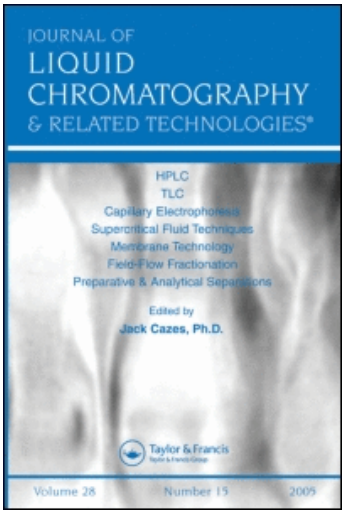

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VALIDATION OF A LC METHOD FOR POLYPHENOLS ASSAY IN CAT'S CLAW (*UNCARIA TOMENTOSA*)

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□ *A reversed-phase LC method was developed and validated for the separation and assay of the main polyphenols in extracts from barks of* Uncaria tomentosa. *The LC method consists of a RP-18 column, in gradient mode (trifluoroacetic acid-methanol) using chlorogenic acid, caffeic acid, and rutin as externals standards and UV-detection at 325 nm. The method showed a good specificity, linearity, precision, and accuracy for standards compounds and for the five major peaks from bark extract. Calibration curves were linear with determination coefficients higher than 0.99. The repeatability and intermediary precision for the five major peaks ranged from 1.09 to 5.60% and 1.25 to 6.28%, respectively. The accuracy values for chlorogenic acid, caffeic acid, and rutin in the bark extract were 97.17, 98.84, and 101.78%, respectively. The LC method was applied successfully to one commercial spray-dried and four different freeze-dried extracts produced from barks and roots of* U. tomentosa.

Keywords column liquid chromatography, polyphenols assay, *Uncaria tomentosa*, validation

INTRODUCTION

Uncaria tomentosa (Willd.) DC. (Rubiaceae), commonly know as Cat's Claw or "Uña de Gato", is a medicinal plant widely spread in the rain forest of Central and South America and largely used in folk medicine for the treatment of rheumatism, arthritis, gastrointestinal disorders, weakness, and viral infections.[1,2] The main bioactive compounds related for this species include alkaloids,[3–5] phenolic,[2] and quinovic acid derivates.[6,7] Several assay methods have been developed focusing the alkaloid fraction.[8–10] However, recent studies have shown the importance of the polyphenols and their relationship with the anti-inflammatory and antioxidant

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properties.[11–13] From an analytical point of view there are few studies regarding the characterization and content assay of phenolic compounds in *U. tomentosa*. In addition, these methods were not designed to provide quantitative evaluation, but only to establish polyphenols profile in samples, without validation issues.[12–14]

In this context, the aim of this study was to develop and validate a LC method for the separation and assay of the main polyphenols occurring in crude extract from barks of *U. tomentosa*. Further, the method was applied to three other different freeze-dried and one spray dried extracts, in order to compare its polyphenol content.

EXPERIMENTAL

Plant Materials

Dried and ground stem barks of *U. tomentosa* were kindly gifted by Laboratorios Induquímica S.A. (Lima, Peru). Roots of *Uncaria tomentosa* were purchased from Quimer Ervas e Especiarias (São Paulo, Brazil). A commercial spray-dried extract (CE) was purchased from one of the most important Brazilian suppliers of cat's claw and used for analytical comparison purposes.

Chemical and Reagents

Methanol (Tedia, USA), trifluoroacetic acid p.a. (TFA) (Vetec, Brazil), and HPLC grade water (Milli-Q system, Millipore, USA) were used for the mobile phase preparation. The chlorogenic acid (Fluka, batch 455159/1, Switzerland), caffeic acid (Extrasynthese, batch 0381024, France), and rutin (Sigma, batch 128K1177, USA) were used as external standards.

HPLC Analysis

The reversed-phase gradient method was performed, employing a Shimadzu liquid chromatography LC 10 Class (Tokyo, Japan) provided with a FCV-10 AL system controller, a LC-10 AD pump system, a SIL-10 A automatic injector (20 μL-loop), and a SPD-10 A ultraviolet-visible detector. The data were processed by LC-10 CLASS software. A Gemini RP-18 (250×4 mm i.d., 5 μm) (Phenomenex, Torrance, USA) column coupled to a pre-column LiChrosorb C-18 (Waters, USA) was used. The main peaks were monitored with a Shimadzu Prominence SPD-M20A (Tokyo, Japan), in the range of 190–800 nm, controlled by LC-Solution Multi-PDA software.

Separation was performed using a gradient elution, which consisted of TFA 0.1% (v/v) (A) and methanol:TFA (99.9:0.1; v/v) (B), as follows: 0–10 min,

30.0% to 40.0% B; 10–18 min, 40.0% to 60.0% B; 18–22 min, 60.0% B; and 22–28 min, 60.0% to 30.0% B. The flow rate was adjusted to 0.9 mL min^{-1}, the wavelength to 325 nm, and the injection volume to 20 μL. The analyses were performed at $23 \pm 1°C$.

The polyphenols were identified, comparing its retention time, and the corresponding UV-spectra with those obtained from the standards compounds.

Standard Curves

Stock solutions of chlorogenic acid (CLA), caffeic acid (CFA) and rutin (RUT) were prepared transferring amounts of the standards substances accurately weighted to 50.0 mL of methanol. Appropriate dilutions were prepared using methanol: TFA 0.1% (50:50 v/v) to yield concentrations ranging from 8.16 to 48.96 μg mL^{-1} for CLA, 0.26 to 1.56 μg mL^{-1} for CFA, and 5.08 to 50.76 μg mL^{-1} for RUT. The samples were filtered through a 0.45 μm membrane filter (Millipore, USA) prior to injection. Each result expresses the mean peak area of three injections.

Samples Preparation

Preparation of the Hydroethanolic Extracts

The extractive solutions of the barks (HB) and of the roots (HR) were prepared by a 4 days-maceration, using an hydroethanolic solution 40% (v/v) and a drug:solvent ratio of 1:10 (w/v). Separately each mixture was pressed, filtered, and concentrated under vacuum at 50°C up to half of their original weights. The concentrates were immediately freeze-dried (Modulyo 4 L, Edwards, USA).

Preparation of the Aqueous Extracts

The extractive solutions of the barks (AB) and of the roots (AR) were prepared, by decoction 4 g-samples of bark and root in 200 mL of water for 45 min, as in the traditional Peruvian medicine.[1] After cooling, both extractive solutions were filtered separately and the final volumes adjusted to 200 mL. The filtrates were immediately freeze-dried (Modulyo 4 L, Edwards, USA).

Freeze-Dried Extract Calibration Curve

Stock solution of HB was prepared dissolving an accurately weighted sample of 1005 mg in 50.0 mL of methanol:water (50:50 v/v). Appropriate dilutions were prepared, using methanol: TFA 0.1% (50:50 v/v) to yield

concentrations of 1.93, 3.87, 5.8, 7.73, and 9.67 mg mL^{-1}. The samples were filtered through a 0.45 μm membrane prior to injection. The results were expressed by the mean of peak areas from three injections.

Method Validation

The validation of the analytical method comprised linearity, repeatability, intermediary precision, and accuracy tests according to the International Conference on the Harmonization (ICH) guideline.[15]

The linearity test comprised regression analysis, ANOVA, and the Durbin-Watson test for residues and the analysis of the confidence intervals for the linear coefficient (constant systematic error). The limit of detection (LOD) and quantification (LOQ) were calculated based on the standard deviation (SD) and the slope (S) of the calibration curves.[15] The system suitability parameters were obtained from chromatograms of the standards solution and HB solutions and the results were expressed by the mean of three determinations for both cases.

The precision parameters, reproducibility, and intermediary precision, were evaluated for all standards compounds, as well as for HB solutions. The repeatability was evaluated by analysis of nine samples in the same day. The intermediary precision was determined by analysis, in triplicate, of three different samples in three consecutive days. The results were expressed as the relative standard deviation (RSD).

The accuracy was evaluated thought the recovery test after spiking known amounts of standards solutions in the HB solution to give final concentrations of 15.50, 23.86, and 32.14 μg mL^{-1} of CLA; 0.62, 0.90, and 1.16 μg mL^{-1} of CFA; and 13.37, 23.34, and 33.29 μg mL^{-1} of RUT.

The quantitative determination peaks areas in the sample chromatograms was performed using the regression equations from the CLA, CFA, and RUT calibration curves. The major constituents were assayed separately, where the peaks corresponding to standards compounds were expressed as such, while the unidentified peaks (coded as peak 1, peak 2, peak 3, and peak 4) were expressed as CLA.

Analysis of the Samples

Samples of the HB, HR, AB, AR, and commercial extract were appropriated diluted with methanol:TFA 0.1% (50:50 v/v) to yield a final concentration of 5.8 mg mL^{-1}. The samples were filtered through a 0.45 μm membrane prior to injection. The results were expressed by the mean of peak areas obtained from three injections.

RESULTS AND DISCUSSIONS

The LC-chromatograms from the standards CLA, CAF, and RUT at 325 nm and their respective UV-spectra are shown in Figure 1. In all cases, both spectra and absorption maxima agreed to those related in the current literature.[16]

The occurrence of CLA, CAF, and RUT could be demonstrated in HB samples after comparison of retention time, purity index similarity above 0.99, and UV-spectra (Figure 2). The two others conspicuous peaks observed in the HB chromatogram were coded as peak 1 and peak 2 and exhibit UV-spectra patterns typical of flavonoids, specifically, flavone and flavonol, respectively.[16]

The system suitability parameters for CLA, CAF, and RUT peaks in the standard and extract solutions are shown in Table 1. The capacity factor (k′), number of theoretical plates (N), and tailing factor (Tf) for both peaks ranged from 1.58 to 5.60, 3372 to 100380, and 0.72 to 1.30, respectively. These results for all parameters evaluated are in accordance with the

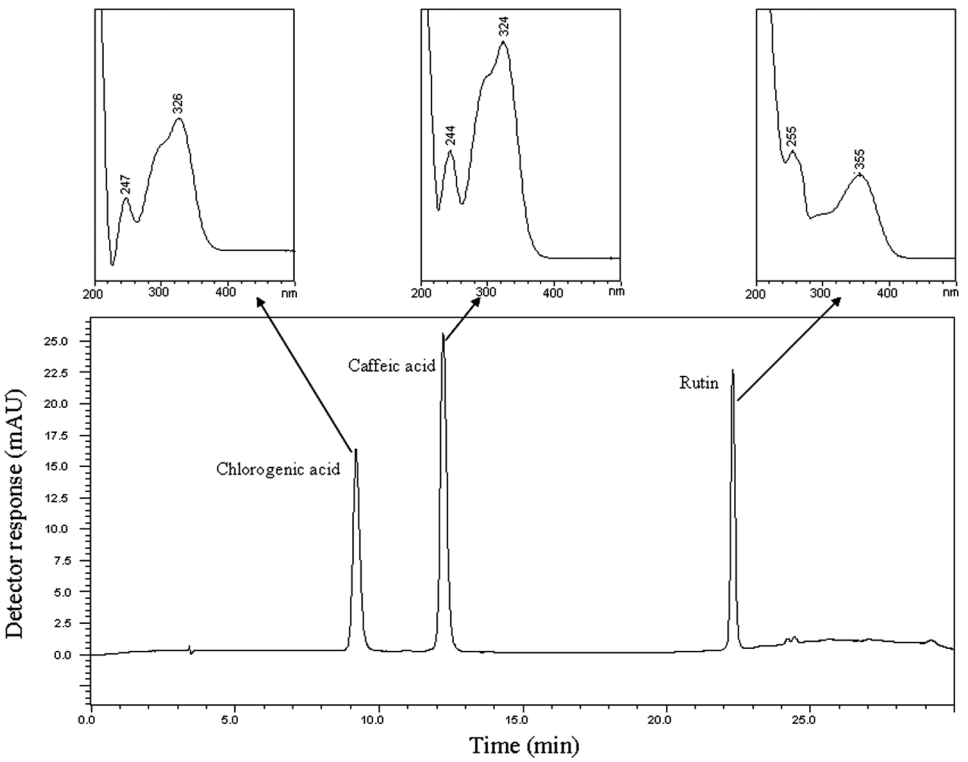

FIGURE 1 Chromatographic profiles of the standards compounds and their respective UV-spectra (200–500 nm).

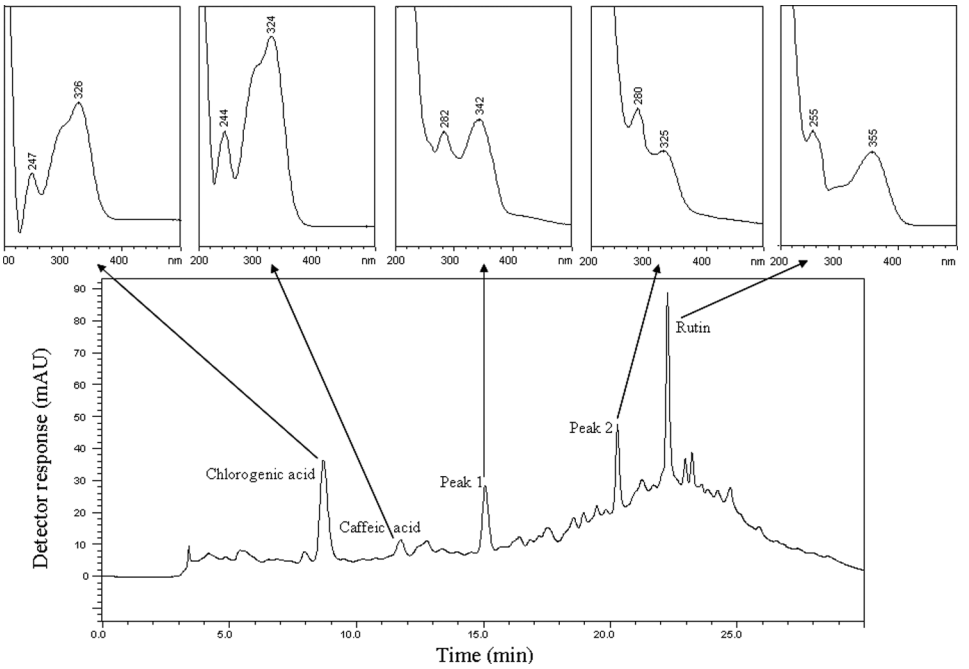

FIGURE 2 LC-Chromatograms and UV-spectra (200–500 nm) showing the presence of chlorogenic acid, caffeic acid, rutin, and two still unidentified flavonoids in HB samples. Detection at 325 nm.

ICH guidelines indicating that the chromatographic system developed showed a suitable performance for the *U. tomentosa* samples analysis.[17]

The results of the regression analysis for the standards compounds CLA, CFA, and RUT are shown in Table 2. The method shown to be linear in the concentration range evaluated for all external standards, with determination coefficients (R^2) higher than 0.999. No evidence of constants systematic error could be assessed after analysis of the confidence limits calculated for the intercept, which included zero. The LOD and LOQ values were clearly below the lowest curve concentration assayed

TABLE 1 System Suitability Parameters for Standards Peaks (Left) and the Five Major Peaks (Right, Italic) Detected in the HB Chromatogram

Peak	k′		N		Tf		Rs	
Chlorogenic acid	1.72	*1.58*	7039	*7400*	1.19	*1.30*	NA	*NA*
Cafeic acid	2.61	*2.51*	13852	*3372*	1.19	*0.72*	7.05	*5.34*
Peak 1	NA	3.48	NA	*15220*	NA	*1.13*	NA	*6.51*
Peak 2	NA	*5.03*	NA	*26094*	NA	*1.16*	NA	*13.96*
Rutin	5.56	*5.60*	100380	*45246*	1.13	*0.99*	28.73	*7.04*

k′: capacity factor; N: number of theoretical plates; Tf: tailing factor; Rs: resolution; NA: not applicable.

TABLE 2 Linearity, LOD, and LOQ Parameters for the Standards Compounds

Substance	R^2	Regression equation	LOD ($\mu g\,mL^{-1}$)	LOQ ($\mu g\,mL^{-1}$)
Chlorogenic acid	0.9998	$y = 30913x + 6513.15$	0.38	1.14
Caffeic acid	0.9996	$y = 107511.2x + 1145.91$	0.02	0.05
Rutin	0.9997	$y = 23082.43x - 5365.81$	0.44	1.35

R^2: regression coefficient; LOD: Limit of Detection; LOQ: Limit of Quantification.

showing a good sensitivity for the standards and suitability for the analytical purposes, according ICH guidelines.[15]

The absence of linearity deviation in the range from 1.93 to 9.67 mg mL^{-1} for all peaks evaluated is demonstrated by the R^2 values higher than 0.99, despite the complex composition of the sample. The confidence limits calculated for the intercept included zero, demonstrating the absence of constant systematic error. No residues autocorrelation was evidenced by the Durbin-Watson test for regression residues in any of the five major peaks here considered, since the all d values calculated were higher than the critical d_U limit ($d_U = 1{,}36$, $\alpha = 0.05$).[18]

The precision of the LC method was evaluated through the repeatability and intermediary precision tests (Table 3). The repeatability for the HB demonstrated that the analyzed peaks presented a RSD range from 1.09 to 5.60%. This data could be considered satisfactory since the majority of similar research data suggested a RSD below to 8% as acceptable.[19] The intermediary precision for the peaks was lower than 6.28%; results up to 15% are considered appropriate when the matrix complexity is taken into account.[20] The results of repeatability and intermediary precision tests of the standards compounds confirmed the method precision with RSD values lower than 2.6% for both precision parameters.

Recovery tests were performed to assess the method accuracy. The results of the recovery tests considering the standard compounds CLA,

TABLE 3 Repeatability and Intermediary Precision for the Main Five Peaks Detected in the HB Chromatogram

	Standards compounds		HB	
Substance	Repeatability RSD (%)	Intermediary precision RSD (%)	Repeatability RSD (%)	Intermediary precision RSD (%)
Chlorogenic acid	0.87	2.60	1.09	1.25
Caffeic acid	1.21	2.51	3.30	6.28
Peak 1	NA	NA	5.60	3.03
Peak 2	NA	NA	3.84	5.80
Rutin	0.32	0.63	3.30	3.83

NA: not applicable.

CFA, and RUT are shown in Table 4. The recovery results were 97.17, 98.84, and 101.78%, respectively, with RSD lower than 3.2% in all analyzed concentrations.

The LC method was applied to compare the polyphenol content in four freeze-dried extracts and one commercial spray-dried extract of *U. tomentosa*. The chromatographic profiles of the dried extracts are shown in Figure 3. Both chromatograms of HB and AB derived from bark (Figures 2 and 3, respectively) were quite similar, as well as for both chromatograms of HR and AR derived from roots. These results indicated that bark and roots afforded a similar LC-profile regardless of the liquid extractor or extraction technique.

In the chromatographic profile of the roots extracts (HR and AR), six major peaks could be detected; two of them were identified as CLA and RUT by UV-spectra comparison. Both root extracts present four characteristic peaks coded as 1, 2, 3, and 4, with UV-spectra closely resembling those currently observed for flavonoids (Figures 2 and 3). Their absorption maxima located between 300–380 nm and 240–280 nm are typical of the cinnamoyl and benzoyl groups observed in derivates of quercetin and isorhamnetin.[16] Peaks 3 and 4 could be detected neither in the aqueous extract from bark nor in the commercial extract, but peaks 1 and 2 are undoubtedly present in both extracts (Figure 3). These results demonstrate the discriminative capacity of developed and validated LC methods when applied to samples obtained from different parts (barks and roots) of the specie.

The similarity of CE chromatographic profile with the HR and AR chromatographic profiles strongly suggests that this extract outcome is from *U. tomentosa* root instead of bark.

The calculated polyphenol content in the different extracts are shown in Table 5. In the roots extracts, the polyphenol content was about 3 times higher than those determined for both barks extracts, probably due to

TABLE 4 Recovery Test for Chlorogenic Acid, Caffeic Acid, and Rutin in HB

Substance	Theoretical concentration ($\mu g\,mL^{-1}$)	Experimental concentration ($\mu g\,mL^{-1}$)	Recovery (%); RSD (%)	Total recovery (%); RSD (%)
Chlorogenic acid	15.50	15.57	100.43; 0.56	97.17; 3.54
	23.86	23.26	97.51; 0.33	
	32.14	30.08	93.57; 0.42	
	0.62	0.65	104.55; 1.71	98.84; 5.67
Caffeic acid	0.90	0.89	98.61; 3.17	
	1.16	1.08	93.34; 0.28	
	13.37	13.28	99.36; 0.65	
Rutin	23.34	23.86	102.21; 0.41	101.78; 2.20
	33.29	34.55	103.77; 0.52	

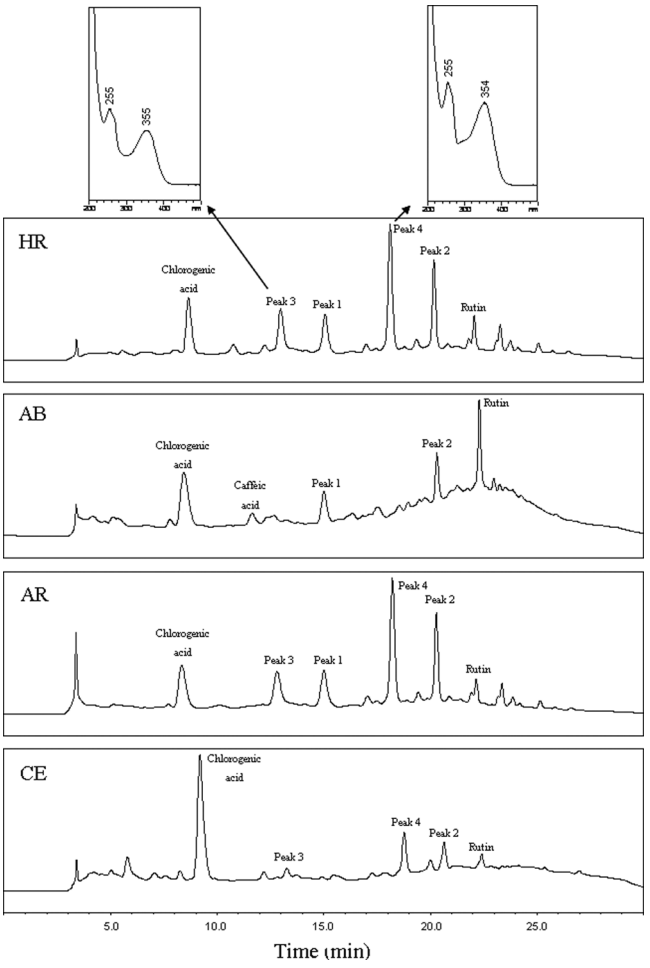

FIGURE 3 LC-chromatograms recorded at 325 nm for the *U. tomentosa* hydroethanolic extract from root (HR); aqueous extract from bark (AB); aqueous extract from root (AR); and commercial extract (CE) along with UV-spectra (200–500 nm) of the peak 3 and peak 4.

presence of the flavonols (peaks 3 and 4) absent in bark extracts. The polarity of both solvents and the extraction method used in this work seem to play a secondary role in the barks extracts and roots extracts, since only small changes were observed in the polyphenol content of those extracts. From an analytical point of view, maceration with hydroethanolic solution was slightly more efficient than decoction with water for the extraction of polyphenols in the analyzed cat's claw barks and roots. In the CE, the polyphenol content was low in comparison with the other freeze-dried extracts, probably due to high concentration of the drying excipients in this spray-dried extract.

TABLE 5 Polyphenol Contents in Different Freeze-dried Extracts (HB, HR, AB, AR) and Commercial Extract (CE)

	Concentration g% (w,w) mean; (RSD%)				
Substance	HB	HR	AB	AR	CE
Chlorogenic acid	0.38; (0.29)	0.84; (2.71)	0.56; (0.55)	0.69; (0.58)	0.30; (0.95)
Caffeic acid	0.02; (1.49)	NA	0.02; (1.10)	NA	NA
Peak 1*	0.16; (0.24)	0.56; (5.89)	0.18; (2.26)	0.50; (3.55)	NA
Peak 2*	0.16; (2.18)	0.78; (0.50)	0.18; (1.00)	0.74; (2.03)	0.02; (1.36)
Peak 3*	NA	0.56; (0.89)	NA	0.51; (2.88)	NA
Peak 4*	NA	1.23; (4.22)	NA	1.21; (1.21)	0.05; (4.91)
Rutin	0.40; (2.09)	0.42; (9.59)	0.40; (2.51)	0.29; (2.14)	0.02; (2.20)
Total	1.12; (0.92)	4.39; (2.33)	1.34; (0.76)	3.94; (0.35)	0.40; (0.50)

NA: not applicable; *Calculated as CLA.

CONCLUSIONS

The LC method developed and validated for assay of polyphenols in hydroethanolic extract from bark of *U. tomentosa* and was specific, linear, precise, and accurate. Furthermore, the LC method was discriminatory when applied to different extracts from bark and roots of the specie. Thus, this easy proposed LC method is suitable for quantification of polyphenols and can be used in the quality control of *U. tomentosa* bark samples, as well as for different bark samples and root samples.

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