Talanta 88 (2012) 502–506

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development of an analytical method using reversed-phase HPLC-PDA for a semipurified extract of *Paullinia cupana* var. *sorbilis* (guaraná)

Traudi Klein, Renata Longhini, João Carlos Palazzo de Mello*

Programa de Pós-Graduação em Ciências Farmacêuticas, Departamento de Farmácia, Universidade Estadual de Maringá, Av. Colombo, 5790, Maringá, PR, BR-87020-900, Brazil

ARTICLE INFO

Article history:
Received 21 June 2011
Received in revised form 4 November 2011
Accepted 7 November 2011
Available online 10 November 2011

Keywords:
Paullinia cupana
HPLC-PDA
Analytical validation
Polyphenols

ABSTRACT

The Neotropical plant 'guaraná' has been widely used in medicine, cosmetics, and industry because of its versatile biological activities. These effects are mainly attributed to the presence of polyphenols. An efficient, precise, and reliable method was developed for quantification of the polyphenols catechin and epicatechin in guaraná extract solution, using HPLC-PDA detection. The ideal conditions for the analysis of a semipurified extract of guaraná (EPA), using solutions of 0.05% TFA–water (phase A) and 0.05% TFA in acetonitrile:methanol (75:25, v v^{-1}) (phase B) as mobile phases were established. Gradient reversed-phase chromatography was performed using a guard cartridge (C18, 4.6 mm × 20 mm, 4 μm) and column (C18, 250 mm × 4.6 mm, 4 μm), flow of 0.5 mL min^{-1} and detection at 280 nm. The main validation parameters of the method were also determined. The method was linear over a range of 18.75–300 μg mL^{-1} for catechin and epicatechin, with detection limits of 0.70 and 0.88 μg mL^{-1} and quantification limits of 2.13 and 2.67 μg mL^{-1}, respectively. The method also showed consistent mean recoveries of 91.3 ± 3.8%, 2.14 RSD and 93.4 ± 3.1, 2.74 RSD of catechin and epicatechin respectively. The relative standard deviations were relatively low: intra-day (0.72% and 0.66% for catechin and epicatechin, respectively) and inter-day (0.93% and 0.75% for catechin and epicatechin, respectively). The semipurified extract showed catechin, epicatechin, and caffeine contents of 180.75, 278.87, and 300.87 μg mg^{-1}, respectively. The results demonstrated the efficiency, precision, accuracy, and robustness of the proposed method. The solutions remained stable for a sufficient time (one week) to complete the analytical process.

1. Introduction

The guaraná plant (*Paullinia cupana* var. *sorbilis* (Mart.) Ducke, Sapindaceae) is widely distributed in the Amazon region and also grows in northeastern Brazil, including the state of Bahia. Its seeds, used in popular medicine, contain large amounts of methylxanthines including caffeine, theophylin and theobromin, saponins, and polyphenols, especially tannins [1,2]. Guaraná extract is used as a stimulant of the central nervous system, in cases of physical and mental stress, and as an antidiarrheal, diuretic, and antineuralgic [1,3]. The antidepressive effect has been reported to be comparable to that of the tricyclic antidepressant imipramine, and with a beneficial effect on cognition, without altering locomotor activity [4–8]. Guaraná extract also shows low toxicity, with antioxidant and antiamnesiac effects [5,6,9–11], potential effect as a chemoprophylactic in carcinogenesis [9], and potential antibactericidal activity against *Streptococcus mutans*, a cause of bacterial dental plaque [3].

Chemical assay of a semipurified fraction of guaraná (EPA) showed the presence of caffeine, epicatechin, catechin, *ent*-epicatechin and procyanidins B1–B4, A2 and C1 [2,3]. This fraction showed an antidepressant effect on animals that received chronic treatment. This activity could not be related to the methylxanthins present, because when caffeine is tested in isolation, the effects differ from those of the EPA fraction. This suggests that the activity results from the presence of other constituents, and the condensed tannins may be the responsible agents; condensed tannins can cross the blood–brain barrier and act on the central nervous system [2,5,6,12]. Previous studies found that the EPA fraction of guaraná caused no toxicity in rats at the smallest dose evaluated (30 mg kg^{-1}) [13].

The potential for using guaraná in a wide range of medicinal applications justifies the interest in the quality control and standardization of its preparations. Capillary electrophoresis [14,15], mass spectrometry, and high-performance liquid chromatography (HPLC) [16,17] have been used to analyze the polyphenols, but the analytical procedures were complex, with long analysis times and dependent on the use of several polyphenols, analytical standards, and expensive reagents. Some analytical methods have employed HPLC to analyze *P. cupana*, but most of them describe the separation of methylxanthines [14,18–20]. Polyphenols, mainly tannins, have been isolated from other plants, but the method is often time-consuming (30–36 min [21]; 50 min [22]; and 55–106 min [23]).

* Corresponding author. Tel.: +55 44 3011 4816; fax: +55 44 3011 5050.
E-mail address: mello@uem.br (J.C.P. de Mello).

0039-9140/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2011.11.023

The aim of the present study was to develop and validate a reversed-phase HPLC-photodiode array (PDA) method for the separation and quantification of the catechin and epicatechin constituents in semipurified extract of guaraná. The main validation parameters of the method were also determined.

2. Experimental

2.1. Chemicals and reagents

Methanol and acetonitrile (J.T. Baker; HPLC grade), water filtered through a Milli-Q apparatus (Millipore), and trifluoroacetic acid (TFA) (J.T. Baker) were used as the mobile phase. Analytical-grade standards of catechin, epicatechin, and caffeine (Sigma) were used as external standards. Procyanidins B1 and B2 were isolated and identified by Ushirobira et al. [2] and Yamaguti-Sasaki et al. [3]. Acetone and ethyl acetate (Merck; analytical grade) were also used.

2.2. Apparatus

High performance liquid chromatography analyses were performed using a Thermo HPLC equipped with pumps and an integral degasser (Finnigan Surveyor LC Pump Plus), PDA spectrophotometric detector module (Finnigan Surveyor PDA Plus Detector), controller software (Chromquest) and autosampler (Finnigan Surveyor Autosampler Plus) equipped with a 10 μL loop and 10 μL injection. Chromatographic separation was accomplished using a Phenomenex® Synergi POLAR–RP 80A stainless-steel analytical column (250 mm × 4.6 mm, 4 μm) and a Phenomenex® C18 guard cartridge system (4 μm, 4.6 mm × 20 mm). The mobile phase used was a gradient system of 0.05% TFA–water (phase A) and 0.05% TFA- acetonitrile:methanol (75:25, v v^{-1}) (phase B), previously degassed using an ultrasonic bath. The gradient system was established and demonstrated in Section 3. Gradient separation was performed at a flow rate of 0.5 mL min^{-1}. Another HPLC analysis was carried out using a different column, a Waters X Bridge™ C18 (100 mm × 4.6 mm, 5 μm) and a Waters X Bridge™ C18 guard cartridge system (5 μm, 4.6 mm × 20 mm).

For the interlaboratory HPLC assay, a different apparatus was used, a Gilson HPLC system consisting of a Model 321 pump, a Model 156 variable-wavelength UV/Vis detector, a Rheodyne manual injection valve with a 10 μL loop, Model 184 degasser, a Model 831 thermostatted column compartment, and Unipoint LC system software.

2.3. Preparation of the EPA extractive solution

Guaraná samples obtained in the municipality of Alta Floresta, state of Mato Grosso, Brazil, were used to prepare the acetone:water (70:30) extractive solution (ES), by turbo extraction (Ultra-Turrax UTC115KT, IKA Works, Wilmington, NC, USA). After the organic solvent was removed, the remaining solid material was lyophilized (EBPC; patent pending PI0006638-9). The EBPC (crude extract) was partitioned with ethyl acetate, resulting in an ethyl-acetate fraction (EPA) [4,13]. The EPA was extracted with solid-phase extraction (SPE). A 2.00 mg portion of EPA was diluted in 1 mL of 20% methanol and was passed through the SPE cartridge and diluted in 25 mL of 20% methanol. A 10 μL aliquot was analyzed by HPLC.

2.4. Method validation

For validation of the analytical method, the guidelines established by the ICH (International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use) and by Brazilian regulation RE 899/2003 of the National Health Surveillance Agency (ANVISA) were employed [24,25].

2.4.1. Linearity

Linearity was determined by the calibration curves obtained from the HPLC analyses of the standard solutions of catechin and epicatechin. The range (interval between the upper and lower concentrations of analyte in the sample) of the appropriate amount of samples was determined. The slope and other statistics of the calibration curves were calculated by linear regression and analysis of variance (ANOVA).

The catechin and epicatechin standards were dissolved in 20% methanol to give concentrations of 18.75, 37.5, 75.0, 150, and 300 μg mL^{-1}. The solutions were filtered through an FHLP01300 20 μm membrane filter (Millipore). Evaluation of each point was conducted in five replicates, and the calibration curve was fitted by linear regression.

2.4.2. Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation (SD) and the slope (S) of the calibration curve based on Eqs. (1) and (2).

$$\mathrm{LOD} = \frac{3.3 \times \mathrm{SD}}{S} \quad (1)$$

$$\mathrm{LOQ}\frac{10 \times \mathrm{SD}}{S} \quad (2)$$

2.4.3. Precision

The precision of the method was determined following ICH guidelines. Precision was evaluated at three levels: repeatability, intermediate precision, and reproducibility. The standard deviation (SD) and relative standard deviation (RSD) of six injections at 100% of the test concentration were evaluated and analyzed intra-day and inter-day, and with different analysts and different apparatus.

2.4.4. Accuracy

The accuracy was determined by recovery analyses, adding measured amounts of catechin (100, 50, and 25 μg mL^{-1}) and epicatechin (100, 50, and 25 μg mL^{-1}) to EPA extractive solution samples. The recovery experiments were performed in triplicate. The recovery data were determined by dividing the value obtained for the sample prepared with the added standard, by the amount added, and then multiplying by 100% [24].

2.4.5. Robustness

The robustness was determined for variations in flow rates, for 0.495 mL min^{-1} and 0.505 mL min^{-1}. The Tukey test of ANOVA was performed to evaluate whether the flow variations altered the results of the HPLC analysis.

2.4.6. Stability

The stability of the EPA extractive solutions was determined over a period of four weeks. A 2.00 mg portion of EPA was diluted in 1 mL of 20% methanol. This solution was passed through the SPE cartridge and diluted in 25 mL of 20% methanol. The samples were stored at room temperature, exposed to light. A 10 μL aliquot was analyzed by HPLC.

2.5. EPA extractive solution quantification

The catechin, epicatechin and caffeine calibration curves were utilized to quantify the EPA extractive solutions. The EPA extractive solutions were analyzed by HPLC in six replicates. The catechin, epicatechin, and caffeine peaks were quantified by linear regression of the standards.

Table 1
Mobile phases and flows tested in separation of EPA extractive solutions.

System	Phase A	Phase B	Flow (mL min^{-1})
A	Water + 5% acetic acid	Methanol + 5% acetic acid	0.8
B	Water + 0.5% phosphoric acid	Methanol + 0.5% phosphoric acid	0.8
C	Water + 0.05% TFA	Acetonitrile + 0.05% TFA	0.5 and 0.8
D	Water + 0.05% TFA	Methanol/acetonitrile (50/50) + 0.05% TFA	0.5
E	Water + 0.05% TFA	Methanol/acetonitrile (40/60) + 0.05% TFA	0.5
F	Water + 0.05% TFA	Methanol/acetonitrile (30/70) + 0.05% TFA	0.5
G	Water + 0.05% TFA	Methanol/acetonitrile (25/75) + 0.05% TFA	0.5
H	Water + 0.05% TFA	Methanol/acetonitrile (75/25) + 0.05% TFA	0.5

TFA = trifluoracetic acid.

Table 2
Curve parameter summary and back-calculation calibration curve concentrations for catechin, epicatechin, and caffeine.

	Catechin	Epicatechin	Caffeine
Linear range (μg mL^{-1})	300–18.75	300–18.75	50–3.125
Detection limit (μg mL^{-1})	0.70	0.88	0.13
Quantification limit (μg mL^{-1})	2.13	2.67	0.39
Regression data*			
N	5	5	5
Slope (*a*)	62438	69637	239600
Standard deviation of slope	1385.35	3152.6	9388.28
Relative standard deviation of slope (%)	2.21	4.53	4.37
Intercept (*b*)	141240	−153220	165590
Correlation coefficient (r^2)	0.9980	0.9918	0.9930

* $y = ax + b$, where x is the concentration of the compound and y is the peak area.

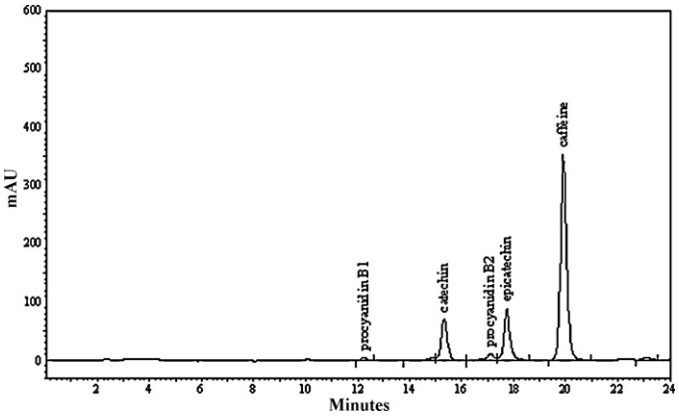

Fig. 1. Chromatogram of EPA extractive solution. Procyanidin B1 (12.24 min), catechin (15.32 min), procyanidin B2 (17.08 min), epicatechin (17.72 min), and caffeine (19.90 min).

3. Results and discussion

In this study, the same mobile phase, column, and other chromatographic conditions were employed throughout. The chromatograms were obtained from several different mobile phases and flows tested (Table 1), in order to establish the ideal conditions for the analysis of the EPA extractive solution. All analyses were performed at 210 and 280 nm. The standard peaks and the EPA multiple peaks were analyzed in the wavelength range of 200–400 nm. The spectra were observed, and the 280 nm wavelength was employed in all subsequent analyses. Different gradient systems and analysis times were tested. System G showed the best performance in the separation of EPA multiple peaks, with a possible shorter analysis time.

Trifluoroacetic acid (TFA) increased the definition of the peaks, compared with acetic and phosphoric acids. System C showed good separation and peak definition. Acetonitrile is an expensive solvent, and we tested mixtures with acetonitrile and methanol. System G gave the best results in the HPLC analysis.

The mobile phases of system G were: Phase A, water plus 0.05% TFA; Phase B, methanol:acetonitrile (25:75) plus 0.05% TFA. The gradient system of the HPLC analysis was established as: 0 min, 80:20 (A:B); 20 min, 74:26 (A:B); 21 min, 80:20 (A:B); 24 min, 80:20 (A:B). The EPA chromatogram obtained at the 280 nm wavelength and 0.5 mL min^{-1} is shown in Fig. 1.

Evaluation of the EPA by HPLC-PDA was indispensable to define certain parameters. By this means, the UV spectra of the catechin and epicatechin peaks of the EPA fraction were obtained (data not shown). Comparison of these spectra indicated that these compounds showed two bands that were very similar to the profile found for the catechin and epicatechin standards.

The Waters X Bridge™ C18 column (100 mm × 4.6 mm, 5 μm) was tested in an attempt to decrease the time required and the volume of solvent used during the analysis. However, under conditions C–H (Table 1) it was not possible to obtain separation of catechin and epicatechin, and therefore this column was not used for the subsequent analyses.

Table 3
ANOVA results for linearity of catechin, epicatechin, and caffeine (SS: sums of squares; df: degrees of freedom; MS: mean squares; *F*: *F* value of the test; Ftab: fixed *F* value).

Catechin					
	SS	df	MS	*F*	Ftab
Model	3.9404×10^{14}	1	3.9404×10^{14}	19098.60	4.098
Residual	7.8401×10^{11}	38	2.0631×10^{10}	Linear	
Lack of fit	1.1604×10^{11}	2	5.8021×10^{10}	3.127043	3.259
Pure error	6.6796×10^{11}	36	1.8554×10^{10}	No lack of fit	
Epicatechin					
Model	4.2450×10^{14}	1	4.2450×10^{14}	4091.133	4.130
Residual	3.5278×10^{12}	34	1.0376×10^{11}	Linear	
Lack of fit	1.3037×10^{11}	2	6.5186×10^{10}	0.613969	3.295
Pure error	3.3975×10^{12}	32	1.0617×10^{11}	No lack of fit	
Caffeine					
Model	8.3685×10^{14}	1	8.3685×10^{14}	6976.483	4.038
Residual	5.8777×10^{12}	49	1.1995×10^{11}	Linear	
Lack of fit	4.1577×10^{11}	3	1.3859×10^{11}	1.167205	2.807
Pure error	5.4619×10^{12}	46	1.1873×10^{11}	No lack of fit	

Table 4
Repeatability and intermediate precision of EPA extract solution.

	RSD%			
	Intra-day	Inter-day	Different analyst	Different apparatus
Catechin	0.72	0.93	0.19	1.52
Epicatechin	0.66	0.75	0.66	1.95

RSD% = relative standard deviation.

For the validation of an analytical method, the ICH guidelines recommend that tests for specificity, linearity, accuracy, precision, LOD, and LOQ of the method be performed [24].

The linearity of the HPLC method, catechin and epicatechin at five concentration levels was investigated. The results are presented in Table 2.

The calibration curves for catechin and epicatechin were linear in the range 18.75–300 μg mL^{-1}. The representative linear equations for catechin and epicatechin were $y = 141240 + 62438x$ ($n = 5$; $r^2 = 0.9980$; RSD = 2.21%) and $y = -153220 + 69637x$ ($n = 5$; $r^2 = 0.9918$; RSD = 4.53%), respectively. According to the Analytical Methods Committee (AMC), a value of regression coefficient close to unity is not necessarily the outcome of a linear relationship, and in consequence the test for the lack of fit should be applied. This test evaluates the variance of the residual values [26]. The ANOVA for catechin and epicatechin linearity is presented in Table 3. The F value for lack of fit was smaller than the tabulated F value for the 95% confidence level ($\alpha = 0.05$), and therefore, according to the ANOVA test, the linear regression showed no lack of fit.

The epicatechin RSD% of the slope was 4.53%. This value is within the limit set by ICH and ANVISA, which is up to 5%. The negative b value was in the 95% confidence interval of the calibration curve by the ANOVA test. These results (RSD% and negative b value) indicate that the reproducibility of the method and compound purity are within acceptable limits. The intercept (b value) confidence interval of the calibration curve of epicatechin was −334848 to 28401.69. The value obtained in the experiments was within the confidence interval (b value was −153220). Similarly to epicatechin, the RSD% of the slope and the b values of the calibration curves of catechin and caffeine were within the limits established by the validation guidelines.

The values of LOD, taken as the lowest absolute concentration of analyte in a sample which can be detected but not necessarily quantified as an exact value under the stated experimental conditions, were 0.70 μg mL^{-1} for catechin and 0.88 μg mL^{-1} for epicatechin. The values of LOQ, taken as the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy under the stated experimental conditions, were 2.13 μg mL^{-1} for catechin and 2.67 μg mL^{-1} for epicatechin.

The repeatability and intermediate precision were determined by evaluation of the precision and the SD and RSD of six determinations at 100% of the test concentration. Repeatability expresses the precision under the same operating conditions over a short interval of time. Intermediate precision, expressed as inter-laboratory variations with different analysts and different apparatus, was evaluated. The results are shown in Table 4. The data were evaluated by one-way ANOVA. Statistical comparison of the results was performed using the P-value of the F-test. Since the P-value of the F-test was always greater than 0.05, there was no statistically significant difference between the mean results obtained from one time of day to another at the 95% confidence level. This procedure was performed to detect any other problems that would be encountered in a reproducibility study. The variations in ambient factors that are expected to occur in practice were simulated, and the results confirmed the precision and reproducibility of the method [27].

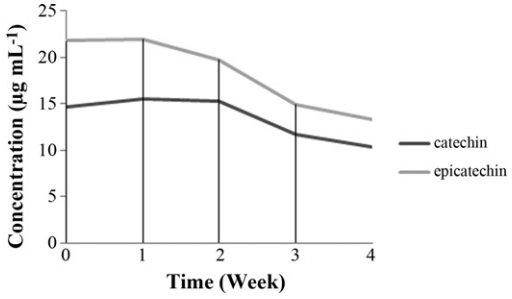

Fig. 2. Four-week evaluation of the stability of the EPA extractive solution.

The accuracy of the HPLC method for the analysis of recovery assay was determined by the preparation of a simulated sample containing a known quantity of catechin and epicatechin. The recovery of an added standard solution at three levels of concentration (100, 50, and 25 μg mL^{-1}) was performed (91.3 ± 3.8%, 2.14 RSD and 93.4 ± 3.1, 2.74 RSD of catechin and epicatechin, respectively). The results refer to the mean of three assays, and they were in good agreement with the results required for complex matrices (80–120%) [24].

The robustness should be evaluated during the development of the HPLC method, and it should demonstrate the reliability of analysis with respect to deliberate variations in the parameters of the methods [24]. The Tukey test evaluates whether a difference exists among the different levels of a factor. At the 5% level, there were no significant differences in the area of the curve and the retention time of catechin and epicatechin when the flow of the mobile phase was varied, from 0.500 mL min^{-1} to 0.495 and 0.505 mL min^{-1}. Therefore, the method proved to be robust for the substances analyzed, under the conditions evaluated.

To demonstrate the stability of the working solutions during the analysis, the EPA extractive solutions were analyzed over a period of four weeks while they were stored at room temperature (22 ± 3 °C) with exposure to natural light. The results are shown in Fig. 2. The retention times and peak areas of the drugs remained almost unchanged, and no significant degradation was observed during the course of one week, suggesting that these solutions remained stable for a sufficient time to complete the analytical process.

For quantification of the EPA extractive solution, the calibration curves of catechin, epicatechin, and caffeine were analyzed. The calibration curves of catechin and epicatechin are shown in Table 2.

The calibration curve of caffeine was linear in the range 3.125–100 μg mL^{-1}. The representative linear equation for caffeine was $y = 165590 + 239600x$ ($n = 5$; $r^2 = 0.9930$; RSD = 4.37%) (Table 2). The ANOVA for caffeine linearity is given in Table 3. These results showed that the curve was linear and there was no lack of fit in the linear regression (Table 3).

The quantification of the EPA extractive solution demonstrated that it contained 14.46 μg mL^{-1} of catechin (180.75 μg catechin mg^{-1} of EPA), 22.31 μg mL^{-1} of epicatechin (278.87 μg epicatechin mg^{-1} of EPA), and 24.07 μg mL^{-1} of caffeine (300.87 μg caffeine mg^{-1} of EPA).

4. Conclusion

A reversed-phase HPLC-PDA method was developed to determine the amount of catechin and epicatechin in the *P. cupana* EPA semipurified extract. Because of the complexity of the extract and in order to eliminate column-blocking compounds, a cleaning step with solid-phase extraction was included in the sample preparation protocol.

The method was validated according to the ICH guidelines and Brazilian regulations. In this study, the HPLC-PDA method proved to

be simple, sensitive, accurate, linear, precise, reproducible, repeatable, specific, and with robust stability. These results indicate that this method is suitable for the determination of catechin and epicatechin in *P. cupana* semipurified extracts.

Acknowledgements

The authors thank the Brazilian agencies CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq, COMCAP/FINEP, and INCT_if for their financial support. CAPES and Fundação Araucária granted fellowships to T. Klein and R. Longhini. Thanks are due to Dr. Janet W. Reid, JWR Associates, Trumansburg, NY, for English revision.

References

[1] A.R. Henman, J. Ethnopharmacol. 6 (1982) 311–338.
[2] T.M.A. Ushirobira, E. Yamaguti, L.M. Uemura, C.V. Nakamura, B.P. Dias Filho, J.C.P. Mello, Lat. Am. J. Pharm. 26 (2007) 5–9.
[3] E. Yamaguti-Sasaki, L.A. Ito, V.C.D. Canteli, T.M.A. Ushirobira, T. Ueda-Nakamura, B.P. Dias Filho, C.V. Nakamura, J.C.P. Mello, Molecules 12 (2007) 1950–1963.
[4] E.A. Audi, J.C.P. Mello, 2000. Fundação Universidade Estadual de Maringá, BR Patent no. PI00066389, Cl. Int. A61P 25/24; A61K 35/78.
[5] F.J. Otobone, A.C.C. Sanches, R.L. Nagae, J.V.C. Martins, S. Obici, J.C.P. Mello, E.A. Audi, Braz. Arch. Biol. Technol. 48 (2005) 723–728.
[6] F.J. Otobone, A.C.C. Sanches, R.L. Nagae, J.V.C. Martins, V.R. Sela, J.C.P. Mello, E.A. Audi, Phytother. Res. 21 (2007) 531–535.
[7] C.M. Roncon, C.B. de Almeida, T. Klein, J.C.P. Mello, E.A. Audi, Planta Med. 77 (2011) 236–241.
[8] E.A. Audi, C.M. Roncon, C.B. Almeida, J.C.P. Mello, J. Eur. Coll. Neuropsychopharmacol. 20 (2010) S274.
[9] E.B. Espinola, R.F. Dias, R. Mattei, E. Carlini, J. Ethnopharmacol. 55 (1997) 223–229.
[10] R. Mattei, R.F. Dias, E.B. Espinola, E.A. Carlini, S.B.M. Barros, J. Ethnopharmacol. 60 (1998) 111–116.
[11] H. Fukumasu, T.C. Silva, J.L. Avanzo, C.E. Lima, I.I. Mackowiak, A. Atroch, H.S. Spinosa, F.S. Moreno, M.L.Z. Dagli, Cancer Lett. 233 (2006) 158–164.
[12] G.A.R. Johnston, P.M. Beart, Br. J. Pharmacol. 142 (2004) 809–810.
[13] T.M. Antonelli-Ushirobira, E.N. Kaneshima, M. Gabriel, E.A. Audi, L.C. Marques, J.C.P. Mello, Food Chem. Toxicol. 48 (2010) 1817–1820.
[14] L.L. Sombra, M.R. Gómez, R. Olsina, L.D. Martínez, M.F. Silva, J. Pharmacol. Biomed. Anal. 36 (2005) 989–994.
[15] M. Kofink, M. Papagiannopoulos, R. Galensa, Eur. Food Res. Technol. 225 (2007) 569–577.
[16] Y. Shen, C. Han, Y. Jiang, X. Zhou, Z. Zhu, X. Lei, Talanta 84 (2011) 1026–1031.
[17] L. Dhooghe, K. Mesia, E. Kohtala, L. Tona, L. Pieters, A.J. Vlietinck, S. Apers, Talanta 76 (2008) 462–468.
[18] F. Marx, J.G. Maia, Quím. Nova 13 (1990) 285–286.
[19] M.L. Athayde, G.C. Coelho, E.P. Schenkel, Phytochemistry 55 (2000) 853–857.
[20] S.A.V. Tfouni, M.C.R. Camargo, S.H.P. Vitorino, T.F. Menegário, M.C.F. Toledo, Rev. Nutr. 20 (2007) 63–68.
[21] S.T. Saito, A. Welzel, E.S. Suyenaga, F. Bueno, Ciênc. Tecnol. Aliment. 26 (2006) 394–400.
[22] M.J. Simirgiotis, G. Schmeda-Hirschmann, J. Food Compos. Anal. 23 (2010) 545–553.
[23] A. Romani, M. Campo, P. Pinelli, Food Chem. 130 (2012) 214–221.
[24] International Conference on Harmonisation, ICH Topic Q2 (R1) Validation of analytical procedures: text and methodology. Available from: http://www.ich.org. 2005.
[25] Brazil, Agência Nacional de Vigilância Sanitária, RE 899/2003-ANVISA, 29 May 2003.
[26] G. Hadad, S. Emara, W.M.M. Mahmoud, Talanta 79 (2009) 1360–1367.
[27] J.A. van Leeuwen, L.M.C. Buydens, B.G.M. Vandeginste, G. Kateman, P.J. Schoenmakers, M. Mulholland, Chemometr. Intell. Lab. Syst. 10 (1991) 337–434.