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Characterization of phenolic compounds and antioxidant activity of ethanolic extracts from flowers of *Andryala glandulosa* ssp. *varia* (Lowe ex DC.) R.Fern., an endemic species of Macaronesia region

Sandra Gouveia, João Gonçalves, Paula C. Castilho*

Centro de Química da Madeira, CCCEE, Universidade da Madeira, Campus Universitário da Penteada, piso 0, 9000-390 Funchal, Portugal

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ABSTRACT

Andryala glandulosa spp. *varia* (Lowe ex DC.) R.Fern. (Asteraceae), is a small shrub that grows in mountains of Madeira Island, Fuerteventura and Lanzarote from Canary Islands. The flowerheads are used traditionally for the treatment of edemas and in homemade dermo-cosmetic preparations.

In this paper the chemical composition of the extracts of this plant, used in folk medicine, and their antioxidant capacity were established; the presence of potentially harmful lactones, so commonly associated with related species used for the same purposes was also evaluated. A reversed-phase high-performance liquid chromatography method (RP-HPLC) coupled with diode-array detection (DAD) and electrospray ionization mass spectrometry (ESI/MS^n) was used for the characterization of phenolic compounds in ethanol extracts of flowers from *A. glandulosa* spp. *varia* collected in Madeira Island. Total phenolic content (TPC) and total flavonoid content (TFC) were established and three assays (DPPH, ABTS and FRAP) were used to measure the antioxidant capacity of the dichloromethane and ethanol extracts.

The dichloromethane extract of *A. glandulosa* contain long linear chain hydrocarbons and esters. In the alcoholic extracts, a total of 16 compounds were characterized based on their UV, mass spectra and HPLC retention time. Quinic acid and luteolin derivatives were found to be the main compounds. Quantification of caffeoylquinic acids (CQA) detected was performed by HPLC-DAD and 5-*O*-CQA and 3,5-*O*-diCQA were the major compounds (with values of 22.40 ± 0.21 and 59.69 ± 1.07 mg/100 g dried plant, respectively). Only the ethanol extract was active, revealing a high radical scavenging capacity and a moderate reducing potential.

The potent antioxidant alcoholic extracts are composed mainly of hydroxycinnamic acid derivatives and flavonoids. The presence of sesquiterpene lactones was not detected. Since lactones are very common among related plants, like arnicas, and known to cause dermatitis and other unwanted effects, this can be an explanation for the preference for *Andryala* over other more easily available alternatives.

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1. Introduction

Andryala glandulosa spp. *varia* (Lowe ex DC.) R.Fern., or Downy Sow Thistle, belongs to the family of *Asteraceae* and is endemic to the archipelagos of Madeira and Canary (Macaronesia Region). This is an herbaceous plant with lanceolate basal leaves and yellow-golden flowers, which occur usually in open places of medium to high altitude (Turland, 1994; Vieira, 1992). The genus *Andryala* L., native of the Mediterranean region, was nested within *Hieracium* subgenus *Pilosella* and has genetic relationships with *Crepis* L. (Gaskin and Wilson, 2007).

Based on field survey, we found out that infusions of the flowers are used in the traditional folk medicine in different formulations. For example, they are used as compresses and washes for inflammation and in hydroalcoholic macerations as antiseptic for wounds. Poultices of the boiled flowers are used as an emollient for spots and to reduce edemas and hematomas.

The same use is given to the flowers of several endemic subspecies of Crepis such as (*Crepis divaricata* (Lowe) F. W. Schultz, *Crepis vesicaria* L. ssp. *andryaloides* and *Crepis noronhaea* Babc.) and also to *Arnica montana* (introduced species), collectively identified as "arnica flowers" (Jardim and Sequeira, 2008).

The phenolic composition of the genus *Andryala* has been poorly studied, as opposed to *Crepis* or *Arnica*, for which a large body of analytical data is available (Kisiel and Michalska, 2001; Zidorn et al., 2008); some studies (Stanojević et al., 2009) on *Hieracium* are available. The few studies relating to *Andryala* species, chemical

* Corresponding author. Tel.: +351 291705102; fax: +351 291705149.
E-mail addresses: alixstone@gmail.com, castilho@uma.pt, marguinha@gmail.com (P.C. Castilho).

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composition concern mostly to their content in sesquiterpene lactones (STL) on the non-polar extracts (Marco et al., 1994). STL are fairly common in Asteraceae aerial parts and are associated with a variety of beneficial biological effects; however they can cause allergic reactions and can be very toxic in high doses.

Phenolic compounds are a class of low molecular weight secondary plant metabolites. Most of these compounds are able to scavenge free radicals such as those produced during cell metabolism (reactive oxygen species (ROS) or free radicals such as hydrogen peroxide, hydroxyl radical and singlet oxygen) that can lead to oxidative stress. Oxidative stress is associated with major chronic health problems like cancer, inflammation, neurodegeneration diseases, heart diseases, aging and also food deterioration (Tsao and Deng, 2004).

Special attention has been paid to plants because they are very rich sources of phenolic compounds.

High performance liquid chromatography coupled with a photodiode-array detector (HPLC-DAD) and with mass spectrometry operating with an electrospray ionization (ESI) source, is an excellent and economical tool for the efficient screening and identification of the main phenolic compounds of plant extracts.

In this work the phenolic composition of the ethanolic extracts of flowers from *A. glandulosa* spp. *varia* was established by HPLC-DAD-ESI/MS^n and the dichloromethane extract was analyzed by GC–MS and FTIR in perusal for STLs.

In addition, the total phenolic and flavonoid contents of the methanol extracts were determined and correlated with the antioxidant capacity established by three different methods (DPPH, ABTS and FRAP assays).

2. Materials and methods

2.1. Chemical reagents

The following reagents were purchased from Merck (Darmstadt, Germany): potassium persulfate (99%), sodium chloride (99.5%), disodium phosphate dodecahydrated (99%), glacial acetic acid (100%), sodium carbonate (p.a.) and ferrous sulfate heptahydrate (99%). 2,2-diphenyl-1-picrylhydrazyl (DPPH) (>95%), Trolox (≥99.8%, HPLC), 2,2′azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (≥99%, HPLC), 2,4,6-Tri(2-Pyridyl)-s-triazine (TPTZ) (≥99.0%, TLC), β-carotene (≥97%, UV), Tween 40 and Folin-Ciocalteu's phenol reagent were purchased from Fluka (Lisbon, Portugal). Potassium chloride (>99.5%), gallic acid (99%, HPLC), potassium acetate (p.a.), rutin (≥98%, HPLC) and ferric chloride hexahydrate (97–100%) were purchased from Panreac (Barcelona, Spain); potassium dihydrogen phosphate (99.5%), aluminium chloride (98%) and sodium acetate trihydrate (pure) were purchased from Riedel-de Haën (Hanover, Germany).

All solvents used for plant extraction were AR grade, purchased from Fisher (Lisbon, Portugal). HPLC-MS grade acetonitrile (99.9%, LabScan, Gliwice, Poland) and ultra-pure water (Milli-Q Waters purification system, EUA) were used for HPLC analysis.

Stock solutions of standard compounds (100 μg/mL) were prepared in ethanol for HPLC-DAD-ESI/MS^n identification and stored in a refrigerator at −20 °C until use. Standards used: caffeic acid (>99%), luteolin (>99%) from Extrasynthese (Lyon, France) and 5-*O*-caffeoylquinic acid (99%) from Acros Organics (Geel, Belgium). 1,3-*O*-dicaffeoylquinic acid, 1,5-*O*-dicaffeoylquinic acid, 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid, 4,5-*O*-dicaffeoylquinic acid and 3,4,5-*O*-tricaffeoylquinic acid (>98% by HPLC for all) were obtained from Chengdo Biopurify Phytochemicals, Ltd. China (Sichuan, China).

2.2. Plant material

The flowers of *A. glandulosa* spp. *varia* were collected in the wild in Madeira Island, in July 2008 and July 2009, at Pico Grande, at an altitude over 1800 m. They were identified by taxonomist Fátima Rocha and vouchers were deposited in the Madeira Botanical Garden Herbarium collection.

2.3. Extraction procedure

Fresh flowers of *A. glandulosa* (450 g) were extracted with dichloromethane (2.5 L) during 10 min, at room temperature. The solution was filtered and concentrated to dryness under reduced pressure in a rotary evaporator (40 °C), yielding 2.60 g of a semi-solid whitish dried extract.

After this first extraction, the flowers were dried, at room temperature, and mill powered.

The flower powder obtained (111 g) was macerated in ethanol (2 × 1 L), at room temperature for 48 h. The extract was decolorized with activated charcoal, filtered and concentrated under reduced pressure in a rotary evaporator (40 °C), to give 28.0 g of a dark yellow oil.

2.4. Lactones determination in dichloromethane extract

2.4.1. TLC analysis

Analytical TLC was performed on silica gel 60 plates, developed with chloroform as eluent and visualized by UV (λ_{max} 254 and 366 nm) and by spraying with Liebermann–Bouchard reagent, with negative response.

2.4.2. GC–MS analysis

The GC–MS analysis for identification of compounds was carried out in a Varian Saturn 3 GC–MS (Ion trap) operating in EI mode and using a HP-5MS column (30 m × 0.25 mm, 0.25 μm film thickness), carrier gas helium, constant pressure 90 kPa, split 1:20. The oven was programmed initially from 70 °C with 2 min hold up time to the final temperature of 230 °C with 5 °C/min ramp. The final temperature hold time was 5 min. The inlet and GC/MS interface temperatures were kept at 250 °C and 280 °C, respectively. The temperature of El 70 eV source was 200 °C with full scan (25–450 *m*/*z*), scan time 0.3 s. The mass spectra of extract components were identified by comparing the mass spectra of the analytes with those of authentic standards from the mass spectra of Wiley 6.0 and Mass Spectra Library (NIST 98).

2.4.3. FTIR

Qualitative FTIR analysis of the dichloromethane extract was performed using a Nicolet Avatar 360 instrument operating in transmission mode within the 4000–400 cm^{-1} interval, with a resolution of 2 cm^{-1}, accumulating 64 spectra, semi-solid samples were deposited over KBr cell windows.

2.5. Phenolic composition of ethanol extract by HPLC-DAD-ESI/MS^n

2.5.1. Sample preparation

Ethanolic extracts were analyzed by HPLC-DAD-ESI/MS^n. For this experiment, a stock solution with concentration (w/v) of 5 mg/mL was prepared by dissolving the extract in initial mobile phase (ACN-H_2O (20:80)). This solution was filtered through 0.45 μm Nylon micropore membranes prior to use. Three assays were performed by injecting aliquots of 10 μL in the HPLC-MS system.

2.5.2. Liquid chromatography

The HPLC analysis was performed on a Dionex ultimate 3000 series instrument (California, EUA) coupled to a binary pump, a diode-array detector (DAD), an autosampler and a column compartment.

Samples were separated on a Phenomenex Gemini C_{18} column (5 μm, 250 × 3.0 mm i.d.; Phenomenex) with a sample injection volume of 10 μL. The mobile phase was mixtures of acetonitrile (A) and water/formic acid (100/0.1, v/v) (B). A gradient program was used as follows: 20% A (0 min), 25% A (10 min), 25% A (20 min), 50% A (40 min), 100% A (42–47 min), 20% A (49–55 min). The mobile phase flow rate was 0.4 mL/min; the chromatogram was recorded at 280 nm and 350 nm and spectral data for all peaks were accumulated in the range of 190–400 nm. Column temperature was controlled at 30 °C.

2.5.3. HPLC-UV-DAD quantification

The analysis was performed with the HPLC system described above using a modified gradient that allowed for the separation of all detected caffeoylquinic acid isomers. The mobile phase consisted of acetonitrile:formic acid (100:0.1, v/v) (A) and water:formic acid (100:0.1, v/v) (B). The gradient program was used as follows: 20% B (0–1 min), 78% B (8–10 min), 76% B (12–14 min), 75% B (16–18 min), 73% B (20 min), 50% B (40 min), 0% B (41–45 min), and 80% B (46–50 min). The flow rate was 0.4 mL/min and the injections volume 10 μL. UV detection was performed at 320 nm.

2.5.4. Mass spectrometry

For HPLC-ESI/MS^n analysis, the Dionex HPLC system describe before was coupled with a Bruker Esquire (Bremen, Germany) model 6000 ion trap mass spectrometer fitted with an ESI source. Data acquisition and processing were performed using Esquire control software. Negative ion mass spectra of the column eluate were recorded in the range *m/z* 100–1000 at a scan speed of 13,000 Da/s. High purity nitrogen (N_2) was used both as drying gas at a flow 10.0 mL/min and as a nebulizing gas at pressure of 50 psi. The nebulizer temperature was set at 365 °C and a potential of +4500 V was used on the capillary. Ultra-high purity helium (He) was used as collision gas at a pressure of 1×10^{-5} mbar and the collision energy was set at 40 V.

The acquisition of MS^n data was made with *auto* MS^n mode, with isolation width of 4.0 *m/z*. For MS^n analysis, mass spectrometer was scanned from 10 to 1000 *m/z* with fragmentation amplitude of 1.0 V and two precursor ions.

2.6. Total phenolic compounds

The content of phenolic compounds of the extracts was determined following the Folin–Ciocalteu method (Zheng and Wang, 2001) with some modifications and using gallic acid as standard. For the calibration curve, 50 μL aliquots of 0.024, 0.075, 0.105, 0.3 and 0.4 mg/mL gallic acid solutions in methanol were mixed with 1.25 mL of Folin–Ciocalteu reagent (diluted ten-fold) and 1 mL of sodium carbonate solution (7.5 g/L). 50 μL of methanolic extract solution (10 mg/mL) were mixed with the same reagents as described above. After incubation for 30 min the absorbance was read at 765 nm. The final results were expressed as gallic acid equivalents per 100 g of plant (mg GAE/100 g).

2.7. Total flavonoid content

Total flavonoid content was measured using a modified method (Akkol et al., 2008). 10 mg of extract was dissolved in 5 mL of methanol. In a 10 mL test tube, 0.5 mL of sample solution, 1.5 mL of methanol, 2.8 mL of water, 0.1 mL of potassium acetate (1 M) and 0.1 mL of aluminium chloride (10% in methanol) were mixed. The decrease in absorbance was measured at 415 nm after incubation at room temperature for 30 min. The total flavonoid content was expressed as milligrams of rutin equivalent per 100 g of plant (mg RUE/100 g).

2.8. Measurement of the antioxidant activity

All UV/Vis absorptions measurements were performed on a PerkinElmer UV-Vis spectrometer Lambda 2 equipped with a water thermostatic cell holder. Glass cells with a 1 cm optical path were used.

2.8.1. $ABTS^{\bullet+}$ radical cation decolorization assay

The antioxidant activity by the method of decolorization of free radical $ABTS^{\bullet+}$ was determined as previously reported (Gouveia and Castilho, 2012).

The plant extracts were dissolved in methanol to yield a concentration of 1 mg/mL. For each analysis, an aliquot of 100 μL methanolic solution was added to 1.8 mL of $ABTS^{\bullet+}$ solution and the decrease of absorbance, at λ = 734 nm, was recorded during 6 min. Results were expressed in terms of μmol Trolox equivalent per 100 g of plant antioxidant capacity (μmol eq. Trolox/100 g plant).

2.8.2. DPPH radical scavenging activity

The antioxidant activity by DPPH method was determined according to (Atoui et al., 2005) with some modifications (Gouveia and Castilho, 2011).

The DPPH radical scavenging effect of the extracts was expressed, based on the Trolox calibration curve, as μmol Trolox equivalent per 100 g of plant (μmol eq. Trolox/100 g plant).

2.8.3. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay, as described by (Benzie and Strain, 1996), was performed with some adjustments as described in our recent paper (Gouveia and Castilho, 2012).

The extracts were dissolved in methanol to yield a final concentration of 1 mg/mL. For each analysis, 30 μL of methanolic solution were added to 180 μL of distilled water and 1.8 mL of FRAP solution. The absorbance of the reaction mixture was recorded at 593 nm in 15 s intervals, during 30 min against methanol as blank. The FRAP results were expressed as mmol Iron(II) sulfate heptahydrate per mg of plant (mmol Fe(II)/mg plant).

2.9. Statistical analysis

All measurements were performed in triplicate and results are expressed as mean ± SD.

Significant differences in antioxidant activity, total phenolic and flavonoid content of the different extracts were determined using one-way ANOVA. The statistical probability was considered to be significantly different at the level of $p < 0.05$.

3. Results and discussion

3.1. TLC, FTIR and GC–MS analysis

The dichloromethane extract was analyzed by TLC, FTIR and GC–MS. The chromatogram obtained in the described conditions showed 4 peaks: two intense ones with very similar mass spectra, without a distinct molecular ion and clusters at intervals of 14 mass units, characteristic of long straight chain hydrocarbons and as such identified by the NIST database; the other two (small) peaks also showed similar mass spectra with clusters at intervals of 14 mass units, with a prominent peak at $C_nH_{2n-1}O_2^+$ in each cluster and a base peak at *m/z* 88, a diagnostic peak for ethyl esters. FTIR spectra of the extract showed sp^3 C—H bands as the main

features with a medium intensity C=O band at 1731 cm^{-1} and moderate C—O band at 1460 cm^{-1}. This is consistent with the GC–MS results, with hydrocarbons being more abundant that esters. The spectra did not show the characteristic lactone band at around 1760 cm^{-1}, thus confirming the TLC data (negative response to the Liebermann–Burchard reagent) No lactones were detected, so this extract of surface components was no longer considered of interest.

3.2. HPLC-DAD-ESI/MS^n analysis

The high antioxidant capacity and phenolic content of the ethanolic extract led us to investigate the phenolic profile of this extract by HPLC-DAD-ESI/MS^n.

Three independent assays were performed for the analysis of the ethanolic extract from *A. glandulosa* by HPLC-DAD-ESI/MS^n and no relevant variation was noticed that can be related to the nature of detected fragments and their relative intensities.

The base peak chromatogram (BPC) profile of ethanolic extract is shown in Fig. 1 and, as can been seen, the majority of the compounds could be well separated.

Whenever it was possible the detected compounds were compared with reference. For unknown compounds, their structures were thus characterized based mainly on their MS^n fragmentation behavior, on HPLC retention times and on studies of their UV spectra.

Different types of compounds showed different UV absorption characteristics bands. Hydroxycinnamic acid derivatives showed two maximum absorption bands at 230–240 nm and 320–330 nm, with a shoulder around 300–310 nm. Peaks corresponding to flavones glycosides show three absorptions at 210–230 nm, 250–280 nm and 330–350 nm. Typical flavonols spectrum exhibit two maxima absorptions at 250–295 nm and 310–370 nm, derived from the aglycone A and B rings, respectively (Gouveia and Castilho, 2009). However, different substitutions of the hydroxyl groups led to alteration in wavelength and relative intensities of these maxima (Olsen et al., 2009).

MS^n fragmentation ions of the 16 compounds detected in ethanolic extract are given in Table 1 and their chemical structures are shown in Fig. 2.

Most of the phenolic compounds detected gave deprotonated molecular ions $[M-H]^-$ of high abundance, which allowed them to be analyzed by tandem MS^n fragmentation.

3.2.1. Identification of hydroxycinnamic acid derivatives (**1**, **4**, **5**, **10** and **11**)

Five hydroxycinnamic acid derivatives were identified by HPLC-DAD-ESI/MS^n. The deprotonated molecular ions, $[M-H]^-$, were abundantly produced under the MS^n conditions for all hydroxycinnamic acid derivatives and the loss of the substitution groups is always referred to this ion.

Compound **1** occurred at retention time of 3.0 min and exhibited a $[M-H]^-$ ion at *m/z* 499 and corresponds to a quinic acid derivative. In the MS^2 spectrum the base peak is a fragment ion at *m/z* 191 [quinic acid$-H]^-$ formed due to the loss of a 308 Da moiety. This moiety can possibly be composed of a caffeoyl group (162 Da) and a coumaroyl group (146 Da). The possibility of hexoside and rhamnose groups was excluded due to the low retention time. The loss of 146 Da was evidenced by the formation of a fragment ion at *m/z* 353 (ca. 13% of base peak) as showed in Fig. 3.

These facts suggest that the two groups should be linked in the same —OH group of quinic acid and the coumaroyl group must be linked to the caffeoyl group. The linkage position of acyl groups in the quinic acid can be determined by the analysis of the $[M-H]^-$ ion MS^2 fragmentation.

When the acyl group is connected to a 3-OH or 5-OH position in quinic acid, the [quinic acid$-H]^-$ ion at *m/z* 191 is the base peak in MS^2 spectrum. The [caffeic acid$-H]^-$ ion at *m/z* 179 is more significant for 3-*O*-caffeoylquinic acids, while for 5-*O*-caffeoylquinic acid it is very weak (<5%) or undetectable. When the acyl group is linked to the 4-OH, the [quinic acid$-H_2O-H]^-$ ion at *m/z* 173 appeared as the base peak in the MS^2 spectrum, due to loss of caffeic acid and a water molecule.

The absence of a fragment ion at *m/z* 179 in the MS^n fragmentation of compound **1** indicates that the substitution group is connected to a 5-OH position. The fragment ion at *m/z* 481 was produced by the loss of H_2O (18 Da).

In MS^3 spectrum, were detected fragment ions at *m/z* 85, 87, 93, 109, 111, 127 (base peak), 129, 153 and 173. According to the fragmentation of compound 5-*O*-caffeoylquinic acid (5-CQA), described by Clifford et al. (2003), in MS^3 spectrum the ions *m/z* 85, 93, 127 and 173 are characteristic of this molecule, which support the idea that we have a substitution in position 5-OH. So, **1** was identified as 5-*O*-coumaroylcaffeoylquinic acid and its fragmentation pathway is represented in Scheme 1.

Compound **4** (t_R = 5.1 min) was detected overlapped with another peak (described later) and showed a $[M-H]^-$ ion at *m/z* 353. Its MS^2 fragmentation showed a fragment ion at *m/z* 191 (base peak). Comparing its HPLC retention time and MS^n fragmentation pattern with a standard solution of 5-*O*-caffeoylquinic acid, they were found to be similar. Therefore, **4** was identified as 5-*O*-caffeoylquinic acid.

Compound **5** (t_R = 7.0 min) showed a $[M-H]^-$ ion at *m/z* 615. Its MS^2 fragmentation showed the loss of 262 Da forming a fragment ion at *m/z* 353. The MS^3 spectrum of this ion showed as base peak a fragment ion at *m/z* 191, which indicates a 5-*O*-caffeoylquinic acid structure. Based on the literature reports (Lin and Harnly, 2008) the fragment of 262 Da is probably composed of a caffeoyl group (162 Da) esterified with a succinyl moiety (100 Da). However, the linkage position of this fragment was not possible to identify based on these MS^n data. Therefore, **5** was identified as dicaffeoylsuccinylquinic acid.

Compound **10** (t_R = 13.0 min) and **11** (t_R = 14.6 min) showed a $[M-H]^-$ ion at *m/z* 515 and were identified as 3,5-*O*-dicaffeoylquinic acid (3,5-diCQA) and 4,5-*O*-dicaffeoylquinic acid (4,5-diCQA), respectively. Both isomers gave as base peak, in MS^2 spectrum, a $[M-H-162]^-$ ion at *m/z* 353 that corresponds to the loss of a caffeyol residue (162 Da). However, their MS^3 and MS^4 spectra of the *m/z* 353 ions were found to be different. Compound **11** gave as base peak in the MS^3 spectrum a fragment ion at *m/z* 173 which indicates the presence of a 4-OH substituted quinic acid. Based on previously reports (Clifford et al., 2003), it is possible to differentiate the 3,4-*O*-dicaffeoylquinic acid from the 4,5-*O*-dicaffeoylquinic acid since the two isomers differ in the intensity of the MS^2 "dehydrated" ion at *m/z* 335 ($[M-H_2O-H^+]^-$). For 3,4-*O*-dicaffeoylquinic acid, the peak at *m/z* 335 is more intense (ca. 15% of base peak). While for 4,5-*O*-dicaffeoylquinic acid this ion is barely detectable (<5% of base peak). The MS^2 spectrum of **11** showed did not exhibited a secondary ion at *m/z* 335, thus, this compound was identified as 4,5-*O*-dicaffeoylquinic acid.

The MS^3 spectrum of **10** showed as base peak a fragment ion at *m/z* 191 ([quinic acid$-H]^-$) and a fragment ion at *m/z* 179 (49.3% of base peak) ([caffeic acid$-H]^-$). Comparing this fragmentation behavior to literature data (Gouveia and Castilho, 2009) **10** was identified as 3,5-*O*-dicaffeoylquinic acid.

Further comparison with reference standards confirmed the identification proposed.

3.2.2. Identification of procyanidin compound (**2**)

Compound **2** (t_R = 4.3 min) showed a $[M-H]^-$ ion at *m/z* 577 characteristics of dimeric procyanidins. The procyanidin B dimers

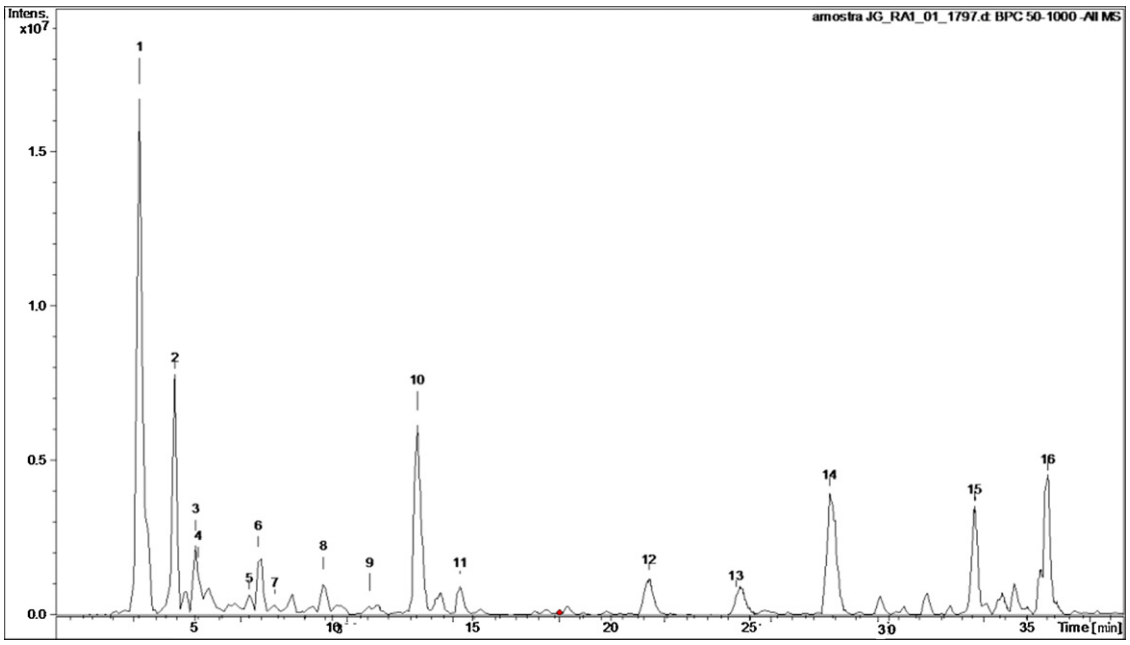


Fig. 1. HPLC-DAD-ESI/MS^n analysis of the methanolic extract of *Andryala glandulosa* spp. *varia* – HPLC-MS negative ion ESI-MS^n base peak chromatogram (BPC) (peak identification according to Table 1).

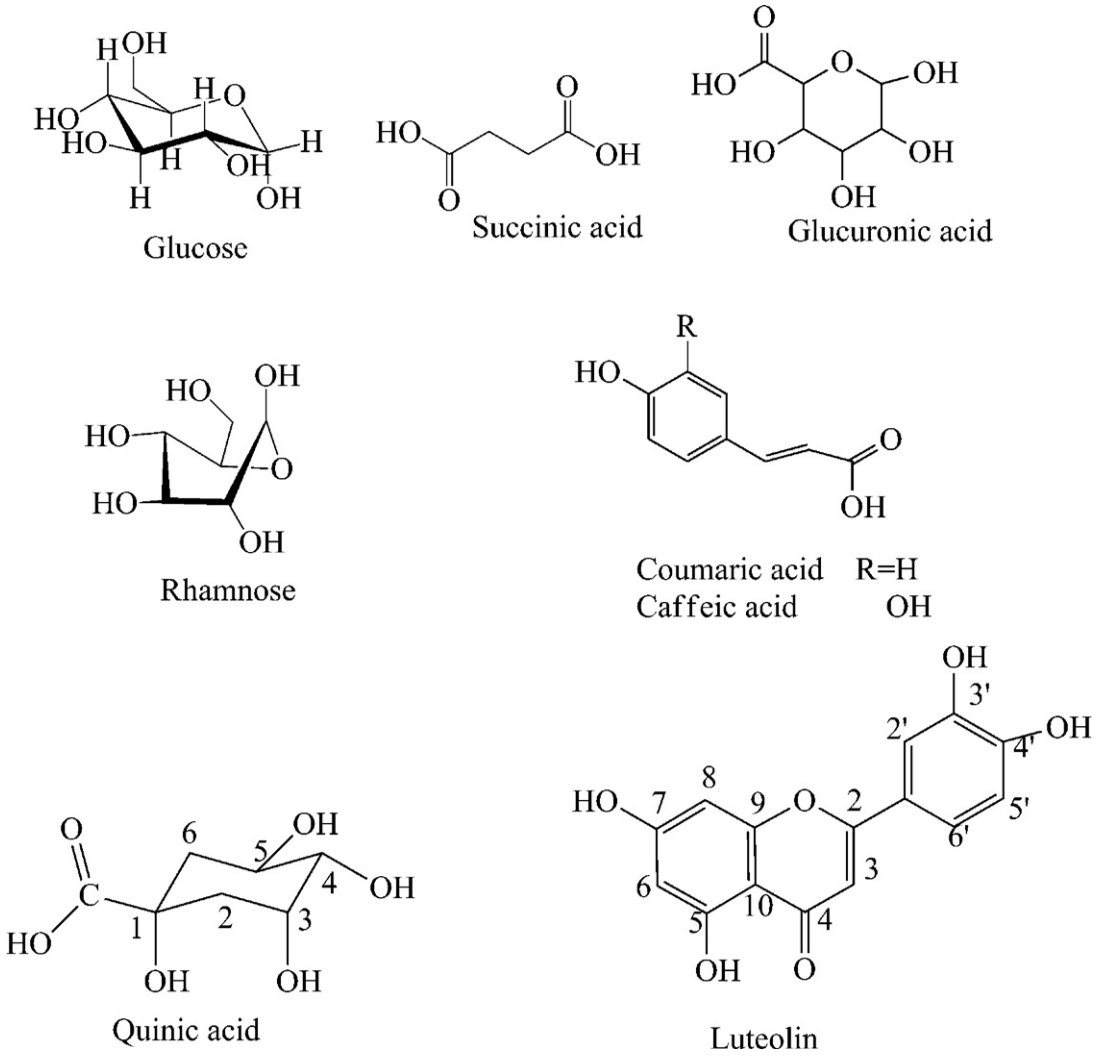


Fig. 2. Chemical structures of phenolic compounds and its substituents in the ethanolic extract of *Andryala glandulosa* spp. varia.

Table 1
Characterization of phenolic compounds of the ethanolic extract of flowers in *Andryala glandulosa* spp. *varia* by HPLC-DAD-ESI/MS^n.

Peak No.	t_R (min)	UV λ_{max} (nm)	$[M-H]^-$ m/z	HPLC-DAD-ESI/MS^n m/z (% base peak)	Identification
1	3.0	278	499.3	MS^2 [499.3]: 173.0 (94.3), 190.9 (100), 353.1 (13.2), 481.1 (18.7) MS^3 [499.3 → 190.9]: 85.2 (37.9), 87.0 (22.5), 93.1 (57.3), 109.1 (21.8), 110.9 (43.5), 127.0 (100), 129.2 (45.4), 152.8 (17.6), 172.9 (18.1)	5-*O*-coumaroylcaffeoylquinic acid
2	4.3	201, 261	577.3	MS^2 [577.3]: 391.1 (14.6), 409.0 (100), 410.0 (15.9) MS^3 [577.3 → 409.0]: 240.9 (13.7), 283.0 (12.4), 301.0 (27.6), 373.0 (19.6), 391.0 (100), 392.0 (10.1) MS^4 [577.3 → 409.0 → 391.0]: 204.9 (72.0), 240.9 (48.0), 246.0 (100), 283.0 (44.0)	Procyanidin
3	5.0	217, 242, 325	519.3	MS^2 [519.3]: 155.0 (11.6), 215.0 (44.7), 259.0 (100), 260.0 (13.6) MS^3 [519.3 → 259.0]: 155.0 (30.1), 215.0 (100), 216.0 (55.0) MS^4 [519.3 → 259.3 → 215.0]: 155.0 (100), 155.9 (30.2)	Unknown
4[a]	5.1	242, 300, 325	353	MS^2 [353]: 191 (100) MS^3 [353 → 191]: 85 (46.1), 93 (100), 111 (52.2), 127 (56.8), 173 (45.7)	5-*O*-caffeoylquinic acid
5	7.0	230, 291, 318	615	MS^2 [615]: 191 (38.6), 353 (100) MS^3 [615 → 353]: 191 (100) MS^4 [615 → 353 → 191]: 85 (100), 127 (69.9), 153 (74.4), 173 (94.5)	Dicaffeoylsuccinylquinic acid
6	7.3	230, 318	471.4	MS^2 [471.4]: 425.2 (100), 426.1 (20.9) MS^3 [471.4 → 425.2]: 175.1 (14.7), 201.0 (22.7), 219.0 (17.9), 245.0 (34.1), 263.0 (100) MS^4 [471.4 → 425.2 → 263.0]: 105.1 (18.8), 109.2 (28.5), 175.0 (23.2), 176.0 (14.8), 201.0 (34.4), 216.9 (19.7), 219.0 (55.6), 245.0 (100), 246.1 (45.5)	Unknown
7	7.9	244, 326	579	MS^2 [579]: 285 (100), 286 (11.4) MS^3 [570 → 285]: 199 (71.4), 217 (48.9), 243 (100)	Luteolin-7-*O*-pentoside-hexoside
8	9.7	245, 326	447	MS^2 [447]: 285 (100), 286 (15.6) MS^3 [447 → 285]: 175 (74.5), 199 (100), 217 (91.6), 241 (40.5), 243 (22.0)	Luteolin-7-*O*-hexoside
9	11.3	243, 326	461	MS^2 [461]: 285 (100), 286 (16.5) MS^3 [461 → 285]: 199 (76.2), 217 (82.3), 243 (100)	Luteolin-7-*O*-glucuronide
10[a]	13.0	217, 243, 326	515.2	MS^2 [515.3]: 353.1 (100), 354.0 (18.0) MS^3 [515.3 → 353.1]: 179.0 (49.3), 190.9 (100) MS^4 [515.3 → 353.1 → 190.9]: 85.2 (96.9), 87.1 (26.7), 93.2 (81.4), 96.2 (11.0), 99.2 (23.0), 110.1 (16.3), 126.9 (44.2), 145.0 (59.1), 152.9 (33.5), 171.0 (28.0), 172.9 (100)	3,5-*O*-dicaffeoylquinic acid
11[a]	14.6	245, 295, 328	515.2	MS^2 [515.2]: 173.0 (20.7), 179.0 (10.1), 202.9 (12.4), 353.1 (100), 354.1 (15.7) MS^3 [515.2 → 353.1]: 135.1 (23.7), 154.9 (15.1), 173.0 (100), 179.0 (86.0), 180.0 (11.6), 191.0 (59.5) MS^4 [515.2 → 353.1 → 173.0]: 93.1 (100), 95.3 (11.0)	4,5-*O*-dicaffeoylquinic acid
12	21.2	–	455.3	MS^2 [455.3]: 409.1 (100), 410.2 (19.7) MS^3 [455.3 → 409.1]: 123.1 (12.6), 132.9 (13.6), 135.0 (21.3), 247.0 (100), 248.0 (18.8), 391.0 (10.9) MS^4 [455.3 → 409.1 → 247.0]: 123.0 (17.5), 165.1 (30.9), 173.0 (26.5), 201.8 (15.8), 203.0 (100), 204.0 (12.0), 219.0 (38.1)	Unknown
13	24.5	–	457.3	MS^2 [457.3]: 249.1 (11.7), 411.2 (100), 412.1 (19.0) MS^3 [457.3 → 411.2]: 249.1 (100), 250.1 (19.6) MS^4 [457.3 → 411.2 → 249.1]: 205.0 (92.5), 206.1 (100)	Unknown
14[a]	27.9	206, 253, 346	285.1	MS^2 [285.1]: 109.0 (18.8), 133.0 (14.8), 149.0 (45.9), 150.9 (70.8), 156.0 (13.2), 172.9 (68.9), 175.0 (100), 189.9 (10.6), 196.9 (11.6), 199.0 (99.0), 199.8 (24.1), 214.0 (76.0), 217.1 (25.0), 217.7 (14.0), 241.0 (17.4), 242.8 (39.3), 243.8 (11.7), 254.9 (47.4), 258.0 (30.9), 267.0 (19.6), 269.8 (14.8) MS^3 [285.1 → 199.0]: 172.0 (100)	Luteolin
15	32.9	269	327.4	MS^2 [327.4]: 165.1 (22.3), 171.0 (86.9), 182.9 (13.9), 201.0 (23.1), 209.1 (27.5), 211.1 (38.2), 221.1 (16.8), 229.1 (100), 239.0 (15.2), 247.2 (22.4), 291.2 (34.7), 309.1 (12.0) MS^3 [327.4 → 229.1]: 85.1 (59.2), 126.9 (24.8), 165.1 (59.2), 167.1 (33.8), 194.0 (96.8), 208.9 (100), 211.0 (25.5) MS^4 [327.4 → 229.1 → 208.9]: 166.0 (100)	Unknown
16	35.7	213, 224, 297, 306	329.4	MS^2 [329.4]: 171.0 (12.8), 183.1 (15.9), 209.1 (15.5), 211.1 (71.8), 229.1 (100), 230.1 (15.5), 293.2 (17.8), 311.2 (20.4) MS^3 [329.4 → 229.1]: 95.6 (21.0), 124.9 (20.6), 127.2 (38.0), 163.0 (12.3), 209.0 (41.3), 210.9 (100) MS^4 [329.4 → 229.1 → 210.9]: 95.2 (30.6), 125.0 (100)	Unknown

[a] Comparison with a reference standard; - Their UV spectra have not been properly observed due to low intensity.

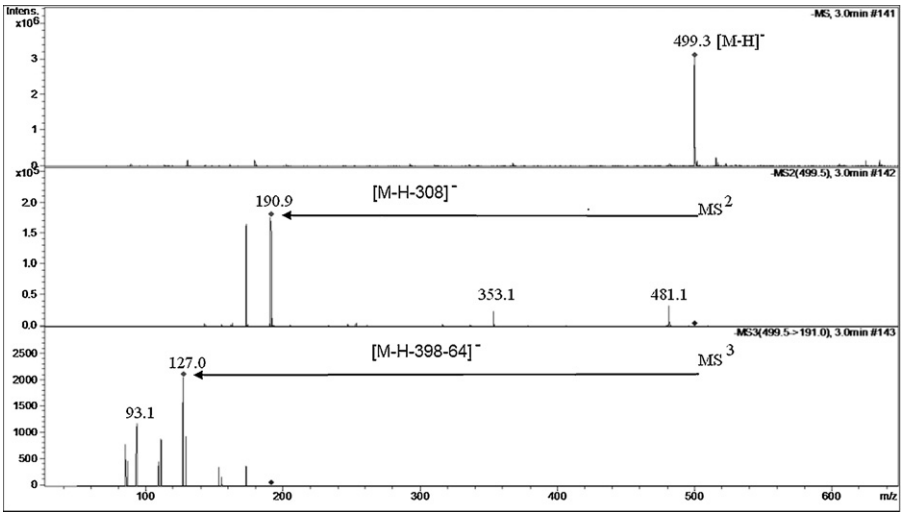


Fig. 3. ESI/MS^n negative mode of compound 1. Sequential fragmentation, MS^n (n up to 3) of the ion at m/z 499.

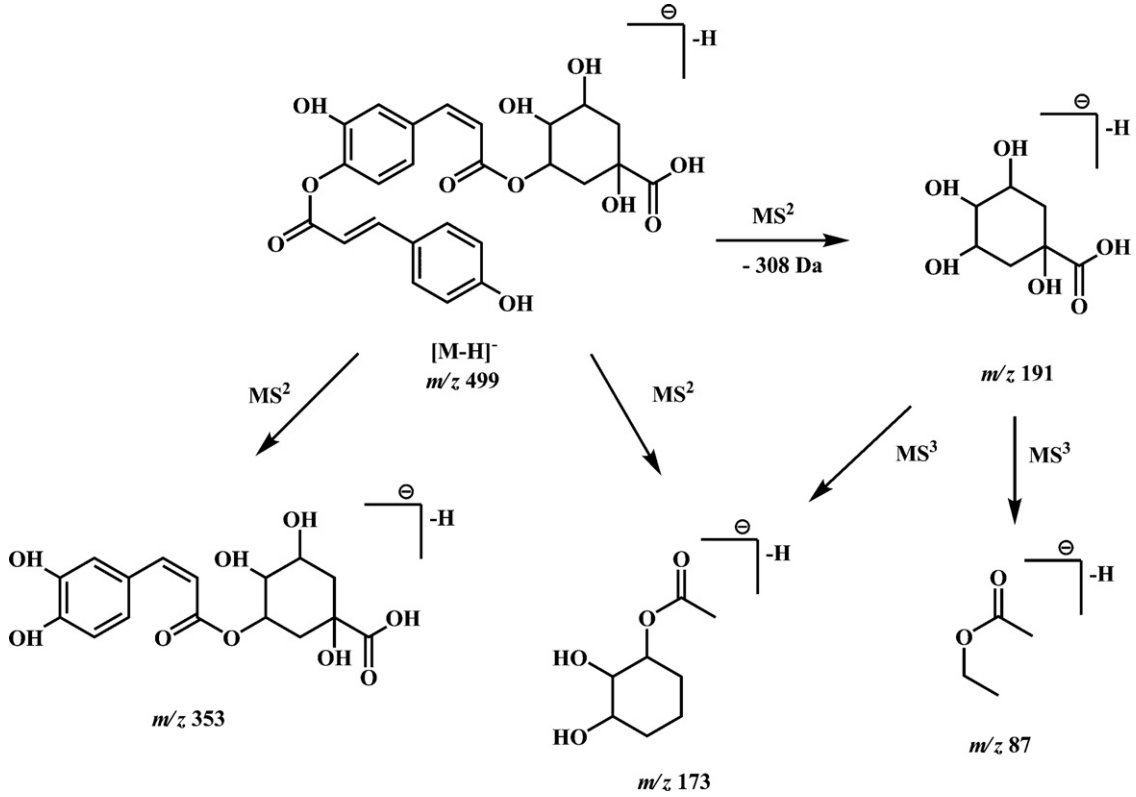
consist of eight stereoisomers composed of catechin and epicatechin monomers that can be divided into two subgroups based on their 4–8 or 4–6 linkage (Eng et al., 2003).

However, the fragmentation pattern obtained for this compound was not found reported in the literature, especially concerning the non-observation of the ion m/z 289 typical of procyanidins B (de Souza et al., 2008; Sun et al., 2007).

The fragmentation in MS^2 gave a mass spectrum with a base peak at m/z 409 that corresponds to the loss of $C_8H_8O_4$ (168 Da). A fragment at m/z 391 was also observed and may correspond to the combined loss of $C_8H_8O_4$ and H_2O ($[M-H-168-18]^-$). MS^3 spectrum contained a base peak ion at m/z 391 that corresponds to the loss of a molecule of water. The ion at m/z 373 (20% of base peak) corresponds to the loss of two molecules of water (36 Da), the m/z 241 (14%) to loss of $C_8H_8O_4$ and the m/z 392 (10%) occur with the loss of a hydroxyl group. The formation of a fragment ion at m/z 283 can be possibly caused by the loss of $C_8H_8O_4$ (168 Da) and C_2H_2O (42 Da) residues. The ion at m/z 301 suggests the loss of a ring $C_6H_6O_2$. In the absence of standard compounds to clarify this structure of this molecule, no further identification was attempted at this point.

3.2.3. *Identification of flavones derivatives (**7**, **8**, **9** and **14**)*

Compound **14** (t_R = 27.9 min) exhibited a $[M-H]^-$ ion at m/z 285 and its MS^n fragmentation formed several fragment ions at m/z 243 ($[M-H-C_2H_2O]^-$), 241 ($[M-H-CO_2]^-$), 217 ($[M-H-C_3O_2]^-$), 175



Scheme 1. Proposed fragmentation pathway for compound 1.

Table 2
Contents of individual phenolic compounds in *Andryala glandulosa* ethanolic extract (mg/100 g of plant material).

Compound	5-*O*	CQA3,5-*O*-diCQA	4,5-*O*-diCQA	Total amount
Ethanolic extract	22.40 ± 0.21	59.69 ± 1.1	3.81 ± 0.031	85.90 ± 1.3

($[M-H-C_3O_2-C_2H_2O]^-$) and 199 ($^{1,3}A^-$). This compound was identified as luteolin by comparison of its MS^n fragmentation pattern with that of a reference standard (data not shown) and literature data (Gouveia and Castilho, 2009).

Compound **7** (t_R = 7.9 min) displayed a $[M-H]^-$ ion at *m/z* 579 and under MS^n fragmentation this ion easily loosed a fragment of 294 Da forming the deprotonated aglycone ion, Y_0^-, at *m/z* 285. Fragmentation of this ion formed fragment ions at *m/z* 243, 199 and 217, characteristics of luteolin. The 294 Da residue is probably composed of a hexoside (162 Da) and a pentoside (132 Da) group. The favored glycosylation position for flavoinds is the 7-OH. Therefore, compound **7** was identified as luteolin-7-*O*-pentoside-hexoside (Cuyckens and Claeys, 2004).

Compound **8** (t_R = 9.7 min) exhibited a $[M-H]^-$ ion at *m/z* 447. When submitted to further fragmentation this ion readily eliminated a hexoside residue (162 Da) to produce the deprotonated aglycone ion, Y_0^-, at *m/z* 285. The MS^3 spectrum of the aglycone ion gave fragments ions at *m/z* 199, 217, 175, 241 and 243 characteristic ions of luteolin as described above. So, compound **8** was characterized as luteolin-7-*O*-hexoside.

Compound **9** (t_R = 11.3 min) showed a $[M-H]^-$ ion at *m/z* 461 and its MS^2 spectrum showed a fragment ion at *m/z* 285 due to the loss of 176 Da, probably a glucuronic acid moiety. The MS^3 fragmentation of the deprotonated aglycone ion, Y_0^-, at *m/z* 285 gave ions characteristic of luteolin (Scheme 2). Comparing these data and literature data (Gouveia and Castilho, 2009) **9** was identified as luteolin-7-*O*-glucuronide.

3.2.4. Other compounds

Other peaks, described as compounds **3**, **6**, **12**, **13**, **15** and **16**, were observed. However, the elucidation of their structures, based solely on UV and MS^n data, could not be completely achieved.

At a retention time of 5.0 min, compound **3** exhibited a $[M-H]^-$ ion at *m/z* 519. The MS^2 spectrum showed an ion at *m/z* 259 from the loss of 260 Da. Its MS^n fragmentation gave ions at *m/z* 215 (loss of 44 Da, probably CO_2) and 155 (loss of 60 Da). It is apparently a dimer but this information is not enough to identify the monomer.

Compound **6** (t_R = 7.3 min) gave a $[M-H]^-$ ion at *m/z* 471 and its fragmentation led to formation of a fragment ion at *m/z* 425 which corresponds to the loss of 46 Da (probably formic acid). The MS^3 and MS^4 spectra showed ions at *m/z* 263 (loss of 162 Da, probably a hexoside residue) and *m/z* 245 (loss of 18 Da, probably H_2O).

Compounds **12** (t_R = 21.2 min) and **13** (t_R = 24.5 min), under fragmentation, exhibited a deprotonated molecular ion $[M-H]^-$ at *m/z* 455 and 457, respectively. Both compounds presented a very similar MS^n pattern and gave the neutral loss of 46 Da (formic acid) in MS^2 spectrum, 162 Da (hexoside residue) in MS^3, and 44 Da (CO_2) in MS^4 fragmentation.

Compounds **15** (t_R = 32.9 min) and **16** (t_R = 35.7 min), under fragmentation, showed a deprotonated molecular ion $[M-H]^-$ at *m/z* 327 and 329, corresponding to $C_{21}H_{30}O_3$ and $C_{21}H_{28}O_3$, respectively. Their MS^2 spectra presented an identical base peak ion at *m/z* 229. In MS^3 spectra, compound **10** showed a base peak at *m/z* 209 (loss of 20 Da) while compound **11** showed a base peak at *m/z* 211 (loss of 18 Da, probably H_2O). In MS^4, compounds **15** and **16** showed a based peak at *m/z* 166 (loss of 43 Da) and *m/z* 125 (loss of 86 Da, 2 times 43 Da), respectively. Since these compounds show a $[M-H]^-$ ion very identical (only differ in 2 Da) and a MS^n very similar, probably the only difference between these compounds is a double bond. Since these are relatively intense peaks in the chromatogram, further studies will be performed in order to determine their relevance for the antioxidant activity of the extract and to achieve their identification. A compound with [M−H] at *m/z* 329 with a similar pattern of fragmentation was detected but not identified in *Fagus sylvatica* and *Betula pendula* (Mammela, 2001).

3.3. HPLC-UV-DAD quantification of caffeoylquinic acids isomers

The three caffeoylquinic acids detected in the HPLC-ESI/MS^n screening were quantified by HPLC-DAD (λ = 320 nm) and Table 2 resumes the results obtained.

As so, 3,5-*O*-dicaffeoylquinic acid is the isomer present in highest amount at 59.69 ± 1.1 mg/100 g plant. This value represents ca. 69% of the total CQA compounds quantified.

5-*O*-dicaffeoylquinic acid level was about half of that found for 3,5-*O*-dicaffeoylquinic acid with a value at 22.40 ± 0.21 mg/100 g dry plant and 4,5-*O*-dicaffeoylquinic acid was found in very low amounts at 3.81 ± 0.031 mg/100 g dry plant, ca. 4.44% of the total amount of CQA isomers quantified.

5-*O*-dicaffeoylquinic acid was also quantified in *Hieracium pilosella* L. methanolic extracts (Stanojević et al., 2009) and the values obtained were ca. 10 times higher than those found *Andryala* flowers. However, the *H. pilosella* extract corresponded to the leaves and roots which are morphological parts, in general, with highest amounts of compounds.

3.4. Total phenolic content (TPC) and total flavonoid content (TFC)

The TPC was determined by the Folin–Ciocalteu method which is a rapid, simple and reproducible method widely used and results are shown in Table 3.

The ethanolic extract gave a remarkably higher TPC value when compared to similar plants, values at 621.2 ± 5.05 mg GAE/100 g. TFC value was also exceptionally high (328.7 ± 1.28 mg RUE/100 g). *H. pilosella*, a plant closely related to *A. glandulosa* was recently studied showing values in the order of 105 mg GAE/100 g and 35 mg RUE/100 g for TPC and TFC respectively (Stanojević et al., 2009).

3.5. Antioxidant capacities

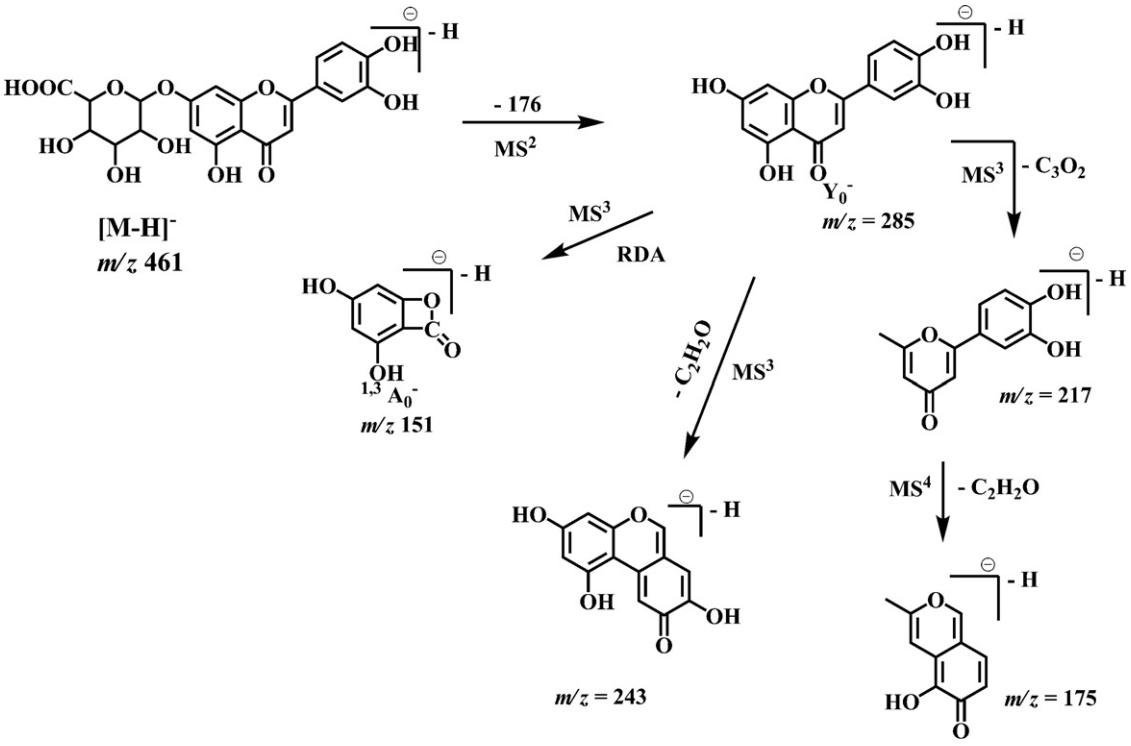
Antioxidants can reduce radicals primarily by two mechanisms: single electron transfer (ET) and hydrogen atom transfer (HAT).

The presence of different antioxidant compounds in the plant extracts makes it relatively hard to quantify each antioxidant activity component separately, so the selected method to measure the

Table 3
TPC, TFC, DPPH, ABTS and FRAP assays results for ethanolic extracts from flowers of *Andryala glandulosa* spp. *varia*.

Assay	Ethanol extract
Total phenolic content (mg GAE/100 g dried plant)	621.2 ± 5.05
Total flavonoid content (mg RUE/100 g dried plant)	328.7 ± 1.28
DPPH (μmol eq. Trolox/100 g dried plant)	1271 ± 10.5
ABTS (μmol eq. Trolox/100 g dried plant)	10,049 ± 139
FRAP (mmol Fe(II)/mg dried plant)	7151 ± 165
Yield (%)	25.2

Values represented as mean ± SD (n = 3).



Scheme 2. Proposed fragmentation pathway for compound 9.

antioxidant activity gave results relative to the total antioxidant activity of the whole sample.

The radical scavenging capacity (RSC) of the ethanol extract of the flowers from *A. glandulosa* was determined by two assays: DPPH and ABTS methods.

In the DPPH assay the methanol extract gave a value at 1271 ± 10.5 μmol eq. Trolox/100 g plant. While for the $ABTS^{\bullet+}$ assay the value obtained was 10,049 ± 138.8 μmol eq. Trolox/100 g plant (Table 3).

The ABTS assay values were higher than the correspondent values by the DPPH method. Higher values in the ABTS assay are mainly due to differences in the sensitivity of the method since ABTS assay measures the antioxidant activity of both hydrophilic and lipophilic antioxidants.

Cai et al. (2004) studied 112 traditional medicinal antioxidant plants (aqueous and alcoholic extracts) using the ABTS method. Comparing alcoholic extracts, *A. glandulosa* extract is one of the most active plants. Among the Asteraceae plants presented in that study, *Chysantheumum morifolium* Ramat is the most active with 731.6 μmol eq. Trolox/100 g dry plant, still more than 10 times less active than *A. glandulosa*. Special attention that studies of different labs must be compared in qualitative way since small variations in experimental conditions can affect drastically the results.

In the FRAP method, the yellow Fe^{3+}-TPTZ complex is reduced to the blue Fe^{2+}-TPTZ complex by electron-donating substances, under acidic conditions. Any electron donating substances with a half reaction of lower redox potential than Fe^{3+}/Fe^{2+}-TPTZ will drive the reaction and the formation of the blue complex forward. In this method the extract showed an increase of absorbance over time (Nilsson et al., 2005). *A. glandulosa*, already confirmed as being a good radical scavenger it was also a good source of compounds with reducing capacity as it can be concluded by the FRAP value at 7151 ± 165 mmol Fe(II)/mg.

Our group published recently a study with *Artemisia Argentea* alcoholic extracts (Gouveia and Castilho, 2011) using the same antioxidant assays described in this paper and we can conclude that *A. glandulosa* flowers extracts present the highest antioxidant capacity.

4. Conclusion

A. glandulosa grows in rather difficult to reach places, accessible only by narrow and sometimes dangerous footpaths. Despite this and the existence of other similar easier to gather plants, *A. glandulosa* extracts are gaining popularity, reaching the urban natural products shops at high prices. From this study it appears that the absence of potentially harmful lactones may be the key issue for the local collectors preference.

In this study, it was reported for the first time, the high antioxidant capacity associated to the characterization of the phenolic composition of the flowers from *A. glandulosa* spp. *varia*.

The results showed that the ethanolic extract was the strongest radical scavenger in both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays, while both extracts displayed in some extent good reducing capacity.

The HPLC-DAD-ESI/MS^n screening was used to determine the phenolic composition of the ethanolic extract of *A. glandulosa*.

Ten compounds were identified or characterized based on the MS^n fragmentation behavior, UV spectra and HPLC retention time making use of the described rules and published data.

The major compounds found in alcoholic extract were quinic acid and luteolin derivatives. The detection of luteolin derivatives is in good agreement with the high TFC value of this extract.

Three caffeoylquinic acid isomers were quantified and 3,5-*O*-dicaffeoylquinic acid was present in highest amounts (59.69 ± 1.07 mg/100 g of plant) which represents more than 69% of the amounts of these three compounds.

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