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Online Identification of Chlorogenic Acids, Sesquiterpene Lactones, and Flavonoids in the Brazilian Arnica *Lychnophora ericoides* Mart. (Asteraceae) Leaves by HPLC-DAD-MS and HPLC-DAD-MS/MS and a Validated HPLC-DAD Method for Their Simultaneous Analysis

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Lychnophora ericoides Mart. (Asteraceae, Vernonieae) is a plant, endemic to Brazil, with occurrence restricted to the "cerrado" biome. Traditional medicine employs alcoholic and aqueous−alcoholic preparations of leaves from this species for the treatment of wounds, inflammation, and pain. Furthermore, leaves of *L. ericoides* are also widely used as flavorings for the Brazilian traditional spirit "cachaça". A method has been developed for the extraction and HPLC-DAD analysis of the secondary metabolites of *L. ericoides* leaves. This analytical method was validated with 11 secondary metabolites chosen to represent the different classes and polarities of secondary metabolites occurring in *L. ericoides* leaves, and good responses were obtained for each validation parameter analyzed. The same HPLC analytical method was also employed for online secondary metabolite identification by HPLC-DAD-MS and HPLC-DAD-MS/MS, leading to the identification of di-*C*-glucosylflavones, coumaroylglucosylflavonols, flavone, flavanones, flavonols, chalcones, goyazensolide, and eremantholide-type sesquiterpene lactones and positional isomeric series of chlorogenic acids possessing caffeic and/or ferulic moieties. Among the 52 chromatographic peaks observed, 36 were fully identified and 8 were attributed to compounds belonging to series of caffeoylferuloylquinic and diferuloylquinic acids that could not be individualized from each other.

KEYWORDS: *Lychnophora*; Asteraceae; secondary metabolite online identification; natural product dereplication; hyphenated techniques; HPLC-DAD; HPLC-DAD-MS/MS

INTRODUCTION

Lychnophora ericoides Mart. (Asteraceae, Vernonieae), as well as all of the *Lychnophora* genus, are Brazilian endemic plants with occurrence restricted to "campus rupestris", which is a particular type of high-altitude areas of the Brazilian "cerrado" (the Brazilian savannas) (*1–3*). Traditional medicine employs alcoholic and aqueous−alcoholic preparations of leaves from this species for the treatment of wounds, inflammation, and pain (*4, 5*). Furthermore, in the Brazilian states of Minas Gerais and Goiás, leaves of *L. ericoides* are also widely used as flavorings for the traditional spirit "cachaça", also known as "pinga" and officially called "aguardente de cana". This cachaça macerate of *L. ericoides* leaves is also taken orally for medical purposes. *L. ericoides* is also popularly known as "arnica da serra", "arnica brasileira", or "falsa arnica" (*4, 5*) in allusion to the similarity of the traditional uses to that of the European arnica (*Arnica montana*, Asteraceae).

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Previous phytochemical investigations of medium polarity extracts of *L. ericoides* leaves yielded sesquiterpene lactones (STL) as the major isolated compounds, in addition to flavonoids (*6–8*). Polar extracts furnished caffeoylquinic acids and di-*C*-glucosylflavones (*4, 5*). *In vivo* experiments indicated that several lignans and the di-*C*-glucosylflavone vicenin-2, isolated from the roots and leaves, respectively, were the main antiinflammatory compounds and that the caffeoylquinic acid derivatives, isolated from both leaves and roots, were the main analgesic compounds (*4, 5, 9*).

Besides the antiinflammatory bioactivity exhibited *in vitro* by the STL present in *L. ericoides* leaf extracts (*10*), such metabolites are also well-known for their cytotoxic activities (*11, 12*), which could be a problem in the case of the orally taken cachaça macerate. Such STL bioactivities have been related to the α,β-unsaturated γ-lactone group that can behave as an acceptor group in Michael addition type reactions with cysteine amino acid residues (*10*).

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Taken together, the bioactivities of *L. ericoides* extracts and secondary metabolites, as well as its popular uses, demand a sensitive analytical technique for the separation, identification, and quantification of the major secondary metabolites in the leaf extracts for more detailed analyses and future studies of infra-specific quali- and quantitative variations of its secondary metabolites. For such purposes, an HPLC-DAD method for the analyses of *L. ericoides* leaf secondary metabolites has been developed. Analytical curves for compounds representing the different classes and polarities of secondary metabolites identified in the *L. ericoides* leaves were constructed and used to validate the extraction and analysis methods. The peak identification was done by HPLC-DAD-MS and -MS/MS and coinjection with authentic standards.

MATERIALS AND METHODS

General Experimental Procedures. *Chemicals*. HPLC grade methanol (MeOH), acetonitrile (MeCN), and acetic acid were obtained from J. T. Baker. Deionized water, 18 mΩ (Milli-Q; Millipore), was used throughout the study.

Equipment. The HPLC-DAD analytical method was developed and validated on a Shimadzu LC-6AD apparatus with a diode array detector (SPD-M10Avp; Shimadzu), coupled with an autoinjector (SIL-10AF; Shimadzu) and controlled by the software CLASS-VP 6.14. A Hypersil LC-18 column (5 μm, 4.6 × 250 mm; Sigma-Aldrich) was coupled with a guard column (4.6 × 10.0 mm) of equivalent material. Analyses and peak identification by HPLC-DAD-MS and -MS/MS were performed on a Shimadzu LC-20A HPLC apparatus with a diode array detector (CBM20A; Shimadzu) coupled to an UltrOTOFq (Bruker Daltonics) ESI-qTOF mass spectrometer.

Plant Material. In order to obtain a method of sample preparation and HPLC analysis that could be virtually applied for the analysis of leaves of any *L. ericoides* specimen, independently of its provenance (collection area), the procedure for sample preparation and HPLC analytical method were developed and optimized taking into account plant material sampled from two individuals from each population bellow.

Branches of plants from five populations of *L. ericoides* Mart. were collected by Dr. Leonardo Gobbo Neto and Norberto P. Lopes at Ibiraci (NPL284; S 20° 20′ 04.6″, W 047° 08′ 22.9″; 1090 m altitude), Delfinópolis (NPL123; S 20° 20′ 55.0″, W 046° 47′ 63.8″; 870 m altitude), Capitólio (NPL225; S 20° 42′ 10.7″, W 046° 17′ 33.6″; 1090 m altitude), São João Batista do Glória (NPL221; S 20° 37′ 54.0″, W 046° 19′ 39.1″; 900 m altitude), and São José da Barra (NPL227; S 20° 38′ 31.6″, W 046° 15′ 31.8″; 1010 m altitude) and identified by Prof. Dr. João Semir, Departamento de Botânica, Instituto de Biologia−UNICAMP, SP, Brazil, where voucher materials were deposited under the codes NPL284, NPL123, NPL225, NPL221, and NPL227.

After collection, plant material was brought to the laboratories and then dried, as soon as possible, at 40 °C under forced ventilation for 48 h and stored in a freezer. In order to check the stability of the secondary metabolite constitution submitted to this process of collection and storage, one plant per population was simultaneously sampled by detachment of two branches: one was collected, dried 24 h after the detachment, and stored for 2 years before submission to the extraction and analyses method; the other branch was frozen with solid CO_2 immediately after the detachment, kept frozen until the drying process, and, just after drying, submitted to the extraction and analysis method.

Sample Preparation for Chromatographic Analysis. Prior to analyses, the branches of *L. ericoides* to be analyzed were redried for 24 h at 40 °C, and then the leaves of each branch were detached and powdered in an analytical mill. Powdered leaves (20 mg) were weighed in a glass vial and extracted in an ultrasonic bath for 10 min with 3 mL of a solution of MeOH−H_2O (9:1) containing the internal standard coumarin (15μg mL^{-1}). The extract (400 μL) was transferred to a centrifuge tube (1.5 mL), followed by the addition of 600 μL of hexane, for a liquid–liquid partition. This mixture was stirred in a vortex and then centrifuged at 1200*g* for 10 min. An aliquot of 200 μL was taken from the hydroalcoholic phase, filtered on a 0.45 μm cellulose acetate membrane, and submitted to HPLC analysis, by injection of 20 μL.

Analytical HPLC Method. The following elution gradient was employed, with a flow rate of 1.5 mL min^{-1}: solvent A = aqueous acetic acid, 2% (v/v); solvent B = MeCN, 88%−MeOH, 10%−acetic acid, 2% (v/v); elution profile = 0−35 min, 8−20% B (linear gradient), 35−65 min, 20−28% B (linear gradient), 65−95 min, 28−38% B (linear gradient), 95−135 min, 38−55% B (linear gradient), 135−145 min, 55−100% B (linear gradient), 145−150 min (column washing), 100% B (isocratic), 150−155 min, 100−8% B (linear gradient), 155−160 min (column equilibration), 8% B (isocratic); the UV-DAD detector was set to record between 210 and 600 nm, and UV chromatograms were recorded at 275 and 325 nm.

HPLC-DAD-MS and HPLC-DAD-MS/MS Analyses. Analyses by HPLC-DAD-MS and -MS/MS were performed using the same column and elution gradient described for the analytical HPLC method. The column eluent was split at a ratio of 3:1, the larger flow going to the DAD detector and the lower one to the mass spectrometer. HPLC-MS TIC chromatograms were recorded between *m/z* 50 and *m/z* 900 in both positive and negative ionization modes, and the mass spectrometer parameters were maintained the same in all analyses: 1000 scans per second; spectrum interval, 2 s; drying gas flow, 5.0 L min^{-1}; drying gas temperature, 180 °C; nebulizer gas pressure, 4 bar. After the HPLC-DAD-MS run, the *m/z* and retention time data obtained for each chromatographic peak were used to determine the HPLC-DAD-MS/MS fragmentation parameters; i.e., for online MS/MS, the retention times and *m/z* of the ion to be fragmented in the collision cell were used in combination as the input for the mass spectrometer software. Collision-induced dissociation (CID) fragmentation was performed using N_2 collision gas on the isolated protonated molecule using collision energies between 10 and 25 eV.

Analytical Curves and Method Validation. For the validation of the analytical method, the parameters, methods, and definitions described by Causon (*13*), by Ribani (*14*), and by the Brazilian Sanitary Vigilance Agency−ANVISA (*15*) were taken into consideration.

With the aim of representing each class and polarity of secondary metabolites identified in the *L. ericoides* leaf extracts and based on the availability of standards, 11 compounds were chosen for the construction of analytical curves and method validation: the di-*C*-glucosylflavones vicenin-2 (6,8-di-*C*-β-glucopyranosylapigenin) and 6,8-di-*C*-β-glucosylchrysin; the chlorogenic acid 3,5-di-*O*-(*E*)-caffeoylquinic acid (all data for this and other chlorogenic acids presented in this paper follow the recommended IUPAC numbering system for cyclitols) (*16*); the STL centratherin, 4,5-dihydro-15-deoxygoyazensolide, 4,5-dihydrolychnopholide, 4,5-dihydroeremantholide C, 16α-(1′-methylprop-1′-*Z*-enyl)eremantholide, and lychnopholide; and the flavanones pinocembrin and pinostrobin. Peak areas were calculated at 325 nm for vicenin-2 and 3,5-di-*O*-(*E*)-caffeoylquinic acid and at 270 nm for the other compounds.

Linear and analytical curves were plotted by the internal standard (IS) method for these compound standards at concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100.0, 200.0, 500.0, and 1000.0 μg mL^{-1}, in triplicate. The detection response was considered linear in an interval of 5% around the medium line plotted by the reason between the detector response (as standard substance area/IS area) at each concentration and the respective concentration versus the concentrations in a logarithmic scale. Analytical curves were then calculated using the concentrations in the linearity interval. Each determination was carried out in triplicate, and the mean of each concentration was used for calculations.

The purity of the standards was confirmed by HPLC analysis performed under the described chromatographic conditions, and for purposes of calculation, it was assumed that each standard was 100% pure.

The validation parameters analyzed were calculated for each standard substance employed for the construction of the analytical curves. The minimum limits of detection were considered the lowest concentrations in which the responses (peak areas) were three times higher than the baseline noise. The minimum limits of quantification were considered to be the lowest concentrations that could be quantified with an error

below 10% in relation to the actual concentration. The maximum limits of quantification were considered to be the upper limit of the linearity of response. The percentage coefficients of variation [i.e., (standard deviation/mean) × 100] were calculated using a standard substance solution at 20.0 μg mL^{-1} in MeOH−H_2O (9:1) for five consecutives analyses (intraassay precision) and for five analyses performed at intervals of 7 days from each other (interassay precision). The accuracies were calculated on the basis of the mean responses of five consecutive analyses, as a percentage bias, i.e., [(measured value − true value)/true value] × 100.

The overall recovery level of the method was calculated by spiking 30.0, 150.0, and 300.0 μg of each standard substance into a matrix consisting of dried and powdered *L. ericoides* leaves (20.0 mg) previously extracted exhaustively (five times) employing the same extraction method described above. The spiked matrices (four replicates for each concentration) and a blank control (only the matrix) were submitted to the extraction and analyses procedures described above. The mean of the responses of each substance was used to calculate the percentage overall recovery for each concentration, by comparing with the responses of standard solutions at the same concentrations, i.e., [response of standard spiked into matrix (processed)/response of pure standard (unprocessed)] × 100.

To check the stability of the extracts after preparation, the responses of the major chromatographic peaks in an extract obtained from the leaves of the specimen collected at Ibiraci were analyzed (in triplicate) 15, 30, 45, and 60 h after preparation, and the mean response of each peak was compared with the responses obtained for the same extract analyzed immediately after the preparation procedure. The results obtained were analyzed as percentage variations found for each peak versus the time after sample preparation (hours), and a variation up to ±5% was considered acceptable.

Chromatographic Peak Identification. Compound identification relied first on UV spectra and reasonable molecular formulas calculated from accurate mass measurements, both obtained from HPLC-DAD-MS analyses, and comparison of these data with the secondary chemistry previously reported for the *Lychnophora* genus (*4–8*). Such data were used to suggest secondary metabolites for each peak. When authentic standard compounds were available, coinjection was also used for peak identity confirmation. HPLC-DAD-MS/MS was subsequently carried out for structure elucidations and to support/confirm the peak assignments. Fundamental information for the structural elucidation by product ion spectra (MS/MS) was obtained from previous reports that identified the key fragments of chlorogenic acid derivatives (C_6C_3 moiety elimination), of goyazensolide STL (side chain elimination and some diagnostic ions) and of flavonoids (C-ring fragmentation pattern), in the positive and/or negative modes of ionization. The fragmentation patterns of caffeoylquinic acids (*17–20*), goyazensolide moiety STL (*21*), flavonoids, and chalcones (*22–25*) were compared with those reported elsewhere, and patterns of eremantholide type STL were compared with spectra obtained by us for authentic standards previously isolated by our group.

The authentic standards used were obtained from the following sources: coumarin for the HPLC internal standard was from Merck; 5-*O*-(*E*)-caffeoylquinic acid was purchased from Acros (Belgium); pinostrobin, pinocembrin, pinobanksin, vicenin-2, 6,8-di-*C*-β-glucopyranosylchrysin, 15-hydroxy-16α-(1′-methylprop-1′-*Z*-enyl)eremantholide, 4,5-dihydroeremantholide C, 4,5-dihydro-16α-(1′-methylprop-1′*Z*-enyl)eremantholide, 16α-(1′-methylprop-1′-*Z*-enyl)eremantholide, centratherin, 4,5-dihydro-15-deoxygoyazensolide, 4,5-dihydrolychnopholide, zexbrevanolide tiglate, lychnopholide, 3,5-, 4,5-, and 3,4-di-*O*-(*E*)-caffeoylquinic acids, and 3,4,5-tri-*O*-(*E*)-caffeoylquinic acid were previously isolated from *L. ericoides* (*4–6*); 3-*O*-acetylpinobanksin and 3-*O*-methylquercetin were previously isolated from *Lychnophora staavioides* (*26*); tiliroside [3-*O*-(6″-*O*-(*E*)-*p*-coumaroyl)-β-glucopyranosylkaempferol] was previously isolated from *Lychnophora passerina* (*27*); 3-*O*-(6″-*O*-(*E*)-*p*-coumaroyl)-β-glucopyranosylisorhamnetin was previously isolated from *Lychnophora pohlii* (*28*); and 15-hydroxyeremantholide C was previously isolated from *Lychnophora rupestris* (*29*).

RESULTS AND DISCUSSION

Chromatographic Peak Identification. Initially, extracts from all of the individual *L. ericoides* plants collected were analyzed by HPLC-DAD-MS, and it was found that almost all of the chromatographic peaks identified in these samples were present in the Ibiraci specimen. Hence, chromatographic peak identification was based on these specimen extracts, and, when necessary, extracts of the other *L. ericoides* individuals collected were analyzed by HPLC-DAD-MS and/or -MS/MS for peak identity confirmation and to obtain MS/MS spectra of compounds present in these specimens in greater amounts than in the Ibiraci specimen. Chromatograms recorded at 270 nm (a wavelength in which all of the peaks could be seen), representative of the developed HPLC-DAD method and presenting the numbered chromatographic peaks, are shown in **Figure 1**. Chromatograms at 325 nm are available as Supporting Information. The retention times and UV, MS, and MS/MS data obtained by HPLC-DAD-MS and -MS/MS analyses are presented in **Table 1**. Chemical structures of the identified compounds are presented in **Figure 2**, and the identification of the chromatographic peaks was performed as follows:

(a) Chlorogenic Acids. Chlorogenic acids are esters of *trans*-cinnamic acids (coumaroyl, caffeoyl, feruloyl, and dimethoxycinnamoyl acids) with quinic acid. The *trans*-cinnamic acids can be esterified at one or more of the hydroxyls at positions 1, 3, 4, and 5 of quinic acid, originating series of positional isomers. Such compounds are secondary metabolites widespread among vascular plants, especially the caffeoylquinic acids, which have already been isolated from *L. ericoides* roots and were expected to be found in the leaf extracts (*5*).

Chromatographic peaks 1, 3, and 4 presented *m/z* 353 as base peaks (bp) in negative ionization mode mass spectra and UV spectra characteristics of caffeoylquinic derivatives (UV max: ≈298 and 325 nm) which, when taken together, suggest positional isomers of a quinic acid (QA) esterified with a single caffeoyl (CAF) unit. The product ion spectra obtained by negative ion MS/MS for precursor ions *m/z* 353 were different from each other, and comparison with the caffeoylquinic acids (CQA) identification keys published by Clifford et al. (*17–19*) led to the individualization of three CQA positional isomers. The product ion spectrum for peak 3 showed *m/z* 173 (dehydrated quinic moiety) as the bp, *m/z* 191 [23% relative intensity (ri); loss of caffeic moiety], and *m/z* 179 (64% ri; loss of quinic moiety). As *m/z* 173 is a diagnostic ion that occurs only in CQA possessing a caffeoyl moiety esterified in position 4 of QA, peak 3 was attributed to 4-CQA (**Figure 2**, structure **3**). The product ion spectrum for peak 1 showed *m/z* 191 (bp) and *m/z* 179 at 51% ri, and that of peak 4 showed *m/z* 191 (bp) and 179 at 4% ri. The greater relative intensity of *m/z* 179 in the product ion spectrum of peak 1, as well as the information that 3-CQA should elute first in a C-18 packing (*17–19*), led to the identification of peak 1 as 3-CQA (**Figure 2**, structure **1**) and, consequently, of peak 4 as 5-CQA (**Figure 2**, structure **4**). Furthermore, the identity of peak 4 was also confirmed through coelution with a 5-CQA authentic standard. The measured accurate masses of the precursor ions of these three peaks (**Table 1**) are also in agreement with the calculated exact mass of protonated and deprotonated CQA ($C_{16}H_{17}O_9$): $[M - H]^-$ 353.0873 and $[M + H]^+$ 355.1029.

Peaks 10, 11, and 12 showed UV spectra identical to the CQA described above and *m/z* 515 as bp in the negative ion mass spectra, suggesting diCQA positional isomers. Their measured accurate masses (**Table 1**) are also in agreement with the calculated exact mass of protonated and deprotonated diCQA

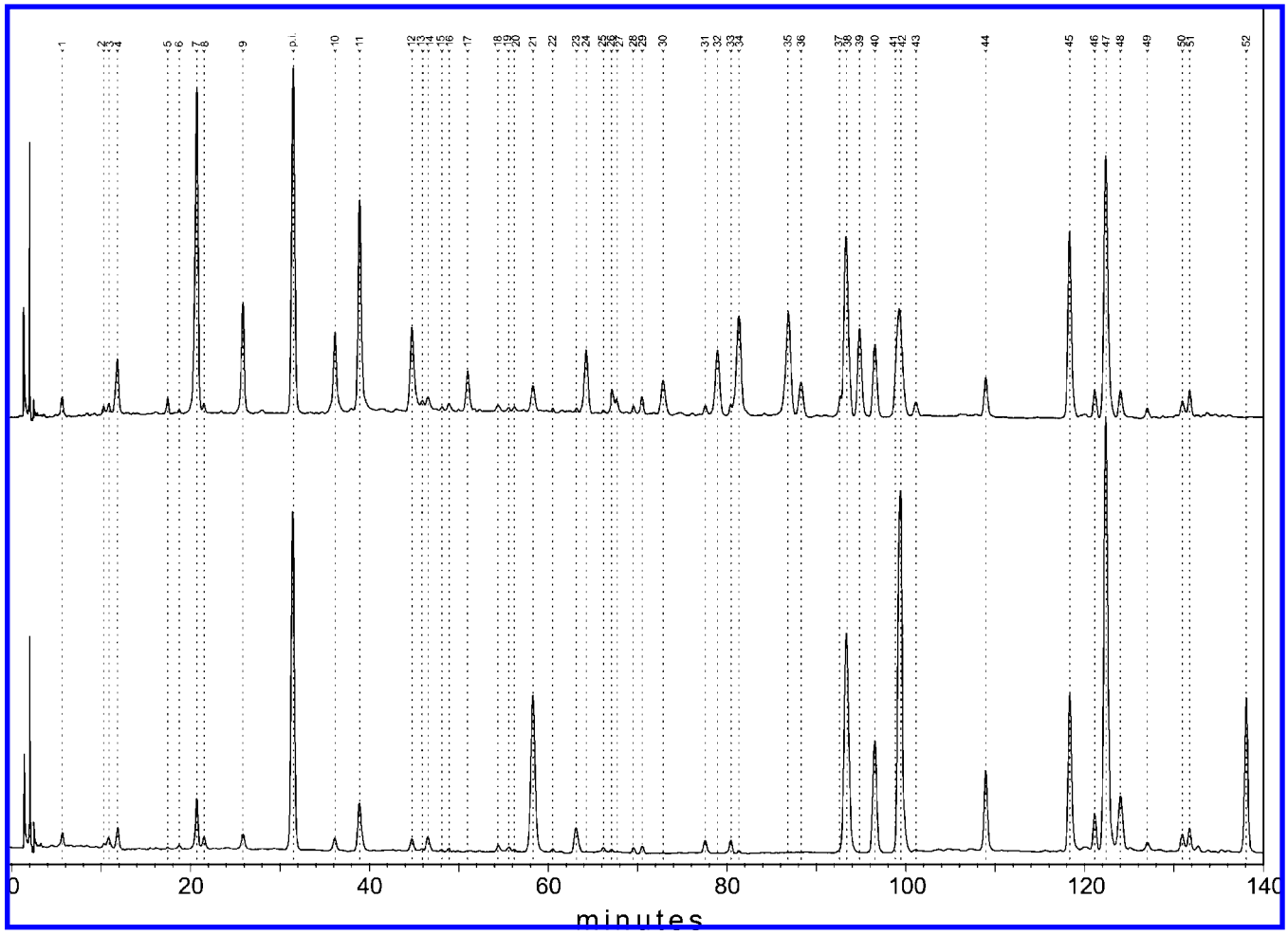

Figure 1. Chromatograms, registered at 270 nm, obtained by employing the developed extraction and HPLC-DAD analysis methods for two *L. ericoides* specimens collected at Ibiraci and Delfinópolis (MG State, Brazil). The chromatographic peak identities are reported in **Table 1** and their chemical structures in **Figure 2**.

($C_{25}H_{27}O_{12}$): $[M - H]^-$ 515.1190 and $[M + H]^+$ 517.1346. Peaks 11 and 12 were attributed respectively to 3,5-diCQA (**Figure 2**, structure **11**) and 4,5-diCQA (**Figure 2**, structure **12**), through coelution with authentic standards of these compounds. Their identity was also confirmed by the negative mode product ion spectra obtained from the fragmentation of their precursor ions *m/z* 515 (*17–19*). As discussed above, *m/z* 173, present in the product ion spectrum of peak 10 (and peak 12, as well), is indicative of acylation at position 4. Furthermore, none of the product ions characteristic of 1,4-diCQA (*m/z* 299 and 203) (*17–19*) are present in the product ion spectrum of peak 10, leading to the identification of this peak as 3,4-diCQA (**Figure 2**, structure **10**).

Employing the same identification strategy above, peak 26 could be attributed to 3,4,5-triCQA (**Figure 2**, structure **26**). Its retention time (coelution) matched that of our 3,4,5-triCQA standard, and its measured accurate mass (**Table 1**) matches the calculated exact masses of a tri-CQA ($C_{34}H_{30}O_{15}$): $[M - H]^-$ 677.1506 and $[M + H]^+$ 679.1663.

The HPLC-DAD-MS and -MS/MS analyses led also to the identification of feruloylquinic acids (FQA) and feruloylcaffeoylquinic acids (FCQA) in the *L. ericoides* leaf extracts. The minor chromatographic peaks 2, 6, and 8 presented, in the negative ion spectra, bp 14 *m/z* larger than CQA in agreement with the calculated exact mass for a FQA ($C_{17}H_{20}O_9$: $[M - H]^-$ 367.1029), as well as UV spectra characteristic of chlorogenic acids. The negative ionization mode fragmentation of the precursor ion *m/z* 367 of peak 6 produced *m/z* 173 as bp. This is a diagnostic ion of acylation in position 4 of quinic acid (*17, 18*) and allows the identification of the compound as 4-FQA (**Figure 2**, structure **6**). On the other hand, *m/z* 193 and *m/z* 191 were found as base peaks in the product ion spectra of peaks 2 and 8, respectively, leading to their identification as 3-FQA (**Figure 2**, structure **2**) and 5-FQA (**Figure 2**, structure **8**), based on the chlorogenic acid identification by Clifford et al. (*17, 18*). The retention times of these three FQA repeat the elution pattern observed for the three CQAs: 3-acylquinic acid elutes first, followed by 4- and then 5-acylquinic acids.

Four other minor chromatographic peaks, 14, 15, 18, and 20, also possessing UV spectra characteristic of chlorogenic acids, presented in negative ion mass spectra bp 14 *m/z*, larger than the diCQA. This is in agreement with the calculated exact mass for a CFQA ($C_{26}H_{26}O_{12}$: $[M - H]^-$ 529.1346). The product ion spectra of the precursor ions *m/z* 529 obtained for these peaks presented product ions in agreement with the expected fragments for CFQA but were not sufficient for the individualization of these peaks; i.e., for the unequivocal identification of the positional isomers responsible for each chromatographic peak, further MS^3 experiments would be required (*17, 18*). Since all positional isomers of both caffeoyl- and feruloylquinic derivatives had been already identified (excluding acylation in position 1 of quinic acid), all of the six possible positional isomers of CFQA (also excluding acylation in position 1) were expected to be present in the *L. ericoides* extracts. Extraction

of *m/z* 529 from the negative ionization HPLC-MS TIC chromatogram produced but six peaks (13−16, 18, and 20) exhibiting bp *m/z* 529 (and accurate masses in agreement with exact masses of CFQA), which were attributed to the CFQA positional isomers. Additionally, in the positive ionization mode, all of the six CFQA peaks could be detected, and their protonated molecule accurate masses were also in agreement with the exact masses of $[CFQA + H]^+$ 531.1502. Positive mode product ion spectra were obtained for these precursor ions *m/z* 513 (i.e., $[CFQA + H - H_2O]^+$), but since product ion spectra in positive mode of chlorogenic acids with the same acyl moieties are very similar to each other, individualization of the positional isomers was not possible. Nevertheless, the presence of both caffeic and ferulic moieties in each of these compounds could be confirmed by the presence, in positive mode product ion spectra, of the diagnostic ions *m/z* 163 and *m/z* 177, which are related to the fragmentation of caffeoyl and feruloyl acyl substituents, respectively.

Chromatographic peak 19 could be attributed to diFQA due to its characteristic UV spectrum and measured accurate masses (14 *m/z* larger than the CFQA and 28 *m/z* larger than the diCQA) in both positive and negative ionization that suggested the molecular formula $C_{27}H_{28}O_{12}$. Comparing the product ion spectrum obtained for the precursor ion *m/z* 543 in the negative ionization mode with the data obtained by Clifford et al. (*19*), this peak could be identified as the 3,4-diFQA (**Figure 2**, structure **19**). It presents the product ions *m/z* 193 and *m/z* 173 which, as discussed above, are diagnostic for the 3- and 4-FQA, respectively, thus indicating esterification of ferulic acid at these positions of quinic acid. As the other two positional isomers of diFQA (i.e., 3,5- and 4,5-diFQA) were also expected, an extraction of *m/z* 543 from the negative ionization HPLC-MS TIC chromatogram produced but three peaks (19, 22, and 25) exhibiting *m/z* 543 as bp. Thus, the minor peaks 22 and 25 were putatively attributed to the other positional isomers of diFQA.

This is the first reported occurrence of 3-CQA and 4-CQA, and of the compounds of the isomeric series of FQA, CFQA, and diFQA in plants of the Lychnophorinae subtribe, in which *Lychnophora* is classified.

(b) Flavonoids. (i) Flavonoid Aglycons. Chromatographic peaks 38 and 47 showed UV spectra typical of dihydroflavonols or flavanones (*30*). Comparison of their retention times and UV spectra with standard compounds permitted the attribution of peaks 38 and 47 to compounds pinocembrin (**Figure 2**, structure **38**) and pinostrobin (**Figure 2**, structure **47**), respectively. The intensity of both compounds was low in the positive ionization HPLC-MS TIC chromatograms, indicating a relative low ionization capacity for them, and hence, a product ion spectrum could be achieved only for peak 47. On the other hand, in negative ionization mode, a product ion spectrum was successfully achieved for peak 38, while peak 47 could not be observed in the HPLC-MS TIC chromatograms. The obtained product ion spectra presented the expected fragmentation patterns for pinocembrin and pinostrobin (*22–24*). Their measured accurate masses (**Table 1**) are also in agreement with the exact masses calculated for pinocembrin ($C_{15}H_{12}O_4$: $[M - H]^-$ 255.0657 and $[M + H]^+$ 257.0814) and pinostrobin ($C_{16}H_{14}O_4$: $[M + H]^+$ 271.0970).

The chromatographic peaks 40 and 45 exhibited nearly identical accurate masses and fragmentation patterns (**Table 1**) to those observed for the flavanones pinocembrin (peak 38) and pinostrobin (peak 47), respectively. However, UV spectra produced by these peaks were very different from those produced by the flavanones, presenting the highest absorbance above 340 nm (**Table 1**) and typical of chalcones (*30*). It is well-known that the isomerization of 2′-hydroxychalcones to the corresponding flavanones occurs in the gas phase, leading to nearly identical mass spectra for these isomeric forms (*25*). Hence, peaks 40 and 45 could be assigned to the isomeric forms of pinocembrin and pinostrobin: the chalcones 2′,4′,6′-trihydroxychalcone (**Figure 2**, structure **40**) and 2′,6′-dihydroxy-4′-methoxychalcone (**Figure 2**, structure **45**), respectively. Signals of both chalcones were relatively low in the positive ionization HPLC-MS TIC chromatograms, but, even so, product ion spectra could be obtained for them. In the negative ionization HPLC-MS TIC chromatograms, both chalcones exhibited strong signals, and their product ion spectra were in agreement with the fragmentation data described in literature (*22–25*). Product ion *m/z* 153, present as bp in the positive ionization product ion spectrum obtained for peak 40 (2′,4′,6′-trihydroxychalcone), is a diagnostic ion for a dihydroxy A ring in flavonoids (*22–24*) (as will be discussed below, in peak 42 identification), thus confirming the assignment of 2′,4′,6′-trihydroxychalcone to this peak. Conversely, in the positive ionization product ion spectrum obtained for peaks 45 (2′,6′-dihydroxy-4′-methoxychalcone) and 38 (the flavanone pinocembrin) the product ion *m/z* 153 was absent, and the A rings with a hydroxyl and a methoxyl group were confirmed by *m/z* 167.

Peak 21 also presented a UV spectrum typical of a dihydroflavonol/flavanone (*30*). It matched exactly the UV spectrum and retention time of a pinobanksin standard previously isolated from *L. ericoides*, and its measured accurate masses (**Table 1**) are in agreement with the calculated exact masses of this compound ($C_{15}H_{12}O_5$), thus confirming the assignment of pinobanksin (**Figure 2**, structure **21**) to peak 21. Peak 42 presented a nearly identical UV spectrum to that obtained for pinobanksin and a bp (*m/z* 313) 42 *m/z* larger than that compound in both negative and positive ionization, which suggests an acetate moiety esterified to pinobanksin. In negative ionization, at 15 eV collision energy (data not shown), only two product ions were produced from parent ion *m/z* 313: losses of neutral acetic acid and C_2H_2O, originating *m/z* 253 and 271, respectively. Otherwise, at higher collision energy (25 eV), further fragmentation occurs, resulting in a product ion spectrum very similar to that obtained for pinobanksin. In the positive ionization mode, the most informative ion produced by the fragmentation of the precursor ion *m/z* 315 is the product ion *m/z* 153, which was attributed to a ^{1,3}A flavonoid fragment, employing here the flavonoid fragmentation nomenclature established by Ma et al. (the ^{1,3}A fragment is relative to the cleavage of the bond at positions 1/3, bonds between O-1 and C-2 and between C-3 and C-4 of ring C, with the charge retention by ring A) (*22, 24*). This product ion confirms the molecular mass and, consequently, the substituents proposed for ring A. Finally, peak 42 was attributed to 3-*O*-acetylpinobanksin (**Figure 2**, structure **42**) by the comparison of its retention time (coelution) and UV spectra with an authentic standard of 3-*O*-acetylpinobanksin and its measured accurate masses (**Table 1**), which are in agreement with the calculated masses for acetylpinobanksin ($C_{17}H_{12}O_6$: $[M - H]^-$ 313.0712 and $[M + H]^+$ 315.0868).

Peak 48 also produced a UV spectrum characteristic of a flavanone or dihydroflavonol (*30*), and its bp accurate mass (**Table 1**) in positive ionization mode suggests the molecular formula $C_{18}H_{17}O_6$ for the protonated molecule, with no other reasonable molecular formula in between a ±15 ppm error interval. This suggests a methyl group in addition to the 3-*O*-acetylpinobanksin chemical structure ($[M + H]^+$ $C_{17}H_{15}O_6$). The

Table 1. Identification of HPLC Chromatographic Peaks of *L. ericoides* Leaf Extracts and Data Taken from HPLC-DAD-MS and HPLC-DAD-MS/MS Analyses[a]

peak	rt (min)	compound	positive ionization TIC chromatogram ions (*m*/*z*)	positive ionization MS/MS	negative ionization TIC chromatogram ions (*m*/*z*)	negative ionization MS/MS	UV max (nm)
1	5.6	3-*O*-(*E*)-caffeoylquinic acid	$[M + H]^+$ 355.1037 (bp), $[MH + Na]^+$ 377, $[MH - QA]^+$ 163	15 eV: 355 → 163 (bp)	$[M - H]^-$ 353.0879 (bp), $[M - CAF]^-$ 191	15 eV: 353 → 191 (bp), 179	299, 324
2	10.6	3-*O*-(*E*)-feruloylquinic acid	$[M + H]^+$ 369.1195, $[M + Na]^+$ 391, $[MH - QA]^+$ 177 (bp)	–	$[M - H]^-$ 367.1040 (bp), $[FER - H]^-$ 193	15 eV: 367 → 193 (bp), 191	299, 325
3	10.9	4-*O*-(*E*)-caffeoylquinic acid	$[M + H]$ 355.1038 (bp), $[M + Na]^+$ 377, $[MH - QA]^+$ 163	15 eV: 355 → 163 (bp)	$[M - H]$ 353.0881 (bp), $[M - CAF - H_2O]^-$ 173	15 eV: 353 → 191, 179, 173 (bp)	300, 325
4	11.8	5-*O*-(*E*)-caffeoylquinic acid	$[M + H]^+$ 355.1035 (bp), $[M + Na]^+$ 377, $[MH - QA]^+$ 163	15 eV: 355 → 163 (bp)	$[M - H]^-$ 353.0879 (bp), $[M - CAF]^-$ 191	15 eV: 353 → 191 (bp), 179	299, 325
5	17.4	lucenin-2 (6,8-di-*C*-β-glucopyranosylluteolin)	$[M + H]$ 611.1615 (bp), $[MH - H_2O]^+$ 593	15 eV: 611 → 593, 575, 557, 545, 539, 527, 497, 491, 473 (bp), 443, 425	$[M - H]^-$ 609.1450 (bp)	25 eV: 609 → 591, 519, 489 (bp), 399, 369	271, 290 sh, 339
6	18.8	4-*O*-(*E*)-feruloylquinic acid	$[M + H]^+$ 369.1195, $[M + Na]^+$ 391, $[MH - QA]^+$ 177 (bp)	–	$[M - H]^-$ 367.1037 (bp), $[M - FER]^-$ 191, $[M - FER - H_2O]^-$ 173	15 eV: 367 → 173 (bp), 191	300, 324
7	20.7	vicenin-2 (6,8-di-*C*-β-glucopyranosylapigenin)	$[M + H]^+$ 595.1655 (bp), $[MH - H_2O]^+$ 577	15 eV: 595 → 577, 559, 541, 529, 523, 511, 481, 475, 457 (bp), 427, 409	$[M - H]^-$ 593.1518 (bp)	25 eV: 593 → 575, 503, 473 (bp), 383, 353	271, 334
8	21.6	5-*O*-(*E*)-feruloylquinic acid	$[M + H]^+$ 369.1193, $[M + Na]^+$ 391, $[MH - QA]^+$ 177 (bp)	–	$[M - H]^-$ 367.1039 (bp), $[M - FER]^-$ 191	15 eV: 367 → 191 (bp) , 173	299, 324
9	25.8	6,8-di-*C*-β-glucopyranosylchrysin	$[M + H]^+$ 579.1708 (bp), $[MH - H_2O]^+$ 561	15 eV: 579 → 561, 543, 525, 513, 507, 495, 465, 459, 441 (bp) , 411, 393	$[M - H]^-$ 577.1570 (bp)	25 eV: 577 → 559, 487, 457 (bp), 367, 337	272, 317
10	36.1	3,4-di-*O*-(*E*)-caffeoylquinic acid	$[M + H]^+$ 517.1355, $[MH - H_2O]^+$ 499 (bp), $[MH - QA]^+$ 163	15 eV: 499 → 319 (bp), 163	$[M - H]^-$ 515.1204 (bp)	15 eV: 515 → 353 (bp), 335, 191, 179, 173	300, 325
11	38.9	3,5-di-*O*-(*E*)-caffeoylquinic acid	$[M + H]^+$ 517.1354, $[MH - H_2O]^+$ 499 (bp)	15 eV: 499 → 319 (bp), 163	$[M - H]^-$ 515.1207 (bp), $[M - CAF]^-$ 353	15 eV: 515 →353 (bp), 191, 179	300, 325
12	44.5	4,5-di-*O*-(*E*)-caffeoylquinic acid	$[M + H]^+$ 517.1357, $[MH - H_2O]^+$ 499 (bp), $[MH - QA]^+$ 163	15 eV: 499 → 319 (bp), 163	$[M - H]^-$ 515.1206 (bp)	15 eV: 515 → 353 (bp), 191, 179, 173	299, 325
13	45.3	caffeoylferuloylquinic acid*	$[M + H]^+$ 531.1510, $[MH - H_2O]^+$ 513 (bp), $[M + Na]^+$ 553	15 eV: 513 → 333, 319, 177 (bp), 163	$[M - H]^-$ 529.1360 (bp)	–	299, 324
14	46.1	caffeoylferuloylquinic acid*	$[M + H]^+$ 531.1510, $[MH - H_2O]^+$ 513 (bp), $[M + Na]^+$ 553	15 eV: 513 → 333, 319, 177 (bp), 163	$[M - H]^-$ 529.1358 (bp), $[M - CAF]^-$ 367	15 eV: 529 → 367 (bp), 335, 193, 173	300, 325
15	48.7	caffeoylferuloylquinic acid*	$[M + H]^+$ 531.1512, $[MH - H_2O]^+$ 513 (bp), $[M + Na]^+$ 553	15 eV: 513 → 333, 319, 177 (bp), 163	$[M - H]^-$ 529.1360 (bp), $[M - CAF]^-$ 367	15 eV: 529 → 367 (bp), 193	299, 324
16	49.6	caffeoylferuloylquinic acid*	$[MH - H_2O]^+$ 513 (bp), $[M + Na]^+$ 553	–	$[M - H]^-$ 529.1361 (bp)	–	299, 324
17	50.9	15-hydroxyeremantholide C	$[M + H]^+$ 363.1447, $[MH - H_2O]^+$ 345 (bp)	15 eV: 363 → 345, 301; 10 eV: 345 → 301, 283, 255 (bp), 203	$[M - H]^-$ 361.1297 (bp)	–	266
18	53.8	caffeoylferuloylquinic acid*	$[M + H]^+$ 531.1507, $[MH - H_2O]^+$ 513 (bp), $[M + Na]^+$ 553	15 eV: 513 → 333, 319, 177 (bp), 163	$[M - H]^-$ 529.1359 (bp), $[M - CAF]^-$ 367	15 eV: 529 → 367 (bp), 173	300, 324
19	55.0	3,4-di-*O*-(*E*)-feruloylquinic acid	$[M + H]^+$ 545.1671, $[MH - H_2O]^+$ 527 (bp), $[M + Na]^+$ 567	15 eV: 527 → 333, 177 (bp)	$[M - H]^-$ 543.1514 (bp)	20 eV: 543 → 367, 349 (bp), 193, 173	300, 325
20	55.7	caffeoylferuloylquinic acid*	$[M + H]^+$ 531.1513, $[MH - H_2O]^+$ 513 (bp), $[M + Na]^+$ 553	15 eV: 513 → 333, 319, 177 (bp), 163	$[M - H]^-$ 529.1359 (bp)	15V: 529 → 353, 191, 179, 173 (bp)	299, 324
21	58.3	pinobanksin	$[M + H]^+$ 273.0757 (bp)	–	$[M - H]^-$ 271.0614 (bp)	23 eV: 271 → 253 (bp), 225, 209, 197, 185, 161	291, 335 sh
22	60.1	diferuloylquinic acid*	$[M + H]^+$ 545.1668, $[MH - H_2O]^+$ 527 (bp), $[M + Na]^+$ 567	15 eV: 527 → 333, 177 (bp)	$[M - H]^-$ 543.1519 (bp)	–	300, 325
23	63.4	3-*O*-methylquercetin	$[M + H]^+$ 317.0654 (bp)	22 eV: 317 → 302 (bp), 301, 274, 273, 245, 229	$[M - H]^-$ 315.0495 (bp)	17 eV: 315 → 300 (bp), 271, 255, 243	255, 268 sh, 295, 355
24	64.2	15-hydroxy-16α-(1′-methylprop-1′-*Z*-enyl)eremantholide	$[M + H]^+$ 377.1610, $[MH - H_2O]^+$ 359 (bp)	12 eV: 377 → 359, 315; 15 eV: 359 → 315, 297, 269, 241, 213, 203 (bp), 175	$[M - H]^-$ 375.1451 (bp)	–	268
25	65.8	diferuloylquinic acid*	$[M + H]^+$ 545.1673, $[MH - H_2O]^+$ 527 (bp), $[M + Na]^+$ 567	–	$[M - H]^-$ 543.1517 (bp)	–	300, 325
26	67.0	3,4,5-tri-*O*-*E*-caffeoylquinic acid	$[M + H]^+$ 679.1673[$MH - H_2O]^+$ 661[$MH -$ CAF-$H_2O]^+$ 499 (bp), $[M + Na]^+$ 701	15 eV: 679 → 661, 499, 163 (bp)	$[M - H]^-$ 677.1521 (bp), $[M - CAF]^-$ 515	15 eV: 677 → 515 (bp), 353, 163	299, 325
27	67.6	centratherin	$[M + H]^+$ 375.1452 (bp), $[M + Na]^+$ 397	15 eV: 375 → 275, 257, 239, 229 (bp), 211, 201, 183	$[M - H]^-$ 373.1272 (bp)	–	269

28	69.3	tiliroside [3-*O*-(6″-*O*-(*E*)-*p*-coumaroyl)-*β*-glucopyranosylkaempferol]	$[M + H]^+$ 595.1435 (bp), $[M + Na]^+$ 617, $[coumaroylhexose]^+$ 309, $[aglycon + H]^+$ 287	17 eV: 595 → 309.0967 291, 287 (bp), 165; 147.0448	$[M - H]^-$ 593.1269 (bp)	20 eV: 593 → 447, 285 (bp)	267, 295 sh, 315, 355 sh
29	71.1	3-*O*-(6″-*O*-(*E*)-*p*-coumaroyl)-*β*-glucopyranosylisorhamnetin	$[M + H]^+$ 625.1555 (bp), $[M + Na]^+$ 647, $[aglycon + H]^+$ 317, $[coumaroylhexose]^+$ 309	17 eV: 625 → 317 (bp), 309.0969, 291, 165, 147.0434	$[M - H]^-$ 623.1375 (bp)	20 eV: 623 → 477, 315 (bp)	267, 295 sh, 315, 355 sh
30	72.9	$C_{19}H_{24}O_6$, possible eremantholide STL	$[M + H]^+$ 349.1659, $[MH - H_2O]^+$ 331 (bp)	18 eV: 349 → 331, 241, 215, 189 (bp), 161; 15 eV: 331 → 241, 229, 189 (bp), 161	–	–	264
31	77.5	unidentified	–	–	–	–	287, 330 sh
32	78.9	4,5-dihydro-15-deoxygoyazensolide	$[M + H]^+$ 347.1501 (bp), $[M + Na]^+$ 369	15 eV: 347 → 261, 243, 233, 215 (bp), 197, 187, 159	–	–	264
33	80.5	possible flavanone or dihydroflavonol, $C_{16}H_{14}O_4$	$[M + H]^+$ 271.0977 (bp)	–	$[M - H]^-$ 269.0808 (bp)	–	283, 325 sh
34	81.3	4,5-dihydroeremantholide C	$[M + H]^+$ 349.1660, $[MH - H_2O]^+$ 331 (bp)	12 eV: 349 → 331, 189 (bp), 161; 15 eV: 331 → 285, 257, 217, 189 (bp), 161	$[M - H]^-$ 347.1497 (bp)	–	265
35	86.8	4,5-dihydro-16α-(1′-methylprop-1′-*Z*-enyl)eremantholide	$[M + H]^+$ 363.1816, $[MH - H_2O]^+$ 345 (bp)	15 eV: 363 → 345, 317, 203 (bp), 189, 161; 15 eV: 345 → 317, 219, 203, 189 (bp), 161	$[M - H]^-$ 361.1644 (bp)	–	265
36	88.3	4,5-dihydrolychnopholide	$[M + H]^+$ 361.1644 (bp), $[M + Na]^+$ 383	10 eV: 361 → 261, 243, 233, 215 (bp), 197, 187, 169	–	–	264
37	92.9	zexbrevanolide tiglate	$[M + H]^+$ 361.1649 (bp), $[M + Na]^+$ 383	10 eV: 361 → 261, 243, 233, 215 (bp), 197, 187, 169	–	–	264
38	93.3	pinocembrin	$[M + H]^+$ 257.0805 (bp)	–	$[M - H]^-$ 255.0650 (bp)	20 eV: 255 → 213 (bp), 211, 185, 171, 169, 151	289, 326 sh
39	94.7	putative 4,5-dihydro-16α-(1′-methylprop-1′-*E*-enyl)eremantholide	$[M + H]^+$ 363.1815, $[MH - H_2O]^+$ 345 (bp)	18 eV: 363 → 345, 203, 189 (bp); 15 eV: 345 → 317, 219, 203, 189 (bp), 161	$[M - H]^-$ 361.1645 (bp)	–	264
40	96.5	2′,4′,6′-trihydroxychalcone	$[M + H]^+$ 257.0809 (bp)	15 eV: 257 → 239, 215, 179, 173, 153 (bp)	$[M - H]^-$ 255.0651 (bp)	20 eV: 255 → 213, 211 (bp), 185, 171, 169, 151	343
41	99.9	16α-(1′-methylprop-1′-*Z*-enyl)eremantholide	$[M + H]^+$ 361.1643, $[MH - H_2O]^+$ 343 (bp)	15 eV: 361 → 343, 215, 187 (bp); 13 eV: 343 → 299, 281, 269, 243, 215, 187 (bp), 159	$[M - H]^-$ 359.1502 (bp)	–	267
42	99.3	3-*O*-acetylpinobanksin	$[M + H]^+$ 315.0875 (bp), $[MH - C_2H_2O]^+$ 273	18 eV: 315 → 273, 255, 227 (bp), 199, 181, 153	$[M - H]^-$ 313.0715 (bp), $[M - HOAc]^-$ 253	25 eV: 313 → 271, 253 (bp), 225, 209, 197	292, 335 sh
43	101.1	lychnopholide	$[M + H]^+$ 359.1489 (bp), $[M + Na]^+$ 381	15 eV: 359 → 259, 241, 231, 213 (bp), 185	–	–	267
44	108.9	$C_{19}H_{20}O_5$	$[M + H]^+$ 329.1379 (bp), $[M + Na]^+$ 351	–	$[M - H]^-$ 327.1236 (bp)	15 eV: 327 → 312 (bp), 179, 164, 147	276
45	118.3	2′,6′-dihydroxy-4′-methoxychalcone	$[M + H]^+$ 271.0979 (bp), $[M + Na]^+$ 293	15 eV: 271 → 167 (bp), 131	$[M - H]^-$ 269.0807 (bp)	20 eV: 269 → 254 (bp), 253, 236, 226, 225, 191, 177, 165	341
46	121.2	putative 3-*O*-propionylalpinone	$[M + H]^+$ 343.1538 (bp), $[M + Na]^+$ 365	10 eV: 343 → 329, 287 (bp), 269, 241, 213	–	–	274, 340 sh
47	122.4	pinostrobin	$[M + H]^+$ 271.0977 (bp), $[M + Na]^+$ 293	15 eV: 271 → 167 (bp), 131	–	–	290, 330 sh
48	124.0	3-*O*-acetylalpinone	$[M + H]^+$ 329.1034 (bp), $[M + Na]^+$ 351	18 eV: 329 → 287, 269, 241, 213 (bp), 195, 167	–	–	287, 330 sh
49	126.8	possible flavanone or dihydroflavonol	–	–	–	–	276, 340 sh
50	130.9	possible flavanone or dihydroflavonol	–	–	–	–	275, 340 sh
51	131.8	possible flavanone or dihydroflavonol	–	–	–	–	276, 340 sh
52	137.8	possible flavanone or dihydroflavonol	–	–	–	–	276, 340 sh

[a] Peak numbers refer to Figure 1. bp = base peak (100% relative intensity); sh = shoulder; QA = quinic acid; CAF = caffeoyl; FER = feruloyl. * = positional isomers of caffeoylquinic acids which could not be distinguished from each other.

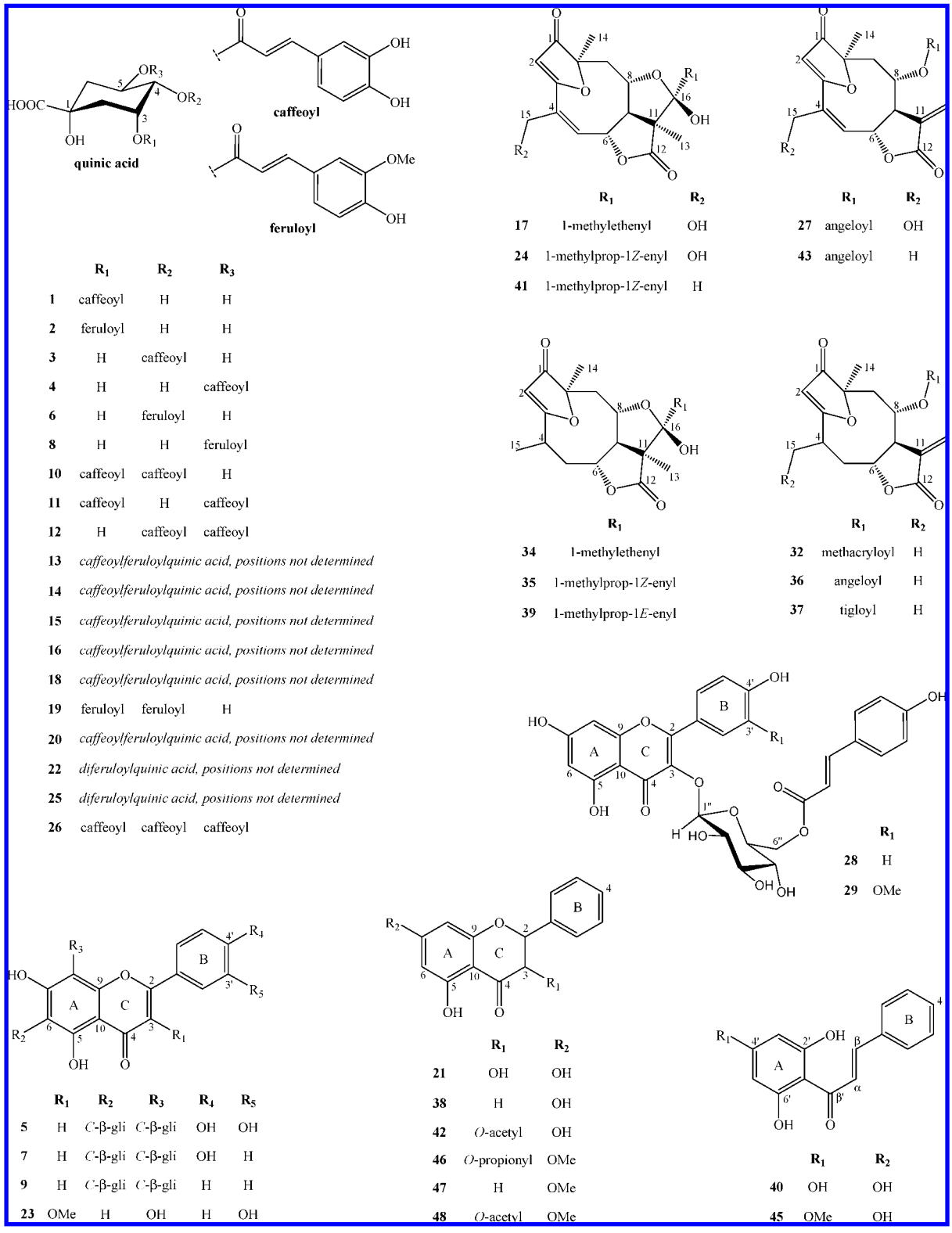

Figure 2. Chemical structures of the compounds identified in the *L. ericoides* leaf extracts. The numbers and identity of the structures correspond to the respective chromatographic peak numbers in **Figure 1** and **Table 1**.

positive ionization product ion spectrum obtained for the precursor ion *m/z* 329 exhibited the same fragmentation pattern observed for 3-*O*-acetylpinobanksin (peak 42), with an increase of 14 *m/z* for each product ion (**Table 1**). Drawing a parallel with the identification of 3-*O*-acetylpinobanksin described above, the absence of product ion *m/z* 153, as well as the presence of *m/*z 167, confirms that ring A possesses the same substitution pattern of pinostrobin (peak 47). Hence, peak 48 could be assigned to putative 3-*O*-acetylalpinone (**Figure 2**, structure **48**).

Similarly, peak 46 also produced a typical dihydroflavonol/flavanone UV spectrum (*30*) and presented a bp in positive ionization mode pointing to the molecular formula $C_{20}H_{22}O_5$, with no other plausible molecular formula in between a ±15 ppm error interval. The positive ionization product ion spectrum obtained for the precursor ion *m/z* 343 revealed a fragmentation

pattern nearly identical to that observed for 3-*O*-acetylalpinone (peak 48) discussed above (**Table 1**). However, the product ion spectrum obtained for peak 46 exhibited the loss of a 74 *m*/*z* fragment from precursor ion *m*/*z* 343, producing a product ion corresponding to the aglycon at *m*/*z* 269, which suggests a propionate ester in substitution of the acetate present in 3-*O*-acetylalpinone. Thus, peak 46 was attributed to putative 3-*O*-propionylalpinone (**Figure 2**, structure **46**).

Peak 23 produced a typical flavone UV spectrum (*30*), and comparison with retention time (coelution) and UV spectrum of an authentic standard of 3-*O*-methylquercetin (**Figure 2**, structure **23**) led to the attribution of this compound to peak 23. Furthermore, its measured accurate masses in both positive and negative ionization modes (**Table 1**) confirmed the molecular formula $C_{16}H_{12}O_7$, and its fragmentation patterns are in accordance with that described by Ma et al. (*31*).

(ii) Di-C-glucosylflavones. Chromatographic peaks 7 and 9 matched exactly the retention times and UV spectra of authentic standards of 6,8-di-*C*-β-glucopyranosylapigenin (vicenin-2) (**Figure 2**, structure **7**) and 6,8-di-*C*-β-glucopyranosylchrysin (**Figure 2**, structure **9**), respectively, and were attributed to these compounds, both previously isolated from *L. ericoides* (*4*). The measured accurate masses of the bp (**Table 1**) match the calculated exact masses for 6,8-di-*C*-β-glucopyranosylapigenin ($C_{27}H_{30}O_{15}$: $[M - H]^-$ 593.1506 and $[M + H]^+$ 595.1663) and 6,8-di-*C*-β-glucopyranosylchrysin ($C_{27}H_{30}O_{14}$: $[M - H]^-$ 577.1557 and $[M + H]^+$ 579.1714). In addition, the product ion spectra obtained in both positive and negative ionization are in agreement with those expected for these di-*C*-glucosylflavones (*22, 32*). Examination of these spectra revealed the fragmentation patterns characteristic of di-*C*-glucosylflavones: loss of H_2O $[(M - H) - 18]^-$ and fragmentation of the sugars producing the ions $[(M - H) - 90]^-$, $[(M - H) - 120]^-$, $[\text{aglycon} + 113]^-$, and $[\text{aglycon} + 83]^-$ as the most intense peaks in the negative ionization spectra, and an intense fragmentation of the sugar units, which is not very useful for structural identification, in the positive mode product ion spectra (*22, 32*).

Both positive and negative ionization spectra produced by peak 5 showed precursor ions 16 *m*/*z* larger than that of vicenin-2 (peak 7), suggesting a similar chemical structure with one more oxygen atom. This was confirmed by its measured accurate masses (**Table 1**), which suggested the molecular formula $C_{27}H_{30}O_{16}$. Product ion spectra obtained in negative ionization mode exhibited exactly the same fragmentation pattern observed for the other two di-*C*-glucosylflavones identified (peaks 7 and 9) presenting, in relation to the product ion spectra of those two compounds, a 16 *m*/*z* (one oxygen atom) or 32 *m*/*z* (two oxygen atoms) difference for all of the product ions. Product ions $[\text{aglycon} + 113]^-$ at *m*/*z* 399 and $[\text{aglycon} + 83]^-$ at *m*/*z* 369 correspond to the aglycon bearing sugar fragments, thus confirming a molecular mass of 286 for the aglycon, which suggests that it is the flavone luteolin. The absence of an $[(M - H) - 60]^-$ product ion, generally produced by the fragmentation of pentose derivatives, as well as the presence of $[(M - H) - 90]^-$ and $[(M - H) - 120]^-$ confirms hexoses as the sugar substituents (*22, 32–34*). These facts, taken together with the presence in the UV spectrum of a band II shift above 270 nm (in accordance with the tetrasubstituted A ring) and a shoulder at 290 nm in band II (characteristic of a 3′,4′-diOH system in flavones), confirmed two hydroxyls in ring B and two hexose substituents at positions 6 and 8, which are, by far, the most common *C*-glycosylation positions in flavones (*22, 30, 34*). Taking together all of these data and comparing with that described by Gattuso et al. (*34*) led to the identification of chromatographic peak 5 as the flavone 6,8-di-*C*-β-glucopyranosylluteolin, also known as lucenin-2 (**Figure 2**, structure **5**).

(iii) Coumaroylglycosylflavonols. Two peaks, 28 and 29, eluting close to each other and presenting UV spectra very similar to each other were attributed to coumaroylglycosylflavonols. First, peak 28 was identified by comparison of retention time (coelution) and UV spectrum with an authentic standard of tiliroside [3-*O*-(6″-*O*-(*E*)-*p*-coumaroyl)-β-glucopyranosyl-kaempferol] (**Figure 2**, structure **28**), previously isolated from other *Lychnophora* species (*27*). Its accurate masses obtained in both positive and negative ionization modes (**Table 1**) are also in agreement with the calculated molecular masses for the protonated and deprotonated tiliroside molecule ($C_{30}H_{26}O_{13}$). Similarly, peak 29 could be attributed to 3-*O*-(6″-*O*-(*E*)-*p*-coumaroyl)-β-glucopyranosylisorhamnetin (**Figure 2**, structure **29**) due to the comparison of its UV spectrum, retention time (coelution), and measured accurate masses with those obtained for an authentic standard previously isolated from another *Lychnophora* species (*28*).

(iv) Unidentified Flavonoid Peaks. Chromatographic peaks 33 produced UV spectra (**Table 1**) typical of flavanones/dihydroflavonols (*30*). Accurate mass measurements (**Table 1**) of its mass spectra bp in both positive and negative ionization suggested the molecular formulas $C_{16}H_{14}O_4$, with no other reasonable formula in a ±15 ppm error interval, for peak 33. For peak 44, by its measured accurate masses (**Table 1**), only the molecular formula could be determined. These peaks have not matched the retention time of any of our standard compounds, and no product ion spectra could be achieved for them. The UV spectrum and molecular formula obtained for peak 33 are coherent with an unique substance identified in plants of the *Lychnophora* genus, 7-hydroxy-4′-methoxyflavone, but due to the unavailability of a standard of this compound, this peak remains unidentified. The molecular formula calculated for peak 44 does not match any compound previously identified in the entire subtribe Lychnophorinae, and this peak could not be identified.

Similarly, peaks 31 and 49−52 all presented UV spectra (**Table 1**) typical of flavanones/dihydroflavonols (*30*). However, no mass spectral data could be obtained for them in both ionization modes, which might be due to their low ionization potential combined with their low concentrations in the extracts.

Among the flavonoids identified here, the majority had been previously identified in *L. ericoides* and/or in extracts of other *Lychnophora* species. The compounds 2′,4′,6′-trihydroxychalcone, 2′,6′-dihydroxy-4′-methoxychalcone, 3-*O*-acetylalpinone, 3-*O*-propionylalpinone, and lucenin-2 are reported here for the first time in a plant of the subtribe Lychnophorinae. The compounds 3-*O*-methylquercetin, tiliroside, and 3-*O*-(6″-*O*-(*E*)-*p*-coumaroyl)-β-glucopyranosylisorhamnetin were already identified in other *Lychnophora* species (*26–28*) and are being reported for the first time in *L. ericoides*. The other flavonoids identified here have been previously isolated from *L. ericoides* extracts (*4, 6–8, 35*).

(c) Sesquiterpene Lactones. (i) Goyazensolide-Type STL. Chromatographic peaks 27, 32, 36, 37, and 43 were attributed respectively to the goyazensolide-type STL centratherin (**Figure 2**, structure **27**), 4,5-dihydro-15-deoxygoyazensolide (**Figure 2**, structure **32**), 4,5-dihydrolychnopholide (**Figure 2**, structure **36**), zexbrevanolide tiglate (**Figure 2**, structure **37**), and lychnopholide (**Figure 2**, structure **43**). Their identification was achieved

by comparison with the retention times (coelution) of authentic standards previously isolated from *L. ericoides* (*6*). Accurate masses obtained for these peaks are in agreement with the calculated exact masses of the protonated molecules of these compounds, and the product ion spectra (**Table 1**) exhibited the same fragmentation patterns and diagnostic ions described by Crotti et al. (*21*).

It should be noted that 4,5-dihydrolychnopholide (peak 36) and zexbrevanolide tiglate (peak 37) possess the same basic structure, differing only in the stereochemistry of the ester linked at C-8 of the STL structure: an angelate (*trans* double bond) in 4,5-dihydrolychnopholide and a tiglate (*cis* double bond) in zexbrevanolide tiglate. However, positive ionization product ion spectra of these two compounds are nearly identical, which indicates that the stereochemistry of the ester double bond has negligible effect in their fragmentation patterns.

Although chromatographic peaks 37 (zexbrevanolide tiglate) and 38 (the flavanone pinocembrin) present very close retention times, they could be easily differentiated and individualized in a HPLC-DAD analysis (even without using the mass spectrometer) through their UV spectra, since the STL does not absorb at 325 nm. The same should be noted for peaks 26 (3,4,5-tri-CQA) and 27 (centratherin).

(ii) Eremantholide-Type STL. Chromatogaphic peaks 17, 24, 34, 35, and 41 were attributed respectively to the eremantholide-type STL 15-hydroxyeremantholide C (**Figure 2**, structure **17**), 15-hydroxy-16α-(1′-methylprop-1′-*Z*-enyl)eremantholide (**Figure 2**, structure **24**), 4,5-dihydroeremantholide C (**Figure 2**, structure **34**), 4,5-dihydro-16α-(1′-methylprop-1′-*Z*-enyl)eremantholide (**Figure 2**, structure **35**), and 16α-(1′-methylprop-1′-*Z*-enyl)eremantholide (**Figure 2**, structure **41**). Their identification was achieved by comparison with retention times (coelution) of authentic standards previously isolated from *Lychnophora* species, obtained as described in the Materials and Methods section. Moreover, such attributions were confirmed by the comparison of the product ion spectra obtained for these peaks (**Table 1**) with product ion spectra obtained for the respective standards, since no fragmentation patterns for this class of compounds are described in literature.

All of the data obtained for peak 39 were nearly identical to that obtained for peak 35 [4,5-dihydro-16α-(1′-methylprop-1′-*Z*-enyl)eremantholide], including the product ion spectrum (**Table 1**). This strongly suggests that, similarly to those observed for the stereoisomers 4,5-dihydrolychnopholide and zexbrevanolide tiglate, both compounds (responsible for peaks 35 and 39) possess the same basic chemical structure, differing in the side chain (the 1′-methylprop-1′-enyl group) configuration. Thus, peak 39 was putatively assigned to the substance 4,5-dihydro-16α-(1′-methylprop-1′-*E*-enyl)eremantholide (**Figure 2**, structure **39**), i.e., the side chain *cis* isomer of the compound attributed to peak 35.

It should be noted that, for all of these eremantholides, the bp observed in the mass spectra obtained from the HPLC-MS TIC chromatogram is relative to the loss of a neutral H_2O molecule (18 *m/z*) from the protonated STL by in source dissociation. The $[M + H]^+$ ions are also observed in all of these spectra, but in a much lower relative intensity than $[MH - H_2O]^+$. On the other hand, in all the goyazensolide mass spectra, the bp is the $[M + H]^+$, and the $[MH - H_2O]^+$ ions were not observed. This observation could be useful for the differentiation of goyazensolide and eremantholide STL by their mass spectra.

The accurate mass measurement of peak 30 (**Table 1**) suggests the molecular formula $C_{19}H_{24}O_6$, with no other reasonable formula within a ±15 ppm error interval. Its UV and product ion spectra exhibited patterns very similar to the other STL, strongly suggesting a STL with an eremantholide moiety because of the first loss of 18 *m/z* ($[MH - H_2O]^+$), as discussed above. Among the compounds previously identified in the *Lychnophora* genus, only the STL 4,5-dihydroeremantholide C (already attributed to peak 34) and eremantholide A match the molecular formula $C_{19}H_{24}O_6$. However, the retention time of peak 30 does not match that obtained for authentic standards of these two compounds. As a result, the compound responsible for peak 30 could not be identified beyond the attribution of a molecular formula.

Although chromatographic peaks 41 [16α-(1′-methylprop-1′-*Z*-enyl)eremantholide] and 42 (the flavonoid 3-*O*-acetylpinobanksin) present very close retention times, coeluting when both are present in the plant extract, they can be identified and differentiated in a HPLC-DAD analysis (without using the mass spectrometer) by their UV spectra, since the STL does not absorb at 325 nm. Peaks 33 (an unidentified flavonoid) and 34 (4,5-dihydroeremantholide C) are also distinguishable from each other due to differences in UV absorbance; i.e., the substance corresponding to peak 33 absorbs at 325 nm and the STL 4,5-dihydroeremantholide C does not.

Among the identified STL, only 15-hydroxyeremantholide C and putative 4,5-dihydro-16α-(1′-methylprop-1′-*E*-enyl)eremantholide were not previously isolated from *L. ericoides* (*6*–*8*), and the former was previously isolated from another *Lychnophora* species, *L. rupestris* (*28*).

HPLC-DAD Analysis Method. As pointed out previously in the Materials and Methods section, the construction of analytical curves for all of the compounds identified in the *L. ericoides* leaf extracts was not viable. Hence, the analytical method validation was performed on the basis of 11 standard substances chosen to represent the diverse classes and polarities of the secondary metabolites identified in *L. ericoides* leaves and thus assuring that their validation results could be representative of the whole extract.

Good linearity was obtained in the concentration range between 2.0 and 200.0 μg mL^{-1} for all of the standard compounds employed for method validation. Thus, the analytical curves for all of the standards were constructed using the responses for concentrations in this range (2.0, 5.0, 10.0, 20.0, 50.0, 100.0, 200.0 μg mL^{-1}). The regression equations found were as follows: vicenin-2 (325 nm), $y = 34.898x - 0.112$ ($R^2 = 0.999$); 6,8-di-*C*-β-glucosylchrysin (270 nm), $y = 51.200x - 0.121$ ($R^2 = 0.999$); 3,5-di-*O*-*E*-caffeoylquinic acid (325 nm), $y = 19.370x + 0.114$ ($R^2 = 0.999$); centratherin (270 nm), $y = 22.443x - 0.095$ ($R^2 = 0.999$); 4,5-dihydro-15-deoxygoyazensolide (270 nm), $y = 28.452x - 0.188$ ($R^2 = 0.999$); 4,5-dihydrolychnopholide (270 nm), $y = 39.114x - 0.116$ ($R^2 = 0.999$); 4,5-dihydroeremantholide C (270 nm), $y = 34.101x - 0.175$ ($R^2 = 0.999$); 16α-(1′-methylprop-1′-*Z*-enyl)eremantholide (270 nm), $y = 63.915x - 0.124$ ($R^2 = 0.999$); lychnopholide (270 nm), $y = 39.269x - 0.176$ ($R^2 = 0.999$); pinocembrin (270 nm), $y = 52.392x - 0.194$ ($R^2 = 0.999$); and pinostrobin (270 nm), $y = 30.642x - 0.063$ ($R^2 = 0.999$). Minimum limits of detection (MinLD), minimum limits of quantification (MinLQ), and maximum limits of quantification (MaxLQ) found (**Table 2**) showed a very good sensibility for the analyses of *L. ericoides* secondary metabolites. Good precision and accuracy were also found for the standard compounds used for the analytical method validation, and the calculated accuracies (Acur) and coefficients of variation for

Table 2. Sensibility, Accuracy, and Precision Validation Parameter Results Obtained for the Analyzed Standard Compounds in the HPLC-DAD Analytical Method Developed: Minimum Limits of Detection (MinLD), Minimum Limits of Quantification (MinLQ), Maximum Limits of Quantification (MaxLQ), Accuracies (Acur), and Coefficients of Variation for Intraassay Precision (Intra-CV) and for Interassay Precision (Inter-CV)

standard compound	MinLD (μg mL^{-1})	MinLQ (μg mL^{-1})	MaxLQ (μg mL^{-1})	Acur (%)	Intra-CV (%)	Inter-CV (%)
vicenin-2	0.2	1.0	500.0	4.8	2.1	3.1
6,8-di-*C*-β-glucosylchrysin	0.2	2.0	500.0	4.5	2.3	2.9
3,5-di-*O*-(*E*)-caffeoylquinic acid	0.2	2.0	1000.0	5.8	2.2	3.7
centratherin	0.2	1.0	500.0	3.8	1.9	3.0
4,5-dihydro-15-deoxygoyazensolide	0.2	1.0	500.0	3.6	2.1	3.4
4,5-dihydrolychnopholide	0.2	0.5	500.0	3.9	1.8	2.8
4,5-dihydroeremantholide C	0.2	0.5	500.0	4.1	2.0	2.9
16α-(1′-methylprop-1′-*Z*-enyl)eremantholide	0.2	1.0	500.0	4.0	1.7	3.2
lychnopholide	0.2	1.0	500.0	3.8	2.2	3.0
pinocembrin	0.2	1.0	500.0	5.9	1.8	3.9
pinostrobin	0.2	1.0	1000.0	7.1	2.0	4.2

Table 3. Overall Recoveries Obtained in the Analytical Method Validation for the Analyzed Standard Compounds at 30, 150, and 300 μg of Spiking in Matrix

standard compound	overall recoveries (%)		
	30 μg	150 μg	300 μg
vicenin-2	96.3	91.2	84.0
6,8-di-*C*-β-glucosylchrysin	95.6	89.8	82.7
3,5-di-*O*-(*E*)-caffeoylquinic acid	97.5	92.4	86.4
centratherin	97.7	91.4	86.2
4,5-dihydro-15-deoxygoyazensolide	97.6	91.6	84.4
4,5-dihydrolychnopholide	97.4	90.5	83.3
4,5-dihydroeremantholide C	96.9	91.5	83.9
16α-(1′-methylprop-1′-*Z*-enyl)eremantholide	96.8	90.3	83.0
lychnopholide	96.4	90.0	83.2
pinocembrin	95.7	89.3	81.4
pinostrobin	94.0	88.1	79.3

intraassay precision (Intra-CV) and interassay precision (Inter-CV) are presented in **Table 2**.

The method's overall recovery was also considered very good. At 150 μg spiked amounts, for example, recoveries around 90% were found for all of the standard compounds employed for the method validation, hence showing that the global extraction procedure (extraction and cleanup processes) developed is efficient and adequate for the analyses of the secondary metabolites of *L. ericoides*. Even at 300 μg spiked amounts, which is much higher than the expected concentrations in the leaf extracts of *L. ericoides*, satisfactory overall recoveries were found. Furthermore, the overall recovery values are similar to each other, revealing the applicability of the method to the different classes of secondary metabolites identified in the MeOH−H_2O extracts, independent of their polarity. The overall recoveries (OR) found at 30, 150, and 300 μg are presented in **Table 3**.

Regarding the stability of the secondary metabolites in the extract solutions, significant alterations were found for some chromatographic peaks only 30 h after sample preparation. Until this time following the extraction cleanup processes, no alterations in the chromatographic peak responses (meaning secondary metabolite concentration) were found. This means that a sample could be analyzed with confidence of its secondary metabolite concentrations up to 30 h after its preparation for HPLC analyses. Vegetal samples stored for up to 18 months were evaluated regarding their secondary metabolite content stability, and no significant alteration in concentrations was found. The largest differences detected were below the inter- and intraassays coefficients of variation, leading to the conclusion that, virtually, no alteration of the secondary metabolite concentrations occur during storage.

In a future perspective, the HPLC-DAD analytical method established here should be employed for quali- and semiquantitative (i.e., total concentration of a group of secondary metabolites based on the analytical curve of a single representative secondary metabolite) analyses of *L. ericoides* infra-specific secondary metabolite variations. Furthermore, the method should also be applied for the dereplication of secondary metabolites of other correlated plant species.

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Supporting Information Available: Chromatograms, registered at 325 nm, obtained by employing the developed extraction and HPLC-DAD analysis methods for two *L. ericoides* specimens collected at Ibiraci and Delfinópolis (MG State, Brazil). This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Coile, N. C.; Jones, S. B. *Lychnophora* (Compositae: Vernonieae), a genus endemic to the Brazilian planalto. *Brittonia* **1981**, *33*, 528–542.

(2) Robinson, H. Generic and subtribal classification of American Vernonieae. *Smithson. Contrib. Bot.* **1999**, *89*, 1–116.

(3) Semir, J. Revisão taxonômica de *Lychnophora* Mart. (Vernonieae, Compositae). Doctoral Thesis, Instituto de Biologia−UNICAMP, Campinas-SP, 1991,515 pp.

(4) Gobbo-Neto, L.; Santos, M. D.; Kanashiro, A.; Almeida, M. C.; Lucisano-Valim, Y. M.; Lopes, J. L. C.; Souza, G. E. P.; LopesN. P. Evaluation of the anti-inflammatory and antioxidant activities−*C*-glucosylflavones from *Lychnophora ericoides* (Asteraceae). *Planta Med.* **2005**, *71*, 3–6.

(5) Santos, M. D.; Gobbo-Neto, L.; Albarella, L.; Souza, G. E. P.; Lopes, N. P. Analgesic activity of di-caffeoylquinic acids from roots of *Lychnophora ericoides* (Arnica da serra). *J. Ethnopharmacol.* **2005**, *96*, 545–549.

(6) Sakamoto, H. T.; Flausino, D.; Castellano, E. E.; Stark, C. B. W.; Gates, P. J.; Lopes, N. P. Sesquiterpene lactones from *Lychnophora ericoides*. *J. Nat. Prod.* **2003**, *66*, 693–695.

(7) Borella, J. C.; Lopes, J. L. C.; Vichnewski, W.; Cunha, W. R.; Herz, W. Sesquiterpene lactones, triterpenes and flavones from *Lychnophora ericoides* and *Lychnophora pseudovillosissima*. *Biochem. Syst. Ecol.* **1998**, *26*, 671–676.
(8) Sargenti, S. R.; Vichnewski, W. Sonication and liquid chromatography as a rapid technique for extraction and fractionation of plant material. *Phytochem. Anal.* **2000**, *11*, 69–73.
(9) Borsato, M. L. C.; Grael, C. F. F.; Souza, G. E. P.; Lopes, N. P. Analgesic activity of the lignans from *Lychnophora ericoides*. *Phytochemistry* **2000**, *55*, 809–813.
(10) Rüngeler, P.; Castro, V.; Mora, G.; Goren, N.; Vichnewski, W.; Pahl, H. L.; Merfort, I.; Schmidt, T. J. Inhibition of transcription factor NF-κB by sesquiterpene lactones: a proposed molecular mechanism of action. *Bioorg. Med. Chem.* **1999**, *7*, 2343–2352.
(11) Schmidt, T. J. Toxic activities of sesquiterpene lactones: structural and biochemical aspects. *Curr. Org. Chem.* **1999**, *3*, 577–608.
(12) Picman, A. K. Biological activities of sesquiterpene lactones. *Biochem. Syst. Ecol.* **1986**, *14*, 255–281.
(13) Causon, R. Validation of chromatographic methods in biomedical analysis: viewpoint and discussion. *J. Chromatogr. B* **1997**, *689*, 175–180.
(14) Ribani, M.; Bottoli, C. B. G.; Collins, C. H.; Jardim, C. S. F.; Melo, L. F. C. Validação em métodos cromatográficos e eletroforéticos. *Quim. Nova* **2004**, *27*, 771–780.
(15) ANVISA—Agência Nacionade Vigilância Sanitária. Resolução° 899. Guia para validação de métodos analíticos e bioanalíticos 2003.
(16) IUPAC. Nomenclature of cyclitols. *Biochem. J.* **1976**, *153*, 23–31.
(17) Clifford, M. N.; Johnston, K. L.; Knight, S.; Kuhnert, N. Hierarchical scheme for LC-MS^n identification of chlorogenic acids. *J. Agric. Food Chem.* **2003**, *51*, 2900–2911.
(18) Clifford, M. N.; Knight, S.; Kuhnert, N. Discriminating between the six isomers of dicaffeoylquinic acid by LC-MS^n. *J. Agric. Food Chem.* **2005**, *53*, 3821–3832.
(19) Clifford, M. N.; Knight, S.; Surucu, B.; Kuhnert, N. Characterization by LC-MS^n of four new classes of chlorogenic acids in green coffee beans: dimethoxycinnamoylquinic acids, diferuloylquinic acids, caffeoyl-dimethoxycinnamoylquinic acids, and feruloyl-dimethoxycinnamoylquinic acids. *J. Agric. Food Chem.* **2006**, *54*, 1957–1969.
(20) Miketova, P.; Schram, K. H.; Whitney, J.; Kearns, E. H.; Timmermann, B. N. Mass spectrometry of 3,5- and 4,5-dicaffeoylquinic acids and selected derivatives. *J. Mass Spectrom.* **1999**, *34*, 1240–1252.
(21) Crotti, A. E. M.; Lopes, J. L. C.; Lopes, N. P. Triple quadrupole tandem mass spectrometry of sesquiterpene lactones: a study of goyazensolide and its congeners. *J. Mass Spectrom.* **2005**, *40*, 1030–1034.
(22) Cuyckens, F.; Claeys, M. Mass spectrometry in the structural analysis of flavonoids. *J. Mass Spectrom.* **2004**, *39*, 1–15.
(23) Fabre, N.; Rustan, I.; Hoffmann, E.; Quetin-Leclerq, J. Determination of flavone, flavonol, and flavanone aglycones by negative ion liquid chromatography electrospray ion trap mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 707–715.
(24) Ma, Y. L.; Li, Q. M.; Van den Heuvel, H.; Claeys, M. Characterization of flavone and flavonol aglicones by collision-induced dissociation tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 1357–1364.
(25) Zhang, J.; Brodbelt, J. S. Structural characterization and isomer differentiation of chalcones by eletrospray ionization tandem mass spectrometry. *J. Mass Spectrom.* **2003**, *38*, 555–572.
(26) Takeara, R.; Albuquerque, S.; Lopes, N. P.; Lopes, J. L. C. Trypanocidal activity of *Lychnophora staavioides* Mart. (Vernonieae. Asteraceae). *Phytomedicine* **2003**, *10*, 490–493.
(27) Chicaro, P.; Pinto, E.; Colepicolo, P.; Lopes, J. L. C.; Lopes, N. P. Flavonoids from *Lychnophora passerina* (Asteraceae): potential antioxidants and UV-protectants. *Biochem. Syst. Ecol.* **2004**, *32*, 239–243.
(28) Grael, C. F. F.; Albuquerque, S.; Lopes, J. L. C. Chemical constituents of *Lychnophora pohlii* and trypanocidal activity of crude plant extracts and of isolated compounds. *Fitoterapia* **2005**, *76*, 73–82.
(29) Cunha, W. R.; Lopes, J. L. C.; Vichnewski, W.; Diaz, J. G.; Herz, W. Eremantholides and a guaianolide from *Lychnophora rupestris*. *Phytochemistry* **1995**, *39*, 387–389.
(30) Markham, K. R. Ultraviolet-visible absorption spectroscopy. In *Techniques of flavonoid identification*; Markham, K. R., Ed.; Academic Press: London, U.K., 1982; pp 36–51.
(31) Ma, Y. L.; Van den Heuvel, H.; Claeys, M. Characterization of 3-methoxyflavones using fast-atom bombardment and collision-induced dissociation tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 1932–1942.
(32) Becchi, M.; Fraisse, D. Fast atom bombardment and fast atom bombardment collision-activated dissociation/mass-analysed ion kinetic energy analysis of *C*-glycosidic flavonoids. *Biomed. Environ. Mass Spectrom.* **1989**, *18*, 122–130.
(33) Caristi, C.; Bellocco, E.; Panzera, V.; Toscano, G.; Vadalà, R.; Leuzzi, U. Flavonoids detection by HPLC-DAD-MS-MS in lemon juices from sicilian cultivars. *J. Agric. Food Chem.* **2003**, *51*, 3528–3534.
(34) Gattuso, G.; Caristi, C.; Gargiulli, C.; Bellocco, E.; Toscano, G.; Leuzzi, U. Flavonoid glycosides in bergamot juice (*Citrus bergamia* Risso). *J. Agric. Food Chem.* **2006**, *54*, 3929–3935.
(35) Gobbo-Neto, L.; Santos, M. D.; Albarella, L.; Zollo, F.; Pizza, C.; Lopes, N. P. Glycosides, caffeoylquinic acids and flavonoids from the leaves polar extract of *Lychnophora ericoides* Mart. (Asteraceae). in press.

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