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Identification of a dicaffeoylquinic acid isomer from *Arctium lappa* with a potent anti-ulcer activity

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ABSTRACT

Leaves of *Arctium lappa* contain several mono- and dicaffeoylquinic acids, as evaluated by liquid chromatography-mass spectrometry. In order to investigate the protection on gastric mucosa against ulcers, rats were treated with fractions from leaf extract prior to ethanol-induced ulcers. The original fraction obtained as ethanol soluble fraction from hot aqueous extract was able to protect de gastric mucosa, and this effect was retained in the ethyl acetate fraction, obtained from liquid/liquid fractionation. The main compound in this fraction was isolated and chemically characterized by nuclear magnetic resonance and mass spectrometry, assisted by isopropylidene derivatization which gave rise a mass increment of 40 units. Therefore, the underivatized compound that had *m/z* 515.119 $[M-H]^-$ was shifted to *m/z* 555.151, being confirmed as 1,3-*O*-dicaffeoylquinic acid, which presented an ED_{50} of 57 μg kg^{-1} on gastric protection, lesser than the therapeutic concentration of omeprazole (40 mg kg^{-1}).

1. Introduction

Arctium lappa L. (Asteraceae), popularly known as "burdock" or "bardana" is considered a medicinal plant, brought from Asia and acclimated worldwide, including Brazil. *A. lappa* is an important detoxifying herb in both Chinese and Western natural medicines [1]. Therapeutic applications are attributed to different parts of the plant, such as roots, leaves, seeds and fruits [2], being used in folk medicine for treatment of intoxications, throat infections, boils, rashes and other chronic skin disorders [1]. The roots and seeds have also been used as diuretic, antipyretic, and blood detoxifier [3,4], whereas leaves are used to treat burns, ulcers, sores and for heat clearing [5].

Roots from *A. lappa* are popular in the Asian cuisine being widely consumed, whereas the leaves are used as infusions or, externally, as a poultice [6]. Although the leaves are rich in phenolic compounds [5], to which many health benefits are associated [7–10], there is a prevalence of investigations on the roots and seeds. Extracts from roots containing several monocaffeoylquinic acids (MCQA) and dicaffeoylquinic acids (DCQA), including several isomers, were reported to have gastroprotective activity [10,11].

Other compounds were also found in *A. lappa*, including arctiin and arctigenin, caffeic acid, chlorogenic acid, cynarin, rutin, quercitrin, quercetin, luteolin, benzoic and *p*-coumaric acid [5,12,13]. They are associated to the medicinal properties of *A. lappa*, such as the lignan arctigenin that exhibited antitumor and antidiabetic activities [14], the sesquilignans isolappaol C, lappaol (C, D, F) and diarctigenin, which have anti-inflammatory activity [15]. Antioxidant activity due to the combined action of chlorogenic acid, *p*-coumaric acid and caffeic acid has also been reported [5], whereas quercetin has been associated to the anticarcinogenic activity [7].

Considering the anti-ulcer activity exhibited for roots extracts, the goal of the present investigation was to evaluate the gastroprotective ability of the leaves from *A. lappa*, with a bioguided fractionation and purification of the gastroprotective agent.

2. Experimental

2.1. Botanical material and chemicals

Commercially processed (dried and milled) leaves of *A. lappa* were obtained in local market. The analyses were developed with type I ultrapure water (Millipore) and the organics methanol, chloroform,

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ethyl acetate, *n*-butanol, acetone and formic acid were chromatographic grade (Tedia). Standards of chlorogenic acid, rutin, caffeic acid and cynarin were analytical grade (Sigma-Aldrich). All solvents and samples were filtered through a 0.22 μm membrane.

2.2. Extraction

The leaves (500 g) were defatted and depigmented with $CHCl_3$-MeOH (2:1, v/v) in a Soxhlet extractor. The dried residue was then extracted with water (2 L) under reflux for 2 h (× 3). The extracts were combined and concentrated to a small volume (200 mL). High molecular weight components were precipitated by addition to cold ethanol (600 mL), and separated by centrifugation (8.000 rpm at 4 °C, 20 min). Ethanol-soluble fraction (ESF, 48.23 g) was concentrated under reduced pressure, and then freeze-dried and stored at −20 °C.

2.3. Liquid/liquid extraction

ESF (30 g) was suspended in distilled water (100 mL) and submitted to liquid/liquid extraction, successively, with chloroform (5 × 100 mL), ethyl acetate (5 × 100 mL) and *n*-butanol (5 × 100 mL). Similar fractions were combined and evaporated to dryness, yielding chloroform (ESF-C, 0.4%), ethyl acetate (ESF-EA, 4%), *n*-butanol (ESF-B, 8%) and aqueous (ESF-AQ, 87.6%) fractions, which were stored at −20 °C. All these fractions were used in animal experiments and in phytochemical investigation.

2.4. Liquid chromatography (LC)

Analytical chromatographic separation was carried out using an Acquity-UPLC™ system (Waters), incorporating a binary pump, sample manager and column oven. The samples were held at room temperature (22 °C) and the column oven at 60 °C. A BEH-C18 column (Waters), with 50 mm × 2.1 mm and 1.7 μm particle size was used.

The mobile phase consisted of H_2O containing 0.1% formic acid (v/v) and methanol. A linear gradient with a flow rate of 0.5 mL min^{-1} was developed by increasing methanol, from 0 to 38% in 10 min, then to 60% at 13 min, returning to the initial condition in 14 min, held 3 min else for column re-equilibration. The samples (2 mg mL^{-1}) were prepared in H_2O-MeOH (7:3, v/v), with 5 μL being injected. Detection was provided by a photodiode array detector (PDA, 200–400 nm) and mass spectrometry (MS).

Semi-preparative chromatography was performed on a high performance liquid chromatography (HPLC) model 1220 Infinity LC (Agilent), equipped with a single channel UV detector and automatic fraction collector. The column was a Symmetry-C18 (Waters) with 50 mm × 7.8 mm i.d. and 5 μm of particle size. The chromatography was developed using H_2O containing 0.1% formic acid (v/v) and methanol. A linear gradient of methanol, from 0 to 50% in 30 min with a flow rate of 1 mL min^{-1}, was developed. The solvent system returns to initial condition in 35 min and other 5 min was used for re-equilibrating the system. The fraction ESF-EA was used for compound purification, being detected at 325 nm for dicaffeoylquinic acids.

2.5. High resolution mass spectrometry (HR-MS)

The HR-MS was developed on an electrospray ionization mass spectrometry (ESI-MS) LTQ-Orbitrap-XL (Thermo-Scientific), operating in the negative and positive ionization, at atmospheric pressure ionization (API). The source temperature was 380 °C, N_2 was used for sample desolvation with sheath gas flow rate at 60 arbitrary units (abu) and auxiliary at 20 abu. The parameters for ionization were: electrospray at 3.5 kV, capillary at −20 V and tube lens at −200 V. Instrument calibration was performed externally prior to each sequence with a calibration solution (100 to 2000 *m/z*). Accuracy raging between 1–3 ppm with resolution set at 7500 FWHM in LC-MS mode. Acquisition was obtained in total ion current (TIC) mode, with *m/z* 100–1000.

2.6. Nuclear magnetic resonance (NMR)

The NMR analyzes were performed on an Avance HD (14.1 T, 600 MHz for ^1H nucleus) using a 5 mm inverse probe. Analysis was developed at 30 °C using MeOD-d_4 as solvent and internal reference for chemical shifts (δ ^{13}C=49.15 and ^1H=3.31, calibrated from tetramethylsilane δ $^{13}C/^1H$=0). 1D experiments (^1H, ^{13}C) and 2D (COSY, HSQC and HMBC) were carried out.

2.7. Chemical modifications of dicaffeoyquinic acids

For acetalation, 5 mg of the ESF-B and ESF-EA fractions were dissolved in 5 mL of dry acetone and lyophilized *p*-toluenesulfonic acid (5 mg). The solution was stirred for 3 h at room temperature (~22 °C) then, the reaction was stopped by adding a solution of aq. NH_4OH to the neutrality, yielding a non-soluble ammonium salt, removed by centrifugation. The soluble was evaporated to dryness under N_2 stream, the samples were dissolved in H_2O-MeOH (1:1, v/v) and analyzed by UHPLC-MS as described above.

In order to evaluate if *cis*-caffeic acid or isomers from quinic acid were presented, the samples ESF-EA and ESF-B (1 mg) were methanolysed by heating in a solution of 1 M MeOH-HCl (80 °C, 2 h). The solvent was evaporated under N_2 stream, then dissolved in acetic anhydride-pyridine (300 μL, 1:1, v/v) and held for 3 h at 80 °C. Methanol (200 μL) was added to stop the reaction and the reagents were evaporated under N_2 stream. The samples were dissolved in acetone and analyzed by gas chromatography-MS (Varian), on capillary columns DB1-MS and DB225-MS (30 m × 0.25 mm), programmed from 50 to 200 °C. The mass spectrometry was performed on an Ion Trap with electron ionization (70 eV). Caffeic and chlorogenic acids were used as standards.

2.8. Animals

Wistar rats (180–200 g) from Federal University of Paraná vivarium were used in the experiments. The animals were maintained under established conditions [10], in a 12 h light/dark cycle, temperature of 22 ± 2 °C. Food and water were freely provided until 15–18 h prior to the experiment, when they were deprived of food. All experiments were in agreement with the "Principles of Laboratory Animal Care" (NIH Publication 85–23, revised 1985), and approved by the local Animal Ethics Committee of Federal University of Paraná (CEUA/BIO-UFPR; approval number 500).

2.9. Induction of acute gastric lesions

Gastric ulcers were induced with ethanol as described previously by Robert and coworkers [16] with minor modifications. Rats were fasted during the night, 18 h prior to the experiment, with free access to water. The animals were orally treated (gavage) with vehicle (water plus Tween™ 1 mL kg^{-1}, ulcerated control group), omeprazole (40 mg kg^{-1}) or ESF (1, 10 and 100 mg kg^{-1}), 1 h before administration of ethanol P.A. (0.5 mL 200 g^{-1}), and then sacrificed 1 h after ethanol administration. To determine the extent of gastric lesions, the stomach was removed, opened, photographed and the gastric injury was measured (mm^2) by computerized planimetry.

The fractions from liquid/liquid fractionation were also evaluated, at the following doses: ESF-C at 0.01 mg kg^{-1}, ESF-EA at 0.15 mg − kg^{-1}, ESF-B at 0.30 mg kg^{-1}, ESF-AQ at 1.99 mg kg^{-1}. Purified DCQA and cynarin were also tested, each at 0.057 mg kg^{-1}. These doses were

calculated according to their yields on fractionation and effective ED_{50}, from ethanol soluble fraction (ESF).

The results were expressed as mean ± standard error of mean (SEM) with 6–8 animals per group. The statistical significance was determined using one-way analysis of variance (ANOVA) followed by Bonferroni's test. Data were considered different at a significance level of $p < 0.05$.

3. Results and discussion

3.1. Overall phytochemical investigation

The lower molecular compounds present in the ethanol soluble fraction (ESF) were analyzed by reversed-phase UHPLC-PDA-MS, in which many caffeoylquinic acids, with UV-absorbance with λ_{max} at ~325 nm with a shoulder at ~293 nm, were identified, such as the monocaffeoylquinic acids, with *m/z* 353.087 $[M-H]^-$, *neo*-chlorogenic (5-*O*-caffeoylquinic, peak 8), chlorogenic (3-*O*-caffeoylquinic, peak 11), *crypto*-chlorogenic (4-*O*-caffeoylquinic, peak 12), and the dicaffeoylquinic acids, 3,4-*O*-dicaffeoylquinic (peak 21), 3,5-*O*-dicaffeoylquinic (peak 21) and 4,5-*O*-dicaffeoylquinic (peak 27) acids, with *m/z* 353.087 $[M-H]^-$, identified according to previous works [17,18] (Fig. 1A, Table 1). The compounds 3,4-DCQA and 3,5-DCQA were resolved with BEH-Phenyl column, with similar conditions (data not shown). Despite the isomerism, when these compounds were fragmented, each isomer exhibited a characteristic fragmentation profile, producing fragment-ions with different ratios (e.g. *m/z* 353.087, 191.056, 179.034, 135.045), in accordance to previous works [17–20]. Flavonoid glycosides and other low abundant peaks were identified on the basis of their UV-absorbance and mass spectra (Table 1).

Two high abundant peaks (13 and 23) with MS^1 at *m/z* 515.119 and MS^2 at *m/z* 353, 191 and 179 and λ_{max} at ~325 nm were identified as dicaffeoylquinic acids. Although the *trans*-caffeic acid is the major component of caffeoylquinic acids, the *cis*-caffeic acid has being reported [21]. In order to determine if the peaks 13 and 23 could be products of these *cis*-isomers, the samples were prepared by methanolysis followed by acetylation for gas chromatography-mass spectrometry (GC–MS) analysis (data not shown). Derivatized chlorogenic acid was used as GC–MS standard and no evidences for *cis*-caffeic acid nor isomeric quinic acid were found.

Therefore, the compounds 13 and 23 were concluded as positional isomers. Thus, considering that the linkage sites on 3,4-, 3,5- and 4,5- had already been found (peaks 21 and 27), compounds 13 and 23 must be linked on position 1 combined with positions 3, 4 or 5. Actually, there is much disarray on the correct way for numbering

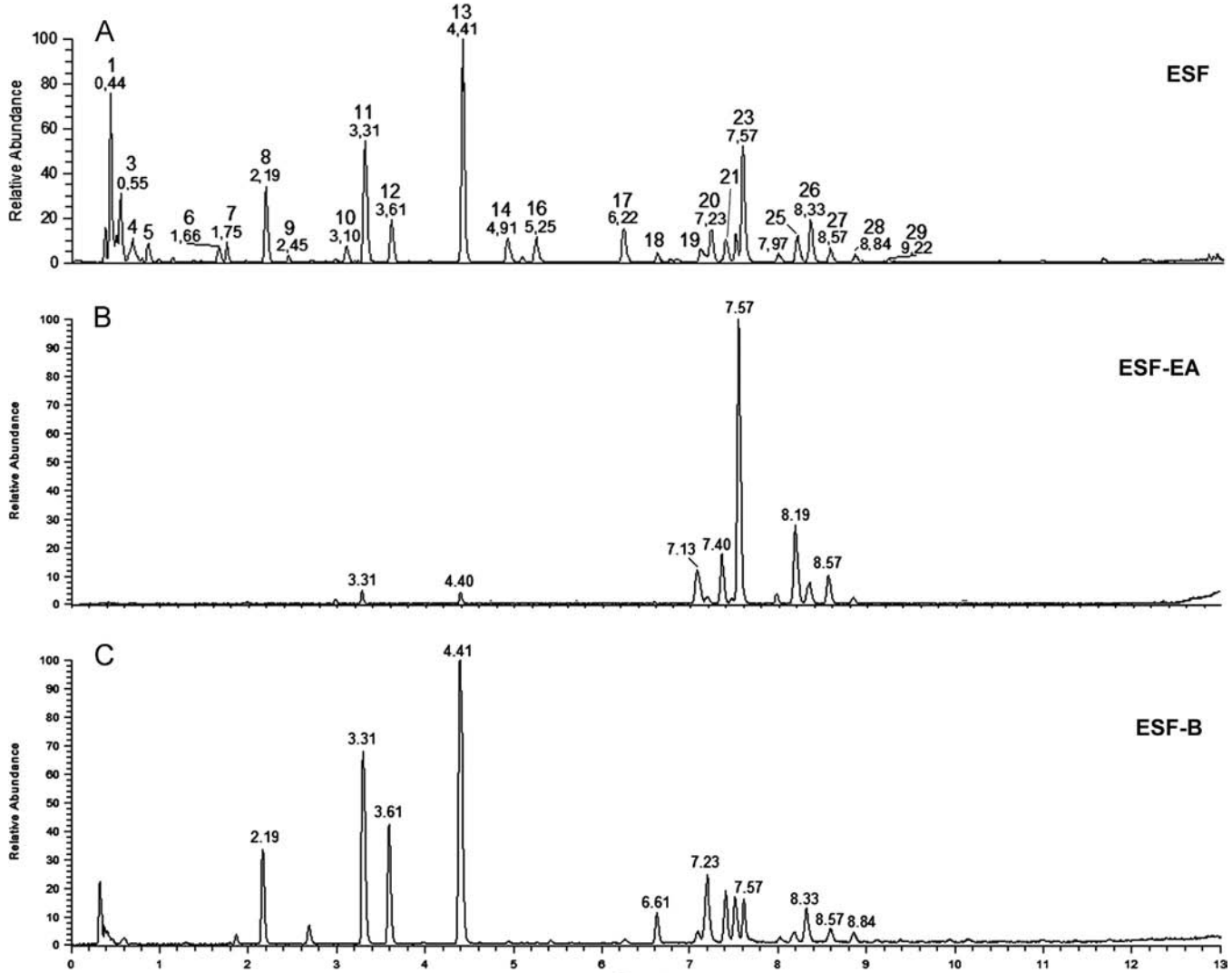

Fig. 1. UHPLC-MS chromatograms of the ethanol soluble fraction from leaves of *A. lappa* (A) and fractions from liquid/liquid extraction that retained the caffeoylquinic acids, ethyl acetate fraction (B) and butanol fraction (C).

Table 1
Composition of the ethanol soluble fraction from leaves of *Arctium lappa*, by UHPLC-MS.

Peak	Rt ($_{min}$)	MS^1 $[M-H]^-$	MS^2 (main)	Identification
1	0.44	191.05610	–	Quinic acid
2	0.51	473.15106	353, 317, 195	unknown
3	0.55	335.04895	167, 124	unknown
4	0.69	290.08775	200, 188, 128	unknown
5	0.86	366.02121	344, 150, 133	unknown
6	1.66	315.07144	254.9, 232.9, 214.9, 152, 108	unknown
7	1.75	353.08742	191, 179, 135	1-*O*-caffeoylquinic acid
8	2.19	353.08744	191, 179, 135	*neo*-chlorogenic acid
9	2.45	485.16851	359, 254.8, 152.9	unknown
10	3.10	349.05870	254.8, 152.9, 144	unknown
11	3.31	353.08743	191, 179, 135	Chlorogenic acid
12	3.61	353.08714	191, 179, 173, 135	*crypto*-chlorogenic acid
13	4.41	515.11952	353, 335, 191, 179,135	1,5-*O*-dicaffeoylquinic acid
14	4.91	341.09047	254.8, 152.9	unknown
15	5.08	467.15847	281, 254.8, 152.9	unknown
16	5.25	465.14268	–	unknown
17	6.22	465.14252	–	unknown
18	6.61	631.12962	353, 191, 179, 173, 161,135	dicaffeoylmaloylquinic acid
19	7.10	463.0877	300, 271, 255, 243	quercetin-hexoside
20	7.22	609.14565	300, 271, 255, 243	Rutin
21	7.38	515.11909	353, 335, 191, 179, 173,135	3,4-*O*-dicaffeoylquinic acid/3,5-*O*-dicaffeoylquinic acid
22	7.49	631.13099	353, 191, 179, 135	dicaffeoylmaloylquinic acid
23	7.57	515.11950	353, 335,191, 179, 135	1,3-*O*-dicaffeoylquinic acid
24	7.97	487.30474	451, 337, 293, 191,135	unknown
25	8.19	447.09299	284, 255, 227	Kaempferol-hexoside
26	8.33	593.15115	285, 284, 255, 227	Kaempferol-rutinoside
27	8.56	515.11952	353, 335, 191, 179, 173, 135	4,5-*O*-dicaffeoylquinic acid
28	8.84	489.10358	442.9, 429, 307, 285, 255, 229	unknown
29	9.22	479.19496	415	unknown

the carbon atoms from quinic acid, causing serious misinterpretations. Here, considering the chlorogenic acid (3-*O*-caffeoylquinic acid) as a marker for the caffeoylquinic acids, the carbon atoms around the quinic acid were numbered accordingly it. Aided by a standard, the peak 13 was identified as cynarin (1,5-*O*-dicaffeoylquinic acid), however the peak 23 could not be directly identified, remaining two possibilities 1,3-*O*-dicaffeoylquinic acid or 1,4-*O*-dicaffeoylquinic acid.

The ESF was able to protect the gastric mucosa against ethanol-induced ulcers (see below). Therefore, in order to evaluate the compounds responsible for the gastric protection, a liquid/liquid fractionation was carried out with ESF. According to Silva et al. [10], the fractions containing the phenolic compounds were considered as potential for such activity. The LC-MS analysis of the fractions revealed that the ethyl acetate (ESF-EA) and the butanol fraction (ESF-B) have concentrated all caffeoylquinic acids. Whereas ESF-EA concentrated mainly the dicaffeoylquinic acids (Fig. 1B), ESF-B concentrated most of monocaffeoylquinic acids (Fig. 1C). An interesting exception was the cynarin (1,5-DCQA) which was separated from other DCQA, remaining in the butanolic fraction, peak 13 (Fig. 1C).

The main compound from ESF-EA was the unknown DCQA, peak 23 (7.57 min), *m/z* 515.119 $[M-H]^-$ (Fig. 1B). Considering that the ESF-EA fraction was the only one active on gastric protection (see below), it may be inferred that the dicaffeoylquinic acids available in this fraction, and mainly that unknown DCQA, can be related to the observed effect. This is in accordance with the study performed by Silva et al. [10], which showed that the crude ethanolic extract from the *A. lappa* roots rich in hydroxycinnamoylquinic acid, mainly dicaffeoylquinic acids, had anti-ulcer activity.

3.2. Purification and identification of unknown dicaffeoylquinic acid

The unknown DCQA was purified from ESF-EA fraction by a semi-preparative reversed-phase liquid chromatography. The purified compound was analyzed by UHPLC-PDA-MS, being ~95% pure, as estimated by the area integration at 325 nm and TIC-MS. The purified DCQA was then analyzed by NMR experiments (^{1}H, COSY, HSQC and HMBC).

The HSQC alone was poorly informative, due to a "pseudo-symmetry" of the substituent groups on the quinic acid ring (i.e. similar substituents with different stereochemistry). However, the ^{1}H/^{13}C signal at 5.36/71.3 ppm was consistent with site for caffeate ester, since this ^{1}H signal is characteristically shifted to higher frequency value when close to an ester linkage. Other hydroxylated ^{1}H/^{13}C signal from quinic acid appeared at 4.29/70.15 ppm and 3.84/73.44 ppm. Since C1 from quinic acid does not contain hydrogen, it was absent on the HSQC spectrum (Fig. 2A).

In the HMBC experiment a signal consistent to the long range coupling of the carbonyl group from caffeic acid at 169.7 ppm, with the ^{1}H at 5.36 ppm from quinic acid confirmed the ester linkage site. The signal of carbonyl group also had a strong coupling with the β-*trans*-hydrogen of caffeic acid. Other intra-ring HMBC correlations were observed (Fig. 2B). With the COSY experiment the chemical shifts of the quinic acid were assigned, however those pseudo-symmetry cited above hindered the precise definition of the actual carbons and hydrogens position. Only the signal of H4 from quinic acid could be identified, because it has two *vic*-CHOH groups, thus the COSY coupling was able to undoubtedly located it at 3.84 ppm, coupled with 5.36 and 4.29 ppm, from H3 and/or H5. COSY couplings of quinic acid moiety also exhibited the correlation between H3/H5 with their vicinal CH_2 groups, at 5.36 ppm with 2.63/2.02 ppm, and the signal at 4.3 ppm with 2.59/2.25 ppm, (Fig. 2C).

The ^{1}H chemical shift from ester linkage was defined by HSQC/HMBC experiments at 5.36 ppm, and this signal on the ^{1}H spectrum appeared as a double-double-doublet, with strong coupling constants at $J_{3ax/2ax}$ and $J_{3ax/4ax}$ ranging 10–12 Hz, and weak coupling at $J_{3ax/2eq}$ ~4 Hz. This was due to the axial conformation of the hydrogen on the ester linkage site, which has strong coupling constant with two

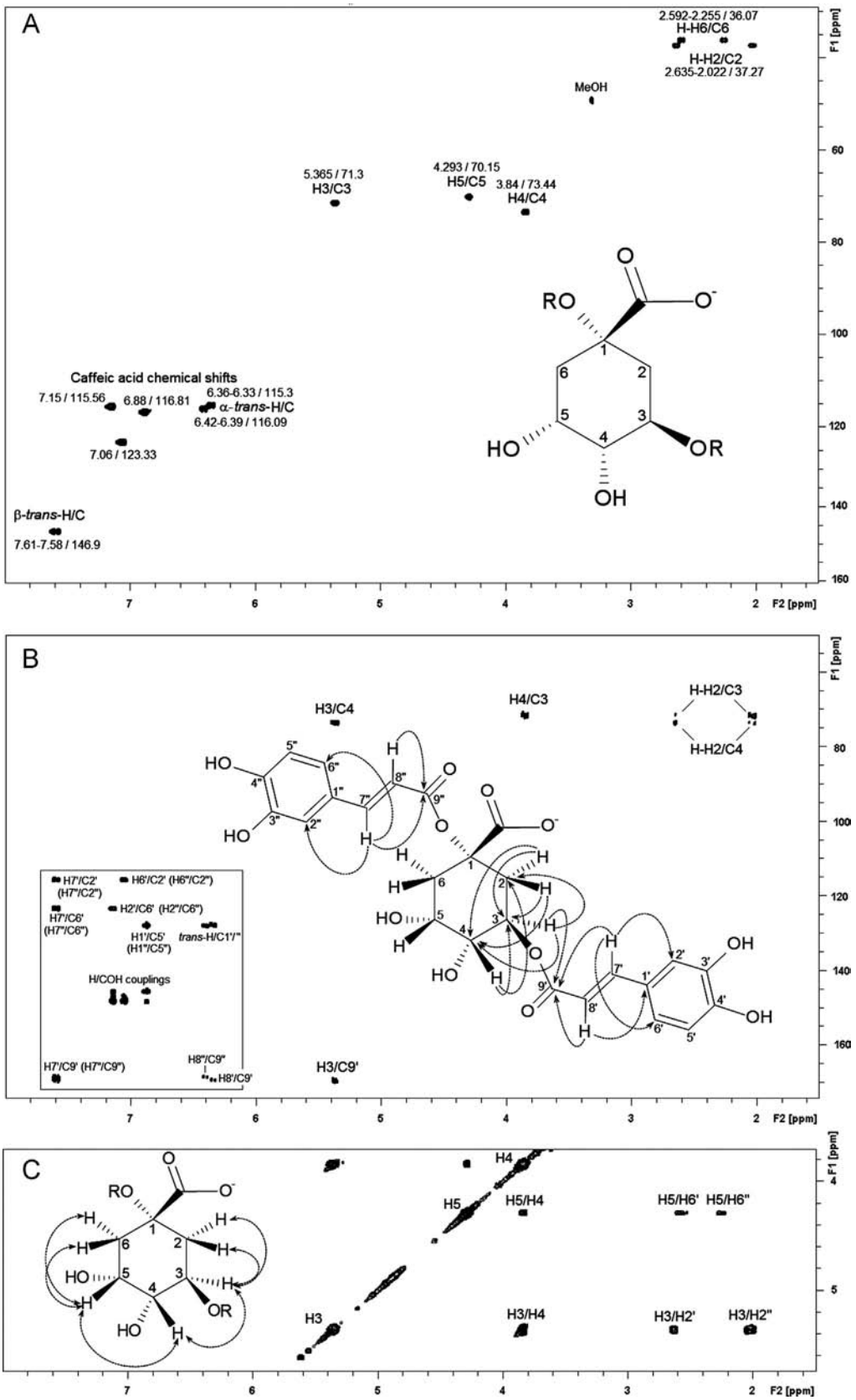

Fig. 2. NMR analysis of purified peak 23. (A) HSQC correlations supported by (B) HMBC and (C) COSY signaling. Arrows indicate the correlations among *vic* ^{1}H–^{1}H on COSY, and long range ^{1}H–^{13}C on HMBC experiments.

other *vic*-axial H2 and H4, and a weak coupling with the equatorial H2 of the quinic acid ring. This information indicates the actual ester linkage site is on C3 (similar to chlorogenic acid), since this carbon has the hydrogen neighborhood at properly conformation to give that chemical shift with those coupling constant. With these details, it was possible to assign all chemical shifts on the HSQC correlations. Also,

the coupling constant obtained for the caffeic acid signals $J_{H/H}$ 6.36/6.33 and 6.42/6.39 were each 15.9 Hz, characteristic of *trans* configuration, confirming the identification of *trans*-caffeic acid, these results are in accordance with previous findings [22–24].

The acetalation reaction, yielding isopropylidene derivatives, can aid in the structural elucidation of many isomers [18], since this reaction employing dry acetone in acidic medium promotes the formation of the *O*-isopropylidene ketal, when there are specific stereochemistry conditions in the molecules containing hydroxyl groups. An exigency for the reaction is the presence of free vicinal hydroxyls in *cis* configuration. Quinic acid has 3 *vic*-hydroxyl groups (on C3, C4 and C5) and those at C4 and C5 are *cis*, whereas C3 and C4 are *trans*. Therefore, the required stereochemistry for isopropylidene formation is achieved when the quinic acid has the hydroxyls on C4 and C5 not attached by the caffeic acids, hence the 1,3-*O*-dicaffeoylquinic acid allows the formation of the 4–5-*O*-isopropylidene derivate, whereas 1,4-DCQA or 1,5-DCQA do not. Therefore, it is possible to promote a selective acetalation reaction in order to identify the position where caffeic acid groups are esterifying on quinic acid.

The result of acetalation reaction with the purified DCQA was verified by LC-PDA-MS. It was observed a lack of peak 23 on its original retention time (7.57 min), followed by the emergency of a novel peak at 12.06 min, which could be attributed to the positive reaction, because of inserting an isopropylidene ketal group decreases the polarity of the molecule, consequently increasing the interaction with the reversed phase column. The PDA detection revealed similar UV-absorbance spectrum of caffeoylquinic acids (~325 nm), however the MS detection produced an ion at *m/z* 555.151 $[M-H]^-$, which was 40 mass units (m.u.) higher than non-derivatized dicaffeoylquinic acid. This has confirmed the positive reaction of the compound/peak 23, since isopropylidene adds 40 m.u. to the molecules. The CID-fragmenstation of the ion at *m/z* 555.151 yields fragment-ions at *m/z* 393.119 and 231.087. The later fragment confirms that the isopropylidene was attached to the quinic acid (40 m.u. higher than *m/z* 191.056 from non-derivatized) (Fig. 3A,B). As previously considered, the 1,4-*O*-dicaffeoylquinic acid does not match the requirements to be reacted, because it lacks free *vic-cis* OH groups. Thus the compound/peak 23 could be confirmed as being 1,3-*O*-dicaffeoylquinic acid.

The acetalation reaction was also performed on standard of chlorogencic acid (3-*O*-caffeoylquinic acid), ESF-EA and ESF-B fractions. In the ESF-EA fraction, again the peak 23 was shifted to 12.06 min with *m/z* 555.151. The chlorogenic acid, standard or that present in the ESF-B was also reactive, being shifted to 8.96 min, with *m/z* increased to 393.119 $[M-H]^-$, with the diagnostic fragment-ion at *m/z* 231.087. As expected, the cynarin (peak 13) was not reactive to the acetalation.

3.3. Gastric protection induced by ESF, fractions and isolated compound

Compounds from *A. lappa* were tested in a model of ethanol-induced acute gastric lesion. The oral treatment of animals with ESF (1, 10 e 100 mg kg^{-1}) reduced the gastric lesions in a dependent dose manner, with ED_{50} of 3.8 mg kg^{-1}. The positive control, omeprazole (40 mg kg^{-1}), promoted 93.7% of gastric protection when compared with ulcerated vehicle group control (C: 205.5 ± 17.7 mm^2) (Fig. 4A).

The fractions from liquid/liquid fractionation were also tested. As expected, the oral administration of absolute ethanol (0.5 mL 200 g^{-1}) produced lesions with 173.9 ± 19.9 mm^2 on the gastric mucosa. However, the oral treatment with ESF-EA fraction (0.15 mg kg^{-1}) or

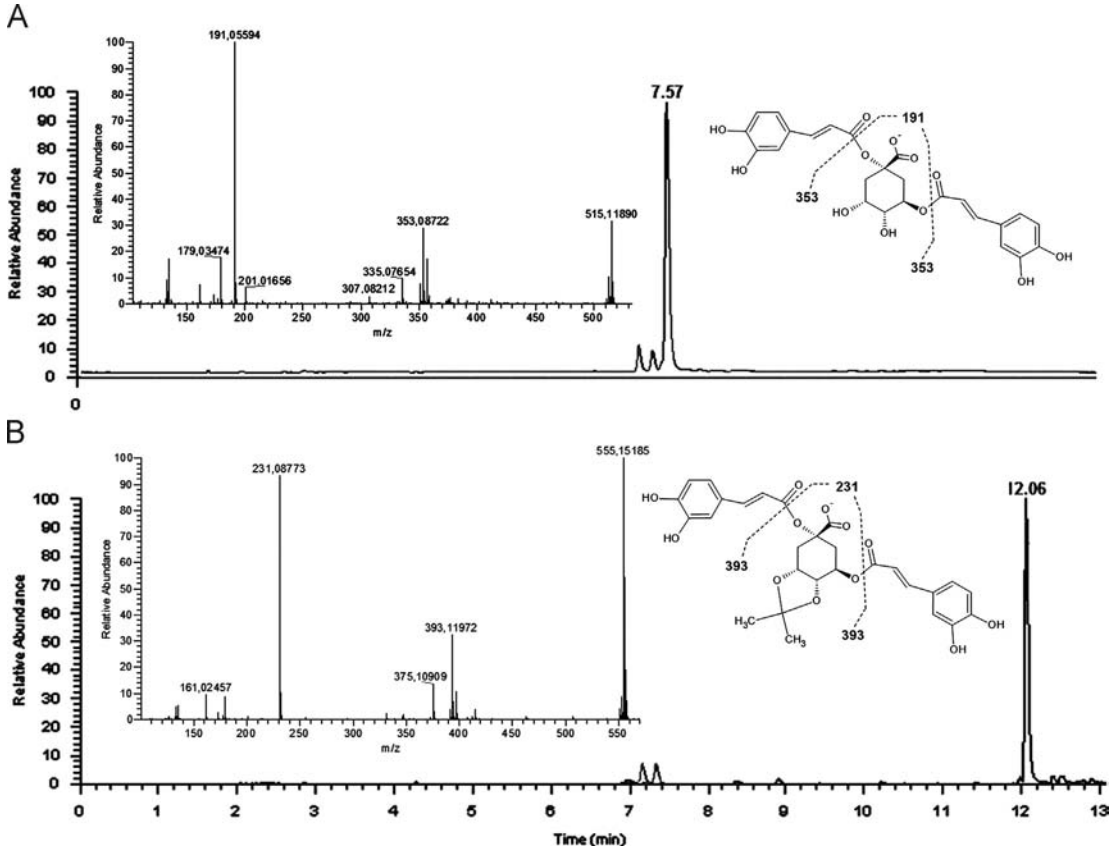

Fig. 3. UHPLC-MS chromatograms of native 1,3-*O*-dicaffeoylquinic acid (A) and their isopropylidene derivative (B). The difference in the retention time is due to the additional hydrophobic site added on quinic acid, with a mass increment of 40 m.u.

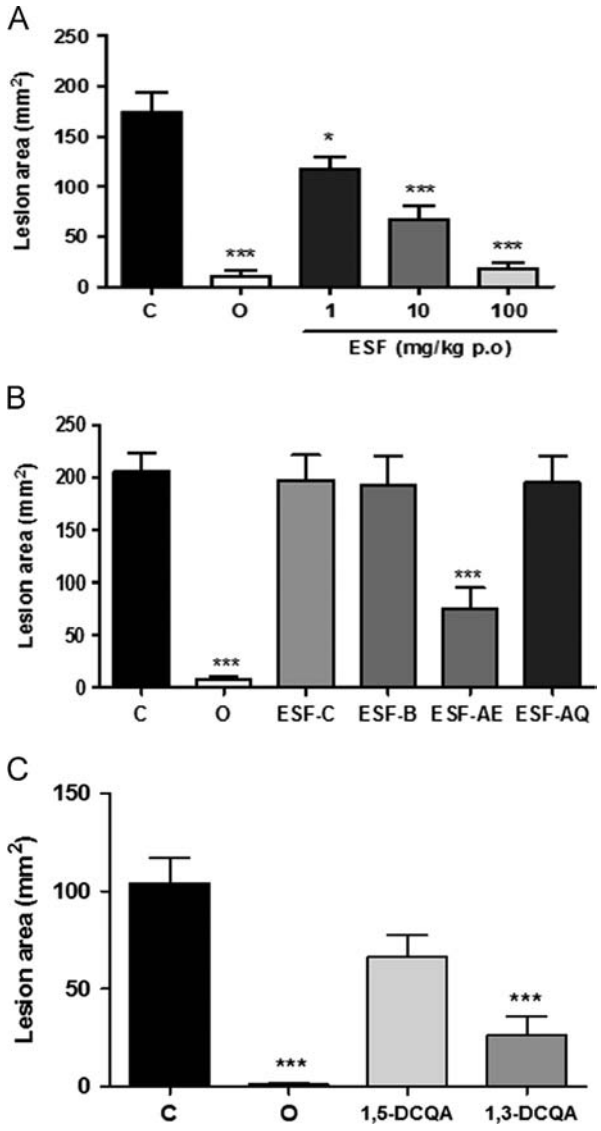

Fig. 4. Effect of *A. lappa* extract, fractions and purified compounds in the ethanol induced gastric ulcer, compared with negative control (c) and omeprazole (o). (A) ESF at 1, 10 and 100 mg kg^{-1}, (B) fraction from liquid/liquid fractionation: ESF-C (0.01 mg kg^{-1}), ESF-EA (0.15 mg kg^{-1}), ESF-B (0.30 mg kg^{-1}) and ESF-AQ (1.99 mg kg^{-1}). (C) Effect of 1,5-DCQA (cynarin, at 0.057 mg kg^{-1}) and 1,3-DCQA (isolated from ESF-EA, at 0.057 mg kg^{-1}). The results are expressed as mean ± S.E. M. Statistical comparison was performed using analysis of variance (one-way ANOVA) followed by Bonferroni's test. * $p < 0.05$ and *** $p < 0.001$, when compared to the negative control group.

the positive control omeprazole (40 mg kg^{-1}) promoted reduction of the gastric lesion in 63.5% and 96.9%, respectively. On the other hand, the ESF-C, ESF-B and ESF-AQ have no effects, confirming that the active compound was concentrated on the ethyl acetate fraction (Fig. 4B).

The 1,3-DCQA isolated from ESF-EA was also evaluated using ethanol-induced acute gastric lesion. In this experiment, oral administration of absolute ethanol (0.5 mL 200 g^{-1}) has injured the gastric mucosa at 104.0 ± 13.0 mm^2. The oral administration of the purified 1,3-DCQA (at 0.057 mg kg^{-1}) was able to protect the gastric from lesion formation in 75%. In contrast, the 1,5-DCQA (cynarin) at same dose, was ineffective. The positive control with omeprazole (at 40 mg kg-1, p.o) inhibited the gastric lesion formation in 99.8%. These results confirms that the 1,3-*O*-dicaffeoylquinic acid is the main responsible compound for the gastroprotective effect observed for *A. lappa* leaves (Fig. 4C).

4. Conclusion

The *Arctium lappa* leaves contain a potent gastroprotective agent, with experimental ED_{50} at 57 μg kg^{-1}, much smaller than the therapeutic dose of omeprazole (40 mg kg^{-1}). The candidates for such activity were the mono- and dicaffeoylquinic acids, which appeared in the leaves as the main phenolic compounds, having several isomers. For a such complex extract, a bioguided fractionation with further purification was successfully developed, and the active compound could be achieved among several isomers, and identified as 1,3-*O*-dicaffeoylquinic acid. Together, NMR and acetalation reaction followed by UHPLC-MS analysis have provided unambiguously details allowing the correct statement of the structure of 1,3-DCQA. Although being an isomer, the cynarin present at high abundance in the butanolic fraction, as well as the authentic standard, was not active at similar doses. Further experiments are at advanced course, with promising results for application of 1,3-DCQA, as preventive and curative agent against gastric lesions.

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