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Original Article

Characterization of hepatoprotective metabolites from *Artemisia annua* and *Cleome droserifolia* using HPLC/PDA/ESI/MS–MS

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ABSTRACT

The hepatoprotective activities of two traditionally used plants, *Cleome droserifolia* (Forssk.) Delile, Cleomaceae, and *Artemisia annua* L., Asteraceae, were recently reported. However, the biologically active metabolites responsible for this activity were not identified. The aqueous extract of *C. droserifolia* aerial parts, and the polar fraction of *A. annua* leaves were screened for their antioxidant activities using the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) assay. The *in vitro* viability of HepG-2 cells treated with CCl_4 and the extracts were assessed by MTT assay. The effects of the extracts on the liver enzymes and the total soluble protein in CCl_4-intoxicated HepG-2 cells were investigated. An HPLC/PDA/ESI/MS–MS based analysis was carried out for extract of *C. droserifolia* and polar fraction of *A. annua*. Both exhibited pronounced free radical scavenging activities (86 and 83%, respectively). Both showed a significant increase in cell viability: 86.43% for the extract of *C. droserifolia* and 79.32% for polar fraction of *A. annua*. Only the extract of *C. droserifolia* (39.6 ± 5.41 and 20.4 ± 6.91 IU/dl, respectively) and polar fraction of *A. annua* (40.8 ± 2.14 and 24.5 ± 3.11 IU/dl, respectively) restored the levels of liver enzymes (aspartate transaminase and alanine transaminase, respectively) compared to the CCl_4 intoxicated group (87.5 ± 4.34 and 34.1 ± 8.12 IU/dl, respectively) and other herbal extracts. More than fifty phenolic secondary metabolites were identified in the extracts under investigation. The significant hepatoprotective activities of both extracts seemed to be strongly connected to their content of hydroxycinnamoyl quinic acids and flavonoids.

Introduction

Hepatitis C is common worldwide. The most affected regions are Eastern Mediterranean and European Regions, with the prevalence of 2.3 and 1.5%, respectively. The incidence of hepatocellular carcinoma in Egypt has doubled in the last 10 years, rising from 4 to 7.2% among chronic liver patients (Abou El Azm et al., 2014). Thus, people worldwide are demanding traditional herbal remedies to protect the liver, in addition to the conventional drugs used for the treatment of HCV.

Recently, *in vitro* systems were shown to be valuable tools for drug discovery. They have been extensively utilized in evaluating the protective effect of plant extracts on liver lesions induced by toxic compounds (Torres-Gonzalez et al., 2011).

The herb *Cleome droserifolia* (Forssk.) Delile, Cleomaceae, has been traditionally used in Egypt as a decoction for the treatment of diabetes. Several studies have validated its antidiabetic effect (Abdel-Kawy et al., 2000; Abdel Motaal et al., 2014). A study carried out by members of our group determined the active mechanism of its isolated terpenoids and flavonol glycosides (Motaal et al., 2011). These isolated compounds also showed significant cytotoxic effects against the MCF7 and HCT116 cell lines (Ezzat and Abdel Motaal, 2012). It was previously reported that the herb contains antioxidant and hepatoprotective active constituents (Nassar and Gamal-Eldeen, 2003; Abdel-Kader et al., 2009).

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Artemisia annua L., Asteraceae, commonly known as sweet sagewort, has been traditionally used in Chinese medicine and has become a valuable source of raw material for antimalarial drugs. El-Askary et al. (2004) succeeded in cultivating *A. annua* in Egypt with a high yield of artemisinin (1% dry weight at the pre-flowering stage, compared to 0.86% dry weight in the Vietnamese cultivar) (Ferreira et al., 1995). Crude extracts of the leaves of *A. annua* were reported to possess antioxidant activities due to the high content of flavonoids (Zheng and Wang, 2001; Bilia et al., 2006). The plant is valuable due to its diverse biological actions, ranging from anti-malarial to anticancer activities (Beekman et al., 1998). Previous reports generally focused on the terpenes of the non-polar fractions, particularly artemisinin (the sesquiterpene lactone). However, little attention was paid to the phenolic compounds of the polar fraction. Accordingly, this work explored the polar fraction of *A. annua*.

Thus, the antioxidant and hepatoprotective activities of the bioactive extracts of *A. annua* and *C. droserifolia* were studied and a qualitative analysis of these hepatoprotective bioactive extracts was carried out using HPLC/PDA/ESI/MS–MS in negative and positive ionization modes. This method was used to detect and characterize the phytochemical compounds, many of which were tentatively characterized for the first time in both plants.

Material and methods

Plant material

The aerial parts of *Cleome droserifolia* (Forssk.) Delile, Cleomaceae, were obtained from the Medicinal Plants Society, Saint Catherine, Sinai in 2010. The plant was authenticated by Assistant Prof. Dr. M. Gebali (Plant Taxonomy and Egyptian Flora Department, National Research Center, Giza, Egypt). Leaves of *Artemisia annua* L., Asteraceae, were obtained from the Experimental Station of Medicinal Plants of the Faculty of Pharmacy, Cairo University in Giza in July 2012. The plant was authenticated by Prof. Dr. Ebrahim A. El-Garf (Professor of Botany, Department of Science, Cairo University) during the flowering stage (September). Voucher specimens for *Cleome* (29-04-2011) and *Artemisia* (13-04-2014) were deposited at the herbarium of the Faculty of Pharmacy, Cairo University, Egypt.

Preparation of extracts and fractions

The air-dried aerial parts of *C. droserifolia* (200 g) were extracted with boiled water (3 × 500 ml) giving a yellowish buff powder extract (Cl-AQ, 70 g). Air-dried leaves of *A. annua* L. (1 kg) were extracted with 70% ethanol by sonication till exhaustion, yielding a dark green residue of (235 g, 23.5% w/w). The alcoholic extract (200 g) was extracted successively with hexane, chloroform, ethyl acetate and *n*-butanol fractions in portions till exhaustion. The combined polar fractions (ethyl acetate, 21.1 g and *n*-butanol, 27.5 g) (ART-CQ) were used in this study.

Fractions of *Trigonella foenum-graecum*, *Rosmarinus officinalis* and *Linum usitatissimum* (FEN-SaP, RO-MC, and Lin-LRF, respectively) were prepared by members of our group (data under publication). FEN-SaP is the butanol fraction of fenugreek, RO-MC is the methylene chloride fraction of rosemary, and Lin-LRF is the lignin-rich fraction of linseed.

General

Kits for the enzymatic assays of aspartate transaminase (AST) and alanine transaminase (ALT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Gibco Laboratories (Life Technologies Inc., Grand Island, NY, USA). All chemicals used were of the highest pure grade available. Human liver hepatocellular carcinoma cell line HepG-2 from American Type Culture Collection (ATCC, Rockville, MD, USA) was delivered from Vaccera, Dokki, Egypt. Plates of 96 wells were purchased from Corning Costar (Cambridge, MA, USA).

Antioxidant activity

This assay depends on the ability of the extracts to scavenge 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical cation, according to Shalaby and Shanab (2013). All extracts (100 μg/ml final concentration) were dissolved in 0.1% DMSO.

Cell culture

The HepG-2 cells were cultured in a DMEM medium supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 10% CO_2 at 37 °C. All cells were between passages 50 and 62.

In vitro *viability of HepG-2 cells*

The HepG-2 cells viability was assessed by MTT assay according to the manufacturer's recommendations (Roche Diagnostics GmbH, Mannheim, Germany). The extracts were tested at 100 μg/ml. The solubilized formazan product was spectrophotometrically quantified at 540 nm with the help of a microplate reader, Power Wave XS (Bio Tek, Winooski, VT, USA) (Ibrahim et al., 2014).

In vitro *hepatoprotective activity*

HepG-2 cells were trypsinized in uniform single cell suspension, having approximately 5×10^5 cells/ml in DMEM, and were then seeded in 21 flasks. Then 20 mM CCl_4 in 0.1% DMSO along with 1 ml buffer were added to the test extract groups, reaching an effective concentration of 4 mM CCl_4 and a final concentration of 100 μg/ml of the extract. After 14 h treatment with CCl_4, the supernatant was used for the analysis of the total soluble protein and the liver enzymes (AST, ALT), using a commercial kit purchased from Biomed Diagnostics (White City, OR) (Gite et al., 2014).

Statistical analysis

Data were presented as mean values ± (SD). Statistical comparisons between groups were performed by one-way analysis of variance (ANOVA), followed by Posthoc Tukey's test (Statistica, StatSoft, USA). Values of $p < 0.05$ were assumed to be statistically significant.

LC/MS of active extracts

The chromatographic analysis was performed on an HPLC Agilent 1200 series instrument equipped with a high performance autosampler, binary pump and PDA detector G 1314 C (SL) (Agilent Technologies, Waldbronn, Germany), the column was Gemini 3 mm C18 110 Å from Phenomenex with dimensions (100 mm × 3 mm i.d., 5 μm) protected with RP C18 100 Å guard column with dimensions (5 mm × 3 mm i.d., 5 μm). The mobile phase consisted of two solvents: 2% acetic acid in purified water (A) and 90% MeOH in purified water (B) at a flow rate of 50 μl/min. The sample was dissolved in 5% MeOH and 2% acetic acid. The mobile phase gradient was: 0–60 min, 5% B; 60–70 min, 50% B; 70–80 min, 90% B; 80–90 min, 5% B. The samples were dissolved in 5% MeOH and 2% acetic acid with a concentration of 1 mg/ml then filtered using a syringe filter with a pore size 0.2 μm. The sample injection volume was 10 μl.

Table 1
In vitro viability of HepG-2 cells treated with CCl_4 and the herbal extracts.

Groups	Treatment	% HepG-2 viability
1	Control (untreated)	100
2	CCl_4	35.23 ± 0.24
3	CCl_4 + Cl-AQ	86.43 ± 0.40
4	CCl_4 + ART-CQ	79.32 ± 1.54
5	CCl_4 + FEN-SaP	63.22 ± 2.1
6	CCl_4 + RO-MC	58.31 ± 0.73
7	CCl_4 + Lin-LRF	42.82 ± 1.15

All extracts were tested at a final concentration of 100 μg/ml.

A Fourier transform ion cyclotron resonance mass analyzer was used, equipped with an electrospray ionization (ESI) system. The mass analyzed is the FT-ICR in the full scan and in trap in ms/ms mode (fragmentation). X-calibur® software was used to control the system. Detection was performed in the negative ion mode applying a capillary voltage of 36 V and a temperature of 275 °C. The API source voltage was adjusted to 5 kV, and the desolvation temperature to 275 °C. Nitrogen was used as a nebulizing gas with a flow adjusted to 15 l/min. The analytical run time was 89 min and the full mass scan covered the mass range from 150 to 2000 *m/z* with resolution up to 100,000 (Handoussa et al., 2013).

Results and discussion

Antioxidant activity

Cl-AQ and ART-CQ extracts were assessed for their ability to scavenge ABTS free radicals, along with FEN-SaP, RO-MC, Lin-LRF, and ascorbic acid as a positive standard control. Both Cl-AQ and ART-CQ extracts exhibited pronounced antioxidant activities (86 ± 2.04% and 83 ± 1.24%, respectively), which were comparable to ascorbic acid (88 ± 2.0%). On the other hand, FEN-SaP (75 ± 0.75%), RO-MC (53 ± 1.35%), and Lin-LRF (61 ± 0.64%) showed moderate activities.

In vitro *hepatoprotective activity*

Recently, *in vitro* models were shown to be valuable tools for drug discovery. Plant extracts have been employed in several applications as protection against liver lesions induced by toxic compounds (Torres-Gonzalez et al., 2011). Pretreatment of the CCl_4 injured HepG-2 cells with the plant extracts increased the percentage of viable cells compared to the toxic cells (Table 1). Notably, Cl-AQ and ART-CQ showed a significant increase in cell viability (86.43 ± 0.40 and 79.32 ± 1.54%, respectively) when compared to the other three herbal extracts.

It was observed that both the Cl-AQ and ART-CQ extracts possessed a preventive role against chloride radical toxicity. The levels of the hepatic enzymes (AST and ALT) were significantly elevated in the CCl_4 group (87.5 ± 4.34 and 34.1 ± 8.12 IU/dl, respectively), compared to the control group (36.8 ± 4.25 and 14.5 ± 3.36 IU/dl, respectively) and the Cl-AQ (39.6 ± 5.41 and 20.4 ± 6.91 IU/dl, respectively) and ART-CQ (40.8 ± 2.14 and 24.5 ± 3.11 IU/dl, respectively) treated groups (Table 2). Also, the protein level was restored in the cells pretreated with Cl-AQ and ART-CQ. The FEN-SaP, RO-MC, and Lin-LRF extracts did not protect the hepatic cells from oxidative free radical intoxication (Table 2).

The ability of the cells to reduce MTT provided an indication of mitochondrial integrity and activity (Maianski et al., 2004). Serum transaminase levels were shown to return to normal through stabilization of the plasma membranes of the injured hepatocytes. Thus, the extracts were protective against liver toxicity, most likely through both the restoration of the functional integrity of the cell membranes with the resulting reduction of transaminases, as well as through the antioxidative mechanism.

Table 2
Effect of extracts on AST, ALT and total soluble protein levels on CCl_4-intoxicated HepG 2 cells.

Groups	Treatment	AST (IU/dl)	ALT (IU/dl)	Protein (g/dl)
1	Control (untreated)	36.8 ± 4.25	14.5 ± 3.36	7.12 ± 0.45
2	CCl_4	87.5 ± 4.34	34.1 ± 8.12	2.62 ± 4.14
3	CCl_4 + Cl-AQ	39.6 ± 5.41	20.4 ± 6.91	6.4 ± 0.22
4	CCl_4 + ART-CQ	40.8 ± 2.14	24.5 ± 3.11	5.18 ± 0.24
5	CCl_4 + FEN-SaP	52.8 ± 1.82	34.7 ± 2.33	4.15 ± 0.71
6	CCl_4 + RO-MC	65.3 ± 1.41	40.2 ± 1.41	4.01 ± 1.52
7	CCl_4 + Lin-LRF	69.3 ± 2.11	45.1 ± 2.12	3.51 ± 0.45

Values were expressed as mean ± SD of triplicates in each group; significant difference noted from groups 1 and 2 at $p < 0.05$. All extracts were tested at a final concentration of 100 μg/ml.

Metabolite profiling of active extracts

This study aimed to identify the phenolic compounds within the bioactive fractions using HPLC/PDA/ESI/MS–MS approach. Data are shown in Tables 3 and 4 for Cl-AQ and ART-CQ, respectively. Peak identification was performed by comparison fragmentation pattern of the precursor ion $[M-H]^-$ and their diagnostic product ions. Negative mode (Figs. 1 and 2) was used, since phenolic molecules are more clearly detected in this mode rather than in the positive ion mode (Figs. 1S and 2S in supplementary material), According to Mittal et al. (2013) it gives better deprotonating process, with optimum ionization, besides the decrement of the signal-to-noise ratio and higher peaks abundance.

Cleome droserifolia *(Cl-AQ)*

Phenolic acid derivatives

Compound (Cd1) showed a base peak anion at *m/z* 355.09. Moreover, the MS^n spectrum corroborated the hypothesis of a caffeoyl-hexuronide derivative, as the characteristic fragment ions of caffeic acid at *m/z* 179 after loss of a hexuronic acid at *m/z* 135 were detected; *m/z* 175 appeared due to the loss of caffeic acid from the base peak and *m/z* 113 peak of a hexuronide acid fragmentation (Fig. 1 and Table 3). Furthermore, the appearance of peak Cd2 at $[M-H]^-$ *m/z* 339.04 showed the carboxylic form of peak Cd1, as they have exactly the same fragmentation pattern, with the exception of the loss of CO_2 (Catarino et al., 2015) so it is identified as a caffeoyl-hexuronide derivative. Moreover, Cd7 was identified as 5-caffeoyl-quinic acid with its deprotonated molecule $[M-H]^-$ *m/z* 353.09. According to Clifford et al. (2003), the position of the caffeoyl substituent was suggested due to its peak ion of deprotonated quinic acid at *m/z* 191 and peak ion at *m/z* 179. Peak Cd11 showed a molecular ion $[M-H]^-$ at *m/z* 597.18, yielding prominent ions at *m/z* 359, 295 and 179. Similar fragments were reported by Chen et al. (2010) for the compound yunnaneic acid F, which is a caffeic acid metabolite previously detected in *Salvia miltiorrhiza* and *Melissa officinalis*. This was considered as a first report in *C. droserifolia* (Aboushoer et al., 2010).

Flavonoids

Several flavonol glycosides were detected in *C. droserifolia* as quercetin-*O*-glucoside-*O*-rhamnoside, which was recognized as peak Cd10; $[M-H]^-$ of *m/z* 609.15, with Ms^n at *m/z*: 447 $[Q+Rha-H]^-$, 463 $[Q+Glu-H]^-$ and 299 $[Q-H]^-$ (Handoussa et al., 2013), this compound was previously reported for the same species (Abdullah et al., 2016).

Additionally, Cd12 revealed the presence of kaempferol-*O*-glucoside-*O*-rhamnoside, having its deprotonated anion $[M-H]^-$

Table 3
Peak assignments using HPLC/PDA/ES-MS^n of metabolites detected in Cl-AQ (negative ion mode). (the structures are shown in Fig. 3S).

Peak (compound)	Identified compounds	Retention time (min)	UV–Vis (λ_{max})	$[M-H]^-$ (m/z)	Error(ppm)	Molecular formula	Fragment ions (m/z)	Peak area%	Reference
Cd1	Caffeoyl-hexuronic acid	2.05	245, 318	355.0883	−3.38	$C_{12}H_{19}O_{12}$	311, 194, 195, 179, 175, 113,	3.1	Catarino et al. (2015)
Cd2	Caffeoyl-hexuronide derv.	2.86	240, 320	339.0935	−4.13	$C_{12}H_{20}O_{11}$	355, 311, 179, 175, 113	0.9	Catarino et al. (2015)
Cd3	(Iso) pentyl dihexose	10.17	258	411.1506	−2.43	$C_{16}H_{28}O_{12}$	249, 205	0.15	Barros et al. (2012)
Cd4	Caffeoyl ferulic acid derivative	17.70	240, 330	408.0429	−3.19	$C_{28}H_9O_4$	315, 303, 179	0.2	El Sayed et al. (2016)
Cd5	Caffeoyl ferulic acid derivative	19.05	240, 330	408.0430	−3.19	$C_{28}H_9O_4$	315, 303, 269, 252, 179	0.35	El Sayed et al. (2016)
Cd6	(Epi) catechin derivative	23.05	264, 320	388.0742	−3.09	$C_{26}H_{13}O_4$	261, 221, 219, 179	0.4	Tentative
Cd7	5-Caffeoylquinic acid	33.99	326	353.0879	−3.40	$C_{16}H_{18}O_9$	191, 179, 161	0.28	Clifford et al. (2003)
Cd8	(Iso) rhamnetin hexoside derv.	40.61	250, 352	655.1476	2.5	$C_{22}H_{21}O_{15}$	315, 301, 179	0.27	Tentative
Cd9	(Iso) rhamnetin	42.44	250, 352	315.2178	−3.81	$C_{16}H_{12}O_7$	217, 300	0.23	Abu-Reidah et al. (2015)
Cd10	Rutin	45.05	282, 355	609.1464	−1.64	$C_{27}H_{30}O_{16}$	463, 447, 301, 299	1.5	Handoussa et al. (2013)
Cd11	Yunnaneic acid F	47.3	297, 330	597.1828	−0.87	$C_{29}H_{26}O_{14}$	579, 359, 295, 179	0.72	Barros et al. (2012)
Cd12	Kaempferol 3-*O*-glucoside 7-*O*-rhamnoside	49.63	254, 366	593.1514	−2.36	$C_{27}H_{30}O_{15}$	431, 269	0.58	Farag et al. (2016)
Cd13	(Iso) rhamnetin rhamnosyl hexoside	50.48	250, 352	623.1619	−1.60	$C_{28}H_{31}O_{16}$	477, 315, 300	0.38	Farag et al. (2016)
Cd14	Quercetin dimethyl ether rutinose	55.61	282, 357	637.1775	−1.57	$C_{29}H_{34}O_{16}$	491, 329, 179, 151	0.6	Simirgiotis (2013)
Cd15	Gallocatechin	60.08	274	305.0667	−3.28	$C_{15}H_{14}O_7$	261, 221, 219, 179, 167, and 165	0.72	Dou et al. (2007)
Cd16	Acetyl di-caffeoylquinic acid	65.76	245, 317	557.2607	−0.77	$C_{27}H_{25}O_{13}$	515, 353, 179	0.53	Lin and Harnly (2010)
Cd17	Flavonoid derivative	67.79	255, 354	523.2918	−3.25	$C_{28}H_{43}O_9$	179, 1047	1.35	Zou et al. (2011)
Cd18	Di-flavonoid derivative	68.77	255, 355	1047.5877	−2.77	$C_{48}H_{56}O_{26}$	523, 179	2.7	Żuchowski et al. (2014)
Cd19	(Iso) rhamnetin-glu-malonate	68.85	270, 360	563.3024	−2.55	$C_{25}H_{24}O_{15}$	315, 301, 179	2.9	Abu-Reidah et al. (2015)
Cd20	Digalloyl deoxyhexose	72.65	274	465.2870	3.01	$C_{20}H_{18}O_{13}$	313, 301, 179, 169	1.98	Abdel-Hameed et al. (2013)

Table 4
Peak assignments using HPLC/PDA/ESI-MS^n of metabolites detected in ART-CQ (negative ion mode) (the structures are shown in Fig. 4S).

Peak (compound)	Identified compounds	Retention time (min)	UV–Vis (λ_{max})	$[M-H]^-$ (m/z)	Error (ppm)	Formula	Fragment ions (m/z)	peak area%	Reference
Aa1	Caftaric acid	2.02	240, 320	311.0620	−3.86	$C_{10}H_{15}O_{11}$	179, 149	0.32	Handoussa et al. (2013)
Aa2	Caffeoylquinic acid derivative	2.22	244, 326	461.2029	1.8	$C_{16}H_{18}O_9$	281, 239, 191, 179, 137	0.75	El Sayed et al. (2016)
Aa3	3-Caffeoylquinic acid	4.32	244, 326	353.0878	−3.12	$C_{16}H_{18}O_9$	191, 179	0.12	Clifford et al. (2005)
Aa4	5-Caffeoylquinic acid	21.10	244, 326	353.0881	−3.97	$C_{16}H_{18}O_9$	191, 179	0.47	Clifford et al. (2005)
Aa5	Apigenin-7-*β*-*O*-glucuronide	22.36	267, 355	445.2082	−3.14	$C_{21}H_{18}O_{11}$	431, 335, 269	0.52	Pereira et al. (2013)
Aa6	4-Caffeoylquinic acid	23.42	244, 326	353.0901	1.25	$C_{16}H_{18}O_9$	173, 179	0.2	Clifford et al. (2005)
Aa7	Digallic acid	27.18	nd	321.0242	−0.31	$C_{14}H_{10}O_9$	169, 125	0.17	Abu-Reidah et al. (2015)
Aa8	5-Feruloylquinic acid	29.36	240, 360	367.1036	−3.54	$C_{17}H_{20}O_9$	190, 172	0.18	Lin and Harnly (2010)
Aa9	3-Feruloylquinic acid	30.83	240, 360	367.1037	−3.81	$C_{17}H_{20}O_9$	192, 179	0.3	Lin and Harnly (2010)
Aa10	Caffeoyl coumaryl glucaric acid	34.48	241, 265	535.1672	2.5	$C_{26}H_{31}O_{12}$	355, 209, 179, 163	0.45	Lin and Harnly (2010)
Aa11	Feruloylquinic acid	35.21	240, 360	367.1037	−3.81	$C_{17}H_{20}O_9$	192, 179	0.4	Lin and Harnly (2010)
Aa12	Caffeic acid derivative	43.03	220, 316	381.1194	2.46	$C_{26}H_5O_4$	353, 337, 179	2.45	Gouveia and Castilho (2011)
Aa13	Vitexin (apigenin 8-*C*-hexoside)	43.48	336	431.0987	−3.48	$C_{21}H_{20}O_{10}$	311, 270, 179	2.5	Barros et al. (2012)
Aa14	Apigenin-7-hexoside	46.41	336	431.0988	−3.48	$C_{21}H_{20}O_{10}$	270, 179	0.28	Barros et al. (2012)
Aa15	4,5-Dicaffeoylquinic acid dimer	48.11	220, 330	1031.2443	0.78	$C_{50}H_{47}O_{24}$	515, 191, 179	2.75	Clifford et al. (2005)
Aa16	4,5-Dicaffeoylquinic acid	49.64	220, 330	515.1200	−3.11	$C_{25}H_{24}O_{12}$	191, 179	2.34	Clifford et al. (2005)
Aa17	Caffeoyl-feruloylquinic acid isomer	51.54	243, 366	529.1353	−2.46	$C_{26}H_{26}O_{12}$	367, 353, 191, 179	1.2	Lin and Harnly (2010)
Aa18	3,5-Dicaffeoylquinic acid dimer	53.59	220, 330	1031.2438	1.26	$C_{50}H_{47}O_{24}$	515, 191, 179	3.2	Clifford et al. (2005)
Aa19	Caffeoyl-feruloylquinic acid isomer	54.46	243, 366	529.1355	−2.83	$C_{26}H_{26}O_{12}$	367, 353, 191, 179	2.97	Lin and Harnly (2010)
Aa20	Caffeoyl-feruloylquinic acid isomer	55.57	244, 365	529.1357	−3.21	$C_{26}H_{26}O_{12}$	367, 353, 191, 179	1.25	Lin and Harnly (2010)
Aa21	3,4-Di-feruloylquinic acid	56.58	254, 367	543.1510	−0.37	$C_{27}H_{28}O_{12}$	349, 299, 193	0.72	Clifford et al. (2005)
Aa22	3,5-Di-feruloylquinic acid	57.01	254, 367	543.1512	−2.21	$C_{27}H_{28}O_{12}$	367, 349	0.79	Clifford et al. (2005)
Aa23	Caffeoyl-feruloylquinic acid isomer	59.82	243, 366	529.1355	−2.83	$C_{26}H_{26}O_{12}$	367, 353, 191, 179	3.5	Lin and Harnly (2010)
Aa24	(Iso) rhamnetin-*O*-hexoside sulphate	64.35	225, 340	557.1669	3.41	$C_{22}H_{21}O_{15}S$	316, 301, 179	1.3	Kargbo et al. (2015)
Aa25	Di-feruloylquinic acid	64.65	254, 367	543.1512	−2.76	$C_{27}H_{28}O_{12}$	367, 349	2.1	Clifford et al. (2005)
Aa26	3,4,5-Tricaffeoylquinic acid	65.38	220, 330	677.1516	−1.48	$C_{34}H_{30}O_{15}$	515, 179	1.6	Gouveia and Castilho (2011)
Aa27	(Iso) rhamnetin-*O*-hexoside sulphate	66.67	225, 340	557.1669	3.41	$C_{22}H_{21}O_{15}S$	316, 301, 179	1.25	Abu-Reidah et al. (2015)
Aa28	Feruloyl dicaffeoylquinic acid	67.06	250, 368	691.1668	2.89	$C_{34}H_{27}O_{16}$	515, 335, 179	3.15	Johnson et al. (2014)
Aa29	Di-feruloyl caffeoylquinic acid	68.79	250, 368	719.1990	−1.39	$C_{36}H_{31}O_{16}$	515, 335, 191, 179	0.7	Johnson et al. (2014)
Aa30	Dicaffeoyl ferulic acid derivative	69.65	243, 366	671.4020	−1.46	$C_{28}H_{22}O_{10}$	529, 179	1.3	Tentative

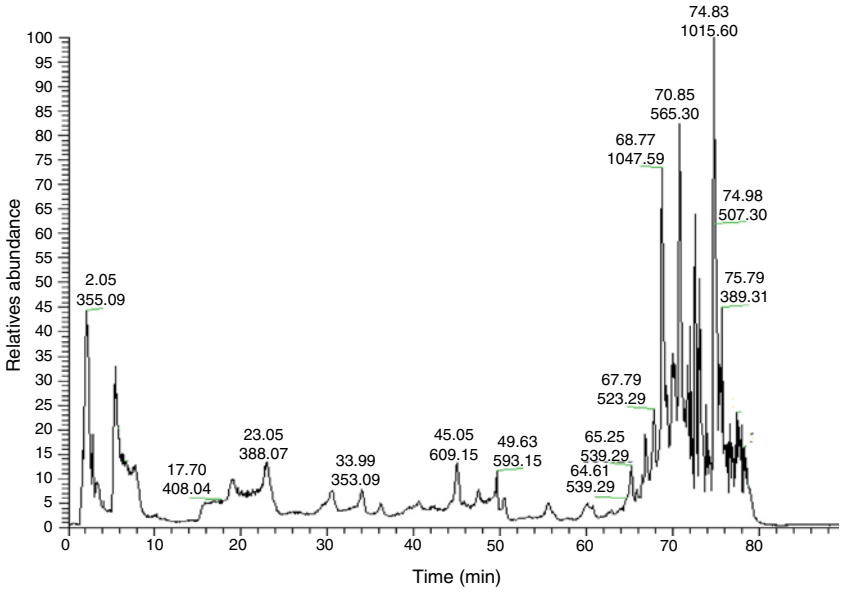

Fig. 1. Negative RP-HPLC/HRESI-PDA/QTOF-MS TI chromatogram of phenolics in Cl-AQ.

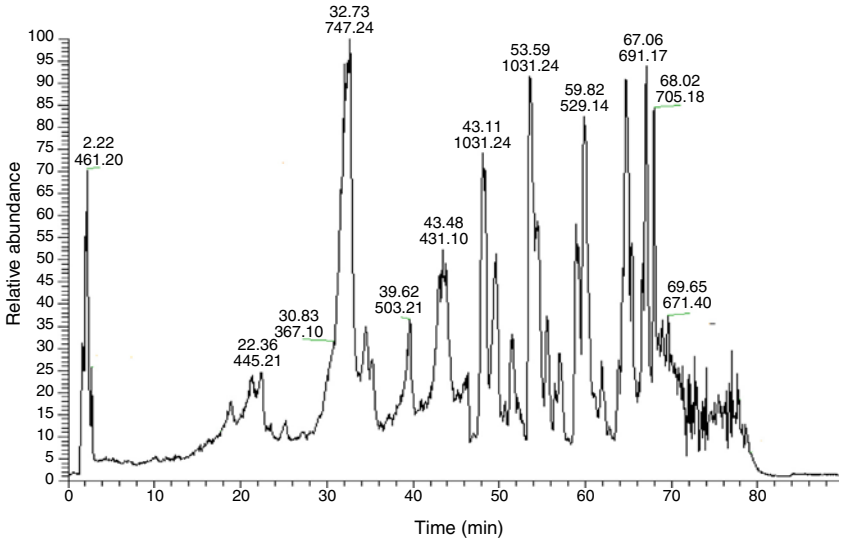

Fig. 2. Negative RP-HPLC/HRESI-PDA/QTOF-MS TI chromatogram of phenolics in ART-CQ.

at *m/z* 593.15 due to $[K+Rha+Glu-H]^-$. Its fragmentation pattern was consistent with that reported in Simirgiotis (2013) *i.e.* *m/z* 431 of $[K+Rha-H]^-$ and *m/z* 447 of $[K+Glu-H]^-$. In addition, Cd13 displayed (*iso*) rhamnetin-*O*-glucoside-*O*-rhamnoside, in which the deprotonated peak $[I+Glu+Rha-H]^-$ at *m/z* 623.16 and its fragmentation pattern showed molecular ions at *m/z*: 461 $[I+Rha-H]^-$, 477 $[I+Glu-H]^-$ and 313 [aglycone ion]$^-$ (Farag et al., 2016). Furthermore, Cd14 showed a $[M-H]^-$ at *m/z* 637.18 which produced MS^n ions at *m/z* 491 (loss of glucose) and *m/z* 329 (loss of rutinose). These were in accordance with the MS data reported for quercetin dimethyl ether with the typical fragments of quercetin *m/z* 179 and *m/z* 151 (Simirgiotis, 2013).

Artemsia annua *(ART-CQ)*

Phenolic acids

Several metabolites belonging to hydroxycinnamoyl quinic acids were detected in *A. annua* bioactive polar fraction, Aa1 showed the cinnamic-type UV spectrum, and a deprotonated anion $[M-H]^-$ at *m/z* 311.06, its main molecular ion peak at *m/z* 179 $[M-H-132]^-$, which is representing the deprotonated caffeic acid moiety (Fig. 2 and Table 4), thus it was identified as caftaric acid, which was previously described in *Vitis vinifera* (Handoussa et al., 2013). Aa2 has a $[M-H]^-$ at *m/z* 461.20, with fragments at *m/z* 281, 239, 179 and 137, which are characteristic of caffeic acid derivatives. In addition to, the presence of an intensively strong peak at *m/z* 191 which is indicative to quinic acid moiety (El Sayed et al., 2016).

Three caffeoyl-quinic acid isomers having the same $[M-H]^-$ at *m/z* 353.09 were detected. 3-caffeoyl-quinic acid was identified for peak Aa3 due to the presence of an intense base peak at *m/z* 191 and a strong peak (50% of base peak) at *m/z* 179. The 5-caffeoyl-quinic acid for peak Aa4 indicated a weak fragment at *m/z* 179, while peak Aa6 was identified as 4-caffeoyl-quinic acid due to a base peak at *m/z* 173 (Clifford et al., 2005). Furthermore, di-caffeoyl-quinic acid isomers were also recognized by their parent ion at *m/z* 515.12. Aa16 was identified as 4,5-di-caffeoyl-quinic acid, which was distinguished from its isomers by its pattern; of undetectable

peak at *m/z* 353 and the presence of a strong recognizable peak (≥50) at *m/z* 179. In addition, its dimer was observed as peak Aa15. Furthermore, Peak Aa18 exhibited another di-caffeoyl-quinic acid dimer, tentatively identified as 3,5-di-caffeoyl-quinic acid dimer, according to Clifford et al. (2005).

Several feruloylquinic acid isomers of $[M-H]^-$ at *m/z* 367.10 as in peak Aa8 that was identified as 5-feruloylquinic acid with a fragmentation pattern; *m/z* 191 for deprotonated quinic acid and *m/z* 172, which is typical for a substituted quinic acid at position number 5 (Lin and Harnly, 2010). It was not identified as isoferuloylquinic acid due to the absence of *m/z* 154 (Lin and Harnly, 2010). Similarly, Aa9 was identified as 3-feruloylquinic acid, owing to the fragmentation pattern; a 3-substituted position could be deduced from the intense peak of *m/z* 179 (Lin and Harnly, 2010). The presence of *m/z* 192 negated the possibility that this compound could be an isoferuloyl isomer.

Based on Gouveia and Castilho (2011), peak Aa12 was tentatively identified as a caffeoyl acid derivative with a deprotonated molecule $[M-H]^-$ at *m/z* 381.11 in the negative ion mode. The MS^n data showed neutral losses to arise peaks of CO_2 (*m/z* 337) and caffeic acid (*m/z* 201) from the deprotonated molecule of (*m/z* 381). The specific structure of the compound eluted at peak Aa12 could not be determined. However, these fragmentations were typically observed for caffeoyl acid derivatives. Peak Aa26 showed a deprotonated molecule at *m/z* 677.15 and its MS^n fragmentation showed three consecutive losses of caffeoyl moieties (162 a.m.u). This is consistent with these fragmentations reported for a 3,4,5-tricaffeoylquinic acid (Gouveia and Castilho, 2011).

Compound Aa10, with a molecular $[M-H]^-$ ion at *m/z* 535.17, proved to be caffeoyl coumaryl glucaric acid. While the MS/MS of *m/z* 535.17 produced the same fragment ions with peaks at *m/z* 197 and 163 characteristic of a coumaroyl moiety, the fragment ions at *m/z* 147, *m/z* 173 and *m/z* 209 indicated a glucaric moiety and *m/z* 179 a caffeoyl moiety. It was not possible to assign the binding position of the moieties, but they may be linked at the C-2, C-3 or C-4 positions of the glucaric acid (Lin and Harnly, 2010).

Flavonoids

Peak Aa5 showed a UV spectrum with maximum absorption at 267 and 335 nm, characteristic for the flavone apigenin. The mass spectrum in the negative ionization mode of this peak showed $[M-H]^-$ at *m/z* 445.21 and at *m/z* 269, corresponding to the loss of a glycuronyl unit; thus, it was identified as apigenin-7-β-*O*-glucuronide (Pereira et al., 2013). It was previously isolated from *A. annua* by Ferreira et al. (2010).

Several caffeoyl acid derivatives (Cd1, Cd2, Cd4, Cd5 and Cd11) were identified for the first time in *C. droserifolia* aerial parts (Table 3). This is the first report of a 3,5-dicaffeoylquinic acid dimer (Aa18), 4,5-dicaffeoylquinic acid (Aa16), 3,4,5-tricaffeoylquinic acid (Aa26), and 3-feruloylquinic acid (Aa9) in *A. annua* leaves (Table 4). Previous studies indicated caffeoylquinic acid derivatives 3,5-di-*O*-caffeoylquinic acid 1-methyl ether and 4,5-di-*O*-caffeoylquinic acid 1-methyl ether, in addition to the well-known hepatoprotective compound 1,5-di-*O*-caffeoylquinic acid; these were isolated from the hepatoprotective fraction of *Inula crithmoides* roots. These compounds significantly decreased the level of four serum biochemical parameters *in vivo* (AST, ALT, ALP, and bilirubin) (Aboul Ela et al., 2012). Both Cl-AQ and ART-CQ are rich in hydroxycinnamoyl quinic acids, which are protective against liver toxicity. The tested extract FEN-SaP was shown to be rich in alkaloids and steroidal saponins, while RO-MC contained phenolic diterpenes, and Lin-LRF had significant lignans; however, these exhibited poor hepatoprotective activities. Thus, our results provide evidence of the hepatoprotective activity of caffeoyl- and feruloylquinic acid derivatives.

Conclusion

The two extracts Cl-AQ and ART-CQ effectively prevented CCl_4-induced acute hepatotoxicity *in vitro*, which proved their potential to ameliorate radical-induced toxicity. An HPLC/PDA/ESI/MS–MS based analysis of the extracts revealed that both Cl-AQ and ART-CQ were rich in flavonoid glycosides, caffeoyl- and feruloylquinic acid derivatives, while the other tested extracts which contained alkaloids, steroidal saponins, phenolic diterpenes and lignans showed little activity. Thus, our study provided further evidence of the hepatoprotective activity of hydroxycinnamoyl quinic acid derivatives and flavonoids.

Authors' contributions

HE and AAM contributed in collecting plant samples and extract preparation. AHE and MWL performed qualitative chemical profiling of the phytoconstituents. HH and AA carried out the data analysis. FB carried out the *in vitro* assays. HE and AAM designed the study and contributed to the critical reading of the manuscript. All authors read and approved the final manuscript.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2018.10.001.

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