proportion of 10% (w/v). The extraction solutions were filtered and transferred to a volumetric flask and diluted to 200 ml. Portions of these solutions were used in the dry residue test according to the German Pharmacopeia (2015). The TP content expressed in pyrogallol was analyzed in the CE (from samples A, B, and C) by spectrophotometry according to the method described by Blainski et al. (2013). The FAE (from samples A, B, and C) were analyzed by HPLC method to compare their chromatographic fingerprints and determine the GC and EGC contents. For thin-layer chromatography (TLC), the CE, FAE (samples A, B and C) and reference substances (gallic acid and gallocathechi – GA and GC, respectively) were prepared in methanol in the concentration of 40 mg/ml, 10 mg/ml and 1 mg/ml, respectively; sample solutions were 5 min in ultrasonic bath and followed to centrifugation (3 min, 12,100 × g, Eppendorf MiniSpin); 40 μl of each sample and 10 μl of reference substances were applied in glass plate (TLC Silica gel 60 F254, 20 cm × 20 cm, Merck) by automatic Sampler (Camag); the elution was with ethyl acetate:formic acid anhydrous:water (90:5:5, v/v) for 15 cm; after drying, the TLC was sprayed with ferric chloride, hexahydrate, 1% in methanol (w/v) and the image was captured by in TLC Visualizer (Camag, system Vision Cats).

*Comparison of extracted liquid and selective for tannins*

Sample A extracts were prepared according to the previously described in extraction preparation with a series of different solutions: hydroethanol 50°GL (Extract A); hydroethanol 70°GL (Extract B); hydroethanol 90°GL (Extract C) and methanol: water (1:1, v/v) (Extract D), as well as acetone: water (7:3, v/v) (Extract E). The extracted solutions were used to determine dry residue (German Pharmacopeia, 2015), and CE were obtained from remaining solutions and used to determine the TP and total tannin (TT) contents (Glasl, 1983). These results were used to compare different extraction solutions and to determine which is more selective for tannins in the context of the turbo-extraction process.

*Antioxidant potential*

Sample B was used to determine the free-radical scavenging capacity of the CE, FAE, and FAQ according to the method described by Amarowicz et al. (2004).

*Statistical analysis*

Data were analyzed by the Statistica® 8.0 program (Copyright StatSoft, Inc. 1984–2007) by a one-way analysis of variance (ANOVA) followed by Tukey's test considering $p < 0.05$ as the significance level, as well as the $T$-test for two groups considering $p < 0.05$ as the significance level. Results were expressed as mean ± standard deviation [residual standard deviation (RSD%)]. To determine the linearity of the method, line correlation tests and residual analysis were made by simple line regression considering $R^2$ values equal or greater than 0.99 and the residual sum of squares were evaluated.

## Results and discussion

*HPLC method development*

The extraction of the target phenolic compounds was performed by liquid–liquid extraction with ethyl acetate. The purification by solid-phase extraction (SPE C8 – 55 μm, 70 Å) was tested and the results demonstrated no difference in the chromatographic profile, for this reason, the solid-phase extraction was not employed for preparation of samples The choice of the detection wavelength is a crucial step in developing an efficient method. The spectra for all the main peaks were investigated by use of the DAD detector; the main peaks of chromatogram profile had a great absorbance at 210 nm (data not shown). In addition, the detection wavelength of 210 nm obtained a sufficiently large number of detectable peaks in the chromatograms. The choice of the C-18 column was based on previous data from the study of phenols (Soares et al., 2004; Lopes et al., 2009; Rosenthal et al., 2014), and there is a preference for a stable column in the acidic pH (1–6) range, considering the acidic mobile phase. For the composition of the mobile phase, acidification with 0.05% (pH 2.21) and 0.1% (pH 1.93) trifluoroacetic acid, 0.2% formic acid (pH 2.47) and 0.2% (pH 1.94) and 0.4% (pH 1.78) phosphoric acid were tested. Acidification with 0.2% phosphoric acid demonstrated a suitable separation of compounds. The spectra for all signals were recorded in the range of 200–400 nm; this allowed for the determination of the suitable purity peak for all eight major peaks (>0.99) and suggest the identification of three substances by spectral similarity, RT and spiked sample with the reference substance: GA (RT = 9.9 min), GC (RT = 13.1 min), and EGC

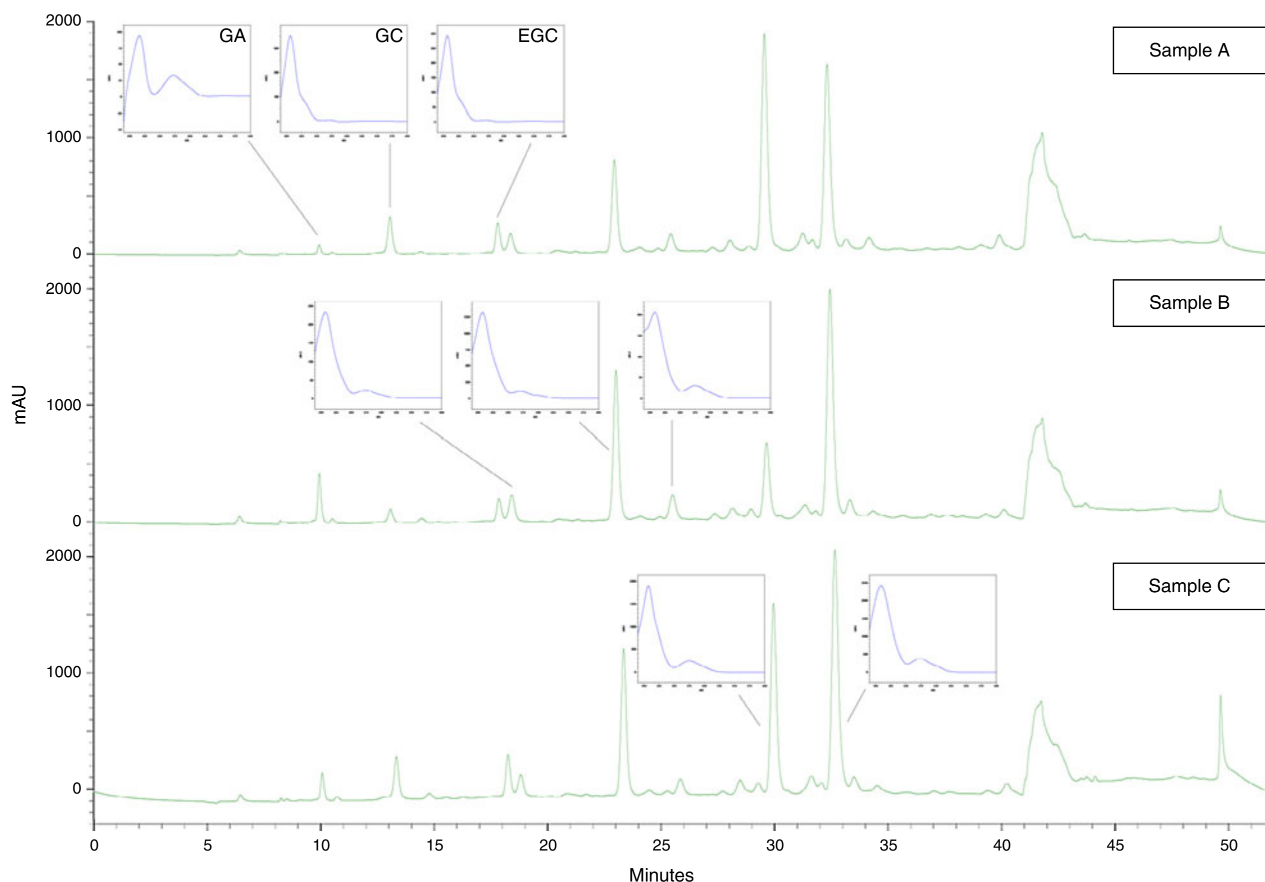


**Fig. 1.** HPLC fingerprint of the ethyl-acetate fraction (FAE) from *Limonium brasiliense* rhizomes extracted with acetone:water (7:3, v/v) for samples A, B, and C. GA, gallic acid; GC, gallocathechin; EGC, epigallocatechin.

(RT = 17.8 min). The GC and EGC were chosen for the validation procedures. The others major peaks (RT = 18.4; 22.9; 25.8; 29.4, and 32.0 min) showed characteristic spectra for condensed tannins with a absorption band at round 275 nm and a shoulder at 305 nm, commonly attributed, respectively, to the A-ring and B-ring from the flavan-3-ols and flavan-3,4-diols units (Vihakas, 2014). Fig. 1 shows the obtained the spectra and chromatographic profiles. The HPLC method carried out in this study was aimed at developing a chromatographic system capable of eluting and resolving phenolic compounds in plant materials. The preliminary investigations were directed toward evaluating the effect of various factors on the system. The factors assessed include sample purification, detection wavelength, the column type, and the composition of the mobile phase. The elimination of high molecular weight phenolic compounds from the plant extract is critically important, because of the interaction of these compounds with the stationary phase. This interaction can seriously damage the analytical column, interfering with the chromatographic process (Lopes et al., 2010).

### *Validation of the HPLC method*

The calibration equation in the linearity test was $y = 1.44 \cdot 10^8 x + 1.2 \cdot 10^7$ ($n = 3$, $r^2 = 0.9980$) for GC and $y = 1.41 \cdot 10^8 x + 1.0 \cdot 10^7$ ($n = 3$, $r^2 = 0.9950$) for EGC. The RSD% of the slopes was 2.3 and 1.8% for GC and EGC, respectively. Table 1 shows the statistical data for the regression equations with analysis of variance and lack of fit calculations for calibration curves of GC and EGC. The calibration curves are adequate for assay determination and statistical data comply with all requirements for linear regression: calculated *F*-value is lower than critical *F*-value, as well as the lack of fit error than sum of pure error, and the ANOVA test is significant.

The specificity of the method was shown by comparing the UV spectra of GC (13.1 min) and EGC (17.8 min) in the reference solution with those from the test solution. The purity of the GC, EGC, and six other main peaks was suitable (>0.99). The degradation of both peaks was suitable for the acid, heat and oxidation and light stress conditions, because they provided the partial degradation, by which was allowed the reliable analyses of the peak purity of the remaining GC and EGC (Table 2). On the other hand, the sample appeared to be very sensitive to the basic condition and there was almost complete degradation of the GC and EGC peaks even under more mild conditions. However, it seems that substances resulting from the degradation induced by basic stress do not interfere with the retention times for GC and EGC. Thus, the chromatography method is considered specific for assay of GC and EGC.

The LOD is defined as the lowest absolute concentration of analyte in solution that can be detected but not necessarily quantified under the stated experimental conditions and was 0.161 μg/ml for GC and 0.238 μg/ml for EGC. The LOQ is defined as the lowest concentration of analyte in solution that can be quantitatively determined with acceptable precision and accuracy. It was 0.536 μg/ml for GC and 0.793 μg/ml for EGC.

The repeatability and the intermediate precision for sample were 2.7% ± 0.086 [3.2%] and 2.8% ± 0.042 [1.5%] for GC, and 2.2% ± 0.126 [5.8%] and 2.3% ± 0.085 [3.7%] for EGC, respectively. By *T*-test, there was no significant difference ($t_{1,16} = -1.85$, $p = 0.08$) for EGC between the results of repeatability and intermediate precision analyses. On the other hand, there was a significant difference ($t_{1,16} = -2.80$, $p < 0.05$) for GC, but the low RSD% (2.9%) for the mean from all samples of GC (2.8% ± 0.080 [2.9%]) showed suitable data

**Table 1**
Statistical data for the regression equations of the calibration curves for gallocatechin (GC) and epigallocatechin (EGC) by the HPLC method.

| Regression analysis | Calibration curve for GC | | Calibration curve for EGC | |
|---|---|---|---|---|
| Slope (SE) | $1.44 \times 10^8$ ($2.29 \times 10^{14}$) | | $1.41 \times 10^8$ ($4.78 \times 10^{14}$) | |
| Intercept (SE) | $1.2 \times 10^7$ ($7.74 \times 10^6$) | | $1.0 \times 10^8$ ($1.1 \times 10^7$) | |
| Regression coefficient ($R^2$) | 0.9980 | | 0.9950 | |
| Calculated $F$-value (critical $F$-value) | 0.30 (3.71) | | 0.30 (3.71) | |
| Sum of pure error | $3.51 \times 10^{16}$ | | $5.09 \times 10^{16}$ | |
| Lack of fit error | $3.48 \times 10^{15}$ | | $5.04 \times 10^{15}$ | |
| Analysis of variance | $F_{1,13} = 6385.7$, $p < 0.001$ | | $F_{1,13} = 2610.8$, $p < 0.001$ | |
| 95% CL Slope | $1.40 \times 10^8$; $1.48 \times 10^8$ | | $1.35 \times 10^8$; $1.47 \times 10^8$ | |
| 95% CL Intercept | $-4.6 \times 10^6$; $2.9 \times 10^7$ | | $7.7 \times 10^7$; $1.2 \times 10^8$ | |
| | SS | MS | SS | MS |
| Regression | $1.13 \times 10^{17}$ | $1.13 \times 10^{17}$ | $9.60 \times 10^{16}$ | $9.60 \times 10^{16}$ |
| Residual | $2.29 \times 10^{14}$ | $1.76 \times 10^{13}$ | $4.78 \times 10^{14}$ | $3.68 \times 10^{13}$ |
| Total | $1.13 \times 10^{17}$ | | $9.64 \times 10^{16}$ | |

SE, standard error; SS, sum of squares; MS, mean square; CL, confidence limits.

**Table 2**
The partial degradation and the reliability analyses of the purity peak for the remaining gallocatechin (GC) and epigallocatechin (EGC) in stress conditions for specificity test.

| Condition for degradation | GC content (%)/purity peak | EGC content (%)/purity peak |
|---|---|---|
| *Normal condition* | 2.8/1.0000 | 2.3/1.0000 |
| *Acid stress* | 2.3/1.0000 | 1.1/1.0000 |
| *Heat stress* | 2.3/1.0000 | 1.8/1.0000 |
| *Oxidation stress* | 2.9/1.0000 | 2.1/1.0000 |
| *Basic stress* | 0.1/0.8771 | 0.2/0.9153 |
| *Light stress* | | |
| Amber | 2.7/1.0000 | 2.1/1.0000 |
| Transparent | 2.4/1.0000 | 1.9/1.0000 |

for the complex matrix, so it is considered, that the method is precise for both substances.

The accuracy of the method was established based on the recovery test for GC and EGC. The mean recovery rate was 102.3% ± 1.54 [1.5%] for GC and 99.7% ± 1.93 [1.9%] for EGC (Table 3). The result is according to the accepted criterion, thus the method has excellent accuracy for herbal drugs.

To ensure that the HPLC method is insensitive to minor changes in experimental conditions, it tests the robustness parameters. When the oven temperature was changed from a gradient to a constant temperature of 24 °C, the resolution retention time (RT) and peak area of the GC and EGC had no significant change. However, the chromatographic profile after 25 min and resolution of these peaks was changed. At a constant temperature of 35 °C, there were alterations in the RT of GC and EGC, and the resolution between EGC and peak at 18.4 min was lost completely. When the eluent flow was changed to 0.6 ml/min, this changed the RT of the main peaks, including for GC and EGC. The resolution for some peaks was also lost, but there was no significant change in peak area for GC and EGC. Regarding the stability of the sample solution, no difference was observed for the samples left in the injector at 10 °C for 30 h. However, there was a significant content decrease of GC (*ca.* 12%) at room temperature. These results demonstrated, that it should procedure the method under determinate conditions and some change can induce to problems with chromatographic profile and resolution.

### *Pharmacognostic quality control assays*

The results of pharmacognostic quality control assays from milled rhizomes (granulometry, total ash, loss on drying and total polyphenols), CE (dry residue and TP), and FAE (GC and EGC) in samples A, B, and C are presented in Table 4.

Sample B has a TP rhizome lower than that of the other samples, but this seems to have no substantial differences among the samples in relation to the dry residue and TP in the CE. The $d_{50}$ determined from the curves shows a value substantially lower for sample B (0.21 mm) in relation to the other samples (0.42 mm and 0.48 mm). However, this lower diameter did not seem to influence the extraction yield, presumably because it is a turbo-extraction process, where happens the breaking of particles. The granulometric distribution is an important factor in obtaining plant extracts, mainly because the extraction yield is closely related to the surface area and particle size in contact with the extraction liquid. Studies to determine the existence of positive and negative influences from the $d_{50}$ on extraction yield are possible through factor analysis (Mello and Petrovick, 2000; Delaporte et al., 2001).

The total ash results range between 4.9 and 5.7% from the three different samples show good consistency for the total ash determination for this species. Total ash is a measure of the quantity of non-volatile inorganic impurities, because it is influenced by contamination or adulteration of plant products (Mukherjee, 2002). The total ash includes those derived from plant tissue (physiological ash) and foreign material, specifically sand and dirt adhering on the surface of the plant (non-physiological ash). The percentage of total ash may vary with the plant species and must be determined individually for each species. For plant drugs, the percentage of total ash is between 3 and 15% for some species described in the Brazilian Pharmacopeia (2010).

The results of loss on drying lesser than 14% show that the drying process was efficient. For some species described in the Brazilian Pharmacopeia (2010), the percentage of loss on drying is between 8 and 14%. The percentage of loss on drying is a parameter that can be used to estimate the efficiency of the plant drying procedure. The excess water in plant drugs promotes the growth of fungi, bacteria or insects and promote hydrolysis of its constituents. For this reason, limits of water content are described for herbs, especially for those that readily absorb water or those for which degradation is promoted by the presence of water. This parameter is critical to determine the stability of the drug during the storage period (Mukherjee, 2002).

Although the TP contents were not so different among the samples (25–33.1%), the GC and EGC contents showed results in a large interval: 0.8–2.7% and 1.2–2.2%, respectively. It was possible to note difference of GA peak area. Thereupon, we can say that the concentrations of GA were approximately 0.5, 2.4, and 1.6% for sample A, B, and C, respectively. These values were calculated from known concentration GA solutions, although there was no validation test for this analyte, but it also showed a large interval among the samples. To ensure the acceptable interval for compound assay, it

**Table 3**
Accuracy results determined by analyzing gallocatechin (GC) and epigallocatechin (EGC) in solutions of known concentration from *Limonium brasiliense*.

| Gallocatechin | | | | Epigallocatechin | | | |
|---|---|---|---|---|---|---|---|
| Theoretical concentration (μg/ml) | Measured concentration (μg/ml) | % Recovery | ($\bar{x} \pm$ sd) [RSD(%)] | Theoretical concentration (μg/ml) | Measured concentration (μg/ml) | % Recovered | ($\bar{x} \pm$ sd) [RSD(%)] |
| *LC* | | | | | | | |
| 3.8266 | 3.9419 | 103.0 | | 3.2580 | 3.1848 | 97.8 | |
| 3.7543 | 3.9246 | 104.5 | 103.5% ± 0.91 (0.9%) | 3.1998 | 3.1698 | 99.1 | 97.8 ± 1.29 (1.3%) |
| 3.6338 | 3.7400 | 102.9 | | 3.1029 | 2.9938 | 96.5 | |
| *IC* | | | | | | | |
| 4.6284 | 4.7329 | 102.2 | | 4.0242 | 4.0083 | 99.6 | |
| 4.7248 | 4.7996 | 101.6 | 102.4% ± 0.88 (0.9%) | 4.1018 | 4.0888 | 99.7 | 100.2 ± 0.87 (0.9%) |
| 4.7007 | 4.8576 | 103.3 | | 4.0824 | 4.1293 | 101.2 | |
| *HC* | | | | | | | |
| 5.5929 | 5.5731 | 99.6 | | 4.9192 | 4.8997 | 99.6 | |
| 5.5929 | 5.7554 | 102.9 | 100.9% ± 1.73 (1.7%) | 4.9192 | 5.0766 | 103.2 | 101.2 ± 1.85 (1.8%) |
| 5.4731 | 5.4883 | 100.3 | | 4.9599 | 4.9921 | 100.7 | |
| *Total ($\bar{x} \pm$ sd) [RSD(%)]* | | 102.3% ± 1.54 (1.5%) | | | | 99.7 ± 1.93 (1.9%) | |

$\bar{x}$, mean (%); sd, standard deviation; RSD, residual standard deviation.

**Table 4**
Results of pharmacognostic quality control assays from *Limonium brasiliense* (samples A, B, and C).

| | Sample A | Sample B | Sample C |
|---|---|---|---|
| Granulometry ($d_{50}$) – MR | 0.42 mm | 0.21 mm | 0.48 mm |
| Total ash – MR[a] | 5.6% ± 0.15 [2.7%] | 5.7% ± 0.07 [1.4%] | 4.9% ± 0.05 [1.0%] |
| Loss on drying – MR[a] | 11.1% ± 0.25 [2.3%] | 12.4% ± 0.28 [2.4%] | 11.9% ± 0.04 [0.4%] |
| PT – MR[a] | 15.8% ± 0.12 [0.8%] | 8.5% ± 0.36 [4.3%] | 9.4% ± 0.11 [1.2%] |
| Dry residue – MR[a] | 39.5% ± 0.63 [1.6%] | 36.1% ± 0.61 [1.7%] | 30.6% ± 0.19 [0.6%] |
| TP – CE[a] | 28.9% ± 0.42 [1.5%] | 25.0% ± 1.94 [0.8%] | 29.1% ± 0.30 [1.1%] |
| GC – FAE[a] | 2.6% ± 0.06 [2.3%] | 0.8% ± 0.03 [3.7%] | 2.7% ± 0.09 [3.2%] |
| EGC – FAE[a] | 1.8% ± 0.03 [1.8%] | 1.2% ± 0.02 [1.9%] | 2.2% ± 0.13 [5.8%] |

[a] $\bar{x} \pm$ sd [RSD(%)]: $\bar{x}$, mean; sd, standard deviation; RSD, residual standard deviation; MR, milled rhizome; CE, crude extract; FAE, ethyl-acetate fractions; TP, total polyphenols; GC, gallocatechin; EGC, epigallocatechin.

recommends the evaluation of a wide number of samples from different year seasons and regions.

Fig. 1 shows the HPLC fingerprints of FAE from samples A, B, and C. There is a good similarity between the samples regarding their peak retention time; however, the peaks have different proportions. Fig. 2 shows the TLC fingerprints of CE and FAE from samples A, B, and C. As well as HPLC fingerprint, it is possible to note a good similarity with difference intensity among the spots. For example, in the sample B, the spot of GA is stronger, but weak spot of GC is fast absent, that it complies with HPLC fingerprint. For complex botanicals, quantitation of a single compound usually does not provide complete information. For this reason, chromatographic fingerprint technology is accepted by the WHO (1991) as a strategy for identification and evaluation of the quality of herbal medicines. This work shows for the first time the chromatographic fingerprints for *L. brasiliense*, and the results seem to provide a reliable and sensitive quality assessment method with characteristic retention time, as well UV spectra of peaks for HPLC method. The HPLC fingerprint analysis can be used for qualitative analysis, especially to identify and standardize plant materials and preparations. This analysis requires good separation and resolution of the complex mixture as well as peak purity control in order to prevent overlapping of the peaks (Rehwald et al., 1994; Ding et al., 2011). HPLC fingerprinting has become an attractive option for the quality control of complex plant mixtures because of its focus on identification, ensuring no adulterants (He et al., 2005), and assessing the stability of the plants, seeing that changes in the HPLC fingerprint can occur in samples by the influence of external factors such as temperature, humidity, and stress conditions (Heigl and Franz, 2003). Several studies have shown that the HPLC fingerprint can be used to characterize plant drugs that have polyphenols as primary

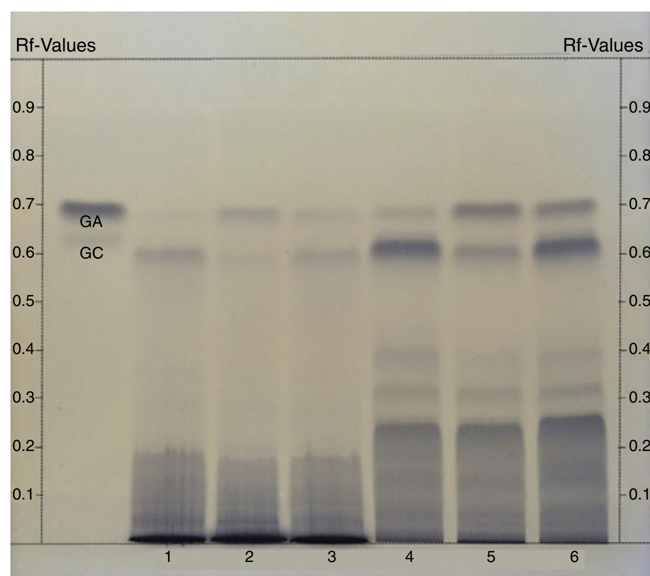


**Fig. 2.** TLC fingerprint of the crude extract (CE) and ethyl-acetate fractions (FAE) from *Limonium brasiliense* rhizomes extracted with acetone: water (7:3, v/v) for samples A, B and C. GA, gallic acid; GC, gallocathechin; 1, 2 and 3, CE from samples A, B and C, respectively; 4, 5 and 6, FAE from samples A, B and C, respectively.

components (Marques et al., 2007; Chou et al., 2009; Lopes et al., 2009, 2010; Simirgiotis and Schmeda-Hirschmann, 2010). The TLC is a classic method for quality control of natural products and it is required for all pharmacopoeia. It is of simple performance and low cost, what makes possible easily its use. As well HPLC fingerprint,

**Table 5**
Results for the comparison of the extraction liquid and selected tannins in sample A.

| | Extract A | Extract B | Extract C | Extract D | Extract E |
|---|---|---|---|---|---|
| TP[b] | 31.0% ± 0.32 [1.0%] | 29.3% ± 0.34 [1.2%] | 30.2% ± 1.12 [3.7%] | 31.3% ± 0.46 [1.5%] | 33.1%% ± 0.64 [2.0%][a] |
| TT[b] | 27.6% ± 0.33 [1.2%] | 25.3% ± 0.36 [1.4%] | 25.9% ± 1.02 [4.0%] | 27.7% ± 0.42 [1.5%] | 29.5% ± 0.60 [2.0%][a] |
| %TT/TP[b] | 88.9% ± 0.27 [0.3%] | 86.6% ± 0.34 [0.4%] | 85.7% ± 0.50 [0.6%] | 88.7% ± 0.21 [0.2%] | 89.2% ± 0.49 [0.6%][a] |
| Dry residue[b] | 36.4% ± 0.41 [1.1%] | 32.2% ± 0.34 [1.1%] | 29.9% ± 0.16 [0.5%] | 36.3% ± 0.56 [1.5%] | 39.5% ± 0.63 [1.6%][a] |

[a] $p < 0.001$.
[b] $\bar{x}$ ± sd [RSD(%)]: $\bar{x}$, mean; sd, standard deviation; RSD, residual standard deviation; TP, total polyphenols; TT, total tannins.

TLC was also adequate to determine a characteristic fingerprint for this herbal drug. Differences among samples A, B and C can be observed, suggesting that it is important hereafter to correlate the fingerprints with the seasonal conditions and possible differences in pharmacological activities.

### *Comparison of extraction liquid and selectivity for tannins*

The preparation of different extracts followed by analysis of the dry residue, TP and TT are shown in Table 5. According to the data, there is a significant increase in dry residue from Extract E [extraction liquid acetone: water (7:3, v/v)] compared with the other extracts ($F_{4,20} = 136.0$, $p < 0.001$). In addition, Extract E shows significantly higher levels of TP ($F_{4,20} = 23.7$, $p < 0.001$) and TT ($F_{4,20} = 37.0$, $p < 0.001$), and comparatively higher TT/TP ratio ($F_{4,20} = 86.0$, $p < 0.001$).

The preparation of different extracts followed by analysis of dry residue and chemical compound content may provide complementary data for quality control and contribute to the choice of the best extraction solvent. According to our data (Table 5), it was determined that the best extraction solvent for the preparation of crude extract of dry rhizomes of the *L. brasiliense* by tubo-extraction process is acetone:water (7:3, v/v). According to Mueller-Harvey (2001), 50–70% aqueous acetone is often the better solvent more than water or methanol to extract hydrolysable tannins. However, methanol or water can extract better tannins of low molecular weight.

Considering the high quantity of tannins among the polyphenols of dry rhizomes from *L. brasilense* and comparing with other plants, it is possible to affirm that it is a representative species for tannins. Commission E (1994) determined tannin content of 3% for *Crataegus oxyacantha* L. and 10% for *Krameria triandra* Ruiz & Pav.; both are classical plant drugs for tannins. *Stryphnodendron obovatum* Benth. and *S. polyphyllum* Mart. have tannin contents of 19 and 12%, respectively (Lopes et al., 2005), and *Stryphnodendron adstringens* (Mart.) Coville has a minimum of 8% of total tannins (Brazilian Pharmacopeia, 2010).

### *Antioxidant potential*

The results for the CE, FAE, and FAQ from sample B were, respectively, 6.87, 5.91, and 6.92 μg/ml. Trolox and Vitamin C were used as controls, and their results were 3.97 and 4.36 μg/ml, respectively. The antioxidant effect of polyphenols is known, however a recent study suggested that *Rosa canina* L. fruit extract, which is rich in polyphenols, may act not only as an antioxidant, but also as a prooxidant in high concentrations, showing that it is necessary to define an optimal concentration of polyphenols for antioxidant activity (Kiliçgün and Altiner, 2010). Although it could be already expected a good antioxidant potential for *L. brasiliense*, since the sample showed a high TP and tannic substances, few studies have related this activity to plants of the genus *Limonium*, and this investigation proposed to show these potential even with the sample with lower phenolic contents.

Aniya et al. (2002) showed a strong antiradical activity ($IC_{50}$ 7.5 μg/ml) by the DPPH method for aqueous extracts from *L. wrightii* (Hance) Kuntze. Murray et al. (2004) showed an $IC_{50}$ of 20 μg/ml for a methanolic extract from *L. brasiliense* by the DPPH assay. The present results exhibited major antioxidant potential for the acetone:water (7:3, v/v) extract and its ethyl-acetate fraction, in comparison with the above result.

## Conclusions

In the absence of updated pharmacopoeia data regarding *L. brasiliense*, these results can be used as quality control parameters and contribute to the pharmacognostic quality of such extracts. The TLC and HPLC fingerprint have been shown to be a valuable tool for the chemical analysis of complex matrices. The validation of a HPLC method offers chromatographic conditions for determination and analysis of GC and EGC. The method was found to be specific and suitable for routine analysis. It is also simple, sensitive, accurate, and reproducible.

## Authors' contributions

AB (Ph.D. student) collected and dried the plant material samples A and C, prepared their voucher specimens, worked on laboratorial tests with both samples, conducted all HPLC and TLC analysis, and wrote and formatted the article. TMAU collected and dried sample B of the plant material, prepared its voucher specimen, worked in laboratorial tests with this sample, and assisted with writing. GG (undergraduate student) assisted in laboratory work with sample B. EVSLM assisted in the project design and reviewed the manuscript. JCPM was responsible for the project concept and supervision of the study, as well as the writing and review of the manuscript.

## Conflicts of interest

The authors declare no conflicts of interest.

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## References

Amarowicz, R., Pegg, R.B., Rahimi-Moghaddam, P., Barl, B., Weil, J.A., 2004. Free-radical scavenging capacity and antioxidant activity of selected plant species from the *Canadian prairies*. Food Chem. 84, 551–562.

Aniya, Y., Miyagi, C., Nakandakari, A., Kamiya, S., Imaizumi, N., Ichiba, T., 2002. Free radical cavenging action of the medicinal herb *Limonium wrightii* from the Okinawa islands. Phytomedicine 9, 239–244.

Antonelli-Ushirobira, T., Blainski, A., Gancedo, N.C., Gaburo, F., Cardoso, K.A.K.C., Leite-Mello, E.V.S., Mello, J.C.P., Milaneze-Gutierre, M.A., 2015a. Morpho-anatomical study of rhizome of *Limonium brasiliense*. Rev. Bras. Farmacogn. 25, 320–327.

Antonelli-Ushirobira, T., Blainski, A., Fernandes, H.G., Moura-Costa, G.F., Costa, M.A., Campos-Shimada, L.C., Salgueiro-Pagadigorria, C.L., Kaneshima, E.N., Becker, T.C.A., Leite-Mello, E.V.S., Mello, J.C.P., 2015b. Acute toxicity and long-term safety evaluation of the crude extract from rhizomes of *Limonium brasiliense* in mice and rats. J. Ethnopharmacol. 174, 293–298.

Anvisa, 2003. Resolução RE n° 899 – Guia para validação de métodos analíticos e bioanalíticos. Agência Nacional de Vigilância Sanitária, Brasilia.

Blainski, A., Lopes, G.C., Mello, J.C.P., 2013. Application and analysis of the Folin Ciocalteu method for the determination of total phenolic content from *Limonium brasiliense* L. Molecules 18, 6852–6865.

Brazilian Pharmacopeia, 2010. Farmacopeia Brasileira, 5th ed. ANVISA, Brasília.

Chou, G., Xu, S.J., Liu, D., Koh, G.Y., Zhang, J., Liu, Z., 2009. Quantitative and fingerprint analyses of chinese sweet tea plant (*Rubus suavissimus* S. Lee). J. Agric. Food Chem. 57, 1076–1083.

Commission, E., 1994. *Kommission E*, Liste der Monographien. Bundesgesundheitsamt-Institut für Arzneimittel, Bundesanzeiger.

Delaporte, R.H., Sánchez, G.M., Cuellar, A.C., Mello, J.C.P., 2001. Control de calidad y actividad antiinflamatoria de las drogas vegetales *Altemantbera brasiliana* (L.) Kuntze y Bouchea flumtnensis (Vell.) Mold. Acta Farm. Bonaer. 20, 39–46.

Ding, Y., Wu, E.Q., Liang, C., Chen, J., Tran, M.N., Hong, C.H., Jang, Y., Park, K.L., Bae, K., Kim, Y.h., Kang, J.S., 2011. Discrimination of cinnamon bark and cinnamon twig samples sourced from various countries using HPLC-based fingerprint analysis. Food Chem. 127, 755–760.

Fenner, R., Betti, A.H., Mentz, L.A., Rates, S.M.K., 2006. Plantas utilizadas na medicina popular brasileira com potencial atividade antifúngica. Rev. Bras. Cienc. Farm. 42, 269–394.

German Pharmacopeia, 2015. Deutsches Arzneibuch. Deutscher Apotheker Verlag, Bonn.

Glasl, H., 1983. Zur Photometrie in der Drogenstandardisierung – 3. Gehaltsbestimmung von Gerbstoffdrogen. Dtsch. Apoth. Ztg. 123, 1979–1983.

Gobbo-Neto, L., Lopes, N.P., 2007. Plantas medicinais: fatores de influência no conteúdo de metabólitos secundários. Quim. Nova 30, 374–381.

He, Z.D., Qiao, C.F., Han, Q.B., Cheng, C.L., Xu, H.X., Jiang, R.W., But, P.P., Shaw, P.C., 2005. Authentication and quantitative analysis on the chemical profile of cassia bark (*Cortex Cinnamomi*) by high-pressure liquid chromatography. J. Agric. Food Chem. 53, 2424–2428.

Heigl, D., Franz, G., 2003. Stability testing on typical flavonoid containing herbal drugs. Pharmazie 58, 881–885.

ICH, 2005. The European Agency for the Evaluation of Medicinal Products. Topic Q2(R1): Validation of Analytical Procedures: Methodology.

Janhs, R.T., Crescente, A.S., 1976. Ensaios farmacológicos e clínicos com a associação do extrato fluido de *Limonium brasiliense* e *N*-acetil-*p*-aminophenol. Trib. Pharm. 44, 105–111.

Kiliçgün, H., Altiner, D., 2010. Correlation between antioxidant effect mechanisms and polyphenol content of *Rosa canina*. Pharmacogn. Mag. 6, 238–241.

Lopes, G.C., Brainski, A., Santos, P.V.P., Diciaula, M.C., Mello, J.C.P., 2010. Development and validation of an HPLC method for the determination of epicatechin in *Maytenus ilicifolia* (Schrad.) Planch., Celastraceae. Rev. Bras. Farmacogn. 20, 789–795.

Lopes, G.C., Bruschi, M.L., Mello, J.C.P., 2009. RP-LC–UV determination of proanthocyanidins in *Guazuma ulmifolia*. Chromatographia 69, 175–181.

Lopes, G.C., Sanches, A.C.C., Nakamura, C.V., Dias-Filho, B.P., Hernandes, L., Mello, J.C.P., 2005. Influence of extracts of *Stryphnodendron polyphyllum* Mart. and *Stryphnodendron obovatum* Benth. on the cicatrisation of cutaneous wounds in rats. J. Ethnopharmacol. 99, 265–272.

Marques, L.C., Pieri, C., Roman-Junior, W., Cardoso, M.L.C., Milaneze-Gutierre, M.A., Mello, J.C.P., 2007. Controle farmacognóstico das raízes de *Heteropteris aphrodisiaca* O. Mach. (Malpighiaceae). Rev. Bras. Farmacogn. 17, 607–615.

Mello, J.C.P., Petrovick, P.R., 2000. Quality control of *Baccharis trimera* (Less.) DC (Asteraceae) hydroalcoholic extracts. Acta Farm. Bonaer. 19, 211–216.

Mueller-Harvey, I., 2001. Analysis of hydrolysable tannins. Anim. Feed Sci. Technol. 91, 3–20.

Mukherjee, P.K., 2002. Quality Control Herbal Drugs: An Approach to Evaluation of Botanicals. Business Horizons, New Delhi, pp. 183–245.

Murray, A.P., Rodriguez, S., Frontera, M.A., Tomas, M.A., Mulet, M.C., 2004. Antioxidant metabolites from *Limonium brasiliense* (Boiss.) Kuntze. Z. Naturforsch. 59c, 477–480.

Nisbet, L.J., Moore, M., 1997. Will natural products remain an important source of drug research for the future? Curr. Opin. Biotechnol. 8, 708–712.

Rehwald, A., Meier, B., Sticher, O., 1994. Qualitative and quantitative reversed-phase high-performance liquid chromatography of flavonoids in *Crataegus* leaves and flowers. J. Chromatogr. A 677, 25–33.

Rosenthal, I., Wolfram, E., Meier, B., 2014. An HPLC method to determine sennoside A and sennoside B in *Sennae fructus* and *Sennae folium*. Pharmeur. Bio. Sci. Notes 2014, 92–102.

Schofield, P., Mbugua, D.M., Pell, A.N., 2001. Analysis of condensed tannins: a review. Anim. Feed Sci. Technol. 91, 21–40.

Schulz, V., Hänsel, R., Blumenthal, M., Tyler, V.E., 2004. Medicinal plants, phytomedicines, and phytotherapy. In: Rational Phytotherapy: A Reference Guide for Physicians and Pharmacists. Springer-Verlag, Berlin/Heidelberg, pp. 1–42.

Simirgiotis, M.J., Schmeda-Hirschmann, G., 2010. Direct identification of phenolic constituents in Boldo Folium (*Peumus boldus* Mol.) infusions by high-performance liquid chromatography with diode array detection and electrospray ionization tandem mass spectrometry. J. Chromatogr. A 217, 443–449.

Soares, L.A., Oliveira, A.L., Ortega, G.G., Petrovick, P.R., 2004. Development and validation of a LC-method for determination of catechin and epicatechin in aqueous extractives from leaves of *Maytenus ilicifolia*. J. Pharm. Biomed. Anal. 36, 787–790.

Vihakas, M., (Doctor thesis) 2014. Flavonoids and Other Phenolic Compound: Characterization and Interaction with Lepidopteran and Sawfly Larvae. University of Turku, Turku, 135 pp. http://www.doria.fi/handle/10024/100939.

WHO, 1991. Guidelines for the Assessment of Herbal Medicines. WHO, Munich.