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Research Article

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Development and validation of an HPLC-PDA method for the determination of flavonoids in *Allamanda species* flowers

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ABSTRACT

This study aims to evaluate the presence of flavonoids in flowers of different Allamanda species and quantify the rutin by high performance liquid chromatography. The gradient method employs a Phenomenex C18 column with methanol, acetonitrile and water acidified with constant flow of 0.8 mL min^{-1} and photo diode array (PDA) detection at 355 nm. It was possible to detect several flavonoids in the flowers extract, including rutin with Rt 29.6 ± 0.5 min. Good linearity (correlation coefficient >0.999) for calibration curve of standard rutin was achieved. The limits of detection and quantitation were 0.25 and 2 µg mL^{-1}, respectively. The accuracy, in terms of recovery percentage, ranged from 98.18% to 100.9%. The following concentrations of rutin in the ethyl acetate fraction were obtained: A. cathartica 18.46 ± 0.04, A. schottii 24.85 ± 0.08 a nd A. blanchettii 45.22 ± 0.08 mg 100g of fresh flo wers. The Allamanda is an excellent font of flavonoids.

Keywords: *Allamanda*, flavonoids, rutin, HPLC, validation method.

INTRODUCTION

Among the plants of great interest are the genus *Allamanda*, which belongs to Apocynaceae family [1]. Numerous biological activities have been reported in this genus, such as fungicide, bactericide, antiviral and antileukemic [2-5]. Between the classes of secondary metabolites isolated are flavonoids, iridoids, terpenoids, coumarins and lignans [6-11].

In Brazil, ten species of this genus are found distributed throughout the national territory. The most common are *A. cathartica* (large and yellow flowers), *A. schottii* (small and yellow flowers) and *A. blanchetti* (large pink / purple flowers) [12]. Flavonoids are mainly found in flowers in this genus. kaempferol, quercetin, rutin have been isolated from *Allamanda schottii* and *Allamanda cathartica* [5,7,8]. While in the leaves the iridoids and terpenoids are the majority [2, 3, 5-9]. Volatile compounds such as linalool and β-cariofilene are isolated too [10].

Among the classes of secondary metabolites with pharmacological potential stand out the flavonoids as rutin, which play a vital role in protection against oxidizing agents, such as ultraviolet rays [13], environmental pollution and chemical in foods, among others. They also act as therapeutic agents in a large number of pathologies such as atherosclerosis, hypertension, attenuation of collagen-induced arthritis and to delay the initiation of Alzheimer's disease [14-17].

This study aims to evaluate the presence of flavonoids in flowers of these species and quantify the flavonoid rutin by high performance liquid chromatography in an attempt to direct the extraction process to obtain better yields of secondary metabolites of interest.

EXPERIMENTAL SECTION

Chemicals

Rutin (95% purity) was obtained from Sigma-Aldrich (Germany). Methanol and acetonitrile were purchased from Tedia ® (HPLC grade). Distilled water was obtained from a Millipore Direct-Q water-purification system and used in all solutions. Other reagents were of analytical grade.

Plant Material

The flowers of *A. cathartica*, *A. schottii* and *A. blanchettii* were collected in Ilhota, Blumenau and Itajaí, cities in the state of Santa Catarina, Brazil on December of 2010. The specimens were deposited in the Herbarium Barbosa Rodrigues in Itajai - SC under codes HBR 52742, 52524, 52525, respectively.

Preparation of extracts

Fresh flowers of *A. cathartica*, *A. schottii*, and *A. blanchettii* (200 g each species) were ground separately in multiprocessor and submitted to maceration with 2000 mL ethanol for seven days and this procedure was performed twice. The crude extracts were obtained by evaporation of the solvent under reduced pressure on rotary evaporator at a temperature below 50°C. Five grams of crude extract were each separately dissolved in methanol: water (90:10) and subjected to liquid-liquid partition with solvents of increasing polarity, thereby obtaining the semi purified fraction of hexane, dichloromethane and ethyl acetate. The ethyl acetate fractions were analyzed by high performance liquid chromatography (HPLC) with emphasis on flavonoids. An aliquot of 1 mg of each fraction were diluted in 1 ml of a mixture of methanol (A), acetonitrile (B) and acidic water (C) (H_3PO_4 0.5% v / v) at a ratio of (A:B:C 70:10:20), respectively. Then the solutions were filtered through a micro-filter of 0.45 micrometers before HPLC analysis.

Standard solution

A stock solution of rutin standard of 0.2 mg mL^{-1} was prepared in methanol. From this solution eight dilutions were performed to prepare standard solutions at concentrations of 2, 4, 8, 12, 20, 50, 100, 150, 200 µg mL^{-1}, 20 µL of each standard solution were used for plotting the standard curve of rutin.

HPLC instrumentation and chromatographic conditions

Chromatographic analysis was performed on a Waters liquid chromatograph® 2996 system, equipped with a 600 quaternary gradient pump, UV detector and Waters ® 717 plus autosampler with injection volume of 20 µL. The analytes were separated on a Phenomenex® Luna-phase C18 analytical column (250 mm x 4.6 mm x 5 µm) protected by a C18 guard column. The column temperature was set at 25 °C. For the development of the analytical method various analysis conditions have been tested, such as varying the mobile phase, gradient and flow. An external standard method was used to determine the concentration by a calibration curve. All solutions were injected in triplicate with constant flow of 0.8 mL min^{-1}. After some modifications in the gradient, a good condition of the mobile phase was obtained by the combination of A:B:C. The gradient elution was programmed as follows: 0-10 min, (10:10:80 to 20:10:70), 10-20 min, (35:10:55), 20- 35 min, (50:10:40), 35-50 min, (70:10:20), 50-60 min, (50:10:40) and 60-65 min, (10:10:80) than return to the initial condition. In this chromatographic condition rutin had retention time (Rt) next to 29.5 ± 0.1 min. The peak area measurements were used for the quantification.

HPLC/UV validation method

The method was validated according to linearity parameters, accuracy, limit of detection (LOD), limit of quantification (LOQ), accuracy and specificity.

To check the method linearity, nine different concentrations of the standard solution were prepared, as described for the Standard Solution, ranging from 2-200 µg mL^{-1}. The solutions were injected in triplicate and monitored at 355 nm. Calibration graphs were plotted subsequently for linear regression analysis of the peak area with concentration.

The method precision was determined by analyzing three standard solutions containing rutin at a concentration of 50.6 mg mL^{-1}. Each analysis was performed five times on the same day, obtaining the total of 15 injections. According to literature, the accuracy can be expressed as the relative standard deviation (R.S.D.) not accepting values above 5% [18, 19].

The detection and quantification limits were obtained by the method of signal-to-noise ratios of 3:1 and 10:1, respectively. Thus, a standard solution of rutin suffered successive dilutions yielding solutions of low concentration and these were injected until the signal-to-noise ratio was of 3:1 to LOD and 10:1 to LOQ [19].

To evaluate the method accuracy a recovery experiment was performed. Four replicates of the ethyl acetate fraction of *A. blanchettii* (1 mg mL^{-1}) were spiked with different amounts of a standard solution containing rutin, (202.4 µg mL^{-1}). The addition of standard solution in the fraction was carried out at different concentrations. Each analysis was performed in triplicate in the same vial and this procedure was performed three times. The recovery percentage was calculated using the relationship of the levels detected (real) to those added (theoretical).

The specificity was analyzed after subtracting the mean concentration of vial 1 from the vial 3. The value obtained was compared with the average concentration of rutin contained in the vial 5 by a rule of three, where the average concentration of the vial 5 is equal to 100%.

Quantification of active constituents

For the markers quantification it was used the external calibration method, which compares the area of the substance to be quantified in the sample with the areas obtained by the injection of solutions of known concentrations prepared from a reference standard of the analyte [19].

The value of the areas obtained in triplicates of the marker was recorded by Empower software. We calculated the mean of triplicates, standard deviation (s) and R.S.D. to check the reproducibility of the data. The values of R.S.D were lower than 5%, analyzes were considered reproducible and by the straight line equation of the analytical curve of the marker were obtained the concentrations of the marker in each species.

Statistical analysis

After rutin quantization the data were interpreted using analysis of variance (ANOVA) using F-test at 5% significance level. The mean values of concentrations of rutin of each fraction of the respective species were compared using Tukey test and adopting the significance level of 5%. Statistical analyzes were performed using the statistical application version 6.0, Statsoft, Inc. STATISTICA.

RESULTS AND DISCUSSION

The yield of extracts obtained after maceration of flowers of the species *A. cathartica*, *A. schottii* and *A. blanchettii* were 7.06, 6.84 and 8.53%, respectively. According to the results there is a similarity in the mass obtained from the species *A. cathartica* and *A. schottii*. However, the *A. blanchettii* showed an increase of mass in relation to other species. The extraction by maceration has the advantage of using cold solvent which minimizes the secondary metabolites decomposition.

The ethanolic extracts of *A. cathartica*, *A. schottii* and *A. blanchettii* was divided into hexane, dichloromethane and ethyl acetate soluble fractions by liquid-liquid partition and the results are shown in Table 1.

Table 1. Yield of the fractions obtained from ethanolic extract (5 g) of the flowers of the Allamanda species

Plant	Hexane (mg)	Dichloromethane (mg)	Ethyl acetate (mg)	Total (mg)
A. cathartica	13.8	72.0	126.3	212.1
A. schottii	481.9	96.6	186.3	764.8
A. blanchettii	150.3	124.3	326.5	601.1

The yields obtained from the sum of the mass fractions for each species did not exceed 764.8 mg (*A. schottii*), it can be said that the substances found in all extracts have very high polarity, keeping in the aqueous fraction. These compounds are probably sugars. Flavonoids and iridoids were the main group of compounds found in the fractions. This information was obtained by thin layer chromatography.

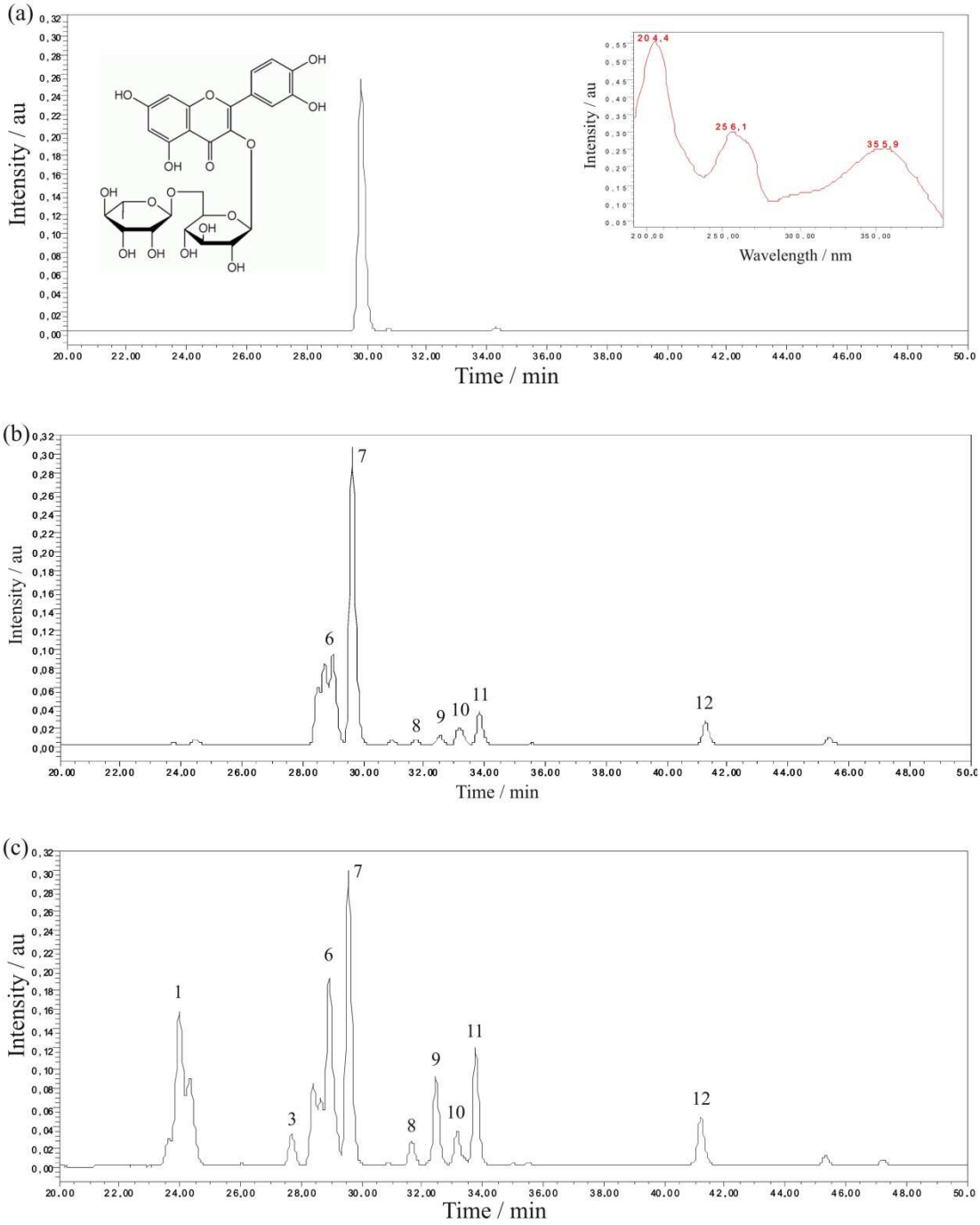
When assessing the mass extracted by the solvents used, it was found that *A. cathartica* and *A. blanchettii* showed higher yield in the ethyl acetate fraction, noting a higher concentration of polar compounds, as flavonoids. On the other hand *A. schottii* had the highest yield in the hexane fraction with higher concentration of nonpolar compounds. These differences indicate very different requirements for the production of secondary metabolites between species. Other factor that could explain these differences is that each species was collected in a different location, with different soil and suffering solar radiation, humidity or insect attack effects in different ways, enabling the differentiation in the production of secondary metabolites.

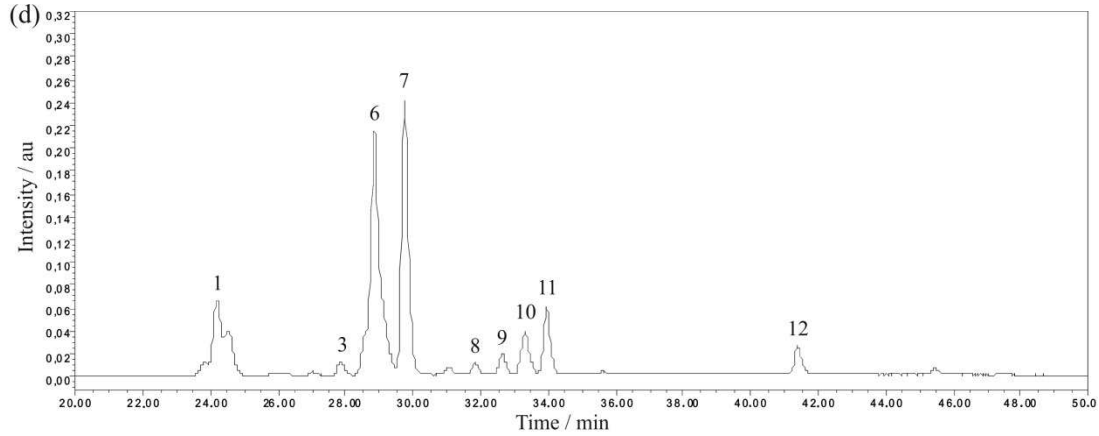
Qualitative analysis by HPLC

In this work, it was developed a sensitive method to analysis of flavonoids in *Allamanda* species flowers and validated for quantitative analysis of rutin. The method showed a satisfactory separation of the flavonoids, mainly rutin, the main component in the ethyl acetate fractions, with good resolution within a short space of time using a gradient method. The flavonoids were detectable under the wavelength of 355 nm.

The figure 1 shows the chromatogram of marker rutin (figure 1A) and ethyl acetate fractions of different species (figure 1 B, C and D) obtained under the same analytical conditions. The marker has chromatographic Rt of 29.5 ± 0.1 min. Gupta et al., [21] describes an HPLC method to analyze rutin content in *Fagopyrum* species and report a retention time for rutin of 8,1 min.

Fig. 1. Chromatographic profile of the standard rutin (A); Chromatogram of the ethyl acetate fraction of flowers *A. Cathartica* (B), *A. Schottii* (C), *A. Blanchettii* (D). For Chromatographic conditions see experimental session





Kuntic et al., [22] validated a rapid, accurate and precise method for determination of rutin in pharmaceutical dosage forms and the retention time of this compound is 2.3 min. However plant extracts are complex mixtures and have several classes of compounds. In the present study we aimed to improve separate all constituents present in the extract. This resulted in an increase of the retention time of the flavonoids.

The peaks named 1, 3, 6, 7, 8, 9, 11 and 12, with retention times of 23.8, 27.7, 28.6, 29.5, 31.6, 32.4, 33. 7 and 41.2 ± 0.1 min (Figure 1), showed characteristic UV profile of flavonoids. All these peaks were found in species *A. schottii* and *A. blanchettii*. However, in *A. cathartica* the peaks 1, 2 and 3 were not detected. The peak 7 shows retention time and UV profile similar to the flavonoid rutin. There was greater similarity to the chromatographic profile of the species *A. schotti* and *A. blanchettii*.

HPLC method validation

The chromatographic method proposed was validated to determine the LOD, LOQ, linearity, intra-day precision and accuracy. Method linearity was checked by calibration curve. The linear regression equation for rutin was expressed as $y = 40300x - 24700$, with correlation coefficient r = 0.9993 and coefficient determining (r^2) 0.9986. Therefore, the r value was found around 1, indicating that the method showed a good linear fit and that the measurement results obtained were directly proportional to analyte concentration in the range 2-200 µg mL^{-1}. The coefficient of determination (r^2) indicated that the response of the straight line equation for calculating the concentration of the rutin marker was 99.86%.

The intra-day precision (repeatability) of the HPLC analytical method proposed expressed as percent R.S.D., was determined by analysis of three standard solutions containing rutin at a concentration of 50.6 µg mL^{-1}. Each analysis was performed five times on the same vial, obtaining the total of 15 injections. The R.S.D. of these three standard solutions were 0.14, 0.21 and 0.69%. Observing the results it was found that the method showed good repeatability, since the R.S.D. value not exceeded 0.69%, which is less than specified by Resolution RE No. 899 [20], that specifies the current limit of the coefficient variance of 5.0%.

Table 2. Recovery test of standard rutin in ethyl acetate fraction of the flowers of *A. blanchettii*

Theore concentration standard (µg mL^{-1})	Área (average) ± s	Area (average) minus the area of the extract ± s	Average concentration recovered ± s (µg mL^{-1})	% Recovery
10.12	685145.3333 ±1775.694	380131.3333 ±4003.769	10.04 ± 0.10	99.21
	683840.3333±1251.208	380873.6667 ±1079.051	10.06 ± 0.03	99.41
	684995.6667 ±1250.233	380667.6667 ±1381.437	10.05 ± 0.03	99.31
50.60	2285714.000 ±7080.571	1980700.000 ±6327.525	49.76 ± 0.16	98.34
	2336426.667 ± 12652.300	2033460.000 ±12756.667	51.07 ± 0.32	100.90
	2281646.333 ±6468.635	1977318.333 ±6934.102	49.68 ± 0.17	98.18
91.08	3889316.000 ±4633.142	3584302.000 ±6649.160	89.55 ± 0.16	98.32
	3887013.333 ±5573.570	3584046.667 ±5501.816	89.54 ± 0.14	98.31
	3887326.000 ±6082.501	3582998.000 ±5356.909	89.52 ± 0.13	98.29
Average ± s				98.92 ± 0.89

s= standard deviation

The LOD and LOQ found for rutin was 0.25 µg mL^{-1} and 2 µg mL^{-1}, respectively. The accuracy, in terms of recovery, was performed by spiking the ethyl acetate fraction with standard at specific concentration and then determined by HPLC method. The results of the recovery assay are shown in Table 2. The mean values for rutin was 98.92 ± 0.89. The R.S.D. was relatively low, about 0.90%. These values are consistent with the acceptance criteria for methodology validation described in the literature, making the methodology validated for the quantification of

the marker in the fractions of the species *A. cathartica*, *A. schottii* and *A. blanchettii*. Correlating the results of recovery test of the rutin, it was found that these are within the acceptable range of variation (70-120%) for the recovery experiments [19]. The specificity was 98.43%.

HPLC-UV quantification of the flavonoid rutin in *Allamanda* flowers

The flavonoid rutin was quantified in the ethyl acetate fraction of the *Allamanda* species flowers using the developed and validated HPLC method. The concentration was determined by the corresponding regression equation. It was found that there are significant differences with $p < 0.05$ (Tukey test) by comparison of the mean concentration of rutin in the three species.

It was found that all mean concentrations of rutin are different from each other. In flowers of *A. cathartica* the concentration was 103.528 ± 0.239 µg mg^{-1} dry fraction, followed by A. schottii 97.423 ± 0.310 µg mg^{-1} dry fraction and the A. blanchettii 81.189 ± 0.137 µg mg^{-1} dry fraction. With these results it was possible to calculate the amount of rutin per 100 grams of fresh flowers. The concentration data were calculated by correlation of the rutin concentration in the ethyl acetate fraction with the mass yield of crude extract. It was obtained the following amounts: *A. cathartica* 18.46 ± 0.04, *A. schottii* 24.85 ± 0.08 and *A. blanchettii* 45.22 ± 0.08 mg 100g of fresh flowers. The results were also interpreted by analysis of variance (ANOVA) using F test at 5% level of significance. Comparing the values, it was observed that the average concentration of rutin in 100 g of fresh flowers were different from obtained by the ethyl acetate fraction, where the highest concentration of rutin was found in *A. blanchettii*, due to the higher yield of the fraction and the crude extract.

Gonçalves et al., [23] developed an HPLC method to quantify rutin in the flowers fractions of *Viola tricolor* in a single run using mobile phase isocratic elution (50: 50 v/v, methanol: water adjusted to pH 2.8 with phosphoric acid), a Merck LiChrospher® 100 RP-8 (5 µm) LiChroCART® column. The contents of rutin present in the butanolic and ethyl acetate fractions were 143.57 ± 8.48 and 33.70 ± 0.81 mg of rutin g dry fraction, respectively.

Rutin has been quantified in various medicinal plants and its content varies with species. For example 8.6% in herbs of *Ruta graveolens*, 3.36% in flowers of *Viola tricolor* and 3.4% in herb of *Rosmarinus officinalis* [24]. Gupta et al., [25] quantify rutin content in 195 accessions of *Fagopyrum* species and obtained a wide range of variation from 6 µg mg^{-1} to 30 µg mg^{-1} dry weight. Therefore, our results suggest that *Allamanda* species, especially its flowers, are a promising source of rutin.

CONCLUSION

Through this work a simple, fast, selective, precise and accurate HPLC-PAD method has been developed for analysis of flavonoids and quantification of rutin in extracts of *Allamanda* flower species. This method can be useful to the pharmaceutical analysis of flavonoids in extracts of plants due to pharmacologically effect important of this class of compounds.

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