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Research Article

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Establishment of an HPLC-PDA method for analysis of derivative products from the flowers of *Allamanda cathartica*

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ABSTRACT

A novel reverse phase high performance liquid chromatographic method with photodiode detection was developed and validated to qualitatively and quantitatively analyze the extracts and products of Allamanda cathartica flowers. The gradient method was carried out with a C18 column using acetonitrile and acidified water as the mobile phase, at a flow rate of 1.0 mL min^{-1} and a total time of 25 min. Plumieride, an iridoide glycoside present in Allamanda species, was used as a marker in the method validation. Four flavonoids also were identified and quantified. Plumieride was detected at 230 nm at a retention time of 9.58 min. Flavonoids peaks were detected at 355 nm and at 10 to 13 min. Samples of extract and nanoemulsions were analyzed. The method was shown to be simple, fast, selective, precise and accurate for A. cathartica flower extracts and nanoemulsions. This method should be useful for the pharmaceutical and cosmetic industry in the analysis of phytoderivatives and products from A. cathartica flowers and may be further adapted to other products containing herbal drug derivatives.

Key words: *Allamanda cathartica*, plumieride, analytical validation, liquid chromatography, rutin.

INTRODUCTION

Allamanda cathartica L. (Apocynaceae) is one of the most studied species of the *Allamanda* genus. This species is popularly known as Alamanda big flower or thimble lady [1] and often it is found in tropical and subtropical regions as an ornamental shrub in gardens [2]. Studies have indicated the potential anti-inflammatory and antioxidant properties of Alamanda flower extracts [3,4].In traditional medicine, an infusion of the bark and leaves is used as a purgative [3]. The leaf extract has displayed anti-inflammatory and healing activities [5].

Phytochemical studies of flowers have reported the isolation of iridoid plumieride, flavonoids such as rutin and sugars [3,6,7]. Iridoids are secondary metabolites with potential therapeutic applications [8,9]. Plumieride is the major compound of the extracts from flowers of *A. cathartica* with potential anti-inflammatory and anti-hypernociceptive activities in models of neuropathic and inflammatory pain [10]. It has been isolated from the genera *Allamanda* [6,11], *Himatanthus*[6,12], *Morinda*[13]and *Plumeria*[14]. Although plumieride has been studied phytochemically and biologically, only one analytical method has been performed on this compound in the *Himatanthus* species; however, the validation was notdescribed[12].

Flavonoids present in the flowers of different species of Allamanda were determined in study of Bonomini et al. [7]. Rutin was identified and used as marker in the HPLC-PDA analytical method with retention time of 29.5 min [7]. Therefore, this study describes the development and validation of a novel analytical method by HPLC-PAD for the analysis of derivative products from the flowers of *A. cathartica* for cosmetic and pharmaceutical applications. The finger print was used for qualitative analysis and plumieride and flavonoids were quantified. Analytical method was

validated using plumieride as marker due it is a majority and pharmacological active compound of the hydroethanolic extract [8,9].

EXPERIMENTAL SECTION

2.1. Reagents and standards

Castor ethoxylate oil (Alkest® CSO 400 – Oxiteno, Brazil) was kindly provided by Brenntag (São Paulo, Brazil), and sorbitan monooleate and triglycerides of caprylic/capric acid (Polymol®, KLK) were purchased from the Biotec (São Paulo, Brazil).Rutin was purchased from Sigma-Aldrich® (São Paulo, Brazil).HPLC grade acetonitrile was purchased from Tedia (Fairfield, Ohio, USA). All other chemicals were of analytical grade. Water was purified using a Milli-Q system (Millipore, Massachusetts, USA). All solutions were filtered through a 0.45 µm membrane (Millipore, Massachusetts, USA).

Plumieride was isolated from *Allamanda cathartica* extract by chromatography. The purity of the compound was assessed by HPLC and NMR analysis. The plumieride presented a purity of > 98% (data non-published).

2.2.Herbal products

Flowers of native specimens obtained from the vegetative propagation of authentic *A. cathartica* were collected in Barra Velha city (Santa Catarina, Brazil) in March2011 and identified by Prof. Renê Artur Ferreira. A voucher specimen was deposited at the Barbosa Rodrigues Herbarium (Itajaí-SC, Brazil) under number HBR 52742.

The flower hydroethanolic extracts were prepared by dynamic maceration with ethanol-water 90:10 (v/v), at a plant: solvent ratio of 10% (w/v), which was stirred for six hours at 300 rpm and then filtered. The extractive solution was concentrated to approximately 20% of dry residue (soft extract)in a bath at 45ºC.

The soft extract 1% (w/w)-loaded nanoemulsion was prepared by the phase inversion method using castor oil ethoxylate, sorbitan monooleate, triglycerides of caprylic/capric acid and water. The water was added to the oil phase (surfactant, oil and soft extract) at 85 °C and 600 rpm. After cooling, the nanoemulsion was stored in plastic vials.

2.3. HPLC analysis

A Shimadzu LC-20AT LC system (Shimadzu, Tokyo, Japan) was used, consisting of a binary pump, photo diode array detector and column oven. The injections (20 µL) were carried out on a Phenomenex (Torrance, California, USA) Luna C18 5 µm (250 x 4.6 mm) RP column.

For method development, different gradient conditions were tried using acetonitrile (ACN)-water (pH 2.7 at 3.5, phosphoric acid or acetic acid) and a flow rate of 0.6 to 1.0 mL min^{-1}. The analyses were monitored at 230 and 355 nm to analyze plumieride and flavonoids, respectively. The method with the best results was of the acetonitrile(A) and acidified water (pH 3.5, acetic acid) (B)gradient method: 15:85 (A:B, v/v) in 0 min; 30:70 in 10 min; 15:85 in 20 min and 15:85 in 25 min; at constant flow of 1.0 mL min^{-1} and a column temperature of30ºC.

2.4. Method validation for the extract assay

At least six individual injections of a standard plumieride solution (100 µg mL^{-1}, methanol)were performed before all measurements to assess the suitability parameters, including the theoretical plates, tailing factor and repeatability of the peak area.

The method was validated according to the ICH guidelines [15]. To determine the linear relationship between peak area and concentration of plumieride, solutions with concentration at the range of 10.0-1000.0 µg mL^{-1} were analyzed. The linearity of plumieride present in the extract was verified with low, medium and high concentration (250, 500 and 1000 µg mL^{-1}), with a theoretical plumieride concentration of 50, 100 and 200 µg mL^{-1}. The linearity equations were calculated by linear regression analysis using Excel®5.0 software.

The LOD and LOQ were calculated based on the standard deviation of the *y*-intercepts of regression lines. The data were evaluated using analysis of variance (ANOVA)($p< 0.05$).

Repeatability (intra-day) and intermediate precision (inter-day) were determined through analysis of the extract sample at 250, 500 and 750µg mL^{-1}. The samples were analyzed in sex tuplicate and the % RSD (relative standard deviation) was determined.

The accuracy of the method was measured through the analyte recovery test [15]. Standard concentrations of about

50, 100 and 150µg mL^{-1} for plumieride were added to diluted matrix samples (extract solution at 50 µg mL^{-1}).

The robustness of the method was evaluated by changing the mobile phase flow, oven temperature and pH of acidified water using extract solution at 500 µg mL^{-1} and plumieride solution at 100µg mL^{-1}. The data were evaluated using single factor ANOVA ($p< 0.05$).

2.5. Method validation for the extract-loaded nanoemulsion

The 1% *A. cathartica* extract-loaded nanoemulsion solution (0.1 g mL^{-1})was prepared using methanol and sonication during 60 min. After filtration, the sample was diluted 1:1 in the mobile phase for obtain a 500 µg mL^{-1} extract solution.

In the selectivity determination, the interference of emulsion excipients was verified at 230 nm and 355 nm using a PDA detector with a 0.1 g mL^{1} solution of the nanoemulsion without extract. The accuracy was analyzed by extraction of the nanoemulsion (1 g) added to 5 mg (low), 10 mg (medium) and 15 mg (high) of the extract with 10 mL of methanol. The method precision was evaluated by nanoemulsion sample preparation in sex tuplicate. The repeatability (intra-day) and intermediate precision (inter-day) were determined.

RESULTS AND DISCUSSION

An analytical method was developed for the quality and quantity control of products derived from flowers of *A. cathartica*. Phytochemical studies in our group have shown that secondary metabolites of medicinal interest are present in flowers, such as iridoid plumieride and flavonoids, in addition to sugars (non-published data). The method developed provided a chromatographic profile with the separation of interesting compounds, allowing the qualitative analysis of extracts (liquid and soft) (Figure 1)and formulations containing these extracts (Figure 3).

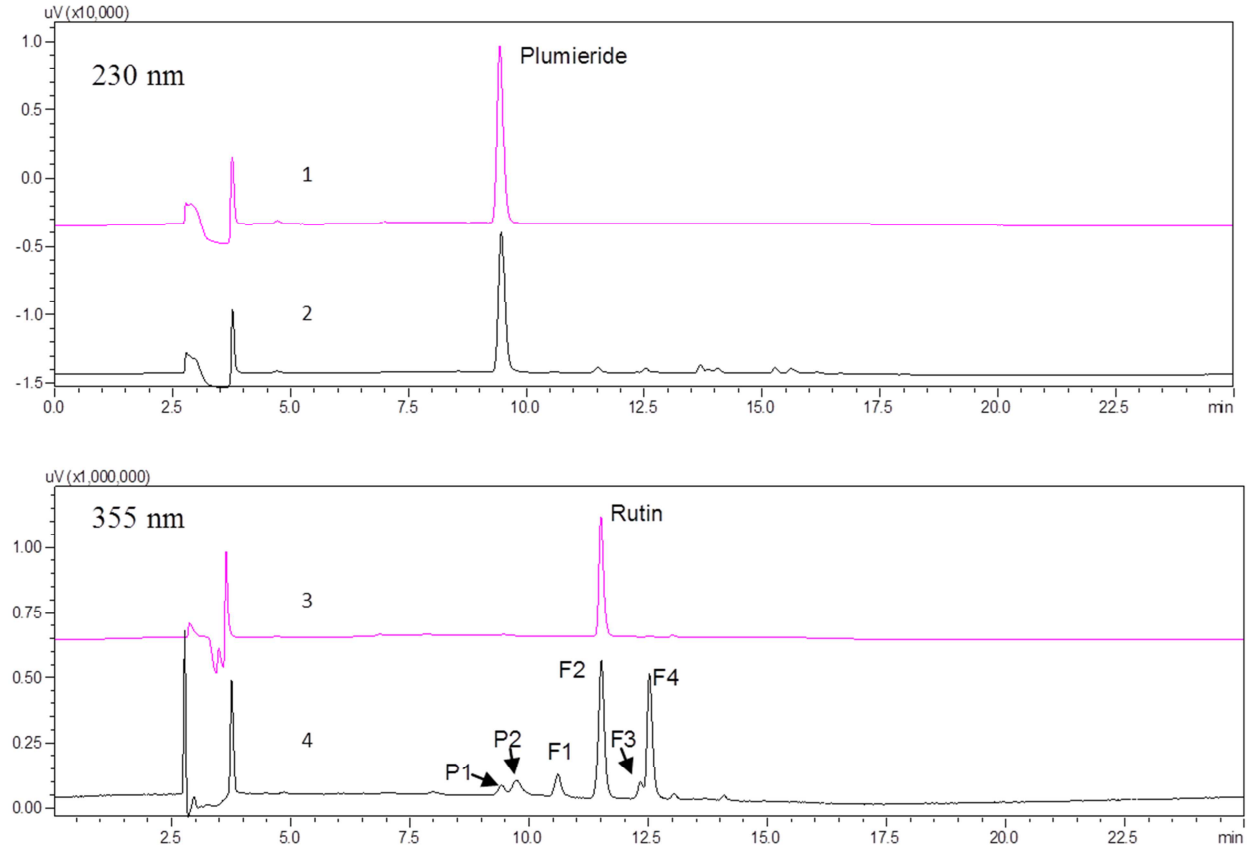

Figure 1 – Chromatographic profile by HPLC method of plumieride (1), rutin (3) and *A. cathartica* extract at 230 nm (2) and 355 nm (4)

To establish the method, different gradients with ACN and acidified water were tested. The best chromatographic

profile was obtained with the gradient ACN and acidified water (pH 3.5, acetic acid), 1.0 mL min^{-1},at 30°C, on a reversed phase column, with detection at 230 and 355 nm for plumieride and flavonoids, respectively. The method was relatively fast (25 min) for a crude hydroethanolic extract from an herbal source. As shown in Figure 1, the retention times for plumieride, flavonoids F1, F2 (rutin), F3 and F4 were approximately 9.58, 10.58, 11.52, 12.32 and 12.51 min, respectively. In 355 nm chromatographic profile, the peaks P1 and P2 were not considered because have not flavonoid UV profile (Figure 2).

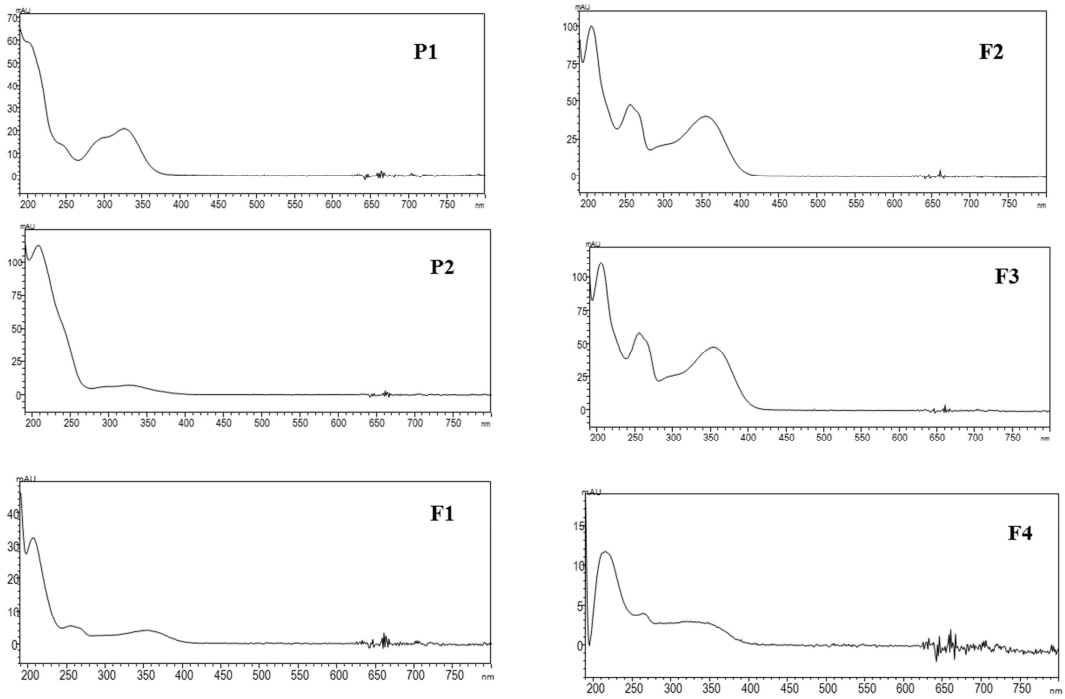

Figure 2 –UV profile of phenolic peaks present in HPLC profile of *Allamanda cathartica* extract

The area of flavonoids F1 to F4 was quantified and the concentration of total flavonoids in the extract was expressed as rutin. As shown in Table 1, the extract contained 21.15 ± 0.60% plumieride, 0.59 ± 0.2% rutin and 1.28 ± 0.06%total flavonoids expressed as rutin. These results show that plumieride was the major compound in the hydroethanolic extract from *A. cathartica* flowers. Plumieride, which isa compound with known pharmacological effect [9], was chosen as marker for validation of the analytical method. The extract, analyzed at different concentrations,showeda similar content of compounds, with an RSD value less than 5%.

The method showed relatively satisfactory separation of compounds. Plumieride showed greater absorptivity at 230 nm than at 355 nm; therefore, 230 nm was chosen for plumieride analysis. The suitability of the method was indicated by the chromatographic parameters relative to the marker peaks: tailing factor 1.08 (<1.2), theoretical plates of about 20789751 (RSD 1.60%) and good system repeatability 2.84% (area of plumieride 100 µg mL^{-1} with RSD% < 3.0) for the six replicates injected daily, making it a suitable method for the quality control of flower extracts of *A. cathartica*.

Table 1- Results of analyze *A. cathartica* extract by HPLC-DAD method

Extract (µg mL^{-1})	PMD (µg/mL)	PMD % (w/w)	RUT (µg mL^{-1})	RUT % (w/w)	TF (µg mL^{-1})	TF % (w/w)
250	54.0	21.62	1.408	0.56	2.964	1.16
500	106.0	21.15	2.988	0.59	6.423	1.28
1000	204.0	20.43	5.986	0.59	12.984	1.30
Average		21.15		0.59		1.28
SRD		2.82		3.37		4.78

Note: PMD – plumieride; RUT – rutin; TF – total flavonoids expresses in rutin.

The method was linear in the range of 10-750 500 µg mL^{-1}, with a coefficient of determination (r^2) greater than 0.99. The average of the three curves resulted in the equation y= 26368x-58577 (r^2 0.999).

The standard error of the regression was 6.626, the $F_{calculated}$ was 36,044.06 and the *p*-value was 0.161. The linearity of the analytical method was statistically significant, i.e., $F_{critical}$ ($4{,}309.10^{-45}$)< $F_{calculated}$. The value of the line

intersecting the y axis was -1.018 to 95% lower and 5.82 to 95%higher, showing that the regression line passes through the origin. The linearity of plumieride present in the extract was verified at the concentrations of 250, 500 and 1000 μg mL^{-1}, with a theoretical plumieride concentration of 50, 100 and 200 μg mL^{-1}. The practical concentration of plumieride was compared with the linearity results and it was observed that the data overlapped. The equation of the linear regression was y = 26368x – 58877 (R^2> 0.99).

The sensitivity of the method for the compound was expressed as the slope of the analytical curve, and by LOQ and LOD values of 1.238 and 0.369 μg mL^{-1}, respectively.

The method showed good inter- and intra-day reproducibility. The RSD for plumieride solution on the same day (repeatability) or on different days (intermediate precision) was less than 2% at all three concentrations evaluated (Table 2).

Table 2. Validation results for intra- and inter-day precision of plumieride solution (n=6)

Spiked concentration (μg mL^{-1})	Intra-day				Inter-day
	day 1		day 2		
	Average area	RSD%	Average area	RSD%	RSD%
150	3641653	0.58	3730246	0.47	1.70
105	2610647	1.33	2622364	0.74	0.32
50	1261498	0.95	1255806	0.96	0.32

The accuracy of the method was assessed through the analyte recovery test [15]. As shown in Table 3, the recovery was nearly 100% for the high and medium concentrations (150 and 100 μg mL^{-1}) and 113% for the 50 μg mL^{-1} solution. The RSD value was less than 5% for all concentrations, showing that the method has adequate recovery and accuracy.

Table 3. Percentage of recovery to evaluate the accuracy of the method

Spiked concentration of plumieride (μg mL^{-1})	Found (μg mL^{-1})	Average Recovery (%)	RSD (%)
50	56.63 (3.15)	113.27	3.15
100	100.63 (0.65)	100.63	4.89
150	153.07 (0.18)	102.05	0.18

The robustness of the method was analyzed by small and deliberate variations in the pH value of the aqueous phase (3.4, 3.5 and 3.6), oven temperature (28, 30 and 32°C) and mobile phase flow rate (0.8, 1.0 and 1.28 mL min^{-1}), as shown in Table 4. The robustness was estimated using the overall mean, standard deviation and %RSD for each variable. Although the calculated *F*-value was higher than the critical *F*-value, the RSD was lower than 5% for the variables temperature and flow. The method was not robust regarding pH changes. The method was found to be partially robust for the assay of plumieride present in extracts of *A. cathartica*.

Table 4. Chromatographic parameters from robustness studies

Parameters	*Average (RSD %) of Plumieride		
	Rt	*Area*	*mg/g*[1]
pH of aqueous phase			
3.4	9.51 (0.01)	2666829 (0.07)	188.76 (0.07)
3.5	9.48 (0.12)	2409087 (0.57)	158.24 (0.57)
3.6	9.51 (0.08)	2585241 (0.16)	159.08 (0.16)
RSD (%)	0.16	5.16	10.30
F_{calc}/F_{crit}	1.99	146.58	596.27
Oven temperature (°C)			
28	9.55 (0.05)	2472057 (0.49)	160.45 (0.49)
30	9.48 (0.12)	2409087 (0.57)	158.24 (0.57)
32	9.45 (0.04)	2506234 (0.28)	163.04 (0.28)
RSD (%)	0.55	2.00	1.50
F_{calc}/F_{crit}	27.68	11.18	6.23
Flow rate (mL min^{-1})			
0.8	11.55 (0.01)	2916973 (0.26)	152.43 (0.26)
1.0	9.48 (0.12)	2409087 (0.57)	158.24 (0.57)
1.2	8.08 (0.06)	2001155 (0.26)	158.22 (0.26)
RSD (%)	18.02	18.79	2.14
F_{calc}/F_{crit}	32522.00	1358.47	17.36

*Rt-retention time; [1]plumieride assay in the A. cathartica extract. *triplicate injection.*

The nanoemulsion systems containing the soft extract of *A. cathartica*(1%) showed a chromatographic profile

similar to that of the extract at the two wavelengths assessed (230 and 355 nm) (Figure 3). The chromatogram of the formulations with and without the extract showed two intense peaks in approximately 16 min at 230 nm; these peaks were not observed for the pure extract, so therefore must be related to the nanoemulsion excipients. However, the method can be considered selective because they did not interfere in the integration peaks used in the quantification of the marker plumieride.

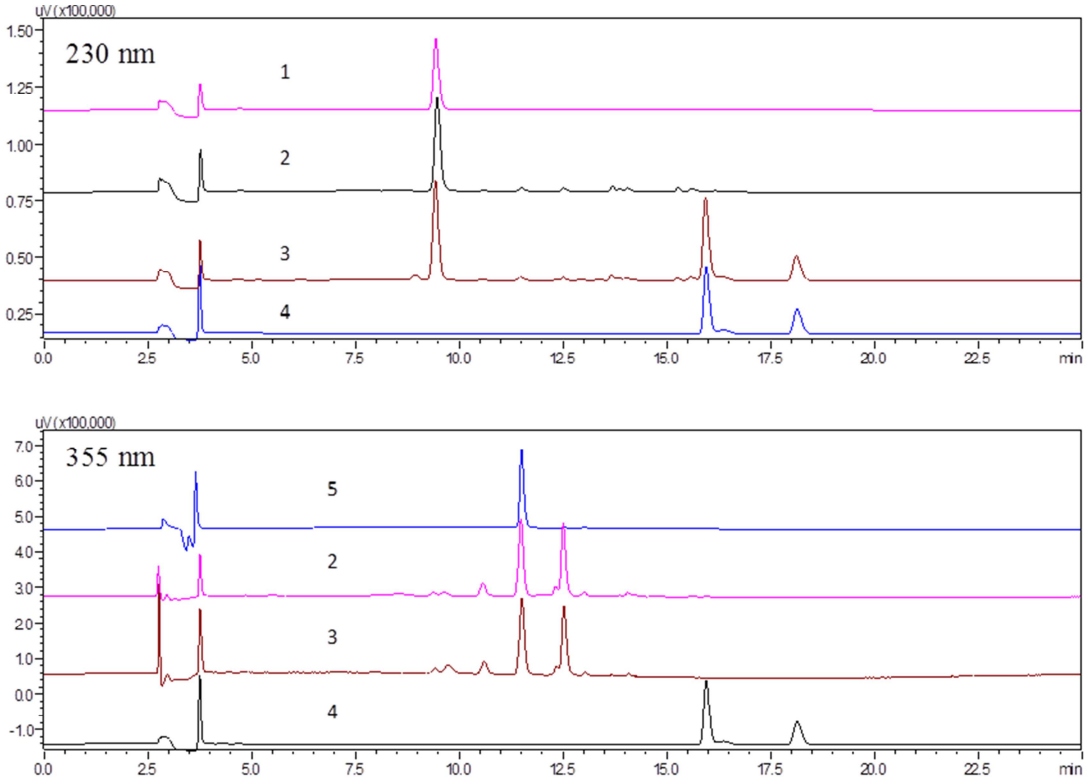

Figure 3 – Chromatographic profile by HPLC method of plumieride (1), *A. cathartica* extract (2), nanoemulsion with (3) and without (4) extract 1% and rutin (5)

The precision of the sample preparation method for the extract-loaded nanoemulsion was assessed for repeatability (intra-day) and intermediate precision (inter-day). The method showed good repeatability with a% RSD of 2.95% on the first day and 3.96% on the second day (table 5). For intermediate precision, a %RSD of 4.90% was obtained, i.e. the method also showed good inter-day precision.

Table 5. Validation results for intra- and inter-day precision and accuracy of plumieride presents in extract loaded nanoemulsion

	Plumieride(%)	RSD %
Precision 100 µg mL^{-1}		
Intra-day		
First day	103.84	2.95
Second day	96.88	3.96
Inter-day	100.36	4.90
Accuracy (%)		
50 µg mL^{-1}	110.82	0.28
100 µg mL^{-1}	97.64	0.31
150 µg mL^{-1}	101.38	1.96

CONCLUSION

A simple, fast, selective, precise and accurate HPLC-PAD method was developed and validated for the assessment of plumieride in *A.cathartica* flower extracts and nanoemulsions containing the extract. This may be useful for the pharmaceutical and cosmetic industry in the analysis of phytoderivatives and products from herbal sources and that

may be further adapted to other products containing the same herbal drug derivatives.

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