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Journal of Pharmaceutical and Biomedical Analysis 36 (2004) 787–790
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Development and validation of a LC-method for determination of catechin and epicatechin in aqueous extractives from leaves of *Maytenus ilicifolia*

L.A.L. Soares[a,b,*], A.L. Oliveira[b], G.González Ortega[b], P.R. Petrovick[b]

[a] *Departamento de Farmácia, Universidade Federal do Rio Grande do Norte, Av. Cordeiro de Farias, s/n, 59010-180, Natal, RN, Brazil*
[b] *Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga, 2752, 906010-000, Porto Alegre, RS, Brazil*

Received 30 June 2004; received in revised form 24 August 2004; accepted 27 August 2004
Available online 12 October 2004

Abstract

A reverse phase-LC method was developed and validated for separation and quantification of catechin and epicatechin in aqueous extractives from leaves of *Maytenus ilicifolia*. The analysis was performed using a C_{18} column with acetic acid–acetonitrile gradient elution. The detection was carried out by UV at 280 nm and the peak identification was based on the retention times and by co-chromatography with reference substances. High coefficients of determination were achieved for both catechin and epicatechin peaks from the standard solutions (0.9996 and 0.9999), as well as from extractives (0.9981 and 0.9982, respectively). The method showed good repeatability (R.S.D. <1.5%), reproducibility (R.S.D. <5%) and good accuracy for both catechin and epicatechin peaks (101.4%, R.S.D. = 1.18% and 100.6%, R.S.D. = 2.07%, respectively).

Keywords: *Maytenus ilicifolia*; Catechin; Epicatechin; LC; Validation; Gradient elution

1. Introduction

Maytenus ilicifolia Mart. ex Reissek. (Celastraceae), is widely used in Brazilian folk medicine for the treatment of gastric ulcers. The efficacy and safety of the extracts were confirmed by pharmacological and clinical studies [1–4]. Actually, many Brazilian pharmaceutical industries produce and commercialize phytopharmaceuticals containing this drug. The availability of validated assay methods is therefore an important part of the quality control of such products, and it is required by Brazilian health authorities for registration of phytomedicines [5].

Notwithstanding the active constituents from leaves of *M. ilicifolia* stays undefined, LC-methods for separation and quantification of different compound such as triterpenes and flavonoids were proposed for the quality control of the raw material [6–8]. However, several authors attribute the anti-ulcer activity to the condensed tannins, which are the main constituents of the aqueous extractives [9,10]. On the other hand, the quantification and separation of condensed tannins in the aqueous extracts by RP-LC method have not yet investigated.

Several LC methods are reported for the separation and determination of catechins in tea and other biological matrixes [11–18]. Although the related RP-LC techniques showed satisfactory results, it is often revealed limitations such as complex elution phases, extensive work for the preparation of the samples, low resolution or long time for total separation [18,19].

Thus, in this paper, a simple and fast LC method was developed and validated for simultaneous separation and determination of catechin and epicatechin in the aqueous extractive from leaves of *M. ilicifolia*.

* Corresponding author. Tel.: +55 84 2154355; fax: +55 84 2154340.
E-mail address: phtech@uol.com.br (L.A.L. Soares).

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doi:10.1016/j.jpba.2004.08.029

Table 1
Gradient elution program (B = acetonitrile:water:acetic acid; 50:49:1; v/v)

Time (min)	B (%)
0	12.5
5	20.0
8	30.0
17	45.0
18	100.0
25	100.0
27	12.5
30	12.5

2. Experimental

2.1. Plant material and extractives

Leaves of *M. ilicifolia*, collected in April 1997, were supplied by the "Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas" of the Campinas State University, SP, Brazil. The dried and ground material was used to prepare aqueous extractives by infusion of the raw material in distilled water (10%, w/v) for 15 min.

2.2. Chemicals and solvents

(+)-Catechin and (−)-epicatechin were purchased from Sigma Co. (St. Louis, MO). The mobile phase was prepared with LC grade solvents. It was composed by acetonitrile (Merck, Darmstat, Germany), water (Milli-Q system, Millipore, Bedford, MA) and acetic acid (ExtraSyntese, São Paulo, Brazil).

2.3. LC system

The analysis was carried out in a liquid chromatograph Shimadzu LC-10A equipped with a pump LC-10AD, an UV–Vis-detector SPD-10A, an autosampler SIL-10A and CLASS-LC10 software (Shimadzu, Kyoto). A Nova-Pak C_{18} RP-column 150 mm × 39 mm i.d., 60 Å (Waters, Milford, MA) protected by a pre-column Shimadzu (10 mm × 4 mm i.d.) packed with Bondapak C_{18} 125 Å (Waters, Milford, MA) was used throughout this study. The peaks were detected at 280 nm. After filtration (0.44 μm, Millipore, Bedford, MA) and degassing with helium, a gradient elution was performed by a dual valve system (FVC-10AL, Shimadzu, Kyoto) varying the proportion of solvent B (acetonitrile:water:acetic acid; 50:49:1; v/v/v) to solvent A (acetic acid 1%; v/v) at a flow rate of 1.5 ml/min. The gradient program is summarized in Table 1.

2.4. Method development

2.4.1. Identification of catechin and epicatechin

The peaks of (+)-catechin and (−)-epicatechin in the extract were identified comparing the retention time against the standards, and through addiction of small amount of the standard substances to the sample.

2.4.2. Calibration curves

2.4.2.1. Calibration curves of the standards. Aqueous solutions of catechin and epicatechin were prepared in the following concentrations: 16, 24, 32, 40, 50 and 80 μg/ml, and 32, 48, 64, 80, 100 and 160 μg/ml, respectively. The solutions were filtered through a 0.45 μm membrane (Millipore-HVHP, Bedford, MA). The calibration curves were made by linear regression and the results represented the averaged of three curves performed by three injections of each concentration.

2.4.2.2. Calibration curve of the extractive. The aqueous extractives from leaves of *M. ilicifolia* was filtered through filter paper (grade 1:10 μm, Whatman, UK) and diluted with distilled water to 8, 12, 16, 20 and 40 mg/ml. The calibration curve was made by linear regression and the results represented the average of three curves performed by three injections of each concentration.

2.4.3. Linearity, precision, accuracy, detection and quantification limits

The method linearity, recovery, precision (repeatability and intermediary precision), detection and quantification limits were evaluated according to the ICH guidelines specifications [20].

The linearity of the curves was estimated by regression using the last square method. The slope, intercept (with respective confidence intervals) and coefficient of determination (R^2) were calculated and evaluated [21].

Thereby, three samples of the extractive solution were spiked with known amounts of each standard (0.48 and 0.96 μg from catechin and epicatechin, respectively). Each sample was injected three times and the amount recovered was calculated.

For the repeatability assay, six diluted solutions at 16 mg/ml were prepared. Each diluted solution was injected in triplicate and the repeatability was evaluated for peak areas and retention times of both catechin and epicatechin through the relative standard deviation (R.S.D.%).

The intermediary precision was calculated from three different concentrations of extractive solution (12, 20 and 40 mg/ml), at three different days. At each day a new extractive solution was prepared and injected in triplicate. The data were expressed as relative standard deviation (R.S.D.%).

2.5. Statistical analysis

The individual data were grouped following each experiment. The mean with the respective deviation was used as a measurement of the central tendency and dispersion (relative standard deviation − R.S.D.%). The mean were compared by Student *t*-test [21].

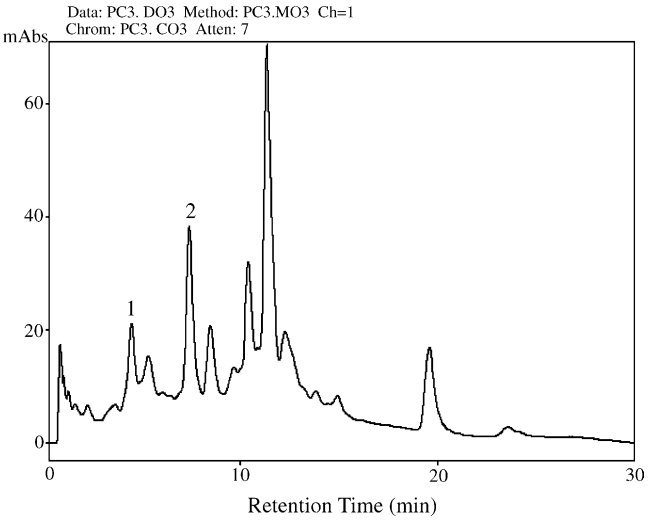

Fig. 1. Chromatogram of *M. ilicifolia* aqueous extractives detected at 280 nm. Peaks: (1) (+)-catechin; (2) (−)-epicatechin.

Table 2
Retention times (min) for both catechin and epicatechin in standard solution and *M. ilicifolia* extractives (n = 3)

Substance	Standard solution	Extractives	*t*-Test
Catechin	4.473 (0.50)	4.415 (1.28)	0.32
Epicatechin	7.402 (0.18)	7.321 (1.13)	0.28

R.S.D. values are given in parentheses; P = 0.95.

3. Results and discussion

The optimized LC conditions were achieved after preliminary assays, where different combination of acetonitrile, water and acetic acid were tested. Fig. 1 shows a chromatogram of the aqueous extractives.

Considering the vegetable matrix complexity, a good separation could be achieved with a total run time of 30 min per injection. Comparing this chromatogram with the chromatogram of standard solution containing both catechin and epicatechin, a coincidence of retention times could be observed (Table 2).

The linearity was evaluated for each standard substance. The calibration curves were obtained by plotting peak areas upon concentrations using six standard solutions. The regression analysis was performed and the resulting parameters are shown in Table 3.

The coefficients of determination for standard curves were greater than 0.999. Thus, the calculated straight line could explain more than 99% of the experimental data. The confidence intervals for both intercept points included zero. Therefore, the result confirms the absence of constant systematic errors. The detection and quantification limits were estimated from the calibration curve slopes. The limits of detection were 0.031 and 0.015 μg/ml for catechin and epicatechin, respectively, demonstrating the method sensitivity. The calculated limits of quantification were respectively 0.093 and 0.045 μg/ml.

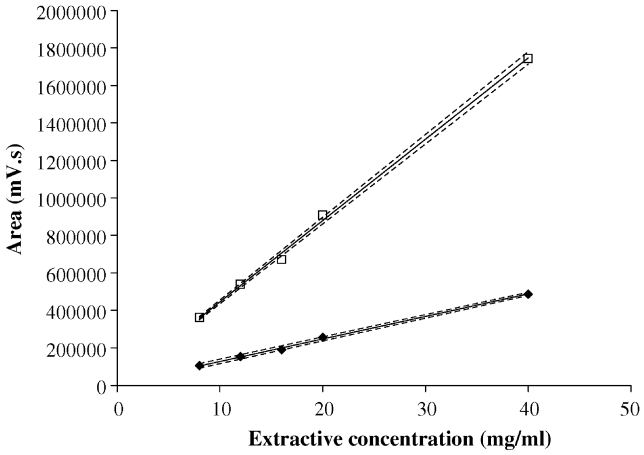

Fig. 2. Calibration curves for both catechin (♦) and epicatechin (□) in *M. ilicifolia* extractives.

Calibration curves for both catechin and epicatechin peaks from aqueous extractives with respective confidence intervals are shown in Fig. 2. The calibration curves were evaluated statistically and the parameters are summarized in Table 4.

As it was observed to standard curves, high coefficients of determination were also obtained for both reference peaks in the aqueous extract. The R^2 values were greater than 0.99, showing that the calculated regression curves could explain significantly the experimental variance.

The assay of recovery or accuracy was performed to evaluate the matrix interference. The differences between the expected and the observed peak areas are shown in Table 5. The results of the accuracy test demonstrated low interference of the matrix onto the recovery of both catechin and epicatechin.

Precision was evaluated through the determination of the repeatability and intermediary precision. The results of repeatability test are presented in Table 6. According to the R.S.D. values obtained from peak retention times and areas during the tests, it could be interpreted that the system showed a satisfactory response with R.S.D. ≤1% [20].

Table 3
Linear regression analysis data for both catechin and epicatechin in standard solutions

Regression parameters	(+)-Catechin	(−)-Epicatechin
Intercept	−22361.33	−20701.1
(Confidence intervals)	(−45945.83 to 1223.174)	(−41850.1 to 447.85)
Slope	8884.74	7894.64
R^2	0.9999	0.9996

Table 4
Linear regression analysis data for both catechin and epicatechin in *M. ilicifolia* extractives

Regression parameters	Catechin	Epicatechin
Intercept	8791	12846
(Confidence interval)	(−33514.1 to 51097.64)	(−135941 to 161632.9)
Slope	11985798.18	43372875
R^2	0.9981	0.9982

Table 5
Recovery results (%) for both reference substances from *M. ilicifolia* aqueous extractives ($n = 3$)

Observations	Catechin	Epicatechin
	Mean (R.S.D.)	Mean (R.S.D.)
1	101.5 (2.14)	100.8 (3.5)
2	102.5 (1.45)	102.5 (0.33)
3	100.2 (1.10)	98.3 (1.44)
Mean (R.S.D.)	101.4 (1.18)	100.6 (2.07)

Table 6
Precision of the assay

	Catechin		Epicatechin	
	Retention (min)	Area (mV s)	Retention (min)	Area (mV s)
Mean (R.S.D.)	4.516 (0.97)	227817 (1.03)	7.400 (0.66)	728935 (0.54)

Repeatability tests for retention times and areas for both catechin and epicatechin in *M. ilicifolia* aqueous extractives ($n = 6$).

Table 7
Intermediary precision tests for retention time and area for both catechin and epicatechin peaks in *M. ilicifolia* aqueous extractives ($n = 3$)

Extractives (mg/ml)	Catechin		Epicatechin	
	Area (mV s)	Retention time (min)	Area (mV s)	Retention time (min)
12.0	153936.89 (4.616)	4.619 (0.608)	539134.56 (4.509)	7.548 (0.708)
20.0	257646.33 (1.620)	4.612 (1.164)	909615.78 (1.909)	7.550 (0.977)
40.0	486951.56 (1.637)	4.605 (1.966)	1744675.22 (2.331)	7.526 (1.606)

R.S.D. values are given in parentheses.

The assay of intermediary precision was performed in order to determine the accumulation of the random errors between different extracts and days. However, the R.S.D. values obtained from both peak areas and retention times of catechin as well as epicatechin were very low, demonstrating that the method showed high reproducibility and thus, suffered low interference of the sample preparation (Table 7).

4. Conclusions

The validation assay of the proposed method showed its suitability for separation and quantification of catechin and epicatechin in the aqueous extractives from leaves of *Maytenus ilicifolia*. No significant variations of peak areas or retention times were detected through the method evaluation. Thus the suggested RP-LC method is simple, rapid and precise, and can be used to quality assurance of the aqueous extractives from *M. ilicifolia*.

Acknowledgments

This work was supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil).

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