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Determination of rutin in *Erythroxylum suberosum* extract by liquid chromatography: applicability in standardization of herbs and stability studies

[Determinación de rutina en el extracto *Erythroxylum suberosum* por cromatografía líquida: aplicación de la normalización de las hierbas y los estudios de estabilidad]

Caio Fernandes Monteiro LEITE[1], Bruno Henrique Monteiro LEITE[1], Ivelone Maria de Carvalho BARROS[2], Sueli Maria GOMES[3], Christopher William FAGG[1], Luiz Alberto SIMEONI[2], Dâmaris SILVEIRA[2] & Yris Maria FONSECA[2]*

[1]Faculdade de Ceilândia, Universidade de Brasília, Campus Ceilândia, Ceilândia-DF, Brazil.
[2]Faculdade de Ciências da Saúde, Universidade de Brasília, Campus Darcy Ribeiro, Brasília-DF, Brazil.
[3]Instituto de Biologia, Universidade de Brasília, Campus Darcy Ribeiro, Brasília-DF, Brazil.
Contactos | Contacts: Yris Maria FONSECA - E-mail address: yrisfonseca@hotmail.com

Abstract*:* In this study, a reverse phase-high performance liquid chromatography (RP-HPLC) technique for determination of rutin in *Erythroxylum suberosum* extract was developed and validated. A regression analysis was performed, with the observation of good linearity (r = 0.9997). The values obtained for precision and accuracy determination are in agreement with ICH guidelines. The detection and the quantitation limits of rutin were 0.19 µg/mL and 0.60 µg/mL, respectively. The results demonstrated that the developed method is a reliable HPLC technique for determination of rutin in *E. suberosum* extract. In addition, the applicability of this method in stability studies and standardization of herbs was investigated.

Keywords: *Erythroxylum suberosum*, rutin, HPLC.

Resumen: En este estudio, la técnica de cromatografía líquida de alta resolución en fase reversa para la determinación de la rutina en el extracto *Erythroxylum suberosum* fue desarrollada y validada. Se realizó un análisis de regresión, con la observación de una buena linealidad (r = 0,9997). Los valores obtenidos para la precisión y la determinación de la precisión están de acuerdo con las directrices ICH. La detección y cuantificación de los límites de la rutina fueron 0,19 µg / mL y 0,60 µg / mL, respectivamente. Los resultados demostraron que el método desarrollado es una técnica fiable de HPLC para la determinación de la rutina en el extracto de *E. suberosum*. Además, se investigó la aplicabilidad de este método en los estudios de estabilidad y la estandarización de hierbas.

Palabras Clave: *Erythroxylum suberosum*, rutina, HPLC

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INTRODUCTION

The Erythroxylaceae family consists of eight genera: *Erythroxylum, Nectaropetalum, Peglera, Pinacopodium, Sethia, Steudelia, Umbellulanthus* and *Aneulophus* (IPNI, 2012). The genus *Erythroxylum* comprises about 230 species, best known for the species *E. coca* L., from which cocaine is derived (Plowman and Hensold, 2004).

The ethnomedicinal uses of *Erythroxylum* species are as stimulants, euphoriants, to reduced fatigue, stomach problems, headache and sore throat. They have also been used against hepatic and renal disorders, as local anesthetic, aphrodisiac, anti-hemorrhagic, anti-inflammatory, for healing and fever (González-Garcia *et al.,* 2005). Pharmacologically, extracts from several species of this genus were studied by evaluating the cytotoxic effect, the antibacterial and antiviral activities, among others (González-Garcia *et al.,* 2005, González-Lavaut *et al.,* 2008).

With respect to the species *E. suberosum*, popularly known as "cabelo-de-negro", it is used by Kayapó people for stomach disorders, as anti-rheumatism and anesthetic remedies. Also, the fruits are useful as food for wildlife (Aquino *et al.,* 2007, Barbosa and Pinto, 2003). Descriptions about pharmacological use of the species *E. suberosum* were not found. However, *Erythroxylum* genus is well-known for the occurrence of pharmacologically relevant tropane derived alkaloids, in addition to flavonoids and terpenoids.

Nascimento *et al.* (2012) reported that from the ethanolic extract from *E. suberosum* branches five diterpenes bearing abietane and *ent*-kaurane- type skeletons, as well as ombuin-3-rutinoside and rutin were identified. Also, tropane alkaloids have not been detected in the crude extracts of either leaves or branches of *E. suberosum* and suggested the usefulness of flavonoids as chemotaxonomic markers for the genus *Erythroxylum* in the Erythroxylaceae.

Rutin has great importance in pharmaceutical industry, due to pharmacological activities (Becho *et al.,* 2009). The pharmacological activities of rutin are well known, as antioxidant (Araújo *et al.,* 2013), improve the strength and permeability of capillaries (Pathak *et al.,* 1991), protects against the functional impairment of the endothelium-dependent vasorelaxation (Fuentes *et al.,* 2013), anti-inflammatory (Yildizoğlu-Ari *et al.,*1991), anti-carcinogenic (Araújo *et al.,* 2013), and others.

In this study, a reverse phase-high performance liquid chromatography (RP-HPLC) technique for determination of rutin in *E. suberosum* extract was developed and validated. In addition, the applicability of this method in stability studies and standardization of herbs were investigated.

MATERIALS AND METHODS

Plants and Chemicals

Erythroxylum suberosum leaves were collected in the tropical savanna ecoregion (Cerrado) of the Federal District – Brazil. The species was identified by comparison with a voucher specimen deposited at the Herbarium of the Universidade de Brasília by number Fagg CW2192. Phosphoric acid of chromatographic grade was supplied by Sigma-Aldrich (St Louis, MO, USA). Acetonitrile (ACN) and methyl alcohol (MeOH) for use in chromatography and spectrophotometry were purchased from Tedia (USA). The water used to prepare the solutions or mobile phase was purified in a Milli-Q-plus System (Millipore®, Bedforte, MA, USA). Rutin (95%) was supplied by ChromaDex® (Irvine, CA, USA).

Preparation of E. suberosum extract

E. suberosum leaves were dried at room temperature (25° C). The process drying end was determined by moisture analyser (Gehaka IV2000, São Paulo, Brazil). Dried leaves showed 5 -10% moisture. The aqueous extract was obtained by infusion (129 g) in distilled water (3 L) at 70° C. The mixture was cooled to 40° C, being filtered and stored at -20° C until lyophilization. Three different extractions were prepared and analyzed by HPLC. All extracts were stored at -20° C until the time of analysis.

Determination of rutin by HPLC

Apparatus and chromatographic conditions

The aqueous extract was analyzed using LaChrom Elite HPLC system (Hitachi, Tokyo, Japan) liquid chromatograph equipped with L2130 pump, L2200 auto-sampler; L2300 column oven was set at 25 °C and a L2455 DAD detector (Hitachi, Tokyo, Japan). The detector was set at 354 nm. Separation was performed by Purospher Star reverse phase C18e column (5 µm, 150 mm x 4.6 mm i.d.) in combination with an appropriate guard column (4 x 4; 5 µm particle size) (Merck, Germany). The eluents used were: aqueous phosphoric acid (1%) (solvent A) and acetonitrile (solvent B). The gradient employed was:

90% A and 10% B for 0 min, 70% A and 30% B for 40 min, 50% A and 50% B for 50 min, 90% A and 10% B for 55 min, at a flow rate of 0.6 mL/min. Data acquisition was performed using EZChrom Elite software (version 3.3.2 SP1 (Scientific Software. Inc.). Sample solution was prepared dissolving 10 mg of aqueous extract into 1.2 mL H_2O and 0.8 mL MeOH. The solution obtained was then filtered. The compounds present in the extract were characterized according to their UV–Vis spectra and identified by their retention times in comparison with those of commercial standards (ChromaDex®). Three different extracts were analyzed by HPLC.

Validation

In the validation studies, the three extracts were prepared with leaves collected in the same seasonal period. Validation was performed following the ICH guidelines (1996). The method was validated considering the parameters linearity, accuracy, precision, limit of detection, limit of quantitation and robustness.

Linearity was checked with standard solutions of rutin in the concentration range of 7.8 – 1000.0 µg/mL. The standard curves were analyzed by linear regression of peak area versus rutin concentration.

Precision was expressed as relative standard deviation (RSD %) and was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by analysis of six replicates of the same extract in the same day under the same experimental conditions. The intermediate precision was studied on three different days.

The accuracy experiments applied the standard addition method (rutin) in the sample (*E. suberosum* extract), using replicates (n = 3) of three different rutin concentrations.

For that, the *E. suberosum* extract solution was prepared by diluting 10 mg of concentrated extract into 1.2 mL of methanol and 0.8 mL of water (150 µg/mL of rutin). Next, 500 µL of this solution were added to 500 µL of rutin solution in the concentration of 90.0 µg/mL, 150.0 µg/mL and 210.0 µg/mL, obtaining rutin theoretical final concentrations of 120.0, 150.0 and 180.0 µg/mL.

Finally, the solutions were filtered and analyzed by the developed HPLC method. Accuracy was expressed as percent of recovery, which was estimated as the relation between the experimental concentrations and the theoretical concentrations $[(C_e/C_t) \times 100]$.

The limit of detection (LOD) and limit of quantitation (LOQ) were determined on the basis of the standard deviation of the response and the slope of the constructed calibration curve. The LOD was expressed as $(3.3 \times \sigma)/S$ and, the LOQ was expressed as $(10 \times \sigma)/S$, where σ is the standard deviation of the response and *S* is the slope of calibration curve.

Robustness was examined by evaluating the influence of small variation in the experimental parameters on the analytical performance of the proposed method. The studied parameters were; variation of wavelength (344 - 364 nm), flow (0.4 - 0.8 mL/min.) and temperature (23 - 27° C). The effects on the parameters retention time and peak area were observed.

Method application

Applicability in stability studies

The appropriate method for stability studies should demonstrate selectivity for detecting analyte in the presence of degradation products. For that, the aqueous extract was subjected to stress testing. For induced degradation study, 17.5 mg of the *E. suberosum* extract was accurately weighed, then 1 mL of the 1 M HCl or 1 M NaOH was added and placed in a water bath maintained at 60° C for 1h. The mixture was cooled and the pH solution was adjusted to about 7.0 with 1 M HCl or 1 M NaOH (Sistla *et al.*, 2005). After that, 1.5 mL of methanol was added to mixture. Then, the obtained products were analyzed for the presence of the intact and degraded drug by the proposed procedure comparing with a control sample.

Applicability in standardization of herbs

Standardization process to obtain the *E. suberosum* extract was evaluated by preparing the aqueous extract four times in the same method described below. The aqueous extract was obtained by infusion (129 g) of the dried plant in distilled water (3 L) at 70° C. The mixture was then cooled to 40° C being filtered and stored at -20° C until lyophilization.

The samples were analyzed by HPLC method and rutin was the marker substance chosen. The precision of the extraction process was expressed as relative standard deviation (RSD %) of rutin content.

RESULTS

Determination of rutin by HPLC

The chromatography profile showed that aqueous leaf extract of *E. suberosum* presents a large number of compounds (Figure 1). Six main peaks were observed. All the peaks have characteristic UV/Vis spectra of flavonols, with λ_{max} between 340 and 370 nm (Arapitsas, 2008). It was possible to identify rutin (peak 3) by comparison with commercial standards (ChromaDex®, CA, USA). In identification of rutin was observed that retentions time of the rutin standard and co-injections with sample were the same. In addition, peak purity in both, rutin standard and co-injections, were 0.9999. The rutin content found in *E. suberosum* extract was about 30 mg/g.

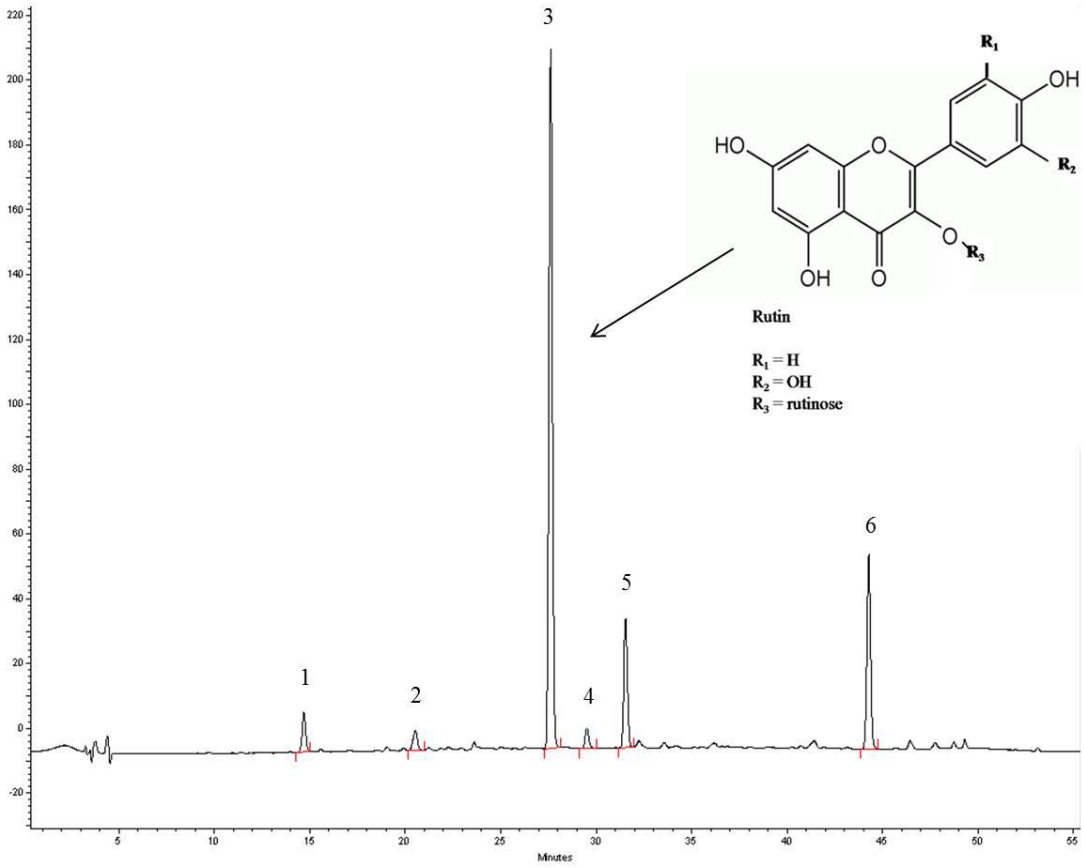

Figure 1
Typical chromatogram of *E. suberosum* extract.

Validation

The chromatographic analysis developed for quantitative determination of this flavonoid was validated according to ICH guidelines (1996). Under the experimental conditions, the linearity was maintained within the concentration range considered. A regression analysis was performed, with the observation of the good linearity (r = 0.9997) and the representative linear equation was:

y = 71400000x + 423500

Table 1 summarizes the results of the regression analysis and shows the slope, intercept and correlation coefficient.

Table 1
Linearity of rutin in the HPLC analysis

	Concentration range (µg/mL)	Slope	intercept	*r*
Rutin	**7.80 - 1000.00**	**71400 ± 409.20**	**423500 ± 178500**	**0.9997**

The results are mean ± SD of three experiments.
***r* – Correlation coefficient.**

The repeatability and intermediate precision were 3.03% and 2.84%, respectively. The mean of peak area, rutin content and the relative standard deviation are reported in Table 2.

Table 2
Precision of HPLC method for quantitation of rutin in *E. suberosum* extract

	Peak area	RSD (%)	Rutin (mg/g)	RSD (%)
Repeatability [a]	**11540512.17 ± 348000**	**3.02**	**31.35 ± 0.95**	**3.03**
Intermediate precision[b]	**11807824.50 ± 336300**	**2.85**	**32.08 ± 0.91**	**2.84**

The results are mean ± SD of six determinations.
[a] Six replicates were assayed on the same day.
[b] Six replicates were assayed on three different days.

The accuracy of the method was determined by adding known amount of rutin standard in known *E. suberosum* extract samples at three levels of concentration. The mean values of the percentage analytical recoveries for the concentration of 120, 150 and 180 µg/mL of rutin were 99.17%, 98.00% and 99.45%, respectively (Table 3).

Table 3
Accuracy of the HPLC method

	Rutin added (µg/mL)	Rutin found (µg/mL)	Recovery (%)
A	**120.0**	**119.0 ± 2.40**	**99.17 ± 2.11**
B	**150.0**	**147.0 ± 1.40**	**98.00 ± 0.98**
C	**180.0**	**179.0 ± 0.20**	**99.45 ± 0.13**

A - Low concentration, B – intermediate concentration and C – high concentration for range calibration.
The results are mean ± SD of three experiments.

The detection and quantitation limits of rutin were 0.19 µg/mL and 0.60 µg/mL, respectively (Table 4).

Table 4
Detection limit (LOD) and quantitation limit (LOQ) of the HPLC method

	Concentration range (µg/mL)	Slope	Standard deviation of y- intercept	LOD (µg/mL)	LOQ (µg/mL)
Rutin	**1.9 – 15.0**	**73350**	**4419**	**0.19**	**0.60**

The results are mean ± SD of three experiments.

No change in retention time was observed when variations of wavelength were established. However, a reduction in peak areas, using wavelength at 344 nm and 364 nm was observed. Flow variations were able to cause changes in retention time and peak area. No change in peak area was found when variations of temperature were established. However, it was observed some changes in retention time, at a temperature of 23° C and 27° C (Table 5).

Table 5
Variation in the experimental parameters on the analytical performance

Wavelength	Peak area	Retention time (min.)
344 nm	**10338804 ± 25872.27**	**27.21 ± 0.01**
354 nm	**10719677 ± 8100.615**	**27.21 ± 0.01**
364 nm	**9716180 ± 27168.91**	**27.21 ± 0.01**
Temperature	**Peak area**	**Retention time (min.)**
23 °C	**10786111 ± 11619.02**	**27.57 ± 0.01**
25 °C	**10737803 ± 31913.98**	**27.21 ± 0.01**
27 °C	**10758930 ± 11036.63**	**26.86 ± 0.02**
Flow (mL/min.)	**Peak area**	**Retention time (min.)**
0.4	**12636885 ± 552967.2**	**32.92 ± 0.23**
0.6	**10737803 ± 31913.98**	**27.21 ± 0.01**
0.8	**6169859 ± 27273.99**	**23.83 ± 0.01**

The results are mean ± SD of three determinations.

Applicability in stability studies

Selectivity was demonstrated by average of the changes in retention time and peak area of rutin in degraded samples (peak 3) (Table 6 and 7). Firstly, it was observed that there was degradation of the sample in the induced acid hydrolysis. It was observed reduction of rutin content and the formation of degradation products, since the sample control showed six main peaks, while samples of the acid hydrolysis showed 10 peaks. Basic hydrolysis was able to degrade the extract too, however all rutin content was degraded. The degradation products did not show retention time similar to retention time of rutin. In addition, all peaks of rutin had good purity index in different analyzed groups.

Table 6
Retention time and wavelength of maximum absorption of the compounds present *E. suberosum* extract after that induced degradation by basic and acid hydrolysis

	Control		Basic hydrolysis		Acid hydrolysis	
Peak	**Retention Time**	**λmax.**	**Retention Time**	**λmax.**	**Retention Time**	**λmax.**
1	**14.10±0.01**	**285; 324**	**-**	**-**	**-**	**-**
2	**19.99±0.01**	**279; 312**	**-**	**-**	**-**	**-**
3	**27.06±0.01**	**255; 353**	**-**	**-**	**27.04±0.03**	**256; 354**
4	**28.94±0.01**	**271; 351**	**-**	**-**	**-**	**-**
5	**30.99±0.01**	**266; 346**	**-**	**-**	**30.98±0.03**	**266; 349**
6	**43.82±0.01**	**255; 353**	**43.84±0.01**	**256; 353**	**40.73±0.03**	**256; 382**
7	**-**	**-**	**-**	**-**	**43.81±0.02**	**255; 354**
8	**-**	**-**	**-**	**-**	**45.85±0.02**	**372; 366**
9	**-**		**48.29±0.01**	**256; 350**	**48.28±0.02**	**255; 354**
10	**-**	**-**	**48.88±0.01**	**266; 343**	**48.86±0.01**	**267; 349**

λmax - wavelength of maximum absorption.
The results are mean ± SD of three determinations.

Applicability in standardization of herbs

Standardization process of obtaining of the *E. suberosum* extract was evaluated preparing the aqueous extract four times in the same form. The precision of the extraction process was 5.7%

DISCUSSION

Plant extracts are composed of many substances and are capable of variation depending on various factors. So, to ensure their efficacy and safety, it is need to standardize these extracts and assure their pharmaceutical quality.

In the present work, RP-HPLC with UV detection and linear gradient mobile phase mode was proposed as a suitable method for quantitative determination of rutin in *E. suberosum* extract. In addition, the applicability of this method in stability studies and standardization of herbs were investigated.

Rutin has been found in several herbal extracts in different amounts, as in *Calendula officinalis* flowers (230.0 µg/mL) (Fonseca *et al.*, 2010), *Viola tricolor* (L) flowers (177.46 mg/g) (Gonçalves *et al.*, 2012), *Rhododendron arboretum* flowers (0.53 mg/g) (Swaroop *et al.*, 2005), and it was found a large amount in the *E. suberosum* extract (30 mg/g).

The chromatographic analysis developed for quantitative determination of this flavonoid was validated according to ICH guidelines (1996) to obtain reproducible analyses with high degree of accuracy and precision in the range of concentrations investigated.

A regression analysis was performed, with the observation of good linearity in a wide concentration range. The values obtained for precision and accuracy determination are in agreement with ICH guidelines (1996), which indicated that the chromatographic conditions used are reliable to quantify the flavonoid

rutin in the evaluated range. The detection and the quantitation limits of rutin were established.

Table 7
Peak area of the compounds present *E. suberosum* extract after that induced degradation by basic and acid hydrolysis

	Peak area		
Peak	**Control**	**Basic hydrolysis**	**Acid hydrolysis**
1	**629912 ± 20581.58**	**-**	**-**
2	**384798 ± 13040.65**	**-**	**-**
3	**11401223 ± 368755.2**	**-**	**342472.7 ± 33634.17[a]**
4	**381710.7 ± 12334.1**	**-**	**-**
5	**2059206 ± 66230.43**	**-**	**120232.3 ± 10562.94**
6	**3475705 ± 104211.1**	**860735 ± 84717.09[a]**	**264790.3 ± 41115.32[a]**
7*	**-**	**-**	**1271992 ± 101284.65**
8*	**-**	**-**	**132322.3 ± 28610.28**
9*	**-**	**101070.3 ± 1188.11**	**125575 ± 11110.85**
10*	**-**	**92435 ± 890.95**	**106033.7 ± 8311.92**

The results are mean ± SD of three determinations.
*** Degradation products.**
Statistical analysis was performed using Student's t-test.
[a] $p < 0.05$ peak area significantly reduced compared by control without hydrolysis

In robustness assay, peak area and retention time changes could be explained: (1) compounds could absorb energy from electromagnetic spectrum with different intensities in relation to each wavelength. Thus, samples and compounds standard should be analyzed under the same conditions. (2) Flow rate is a parameter that can influence interactions of substances with a stationary phase and separations of compounds. (3) Temperature is a parameter that can influence the viscosity of eluents, interactions of substances with stationary phase and separations of compounds. Despite small changes in the peak area and retention time, the flow rate, the temperature and the wavelength are analytical parameters that should be controlled to get better analytical performance of the proposed method. The analytical parameter: wavelength of 354 nm, flow rate of 0.6 mL/min. and temperature of 25° C proved best conditions for separating compounds present in *E. suberosum* extract.

The aqueous extract was subjected to stress testing by acid and basic hydrolysis, to check whether the method is able to separate impurities and degradation products of the reference compound rutin. The results showed that proposed method demonstrates selectivity for detecting rutin in the presence of their degradation products.

Extractive process was accurate and able to obtain the extract of *E. suberosum* with rutin content uniform.

CONCLUSION

The developed method is a reliable HPLC technique for determination of rutin in *E. suberosum* extract. This described method may be applied to the standardization of herbs, extracts or phytomedicines commercialized, as well as to perform stability studies of this plant drug.

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