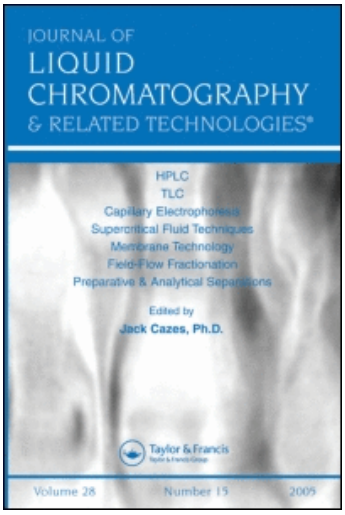

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[a] Pós Graduação em Ciências Farmacêuticas, Departamento de Farmácia e Farmacologia, Universidade Estadual de Maringá, [b] Laboratório de Microbiologia Aplicada aos Produtos Naturais e Sintéticos, Departamento de Análises Clínicas, Universidade Estadual de Maringá, [c] Instituto de Botânica de São Paulo,

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QUANTITATIVE AND QUALITATIVE ANALYSIS OF (-) MAMMEA A/BB COUMARIN IN EXTRACTS OF *CALOPHYLLUM BRASILIENSE* CAMBESS (CLUSIACEAE) BY HPLC

M. A. Brenzan,[1] C. V. Nakamura,[2] B. P. D. Filho,[2] T. Ueda-Nakamura,[2] M. C. M. Young,[3] M. L. Mitsui,[1] and D. A. G. Cortez[1]

[1]*Pós Graduação em Ciências Farmacêuticas, Departamento de Farmácia e Farmacologia, Universidade Estadual de Maringá*
[2]*Laboratório de Microbiologia Aplicada aos Produtos Naturais e Sintéticos, Departamento de Análises Clínicas, Universidade Estadual de Maringá*
[3]*Instituto de Botânica de São Paulo*

□ *A simple, rapid, and specific high performance liquid chromatographic (HPLC) method was established for analysis of the bioactive compound (-) mammea A/BB in C. brasiliense leaves. In the present study, a method was developed and validated for quantitative determination of (-) mammea A/BB in extracts of C. brasiliense Cambess (Clusiaceae). The analyses were carried out on a Metasil ODS column at 30°C by gradient elution using different concentrations of acetonitrile–water as the mobile phase, flow rate of 0.6 mL/min and detection wavelength of 280 nm. The validation using (-) mammea A/BB as the standard demonstrated that the method shows linearity (linear correlation coefficient = 0.9989), precision (relative standard deviation <1.35%) and accuracy (mean recovery = 88.6%) in the concentration range 15.56–250.0 μg/mL. /mL. The limit of detection (LOD) and limit of quantification (LOQ) were 7.61 and 25.36 μg/mL, respectively. This method allowed the identification and quantification of (-) mammea A/BB in extracts obtained from the leaves, stems, and fruits by maceration. The extracts showed different chromatographic profiles: the leaves presented the highest concentration of (-) mammea A/BB, and the dichloromethane extract of leaves showed a larger quantity of this compound than did the hydroethanolic extract. This HPLC method is suitable for routine quantitative analysis of (-) mammea A/BB in extracts of C. brasiliense and phytopharmaceuticals containing this herb.*

Keywords calophyllum brasiliense, HPLC, (-) mammea A/BB, validation

INTRODUCTION

Calophyllum brasiliense Camb. (Clusiaceae) is popularly known as 'Guanandi.' It has proven to be a rich source of bioactive compounds,

Correspondence: D. A. G. Cortez, Departamento de Farmácia e Farmacologia, Universidade Estadual de Maringá; Avenida Colombo 5790, BR-87020-900, Maringá, PR, Brazil. E-mail: dagcortez@uem.br

including coumarins,[1,2] xanthones,[3,4] triterpenoids,[2] and biflavonoids,[5] and it has been used in folk medicine for the treatment of rheumatism, varicose veins, hemorrhoids, and chronic ulcers.[6] The (-) mammea A/BB has important biological activity, mainly against protozoans and tumors.[7] Recent studies have shown that mammea A/BB has high cytotoxic activity against some tumor cell lines.[2,8] In our previous work, we have shown that the extracts, fractions, and mainly coumarin (-) mammea A/BB isolated from *C. brasiliense* leaves show significant molluscicidal activity against the snail *Biomphalaria glabrata*[9] and we also reported their potent antileishmanial activity *in vitro* against *Leishmania amazonensis* and *L. braziliensis*.[10,11] Moreover, recently we demonstrated antileishmanial activity of (-) mammea A/BB derivatives against *L. amazonensis*.[12] In all these studies, the extract and (-) mammea A/BB were safe. Also, in a recent work, mammea A/BB isolated from *C. brasiliense* leaves showed trypanocidal effects *in vitro* against *Trypanosoma cruz*.[13] Based on these biological effects, mainly the antileishmanial activity showed by the dichloromethane extract from *C. brasiliense* leaves and the mammea A/BB isolated from them, *C. brasiliense* can be used to produce a topical phytopharmaceutical to treat cutaneous and mucocutaneous leishmaniasis. Therefore, it is necessary to validate a method for the quantification of (-) mammea A/BB in *C. brasiliense* extracts. To the best of our knowledge, there is no other study on the quantitative analysis of (-) mammea A/BB coumarin in *C. brasiliense* extracts by high performance liquid chromatography (HPLC).

The HPLC method is showing increasing importance for qualitative and quantitative analysis of plant extracts, and is useful for quality control of phytochemicals.[14–18] However, validated quality control methods need to be developed, because the validation of analytical procedures is an essential step in the registration of a new phytopharmaceutical. Thus, validation should be regarded as part of an integrated concept to ensure the quality, safety, and efficacy of pharmaceuticals.[19,20]

Therefore, the aim of the present study was to validate the chromatographic HPLC method for qualitative and, mainly, quantitative analysis of (-) mammea A/BB in extracts from different parts of *C. brasiliense*.

EXPERIMENTAL

Plant Material

Calophyllum brasiliense was collected on Cardoso Island, July 2000, in the state of São Paulo, Brazil. The plant material was collected and identified by Prof. Dra. Maria Claudia M. Young. A voucher specimen (SP 363818) is deposited and authenticated at the Herbarium of the Instituto de Botânica de São Paulo, São Paulo, Brazil.

Plant Extraction and Purification of Coumarin Stardard

Leaves were dried at room temperature and then powdered (985 g). To purify the (-) mammea A/BB, the extract of *C. brasiliense* leaves was prepared by exhaustive maceration in EtOH-H_2O (90:10) (4×6 L) at room temperature, filtered, and concentrated under vacuum at 40°C to obtain an aqueous extract and a dark-green residue. The residue from the crude extract, stored in glass bottles, was dissolved with CH_2Cl_2 (200 mL), and the organic solvent was completely removed at room temperature, yielding a CH_2Cl_2 extract (30.9 g). Subsequently, the CH_2Cl_2 extract was chromatographed in a vacuum silica gel column (40×8.0 cm) with hexane (1000 mL), hexane-CH_2Cl_2 (50:50), CH_2Cl_2; CH_2Cl_2-EtOAc (90:10 to 50:50); EtOAc, MeOH, and finally, MeOH-H_2O (90:10). Next, the hexane fraction (5.0 g) was rechromatographed on a silica gel column chromatograph (40×2.0 cm) using hexane; hexane-CH_2Cl_2 (98:2 to 50:50); CH_2Cl_2; CH_2Cl_2-EtOAc (98:2 to 50:50); EtOAc, and finally, MeOH. This procedure yielded the compound (-) mammea A/BB (65 mg), as described in our previous studies (Figure 1).[9–12]

Preparation of Extracts

Leaves, stems, and fruits were dried at room temperature and then powdered (250 g). For HPLC analyses the leaves, stems, and fruits was prepared by maceration in EtOH-H_2O (90:10) for 5 days at room temperature. The extracts were filtered, and concentrated under vacuum at 40°C, and lyophilized. In addition, the CH_2Cl_2 extract of leaves used in HPLC analysis was obtained as described above.

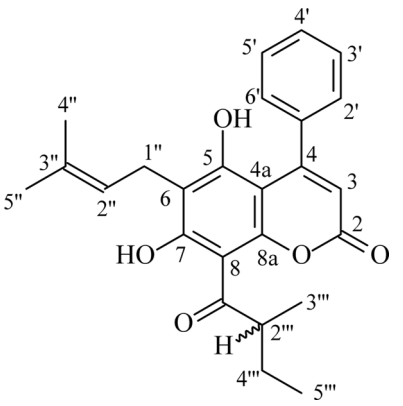

FIGURE 1 Structure of bioactive compound (-) mammea A/BB of *C. brasiliense* leaves.

Structure Elucidation of (-) Mammea A/Bb

The structure of (-) mammea A/BB was identified by comparing NMR data with those in the literature[9] (Figure 1). NMR spectra were recorded on a Bruker DRX-400 spectrometer at 300 MHz (^{1}H, COSY) and 75.5 MHz (^{13}C, DEPT) using deuterated solvent ($CDCl_3$). They were obtained with TMS as the internal standard and constant temperature of 298 K. In addition, a mass spectrum was obtained by a (EI) Shimadzu CG/MS 17 A QP 5000 mass spectrometer equipped with a DB5 column (30 m; 0.32 μm), and α_D were obtained with a Perkin-Elmer Model 241 polarimeter at 20°C at 589 nm, using CH_2Cl_2.

^{1}HNMR (300 MHz, $CDCl_3$/TMS) δ (ppm): 14.57 s (1H, OH-7), 7.42–7.46 m (2H, H-2′, H-6′), 7.55–7.59 m (3H, H-3′, H-4′, H-5′), 6.00 s (1H, H-3); 5.07–5.12 m (1H, H-2″); 3.96 sex (2H, J= 6.9 Hz, H-2‴), 3.3 d (2H, J= 6.9 Hz, H-1″), 1.83–2.01 m (1H, H-4a‴), 1.70 sl (3H, H-4″), 1.65 d (3H, J= 1.2 Hz, H-5″), 1.43–1.55 m (1H, H-4b‴), 1.29 d (3H, J= 6 Hz, H-3‴), 1.02 t (3H, J= 6.6 Hz, H-5‴). ^{1}HNMR (75.5 MHz, $CDCl_3$/TMS) δ (ppm): 158.8 (C-2), 112.4 (C-3), 154.4 (C-4), 154.4 (C-4a), 157.2 (C-5), 112.8 (C-6), 107.1 (C-7), 104.4 (C-8), 155.9 (C-8a), 137.0 (C-1′), 127.7 (C-2″), 129.8 (C-3′), 130.4 (C-4′), 129.8 (C-5′), 127.7 (C-6′), 21.9 (C-1″), 121.0 (C-2″), 134.3 (C-3″), 18.1 (C-4″), 25.9 (C-5″), 210.8 (C-1‴), 47.2 (C-2‴), 16.8 (C-3‴), 24.7 (C-4‴), 12.0 (C-5‴); $[\alpha]_D$ = - 12 (c 0,14, $CHCl_3$), EI MS m/z (rel. int. %): 406 (9,2) $[M]^+$, 349 (31), 293 (100), 171 (19), 115 (23), 55 (39).

HPLC Analysis

Reagents and Chemicals

Acetonitrile (HPLC grade from OmniSolv EM Science,Gibbstown, NJ) and ultrapure water (Milli-Q system, Millipore, Bedford, USA) were used for the mobile phase preparation. Methanol (HPLC grade from OmniSolv EM Science, Gibbstown, NJ) was used for samples preparation. The (-) mammea A/BB isolated from *C. brasiliense* leaves was used as external standard.

Sample Preparation

To obtain the stock solutions, (-) mammea A/BB was accurately weighed and dissolved in methanol at a concentration of 1000 μg/mL. Also, the leaves, stems, and fruits of *C. brasiliense* crude extracts were accurately weighed and dissolved in methanol at a concentration of 10000 μg/mL. The solutions were filtered through a 0.45 μm membrane filter (Millipore, São Paulo, Brazil) for further analysis.

Instrumentation and Chromatographic Conditions

The analyses were carried out using a Shimadzu LC-10 liquid chromatograph equipped with quaternary pump (LC-10 AD), automatic injection valve (Rheodyne) com loop de 20 μL, degasser (DEU-14), thermostatted column compartment from (CTO-10Avp), and a detector UV/vis (SPD-10A), controlled by CLASS LC-10 Software. A Metasil ODS column, 5 μm, 150 × 4.6 mm maintained at 30°C was used in the chromatographic analysis. The separation was carried out in a gradient system, using as mobile phase a mixture of acetonitrile-water 5:95 v/v a 55:45 (0–10 min.), 55:45 v/v a 80:20 (10–20 min.), 80:20 v/v a 100% acetonitrile (20–30 min.) at 100% of acetonitrile (30–40 min.), with flow rate of 0.6 mL/min. The detection was carried out at 254 nm and the running time was 40 min. These conditions were optimized in our previous work.[9,11] The sample injection volume was 20 μL. Three determinations were carried out for each sample. The statistical analyses of the data were performed by Statistica 6.0 software (Statsoft Inc., Tulsa, OK, USA).

Validation Parameters

Linearity

The linearity of the calibration curve for the (-) mammea A/BB was established by the external standard method, based on five concentrations. Stock standard solution at a concentration of 1000 μg/mL was dissolved with methanol yielding concentrations of 15.56 μg/mL, 31.12 μg/mL, 62.25 μg/mL, 125.0 μg/mL, and 250.0 μg/mL. Three determinations were carried out for each solution. The calibration curves were obtained by plotting the peak area of the (-) mammea A/BB versus the concentration of the standard solutions. The statistical parameters of the calibration curve as slope, intercept, and correlation coefficient were calculated by linear regression analysis.

Precision

The repeatability of the method was evaluated for intra-day while the intermediate precision was determined for inter-days (on 2 non-consecutive days). The standard solutions were analyzed at three concentrations (15.56 μg/mL, 62.25 μg/mL, and 250.0 μg/mL). Three determinations were carried out for each solution. The relative standard deviation (R.S.D.%) within the measurements of the concentrations of (-) mammea A/BB was used to evaluate the repeatability and intermediate precision.

Limit of Detection and Quantification

The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the calibration curve of the standard (-) mammea

A/BB. The LOD and LOQ were measured based on a signal-to-noise ratio (S/N) at about 3 and 10, respectively.

Recovery

In order to evaluate the accuracy of this method, a recovery test was performed by adding standard solutions of (-) mammea A/BB at the five concentration levels (7.78 μg/mL, 15.56 μg/mL, 31.12 μg/mL, 62.25 μg/mL, and 125.0 μg/mL) to dichloromethane extract of *C. brasiliense* leaves (2 mg/ml) with a known content of this compound. Three determinations were carried out for each solution. The recovery was calculated as a percentage by subtracting the values obtained for the control matrix preparation from those samples that were prepared with the added standards, divided by the amount added and then multiplied by 100.

Selectivity

Selectivity and peak purity were analyzed by comparison of retention times and UV spectra with the reference compound ((-) mammea A/BB).

Stability of the Analyte During Analysis

The stability was evaluated with standard solutions and sample solutions of *C. brasiliense* leaves extract, which were stored at 4°C, and at room temperature during 72 h. The solutions were each analyzed at 24 h. The results obtained were compared with results of recently prepared analysed solutions.

RESULTS AND DISCUSSION

Optimization of the Chromatographic Conditions

The fingerprint assay method for dichloromethane extract of *C. brasiliense* leaves was established in our previous work.[11] Before validating the HPLC method, several parameters were optimized to select the proper conditions to obtain better resolution of the peaks for the compounds. To optimize the mobile phase, different compositions of acetonitrile in water were tested. The acetonitrile–water ratio 5:95 v/v to 55:45 (0–10 min), 55:45 v/v to 80:20 (10–20 min), 80:20 v/v to 100% acetonitrile (20–30 min), and 100% acetonitrile (30–40 min) was shown to be adequate, because the chromatogram obtained displayed good resolution. Flow rates of 0.6 mL/min and 0.7 mL/min were studied. The value of 0.6 mL/min allowed good separation of the peaks, with an analysis time of 40 min. The temperature was evaluated at 30°C and 50°C. The separation of the compound peaks was further improved when the column temperature was 30°C. Therefore,

the chromatographic conditions chosen for the analysis were: Metasil ODS column; mobile phase acetonitrile–water 5:95 v/v to 55:45 (0–10 min), 55:45 v/v to 80:20 (10–20 min), 80:20 v/v to 100% acetonitrile (20–30 min) and 100% acetonitrile (30–40 min); flow rate: 0.6 mL/min; temperature: 30°C, 40 min runtime, and detection: 254 nm.

Validation

For the validation of the analytical method, the guidelines of the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use were followed.[21] The (-) mammea A/BB was used as the standard, because it was the majority compound in extracts of *C. brasiliense* leaves.[11] Moreover, (-) mammea A/BB and dichloromethane extracts show potent antileishmanial activity,[10–12] and may be used to prepare a topical phytopharmaceutical to treat leishmaniasis.

Linearity

An excellent linear relationship between the corresponding peak areas and the concentration of (-) mammea A/BB in the range 15.56–250.0 μg/mL was achieved, as confirmed by the correlation coefficient of 0.9989. The validating parameters of the calibration curve, including the linearity range, slope, intercepts, and correlation coefficients obtained by linear regression analysis are described in Table 1.

Precision

The method's precision was evaluated in terms of repeatability and intermediate precision, by performing analyses in triplicate for each concentration level (15.56 μg/mL, 62.25 μg/mL, and 250.0 μg/mL). The repeatability test showed R.S.D. values lower than 1.35%, and the intermediate precision, evaluated inter-day, displayed R.S.D. between 0.78% and 2.25% (Table 2). These results were very good, because the majority of phytochemicals show R.S.D. values lower than 5%, according to the literature.[22]

TABLE 1 Linearity Parameters for the Calibration Curve of (-) Mammea A/BB

Compound	Linearity Range (μg/mL)	Slope (a)	Intercept (b)	(r^2)
(-) mammea A/BB	15.56–250.0	41707	212569	0.9989

r^2, correlation coefficient.

TABLE 2 Repeatability and Intermediate Precision Data for the Determination of (-) Mammea A/BB by HPLC

Compound	Concentration (μg/mL)	Repeatability (R.S.D.%)	Intermediate Precision (R.S.D.%)
(-) mammea A/BB	15.56	1.35	1.55
	63.25	0.86	2.25
	250.0	0.87	0.78

R.S.D., relative standard deviation. For each sample $n = 3$.

Limit of Detection and Quantification

The limit of detection is defined as the smallest quantity of (-) mammea A/BB that is detectable in a sample, but not necessarily quantifiable under the stated experimental conditions; it was 7.61 μg/mL. The limit of quantification is defined as the smallest quantity of compound in a sample that is quantifiable with acceptable precision and accuracy; this limit was 25.36 μg/mL.

Recovery

The accuracy of this method was evaluated by means of the recovery test. Table 3 shows the recovery data, which were obtained from the relationship between the amount of added standard and the amount detected. The method produced a mean recovery of 88.6% with R.S.D. below 1.24% for all analyzed concentrations, confirming the accuracy of the method. In this analysis, a recovery between 70 and 120% is acceptable.[23]

Selectivity

In order to evaluate the selectivity of the method and peak purity, was compared the retention times and ultraviolet spectra of all the peaks of the dichloromethane extract of *C. brasiliense* leaves with the (-) mammea A/BB chromatograms. Thereby, it was possible to identify (-) mammea

TABLE 3 Results of the Recovery Test for (-) Mammea A/BB in Extract of *C. brasiliense* Leaves

Compound	Spiked Concentration (μg/mL)	Recovery (%) (Mean ± S.D.)	Mean ± S.D.	CV (%)
(-) mammea A/BB	7.78	106.05 ± 0.00	88.60 ± 0.56	0.63
	15.56	90.83 ± 0.00		
	31.13	85.69 ± 0.00		
	62.25	80.80 ± 0.70		
	125.00	79.58 ± 1.24		

S.D. standard deviation; R.S.D. relative standard deviation. For each sample $n = 3$.

A/BB in the dichloromethane extract with a retention time of 26.2 min (Figures 2a–b).

Stability of the Analyte During Analysis

For the stability test, the same solutions of (-) mammea A/BB and dichloromethane extract were analyzed every 24 h, up to 72 h. The analyte in solution did not show any appreciable change in the chromatographic profile for at least 72 h. The R.S.D. of the compound (-) mammea A/BB changed by 4.92% in the (-) mammea A/BB solutions, and by 2.76% in the dichloromethane extract. No degradation products were observed in the chromatogram, confirming the stability of the samples under the study conditions.

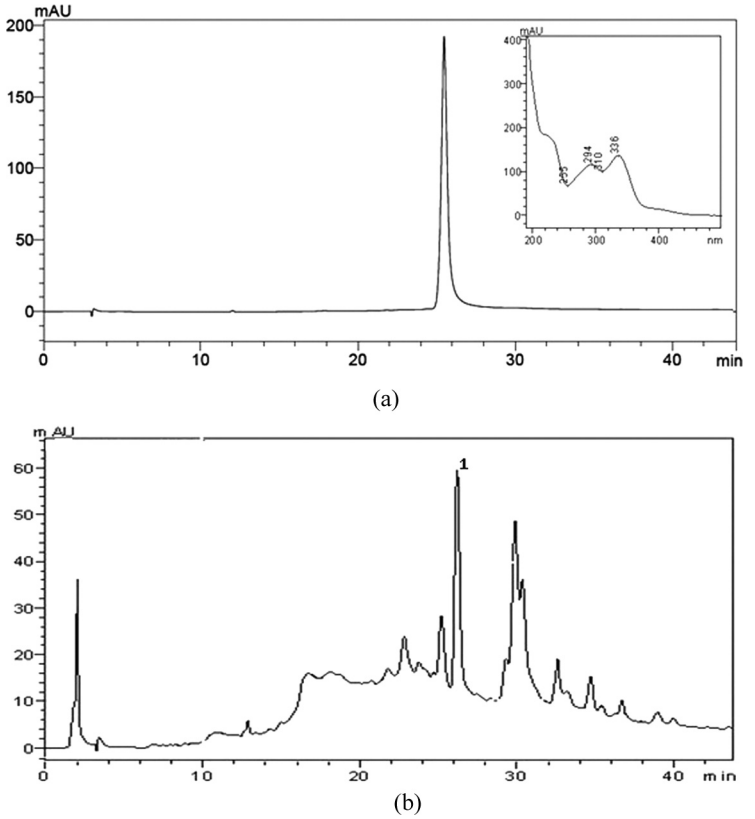

FIGURE 2 (a) Chromatogram of the standard (-) mammea A/BB (RT = 26.2 min.) and UV spectrum in above. (b) Chromatogram of dichloromethane crude extract of *C. brasiliense* leaves (1) (-) mammea A/BB (RT = 26.2 min.). *Chromatographic conditions*: Metasil ODS column; mobile phase: acetonitrile-water 5:95 v/v a 55:45 (0–10 min.), 55:45 v/v a 80:20 (10–20 min.), 80:20 v/v a 100% de acetonitrila (20–30 min.) e 100% de acetonitrila (30–40 min.); flow rate of 0.6 mL/min; temperature: 30°C; detection: 254 nm.

Analysis of Leaves, Stems and Fruit Extracts of *C. Brasiliense*

The retention times of the standard (-) mammea A/BB and the UV spectrum were used to identify the corresponding peaks in the extracts of *C. brasiliense*. For determination of the (-) mammea A/BB content in different extracts of *C. brasiliense*, the regression equation $y = 41707x - 212569$ was used. Figures 3a–c shows the chromatograms of the extracts of *C. brasiliense* obtained from leaves, stems, and fruits, respectively.

The extracts displayed different chromatographic profiles, and there were differences in the concentrations of (-) mammea A/BB. Table 4 shows the content of this compound in the different parts of this plant. As can be

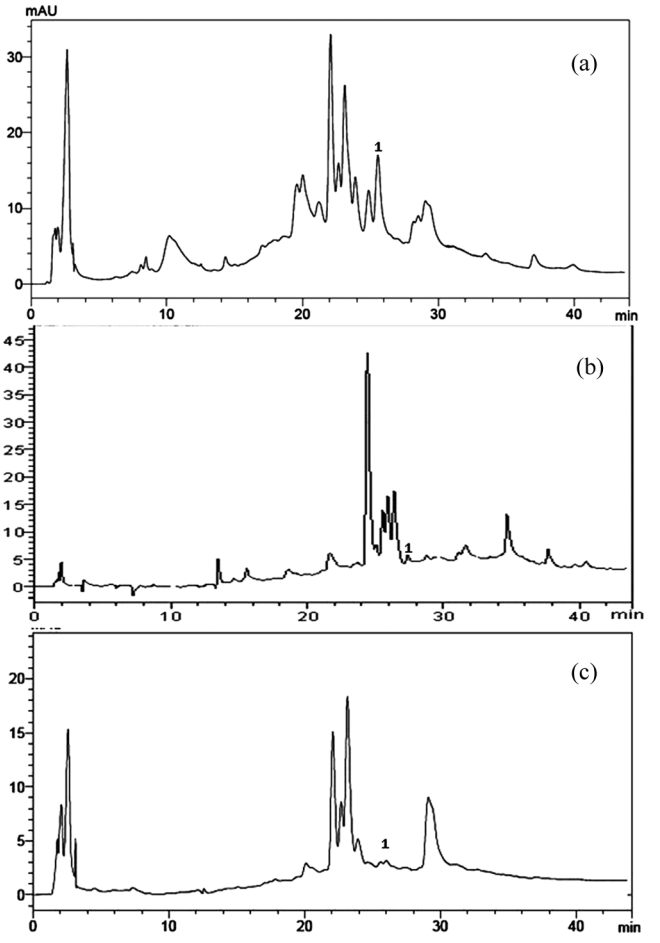

FIGURE 3 Chromatograms of hydroethanolic *C. brasiliense* extracts: (a) leaves; (b) stems; (c) fruits. *Chromatographic conditions*: Metasil ODS column; mobile phase: acetonitrile-water 5:95 v/v a 55:45 (0–10 min.), 55:45 v/v a 80:20 (10–20 min.), 80:20 v/v a 100% de acetonitrila (20–30 min.) e 100% de acetonitrila (30–40 min.); flow rate of 0.6 mL/min; temperature: 30°C; detection: 254 nm. (1) (-) mammea A/BB.

TABLE 4 Quantification of (-) Mammea A/BB in Hydroethanolic Extracts of the Leaves, Stem and Fruits of *C. brasiliense* by HPLC

Material	(-) Mammea A/BB (μg/mg of Extract)
Leaves	$6.19^{*} \pm 0.27$
Stems	$0.36^{*} \pm 0.67$
Fruits	nd

$^{*}p < 0.001$, $n = 3$, nd = not determined.

seen, the leaves showed a higher concentration of (-) mammea A/BB than the stems, and this difference was significant ($p < 0.001$). It was not possible to quantify (-) mammea A/BB in fruits, because they contained only a small quantity of this compound.

In order to obtain quantitative extraction of (-) mammea A/BB, leaves were extracted in dichloromethane and in ethanol-water (9:1). This is important, because in our previous study the dichloromethane extract showed potent antileishmanial activity. These extracts showed different chromatographic profiles (Figures 2b, 3a) and by comparing the peak area of (-) mammea A/BB, we found that the peak area of the compound reached its highest values when dichloromethane was employed in the extraction. The results are given in μg of (-) mammea A/BB per mg of extract; the dichloromethane extract contained 20.57 ± 0.94, whereas the hydroethanolic extract contained 6.19 ± 0.27 μg/mg. The difference between the contents of (-) mammea A/BB in these extracts was significant ($p < 0.001$). This experiment demonstrated that dichloromethane was the most suitable solvent to extract the highest concentration of the bioactive compound (-) mammea A/BB.

The developed and validated method was then successfully applied to quantify the bioactive compound (-) mammea A/BB in different samples of *C. brasiliense*. This study is important because it is the first quantitative analysis of (-) mammea A/BB coumarin in *C. brasiliense* extracts by high performance liquid chromatography (HPLC).

CONCLUSION

The HPLC method developed allowed the detection and quantification of the compound (-) mammea A/BB in extracts of *C. brasiliense*. The validation procedure demonstrated that the method showed good linearity, repeatability, precision, accuracy, and limits of detection and quantification in the range studied. This procedure confirms that the technique affords a reliable analysis of (-) mammea A/BB, and is appropriate for the quality control of extracts and phytopharmaceutical preparations containing

C. brasiliense. Moreover, the validated method complies with regulatory requirements if the plant is to be used by the pharmaceutical industry.

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