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Development and validation of a high performance liquid chromatography-diode array detection (HPLC-DAD) method for the quantification of rutin and isoquercetin in *Morus nigra* L. (Moraceae)

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***Morus nigra* L. is widely used by the population of the São Francisco Valley (Brazil) for the treatment of several diseases such as hypertension, diabetes, arthritis etc. The objective of this study was to develop and validate an analytical method that is capable of identifing and quantifing flavonoids in the ethanolic crude extract (Mn-EtOH) and its ethyl acetate phase (Mn-AcOEt) obtained from *M. nigra* leaves using high performance liquid chromatography (HPLC). The plant material was collected and processed to obtain Mn-EtOH, which was subjected to a process of liquid/liquid partition, from which the AcOEt phase was obtained. The method was developed using an reverse phase (RP) C-18 column (250 × 4.6 mm, 5 µm) as the stationary phase. A mixture of acidified water (0.01% trifluoroacetic acid): acetonitrile was used as the mobile phase in a gradient system with a flow rate of 0.8 mL/min at a wavelength of 340 nm for the detection and determination. The method developed was considered to be specific: linear (R^2 > 0.99), selective, accurate, and robust.**

Key words: Flavonoids, isoquercetin, rutin, morus.

INTRODUCTION

Moraceae is a family of a flowering plant that comprises about 40 genera and 1400 species. Mulberry (*Morus* species) has been domesticated over thousands of years and has been adapted to a wide area of tropical, subtropical, and temperate zones of Asia, Europe, North and South America, and Africa. *Morus nigra* L. (Moraceae), known as ‘‘black mulberry” belongs to the genus *Morus* and is found in Africa, South America and in

Asia, possessing a wide range of medicinal uses and can be used either as single or associated drug to treat different ailments (Freitas et al., 2016).

The species under study, *M. nigra* L., is originally from the far east, being brought to the region of Vale do São Francisco, Northeastern Brazil, by Japanese immigrants, a place in which it has adapted well to the climate and soil conditions (Oliveira et al., 2013). In this region, it is popularly known as “amora-miúra”, being widely used for the treatment of diabetes, cholesterol, cardiovascular problems and gout (Oliveira et al., 2013); it has anticâncer (Qadir et al., 2014), antimicrobial (Mazimba et al., 2011), and hepatoprotective activities (Mallhith et al., 2014). Another study described that the total flavonoids found in black mulberry fruits possess antiinflamatory and analgesic effects (Chen et al., 2016).

In view of the need to encourage the undertaking of studies and development and use of the herbal medicines, the Brazilian government issued the Decree n° 5813 in 2006, which deals with the "National Policy of Medicinal Plants and Herbal Medicines" (Brasil, 2006). In 2009, the Ministry of Health disclosed the National List of Medicinal Plants of Interest (RENISUS), to the Unified Health System (SUS) being the *Morus* genus among the plants included in this list. It is noteworthy that the availability of validated assay methods is an important part of the quality control of these products, and such assays are required by the Brazilian health authorities for registration of phytomedicines (Lopes et al., 2010).

Thus, considering the absence of studies with the species cultivated in the region of Vale do São Francisco, in the city of Casa Nova, BA, this work was conducted with the objective to develop and validate one analytical method to identify and quantify the flavonoids rutin and isoquercetin in extracts of leaves of *M. nigra* L.

MATERIALS AND METHODS

Plant

Leaves of *M. nigra* L. were collected in January, 2013 in the city of Casa Nova, Fazenda Ouro Verde (Ouro Verde Farm), Bahia (S 9°16’15”; W 40°51’44”). Voucher specimens were deposited at the Vale do São Francisco Herbarium (HVASF) of the Universidade Federal do Vale do São Francisco (UNIVASF) under the number 1764.

Extract preparation

The dried and powdered plant material (3 kg) underwent exhaustive maceration with 95% ethanol until depletion of the plant drug. The extractive solution was subjected to a solvent distillation process in a rotary evaporator under reduced pressure and average temperature of 50°C. 57.24 g of the ethanolic extract of *M. nigra* (Mn-EtOH) was obtained. Mn-EtOH was subjected to a liquid-liquid extraction. The extract was suspended in a water and methanol mixture (8:2) and the solution was stirred for 50 min. The solution was extracted with hexane (HEX), chloroform ($CHCl_3$), and ethyl acetate (AcOEt), in an increasing polarity order. The organic solvent was removed from each extractive solution under reduced pressure in a rotary evaporator. Thus, the organic phase extracts Mn-HEX, Mn-$CHCl_3$, and Mn-AcOEt were obtained.

Chemicals and reagents

Rutin and isoquercetin (Sigma, USA) of the highest grade (purity > 98.0%) were used as the external standards. High performance liquid chromatograpgy (HPLC)-grade methanol and acetonitrile were purchased from Tedia (Fairfield, OH, USA). Ultrapure water was obtained from Milli-Q Gradient® (Smart, Healforce, USA), having conductivity less than 0.6 µS/cm in all the analyses. Ethanol, methanol, hexane, chloroform, and ethyl acetate, all for analytical purpose grade, were obtained from Synth (Brazil).

Instrumentation and chromatographic conditions

All the chromatographic analyses were carried out in the Central de Análises de Fármacos, Medicamentos e Alimentos, at UNIVASF. The analytical method was developed and validated using a Shimadzu® chromatograph equipped with a quaternary pump system (model LC, 20 ADVP), degasser (model DGU, 20A), photodiode array (PDA) detector (model SPD, 20AVP), thermostated column compartment (model CTO, 20ASVP), autosampler (model SIL, 20ADVP), and a controller (model SCL, 20AVP). All data were analyzed through Shimadzu® LC solution 1.0 software (Japan). Study solutions were submitted individually to analysis under the following chromatographic conditions: RP C-18 column (250 × 4.6 mm), 5 µm particle size (Thermo Scientific® Hypersil). The mobile phase consisted of water (0.01% trifluoroacetic acid [TFA]) as solvent A and acetonitrile as solvent B. Separations were effected by a linear gradiente as follows: 0 min 0% B; 40 min 40% B; 60 min 0% B. The mobile phase flow rate was 0.8 mL/min and the injection volume was 5 µL. The chromatographic runs were carried out at 37°C. UV detection was performed at 340 nm.

Sample preparation of extracts

Stock solutions (10 mg/mL) were prepared from 250 mg of the Mn-EtOH and Mn-AcOEt extracts, which were transferred to 2 different 25 mL volumetric flasks and solubilized with HPLC grade methanol. Sonication for 6 min was used to optimize the solubilization process. Seven different concentrations ranging from 800 to 1200 µg/mL were prepared from the stock solutions in order to perform the analyses.

Standard solutions preparation

Rutine (RUT, 200 µg/mL) and isoquercetine (ISO, 600 µg/mL) stock

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solutions were prepared using HPLC grade methanol as the solvent and sonication. The working solutions ranged from 1.44 to 2.16 µg/mL and from 4.08 to 6.12 µg/mL for rutine and isoquercetine, respectively, using HPLC grade methanol as the diluting solvent.

Validation method

For the validation of the analytical method, the following parameters were determined: specificity, linearity, limit of detection and limit of quantification, precision (repeatability and intermediate precision), accuracy and robustness, in accordance with the standards set by RE n° 899/03 of ANVISA (Brasil, 2003). Analyses were performed in triplicate and the reliability of the parameters were assessed by analyzing the coefficient of variation (CV%) or standard deviation (SD), not being admitted above 5% for analytical standards and 15% for plant extracts (ICH, 2005). Data were statistically analyzed one-way or two-way analysis of variance (ANOVA) if applicable. Statistical analysis and graphs were obtained in OriginPro8®.

Specificity

The specificity was demonstrated by analysing a 1000 µg/mL solution of *Baccharis trimera* lyophilized extract under the same chromatographic conditions of the extracts under study (Mn-EtOH and Mn-AcOEt) and reference substances RUT and ISO. The chromatograms obtained were compared regarding its substances retention times and UV spectra.

Linearity

The extracts solutions were prepared (Mn-EtOH and Mn-AcOEt) in 7 different concentrations from 800 to 1200 µg/mL, corresponding to 80 to 120% working range. Analytical standards were also evaluated at 7 concentrations. However, RUT was evaluated in the range of 1.44 to 2.16 µg/mL and ISO 4.08 to 6.12 µg/mL. All analyses were performed in triplicate to obtain the calibration curve from the average of these values. The analysis of linear regression was performed by the method of least squares to obtain the equation of the line and the correlation coefficient.

Limit of detection (LOD) and limit of quantification (LOQ)

These parameters were determined from calibration curves and the LOD and LOQ values were calculated using the following equations (BRASIL, 2003):

$$LOD = SDa \times 3/IC \quad (1)$$
$$LOQ = SDa \times 10/IC \quad (2)$$

where SDa is the standard deviation of the intercept with the Y axis, obtained from the average of the three linearity curves and IC is the slope of the line of the respective calibration curves.

Accuracy

This parameter was qualitatively evaluated by the method of addition of standard, where known concentrations of RUT and ISO were added to extracts Mn-EtOH and Mn-AcOET, with the purpose of observing the increase of the intensity of the respective peaks in the chromatograms.

Precision

Precision method was evaluated in terms of repeatability and intermediate precision in accordance with RE 899/2003 (Brasil, 2003). For repeatability, 6 different solutions (1000 µg/mL) from each extract (Mn-EtOH and Mn-AcOEt) prepared by the same analyst were assayed. The intermediate precision was evaluated by analyzing 2 different solutions of the same extract and with the same concentration (1000 µg/mL) in 2 different days by 2 different analysts.

Robustness

The robustness of the method was evaluated through the change in its detection wavelength (from 340 to 342), solvent manufacturers, and mobile phase composition. The obtained data were compared to those from the original method proposed.

RESULTS AND DISCUSSION

During this study, a method was developed by using the HPLC technique in order to obtain a chromatographic system that was able to elute and provide good resolution in the separation of compounds from extracts (Mn-EtOH and Mn-AcOEt) obtained from leaves of *M. nigra* L. For this purpose, in a preliminary way, the chromatographic conditions of the method developed by Tallini et al. (2015) were selected, in which modifications were made. Tallini et al. (2015) performed the identification and quantification of kaempferol and quercetin in hydrolysed extracts of *M. nigra* using 0.01% TFA as the mobile phase A and 0.08% ACN as the mobile phase B. Moreover, Hunyadi et al., (2012) conducted a study to quantify chlorogenic acid, rutin, and isoquercetin in *M. alba* extracts using these same solvents as mobile phase.

Consequently, the chromatographic conditions used in this study, including the composition of the mobile phase, as well as the gradient condition, and the analysis time were also effective for the identification and quantification of substances rutin and isoquercetin in Mn-EtOH and Mn-AcOEt.

The chromatograms were obtained at a detection wavelength of 340 nm, since the phenolic compounds show maximum absorption around this wavelength, and also for achieving better chromatographic profiles, once in the 254 nm other peaks were evident. Initially, a phytochemical screening was performed on Mn-EtOH and Mn-AcOEt. The obtained chromatogram showed intense and well-resolved peaks. In order to identify the substances present in the extract, 20 phytochemical standards were subjected to the same chromatographic conditions.

The retention time and UV spectra obtained were compared to those obtained from the extracts aiming to identify the substances. In the chromatogram of Mn-EtOH, the presence of 19 well-resolved peaks were

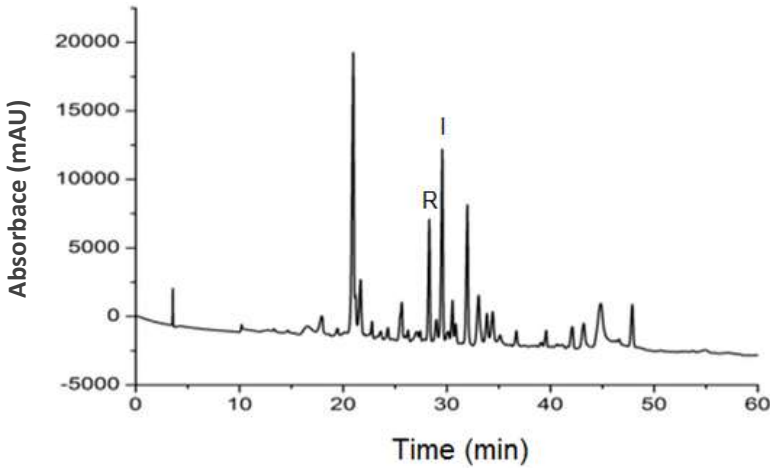

Figure 1. Chromatogram of Mn-EtOH.

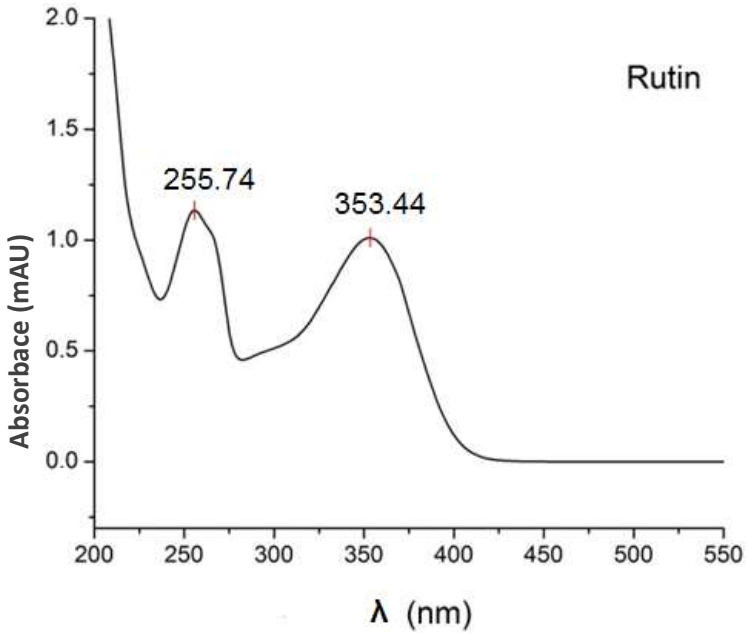

Figure 2. UV spectra of rutin.

observed with purity ≥98% (Figure 1). Only 2 of the substances were identified: rutin (R) (peak 1) and isoquercetin (I) (peak 2), with retention time of 28.3 and 29.3 min, respectively. The peaks identity was confirmed by comparing the UV spectra obtained from the samples with those from the standards (Figures 2 and 3).

In the chromatogram of Mn-AcOEt (Figure 4), 11 well-resolved peaks were observed with a purity ≥98%. As in the Mn-EtOH chromatogram, rutin and isoquercetin were also identified with the same retention times.

It is well known that the UV spectra of flavones and flavonols in methanol present 2 bands of increased absorption in the 240 to 400 nm region. The first band, or band I, stands between 300 and 380 nm, while the second band, or band II, stands between 240 and 280 nm. Band I is related to the flavonoids B ring absorption, while band II is related to the A ring.

Freitas et al. (2016) used the same technique as this study and the analysis revealed the presence of chlorogenic acid, rutin and its major compound, isoquercitrin. In this study, inhibitory activity of tyrosinase by extracts from *M. nigra* leaves as well as the

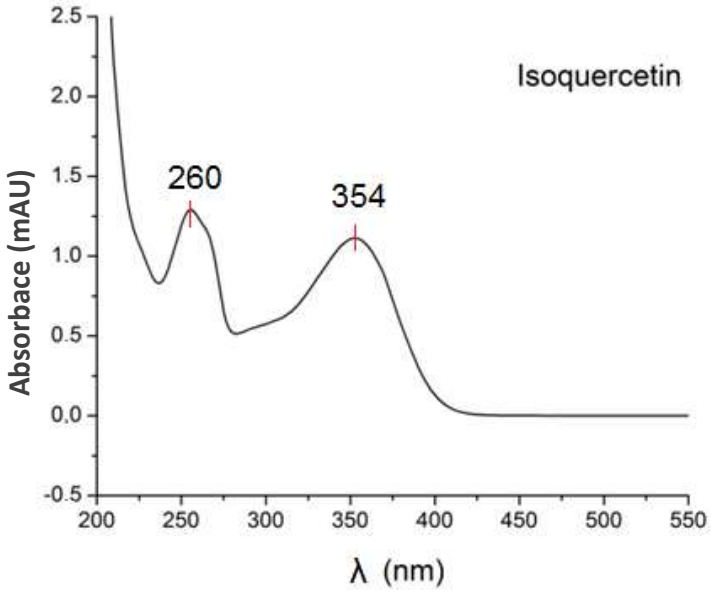

Figure 3. UV spectra of isoquercetin.

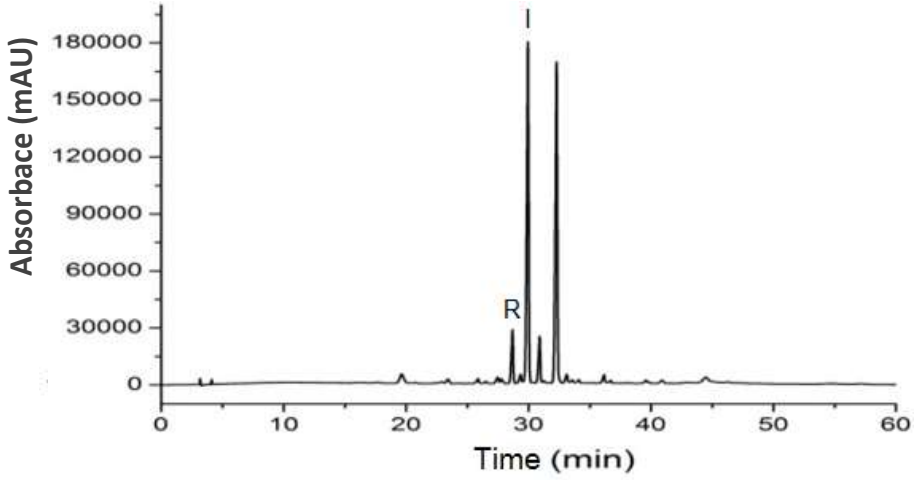

Figure 4. Chromatogram of Mn-AcOEt.

characterization of its chromatographic profile and cytotoxicity was evaluated to obtain a new therapeutic option from a natural source.

Validation of the analytical method

Specificity of the method

The developed method was specific, as shown in Figure 5, which brings the overlapping between the chromatograms of species *B. trimera* and RUT and ISO standards. It is noteworthy that all analyses were performed under the same chromatographic conditions. No peak was observed on the *B. trimera* chromatogram eluting at the same retention time of the RUT and ISO standards, which confirms the specificity of the analytical method.

System suitability tests are integral parts of liquid chromatography methods once they are applied in order to verify if the resolution and reproducibility of the chromatographic system are adequate for the analyses to

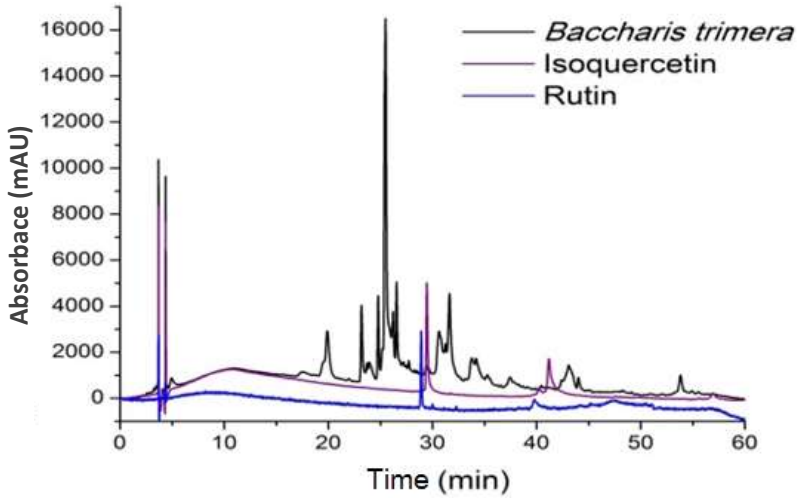

Figure 5. Evaluation of the specificity of the method.

Table 1. Chromatographic parameters of Mn-EtOH and Mn-AcOE.

Sample		Peak resolution	Tailing factor	Theoretical plates
Mn-EtOH	RUT	3. 48	1.130	1356371
	ISO	1.702	1.153	1809392
Mn-AcOEt	RUT	3.5	1.133	1941962
	ISO	1.150	1.652	1848387

Table 2. Linear equation, linearity, limit of detection (LOD) and quantification (LOQ) of rutin and isoquercetin in Mn-EtOH and Mn-AcOEt by HPLC-DAD.

Extract	Flavonoid	Linear equation	R^2	LOD (µg/mL)	LOQ (µg/mL)
Mn-EtOH	Rutin	y=39.456x - 12241	0.9877	766.97	2556.34
	Isoquercetin	y=85.17x - 19714	0.9966	744.46	2481.53
Mn-AcOEt	Rutin	y=343.81x - 82576	0.9866	370.78	1235.93
	Isoquercetin	y=2621.2x - 723284	0.9911	410.62	1368.80

be performed. The limits recommended are asymmetry factor (T) less or equal to 2.0 and the number of theoretical plates (N), generally, higher than 2000 by column (Brasil, 2010). The number of theoretical plates is an indication of the efficiency of the column and the resolution (R) is the chromatographic parameter that indicates the degree of separation between 2 substances in a mixture. The area or peak height are proportional to the amount of the eluted substance. The asymmetry factor (T) indicates the symmetry of the peak and it is equal to 1 when the peak is perfectly symmetrical (Brasil, 2010). Therefore, the suitability of the system was proven through the analysis of the aforementioned parameters. The values were calculated automatically by the equipment's software and all were within the limits specified in the literature shown in Table 1.

Linearity

This parameter was determined by constructing calibration curves of the extracts (Mn-EtOH and Mn-AcOEt) in the range of 800 to 1200 µg/mL, which correspond to the range of 80 to 120% of the working concentration. The results are shown in Table 2.

The correlation coefficients (R^2) were obtained from

Table 3. Analysis of variance (ANOVA) for the curves of the extracts under study.

Sample			Residue	Regression	Total
		SQ	71799060.3	705088203	776887263
		G*l*	2	18	20
	RUT	MQ	35899530	39171567	-
		F cal	0.916469	-	-
		F tab	3.554557	-	-
Mn-EtOH					
		SQ	206267179.1	4567116251.1	4773383430
		G*l*	2	18	20
	ISO	MQ	2880580921	2786696905	-
		F *cal*	1.0337	-	-
		F *tab*	3.5546	-	-
		SQ	5761161842	50160544295	55921706137
		G*l*	2	18	20
	RUT	MQ	2880580921	2786696905	-
		F *cal*	1.0337	-	-
		F *tab*	3.5546	-	-
Mn-AcoEt					
		SQ	383015909816.7	2597481198640.3	2.9805E+12
		G*l*	2	18	20
	ISO	MQ	191507954908.3	144304511035.6	-
		F *cal*	1.3271	-	-
		F *tab*	3.5546	-	-

linear regression analysis greater than 0.99 and 0.98, for ISO and RUT, respectively, in both Mn-EtOH and Mn-AcOEt extracts. The higher difficulty in obtaining more accurate and precise results with complex matrices, such as herbal extracts, is taken in consideration when validating a method by its correlation coefficient. Although, the rutin correlation coefficient was lower than that of isoquercetin, these results demonstrate that there is linearity within the choosen working range. It is believed that the lower correlation coefficient obtained for rutin is directly related to its lower concentration in both extracts when compared with isoquercetin.

Therefore, these results complies with the Brazilian regulatory agency legislation regarding the matter (Brasil, 2003). This parameter was also assessed by analysis of the calibration curve of the substances identified in the extracts using the method of least squares and ANOVA (Table 3). All analyses showed satisfactory results with values of F calculated below the critical F values.

LOD and LOQ

The LOD and LOQ results were obtained from 3 different calibration curves for each standard (RUT and ISO) for Mn-EtOH and Mn-AcOEt. The Mn-EtOH results, LOD, found were 766.97 and 744.46 µg/mL for RUT and ISO, respectively. The LOQ were 2556.34 and 2481.53 µg/mL for RUT and ISO, respectively (Table 2).

The Mn-AcOEt results, LOD, found were 370.78 and 410.62 µg/mL for RUT and ISO, respectively. The LOQ were 1235.93 and 1368.80 µg/mL for RUT and ISO, respectively (Table 2). The results show that the proposed method has an adequate sensitivity to detect and quantify the analysed substances.

Accuracy

The proposed method accuracy was measured by the standard addition method. In this procedure, an amount of the analyte is added to a known concentration solution of the same analyte and the final concentration is measured. The amount of the substance recovered from the sample reflects the accuracy of the method. However, the objective of this study was to verify this parameter in a qualitative way, being possible to verify the increase of intensity of the peaks referring to rutin and isoquercetin in

Table 4. Results obtained in the intermediate precision analysis.

Sample		Analyst	Day 1 (µg/mL)	Day 2 (µg/mL)	F
Mn-EtOH	RUT	1	4.81	4.47	F *cal* 2.02
		2	4.68	4.19	F *cri* 6.60
	ISO	1	12.15	11.2	F *cal* 5.04
		2	11.42	10.16	F *cri* 6.60
Mn-AcOEt	RUT	1	4.81	4.47	F *cal* 3.52
		2	4.68	4.19	F *cri* 6.60
	ISO	1	12.15	11.20	F *cal* 0.003
		2	11.42	10.16	F *cri* 6.60

Table 5. Results of the robustness tests of Mn-EtOH.

Parameter	Variable	Mean (µg/mL) ± DP (%)	Fcal	Fcri
Detection wavelength	λ = 340 nm	RUT 4.46 ± 0.93	1.86	7.70
		ISO 10.93 ± 6.57	0.04	7.70
	λ = 342 nm	RUT 4.52 ± 1.25	1.86	7.70
		ISO 11.05 ± 6.12	0.04	7.70
Brand solvent	J.T.Baker®	RUT 4.68 ± 4.7	3.43	7.70
		ISO 11.43 ± 3.72	6.49	7.70
	Carlo Erba®	RUT 4.42 ± 2.33	3.43	7.70
		ISO 10.68 ± 2.52	6.49	7.70

the two extracts evaluated.

Precision

The method repeatability was evaluated through the assay of 6 different solutions with the same concentration for each extract. Mn-EtOH extract presented a relative standard deviation (RSD) equal to 8.92 and 8.45% for rutin and isoquercetin, respectively. Mn-AcOEt extract presented a RSD equal to 5.8 and 1.8% for rutin and isoquercetin, respectively. The intermediate precision was evaluated by 2 different analysts using the same equipment within 2 consecutive days. The results are shown in Table 4.

Taking the obtained results into consideration, the proposed method can be considered precise once the relatives standards deviations (RSD) found were smaller than 15%, considering plant extracts. In addition, the RUT and ISO content were higher in Mn-AcOEt than in Mn-EtOH, which was already expected considering the polarity of the solvent used to extract the substances from the etanolic extract.

Robustness

Robustness tests are very useful, since they are capable of displaying factors that can influence significantly the response of the studied method. In this study, changes were performed on the brand of the solvent used for HPLC analyses and the wavelength of detection (Table 5).

Conclusions

The developed method was suitable for extract quatitative analysis, indicating the presence of the flavonoids rutin and isoquercetin in Mn-EtOH and Mn-AcOEt.

The chromatograms showed other intense and resolved peaks, which suggest the presence of other phenolic compounds and the need of further studies to identify them. The validation step was successfully executed with all parameters being evaluated. The validation of the method will allow the continuation of the studies involving *M. nigra* extracts in order to compare the influence of different extraction methods on the content of these substances and in an attempt to correlate the quantified substances in the extract with potential pharmacological activities.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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