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Original article

Hyphenated LC-ABTS$^{\cdot+}$ and LC-DAD-HRMS for simultaneous analysis and identification of antioxidant compounds in *Astragalus emarginatus* Labill. extracts

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ABSTRACT

The compounds in leaf and stem extracts of *Astragalus emarginatus* Labill. (AEL), a plant species used in traditional Lebanese medicine, were investigated for antioxidant properties. First, the activity of various extracts was assessed using the Trolox equivalent antioxidant capacity, oxygen radical absorption capacity, and 2,2-diphenyl-1-picryl-hydrazyl-hydrate assays. The extract obtained using 30% ethanol showed the greatest activity. The antioxidant compounds in this extract were screened using a hyphenated high-performance liquid chromatography-2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate) radical (ABTS$^{\cdot+}$) system before being separated by ultra-high-performance liquid chromatography and identified using high-resolution mass spectrometry and ultra-violet-visible diode array detection. Approximately 40 compounds were identified. Hydroxycinnamates (caffeic, ferulic, and *p*-coumaric acid derivatives) and flavonoids (quercetin, luteolin, apigenin, and isorhamnetin derivatives) were the two main categories of the identified compounds. The active compounds were identified as caffeic acid derivatives and quercetin glycosides. In addition, the catechol moiety was shown to be key to antioxidant activity. This study showed that AEL is a source of natural antioxidants, which may explain its medicinal use.

1. Introduction

Oxidative stress is a major player in the genesis and development of age-related diseases, such as cancer as well as cardiovascular and neurodegenerative disorders. Oxidative stress occurs when excessive build-up of reactive oxygen species such as hydroxyl radicals, hydrogen peroxide, singlet oxygen ($^1O_2$), and superoxide ($O_2^{\cdot-}$) in the human body results in the damage to cell constituents, such as proteins, lipids, and DNA [1,2]. Supplementation of the endogenous antioxidant system by exogenous antioxidant compounds tends to limit and overcome this process. Plants generally synthesize a variety of compounds with antioxidant properties such as flavonoids, phenolic acids, alkaloids, terpenoids, and carotenoids as a part of their defense system against environmental stress. Therefore, plants are a source of natural antioxidants that can potentially be used to combat oxidative stress in humans [3,4].

With approximately 2000 to 3000 documented species, *Astragalus* is the largest genus of the angiosperm group and a member of the legume family (Fabaceae). It is mostly found in regions with temperate and arid climates in Asia, Europe, and North America [5,6]. *Astragalus* species are annual, perennial, stemmed herbs, and shrubs [7]. *Astragalus* root (also called *Astragali Radix* or Huang Qi in Chinese) has been used in traditional medicines, particularly Chinese medicine, for thousands of years, and it is still used today to treat diseases and conditions, such as inflammation, immune system disorders, tumors, diabetes, hypertension, leukemia, and gastric ulcers [8,9]. *Astragalus* extracts also exhibit antioxidant, antibacterial, and antifungal activities [10]. Owing to this, two *Astragalus* species, namely, *Astragalus mongholicus* and *Astragalus membranaceus*, have been included in the Chinese Pharmacopeia

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and five other major pharmacopeias [11]. Flavonoids, polysaccharides, and saponins have been shown to be the main active ingredients of *Astragalus* species [12–14].

As in other parts of the world, *Astragalus* species are used in Lebanon to treat a number of diseases. Arnold et al. [15] described the use of four species as follows: *Astragalus hermoneus* Boiss. is used as an astringent, whereas the roots of *Astragalus coluteoides* Will., *Astragalus oleaefolius* DC., and *Astragalus cruentiflorus* Boiss. are used to prepare oral decoctions for the treatment of diabetes and jaundice. Nonetheless, with a few exceptions, such as the study by Kanaan et al. [16], which reported the antibacterial and antibiofilm activities of *Astragalus angulosus*, our knowledge of the therapeutic uses of *Astragalus* species in Lebanese medicine is limited to ethnobotanical reports [17].

In the present study, we investigated the antioxidant activity of *Astragalus emarginatus* Labill. (AEL), which is one of the 56 species of *Astragalus* endemic to Lebanon and Syria as listed by Tohmé and Tohmé [18] and is widely used in traditional medicine. High-performance liquid chromatography (HPLC) coupled with an online post-column antioxidant system using the 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate) radical ($ABTS^{\cdot+}$) assay was employed in these studies. The isolated compounds with antioxidant properties as well as other compounds were subsequently identified by ultra-performance liquid chromatography coupled with diode array detection and high-resolution mass spectrometry (UHPLC-DAD-HRMS).

2. Materials and methods

2.1. Collection and preparation of plant dry material

The aerial parts (leaves and stems) of AEL were collected from the Shouf Biosphere Reserve in the Spring of 2018. Botanical authentication was performed by the National Council for Scientific Research in Lebanon. After being washed with distilled water, plant samples were air-dried in a shaded location at 25 °C and then ground using an Ultra Centrifugal ZM 200 mill (Retsch, Eragny-sur-Oise, France) set at 6,000 r/min and fitted with a 1 mm sieve. The resulting powder was stored in sealed plastic bags in a dry environment.

2.2. Chemicals and reagents

Caftaric acid, 2,2-azino-bis-3-ethylbenzthioazolin-6-sulfonic acid, Trolox, butylated hydroxytoluene, 2,2-diphenyl-1-picrylhydrazyl, fluorescein, 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH), disodium phosphate, and sodium chloride were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Potassium chloride and potassium persulfate were purchased from VWR (Fontenay-sous-Bois, France), and potassium dihydrogen phosphate was purchased from Merck (Darmstadt, Germany). Isoquercitrin, rutin, luteolin-7-*O*-glucoside, isorhamnetin-3-*O*-rutinoside, and apigenin-7-*O*-glucoside were purchased from Extrasynthese (Genay, France).

Ultrapure water (18.2 MΩ·cm) was used for extraction and chromatography and was prepared using a Millipore Synergy system (Molsheim, France). Hydrochloric acid, acetonitrile (ACN), ethyl acetate, and ethanol (EtOH) were purchased from Fisher Scientific (Illkirch-Graffenstaden, France). Methanol (MeOH) and chloroform were obtained from Carlo Erba (Val-de-Reuil, France). Formic acid was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

2.3. Preparation of reagents and stock solutions

A stock solution (1000 μM) of Trolox was prepared in ACN:H_2O (50:50, *V/V*). Serial dilutions in the range of 5-1000 μM were made for use during the construction of calibration curves for the oxygen radical absorption capacity (ORAC) and the Trolox equivalent antioxidant capacity (TEAC) assays. Phosphate buffer saline (PBS, 1×) was prepared by mixing sodium chloride (137 mM), disodium phosphate (10 mM), potassium phosphate (1.76 mM), and potassium chloride (2.7 mM). A stock solution of the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) cation radical ($ABTS^{\cdot+}$) was prepared by mixing 7 mM ABTS and 2.5 mM potassium persulfate in water. It was then kept in the dark at 4 °C for at least 12 h to allow for the generation of $ABTS^{\cdot+}$. Prior to use, this stock solution was diluted in PBS (1×) to obtain a final working concentration of 0.14 mM ABTS.

2.4. Preparation of AEL extracts

Six solvents covering a range of polarity indices (water (*P'*=10.2), acidified water at pH 3 (*P'*=10.2), 70% MeOH (*P'*=6.58), 70% EtOH (*P'*=6.07), ethyl acetate (*P'*=4.4), and chloroform (*P'*=4.1)) were used to obtain AEL extracts. One gram of the plant powder was individually mixed with 15 mL of the extraction solvent and sonicated for 30 min at 25 °C in a Fisherbrand 15051 ultrasonic bath (Fisher Scientific, Loughborough, UK; 37 kHz, 280 W, ultrasonic peak max. 320 W, standard sine wave modulation). The mixture was then filtered through a 125 mm filter paper using a Buchner vacuum filtration system (Dutscher, Brumath, France). After extraction with 70% MeOH and 70% EtOH, the organic solvents were removed using a VV 2000 rotary evaporator (Heidolph Instruments GmbH, Schwabach, Germany) and the residual water was dried using a Free Zone 4.5 freeze-dryer (Labconco, Kansas City, MO, USA) to yield dry extracts. In the extraction procedures that used ethyl acetate and chloroform, dry extracts were obtained after complete removal of solvents in a rotary evaporator. For the water and acidified water extractions, the filtrates were directly freeze-dried to obtain dry extracts. All dry extracts were stored at −20 °C until use. Stock solutions (2.0 mg/mL) were prepared from dry extracts using H_2O, ACN:H_2O (50:50, *V/V*), and ACN as solvents for water and acidified water extracts, 70% MeOH and 70% EtOH extracts, and chloroform and ethyl acetate extracts, respectively. Dilutions for the antioxidant activity tests were carried out using the corresponding solvents.

To optimize the extraction conditions, different concentrations of EtOH (10%, 30%, 50%, and 70%) and different extraction time (5, 15, 30, and 60 min) were used to investigate the effect of these parameters on the extraction efficiency of compounds from AEL. Following ultrasonic extraction, the filtered extracts (200 μL) were assessed for their antioxidant activity using the HPLC-$ABTS^{\cdot+}$ assay, as described in Section 2.8. For the complete extraction of compounds from AEL, successive extractions were carried out using 30% EtOH at a sonication time of 30 min. Successive extracts were pooled, dried, and stored as previously described. All experiments were performed in triplicate.

2.5. TEAC assay

The TEAC assay was used to assess the antioxidant capacity of the AEL extracts in 96-well microplates as described by Re et al. [19] with slight modifications. Ten microliters of each sample solution (blank (PBS), Trolox, and extracts) was deposited in the wells in triplicate. After adding 200 μL of the $ABTS^{\cdot+}$ solution (7 mM), the mixtures were incubated at 37 °C for 10 min, and their absorbance

values were determined at 734 nm using a Varioskan plate reader (Thermo Fisher Scientific, Waltham, MA, USA).

A calibration curve was constructed using Trolox over a concentration range of 10–800 μM. The antioxidant capacity of each AEL extract was expressed in μmol of Trolox equivalent (TE) per gram of the dry extract (μmol TE/g dry extract). The experiment was independently repeated three times.

2.6. ORAC assay

The ORAC assay was used to assess the antioxidant capacity of the AEL extracts in 96-well dark microplates as described by Ou et al. [20]. Fluorescein (150 μL) in PBS (1×) (2.8×10^{-5} mg/mL) was mixed in wells with 10 μL of each sample solution (blank (PBS), Trolox, and extracts) in triplicate. After 10 min of incubation at 37 °C, the reaction was started by the automated addition of 30 μL of a AAPH solution (41.5 mg/mL in PBS (1×)). The intensity of fluorescence at 518 nm was measured using a Varioskan plate reader every minute for 2 h at an excitation wavelength of 485 nm. The decay in fluorescence as a function of time was represented by descending curves, and the area under the curve (AUC) for each sample, including that of the blank, was calculated. The net AUC was then calculated by subtracting the AUC of the sample from that of the blank. As in the TEAC assay, Trolox was used as a reference compound, and its calibration curve was constructed (5–1000 μM). The antioxidant capacity of each extract was expressed as μmol TE/g dry extract. The experiment was independently repeated three times.

2.7. Radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, as described by Hassan et al. [21], was performed to measure the radical scavenging activity of the 30% EtOH AEL extract. Briefly, 1.0 mL of the extract at different concentrations (25–400 μg/mL) was added to 1.0 mL of the freshly prepared DPPH solution (0.15 mM in MeOH). The mixtures were briefly vortexed and incubated for 30 min in the dark at 25 °C. The absorbance at 517 nm, monitored using a Hitachi U-2900 double-beam spectrophotometer (Hitachi Corporation, Tokyo, Japan), was determined. Butylated hydroxytoluene (BHT) was used as the positive control. The DPPH radical scavenging activity (RSA) was expressed as percentage inhibition, calculated using the following equation:

$$\mathrm{RSA}(\%) = \left[\left(A_{\mathrm{control}} - A_{\mathrm{sample}}\right)/A_{\mathrm{control}}\right] \times 100$$

where A_{control} and A_{sample} represent the absorbance of the reaction mixture at 517 nm in the absence and presence of a test sample, respectively. The sample concentration yielding 50% inhibition (IC_{50}) was calculated based on the curve of RSA versus sample concentration. All experiments were performed in triplicate.

2.8. Online HPLC-ABTS$^{\cdot+}$ assay

The screening of antioxidants present in the 30% EtOH AEL extract was performed using an HPLC-ABTS$^{\cdot+}$ online system according to Leitao et al. [22]. Chromatographic separation was achieved at 25 °C using a C_{18}-PFP analytical column (3 μm, 250 mm × 4.6 mm; Advanced Chromatography Technologies Ltd., Aberdeen, Scotland). The mobile phase consisted of H_2O (A) and ACN (B), both containing 0.1% formic acid, and was delivered at 1 mL/min as follows: 2%–10% B (0–8 min), 10%–10% B (8–15 min), and 10%–45% B (15–29 min). 20 mL of sample solution was injected, and UV detection was carried out at 325 nm. The ABTS$^{\cdot+}$ solution was delivered via a second pump at 0.5 mL/min and mixed with the eluent from the analytical column. The mixture passed through the reaction coil and then through a second detector, where the discoloration was monitored at 412 nm. Compounds with antioxidant activity displayed negative peaks. The sum of the negative peak areas was used to evaluate the overall activity of the AEL extracts.

2.9. UHPLC-DAD-HRMS

The separation and identification of antioxidants and other components of the 30% EtOH AEL extract were performed using an Acquity UHPLC system (Waters, Guyancourt, France) connected to a Bruker micrOTOF-Q II mass spectrometer (Bruker, Karlsruhe, Germany) with an electrospray ionization source (ESI). For chromatographic separation, a sample volume of 5 μL was injected through a C_{18}-PFP column (1.7 μm, 100 mm × 2.1 mm, 130 Å; Advanced Chromatography Technologies Ltd., Aberdeen, Scotland). The mobile phase consisted of H_2O containing 0.1% formic acid (A) and ACN containing 0.05% formic acid (B) and was delivered at 0.3 mL/min as follows: 5%–12% B (0–10 min), 12%–20% B (10–11 min), 12%–25% B (11–16 min), and 25%–25% B (16–19 min).

Mass spectrometry (MS) was carried out in negative scan mode from *m/z* 100–1000. The ESI settings were as follows: capillary tension, 4500 V; end plate offset, 500 V; drying gas (N_2) flow rate, 9.0 L/min; drying gas temperature, 200 °C; nebulizer pressure, 280 kPa. For MS/MS experiments, nitrogen was used at collision cell energy of 7.0 eV and collision radio-frequency of 200 Vpp. Two collision energies, 10 and 50 V, were used for the compounds eluting in the range of 0–14 min and 14–28 min, respectively. The operating software was micrOTOF 3.0, combined with Hystar 3.2 and the data analysis software was Compass Data Analysis 4.0 (Bruker, Karlsruhe, Germany). Mass spectrometer calibration was performed over a mass range of *m/z* 100–1000 using a sodium formate solution.

3. Results and discussion

3.1. Selection of the extraction solvent

The efficiency of six solvents (water, acidified water, 70% MeOH, 70% EtOH, chloroform, and ethyl acetate) to extract antioxidants from AEL was assessed using the ORAC and TEAC assays. In the ORAC assay, the decrease in fluorescence from the damage to fluorescein by peroxyl radicals produced by AAPH was monitored. This translates the RSA of an antioxidant via hydrogen atom transfer. The presence of antioxidants protects fluorescein from oxidative damage, resulting in an extended fluorescence time [23]. On the other hand, the TEAC assay is based on electron transfer and measures the ability of an antioxidant to scavenge ABTS$^{\cdot+}$ radicals, which leads to a decrease in color intensity [23]. The obtained data showed that the observed antioxidant activity of the AEL extracts was highly dependent on the polarity of the extraction solvent (Fig. 1). The antioxidant activity of extracts obtained using 70% MeOH and 70% EtOH was approximately 1.4 and 5 times higher than that of those obtained using water and acidified water, and chloroform and ethyl acetate, respectively. Consistent results were obtained using both the ORAC and TEAC assays.

The antioxidant activity observed for AEL extracts is not surprising, because such activity has also been reported in other *Astragalus* species in previous studies [24–26]. Our data indicated that the antioxidant activity of AEL could mainly be attributed to

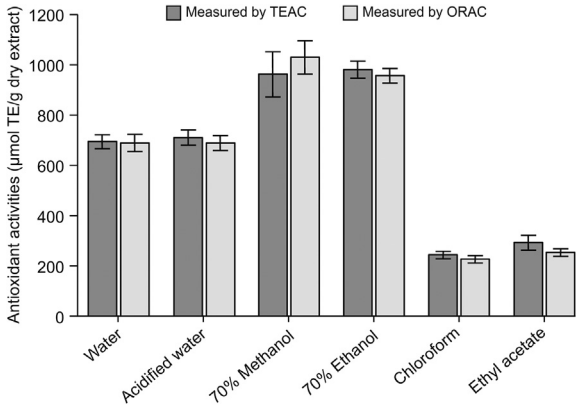

Fig. 1. Antioxidant activities of extracts from *Astragalus emarginatus* Labill. (AEL) obtained using various solvents. Activity was measured by Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorption capacity (ORAC) assays and is expressed as μmoles of Trolox equivalents (TE) per gram of extract.

polar compounds. EtOH/water and MeOH/water mixtures showed similar efficiencies in extracting antioxidant molecules from AEL. Therefore, the hydroethanolic mixture was selected for further investigation of the antioxidant potential of AEL extracts.

3.2. Optimization of extraction conditions

During the initial extraction trials, the use of 70% EtOH for 30 min was selected as the extraction condition as it provided the highest yield recovery of antioxidant compounds. Subsequently, extraction using EtOH was optimized by investigating various proportions of EtOH in water and extraction time (sonication intervals). Fig. 2 shows the obtained activity data of the extracts, expressed as the sum of the negative peak areas from the online HPLC-ABTS$^{\cdot+}$ assay as a function of the extraction time. Extraction using 30% EtOH yielded the highest activity value, whereas the lowest value was obtained when 70% EtOH was used. This indicated that among the tested solvents, 30% EtOH was the most suitable for the extraction of antioxidants from AEL. In addition, extraction durations beyond 30 min did not yield significantly higher antioxidant activity. Consequently, extraction with 30% EtOH for 30 min was selected as the optimal condition.

Fig. 3 shows the total antioxidant activity of AEL extracts as a function of the number of successive extractions using 30% EtOH for 30 min. Approximately 85% of the total antioxidants (represented by the measured activity) were recovered following the first

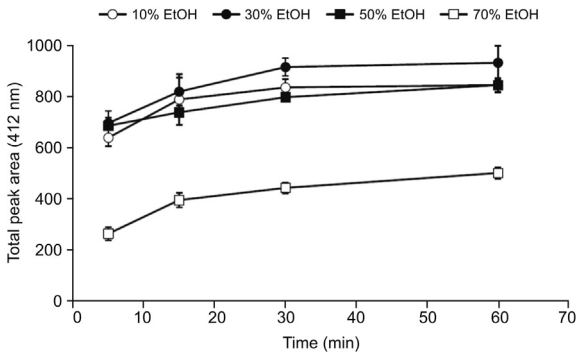

Fig. 2. Extraction of antioxidants from AEL as a function of time using different concentrations of ethanol (EtOH) in water: 10% EtOH, 30% EtOH, 50% EtOH, and 70% EtOH. The presence of antioxidants was assessed using the HPLC-ABTS$^{\cdot+}$ assay as the sum of peak areas at 412 nm. ABTS$^{\cdot+}$: 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate) radical.

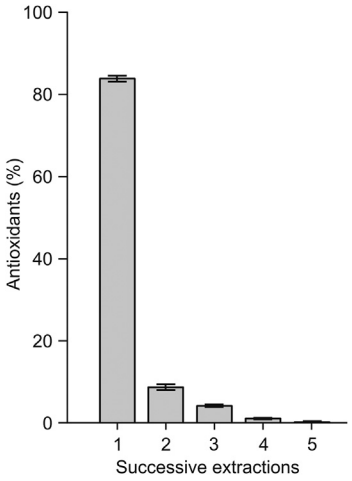

Fig. 3. Percentage of total antioxidants extracted from AEL as a function of the number of successive extractions using 30% EtOH for 30 min. The presence of antioxidants was assessed using the HPLC-ABTS$^{\cdot+}$ assay as the sum of peak areas at 412 nm.

extraction, and two additional extractions were needed to increase the recovery to 98%. This indicates that three extractions were sufficient to recover nearly all antioxidants from AEL under the selected conditions.

3.3. Antioxidant activity measured by DPPH assay

The DPPH assay, a well-established method for the assessment of free RSA through hydrogen transfer [27], was applied to AEL extracts. The assay was performed using BHT as a positive control and the results were expressed as IC_{50} values, with lower values indicating higher antioxidant activity. It was shown that the AEL extracts exhibited significant RSA with an IC_{50} of 102 ± 4.4 μg/mL, as calculated from data in Table 1. The IC_{50} of BHT was determined to be 18.5 ± 2.0 μg/mL. Adigüzel et al. [24] used the same assay to evaluate the antiradical activity of MeOH extracts from the aerial parts of 13 *Astragalus* species. The obtained IC_{50} values ranged between 68.8 and 400.4 μg/mL with an IC_{50} of 19.8 μg/mL for BHT as the positive control. In comparison, the 30% EtOH extract from aerial parts of AEL showed a higher antiradical activity than the MeOH extracts of most investigated species of *Astragalus*.

3.4. Separation and identification of antioxidants and other isolated compounds

HPLC-ABTS$^{\cdot+}$, a hyphenated system consisting of an HPLC and a post-column ABTS$^{\cdot+}$ assay, was used to identify the antioxidant molecules present in the 30% EtOH AEL extract. The individually separated antioxidant compounds reacted with ABTS$^{\cdot+}$ radicals and appeared as upside-down or negative peaks at 412 nm following the discoloration of the radical solution. Two chromatograms were obtained: a regular chromatogram of all analytes that absorbed UV at 325 nm and a radical scavenging chromatogram at 412 nm (Fig. 4A). It can be noted that the majority of the peaks observed at 325 nm gave rise to corresponding negative peaks, which means that the detected compounds had antioxidant activity. Among them, five major negative peaks were observed (peaks Nos. 2, 13+14, 24, 35, and 36 with respective retention time of 10.0, 14.8, 21.3, 24.1, and 25.1 min) representing about 73% of the total negative peak area.

Examination of the UV spectra of all chromatographic peaks revealed that the detected molecules were mainly phenolic compounds, either hydroxycinnamic acid derivatives (HCAD) eluted

Table 1
Radical scavenging activity of the 30% EtOH extract of *Astragalus emarginatus* Labill. (AEL) as determined by the 2,2-diphenyl-1-picrylhydrazyl assay.

Concentration (μg/mL)	Radical scavenging activity (%)
25	10 ± 0.3
50	22 ± 1.6
100	46 ± 0.2
200	75 ± 0.8
400	95 ± 3.4

before 24 min (peaks Nos. 1–34) showing specific UV absorption from 310 to 328 nm and ca. 300 nm or flavonoids eluted after 24 min with typical UV absorption from 328 to 355 nm and ca. 265 nm (peaks Nos. 35–43) (Fig. 4). In addition, the negative peak areas corresponding to HCAD accounted for 80% of the total area, while flavonoids accounted for only 20%. These results showed that the antioxidant activity of the 30% EtOH AEL extract was mainly from HCAD. This contradicts a report by Pistelli et al. [28], who designated flavonoids as the main antioxidants in the aerial parts of *Astragalus* species. Nonetheless, there has been much focus on comparing the activity of flavonoids to that of HCAD [29–31].

To identify the antioxidants and other compounds extracted from AEL, the 30% EtOH extract was subjected to UHPLC-DAD-HRMS. Molecular identification was achieved based on comparing UV, MS, and MS/MS spectra with databases in literature, and if possible, with data obtained from analyzing available authentic standards [32]. As shown in Fig. 5, the obtained UV (325 nm) chromatographic profile was similar to that observed from the HPLC-ABTS$^{\cdot+}$ assay (Fig. 4), although the retention time and retention time intervals were different. As a result, the peaks observed from the HPLC-ABTS$^{\cdot+}$ assay corresponding to the antioxidant compounds were easily identified by UHPLC-DAD-HRMS chromatography.

Compounds corresponding to peaks 1–5, 7–9, 13, 15, 16, and 18 showed UV absorption spectra with characteristics of two hydroxycinnamic acids: caffeic or ferulic acid (λ_{max} 325–328 and ca. 295–300 nm). Their mass spectra exhibited a common

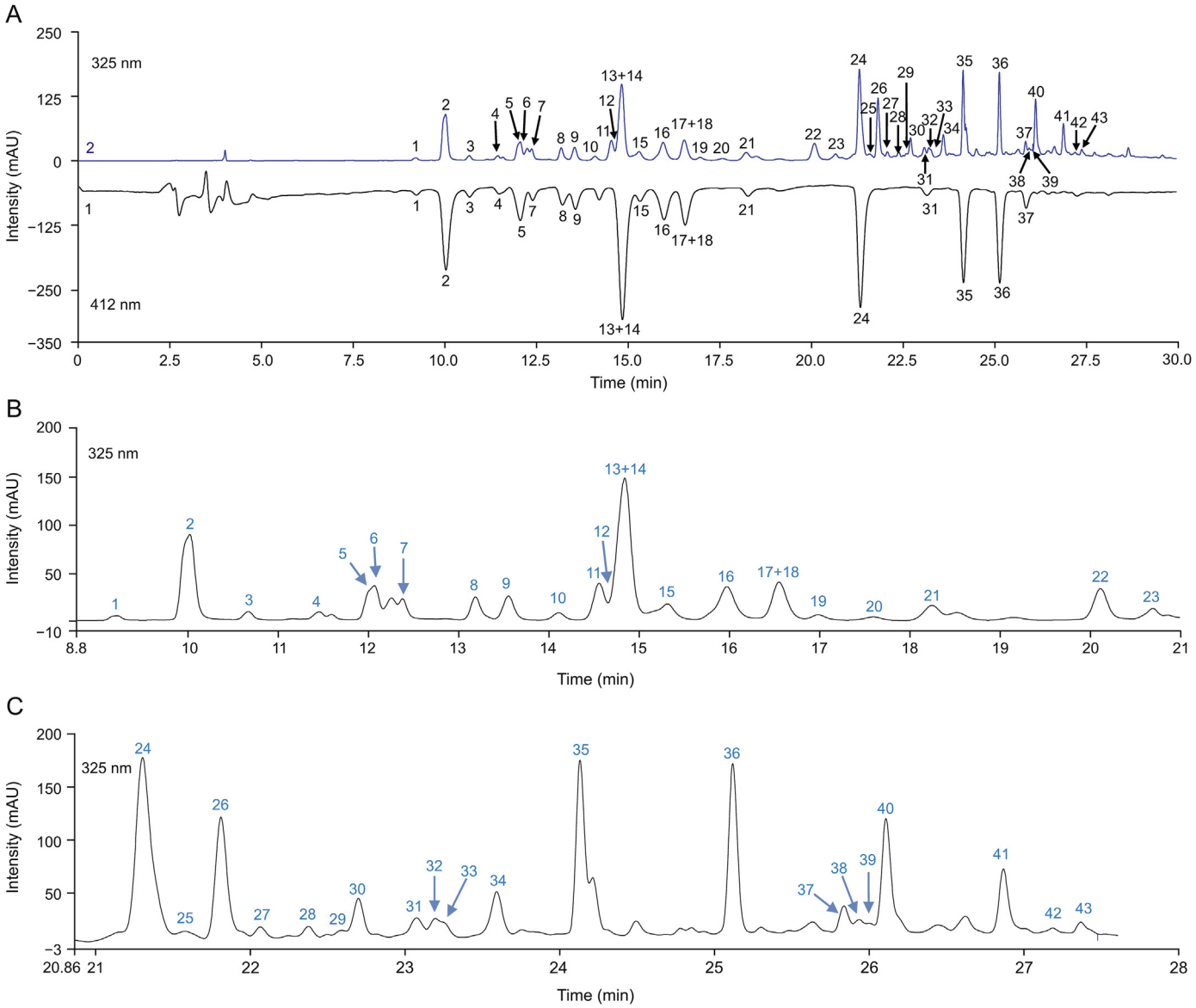

Fig. 4. UV (upper line) and antioxidant (under line) chromatograms of AEL extract obtained using (A) 30% EtOH and (B and C) the zoomed views of the UV chromatogram.

pseudomolecular ion $[M-H]^-$ at *m/z* 371. The HRMS analysis of each ion proposed the same molecular formula as $C_{15}H_{15}O_{11}$. The main product ion at *m/z* 371 was *m/z* 209 ($C_6H_9O_8$), which originated from the cleavage of a caffeoyl moiety ($C_9H_6O_3$, 162 Da) and corresponded to hexaric acid [33]. Since hexaric acid has four OH groups in its structure, it can form different diastereoisomeric caffeoyl esters [34]. Compounds corresponding to peaks 1–5, 7–9, 13, 15, 16, and 18 were tentatively identified as caffeoylhexaric acid isomers. Table 2 summarizes the UV and mass spectra characteristics of the main components in the 30% EtOH AEL extract, as well as their identities. The corresponding chemical structures are depicted in Fig. 6.

Compounds corresponding to peaks 6, 10, 12, 19, and 22 showed UV spectra similar to those of *p*-coumarate (λ_{max} 310–315 nm) [35], with an $[M-H]^-$ ion at *m/z* 355 ($C_{15}H_{15}O_{10}$) and MS/MS fragments at *m/z* 209 (hexaric acid) [M–H-*p*-coumaroyl]$^-$ and *m/z* 191 [M–H-*p*-coumaroyl-H_2O]$^-$. These compounds were tentatively identified as *p*-coumaroylhexaric acid isomers (Table 2 and Fig. 6). Other less intense peaks at *m/z* 355 were also detected, and were consistent with previously published data in which eight different isomers of *p*-coumaroylhexaric acid were identified in *Eupatorium cannabinum* subsp. *cannabinum*, *Leonurus japonicus*, and *Leonurus cardiac* [36].

Compounds corresponding to peaks 11, 20, and 26 showed UV spectra with λ_{max} values from 323 to 328 and ca. 300 nm, and a pseudomolecular ion $[M-H]^-$ at *m/z* 385 ($C_{16}H_{17}O_{11}$). The observation of product ions at *m/z* 209 and 191 indicated the presence of hexaric acid in the compounds, and the loss of 176 Da (385–209=176 Da) corresponded to a feruoyl moiety. Based on these elements and the fact that caffeic and ferulic acids have similar UV spectra [37], these compounds were tentatively identified as feruloylhexaric acid isomers.

Compounds corresponding to peaks 14 and 24 showed the same UV spectrum (λ_{max} ca. 240–244, ca. 300, and 327–328 nm), the same $[M-H]^-$ (*m/z* 311 ($C_{13}H_{11}O_9$)), and the same product ions (*m/z* 179 and 149) as those of the standard compound caffeoyltartaric acid (caftaric acid). Based on retention time, peak 14 was conclusively identified as caftaric acid (retention time=5.48 min), and peak 24 (retention time=8.06 min) was tentatively identified as an isomer of caftaric acid [38] (Table 2 and Fig. 6).

Compounds corresponding to peaks 17 and 21 exhibited an $[M-H]^-$ ion at *m/z* 385 ($C_{16}H_{17}O_{11}$), and UV spectra with λ_{max} values from 326 to 328 and ca. 300 nm. Although these data were the same as those observed for compounds 11, 20, and 26, the MS/MS fragmentation behavior was different. A basic fragment ion at *m/z*

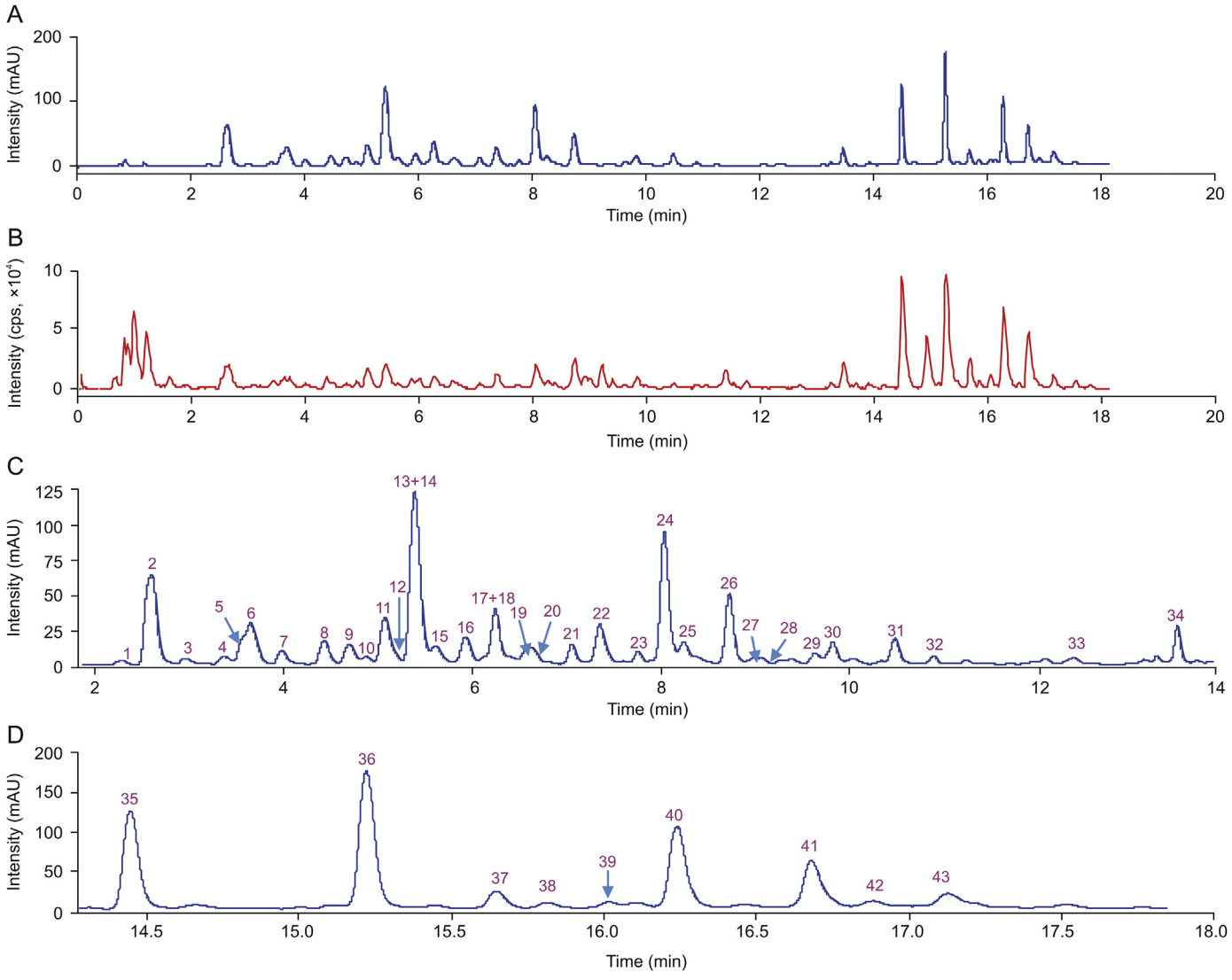

Fig. 5. (A) UV (325 nm) and (B) mass spectrometry chromatograms of the 30% EtOH extract of AEL and (C and D) the zoomed views of the UV chromatogram.

Table 2
Identification of the compounds present in the 30% EtOH extract of AEL by ultra-performance liquid chromatography coupled with diode array detection and high-resolution mass spectrometry. Peak numbers are shown in Figs. 5 and 6.

Peak No.	Retention time (min)	λ (nm)	Formula	Mass (*m/z*)		Error (ppm)	Mass of fragments (*m/z*)	Identified compound
				Theoretical value	Experimental value			
1[a]	2.33	295, 325	$C_{15}H_{15}O_{11}$	371.0620	371.0606	3.8	209.0300	Caffeoylhexaric acid isomer
2[a]	2.65	240, 300, 325	$C_{15}H_{15}O_{11}$	371.0620	371.0608	3.2	209.0295	Caffeoylhexaric acid isomer
3[a]	3,00	295, 325	$C_{15}H_{15}O_{11}$	371.0620	371.0603	4.4	209.0277	Caffeoylhexaric acid isomer
4[a]	3.35	300, 324	$C_{15}H_{15}O_{11}$	371.0620	371.0610	2.6	209.0303	Caffeoylhexaric acid isomer
5[a]	3.64	300, 324	$C_{15}H_{15}O_{11}$	371.0620	371.0615	1.3	209.0303	Caffeoylhexaric acid isomer
6	3.74	313	$C_{15}H_{15}O_{10}$	355.0671	355.0661	2.7	209.0294, 191.0209	*p*-coumaroylhexaric acid isomer
7[a]	4.03	300, 325	$C_{15}H_{15}O_{11}$	371.0620	371.0615	1.3	209.0290	Caffeoylhexaric acid isomer
8[a]	4.48	245, 300, 327	$C_{15}H_{15}O_{11}$	371.0620	371.0610	2.7	209.0291	Caffeoylhexaric acid isomer
9[a]	4.73	245, 302, 327	$C_{15}H_{15}O_{11}$	371.0620	371.0612	2.0	209.0289	Caffeoylhexaric acid isomer
10	4.91	310	$C_{15}H_{15}O_{10}$	355.0671	355.0666	1.2	209.0285, 191.0248	*p*-coumaroylhexaric acid isomer
11	5.11	300, 324	$C_{16}H_{17}O_{11}$	385.0776	385.0764	3.3	209.0308, 191.0211, 147.0297	Feruloylhexaric acid isomer
12	5.19	315	$C_{15}H_{15}O_{10}$	355.0671	355.0672	−0.3	209.0322, 191.0179	*p*-coumaroylhexaric acid isomer
13[a]	5.42	242, 300, 328	$C_{15}H_{15}O_{11}$	371.0620	371.0616	0.9	209.0298	Caffeoylhexaric acid isomer
14[a]	5.48	240, 300, 327	$C_{13}H_{11}O_{9}$	311.0409	311.0399	3.2	179.0340, 149.0085	Caftaric acid[b]
15[a]	5.62	300, 325	$C_{15}H_{15}O_{11}$	371.0620	371.0603	4.4	209.0281	Caffeoylhexaric acid isomer
16[a]	5.96	295, 327	$C_{15}H_{15}O_{11}$	371.0620	371.0617	0.8	209.0283	Caffeoylhexaric acid isomer
17[a]	6.27	240, 300, 328	$C_{16}H_{17}O_{11}$	385.0776	385.0770	1.6	223.0447	Caffeoylhexaric acid derivative
18[a]	6.30	245, 300, 327	$C_{15}H_{15}O_{11}$	371.0620	371.0614	1.5	209.0313	Caffeoylhexaric acid isomer
19	6.60	313	$C_{15}H_{15}O_{10}$	355.0671	355.0667	1.1	209.0326, 191.0159	*p*-coumaroylhexaric acid isomer
20	6.67	300, 323	$C_{16}H_{17}O_{11}$	385.0776	385.0768	2.1	209.0299, 191.0197	Feruloylhexaric acid isomer
21[a]	7.08	300, 326	$C_{16}H_{17}O_{11}$	385.0776	385.0764	3.3	223.0462	Caffeoylhexaric acid derivative
22	7.37	313	$C_{15}H_{15}O_{10}$	355.0671	355.0670	0.2	209.0300, 191.0194	*p*-coumaroylhexaric acid isomer
23	7.42	310	$C_{13}H_{11}O_{8}$	295.0459	295.0458	0.1	163.0391, 149.0322	Coutaric acid isomer
24[a]	8.06	244, 300, 328	$C_{13}H_{11}O_{9}$	311.0409	311.0408	0.2	179.0350, 149.0085	Caftaric acid isomer
25	8.26	314	$C_{16}H_{17}O_{10}$	369.0822	369.0816	3.1	223.0456, 205.0354, 129.0183	*p*-coumaroylhexaric acid derivative
26	8.74	300, 328	$C_{16}H_{17}O_{11}$	385.0776	385.0772	1.0	209.0278, 191.0191, 147.0281	Feruloylhexaric acid isomer
27	8.96	325	$C_{14}H_{13}O_{9}$	325.0560	325.0563	0.5	193.0486	Fertaric acid isomer
28	9.08	310	$C_{16}H_{17}O_{10}$	369.0822	369.0822	1.5	223.0464, 205.0379, 129.0189	*p*-coumaroylhexaric acid derivative
29	9.65	312	$C_{13}H_{11}O_{8}$	295.0459	295.0556	1.1	163.0355, 149.0093	Coutaric acid isomer
30	9.83	300, 327	$C_{17}H_{19}O_{11}$	399.0933	399.0920	3.2	223.0484, 205.0354	Feruloylhexaric acid derivative
31	10.48	313	$C_{13}H_{11}O_{8}$	295.0459	295.0557	1.0	163.0383, 149.0090	Coutaric acid isomer
32	10.88	300, 326	$C_{17}H_{19}O_{11}$	399.0933	399.0919	3.4	223.0496, 205.0328	Feruloylhexaric acid derivative
33	12.36	300, 327	$C_{14}H_{13}O_{9}$	325.0560	325.0549	4.9	193.0513	Fertaric acid isomer
34	13.45	300, 325	$C_{19}H_{23}O_{12}$	443.1195	443.1203	−1.8	267.0713	Ferulic acid derivative
35[a]	14.7	255, 267, 354	$C_{32}H_{37}O_{20}$	741.184	741.1920	−4.9	609.1437, 301.0324, 300.0273, 271.0262, 255.0311	Quercetin-3-(2G-xylosylrutinoside)
36[a]	15.24	255, 267, 355	$C_{27}H_{29}O_{16}$	609.1461	609.1478	−2.8	301.0345, 300.0280, 271.0258, 255.0300	Rutin[b]
37[a]	15.66	255, 267, 303, 353	$C_{21}H_{19}O_{12}$	463.0882	463.0890	−1.7	301.0305, 300.0264, 271.0242, 255.0242	Isoquercitrin[b]
38	15.83	268, 346	$C_{21}H_{19}O_{11}$	447.0933	447.0934	−0.3	285.0384, 284.0336	Luteolin-7-*O*-glucoside[b]
39	16.03	266, 334	$C_{27}H_{29}O_{15}$	593.1512	593.1524	−2.1	285.0406, 284.0322	Luteolin-7-*O*-rutinoside
40	16.25	254, 267, 355	$C_{28}H_{31}O_{16}$	623.1618	623.1631	−2.2	315.0510, 314.0429, 300.0258, 299.0203, 271.0254, 243,0275	Isorhamnetin-3-*O*-rutinoside[b]
41	16.69	254, 267, 354	$C_{22}H_{21}O_{12}$	477.1038	477.1030	1.8	314.0432, 300.0249, 299.0217, 285.0405, 271.0247, 243.0298	Isorhamenetine-3-*O*-glucoside or galactoside
42	16.88	268, 328	$C_{21}H_{19}O_{10}$	431.0984	431.0976	1.7	269.0468, 268.0339	Apigenin-7-*O*-glucoside[b]
43	17.16	268, 340	$C_{24}H_{21}O_{14}$	533.0937	533.0943	−1.1	489.1016, 285.0397, 284.0330	Luteolin-7-*O*-(6″-*O*-malonyl)-glucoside

[a] Antioxidant compound.
[b] Confirmed by the use of an authentic standard.

223 ($C_7H_{11}O_8$) was obtained instead of *m/z* 209 ($C_6H_9O_8$), showing a loss of 162 Da corresponding to a caffeoyl moiety ($C_9H_6O_3$), consistent with the UV data. Therefore, these compounds were postulated to be caffeates with a possible hexaric acid derivative as the alcohol moiety and a greater number of CH_2 groups in their structures. Since 2,4,5-trihydroxy-3-methoxy hexanedioic acid ($C_7H_{12}O_8$) is a natural organic acid, compounds 17 and 21 were tentatively classified as caffeoylhexaric acid derivatives.

Compounds corresponding to peaks 23, 29, and 31 showed UV spectra with λ_{max} values from 310 to 313 nm and a pseudomolecular ion $[M-H]^-$ at *m/z* 295 ($C_{13}H_{11}O_8$). These compounds displayed both tartaric (149 Da) and *p*-coumaric acid (163 Da) fragments and were thus tentatively identified as coutaric acid isomers, in agreement with the published data [39,40].

Compounds corresponding to peaks 25 and 28 exhibited characteristic UV spectra of *p*-coumarates (λ_{max} 310–314 nm) with a common $[M-H]^-$ ion at *m/z* 369.0822 ($C_{16}H_{17}O_{10}$). MS/MS fragmentation resulted in three fragments at *m/z* 223 ($C_7H_{11}O_8$) $[M-H\text{-}p\text{-coumaroyl}]^-$, *m/z* 205 $[M-H\text{-}p\text{-coumaroyl-}H_2O]^-$, and *m/z* 129 $[M-H\text{-}p\text{-coumaroyl}-H_2O-C_2H_4O_3]^-$. Since the fragment at *m/z* 223 ($C_7H_{11}O_8$) was the same as that observed for compounds 17 and 21, compounds 25 and 28 could tentatively be identified as *p*-coumaroylhexaric acid derivatives (Table 2 and Fig. 6). The loss of $C_2H_4O_3$ for the fragment at *m/z* 129 corresponded to the end structure of the hexaric acid derivative, $CH_2(OH)COOH$.

Compounds corresponding to peaks 27 and 33 yielded a molecular ion of *m/z* 325 ($C_{14}H_{13}O_9$) and UV spectra with the characteristics of caffeoyl or feruloyl derivatives (λ_{max} 325–327 nm). A basic product ion at *m/z* 193 ($C_{10}H_9O_4$), resulting from the elimination of the tartaric acid moiety ($C_4H_4O_5$), corresponded to ferulic acid. Therefore, these compounds were tentatively classified as

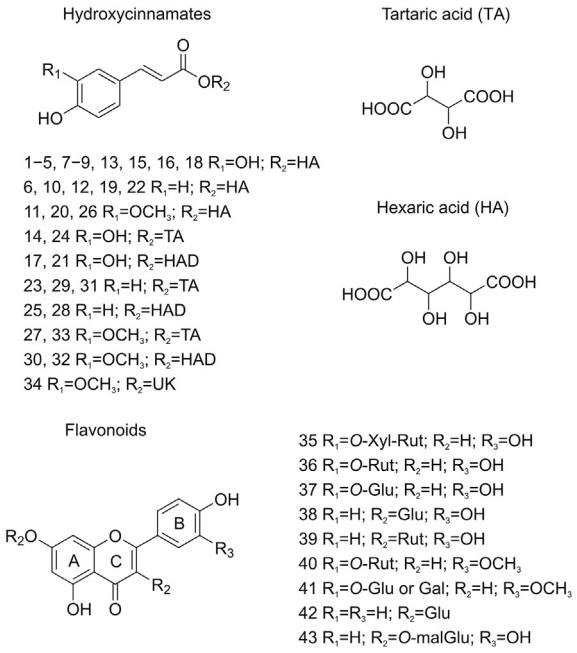

Fig. 6. Chemical structures of compounds contained in the 30% EtOH extract of AEL as identified by ultra-performance liquid chromatography coupled with diode array detection and high-resolution mass spectrometry. HA: hexaric acid; TA: tartaric acid, HAD: hexaric acid derivative; UK: unknown; Xyl: xylose; Rut: rutinose, Glu: glucose; Gal: galactose; malGlu: (6″-*O*-malonyl)-glucose.

fertaric acid isomers, which have been identified in other plants (*Asteraceae*) and wine [39,41].

Compounds corresponding to peaks 30 and 32 displayed characteristic UV spectra of caffeoyl or feruloyl derivatives (λ_{max} 326–327 and ca. 300 nm) with a common $[M-H]^-$ ion at *m/z* 399.0933 ($C_{17}H_{19}O_{11}$). Their MS/MS patterns were similar to those of compounds 17 and 21 (caffeoylhexaric acid derivatives). The same fragment ions (*m/z* 223) were observed as for compounds 17 and 21, suggesting a loss of a feruloyl moiety (399–223=176 Da), which was further fragmented to *m/z* 205 through elimination of a water molecule [42]. Based on these results, compounds 30 and 32 were tentatively identified as feruloylhexaric acid derivatives (Table 2 and Fig. 6).

The compound corresponding to peak 34 exhibited a pseudomolecular ion at *m/z* 443.1195 ($C_{19}H_{23}O_{12}$) and a basic product ion at *m/z* 267 ($C_9H_{15}O_9$) presenting a loss of a feruloyl moiety (443–267=176 Da). Moreover, its UV data (λ_{max} 325 and 300 nm) strongly supported the presence of a feruloyl moiety in its structure. Compound 34 was tentatively identified as a ferulic acid derivative. Unfortunately, the alcohol part of the ester could not be proposed using the known data.

Compounds corresponding to peaks 35–37 showed UV λ_{max} values of 255, ca. 267 and from 353 to 355 nm, suggesting that these three compounds could be classified as flavonoids. They displayed a common product ion at *m/z* 301, corresponding to an aglycone moiety. Therefore, they were identified as quercetin derivatives. The MS spectra of these compounds exhibited pseudomolecular ions $[M-H]^-$ at *m/z* 741.1920, 609.1478, and 463.0890, with the following corresponding molecular formulae: $C_{32}H_{37}O_{20}$, $C_{27}H_{29}O_{16}$, and $C_{21}H_{19}O_{12}$. The product ions at *m/z* 301 showed a neutral loss of 440, 308, and 162 Da, which probably corresponded to the 2G-xylosylrutinose, rutinose, and glucopyranose (or galactose) moieties for compounds 35, 36, and 37, respectively, suggesting that these compounds were *O*-glycosides (Table 2 and Fig. 6). Thus, peak 36 was identified as rutin, and the result was further confirmed by the retention time, UV spectrum, and MS/MS data of the authentic standard. A product ion at *m/z* 609.1437, corresponding to the rutin moiety, was observed for peak 35, indicating a loss of 132 Da, which could be attributed to a xylose moiety. Moreover, the rest of the product ions were similar to those of rutin, suggesting tentative identification of quercetin-3-(2G-xylosylrutinoside) for peak 35. Peak 37 was identified as either isoquercitrin (quercetin-3-*O*-glucoside) or hyperoside (quercetin-3-*O*-galactoside). The use of an authentic standard confirmed the identification of compound 37 as isoquercitrin.

Compounds corresponding to peaks 38, 39, and 43 presented similar UV spectra (λ_{max} 334–346 and ca. 266–268 nm). Compound 38 showed a pseudomolecular ion at *m/z* 447.0934 ($C_{21}H_{19}O_{11}$) and MS/MS fragment ions at *m/z* 285 and *m/z* 284 originating from the loss of a hexose moiety $[M-H-162]^-$. It was identified as luteolin-7-*O*-glucoside and further confirmed using an authentic standard. Compound 39 exhibited a pseudomolecular ion at *m/z* 593.1524 ($C_{27}H_{29}O_{15}$) and had a fragmentation pattern similar to that of compound 38 (Table 2). The presence of fragment ions at *m/z* 285 and *m/z* 284 generated from the loss of the rutinose moiety $[M-H-308]^-$ allowed for the identification of this compound as luteolin-7-*O*-rutinoside [43]. Compound 43, presenting a pseudomolecular ion at *m/z* 533.0943 ($C_{24}H_{21}O_{14}$), produced fragment ions at *m/z* 285, 284 as well as trace amounts at *m/z* 489. The presence of the fragment ion at *m/z* 489 in the MS spectrum (fragmentation in the source) resulted from the loss of CO_2 $[M-H-44]^-$ and this suggested the presence of a malonyl moiety in the molecule [44]. Based on these results, compound 43 was tentatively identified as luteolin-7-*O*-(6″-*O*-malonyl)-glucoside, which has already been identified in *Chrysanthemum morifolium* [45].

The compound corresponding to peak 40 showed a pseudomolecular ion at *m/z* 623.1631 ($C_{28}H_{31}O_{16}$) and the UV characteristics of an isorhamnetin glycoside (λ_{max} 355, ca. 267 and 254 nm). The MS/MS fragment ion at *m/z* 315 was assigned to isorhamnetin due to the loss of a rutinose moiety $[M-H-308]^-$. Further dissociation of the aglycone moiety yielded ions at *m/z* 300 following the loss of a methyl group. Based on the above analysis, this compound was identified as isorhamnetin-3-*O*-rutinoside, which was confirmed using an authentic standard.

Compound 41 showed a pseudomolecular ion at *m/z* 477.1030 ($C_{22}H_{21}O_{12}$), a UV spectrum (λ_{max} 354, ca. 267 and 254 nm), and an MS/MS fragmentation pattern similar to that of compound 40 (Table 2). A fragment ion of *m/z* 314 was obtained, which corresponded to the loss of a hexose moiety $[M-H-162]^-$, probably glucose or galactose. Compound 41 was tentatively identified as either isorhamnetin-3-*O*-glucoside or isorhamnetin-3-*O*-galactoside [46] (Fig. 6). Nevertheless, further investigations using authentic standards are necessary for ensuring full identification.

The compound corresponding to peak 42 exhibited a pseudomolecular ion at *m/z* 431.0976 ($C_{21}H_{19}O_{10}$). In addition, a typical UV spectrum of apigenin glycoside (λ_{max} 328 and ca. 268 nm) and a basic product ion at *m/z* 268, corresponding to the radical anion of apigenin $[Y_0-H]^-$, were observed. The fragments observed at *m/z* 269, though much less intense, suggested the loss of a hexose $[M-H-162]^-$. Therefore, compound 42 was identified as apigenin-7-*O*-glucoside, an identity that was confirmed using an authentic standard.

As previously mentioned, the 30% EtOH AEL extract mainly contained two classes of phenolic compounds, hydroxycinnamate and flavonoid derivatives. Hydroxycinnamates are formed by cross formation between three hydroxycinnamic acids (caffeic, ferulic, and *p*-coumaric acids) and three polyhydroxy organic acids (hexaric acid, tartaric acid, and hexaric acid derivatives). Consequently, nine categories of hydroxycinnamates were tentatively identified, with some

having more stereoisomers or diastereoisomers than others. Further confirmation of some of the structures is needed after complex isolation, purification, and structural identification, and/or with the use of authentic compounds that are not yet commercially available. To the best of our knowledge, hydroxycinnamate derivatives linked to hexaric acid or tartaric acid were identified for the first time in the *Astragalus* genus.

The main flavonoids detected were quercetin derivatives (compounds 35, 36, and 37) and isorhamnetin derivatives (compounds 40 and 41). Only trace amounts of three luteolin derivatives (compounds 38, 39, and 43) and one apigenin derivative (compound 42) were detected.

The major antioxidants in the 30% EtOH AEL extract were identified as caffeic acid derivatives (peaks 2, 13+14, and 24) and quercetin glycosides (peaks 35 and 36). These compounds had a common catechol moiety in their structure. The individual antioxidant activity as determined by the HPLC-ABTS$^{\cdot+}$ assay appeared to be completely or partially related to the presence of this catechol structure. Substitution of one of the two catechol OH groups might make the compound much less active or inactive [47]. For example, compounds 2 (caffeoylhexaric acid isomer) and 26 (feruloylhexaric acid isomer) exhibited similar UV and MS peak intensities. However, compound 2 showed strong antioxidant activity, whereas compound 26 showed no activity. The only structural difference between these two compounds is the substitution of one of the catechol OH groups in compound 2 with $-OCH_3$, yielding compound 26. Another example of the importance of the catechol moiety in antioxidant activity is the fact that two quercetin derivatives with a catechol moiety (ring B), compounds 36 (rutin) and 37 (isoquercitrin), showed strong antioxidant activity, while compound 40 (isorhamnetin-3-*O*-rutinoside), where one OH group of the catechol structure in ring B was replaced with $-OCH_3$, was far less active.

4. Conclusions

This study demonstrated the antioxidant activity of extracts from aerial parts of AEL using different assays, i.e., TEAC, ORAC, DPPH, and online HPLC-ABTS$^{\cdot+}$. Approximately 40 compounds belonging to two families of compounds, hydroxycinnamate and flavonoid derivatives, were identified or tentatively identified by UHPLC-DAD-HRMS. Among these compounds, caffeic acid derivatives and quercetin glycosides showed strong antioxidant activities, with the catechol moiety being the key structural feature for the observation. The reported data indicate that AEL is a potential source of natural antioxidants. However, further investigations are needed to assess the biological activity of this plant in relation to its use in traditional medicine.

CRediT author statement

Abir Chamandy: Investigation, Formal analysis, Visualization, Writing - Original draft preparation; **Minjie Zhao:** Validation, Data curation, Supervision; **Hassan Rammal:** Resources, Writing - Reviewing and Editing; **Saïd Ennahar:** Conceptualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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