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Development And Validation Of A Liquid Chromatography Mass Spectrometry Method For The Determination Of The Anti-Inflammatory 6,8-Di-C-β-D-Glucosylapigenin In Species Of *Lychnophora*

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ABSTRACT

The flavonoid 6,8-di-C-β-D-glucosylapigenin attracts major interest due to its well-demonstrated anti-inflammatory effect. In this study, a method was developed and validated for determination of the anti-inflammatory flavonoid 6,8-di-C-β-D-glucosylapigenin in crude plant extract from different *lychnophora* specie. For this purpose, it was applied the time-of-flight (QTOF) liquid chromatography mass spectrometry interfaced with electrospray ionization (LC-ESI-MS). The LOD and LOQ were found to be 0.01 and 0.10 μg/mL, respectively. Calibration curves were linear, ranging from 0.1 to 10 μg/mL with values for the coefficient of correlation >0.99. The recoveries at three levels of concentration (0.5, 5.0 and 8.0 μg/mL) ranged from 90 to 97 %. High quantities of 6,8-di-C-β-D-glucosylapigenin were found in all species evaluated. The developed method may be used in the selective analysis of plants and to certify the quality of the popular medicinal uses of the extract.

KEYWORDS

6,8-di-C-β-D-Glucosylapigenin;
Anti-inflammatory;
LC-ESI-MS;
Validation;
Lychnophora.

INTRODUCTION

Lychnophora (Asteraceae) is an endemic brazilian genus with restrict occurrence at 'cerrado' and its species are popularly used to the treatment of inflammation, pain and rheumatism[1,2]. According to Robinson this genus comprises 34 species and they are popularly known as 'arnica da serra', 'arnica brasileira' or 'falsa arnica'[3-5]. Chemical investigation of this genus afforded several sesquiterpenoids,

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diterpenoids, triterpenoids, sesquiterpene lactones, steroids, polyacetylenes, lignans and flavonoids[6,5]. The bioguide fractionation indicates that the polar fractions of leaves and roots have a correlation of the traditional used in folk medicine. Among all the isolated compounds of the aerial parts of *L. ericoides* Mart., only 6,8-di-C-β-D-glucosylapigenin showed *in vivo* anti-inflammatory activity[7]. This compound is commonly known as vicenin-2 and it is a water soluble flavonoid which attracts major interest by its high concentration in the folk preparations. Moreover, the anti-inflammatory action is not related to its anti-oxidant activity, and all the other isolated flavonoids were inactive in the applied experimental conditions[7]. It is known that other active compounds should exist in the plant that may contribute to the anti-inflammatory effect. However, systematic investigation was did not indicate significant anti-inflammatory activity to the other isolated compounds of *L. ericoides* Mart. specie[8,9].

Vrinda and Devi demonstrated that orientin and vicenin-2 flavonoids give efficient protection to human peripheral lymphocyte chromosomes against clinically relevant radiation doses[10]. Recently, Santos et al. investigated the effect of the vicenin-2 flavonoid and caffeoylquinic acids on inflammatory mediators production by culture cells, showing that this flavonoid is able to dose-dependently inhibit the production of PGE_2[11].

Among the several methods used for the determination of antioxidant compounds, the coupling of liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) became an important technique for analyses of compounds present in material from a variety of natural product sources, providing powerful analytical tools for the efficient of the constituents in plant extracts rapidly and accurately[12-14]. LC-ESI-MS combines the efficient separation capabilities of LC and the great power in structural characterization of mass spectrometry (MS), and provides a new tool in phytochemistry due to its sensitivity, rapidity, and low levels of sample consumption[15]. As part of our program devoted to the investigation of commercial phytomedicines, we developed and validated a LC-ESI-MS method to quantify the main c-glucosyl favone from different *lychnophora* species.

EXPERIMENTAL

Chemicals and reagents

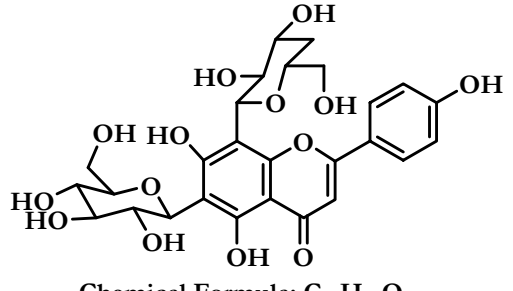
Acetonitrile (MeCN), methanol (MeOH) and ethanol were HPLC-grade (J.T.Baker). MeCN was passed through Millipore filters (0.50 μm FH; Bedford, MA, USA). Acetic acid was obtained from Fluka (Buchs, Switzerland). The water used for the mobile phase preparation was purified with Milli-Q plus system (Millipore, USA). Due to the absence of a commercial standard, 6,8-di-C-β-D-glucosylapigenin (Figure 1) was isolated from *L. ericoides*. The identities of the isolated standard were confirmed with spectral (UV, MS, and NMR) analyses[7]. Stock standard solutions (1mg/mL) were prepared in MeOH and composite working standard solutions were prepared by diluting the stock solutions as required.

Plant material

Leaves of *lychnophora ericoides*, *lychnophora pohlii*, *lychnophora pseudovilosissima*, *lychnophora vilosissima* and *mikania glomerata* were obtained in the Herbarium of Faculdade de Filosofia Cienciase Letras de Ribeirao Preto - Universidade de Sao Paulo.

Extraction of plant material

The ethanolic extract of each species was obtained from dried leaves (20 mg) was submitted to ultrasonic maceration for 10 min at room temperature using ethanol/water (3:7) solution (3 mL). The extracts were filtered through a 0.45 μm RC-membrane (Sartorius) and, after dilution, injected (20 μL) into HPLC system.



Chemical Formula: $C_{27}H_{30}O_{15}$
Exact Mass: 594,15847

Figure 1: Chemical structure of 6,8-di-C-β-D-glucosylapigenin

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LC and MS

Initially, the conditions of analyses were tested using on a Shimadzu liquid chromatograph-equipped with two solvent pumps (LC-6 AD), a photodiode array (PDA) detector (SPD-M10Avp), a 7125 Rheodyne injector with a 20 μ loop and SCL-10Avp system controller. After this, the analyses were performed on a Shimadzu liquid chromatograph equipped with two solvent pumps (LC-20 AD), a photodiode array (PDA) detector (SPD-M20A), a 7125 Rheodyne injector with a 20 μL loop and CBM-20A system controller. The HPLC separation was performed with a VP-ODS-18 (150 mm × 2 mm, Shimadzu, Japan) column and a pre-column packed with GPV-ODS C-18 (5mm × 2mm, Sigma-Aldrich, USA) eluted with a linear gradient of MeCN containing 2% acetic acid (solvent B): water 2% acetic acid (solvent A). Conditions utilized for analysis of extracts: 0-2 min, 12 % B; 2-5 min, 12-25 % B; 5-7 min, 25-60 % B; 7-9 min, 60 % B; 9-18 min, 60-12 % at a flow 0.7 mL/min. The UV spectra (DAD) were recorded between 240 and 370 nm.

The MS system used was a quadrupole time-of-flight instrument (UltrOTOF-Q, Bruker Daltonics, Billerica, MA), equipped with an ESI positive ion source. The analyses were performed with the mass spectrometer in full scan mode. The best conditions detection were: capillary voltage 3900 V; dry gas temperature, 180°C ; dry gas flow, 4 L/h; nebulizer gas, nitrogen. A split 1:3 the HPLC eluent was used to introduce the sample into the stainless steel capillary probe, where the compound was ionized.

Procedure validation

For quantification, the external standard method was employed using the purified 6,8-di-C-β-D-lucosylapigenin as standard. A five-point calibration curve was constructed by plotting peak area versus 6,8-di-C-β-D-glucosylapigenin concentrations. Analysis of calibration standard at each concentration was performed in triplicate. Slope intercept and correlation coefficient were calculated by linear regression. The intra- and inter-day accuracy and precision were studied by performing three separate analyses per day for 3 days with 6,8-di-C-β-D-glucosylapigenin (0.5, 5.0 and 8.0 μg/mL). The limit of detection (LOD) was measured by signal to noise ratio (S/N) = 3 and a limit of quantification (LOQ) measured by means of S/N = 10.

RESULTS AND DISCUSSION

Development of LC-ESI-MS conditions

Looking to quantify the anti-inflammatory flavonoid 6,8-di-C-β-D-glucosylapigenin in different species of *lychnophora* used in brazilian folk medicine, the parameters of MS were optimized in order to obtain more abundant molecular ion. Analyses of the balance of protonated or deprotonated and cationazed or anionized compound or other adduct ion species should ideally be of high intensity, allowing for low concentration of analyte to be detected in complex matrices. The intensity of ion species was therefore the main criteria for optimization. Initially, 6, 8-di-C-β-D-glucosylapigenin standard was directly introduced in the MS detector using the ESI ionization. ESI source was tried for the ionization of the compound both in positive and negative ion modes by infusion of standard solution at 1 μg/mL. ESI MS full scan spectra showed molecular ion at m/z 595 $[M+H]^+$ and m/z 593 $[M-H]^-$ with positive and negative modes, respectively. In this study, it was found that positive ion detection mode offered a higher sensitivity than the negative mode applying acidic mobile phase.

During the optimization of the method, several LC conditions and solvents system were evaluated. The LC separation was performed using a C-18 column. The addition of acidic modifiers such as acetic acid to the mobile phase was found to improve the resolution of the peak. In general, the addition of acidic mobile phase (acetic acid or formic acid) led to significant enhancement of the $[M+H]^+$ ions.

In order to reduce chromatographic separating time, MeCN was chosen as the organic solvent. The mobile phase composed of MeCN with 2% acetic acid and water with 2% acetic acid with a flow rate of 0.7 mL/min was selected in the report, which resulted on short chromatographic time. Photodiode array detection revealed the two main absorption bands characteristic of compound, maxima at approximately 330 and 271 nm.

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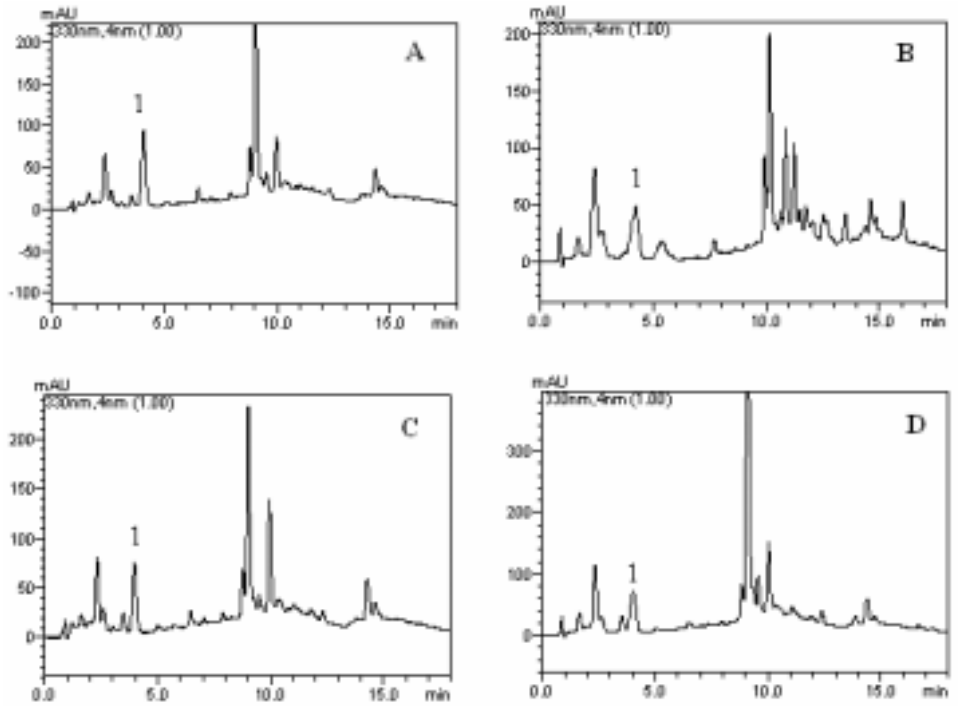

Figure 2: LC-UV chromatogram profile (330 nm) from species of *lychnophora*. Peak 1 is attributed for 6,8-di-C-β -D-glucosylapigenin. A = *L.vilosissima*, B = *L.ericoides*, C = *L.pseudovilosissima*, D = *L.polhii*

The ethanolic extract was previously filtered and directly injected into LC-UV and LC-MS. None clean-up strategies was adopted for ethanolic extracts because the compound target eluted first not interfering with the other constituents of the matrix.

Figure 2 presents the LC-UV profile obtained for ethanolic extract of *lychnophoras* specie studied. Under the previously described conditions, 6,8-di-C-β-D-glucosylapigenin flavonoid eluted at a retention time of 4 min and the elution of all compounds of the sample was completed within 15 min. Chromatographic profiles showed a good separation of the analyte in a short time.

Method validation

The validation parameters were evaluated through estimation of linearity, precision (intra and inter day) and accuracy of the method. The linearity of an analytical method is its ability, within a definite range, to obtain results directly proportional to the concentrations (quantities) of the analyte in the sample. The calibration curve for 6,8-di-C-β-D-glucosylapigenin was performed with five different concentrations by plotting the peak area versus concentration. Linear regression analysis was obtained by the external standard method. The following equation was obtained y = 3676.0635 + 22998.3156x, r^2 = 0.9994. The correlation coefficient (r^2) calculated for the regression line demonstrates the good relationship between concentration and peak area in the concentration range 0.1 - 10 μg/mL, indicating an excellent linearity.

The values of LOD and LOQ were found to be 0.01 and 0.1 μg/mL, respectively. In this study, the values of limits were considered satisfactory to analyses of 6,8-di-C-β-D-glucosylapigenin in plants.

For quantitative study, *mikania glomerata* specie was selected as a blank. LC-UV and LC-ESI-MS analysis of the crude extract of *mikania glomerata* was not detected 6,8-di-C-β-D-glucosylapigenin. Representative chromatogram of the blank crude extract of *mikania glomerata* is presented on figure 3. The peaks 1, 2 and 3 that appear in the total ion current (TIC) of the blank correspond to the compounds present in the blank and are shown with the m/z given in parenthesis. Still, these peaks are not identi-

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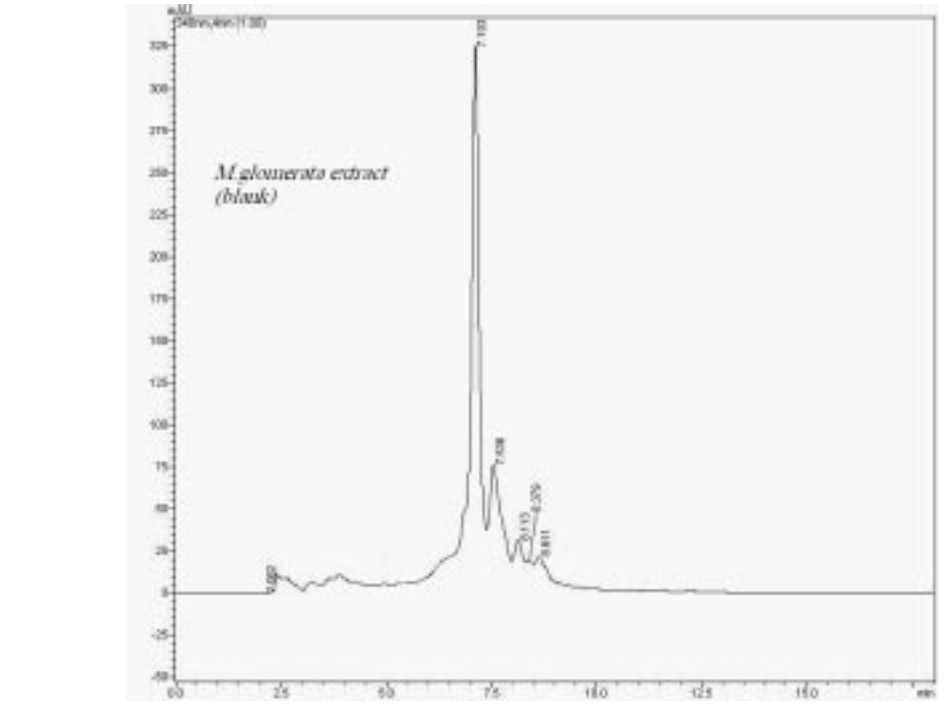
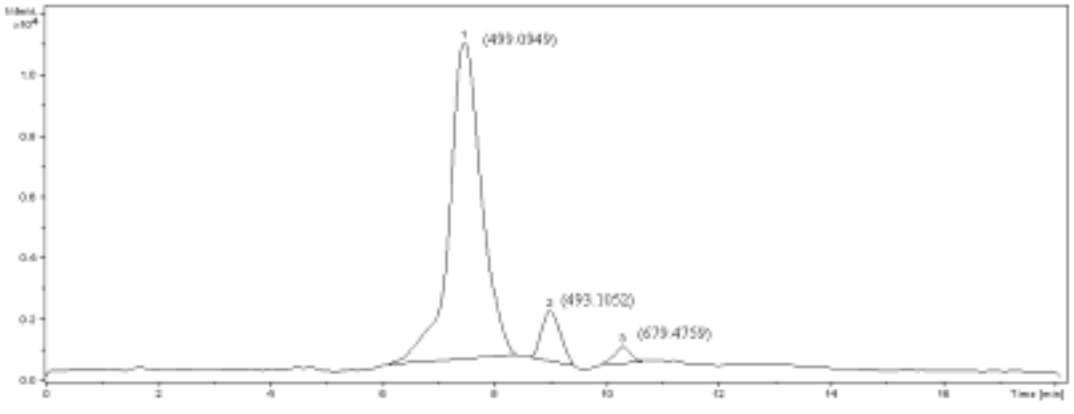


(a)



(b)

Figure 3: Representative LC-UV (a) and TIC chromatogram (b) from a crude extract of *mikania glomerata* (blank). The numbers in parenthesis are the values of the m/z obtained to peaks 1, 2 and 3

fied.

The precision of the LC-ESI-MS was investigated by intra-day and inter-day analysis. 0.5, 5.0 and 8.0 μg/mL of 6,8-di-C-β-D-glucosylapigenin were spiked in blank extract and analyzed. The intra-day and inter-day precision at three different concentrations were evaluated as the coefficient variation CV% of the mean of all determinations at each concentration level. The overall precision ranged from 1.8 to 3.4 %.

In terms of accuracy, the LC-ESI-MS method was found to be satisfactory, with recovery percentages calculated from the triplicate. The values of recovery obtained at three different fortification levels were 97, 90 and 96 %, respectively. The overall accuracy ranged from 3.1 to 6.3. The accuracy and precision data are summarized in TABLE 1.

Application to the method

In order to check the applicability of the developed method, different species of *lychnophora* were evaluated. These species were chosen due to their

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TABLE 1: Precision and accuracy of the method to quantify 6,8-di-C-β-D-glucosylapigenin in crude plant extract (n = 3 days, 3 replicates per day)

Concentration (μg/mL)	Accuracy (%)	Intra-day precision (CV%)	Inter-day precision (CV%)
0.5	3.1	2.8	3.3
5	6.3	2.3	3.4
8	4.1	1.8	2.2

TABLE 2: Content of 6, 8-di-C-β-D-glucosylapigenin flavonoid in different species of *lychnophoras* leaves

Species of lychnophoras	mg 6, 8-di-C-β-D-glucosylapigenin/g of plant
L. ericoide	0.74 ± 0.01
L. pohlii	0.70 ± 0.03
L. pseudovilosissima	0.64 ± 0.02
L. vilosissima	0.66 ± 0.01

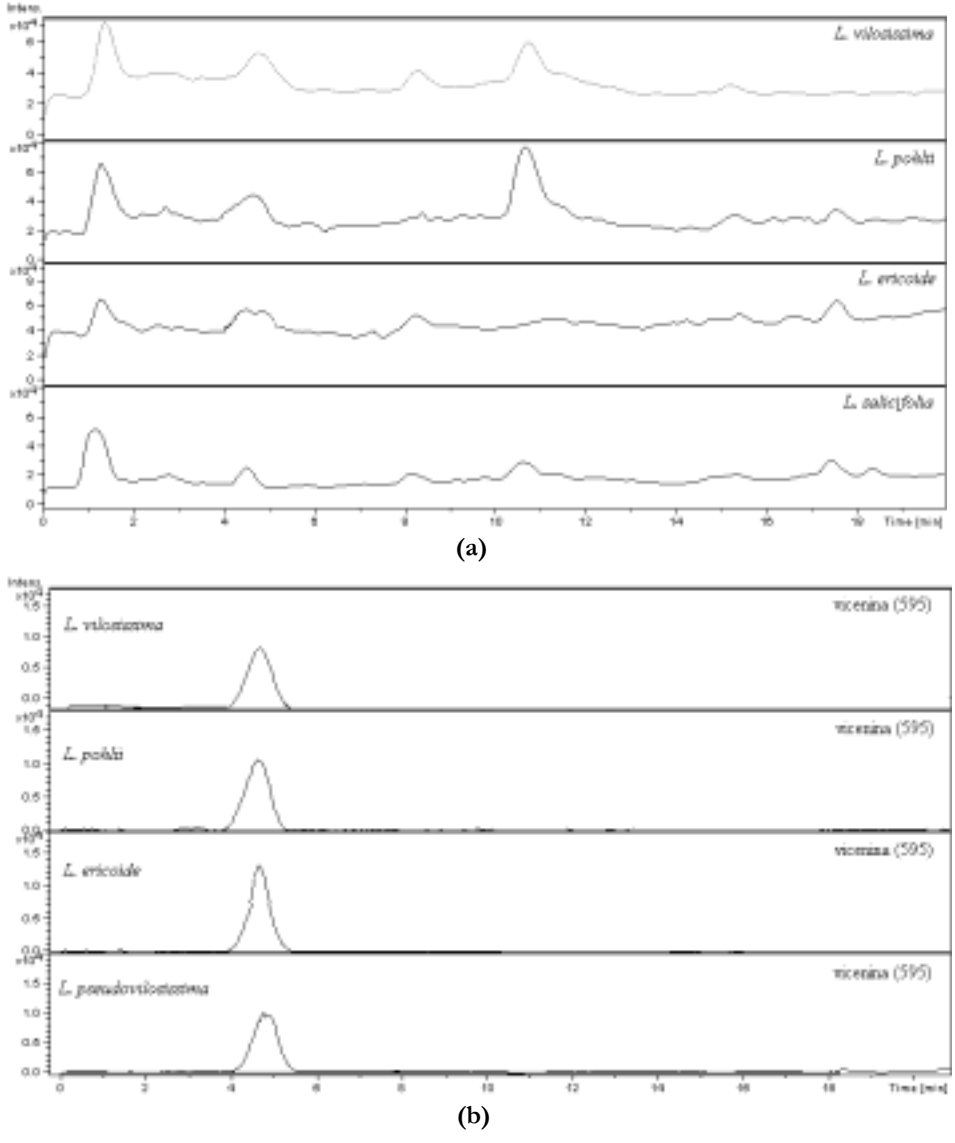

Figure 4: TIC chromatogram (a) and extracted ion chromatogram (m/z 595) (b) from different species of *lychnophoras* studied

wide commercial reach as a brazilian medicinal plant which is usually used to analgesic and anti-inflammatory.

Concentration of plant extracts samples were calculated based on the integrated of peak area of the $[M+H]^+$ chromatogram for 6,8-di-C-β-D-glucosylapigenin extracted from the total ion current (TIC). By extract ion chromatogram is possible to select

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the compound of interest and the quantification of individual compound (considering different molecular weight) can be made without the need for complete chromatography resolution. This selectivity obtained by removing individual ion can be observed in figure 4 and the results of quantification are given in TABLE 2.

All four *lychnophoras* specie analyzed contained 6,8-di-C-β -D-glucosylapigenin. High quantities were found in *L.ericoide*, *L.pohlii*, *L.vilosissima* and the minor quantity was obtained in *L.pseudovilosissima*. All specie showed great potential to extraction of active compound 6,8-di-C-β-D-glucosylapigenin. The values obtained justify the use of these species in folk medicine as anti-inflammatory agents.

CONCLUSION

LC-ESI-MS method described herein proved suitable for the analysis of 6,8-di-C-β-D-glucosylapigenin in leaves extracts, and can be easily applied to routine analyses of plant extracts. Furthermore, the method may be used as an alternative to certify the quality of the popular medicinal uses of the extract and in the selective analysis of plants.

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