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Original Article

Chromatographic profiles of extractives from leaves of *Eugenia uniflora*

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ABSTRACT

Eugenia uniflora L., Myrtaceae, popularly known as "pitanga", is used in traditional medicine due the properties attributed to its chemical content, these being mainly hydrolysable tannins and flavonoids. This study provides a qualitative and quantitative evaluation of chemical profile from leaves of *E. uniflora*. The HPLC analysis was carried out on a C_{18} column (4.6 mm × 250 mm, 5 μm) by gradient elution with methanol and water (acidified with trifluoracetic acid); and silica gel Plates 60-F $_{254}$ with 10–12 μm and 5–6 μm particles, respectively for TLC and High HPTLC analysis. The chromatographic data obtained from HPLC, TLC and HPTLC presented bands and peaks related to flavonoids (myricitrin and derivatives) and tannins (gallic and ellagic acids), which were observed from different samples. The chromatographic similarities enabled the building of a typical fingerprint for the herbal material. The similarity analysis of the sample data by Pearson correlation showed *R* values >0.9 among peaks (HPLC) and bands (HPTLC). In addition, the analytical methodology developed by HPLC enabled the satisfactory quantification of marker substances [ellagic acid = 0.22% and 0.20% (m/m); gallic acid = 0.20% and 0.43%; myricitrin = 0.42 and 1.74% (m/m) in herbal drug and crude extract, respectively]. The procedure was also validated in accordance with the assays required by Brazilian legislation. Thus, the HPTLC and HPLC methods developed in this study provide helpful and simple tools for the quality evaluation both qualitatively and quantitatively of raw materials and extractives from leaves of *E. uniflora*.

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Introduction

Eugenia uniflora L., a species belonging to the Myrtaceae family, is native to Brazilian flora, and is distributed around the Brazil territory and several Latin American countries (Rattmann et al., 2012; Rutz et al., 2013; Santos et al., 2015). It is popularly known as "pitangueira" or "pitanga" and has been widely used in folk medicine, mainly for the treatment of rheumatism, hypertension, inflammation and digestive disorders (Rattmann et al., 2012; Soares et al., 2014). Due to the activities attributed to the species, *E. uniflora* was included in the list of medicinal plants of interest to the Brazilian Public Health System (Renisus) (Ministério da Saúde, 2008), and in order to improve the specie studies it is included as a valid therapeutic alternative in basic health care. Furthermore, several studies of biological properties have been carried out, demonstrating its many biological activities, such as: antioxidant (Garmus et al., 2014); diuretic, antihypertensive (Consolini et al., 1999; Consolini and Sarubbio, 2002); antipyretic, anti-noceptive (Amorim et al., 2009); anti-rheumatic (Schmeda-Hirschmann et al., 1987); hypocholesterolemic and hypoglycemic (Arai et al., 1999); antifungal (Ferreira et al., 2013); inhibitor of virulence factors in *Candida* sp. (Silva-Rocha et al., 2015); hepatoprotective and anti-depressive (Victoria et al., 2013a, 2013b); anti-trypanosome and leishmanicide (Santos et al., 2012, 2013); and, cytoprotective against heavy metal damage (Cunha et al., 2016), thus evidencing the pharmacological relevance of the species.

Regarding the chemical composition, several studies of *E. uniflora* reported the presence of polyphenols being mainly compounds, among which were evidenced: gallic acid, ellagic acid, macrocyclic tannins and flavonoid glycosides. In addition, anthracene derivatives, terpenes, steroids and essential oils have also been described in the species (Mei-Hsien et al., 1997; Fiúza et al., 2008; Rattmann et al., 2012). According to Schmeda-Hirschmann et al. (1987) and Rattmann et al. (2012) the flavonoids present in the species exhibit potential inhibiting activity of the enzyme xanthine-oxidase and pro-inflammatory factors, like neutrophil influx and cytokines and COX-2 expression. As well as this, Mei-Hsien et al. (1997) reported that the macrocyclic tannins of the *E. uniflora* are responsible for the inhibition of Epstein Barr Virus (EBV), DNA polymerase and cytotoxic activity. EBV is

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a human B lymphotropic herpes virus, which has been associated with nasopharyngeal carcinoma. In addition, Garmus et al. (2014) have correlated the polyphenols content with the antioxidant activity, confirming the therapeutic importance of *E. uniflora* polyphenols. Although there are several studies on the biological activities and the chemical constitution of *E. uniflora*, the development of simple and validated methodologies for the quantification of marker substances remain necessary in order to improve the quality control. Besides the quantitative analysis, the qualitative analysis of herbal materials and extractives by chemical profiles or fingerprints is well known in the identification or authentication of drug materials. However, the chromatographic fingerprint plays an even more important role in terms of quality, safety and efficacy of herbal materials due to the synergetic effect of several phytocompounds (Bansal et al., 2014). Thus, the fingerprint can be considered the most representative analytical approach for the mixture of substances present in the herbal matrix (Goodarzi et al., 2013; Liang et al., 2004).

There are several analytical tools used in fingerprint analysis, but among the chromatography techniques used the high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC) are the most common in the analysis of plant matrices (Zhang et al., 2005; Nicoletti, 2011; Tistaert et al., 2011; Goodarzi et al., 2013; Bansal et al., 2014).

In this context, the objective of this work was to evaluate chromatographic profiles by HPLC-DAD and HPTLC from major compounds, as well as examine the quantification of markers in the leaves from *E. uniflora*.

Materials and methods

Reagents, solvents and standards

The solvents, reagents and standards used were: ultrapure water (Elga®), HPLC grade methanol (JTBaker® and Tedia®), gallic acid (Vetec®), ellagic acid (Sigma®), myricitrin (Sigma®), natural reagent A (NEU, diphenylboril acid 2-ethylamine ester, Sigma®), formic acid (Cinetica®), ethyl acetate (Vetec®), trifluoroacetic acid, ethanol, methanol (Dinamica®).

Herbal drug

The herbal drug (leaves of *Eugenia uniflora* L., Myrtaceae) was collected in "Porto de Galinhas/Ipojuca" (8°30′12.2″S 35°00′21.9″W – PE, Brazil) and identified in the herbarium of the Agronomic Institute of Pernambuco under registration number 89899. This authentic sample was used as reference material (S1). In order to obtain the chromatographic profile, nine samples were collected from different localities (Olinda/S2/S7; Recife/S3/S9, Paulista/S4, Camaragibe/S5, Igarassu/S6, Limoeiro/S8, Ipojuca/S10, Pernambuco, Brazil). After collection, the samples were stabilized at 40 °C for 7 days using a circulating air oven (Luca-82/480, Lucadema®) and ground in a Willye-type knife mill (TE-680, Tecnal®).

Preparation of crude extract (CE) and fractions

The different dried and ground samples were extracted by turbo extraction in the proportion of 10% (w/v), using acetone: water as solvent (7:3, v/v). The extract was filtered, concentrated under reduced pressure (RV10 Basic, IKA®), frozen and then lyophilized (L101, Liotop®) to obtain the crude extract (CE). The CE was dissolved in water (1 g/10 ml) and partitioned six times with 10 ml hexane. The aqueous residue was partitioned twelve times with 10 ml ethyl acetate. The final aqueous residue and the other fractions were concentrated, frozen and lyophilized, to obtain hexane fraction (HF), ethyl acetate fraction (EAF) and aqueous fraction (AqF) (Ramos et al., 2017).

High performance liquid chromatography (HPLC)

Herbal drug (HD)

Herbal drug (1 g) was refluxed using 30 ml of ethanol 80% (v/v) at 85 ± 3 °C for 30 min. The extract was filtered through cotton into 100 ml volumetric flask. The cotton and drug residue were re-extracted twice for 15 min with 30 ml of hydroalcoholic solution. The filtrates were collected in the 100 ml volumetric flask and the volume adjusted with the hydroalcoholic solution. Appropriate dilutions were performed for HPLC analysis.

Crude extract and fractions

Crude extract (50 mg) and 50 mg of each fraction (AqF, EAF and HF) were weighed and transferred to 25 ml volumetric flasks. Then, 20 ml of ultrapure water (Elga®) was added and the flasks were brought to the ultrasonic bath (Ultracleaner, Unique®) for 15 min for complete dissolution. The volume was made up with ultrapure water. After appropriate dilution, the samples (CE and fractions) were filtered into vials through a 0.45 μm PVDF membrane (Macherey-Nagel®).

Stock solutions of standards

Gallic acid: gallic acid (GA, 2.5 mg) was weighed and dissolved with ultrapure water in the 25 ml volumetric flask, resulting in a stock solution of 100 μg/ml.

Ellagic acid: ellagic acid (EA, 5 mg) was weighed and dissolved in dimethylsulfoxide in the 10 ml volumetric flask, which was then sonicated for complete dissolution for 30 min. The obtained concentration of 500 μg/ml solution was diluted in methanol: water (3:2, v/v), resulting in a stock solution concentration of 100 μg/ml.

Myricitrin: myricitrin (MYR, 2.5 mg) was weighed and dissolved with ethanolic solution 50% (v/v) in the 25 ml volumetric flask, resulting in a stock solution of 100 μg/ml.

After appropriate dilution, the solutions were filtered through a 0.45 μm PVDF membrane (Macherey-Nagel®).

LC-analysis and calculations

The LC-analysis of the herbal drug and extractives from leaves of *E. uniflora* were carried out in a HPLC (Ultimate 3000, Thermo Fisher Scientific®), coupled with diode array detector (DAD – 3000 (RS); Thermo Fisher Scientific®), equipped with a binary pump (HPG3x00RS, Thermo Fisher Scientific®), a degasser and autosampler equipped with a 20 μl loop (ACC 3000, Thermo Fisher Scientific®). The software Chromeleon 6.8 (Dionex, Thermo Fisher Scientific®) was used for data analysis and processing. The analyses were performed at wavelengths of 254 nm, 270 nm and 350 nm, after separation by using a C_{18} column (250 mm × 4.6 mm i.d., particle size 5 μm, Dionex®) equipped with pre-column (C_{18}, 4 mm × 3.9 μm, Phenomenex®), and the column oven was adjusted to 21 ± 2 °C. The mobile phase consisted of ultrapure water (A) and methanol (B), both acidified with 0.05% trifluoroacetic acid (TFA), under flow adjusted to 0.8 ml/min. A gradient method was performed following the proportions: 0–10 min, 10–25% B; 10–15 min, 25–40% B; 15–25 min, 40–70% B; 25–30 min, 75% B; 30–31 min, 75–10% B.

Qualitative analyses were performed for the herbal drug, extractives (CE and fractions) and the reference materials. In regard to the quantitative determinations of the markers, calibration curves were developed for each reference substance (gallic acid, ellagic acid and myricitrin). In addition, the total flavonoid content was performed by the sum of the peak areas of the flavonoid substances and the results expressed in w/w% of myricitrin.

Validation of the analytical methodology

The HPLC method was validated according to the parameters recommended by the ICH (International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceutical Products for Human Use – Q2/2005) and the National Agency of Sanitary Vigilance – Anvisa (RE 899/2003) (Anvisa, 2003; ICH, 2005).

High efficiency thin layer chromatography (HPTLC)

Herbal drug

Herbal drug (2 g) was weighed in an erlenmeyer and extraction was performed by decoction using 20 ml of methanol for 5 min. After extraction, the extractive solution was cooled and filtered with cotton.

Crude extracts, fractions and standards

The samples were diluted in methanol at a concentration of 3 mg/ml and the standards at a concentration of 1 mg/ml. The solubilization was performed in vortex (Lab Dancer, IKA®).

TLC and HPTLC analysis

Silica gel Plates 60-F_{254} with 10–12 μm particles (Macherey-Nagel®) for TLC and silica gel 60-F_{254} plates with 5–6 μm particles for HPTLC (Merck®) were used for analysis. The samples were applied using a semi-automatic apparatus (Linomat V, Camag®) controlled by Wincats® software (Camag®). 30 μl of each sample and 15 μl of each standard were applied in bands of 10 mm in width with 5 mm between them. The chromatograms were developed in a twin trough vertical glass chamber (20 cm × 10 cm, Camag®) after saturation for 30 min with the mobile phase 90:5:5 (ethyl acetate:formic acid:water, v/v/v). After this process, the plates were derivatized with natural reagent A (NEU + PEG) and evaluated under UV at 365 nm. The image acquirements and UV observations were performed using MultiDoc – It Imaging System® (Model 125) with UVP® software and a Canon® camera (Rebel T3, EOS 1100 D).

Correlation analysis of the chemical profiles

The preliminary correlation analyses were performed for chromatographic data (HPLC or HPTLC) among the several samples from leaves of *E. uniflora*. The peak relative retention times from HPLC chromatograms and the band retention factors from HPTLC plates were used to calculate the Pearson's correlation coefficients that show the degree of linear dependence between the variables. The relative retention times were calculated by the ratio of the retention times of the chromatogram samples and the retention time of the majority peak (myricitrin). The calculations were carried out by using the software Excel® (Microsoft®) (Goodarzi et al., 2013; Li et al., 2015; Tistaert et al., 2011).

Results

Development of the method by HPLC

The LC-methodology was developed by evaluating several modifications in the method parameters, such as the mobile phase (composition and elution gradient) and column length, until achieving the condition of maximal efficiency for separation of the markers, whose peaks show resolution and symmetry. Thus, the best chromatographic performance was obtained using a mobile phase containing water and methanol, both acidified to prevent ionization and high interaction with the stationary phase (Mesquita et al., 2015; Ferreira et al., 2016).

The developed LC-method was able to separate and detect the gallic (GA) and ellagic (EA) acids, as well as the flavonoid myricitrin (MYR), the presence of these substances was confirmed by the comparison of the chromatographic data (retention times and UV-spectrums) and the increase of the peak areas after fortification. The similarities of the retention times obtained for the samples (HD and CE), as well for standard substances (gallic acid, ellagic acid and myricitrin), can be visualized through the overlapping of the chromatograms, as shown in Fig. 1.

The gallic acid showed a mean retention time of 8.7 min and maximum absorption at 271.1 and 271.9 nm, while the ellagic acid had a mean retention time of 25.1 min and two measurements for maximum absorption, the first at 254.1 nm and the second at 366.0 nm. In regard to the myricitrin, the major phytocompound of *E. uniflora*, the maximum absorption was observed at 355.0 nm and 260.0 nm, for bands I and II, respectively. At this stage, the specificity of the method was also determined from the absence of deviations of maximum absorption during fortification, with evidence of peaks with high purity and absence of co-elution substances.

Validation of the HPLC method for quantification of gallic acid, ellagic acid and myricitrin

Linearity and limits of detection and quantification

Analytical curves were obtained for standards and samples at different concentration ranges. In all cases, the statistical analysis of the curves by linear regression showed determination coefficients higher than 0.99, demonstrating that the analytes presented linear behavior for the concentration ranges studied. In addition, the analysis of variances was performed for all samples, using *F* test for lack-of-fit. The results indicated that there no was lack of adjustments for the models. The limits of detection and quantification were calculated from the regression data of the sample curves (Table 1).

Precision

The precision were evaluated at two levels: repeatability and intermediate precision, using recently prepared samples (HD and CE) and standard solutions (Table 2). The lowest variability was also observed for the combined analysis of the data (days and analysts), and the total relative standard deviation was less than 5% for all analytes (samples and standards). The results are within the limits recommended by the literature (Anvisa, 2003; ICH, 2005) and ensure the reproducibility of the analytical method.

Accuracy

Accuracy was evaluated through recovery trials, where the samples (HD and CE) were fortified using three concentrations of each standard (gallic acid, ellagic acid and myricitrin), established according to the respective calibration curves. For the sample of HD, the recovery ranged between 86.40 and 90.13% for gallic acid; 88.67 and 93.10% for ellagic acid; and, between 97.03 and 103.22% for myricitrin. Regarding the sample of CE, the gallic acid showed recovery between 99.04 and 107.71%; ellagic acid ranged between 97.07 and 107.01%; and, the flavonoid myricitrin presented recovery between 98.90 and 109.64%. These data demonstrate that the developed LC-method demonstrated satisfactory accuracy in accordance to the specifications recognized by the literature (Anvisa, 2003; ICH, 2005).

Robustness

The robustness was evaluated during the development of the LC-method, where small changes were made such as: reagent supplier;

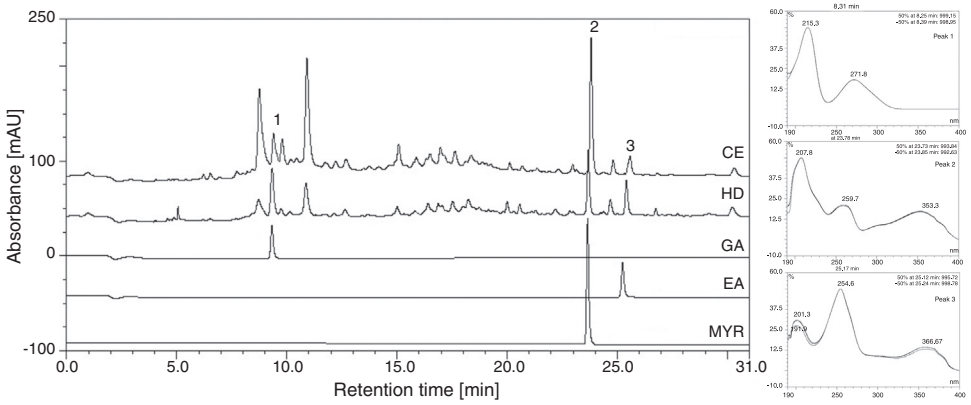

Fig. 1. Chromatograms obtained for myricitrin (MYR), ellagic acid (EA), gallic acid (GA), herbal drug (HD) and crude extract (CE). Detection at 270 nm.

Table 1
Linearity data, detection and quantification coefficients obtained for the samples analyzed.

Parameters	Matrix								
	Standards			HD			CE		
	GA	EA	MYR	GA	EA	MYR	GA	EA	MYR
Concentration range (μg/ml)	1.9–3.1	2.0–6.0	8.0–12.0	1600–2400			800–1200		
R^2	0.9963	0.9954	0.9916	0.9942	0.994	0.9926	0.9949	0.9958	0.9945
Slope	2.6711	6.2681	1.4816	0.0024	0.0082	0.0048	0.0049	0.0092	0.0242
Linear coefficient	−4.131	−5.671	−2.200	4.476	−1.607	0.921	−0.790	−1.013	−0.995
Limit of Detection (μg/ml)	–	–	–	0.18	0.82	0.94	0.94	0.61	1.77
Limit of Quantitation (μg/ml)	–	–	–	0.56	2.50	2.86	2.86	1.84	5.18

HD, herbal drug; CE, crude extract; GA, gallic acid; EA, ellagic acid; MYR, myricitrin.

Table 2
Precision data for herbal drug (HD) and crude extract (CE) from leaves of *Eugenia uniflora*.

Matrix	Substance	Parameters	Content (%; w/w) Mean ± SD (RSD%)	
HD	GA	Repeatability	0.200 ± 0.0008 (0.76)	
		Intermediate precision	Day 1	Day 2
		Analyst 1	0.199 ± 0.0041 (4.09)	0.203 ± 0.0008 (0.79)
		Analyst 2	0.192 ± 0.0007 (0.73)	0.203 ± 0.0010 (0.96)
	EA	Repeatability	0.221 ± 0.0036 (1.64)	
		Intermediate precision	Day 1	Day 2
		Analyst 1	0.219 ± 0.0081 (3.68)	0.197 ± 0.0063 (3.23)
		Analyst 2	0.206 ± 0.0003 (0.16)	0.203 ± 0.0059 (2.89)
	MYR	Repeatability	Day 1	Day 2
		Intermediate precision	0.424 ± 0.0044 (2.04)	
		Analyst 1	0.430 ± 0.0129 (3.00)	0.426 ± 0.0061 (1.42)
		Analyst 2	0.407 ± 0.0004 (0.11)	0.430 ± 0.0007 (0.31)
CE	GA	Repeatability	0.429 ± 0.0043 (1.00)	
		Intermediate precision	Day 1	Day 2
		Analyst 1	0.433 ± 0.0169 (3.91)	0.462 ± 0.0037 (0.80)
		Analyst 2	0.462 ± 0.0037 (0.80)	0.470 ± 0.0111 (2.35)
	EA	Repeatability	0.201 ± 0.0027 (1.36)	
		Intermediate precision	Day 1	Day 2
		Analyst 1	0.196 ± 0.0043 (2.22)	0.192 ± 0.0031 (1.61)
		Analyst 2	0.200 ± 0.0010 (0.50)	0.191 ± 0.0063 (3.28)
	MYR	Repeatability	Day 1	Day 2
		Intermediate precision	1.775 ± 0.0093 (0.52)	
		Analyst 1	1.780 ± 0.0227 (1.27)	1.730 ± 0.0142 (0.82)
		Analyst 2	1.740 ± 0.0050 (0.29)	1.712 ± 0.0417 (2.44)

GA, gallic acid; EA, ellagic acid; MYR, myricitrin.

mobile phase: flow and pH; and column temperature. The results are summarized in Table 3.

The data suggests the stability of the performance method for all reference compounds studied in both herbal samples (HD and CE). However, the reduction of the pH of mobile phases increases a tail factor, resulting in fairly asymmetric peaks, and decreases the calculated contents of EA and MYR. This was especially noticeable in the EA content, which presented statistically significant values, and what is more, this behavior was observed for both the matrices analyzed (HD and CE). In the case of MYR, the decrease does not compromise the performance. According to (del Moral et al., 2007) EA have a higher affinity with stationary phase in low pH, due to the deprotonation and formation of an "ionic-pair" with the stationary phase. Therefore, the pH must be adjusted to values low enough to avoid the deprotonation of the EA, these being in the range of 3–4. This study may explain the results obtained in this work, where in pH A3.0/B4.0 there is maintenance of symmetry and EA content, and in pH A2.0/B3.0 an affinity increase of EA with stationary

Table 3
Robustness assay for herbal drug (HD) and crude extract (CD) of leaves from *Eugenia uniflora*.

Matrix	Parameters	Variation	Content (%; w/w) mean (RSD%)		
			GA	EA	MYR
HD	Supplier	1	0.199 (0.25)	0.166 (0.23)	0.427 (0.18)
	methanol	2	0.203 (0.39)	0.164 (2.05)	0.422 (0.82)
	Mobile phase	0.790	0.200 (2.16)	0.175 (1.15)	0.432 (1.59)
	flow (ml/min)	0.810	0.213 (3.14)	0.170 (1.80)	0.445 (4.27)
	pH of mobile	A2.0/B3.0	0.203 (0.38)	0.128 (4.48)	0.396 (1.83)
	phase	A3.0/B4.0	0.205 (0.71)	0.164 (1.80)	0.422 (0.82)
	Temperature	22	0.203 (0.39)	0.161 (2.05)	0.422 (0.83)
	(°C)	24	0.204 (0.99)	0.149 (3.23)	0.427 (1.42)
CE	Supplier	1	0.459 (4.23)	0.192 (1.61)	1.740 (0.29)
	methanol	2	0.464 (0.92)	0.196 (2.87)	1.728 (1.57)
	Mobile phase	0.790	0.485 (4.26)	0.186 (3.74)	1.749 (1.28)
	flow (ml/min)	0.810	0.497 (0.63)	0.188 (0.52)	1.770 (1.22)
	pH of mobile	A2.0/B3.0	0.495 (0.72)	0.183 (4.89)	1.681(1.58)
	phase	A3.0/B4.0	0.493 (3.74)	0.192 (1.83)	1.740 (0.29)
	Temperature	22	0.459 (4.23)	0.195 (1.53)	1.745 (0.28)
	(°C)	24	0.463 (0.80)	0.193 (1.25)	1.730 (0.82)

GA, gallic acid; EA, ellagic acid; MYR, myricitrin.

Table 4
Marker contents: gallic acid (GA), ellagic acid (EA), myricitrin (MYR) and total flavonoids (TFC) obtained by HPLC in the different *Eugenia uniflora* matrices.

Samples	GA	EA	MYR	TFC[a]
HD	0.200 (0.76)	0.221 (1.64)	0.424 (2.04)	0.448 (0.73)
CE	0.459 (1.99)	0.200 (2.72)	1.775 (0.52)	1.937 (1.23)
AqF	0.365 (3.01)	0.035 (3.90)	0.059 (2.91)	0.107 (4.52)
EAF	0.899 (0.84)	0.323 (4.05)	6.587 (0.52)	7.611 (0.48)
HF	0.058 (4.32)	0.060 (0.87)	1.922 (0.55)	2.238 (1.28)

[a] Expressed in myricitrin; content (%m/m) mean ± standard deviation (relative standard deviation).

phase occurs that results in tail factor, asymmetry and decrease of content, which could be associated with the deprotonation of EA. The concentration of EA in the herbal drug also declines after the increase of the column temperature, but this does not compromise the performance. Thus, when the analytical adjustments of pH and column temperature were taken into account, the performance of the LC-method showed the necessary robustness for the analytical routine.

In addition, the quantification of GA, EA and MYR was also performed on the fractions, which allowed for the evaluation of the efficacy of the fractionation operation. The procedure resulted in the enrichment of the ethyl acetate fraction when the substances of interest were observed. The data are presented in Table 4.

HPLC fingerprint

The analysis of the fingerprints by HPLC from the several matrices of *E. uniflora* (HD, CE and fractions) showed characteristic regions detected in the intervals between 7 and 11 min, and from 22 to 27 min, where at least seven major peaks were observed in the chromatograms (Fig. 2). Two peaks corresponding to gallic acid and ellagic acid and the other four peaks were attributed to flavonoids (due to the UV–vis spectrum). The flavonoids were named as flavonoids 1, 2, 3 and 4 according to order of detection. Flavonoid 2 was identified as myricitrin, and flavonoid 4 was only detected in the ethyl acetate fraction. With reference to the literature, the presence of quercetin and quercitrin have also been reported in leaves from *E. uniflora* (Schmeda-Hirschmann et al., 1987; Schmeda-Hirschmann, 1995; Rattmann et al., 2012). In the fortification analysis, it was not possible to confirm the presence of quercetin in the samples. While in relation to quercitrin, in comparison with the scanning spectra of this substance present in the literature with those obtained in the sample, similarities were evidenced, but the presence was not confirmed (Mabry et al., 1970; Ma et al., 2011).

HPLC fingerprint analysis

The comparative analysis of different crude extracts from *E. uniflora* leaves was carried out in order to optimize a fingerprint to detect the similarities, which could be used for quality control purpose or as indicative of biological properties. The authentic sample (S1 – Porto de Galinhas) was used to build the typical profile and the reference profile was compared with the other samples (S2–S10; Olinda/S2/S7; Recife/S3/S9, Paulista/S4, Camaragibe/S5, Igarassu/S6, Limoeiro/S8, Ipojuca/S10, Pernambuco-Brazil) to confirm the robust regions of the fingerprints. Regarding the fingerprinting by HPLC (Fig. 3), it was possible to observe several regions of similarities among the samples. The chemical profiles showed between 35 and 38 common peaks in the samples. Nevertheless, at least seven peaks were observed in all samples and presented the resolutions and areas with acceptable quantitative performances.

The evaluation of the fingerprint similarities was carried out by Pearson's correlation analysis. This correlation approach for analysis of similarities between herbal fingerprints has been extensively reported. In this work, the analysis was performed by using relative retention times or relative area of the fingerprint peaks (marker and/or representative of unknown compounds) (Tistaert et al., 2011; Goodarzi et al., 2013; Li et al., 2015; He et al., 2016; Lebot et al., 2016).

The correlation coefficients higher than 0.9 calculated for the analysis of fingerprints from the samples of *E. uniflora* (Table 5), can be explained by the important degree of overlapping in chromatograms (Fig. 3). With regard to the substances and their classes found in all samples (S1–S10), it could be shown that the fingerprint proposed can play an important role in the identification/authentication of drug materials.

The validated analytical LC-procedure allowed for the quantification of the multiple peaks in the chromatographic fingerprint. Thus, the quantitative determinations of standards (gallic acid, ellagic acid and myricitrin) and total flavonoids (sum of flavonoid peaks) were performed on the herbal samples. The chemical contents are summarized in Table 6.

The consistency of the chemical diversity plays an important role in the identification; however, the constancy of the chemical contents is responsible for the biological properties. As discussed above, in spite of the presence of chemical markers in all sam-

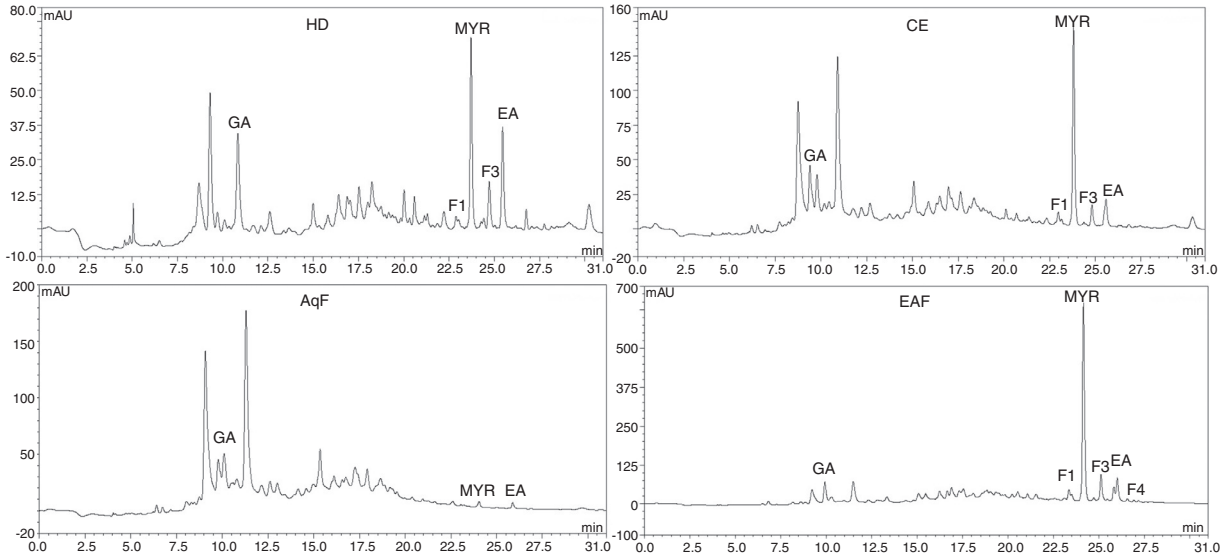

Fig. 2. Chromatograms obtained for the different matrices of the reference material of *Eugenia uniflora*: herbal drug (HD); crude extract (CE); aqueous fraction (AqF); and ethyl acetate fraction (EAF). GA: gallic acid; EA: ellagic acid; MYR: myricitrin; F1, F3 and F4: flavonoids.

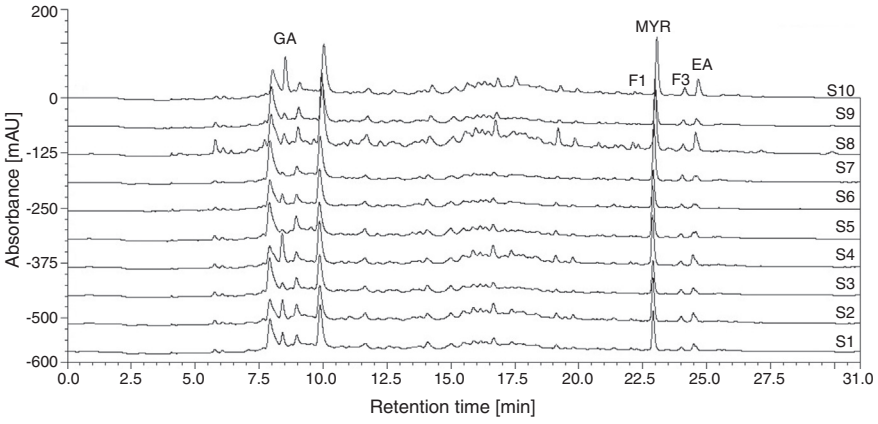

Fig. 3. HPLC fingerprint for different samples of crude extracts of the leaves of *Eugenia uniflora*. GA: gallic acid; EA: ellagic acid; MYR: myricitrin; F1, F3 and F4: flavonoids.

Table 5
Marker contents: gallic acid (GA), ellagic acid (EA), myricitrin (MYR) and total flavonoids (TFC) obtained by HPLC from the different samples of leaves (S1–S10) from *Eugenia uniflora*.

Samples	GA	EA	MYR	TFC[a]
S1	0.459 (1.99)	0.200 (2.72)	1.741 (1.86)	1.937 (1.23)
S2	0.475 (4.52)	0.116 (0.37)	0.811 (4.86)	1.168 (3.20)
S3	0.174 (4.55)	0.102 (4.65)	0.723 (1.41)	1.069 (2.15)
S4	0.723 (1.88)	0.171 (4.04)	1.508 (1.12)	1.620 (1.01)
S5	0.099 (5.52)	0.113 (4.71)	1.163 (4.10)	1.363 (2.94)
S6	0.260 (1.73)	0.075 (8.78)	0.780 (0.45)	0.902 (1.58)
S7	0.069 (1.46)	0.098 (1.29)	1.197 (1.18)	1.392 (2.47)
S8	0.295 (8.79)	0.290 (1.89)	1.581 (2.12)	1.889 (1.79)
S9	0.164 (2.03)	0.101 (1.21)	0.742 (1.48)	0.871 (1.51)
S10	1.005 (0.76)	0.245 (2.16)	1.222 (1.89)	1.465 (1.61)

S1, Porto de Galinhas; S2, Olinda; S3, Recife; S4, Paulista; S5, Camaragibe; S6, Igarassu; S7, Olinda; S8, Limoeiro; S9, Recife; S10, Ipojuca.

[a] Expressed as myricitrin; content (%m/m) mean (relative standard deviation).

Table 6
Correlation analysis of HPLC data among the different crude extracts from samples of leaves (S1–S10) of *Eugenia uniflora*.

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
S1	1									
S2	0.985	1								
S3	0.990	0.996	1							
S4	0.981	0.998	0.993	1						
S5	0.989	0.998	0.997	0.996	1					
S6	0.988	0.987	0.987	0.981	0.989	1				
S7	0.987	0.995	0.993	0.992	0.994	0.996	1			
S8	0.984	0.994	0.988	0.994	0.993	0.989	0.992	1		
S9	0.991	0.992	0.998	0.988	0.992	0.990	0.995	0.984	1	
S10	0.991	0.997	0.994	0.995	0.996	0.993	0.996	0.995	0.998	1

S1, Porto de Galinhas; S2, Olinda; S3, Recife; S4, Paulista; S5, Camaragibe; S6, Igarassu; S7, Olinda; S8, Limoeiro; S9, Recife; S10, Ipojuca.

ples, the quantitative analysis showed significant variation in their contents. Higher variation was detected for gallic acid (ranging from 0.069 to 1.005%; RSD = 80.5%), followed by ellagic acid (ranging from 0.075 to 0.290%, w/w; RSD = 47.9%), myricitrin (ranging from 0.723 to 1.741%, w/w; RSD = 32.7%) and total flavonoid content (ranging from 0.871 to 1.937%, w/w; RSD = 27.38%). The inconsistency of the chemical contents can be easily explained by the biological variability of the materials, but can be also be explained by several factors such as geographic origin, edaphoclimatic conditions and plant development (Govindaraghavan et al., 2012).

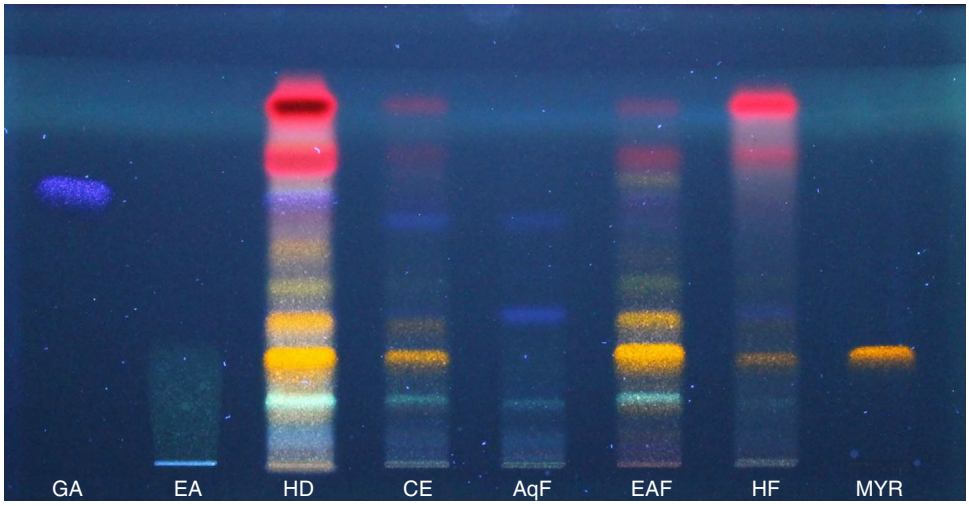

Fig. 4. HPTLC fingerprint obtained for herbal drug (HD) and derivatives (crude extract – CE; AqF: aqueous fraction; EAF: ethyl acetate fraction; and HF: hexanic fraction) from leaves of *Eugenia uniflora*. The plate was observed at ultraviolet 366 nm after derivatization with NEU + PEG. GA: gallic acid; EA: ellagic acid; MYR: myricitrin.

High efficiency thin layer chromatography

With reference to the chromatographic analysis by HPTLC of samples and standards, the TLC-fingerprints are presented in Fig. 4. The standards of gallic acid (GA; deep blue band; R_f 0.71) and myricetrin (MYR; yellowish/orange band; R_f 0.34) were easily resolved either in standard solutions or in the samples (HD, CE and fractions). On the other hand, the ellagic acid (EA; blue/greenish band; R_f 0.38) showed a tailing effect which made difficult the standard identification in the fingerprints of samples. The chromatogram of the reference sample presented a series of known and unknown bands. In total, the HD sample showed twelve bands, and CE and fractions samples showed from 2 to 12 bands (CE – 10 bands; AqF – 2 bands; EAF – 12 bands; FH – 7 bands).

The analysis of the sample chromatograms revealed the presence of gallic acid, myricitrin and suggested the presence of ellagic acid. This presence could be due to the tail presented by the EA, and this tail may be related to the intermediate polarity of the elution system, commonly used for polyphenols research. In this case, the ellagic acid may have an affinity with the two phases (mobile and stationary), resulting in a large tail.

Additionally, there are several bands of typical flavonoid-like color and elution. Concerning the fingerprints of the drug derivatives (CE and fractions), the crude extract showed a similar profile to the herbal drug but with less intense bands, as expected. The fractionation of the crude extract provide fractions with typical and enriched profiles in comparison with the starting material (CE). Thus, the aqueous fraction (AqF) showed the intensification of marker of hydrolysable tannins (GA and EA), while the ethyl acetate fraction presented a significant improvement of the flavonoids concentration, particularly of myricitrin.

The analysis of samples from Eugenia uniflora *by HPTLC*

The TLC was used to develop and optimize the chromatographic conditions for analysis of the crude extract from *E. uniflora*. Afterwards, the same methodological conditions were used to perform the analysis by HPTLC. The optimized TLC conditions allow for the resolution of the chromatograms, especially the bands of reference substances, with satisfactory reproducibility in the inter-samples analysis (Fig. 5A). The analysis by HPTLC showed the improvement in the efficiency of separation of bands observed in the CE (Fig. 5B). Since the same chromatographic conditions were used for both analyses, the improvement in separation performance observed in the chromatograms of Fig. 5A and B, can be attributed to the plates, whose smaller particle size at the stationary phase increases the surface contact and interaction of the substances under analysis, providing a better separation (Nicoletti, 2011; Patil et al., 2013; Hosu et al., 2015).

The correlation analysis was carried out only with the largest markers (gallic acid, ellagic acid and myricitrin), which were clearly separated and identified by the HPTLC-systems in the samples. The Pearson correlation coefficients were used to verify the similarities between the R_f substances in the ten samples. The data showed higher correlation for the profiles from samples with values higher than 0.98 (Table 7). According to these results, it is possible to suggest that the chemical markers selected for the analysis of extractives from *E. uniflora* are representative of its polyphenolic composition (tannins and flavonoids). Additionally, the compounds can be also detected and quantified by HPLC, improving the possibilities of standardization of extracts from *E. uniflora* leaves.

Discussion

The analytical methodology developed by HPLC was presented as a simple and useful tool for qualitative and quantitative analysis of the herbal drug and derivatives of *E. uniflora*. This method is more common due to its good peak forms and resolutions, short time of analysis, and advantageous mobile phases in comparison to acetonitrile, the most commonly used organic modifier in reverse phase chromatography (Rattmann et al., 2012; Cunha et al., 2016). In addition, the validation of the demonstrated method's satisfactory performance in respect to the technical requirements of the current literature ensures that the analytical responses can be reproduced easily for qualitative and quantitative analysis.

The HPTLC analyses have demonstrated many polyphenol bands, showing flavonoids and gallic acid related bands with a high resolution, but a low resolution was shown for ellagic acid, which presented a large tail, making it difficult to identify the EA. This is in contrast with the analysis of (Syed and Khan, 2016), which presented fingerprints that exhibited a well delimited band for ellagic acid. This better resolution can be attributed to the mobile phase used, with a higher apolar characteristic (toluene, chloroform, ethyl acetate and formic acid, 2:6:6:2, v/v/v/v), resulting in a greater affinity with the polar stationary phase. However, this apolar elution system may not apply well to other polyphenols or to simultaneous analysis of several polyphenols due to their polar/intermediate nature, making it difficult to analyze them. This

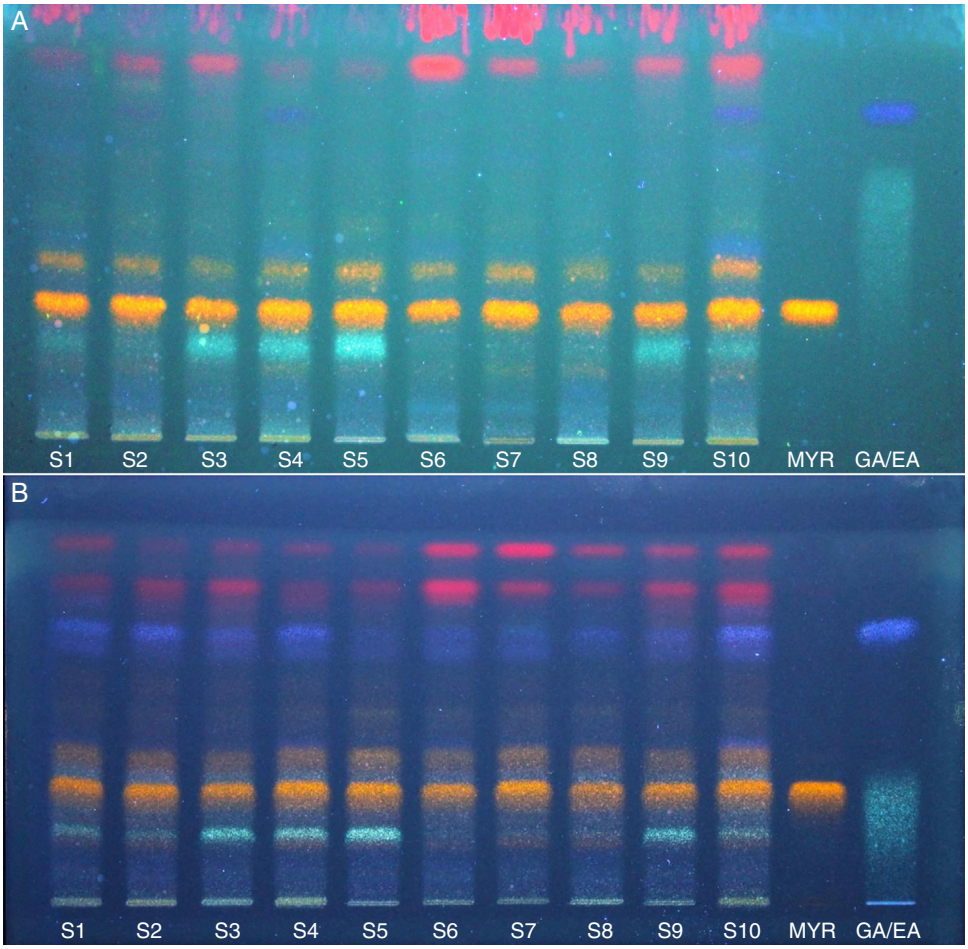

Fig. 5. Fingerprints by TLC (A) and HPTLC (B) of crude extracts from several samples of leaves from *Eugenia uniflora* (S1–S10). The plates were observed at ultraviolet 366 nm after derivatization with NEU + PEG. S1–S10: crude extracts from several drug samples; GA: gallic acid; EA: ellagic acid; MYR: myricitrin.

Table 7
Pearson correlation analysis of HPTLC data from different samples of leaves (S1–S10) of *Eugenia uniflora*.

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
S1	1									
S2	0.9988	1								
S3	0.9997	0.9992	1							
S4	0.9998	0.9979	0.9992	1						
S5	0.9994	0.9991	0.9994	0.9988	1					
S6	0.9890	0.9844	0.9865	0.9904	0.9872	1				
S7	0.9895	0.9861	0.9870	0.9903	0.9878	0.9987	1			
S8	0.9864	0.9812	0.9831	0.9883	0.9845	0.9990	0.9979	1		
S9	0.9863	0.9815	0.9834	0.9880	0.9837	0.9989	0.9979	0.9989	1	
S10	0.9836	0.9793	0.9807	0.9855	0.9812	0.9978	0.9968	0.9983	0.9993	1

S1, Porto de Galinhas; S2, Olinda; S3, Recife; S4, Paulista; S5, Camaragibe; S6, Igarassu; S7, Olinda; S8, Limoeiro; S9, Recife; S10, Ipojuca.

makes the intermediate system used the best choice for analysis of derivatives of *E uniflora*.

The qualitative approach using chromatographic fingerprints is a widely used tool for identification of plant species, due to its ability to provide a large amount of chemical information from complex matrices. Thus, the fingerprints play a major role in the characterization/identification of the chemical markers and other unknown compounds present in the complex matrix. Among the chromatographic techniques used for herbal fingerprinting, HPLC and HPTLC are the most widely used (Gu et al., 2004; Li et al., 2010, 2015; Hosu et al., 2015; He et al., 2016; Syed and Khan, 2016). The identification of the chromatographic regions and the evaluation of similarities and differences are essential in the characterization of typical chemical profiles, which are essential to perform the quality control and to correlate the chemical content with the species biological activities. In this work, the typical profiles of the authentic sample were initially acquired by HPLC and HPLTC, which evidenced characteristic chromatographic regions containing the peaks and bands of chemical markers. The chromatographic analysis of the different samples showed a clear similarity among the samples using both techniques (Figs. 3 and 5). The similarity of the method responses was confirmed by the high values of the Pearson correlation coefficients (>0.9). Only a correlation analysis of R_f did not provide specific data, thus, image analysis is an important complementary parameter to help determine between chemical profiles from different sources. In this case, a preliminary analysis of the HPTLC-band intensities of the chemical markers (gallic acid, ellagic acid and myricitrin), suggested a positive correlation

with the quantitative data obtained by HPLC (Table 7 and Fig. 5).

Notwithstanding the simple correlation approach, the Pearson analysis of LC-peaks and respective TLC-bands showed the important similarity of the analytical performance of the chemical markers by both chromatographic techniques (HPLC and HPTLC), reinforcing the use of the developed fingerprint as a simple complementary tool for identification or authentication of drug materials, extractives and fractions from leaves of *E. uniflora*.

Conclusions

HPLC and HPTLC are widely used techniques for the evaluation of fingerprints, quality control and authenticity of herbal drugs. In this work, chromatographic procedures by HPLC and HPTLC for fingerprinting drug material, extractives and fractions from leaves of *E. uniflora* were developed and evaluated. Three chemical markers (gallic acid, ellagic acid and myricitrin) were separated and identified either by LC or by TLC chromatography. The Pearson correlation analysis was used to evaluate the strength of the relationships between chromatographic performances of the markers. The correlation coefficients ($R > 0.9$) suggested a high similarity between the chromatograms and reinforce the potential of both fingerprints as tools for identification or authentication of herbal products from leaves of *E. uniflora*. Additionally, the fractionation of the crude extract allowed for the enrichment and characterization of fractions with interesting biological potential, when considering the activities described for the species. To conclude, the LC-method was validated for the quantitative analysis of the markers, providing reliable results for the evaluation of raw materials and products derived from leaves of *E. uniflora*. The qualitative and quantitative information obtained by HPLC and HPTLC supplied data on substances currently correlated with biological properties, which is pivotal to the chemical standardization of herbal products and ensures the therapeutic efficacy of the species.

Authors' contributions

ICFB (Ph.D. student) collected and dried the plant material samples, worked on laboratorial tests, conducted all analysis and wrote the article. RTMR (undergraduate student) assisted in laboratory work in all experiments. MRAF assisted in the project design and reviewed the manuscript. LALS 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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