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Development and validation of a HPLC method for the quantification of three flavonoids in a crude extract of *Dimorphandra gardneriana*

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Abstract: A method for separation and quantification of three flavonoids by reverse-phase high performance liquid chromatography (HPLC) was developed and validated. Flavonoids present in a crude methanolic extract of the inner bark of *Dimorphandra gardneriana* Tul., Fabaceae, were analyzed. Rutin, isoquercitrin and quercetin were used as calibration standards. The analysis was performed using a Thermo Scientific Hypersil C18 column (250 x 4.0 mm i.d., 5 µm particle size), as stationary phase, with a flow rate of 0.8 mL/min and detection at a wavelength of 356 nm. The proposed method was validated by resolution RE No. 899/2003 of the National Health Surveillance Agency. In this study, an excellent linearity was obtained with r higher than 0.99. Besides, the chromatographic peaks showed good resolution. With other validation data, including precision, specificity, accuracy and robustness, this method demonstrated good reliability and sensitivity, and can be conveniently used for the quantification of rutin, isoquercitrin and quercetin in crude methanolic extract of *D. gardneriana* pods. Furthermore, there are the advantages of easy sample preparation and short time between each injection.

Introduction

Dimorphandra gardneriana Tul., Fabaceae, known as "fava-d'anta" and "faveiro" is a Brazilian native leguminous tree, naturally found in the states of Maranhão, Piauí, Ceará, Pernambuco, Bahia, Pará, Goiás, Mato Grosso and Minas Gerais. The seed pod of "fava-d'anta" is one of the sources for the extraction of rutin on an industrial scale. Rutin belongs to an important class of flavonoids, vital in their ability to increase the strength of the capillaries and to regulate their permeability (Montano et al., 2007).

Rutin may be used in the treatment and prevention of small varicose veins. This substance is also used in mesotherapy or intradermotherapy to stimulate circulation in treatments against cellulite. It has been used for preparing patients with jaundice for surgery (Silva et al., 2007).

Quercetin is another substance extracted from *D. gardneriana* and of great interest in the pharmaceutical industry. It is a natural polyphenolic antioxidant, present in vegetables, fruits and juices. Chemically, quercetin is an aglycone of rutin and other glycosides. It is a powerful antioxidant and free radical scavenger (Filho et al., 2001). Another substance that may be found in *D. gardneriana* is isoquercitrin. It has been shown to play protective roles against lipid peroxidation and oxidative stress (Silva et al., 2008).

However, one of the impediments in the acceptance of herbal products worldwide is the lack of standard quality control profiles (Shinde et al., 2009). Chemical and chromatographic techniques may be used to aid in the identification of an herbal material or extract. Chromatographic techniques such as HPLC, TLC, GC and capillary electrophoresis and spectroscopic methods such as IR, NMR and UV may also be used for fingerprinting (Patra et al., 2010).

In order to control the quality of herbal drugs in a better way, we must develop new techniques and terms to the maximum extent. The development and validation of an efficient analytical method is an integral part of the quality control of the source material, to guarantee the safety and effectiveness of the resulting compound (Hefnawy et al., 2006).

Thus, considering the pharmacological potential of *D. gardneriana* and the lack of specifications for the quality control of this plant raw material, which is a pre-requisite for the production and registration of

phytomedicines (Anvisa, 2010), the objective of the present study was to develop and validate a method for the separation and quantitative analysis of rutin (**1**), quercetin (**2**) and isoquercitrin (**3**) by HPLC obtained from a methanolic extract of the inner bark of pods of *D. gardneriana.* This will provide the scientific basis for the quality control of extracts prepared from the inner bark of this plant. The choice of these three flavonoids was based on other studies, which reported the presence of these compounds in other species of *Dimorphandra* (Sousa et al., 1991; Ferreira et al., 2001; Lucci & Mazzafera, 2009). The method was validated according to regulation RE 899/2003 of the National Health Surveillance Agency, Brazil (Anvisa, 2003). The following validation characteristics were assessed: specificity, linearity, limit of detection and quantification, accuracy, precision and robustness.

Materials and Methods

Plant material

Pods of *Dimorphandra gardneriana* Tul., Fabaceae, were collected between June and July of 2010 in the city of Crato, State of Ceará, Brazil (Line E, Floresta Nacional do Araripe). The plant material was identified by Prof. Dr. Maria Arlene Pessoa da Silva from the Department of Biological Sciences of Universidade Regional do Cariri. Exsiccates (number 18639) of the species were deposited in the Herbarium Caririense Dárdano de Andrade Lima in the same institution.

Preparation of the extract

The plant material was submitted to a drying process at room temperature. The bark and seeds were then removed. The inner bark (1 kg) was separated and triturated. The powder was weighed and extracted with 2 L methanol, at room temperature, for 72 h. Afterwards, the liquid was filtered and concentrated using a rotary evaporator under reduced pressure to obtain 18 g of crude extract.

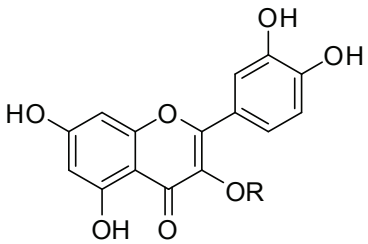
Chemicals and reagents

All reagents and solvents were analytical and HPLC grades (Tedia, USA), except phosphoric acid (Vetec, Brazil) and sodium dihydrogen phosphate (Caledon, Canada). Ultra-pure water obtained using a Milli-Q Gradient® apparatus (Millipore, USA) with conductivity of 0.60 µS/cm was used in all experiments. Rutin, quercetin and isoquercitrin (Sigma, USA) of the highest grade (purity>98.0%) were used as the external standards.

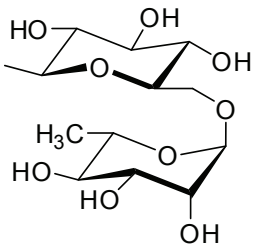
Instrumentation and chromatographic conditions

The analyses were carried out using an HPLC system (Shimadzu, USA) consisting of a solvent delivery pump (Model LC-10 ADvp), a variable wavelength UV/VIS detector (Model SPD 10 AVP), a manual injection valve (Rheodyne®, USA) with a 20 µL loop, and degasser (DGU 14A). Data collection and analyses were performed using CLASS-VP™ System Software. A gradient elution was performed on a Thermo Scientific Hypersil C_{18} column (250 x 4.0 mm i.d., 5 µm particle size) (Thermo Scientific, USA). The mobile phase consisted of two different solutions, solution A and solution B. Both solutions consisted of tetrahydrofuran (THF) and sodium dihydrogen phosphate buffer (15.6 g/L) adjusted to pH 3.0 with phosphoric acid, where the proportion of THF and buffer was 5:95 for solution A and 40:60 for solution B. All solutions were degassed and filtered through a 0.45 µm pore size filter (Millipore, USA). Separations were effected by a gradient elution program as follows: from 0 to 10 min, solution B followed a linear change from 50% to 100%; from 10 to 15 min, B was isocratic at 100%; from 15 to 16 min, B linearly changed from 100% to 50% and from 16 to 20 min, B was isocratic at 50%. The mobile phase flow rate was 1 mL/min and the injection volume was 20 µL. UV detection was performed at 356 nm.

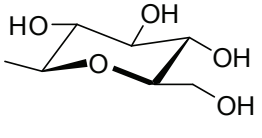
Using these chromatographic conditions, it was possible to confirm the retention time of rutin,



1 R=a
2 R=H
3 R=b



a



b

quercetin and isoquercitrin by injection of each standard separately.

Sample preparation

In the present study, 100 mg of crude extract were dissolved in 100 mL methanol. This sample was submitted to sonication to speed dissolution of the particles. Next, 2 mL were transferred to a suitable volumetric flask and diluted to 100 mL with mobile phase (solution B). A solution was obtained with a known concentration of 0.02 mg/mL.

Preparation of standard solution

Accurately weighed appropriate amounts of the reference compounds (rutin RUT; quercetin QUE; isoquercitrin ISO) were mixed and dissolved in methanol in a 200-mL volumetric flask, to obtain a stock solution. The concentration of the three compounds in this solution was 1000 µg/mL (RUT), 166.7 µg/mL (ISO) and 13.3 µg/mL (QUE). Besides, external standards were established at five data points covering the concentration range of each compound according to the level estimated in the plant sample. Working solutions were prepared by stepwise dilution of the stock solution with solution B of mobile phase.

Method validation

In the validation of the analytical method used for the quantification of rutin, quercetin and isoquercitrin in the methanolic extract of *D. gardneriana*, the following parameters were determined: specificity, linearity, sensitivity, accuracy, precision and robustness.

Specificity

Specificity is the ability of a method to discriminate between the study analyte(s) and other components in the sample. Specificity of the HPLC method is demonstrated by the separation of the analytes from other potential components such as impurities, degradants, or excipients (Dong, 2006). In this study, the specificity was demonstrated by running a procedural blank, where 2 mL of methanol were transferred to a suitable volumetric flask and diluted to 100 mL with mobile phase (solution B). In addition, the resolution between the peaks of the main flavonoids that could be found in methanolic extracts of *D. gardneriana* was determined by analysis of chromatograms of the standard solution and the sample solution. This resolution was calculated by Shimadzu CLASS-VP® software version 6.12 SP2.

Linearity

The linearity between peak area and concentration was analyzed using three calibration curves obtained with standard solutions at five different concentrations of each standard RUT, QUE and ISO. The concentrations of the three compounds in the solution that was considered 100% was 15 µg/mL (RUT), 2.5 µg/mL (ISO) and 0.2 µg/mL (QUE). The other concentration levels used to construct calibration curves were 80, 90, 110 and 120 % of the concentration mentioned above (Anvisa, 2003).The data for peak area versus drug concentration were treated by linear regression analysis.

Sensitivity

The limit of detection (*LOD*) and the limit of quantification (*LOQ*) were determined from the calibration curves of the RUT, QUE and ISO standards. *LOD* was calculated according to the expression DPx3/IC, where DP is the standard deviation of the response and IC is the slope of the calibration curve. LOQ was established by using the expression DP x10/IC (Anvisa, 2003).

Accuracy

The accuracy was evaluated by means of recovery assays carried out by adding known amounts of the RUT and ISO standard to the sample, at three different levels (5%, 10% and 15%) of the initial concentration of the sample. For evaluation of recovery of quercetin standard was added to the sample whose final concentration were 0.180, 0.200 e 0.220 µg/mL of this compound. Each solution was injected in triplicate. Average recoveries were calibrated by the formula recovery (%) = {(amount found - original amount)/amount spiked} x 100.

Precision

The precision of the method was investigated with respect to repeatability, intermediate precision (inter-day variation) and reproducibility by determination of standard solution at 100% of the test concentration. To assess the intra-day precision (repeatability) of the method, the sample was injected six times within a day. The inter-day precision was determined with the sample assayed on different days and by another analyst. Reproducibility was determined by injection of the sample six times and consequently comparing the results in two different laboratories. The main laboratory was the Quality Control, Farmace Indústria Químico-Farmacêutica Cearense, where all the tests were performed for this validation, while the other was the Central Analítica, Universidade Federal do Ceará, Campus Cariri. Precision was expressed as the relative

standard deviations (% RSD) of the concentrations of each compound, RUT, QUE and ISO.

Robustness

Three sample solutions were prepared and analyzed under the conditions established and by changing the wavelength parameter from 356 nm to 358 nm, by using columns from different suppliers and by changing the pH of the mobile phase from 3.0 to 3.1 (Anvisa, 2003).

Statistical analysis

The data were submitted to statistical analysis using GraphPadPrism5® and Excel® software.

Results and Discussion

The HPLC method carried out in this study was aimed at developing a chromatographic system, capable of eluting and resolving flavonoid compounds in a crude methanolic extract of *Dimorphandra gardneriana* Tul., Fabaceae. In the development of the HPLC method for determination of rutin (**1**), quercetin (**2**) and isoquercitrin (**3**) in a methanolic extract of *D. gardneriana*, several solvent systems (methanol-water, acetonitrile-water, tetrahydrofuran-buffer) and separation columns Thermo Scientific Hypurity C_8 column (250 x 4.6 mm i.d., 5 µm particle size), Thermo Scientific Hypersil C_{18} column (250 x 4.0 mm i.d., 5 µm particle size, Phenomenex Hyperclone BDS C_{18} (100 x 4.6 mm i.d., 5 µm particle size), Restek Pinnacle II C_8 (250 x 4.6 mm i.d., 5 µm particle size) were evaluated and compared. The Thermo Scientific Hypersil C_{18} column provided better separation of the plant extract than with other specifications or brands of columns.

The choice of detection wavelength was determined by performing a screening with 10 ppm of rutin, major compound, in methanol in a spectrophotometer UV/VIS. The UV spectra were recorded from 220 to 360 nm and exhibited maximum wavelengths at 254 nm and 356 nm. It was carried out an analysis on HPLC with the two wavelengths and which provided better response even for the others compounds (quercetin and isoquercitrin) was at a wavelength of 356 nm.

The optimization of the chromatographic conditions led to a good resolution of adjacent peaks of the flavonoids in this study when compared to other methods previously described for the quantification of these compounds in crude plant materials (Chou et al., 2009; Batista et al., 2010).

The results for quantification of the flavonoids in the sample were 14.506 µg/mL of rutin and 2.220 µg/mL of isoquercitrin , which means, 72.53 and 11.10% of each compound contained in the crude extract (20 µg/mL), respectively. Quercetin was not found in concentrations levels possible to be measured. It is important to note that this is the first report of quantification of flavonoids in crude extracts of *D. gardneriana.*

The specificity of the method was evaluated by analysis of the blank, standard and sample solution chromatograms (Figure 1). Good separation between the peaks of RUT, QUE and ISO was achieved, with the retention times, 7.5 min for rutin, 9 min for isoquercitrin and 16.5 min for quercetin. Furthermore, the chromatographic peaks showed good resolution (around 5.7 between RUT and ISO and approximately 27 between RUT and QUE). In relation to assimetry, the peaks showed values between 1.19 and 1.29 for RUT and ISO and 1.48 for asymetry of the peak of QUE.

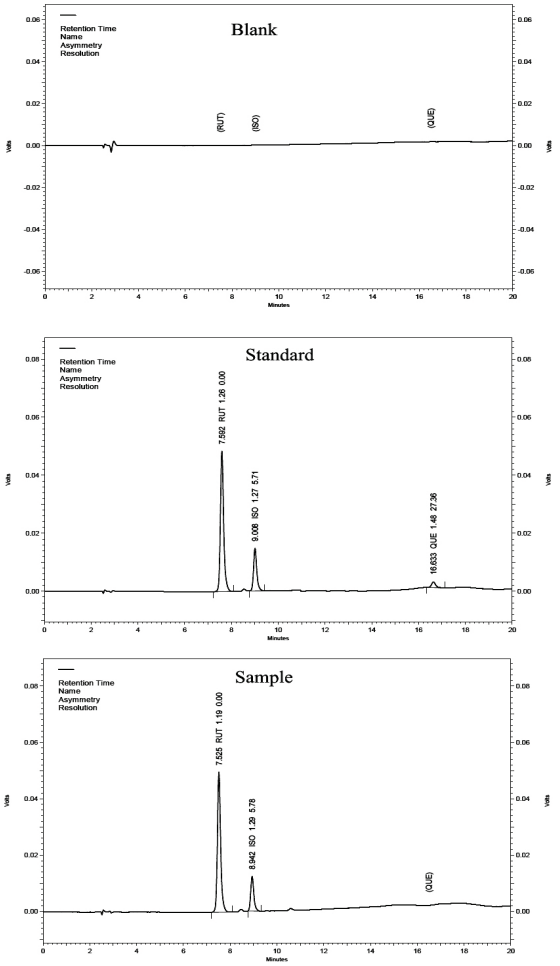


Figure 1. Chromatograms of the blank, standard and sample solution performed on a Thermo Scientific Hypersil C_{18} column (250 x 4.0 mm i.d., 5 µm particle size) at 356 nm.

Linearity was evaluated by the correlation coefficient r, and all values for the three compounds were greater than 0.995, showing that responses for the standard in the concentration ranges examined (from

80 to 120%) were linear. Besides, according to Anvisa (2003), the minimum acceptable correlation coefficient is 0.990.

As shown in Table 1, the LOD values were 0.09 µg/mL, 0.02 µg/mL and 0.02 µg/mL for the compounds RUT, ISO and QUE, respectively, while the LOQ values were 0.29 µg/mL, 0.07 µg/mL and 0.05 µg/mL.

The recovery of the compounds RUT, QUE and ISO was determined by spiking the crude extract with known amounts of RUT, QUE and ISO standards. Recovery of each substance was obtained from the calculated amount found and original amount. The results are presented in Table 2 and conform with the recommendations of Anvisa (Anvisa, 2003).

The data of the precision are shown in Table 3. The results display a coefficient of variation less than that recommended by RE 899 (Anvisa, 2003) whose limit is 5%. Also, there were no significant differences between assay results, indicating that the precision of the proposed method was satisfactory.

Table 1. Calibration curve parameters, limit of detection (LOD), limit of quantification (LOQ) for rutin, isoquecitrin and quercetin.

Compound	Calibration curve equation	Correlation coefficient (r)	Linear range (µg/mL)	LOD(µg/mL)	LOQ(µg/mL)
rutin	y = 32844.31x + 178861.53	0.9987	12-18	0.09	0.29
isoquercitrin	y = 58813.51x + 52111.13	0.9983	2-3	0.02	0.07
quercetin	y = 128566.83x + 25130.40	0.9982	0.16-0.24	0.02	0.05

Table 2. Results of accuracy determination by analyzing of the rutin, quercetin and isoquercitrin of known concentrations.

Compound/Initial concentration	Theoretical concentration after dilution added in the extract (µg/mL)	Amount recovered (µg/mL)	Recovery (%)	Mean (%)	RSD (%)
Rutin (Concentration measured in the sample = 14,506 µg/mL)	0.725	15.315	100.55		
		15.753	103.43	101.92	1.42
		15.504	101.79		
	1.45	15.875	99.49		
		16.124	101.05	100.28	0.78
		15.987	100.19		
	2.175	16.735	100.32		
		16.789	100.65	100.20	0.51
		16.621	99.64		
Isoquercitrin (Concentration measured in the sample = 2,220 µg/mL)	0.111	2.365	101.46		
		2.305	98.88	99.79	1.45
		2.308	99.01		
	0.222	2.451	100.37		
		2.467	101.02	101.05	0.69
		2.485	101.76		
	0.333	2.580	101.06		
		2.571	100.71	100.09	1.38
		2.515	98.51		
Quercetin* (Concentration measured in the sample lower than LOQ and LOD)	0.180	0.183	101.67		
		0.185	102.78	103.33	1.94
		0.189	105.56		
	0.200	0.205	102.50		
		0.208	104.00	102.33	1.72
		0.201	100.50		
	0.220	0.225	102.27		
		0.228	103.64	102.12	1.56
		0.221	100.45		

*Since the concentration of quercetin measured was lower than LOQ and LOD to calculate the recovery its initial concentration was considered as 0.00 µg/mL).

Table 3. Results of the repeatability, intermediate precision and reproducibility tests.

Compound	Repeatability	RSD (%)
	Mean (µg/mL)±standard deviation (n=6)	
rutin	15.062±0.083	0.551
isoquercitrin	2.491±0.007	0.294
quercetin	0.201±0.003	1.457
Compound	Intermediate precision	RSD (%)
	Mean (µg/mL)±standard deviation (n=12)	
rutin	15.076±0.103	0.692
isoquercitrin	2.494±0.013	0.473
quercetin	0.200±0.002	1.170
Compound / Laboratory	Reproducibility	RSD (%)
	Mean (µg/mL)±standard deviation (n=6)	
rutin / Farmace	15.065±0.101	0.668
UFC	15.213±0.121	0.621
isoquercitrin / Farmace	2.494±0.020	0.798
UFC	2.594±0.044	1.699
quercetin / Farmace	0.201±0.003	1.587
	0.202±0.002	1.047

Robustness was evaluated to ensure that the HPLC method is insensitive to small changes in the experimental conditions. In this study, the wavelength, column supplier and pH of the mobile phase were changed. None of the modifications caused any significant change in the resolution or response of the RUT, QUE and ISO peaks.

All results were displayed according to the Guide for Validation of Analytical and Bioanalytical Methods RE 899 (Anvisa, 2003).

The quantitative method developed here was successfully applied in the simultaneous analysis of three different compounds in a crude methanolic extract of *D. gardneriana.* Taking into account the results obtained in this study, the proposed method can be conveniently used for the analysis of rutin, isoquercitrin and quercetin in crude methanolic extracts of *D. gardneriana* pods.

The proposed method demonstrated high specificity at 356 nm detection for the pod extract of *D. gardneriana* showing reliability in the quantification of RUT, ISO and QUE. Furthermore, the method has the following advantages: rapid extraction, easy sample preparation and short time between injections. In summary, the method above can be considered specific, exact, precise, linear, robust and easy to perform.

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