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Stryphnodendron adstringens (Mart.) Coville (Fabaceae) proanthocyanidins quantitation by RP-HPLC

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Stryphnodendron adstringens (Mart.) Coville (barbatimão) is a tree belonging to the Fabaceae family, and it is commonly found in the southeastern Brazilian cerrado. The stem bark of this tree is traditionally used as an anti-inflammatory and antiseptic agent to treat leucorrhea and diarrhea, as well as to promote wound healing (owing to the presence of proanthocyanidins). Proanthocyanidins were obtained from the ethanolic extract of *S. adstringens* stem bark and assessed by reversed phase–high performance liquid chromatography with an ultraviolet/diode array detector. The identified compounds included gallic acid, catechin, gallocatechin (GC), epigallocatechin, and epigallocatechin gallate (EGCG). The selected markers, GC and EGCG, were simultaneously used for chromatographic validation (linearity range: 30-330 ng, equivalent to 3-33 μg/mL; r>0.998). The method showed precision (intra-day relative standard deviation [RSD]: 1.72% for GC; 1.16% for EGCG; inter-day RSD: 1.74%–2.60% for both markers), accuracy, robustness, and selectivity. The limits of detection and quantitation were 0.29 μg/ml and 0.89 μg/ml for GC, and 0.88 μg/mL and 2.67 μg/mL for EGCG, respectively. In addition, *S. obovatum* was evaluated and showed an average of 12.2 μg/mL for GC (equivalent to 1.22% w/w) and 14.2 μg/mL for EGCG (equivalent to 1.42% w/w) in the ethanolic extract. The quantitative results were compared to those obtained for *S. adstringens*, which showed that the markers are present in both species.

Uniterms:*Stryphnodendron adstringens*/phytochemistry. Fabaceae/phytochemistry. Barbatimão/extrato vegetal/evaluation. Proanthocyanidins/evaluation. RP-High performance liquid chromatography/análise quantitativa.

Stryphnodendron adstringens (Mart.) Coville (barbatimão) é uma árvore da família Fabaceae encontrada no cerrado do sudeste do Brasil. As cascas do caule das espécies são tradicionalmente utilizadas como agente antiinflamatório e antisséptico para o tratamento de diarreia e leucorreia, bem como para promover a cicatrização de feridas, devido à presença de proantocianidinas. Estas substâncias obtidas a partir do extracto etanólico (EE) de cascas do caule de *S. adstringens* foram avaliadas por cromatografia líquida de alta eficiência de fase reversa. As substâncias identificadas foram ácido gálico, catequina, galocatequina (GC), epigalocatequina e galato de epigalocatequina (EGCG). Os marcadores selecionados, GC e EGCG, foram utilizados simultaneamente para validação cromatográfica (intervalo de linearidade 30-330 ng, equivalente a 3-33 μg/mL, r>0,998). O método mostrou precisão (intra-dia DPR 1,72% para GC; 1,16% para EGCG; inter-dia DPR 1,74%–2,60% para ambos os marcadores), exatidão, robustez e seletividade. Os limites de detecção e de quantificação de 0,29 μg/mL e 0,89 μg/mL para a GC, e 0,88 ng/mL e 2,67 μg/mL para EGCG, respectivamente. Outra espécie (*S. obovatum*) avaliada, mostrou valores médios 12,2 μg/mL de GC (equivalente a 1,22% p/p) e 14,2 μg/mL de EGCG (equivalente a 1,42% p/p) encontrados no EE. Os resultados quantitativos comparam-se aos de *S. adstringens* e mostram que tais marcadores estão presentes em ambas as espécies.

Unitermos: *Stryphnodendron adstringens*/fitoquímica. Fabaceae/fitoquímica. Barbatimão/extrato vegetal/avaliação. Proantocianidinas/evaluation. Cromatografia líquida de alta eficiência/análise quantitativa.

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INTRODUCTION

Stryphnodendron adstringens (Martius) Coville, Fabaceae, is among the most widely used plants for wound treatment in traditional medicine (Forero, 1972). Its registry has been deposited in the Missouri Botanical Garden (MOBOT) nomenclature database (Missouri, 2009). This plant was described in the 19th century by naturalists who traveled throughout southeast Brazil, in particular, the state of Minas Gerais (Brandão *et al.*, 2008). Popularly known in Brazil as barbatimão, infusions of the stem bark of *S. adstringens* are used as an anti-inflammatory or antiseptic agent, as well as in the treatment of leucorrhea, diarrhea, and wound healing. Previous studies have described anti-ulcerogenic, antioxidant, anti-tyrosinase, antimicrobial, trypanocidal, leishmanicidal, and molluscicidal properties of *Stryphnodendron* species (Audi *et al*., 1999; Baurin *et al*., 2002; Bezerra *et al*., 2002; Herzog-Soares *et al*., 2002; Rebecca *et al*., 2003; Lopes *et al*., 2005; Luize *et al*., 2005; Ishida *et al*., 2006; Souza *et al*., 2007).

Rebecca *et al.* (2003) suggested that a long period of *per os* (p.o.) administration of barbatimão aqueous extract in mice promotes its toxic effects. On the other hand, Costa *et al.* (2010) and Sousa *et al*. (2003) reported that the extract of *S. adstringens* and its proanthocyanidin polymer-rich fraction did not show genotoxicity.

A variety of compounds have been isolated from the *Stryphnodendron* genus, including triterpenes and, mainly, tannins (prodelphinidins and prorobinetinidins) (Mello, Petereit, Nahrstedt, 1996a; Mello, Petereit, Nahrstedt, 1996b; Mello, Petereit, Nahrstedt, 1999; Lopes *et al.*, 2008).

Official monographs of *S. adstringens* can be found in the Brazilian Pharmacopeia, in which the ultraviolet-visible spectrophotometry (UV-Vis) technique for determining tannin content is described (Farmacopeia, 2012). This technique is also described in the British Pharmacopoeia (British, 2011) and the European Pharmacopoeia (European, 2011). Usually described in official compendia, either the UV-Vis Folin-Denis or Ciocalteu methods are based on total phenolics and tannins. These methods require variable sources of a protein binding agent and a waiting period for the formation of the color compound (Schofield, Mbugua, Pell, 2001). Although these methods are efficient, several limitations make their routine use difficult. Furthermore, the diverse structures found in tannins causes difficulties in their quantitation.

Previously reported methods for the determination of tannins involve sample preparation with solid-phase extraction or by liquid-liquid extraction (Nascimento *et al.*, 2008; Lopes *et al.*, 2009; Isler *et al.*, 2010; Lopes *et al.*, 2010; Castilho *et al.*, 2011). Another method involving high performance liquid chromatography (HPLC) that employs several sample preparation steps such as liquid-liquid and solid-phase extractions has recently been updated for the barbatimão monograph in the Brazilian Pharmacopeia (Farmacopeia, 2012).

Condensed tannins can precisely be quantified by HPLC by using either normal or reverse-phase columns. However, the separation of higher polymeric tannins (i.e., larger than tetramers) has not been accomplished, as reported by Waterhouse, Price, Maccord (1999), because the presence of many isomeric tannins with similar polarities results in overlapping peaks with similar retention times.

In this study, we describe a specific, adequate reversed phase-HPLC (RP-HPLC) method using linear-gradient elution with UV/DAD detection for determination of proanthocyanidins in routine analyses. This method was developed and validated using the selected biomarkers, gallocatechin (GC) and epigallocatechin gallate (EGCG), simultaneously. The active compounds were determined in stem bark extracts of *S. adstringens* and *S. obovatum*.

MATERIAL AND METHODS

Chemicals

Acetonitrile and methanol (HPLC grade) were purchased from Tedia (Fairfield, OH, USA) and J.T. Baker (Phillipsburg, NJ, USA), respectively. Concentrated phosphoric acid (85% w/v, Merck, Darmstadt, Germany) and commercial ethanol (96% [v/v]) were used. Ultrapure water was obtained using a Milli-Q plus system (Millipore, Milford, MA, USA). HPLC-grade reference substances used were gallic acid (GA; 98%, Acrós Organics, Geel, Belgium); quercetin (Q; 95%, Aldrich, Milwaukee, WI, USA); epigallocatechin (EGC; 90%, Fluka, Milwaukee, WI, USA); procyanidin B2 [epicatechin-(4β®8)-epicatechin; P, Extrasynthese, Genay, France]; and catechin (C; minimum 98%), gallocatechin (GC; minimum 98%), and epigallocatechin gallate (EGCG; minimum 95%) purchased from Sigma (Milwaukee, WI, USA).

Plant Material

The stem bark of *S. adstringens* (Martius) Coville, Fabaceae, was collected in 2007 from the southeastern state of Minas Gerais in the town of Santa Cruz de Minas (geographical positioning S21°06′42.6″, WO44°13′19.6″; S21°06′42.1″, WO44°13′19.8″; S21°06′39.8″, WO44°13′13.8″) and Ritápolis, (S21°00′44.5″, WO44°18′48.1″; S21°00′44.5″, WO44°18′43.7″;

S21°00′45.2″, WO44°18′42.1″). The samples were identified by Prof. José R. Stehmann, Ph.D. (Botanical Department of Biological Sciences Institute, BDBSI), and a voucher specimen was deposited at the BDBSI herbarium (coded as BHCB111231).

The stem bark of *S. obovatum* Benth. (Fabaceae) was collected from the central state of Mato Grosso do Sul, in Campo Grande city (S20°24′37.4″ and WO54°36′52.5″). The species was identified by Arnildo Pott, Ph.D. (National Center for Research on Beef Cattle, CNPGC EMBRAPA, Campo Grande, MS), and a voucher specimen (32997) was deposited at the CGMS herbarium of the Federal University of Mato Grosso do Sul.

Sample preparation

The stem bark samples of *S. adstringens* and *S. obovatum* were separately dried in an oven at 40 °C until a constant weight was obtained. They were then ground, and 170 g (in triplicate) was extracted (200 ml, 96% [v/v] commercial ethanol) by percolation for 2 weeks at an ambient temperature with the solvent changed every 24 h. The solvent from the ethanol extracts (EE) was removed in a rotavapor at 40 °C, yielding a dark brown solid with 44.82% [w/w] (76.19 g) and 45.50% [w/w] (77.35 g) dry mass for *S. adstringens* and *S. obovatum*, respectively.

Ethanolic extract purification

EE was purified by liquid-liquid extraction by using ethyl acetate:butanol:2-propanol:water (3.5:0.5:1.0:4.5). An aliquot of EE (250 mg) was accurately weighed, transferred to a separation funnel, and vigorously extracted 3-fold with the solvent system. The first organic (OF1) and aqueous fractions (AF1) were separately collected. AF1 was again extracted with the same amount of the saturated organic phase of the solvent system to yield the OF2 fraction. The final combined organic and aqueous fractions were separately evaporated under warm air stream (40 °C) to dry the residues.

Analysis of extracted samples

EE samples from the 2 different species of barbatimão (*S. adstringens* and *S. obovatum*) were evaluated (in duplicate) using the validated method. Both extracts were purified by the described liquid-liquid partition. An aliquot of the combined OF (100 mg) from each species was accurately weighed in a volumetric flask (10 mL), and the volume was adjusted with methanol. Thereafter, a 1-mL aliquot was transferred to a 10-mL volumetric flask, and the volume was completed with purified water.

Analytical conditions

Chromatographic analyses were carried out using a HP1100 system (Agilent, Santa Clara, USA) coupled to a quaternary pump, an auto sampler, and a programmable ultraviolet photodiode array detector (UV/DAD). A HPChemStation for LC3D systems software (Rev. B.02.01-SR2[260] 2001-2006) was used to assess the data. A reversed-phase pre-column C18 (XDB Zorbax®, 4 × 4 mm I.D.; 5 µm, Agilent, Santa Clara, USA) was attached to a C18 column (LiChrospher®100, 250 × 4 mm I.D.; 5 µm, Merck, Darmstadt, Germany) at 40 °C. After filtration with 0.45-µm PTFE membrane, 10 mL of the standard and sample solutions were automatically injected into the system at a flow rate of 1 mL/min and detected at a wavelength of 210 nm. UV/DAD spectral lines were recorded for peak purity and identification in the wavelength range of 190–400 nm, and substance elutions were confirmed by co-elution of the standards. The degassed mobile phase consisted of aqueous 0.1% phosphoric acid (solution A) and 0.1% phosphoric acid in acetonitrile (solution B). The linear gradient used was from A-B (95:5 [v/v]) to A-B (60:40 [v/v]) in 60 min, which was followed by cleaning and reconditioning of the column in 15 min. The tannin retention time (t_R), retention factor (k), resolution (R), column efficiency (N), and tailing factor (T) were assessed.

Standard solutions

Circa 5 mg, accurately weighed for each of the reference substances (i.e., GA, C, Q, EGC, P, GC, and EGCG) were individually transferred to 5-mL volumetric flasks. Methanol was added to the standards to achieve a volume of 5 mL.

Validation

National guidelines for the validation of analytical and bioanalytical methods (RE 899/2003), and international ICH guideline requirements for analytical methods were adhered to for evaluation of the method performance (Agência, 2003; International, 1996).

Selectivity

The purity of the elution peaks for the reference substances and extracts was assessed by UV/DAD spectra plus the chromatographic system suitability parameters k′, R, N, and T.

Linearity

A standard stock solution containing 1 mg/mL each of GC prepared in 10% (v/v) methanol. Aliquots of this solution (3, 9, 15, 21, 27, and 33 mL, in triplicate) were injected. Separate calibration curves were evaluated by plotting their injected concentration (3–33 µg/mL equivalent to 30–300 ng) versus peak areas for both GC and EGCG. Data were submitted for regression analysis and compared by ANOVA and Tukey tests.

Precision

The intra-day precision (repeatability) was evaluated at 100% of the working concentration (15 µg/mL, n=6). Similarly, the inter-day precision (n=12) was evaluated for 2 different days by different analysts. The concentration of GC or EGCG in the organic fraction of the EE was determined, and the relative standard deviation (RSD) was calculated.

Accuracy

Stock solutions (1 mg/mL) of each reference (i.e., for EGCG and GC) were added to the dry extracts before purification to obtain 3 different working concentrations (60%, 100%, and 140%). Accuracy was also tested after purification at the same concentration levels by the addition of adequate volumes (20 µg/mL) of each reference solution to the OF. Samples were prepared in triplicate, and the recovery percentage for each level was determined using the standard addition method.

Detection and quantitation limits

The limit of detection (LOD) and limit of quantitation (LOQ) for GC and EGCG were estimated on the basis of the ratio of standard deviation of the y-intercept (s_a) of the regression lines and the slope (b) of the calibration curve by using the equations LOD = $3.3s_a/b$ and LOQ = $10s_a/b$, respectively.

Robustness

Six sample solutions at a working concentration of 100% were prepared and analyzed under the established conditions, as well as under intentionally varied conditions of analytical parameters (e.g., chromatographic column supplier and gradient slope). The GC and EGCG marker contents and RSD were determined for each condition. Data were submitted for statistical analysis (ANOVA, a = 0.05, Tukey test).

RESULTS AND DISCUSSION

Development and optimization of the chromatographic method

The EE samples of *S. adstringens* were initially tested along different linear gradient conditions (results not shown), considering that baseline drifting was not desirable because it can cause a decrease in analytical accuracy and precision. The tested solvent systems (water:ACN with either acetic or phosphoric acid in both solvents and water:ACN with no added acid) indicated that a decrease in the mobile phase pH clearly affected peak shape in a desired fashion, leading to an increase in peak symmetry. Thus, phosphoric acid in the mobile phase was more effective than acetic acid. The effect of temperature on analyte retention and selectivity was tested by setting the column temperature to 25 °C, 30 °C, or 40 °C, and a better separation was obtained at 40 °C. The wavelength at 210 nm showed the greatest signal intensity for tannin peak evaluation. A small value of the slope of the linear gradient A–B (95:5→60:40 [v/v]) led to a complete resolution of the main peaks with a low baseline drift in a total run time of 60 min.

Sample purification and elution pattern

The purification step, performed as a sample cleanup to eliminate the cluster of polar compounds in the matrix, was critical and considered necessary for vegetal complex matrices. The EE of *S. adstringens* was purified by liquid-liquid extraction by using an adequate, previously developed solvent system (Nascimento *et al.*, 2008). The yields were 36% and 58% [w/w] for OF (Figure 1a) and AF (Figure 1b), respectively.

Five (GA, C, GC, EGC, and EGCG) of the 7 reference substances were selected to assess EE eluate of the 2 barbatimão species stem bark samples. The presence of the reference substances in the EE was also confirmed by retention time (t_R) co-elution experiments and UV/DAD. The chromatographic results showed a profile with the identification of 3 main peaks (GA, GC, and EGCG) with good resolution. Two other identified peaks eluted together with R<1.5 (EGC, t_R 12.33 and C t_R 14.22). GA and C were present in very low concentrations in the extract. EGC did not yield adequate resolution (R 1.124) since it eluted with adjacent peaks. There was a major unidentified peak (t_R 14.56 min), which co-eluted (R 1.311) with C in the chromatogram. This major peak was assumed to

be a substance of the proanthocyanidin group because of the presented UV/DAD spectrum profile. It has not been identified or characterized in this study, as such an investigation is beyond the scope of this work. Although this substance constituted a major peak and could be selected as a good marker, it is neither well resolved nor commercially available. Thus, if chosen for use as a marker, it would prove to be impractical for use in routine analyses. It requires more investigation for a better-fit characterization in the near future. Quercetin and procyanidin B2 were not detected under the described conditions.

The elution patterns of GC (t_R 7.561 min, R 1.514, k′ 2.70) and EGCG (t_R 19.580 min, R 1.964, k 8.23) were more than satisfactory. Therefore, they were selected as markers for quantitation. It is noteworthy to report that the selected markers, GC and EGCG, remained in the OF (Figure 1a) rather than in the AF (Figure 1b).

Validation

The spectral purity of the proanthocyanidins derivative peaks was assessed using the UV/DAD system. The peak purity of the standard elution was higher than 98.5% for all phenolic derivatives, thus confirming that the analyte peak is attributed to only one component. The resolution (R) obtained was greater than 7 for all reference peaks, representing adequate separation of the components in the standard chromatogram.

In the EE sample of *S. adstringens*, the resolution obtained for GC or EGCG peaks was greater than 1.5, a value that indicates good separation. Therefore, the results attest that the method is selective for the determination of tannins in *S. adstringens*.

Linear regression analyses were performed by the least squares method by using external standards. The calibration curve and parameters correlation coefficient, slope, intercept, standard error of the slope, standard error of the intercept, and relative standard deviation (*r*; *b*; *a*; *sb*; *sa,* RSD) at 95% confidence level interval for each marker are shown in Table I. Equivalent concentration and mass ranges were 3–33 µg/mL or 30–330 ng, respectively.

A linear relationship (*r*>0.99) was found for the calibration curve plots of GC or EGCG concentration *versus* peak areas in the assayed range. Graphical

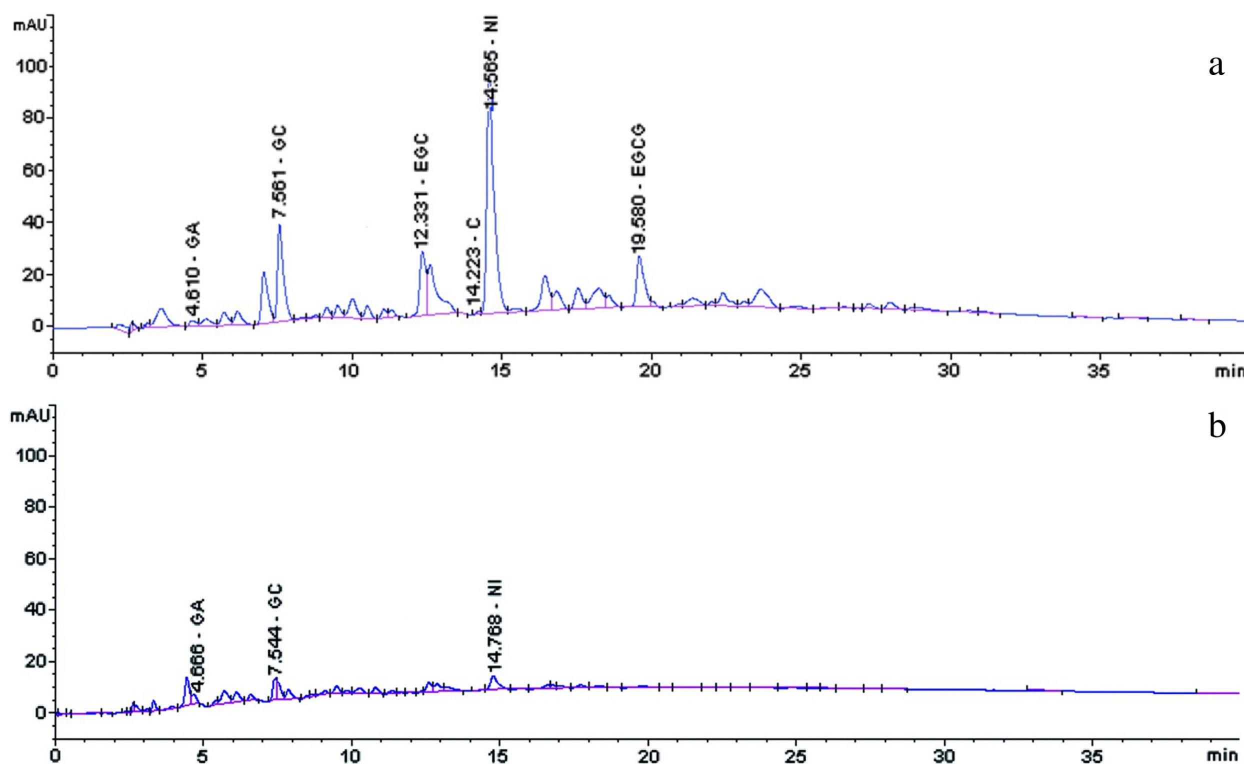

FIGURE 1 Chromatograms of *S. adstringens* stem bark **(a)** organic fraction (OF) containing the majority of the identified peaks (GA, gallic acid; GC, gallocatechin; EGC, epigallocatechin; C, catechin; EGCG, epigallocatechin gallate) and a non-identified (NI) substance peak; **(b)** aqueous fraction (AF) shows minority of eluted peaks from EE of *S. adstringens*. Conditions: pre-column C18 (XDB Zorbax®, 4 × 4 mm I.D.; 5 µm), attached to a C18 column (LiChrospher®100, 250 × 4 mm I.D.; 5 µm); linear gradient elution, A–B (95:5% [v/v]) to A–B (60:40% [v/v]) in 60 min with 0.1% phosphoric acid (A) and 0.1% phosphoric acid in acetonitrile (B); 1 mL/min; 4 °C; 10 mL; λ = 210 nm.

TABLE I - Linearity results for gallocatechin (GC) and epigallocatechin gallate (EGCG) calibration curves in the concentration range 3-33 mg/mL by RP-HPLC-UV gradient elution (n=18). Conditions see Figure 1

Statistical parameters	GC	EGCG
Correlation coefficient (r)	0.9998	0.9986
Slope (b) ± s_b	16.03±0.07	10.94±0.14
Intercept (a) ± s_a	-2.78±14.33	-23.14±29.19
95% CI[a] of intercept	-33.16 to 27.60	-85.02 to 38.74
RSD (%)	1.04	3.15

[a] confidence interval

examination of the data and the residuals showed linearity and homocedasticity, respectively. Table II shows the results for the application of this method for identifying each marker (GC, 13.15±0.23 µg/mL and EGCG, 13.40 ± 0.15 µg/mL) in the EE of *S. adstringens*. Intra-day precision (n=6) RSD results ranged from 1.16% to 1.72%, and the inter-day (n=12) RSD values ranged from 1.74% to 5.01% for *S. adstringens* markers when considering different days and analysts. The acceptance criteria for precision were highly dependent on the type of matrix and the amount of active compounds assessed in the analysis. An RSD value ≤5% is usually considered acceptable for the results of drug determination in dosage forms (Agência, 2003). However, for biological and vegetal complex matrices, higher values (e.g., up to 15%) are considered acceptable (Grdinic, Vukovic, 2004). Hence, the method shows satisfactory precision for intra-assay (repeatability) and inter-assay (intermediate precision) assessments.

The average recovery (n=3) of the standard addition before liquid-liquid extraction (purification) for the 3 concentration levels (60%, 100%, and 140%) was 78.58% (RSD 18.75%) for GC, and 101.84% (RSD 2.32%) for EGCG. Higher RSD values for GC indicate the importance of the purification step in determining recovery rates. Additionally, the average recovery (n=3) of the standards placed directly onto OF (after purification) was 76.50% (RSD 4.53%) for GC and 104.98% (RSD 3.21%) for EGCG (Table III). Therefore, GC seems to be more labile than the more stable ester form (i.e., EGCG).

Lower GC recoveries with greater RSD values were observed. This may be indicative of degradation throughout the analytical procedure. Certain factors, including light exposition and temperature, may promote degradation, which has been previously reported for flavan-3-ols (Friedman, Jurgens, 2000; Chen *et al.*, 2001; Wang, Zhou, Wen, 2006). Moreover, 0.2% ascorbic acid and 0.02% EDTA were added to both the standard and sample solutions in order to prevent secondary metabolite degradation in the determination of antioxidants in botanicals, supplements, and beverages by RP-HPLC-UV/DAD gradient elution with detection at λ 254 nm (Ulluci *et al.*, 2012). Desirable analyte recovery rates near 100% are probably achievable with the addition of ascorbic acid and EDTA, but lower values are acceptable since the results provide linearity, precision, and selectivity (International, 1996; Agência, 2003). Moreover, lower recovery rates are acceptable considering the complexity of the matrix and lability of the analyte (Dhooghe *et al.*, 2008; Serpe *et al.*, 2010). Therefore, precision with an RSD value lower than 4.6% and recovery higher than 76% for GC indicate that the developed method provided precise and acceptably accurate results in the EE of *S. adstringens* (Agência, 2003).

The developed method is considered precise and accurate for EGCG because recovery rates obtained were

TABLE II - Summary results for intra-day (n=6) and inter-day (n=12) precision for gallocatechin (GC) and epigallocatechin gallate (EGCG) determination in *S. adstringens* organic fraction (OF)

Precision	Analyst	Day	Concentration, µg/mL [*x*±*s* [a] (RSD%)]	
			GC	EGCG
Intra-day	1	1	13.15±0.23 (1.72)	13.40±0.15 (1.16)
Inter-day	1 and 1	1-2	13.06±0.23 (1.74)	13.30±0.35 (2.60)
	1 and 2	1-2	13.76±0.69 (5.01)	13.18±0.36 (2.76)

[a] average±standard deviation

TABLE III - Precision and recovery results (n=3) of gallocatechin (GC) and epigallocatechin gallate (EGCG) before and after purification of organic fraction from *S. adstringens* stem bark EE

Marker	Level (%) [a]	%Recovery [b] [$x \pm s^d$ (%RSD)]	%Recovery [c] [$x \pm s^d$ (%RSD)]
GC	140	70.06±2.10 (3.00)	75.81±0.43 (0.57)
	100	67.94±4.80 (7.07)	74.73±4.77 (6.38)
	60	97.75±3.31 (3.39)	78.95±3.26 (4.13)
Average		78.58±14.73 (18.75)	76.50±3.46 (4.53)
EGCG	140	99.34±2.10 (2.11)	103.24±0.72 (0.70)
	100	102.79±1.15 (1.12)	103.91±1.09 (1.05)
	60	103.39±1.53 (1.48)	107.78±5.06 (4.70)
Average		101.84±2.37 (2.32)	104.98±3.37 (3.21)

[a] theoretical concentration level, [b] before purification, [c] after purification; [d] average±standard deviation

close to 100% and RSD average values were lower than 3.5% in both experiments, before and after the purification step (Table III).

The estimated LOD obtained for GC and EGCG were 0.29 and 0.88 µg/mL, respectively, whereas the limits of quantitation were 0.89 and 2.67 µg/mL for GC and EGCG, respectively. The low values of LODs and LOQs demonstrate the high sensitivity of the method presented in this study.

Statistical analysis showed no significant differences between the intentional variations in analytical conditions relative to the established conditions. The *p*-values were greater than the critical value (p>0.05), thus indicating no statistical significant difference. Hence, the method was robust for different column suppliers (GC, p=0.9933; EGCG, p=0.1037), as well as for different values of the gradient slope applied by alterations in the mobile phase composition, for instance, 4.8% to 41.0% ACN (GC, p = 0.5188; EGCG, p = 0.3220) and 5.2% to 39.0% ACN (GC, p=0.9821; EGCG, p=0.1525).

S. obovatum method application

The OF of the EE of *S. obovatum* was also analyzed. The average tannin content was 12.2 µg/mL (equivalent to 1.22% w/w) and 14.2 µg/mL (equivalent to 1.42% w/w) for GC and EGCG, respectively. The values of the quantitative results were similar to those for *S. adstringens* (Table II). They also show that such markers are present in both species (Figure 2) and are quantified with minimal variation between species. Therefore, the method is also adequate for tannin determination in *S. obovatum*.

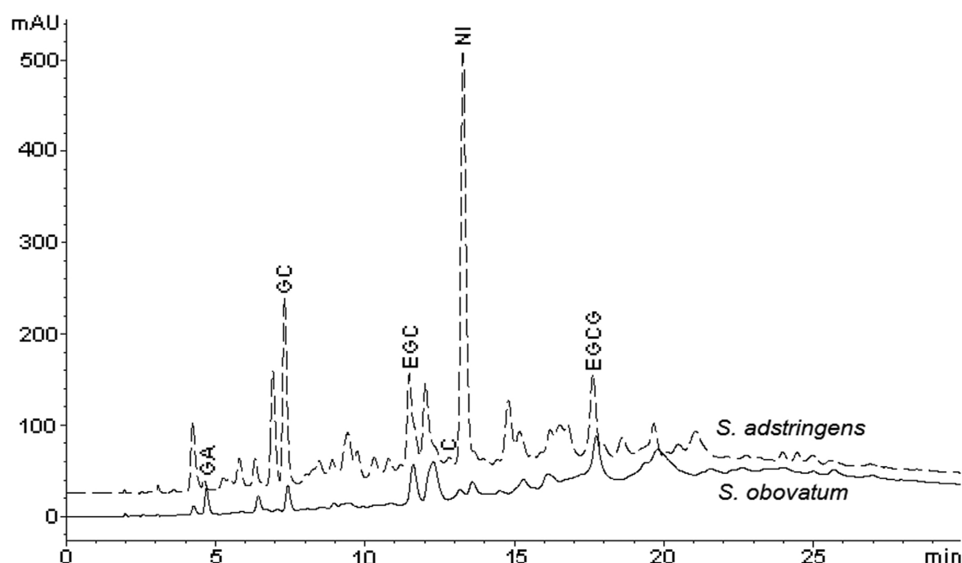

FIGURE 2 Overlaid chromatograms of organic fractions (OF) showing the presence of proanthocyanidins (GA, gallic acid; GC, gallocatechin; EGC, epigallocatechin; C, catechin; EGCG, epigallocatechin gallate) and a non-identified (NI) substance peak from the stem bark EE of *S. adstringens* and *S. obovatum*. Conditions: see Figure 1.

CONCLUSION

In this study, we described the development of an extractive analytical method for the determination of tannins in plant-derived products. The data showed that it is possible to eliminate major interferences with a single liquid-liquid purification step only by using an efficient solvent system for sample cleanup, as well as for the identification and quantification of specific tannin markers. Interestingly, the described chromatographic method allows for the analysis of the characteristic tannins (monomer markers) instead of total phenolics, as reported by compendial methods.

The developed, extractive analytical method employing RP-HPLC-UV/DAD (l=210 nm) was shown to be specific, linear, precise, accurate, and robust for GC and EGCG determination in barbatimão (*S. adstringens*) stem bark extracts, as well as for tannin determination (e.g., in *S. obovatum*).

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REFERENCES

AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA (Brasil). Resolução n.899, de 29 de maio de 2003. Guia para validação de métodos analíticos e bioanalíticos. *Diário Oficial da União*. Poder Executivo, Brasília DF, 02 de Junho de 2003. Available at: <www.anvisa.gov.br/legis/resol/2003/re/899_03re.htm>. Accessed on: March 2012.

AUDI, E.A.; TOLEDO, D.P.; PERES, P.G.; KIMURA, E.; PEREIRA, W.K.V.; MELLO, J.C.P.; NAKAMURA, C.V.; ALVES PRADO, W.; CUMAN, R.K.N.; BERSANI-AMADO, C.A. Gastric antiulcerogenic effects of *Stryphnodendron adstringens* in rats. *Phytother. Res.,* v.13, n.3, p.264-266, 1999.

BAURIN, N.; ARNOULT, E.; SCIOR, T.; DO, Q.T.; BERNARD, P. Preliminary screening of some tropical plants for anti-tyrosinase activity. *J. Ethnopharmacol.,* v.82, n.2-3, p.155-158, 2002.

BEZERRA, J.C.B.; SILVA, I.A.; FERREIRA, H.D.; FERRI, P.H.; SANTOS, S.C. Molluscicidal activity against *Biomphalaria glabrata* of brazilian cerrado medicinal plants. *Fitoterapia,* v.73, n.5, p.428-430, 2002.

BRANDÃO, M.G.L.; ZANETTI, N.N.S.; OLIVEIRA, P.; GRAEL, C.F.F.; SANTOS, A.C.P.; MONTE-MÓR, R.L.M. Brazilian medicinal plants described by 19th century European naturalists and in the official pharmacopoeia. *J. Ethnopharmacol.,* v.120, n.2, p.141-148, 2008.

BRITISH PHARMACOPOEIA. London: Her Majesty's Stationery Office, 2011. p.A297.

CASTILHO, R.O.; NASCIMENTO, A.M.; VIANNA-SOARES, C.D.; GUEDES, P.T. Extractive analytical method for tannins determination on raw materials and plant products. WO. Pat.2011017792, A1. Pat.20110217, CAN. Pat.154:233148, AN Pat.2011:205998, 2011. 37 p.

CHEN, Z.; ZHU, Q.Y.; TSANG, D.; HUANG, Y.Z. Degradation of green tea catechins in tea drinks. *J. Agric. Food Chem.,* v.49, n.1, p.477-482, 2001.

COSTA, M.A.; ISHIDA, K.; KAPLUM, V.; KOSLYK, E.D.A.; MELLO, J.C.P.; UEDA-NAKAMURA, T.; FILHO, B.P.D.; NAKAMURA, C.V. Safety evaluation of proanthocyanidin polymer-rich fraction obtained from stem bark of *Stryphnodendron adstringens* (barbatimão) for use as a pharmacological agent. *Regul. Toxicol. Pharmacol.,* v.58, n.2, p.330-335, 2010.

DHOOGHE, L.; MESIA, K.; KOHTALA, E.; TONA, L.; PIETERS, L.; VLIETINCK, A.J.; APERS, A. Development and validation of an HPLC-method for the determination of alkaloids in the stem bark extract of *Nauclea pobeguinii*. *Talanta,* v.76, n.2, p.462-468, 2008.

EUROPEAN PHARMACOPOEIA. 7.ed. Strasbourg: Directorate of Quality of Medicines of the Council of Europe, 2011. 243 p.

FARMACOPEIA BRASILEIRA. 5.ed. Brasília: ANVISA, 2010. Available at: <http://www.anvisa.gov.br/hotsite/cd_farmacopeia/index.htm.>. Accessed on: August 2012.

FORERO, E. Studies in *Stryphnodendron* (Leguminosae: Mimosoideae) including two new taxa. *Brittonia,* v.24, n.2, p.143-147, 1972.

FRIEDMAN, M.; JURGENS, H.S. Effect of pH on the stability of plant phenolic compounds. *J. Agric. Food Chem.*, v.48, n.6, p.2101-2110, 2000.

GRDINIC, V.; VUKOVIC, J. Prevalidation in pharmaceutical analysis. Part I. Fundamentals and critical discussion. *J. Pharm. Biomed. Anal.*, v.35, n.3, p.489-512, 2004.

HERZOG-SOARES, J.D.; ALVES, R.K.; ISAC, E.; BEZERRA, J.C.B.; GOMES, M.H.; SANTOS, S.C.; FERRI, P.H. Atividade tripanocida *in vivo* de *Stryphnodendron adstringens* (barbatimão verdadeiro) e *Caryocar brasiliensis* (pequi). *Rev. Bras. Farmacogn.*, v.12, suppl.1, p.1-2, 2002.

INTERNATIONAL CONFERENCE ON HARMONISATION. Validation of analytical procedures: text and methodology Q2B (R1). ICH, 2005. Available at: <http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf>. Accessed on: March 2012.

ISHIDA, K.; MELLO, J.C.P.; CORTEZ, D.A.G.; DIAS-FILHO, B.P.; UEDA-NAKAMURA, T.; NAKAMURA, C.V. Influence of tannins from *Stryphnodendron adstringens* on growth and virulence factors of Candida albicans. *J. Antimicrob. Chemother.*, v.58, n.5, p.942-949, 2006.

ISLER, A.C.; LOPES, G.C.; CARDOSO, M.L.C.; MELLO, J.C.P.; MARQUES, L.C. Development and validation of a LC-method for the determination of phenols in a pharmaceutical formulation containing extracts from *Stryphnodendron adstringens*. *Quím. Nova*, v.33, n.5, p.1126-1129, 2010.

LOPES, G.C.; BLAINSKI, A.; SANTOS, P.V.P.; DICIAULA, M.C.; MELLO, J.C.P. Development and validation of an HPLC method for the determination of epicatechin in *Maytenus ilicifolia* (Schrad.) Planch., Celastraceae. *Rev. Bras. Farmacogn.*, v.20, n.5, p.789-795, 2010.

LOPES, G.C.; MACHADO, F.A.V.; TOLEDO, C.E.M.; SAKURAGUI, C.M.; MELLO, J.C.P. Chemotaxonomic significance of 5-deoxyproanthocyanidins in *Stryphnodendron* species. *Biochem. Syst. Ecol.*, v.36, n.12, p.925-931, 2008.

LOPES, G.C.; SANCHES, A.C.C.; NAKAMURA, C.V.; DIAS FILHO, B.P.; HERNANDES, L.; MELLO, J.C.P. Influence of extracts of *Stryphnodendron polyphyllum* Mart. and *Stryphnodendron obovatum* Benth. on the cicatrisation of cutaneous wounds in rats. *J. Ethnopharmacol.,* v.99, n.2-3, p.265-272, 2005.

LOPES, G.C.; SANCHES, A.C.C.; TOLEDO, C.E.M.; ISLER, A.C.; MELLO, J.C.P. Determinação quantitativa de taninos em três espécies de *Stryphnodendron* por cromatografia líquida de alta eficiência. *Braz. J. Pharm. Sci.,* v.45, n.1, p.135-143, 2009.

LUIZE, P.S.; TIUMAN, T.S.; MORELLO, L.G.; MAZA, P.K.; UEDA-NAKAMURA, T.; DIAS-FILHO, B.P.; CORTEZ, D.A.G.; MELLO, J.C.P.; NAKAMURA, C.V. Effects of medicinal extracts on growth of *Leishmania* (L.) *amazonensis* and *Trypanosoma cruzi*. *Braz. J. Pharm. Sci.,* v.41, n.1, p.1-10, 2005.

MELLO, J.C.P.; PETEREIT, F.; NAHRSTEDT, A. Flavan-3-ols and prodelphinidins from *Stryphnodendron adstringens*. *Phytochemistry,* v.41, n.3, p.807-813, 1996a.

MELLO, J.C.P.; PETEREIT, F.; NAHRSTEDT, A. Prorobinetinidins from *Stryphnodendron adstringens*. *Phytochemistry*, v.42, n.3, p.857-862, 1996b.

MELLO, J.C.P.; PETEREIT, F.; NAHRSTEDT, A. A dimeric proanthocyanidin from *Stryphnodendron adstringens*. *Phytochemistry*, v.51, n.8, p.1105-1107, 1999.

MISSOURI BOTANICAL GARDEN. *Nomenclatural database*. Available at: <http://mobot.mobot.org/W3T/Search/vast.html.>. Accessed on: December 2009.

NASCIMENTO, A.M.; CASTILHO, R.O.; BRANDÃO, M.G.L.; VIANNA-SOARES, C.D. *Desenvolvimento de método por CLAE para determinação de taninos condensados de Stryphnodendron adstringens*. XXXI Encontro Anual da Sociedade Brasileira de Química: Águas de Lindóia, 2008. Available at: <http://sec.sbq.org.br/cdrom/31ra/resumos/T1757-2.pdf.>. Accessed on: March 2012.

REBECCA, M.A.; ISHII-IWAMOTO, E.L.; GRESPAN, R.; CUMAN, R.K.N.; CAPARROZ-ASSEF, S.M.; MELLO, J.C.P.; BERSANI-AMADO, C.A. Toxicological studies on *Stryphnodendron adstringens*. *J. Ethnopharmacol.,* v.83, n.1-2, p.101-104, 2002.

REBECCA, M.A.; ISHII-IWAMOTO, E.L.; KELMER-BRACHT, A.M.; CAPARROZ-ASSEF, S.M.; CUMAN, R.K.N.; PAGADIGORRIA, C.L.S.; MELLO, J.C.P.; BRACHT, A.; BERSANI-AMADO, C.A. Effect of *Stryphnodendron adstringens* (barbatimão) on energy metabolism in the rat liver. *Toxicol. Lett.,* v.143, n.9, p.55-63, 2003.

SCHOFIELD, P.; MBUGUA, D.M.; PELL, A.N. Analysis of condensed tannins: a review. *Anim. Feed Sci. Technol.,* v.91, n.1-2, p.21-40, 2001.

SERPE, F.P.; ESPOSITO, M.; GALLO, P.; SERPE, L. Optimisation and validation of an HPLC method for determination of polycyclic aromatic hydrocarbons (PAHs) in mussels. *Food Chem.,* v.122, n.3, p.920-925, 2010.

SOUSA, N.C.; CARVALHO, S.; SPANÓ, M.A.; GRAF, U. Absence of genotoxicity of a phytotherapeutic extract from *Stryphnodendron adstringens* (Mart.) Coville in somatic and germ cells of *Drosophila melanogaster*. *Environ. Mol. Mutag.,* v.41, n.4, p.293-299, 2003.

SOUZA, T.M.; SEVERI, J.A.; SILVA, V.Y.A.; SANTOS, E.; PIETRO, R.C.L.R. Bioprospection of antioxidant and antimicrobial activities in the bark of *Stryphnodendron adstringens* (Mart.) Coville (Leguminosae–Mimosoideae). *Rev. Ciênc. Farm. Básica Apl*., v.28, n.2, p.221-226, 2007.

ULLUCI, P.A.; BAILEY, B.A.; THOMAS, D.H.; ACWORTH, I.N. The spectro-electro array: a novel platform for the measurement of secondary metabolites in botanicals, supplements, and beverages. *LC-GC North America*, v.30. n.6, p.492-503, 2012.

WANG, R.; ZHOU, W.; WEN, R.H. Kinetic study of the thermal stability of tea catechins in aqueous systems using a microwave reactor. *J. Agric. Food Chem.,* v.54, n.16, p.5924-5932.
2006.

WATERHOUSE, A.L.; PRICE, S.F.; MACCORD, J.D. Reversed-phase high-performance liquid chromatography methods for analysis of wine polyphenols. *Methods Enzymol.,* v.299, p.113-121, 1999.

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