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Comparative HPLC-DAD-ESI(+)MS Fingerprint and Quantification of Phenolic and Flavonoid Composition of Aqueous Leaf Extracts of *Cornus mas* and *Crataegus monogyna*, in Relation to Their Cardiotonic Potential

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Abstract

Our study aimed to compare the phenolic and flavonoid composition of two medicinal plants from the wild flora of Romania, traditionally known to be efficient in preventing cardiotoxicity: *Cornus mas* and *Crataegus monogyna*. As most previous studies have focused on fruits, our investigations aimed to fingerprint and quantify the two classes of compounds in aqueous leaf extracts, based on HPLC-DAD-ESI(+)MS analysis. The specific HPLC-DAD fingerprint was coupled with UV spectra at 280 and 340 nm to discriminate between three subclasses of compounds: two hydroxycinnamic acids and a flavonoid glycoside. While the *C. monogyna* extract contained more than 98% vitexin isomers (2'- and 4'-O-rhamnoside), the *C. mas* extract was very complex, containing a mixture of phenolic derivatives and flavonoid glycosides. The spectral patterns of *C. mas*, combined with the molecular mass and specific fragmentations allowed the identification of epi-catechin, coumaric and caffeic acids and quercetin derivatives. Based on LC-MS peak area and parallel calibrations with gallic acid and rutin, the mean concentration for flavonoids in *C. mas* was 13 mg/100 ml, while aprox. 65 mg/100 ml in *C. monogyna* extracts. Using the Folin method, the total phenol content was 105 mg/100 ml in *C. mas* and around three times less in the *C. monogyna* extract, while the antioxidant activity was increased only 1.5-1.7 times in *C. mas* extract compared to *C. monogyna*. These findings suggest a higher stability and potential of flavonoids to act as antioxidants in hydrophilic environment.

Keywords: antioxidants, high performance liquid chromatography, medicinal plants, UV spectra, mass fragmentation, total phenolics

Introduction

Medicinal plants are used since centuries, as such or as ingredients in a large variety of herbal mixtures or food supplements to prevent different diseases. Their composition in bioactive molecules versus efficacy is a key problem, according to present quality requirements, needed worldwide for their authorization and standardization (Wichtl, 2004; Ramawat *et al.,* 2009; Verpoorte, 2009; Gong *et al.,* 2009; Yadav and Dixit, 2008).

Generally, leaves or fruits of medicinal plants are good sources of phenolics, especially flavonoid glycosides with high antioxidant capacity which may prevent or reduce the risk of heart disease (Andersen and Makham, 2006).

Crataegus species, with the generic name "hawthorn", are known traditionally to prevent and treat mild heart diseases, flavonoids such as vitexin derivatives being the main constituents responsible for their biological activities (Burta *et al.,* 2008). By their positive inotropic effect, *Crataegus* extracts stimulate the activation of heart muscle cells, also regulating the blood flow in the coronary arteries (Ammon and Haendel, 1981). *Crataegus* extracts were extensively tested in the last 50 years for their composition and cardiotonic activity in vitro (Ammon, 1981), especially the fruits but also the leaves (Celebi-Kocyildiz *et al.,* 2006). More than 20 species included in the genus Crataegus from Turkey were characterized and data about their fruit composition were reported (Meriçli and Ergezen, 1994, Melikoglu *et al.,* 1999, 2000, 2004; Dönmez, 2004). Few data about the composition of leaves were reported, e.g. five flavonoids were found in Crataegus davisii (hyperoside, vitexin 2"- rhamnoside, vitexin 4'-rhamnoside, rutin, quercetin) together with chlorogenic acid and crataequinone B (Sozer *et al.,* 2006). Belkhir *et al.*

(2013) reported hyperoside in *Crataegus monogyna* leaves while higher quantities of procyanidin B2 in fruits. Simirgiotis MJ (2013) identified 23 compounds in *Crataegus monogyna* fruits, mainly flavonoid glycosides, phenolic acids, anthocyanins and flavonoid aglycons and an antioxidant activity expressed as 3.61 ± 0.01 μg/mL by the DPPH assay. High total phenolic concentration of 55.2 mg gallic acid equivalents (GAE)/g dry weight and an antioxidant capacity of 81.9% was reported also by Çaliskan *et al.* (2012).

Hawthorn (*Crataegus monogyna* Jacq., Rosaceae Family) were subjected to evaluation as potential sources of antioxidant phytochemicals on the basis of their total content of phenolics, levels of phenolic acids, and in vitro antiradical activity (Öztürk and Tunçel, 2011). The highest total phenolic content (343.54 mg of gallic acid equivalents/g) and the highest DPPH radical scavenging activity as the inhibition percentage (60.36%) were obtained in ethyl acetate extract from hawthorn leaves with flower. In addition, the highest phenolic acid content was measured in the extracts of hawthorn leaves with flowers: protocathechuic (108-128 mg/100g), p-hydroxy benzoic (141-468 mg/100 g), caffeic (137-3,580 mg/100 g), chlorogenic (925-4,637 mg/100 g), ferulic (3,363-3,462 mg/100 g), vanillic (214 mg/100 g), and syringic (126 mg/100 g) acids. The ethnomedicinal and phytopharmacological potential of leaves, flowers, berries, bark of *Crataegus oxyacantha* Linn. was reviewed recently (Kashyap *et al*., 2012).

Cornus mas (Cornelian cherry dogwood) belongs to the family *Cornaceae* (Rop *et al.*, 2010) which grows in Asia and Europe, and is recently cultivated in Turkey (Demir, 2003; Kalyoncu *et al.*, 2009, Gulcin *et al.*, 2005; Ercisli, 2004) being highly tolerant to diverse abiotic and biotic conditions. It is also a medicinal plant with cardiotonic effect and anticancer potential, mentioned for the treatment of inflammation (Yilmaz *et al.*, 2009). The fruits are a rich source of phenolics (anthocyanins, flavonoids) and antioxidants (butyl hydroquinone, butylated hydroxyanisole and butylated hydroxytoluene), in addition to their high content of vitamins E, B2, B1, C, as well as oxalic acid (Pantelidis *et al.*, 2007; Gulcin *et al.*, 2005). In Turkey, the fruits are processed to produce syrups, juices, jams, spirits and other traditional products (Rop *et al.*, 2010; Kalyoncu *et al.*, 2009).

The cardioprotective effect of *Cornus mas* fruits extract was recently evaluated in a rat model with acute cardiotoxicity, induced by a single dose of carbon tetrachloride (CCl_4) (1 ml kg^{-1} intraperitoneal) (Eshaghi, 2012). Pre- and post-treatment with *C. mas* fruits extracts (300 and 700 mg kg^{-1}) significantly decreased the levels of serum lactate dehydrogenase, serum creatine kinase and myocardial lipid peroxides, increasing the myocardial endogenous antioxidants (glutathione peroxidase, superoxide dismutase and catalase). The results of biochemical observations in the serum and heart tissues were supplemented by histological examination of rats' heart sections to confirm the effect on myocardial injury. This study provides the first evidence that *Cornus mas* fruit extract exhibits cardioprotective properties alleviating myocardial injury and enhancing antioxidant defense against cardiotoxicity in rats (Eshaghi *et al.*, 2012).

Considering that antioxidants decrease peroxidation of membrane lipids, stimulate antioxidant enzymes in defense cascades and up-modulate the bioenergetic state of cardiac tissue, sources rich in flavonoids such as *Crataegus sp.* and *Cornus mas* may be used as preventive and curative agents against cardiotoxic drugs. Recently, the antioxidant activity of *Cornus mas* fruits was investigated by various antioxidant assays in Turkey (Tural and Koca, 2008; Ersoy *et al.*, 2011), in Greece (Pantelidis *et al.*, 2007) and in other countries (Pawlowska *et al.*, 2010; Popovic *et al.*, 2012). The DPPH scavenging activity was high and significantly increased by salycilic acid treatment, due to significant increase of the total phenols, flavonoids, anthocyanins and ascorbic acid content, and of phenylalanine ammonia-lyase (PAL) enzyme activity (Dokhanieh *et al.*, 2013). Capanoglu *et al.* (2011) investigated the phytochemical composition of Sour cherry (*Prunus cerasus*), Laurel cherry (*Prunus lauracerasus*), and Cornelian cherry (*Cornus mas*) fruits in relation to the antioxidant capacities. Fruit flesh was evaluated for procyanidin concentration, subunit composition and degree of polymerization, for anthocyanin composition and for total antioxidant capacity, total phenolic content and total flavonoid content. High concentrations (up to 1 g per 100 g dry weight) of long-chain procyanidins were found in Laurel cherry, whereas concentrations of procyanidins in Cornelian cherry were 25 times lower.

Polyphenolic derivatives, some of the most important phytochemicals found in medicinal plants, are a large family of secondary metabolites with various roles in plant defense and with demonstrated antioxidant activity and beneficial health effects (Scalbert *et al.*, 2005; Halliwell, 2007). Important progress was registered in the last decade regarding their extraction, identification and quantification of bioactive molecules, needed for an adequate quality control of herbal medicines (Fan *et al.*, 2006; Gong *et al.*, 2006, 2009; Giri *et al.*, 2010). Many analytical protocols to separate and identify phenolic acids, flavonoids and their glucosides, catechins, tannins (Santos-Buelga and Williamson, 2003) or to quantify total phenolics (Singleton, 1999) have been elaborated.

The most advanced technique used today is high performance liquid chromatography (HPLC) coupled with mass spectrometry (MS), a powerful identification technique, based on specific fragmentation of each separated molecule (Socaciu *et al.*, 2009; Mattoli *et al.*, 2011; Khoddami *et al.*, 2013). Although organic solvent (methanol) extraction is the main method used to extract phenolics, for medical studies aqueous extraction using microwaves or ultrasounds is an ecologic alternative. The resulted extracts can be tested either *in vitro* or *in vivo* without unwanted side effects. Many sources indicate good correlations between phenolics' content and antioxidant capacity evaluated by different techniques (Benzie and Strain, 1999; Brand-Williams *et al.*, 1995).

Crataegus monogyna and *Cornus mas* are common species of Romanian flora, traditionally known as cardiotonics, characterized until now especially for their total phenolic content and antioxidant activity in relation

to their nutritional qualities (Nichita *et al.*, 2009; Stef *et al.*, 2010; Rosu *et al.*, 2011; Rizea *et al.*, 2012).

This study aimed to characterize comparatively the cardioprotective potential of *Cornus mas*, compared to *Crataegus monogyna*, by their specific fingerprint in aqueous leaf extracts, to quantify their phenolic concentration and to elucidate in detail their phenolic composition using advanced chromatographic analysis coupled with diode-array detection (280 and 340 nm) and ESI (+) Mass spectrometry. The antioxidant capacity of these extracts was also determined and correlated with the phenolic composition and concentration.

Materials and methods

Plant materials and sample preparation

Fresh leaves of *Cornus mas* and *Crataegus monogyna* were harvested from the Transylvanian hilly region in June 2012. Triplicate aliquots of 15 g fresh leaves (stored at -20°C before analysis) were used to prepare infusions in 100 ml distilled water in a sonication bath for 30 min, and then centrifuged 10 min at 2000 rot/min. The supernatant was collected and filtered through a 0.45 μm filter before UV-Vis, HPLC-DAD –ESI (+)-MS analysis.

Total phenolics determination

To determine the concentration of total phenolic derivatives, 5 ml Folin reagent was added to aliquots of 1 ml filtered extracts from each plant, homogenized and then mixed with 15 ml Natrium carbonate 7,5%, according to the method Folin Ciocâlteu adapted by Singleton (1999). After 2 h, the absorbance of the solution was recorded at λ = 750 nm against a blank. In parallel, using the same technique, a calibration curve was built using pure gallic acid as standard, in the range of 10 to 150 mg/100 ml distilled water. The results were expressed in mg gallic acid equivalents (GAE)/100 ml extract.

Antioxidant activity determined by DPPH method

DPPH radical is used to determine the antioxidant activities of many plant extracts and compounds (Brand-Williams *et al.*, 1995), the method being based on a decrease of absorption of the dark violet DPPH solution in the presence of an antioxidant which acts as H donor. Briefly, aliquots of 100 μl of each sample were mixed with 1.4 ml of DPPH solution (80 μM) and 1 ml ethanol.

The homogenate was shaken vigorously and the decrease in the absorbance of the resulting solution was monitored at 515 nm for 5 min on a spectrophotometer (Jasco V 530) The percentage of scavenging effect of different extracts against DPPH radicals was calculated using the following equation:

DPPH scavenging effect (%) = $[(A_0 - A_s) \times 100] / A_0$

where A_0 is absorbance of the blank, and A_s is sample absorbance.

The capacity of extracts to scavenge the DPPH radical was expressed also in mmol Trolox eq. ml^{-1} d.w.

HPLC-DAD-ESI(+) MS Analysis

The triplicate extracts from each plant were filtered by a Millipore filter of 0.45 μm and injected in a Eclipse column, XDB C_{18}, (4.6 × 150 mm, 5 μm) at 25 °C, using 20 μl injections from each sample.

The separation was made on a Agilent 1200 HPLC device with Diode Array (DAD) coupled with MS detector single quadrupole Agilent 6110. The mobile phases were water: 0.1% acetic acid in acetonitrile (99:1) (solvent A) and 0.1% acetic acid in acetonitrile (solvent B) at a flow of 0.5 ml min^{-1}.

The gradient applied was as follows: % B = 5% (min 0-2), from 5% to 40% (min 2-18 min), from 40% to 90% (min 18-20), then isocratic 4 min and decrease from 90% to 5% (min 24-25). For MS fragmentation, the ESI(+) module was applied, with a capillary voltage of 3,000 V, at 350°C and nitrogen flow of 8 l min^{-1}. Two levels of energy were used to obtain 50 or 100 fragments in the range m/z: 100-1000 Da.

To identify the peaks separated by HPLC-DAD, a mix of 15 pure standards of phenolic derivatives (provided from Sigma Aldrich) was used in parallel, their identification being made by comparison with HPLC chromatograms of pure individual phenolic acids (Tab. 1).

Statistical Analysis

Each sample was analyzed in triplicate (n=3) and all analyses were made in duplicate. Data were subjected to one-way analysis of variance (*ANOVA*) and comparison between means was determined according to Tukey's test. Significant differences were accepted at $P \leq 0.05$. All the statistical tests were generated with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com).

Results and discussion

Total phenolics and antioxidant capacity

Total phenolics in both *Crataegus monogyna* and *Cornus mas* extracts, expressed in gallic acid equivalents (mg GAE/100 ml), as well as the antioxidant capacity expressed as DPPH scavenging effect (%) and in equivalents of Trolox (mmols Trolox ml^{-1} extract) were demonstrated (Tab. 1). Total phenolic content was significantly superior in *Cornus* vs *Crataegus* extract, (around three times, $P<0.005$). Considering the 15% of leaf concentration in the extract, the corresponding concentrations expressed in GAE/100 g leaf powder were 700 mg GAE/100 g *Cornus mas* and 206.6 mg/ 100 g *Crataegus monogyna*. The antioxidant activity was also significantly higher (1.5-1.7 times) for *Cornus* vs. *Crategus* extract.

Our data are in good agreement with other reported results (Roşu et al., 2011, Stef et al., 2010, Scalbert, 2005), although difficult to compare, the extracts being obtained in methanol, which has a higher extraction potential and mainly from fruits and not leaves. Rop et al. (2010) who found in Cornus mas mean values of 800 mg GAE/ 100 g of

fresh mass, with high correlations between polyphenols and antioxidant activity in fruits of the cultivars was observed (r^2 = 0.970 for DPPH test. Yilmaz *et al.* (2009) found also total phenolics of 7480 mg GAE/100 g dry fruit, while Capanoglu *et al.* (2011) found procyanidins B of around 40 mg/100 g fresh fruit.

HPLC–DAD-ESI(+)MS separation and calibration of a mixture of pure phenolics and flavonoids

HPLC-DAD chromatogram of a mixture of 15 different phenolic molecules (aglycons) found in medicinal plants (Fig. 1). The HPLC-DAD-ESI(+)MS data obtained after the separation of the phenolic derivatives which had the same concentration each, 6.66 µg ml^{-1} (Tab. 2). The

Tab. 1. Total phenolics in the extracts (mg GAE /100 ml), the antioxidant capacity expressed as DPPH scavenging effect (%) and in mmol Trolox eq ml^{-1}. All data were obtained from triplicate samples

Sample extract	mg GAE/100 ml	DPPH scavenging effect (%)	mmol Trolox eq. ml^{-1}
Cornus mas	105.03 ± 7.28	79 ± 3.55	0.260 ± 0.003
Crataegus monogyna	31.50 ± 2.60	50 ± 2.20	0.156 ± 0.001

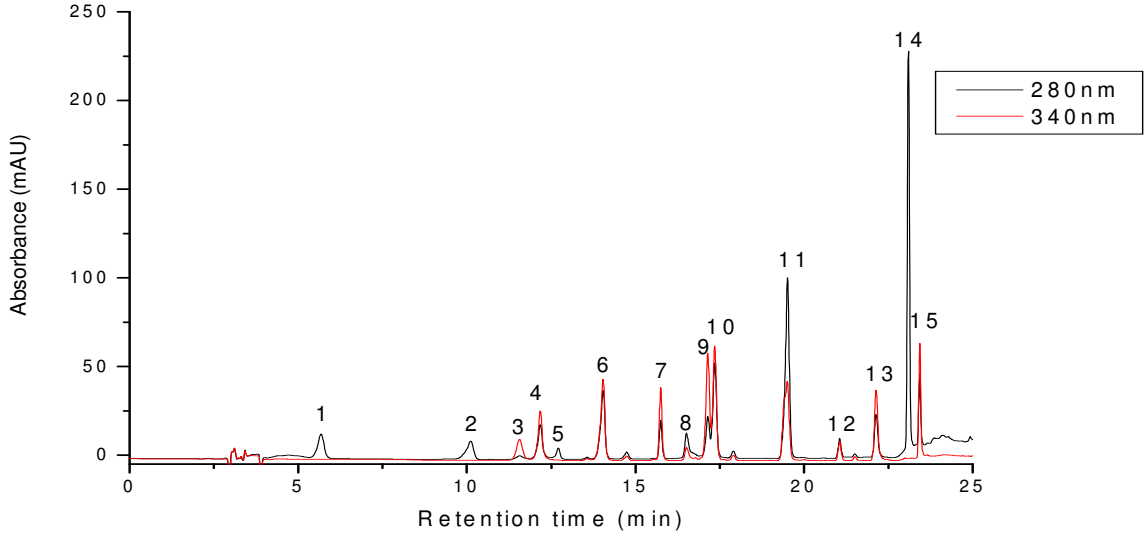

Fig. 1. HPLC-DAD separation of 15 pure standards of phenolic acids and flavonoids at similar concentrations and their comparative absorption intensities at 280 and 340 nm, useful to discriminate between phenolic and flavonoid structures

Tab. 2. HPLC-DAD-ESI(+) MS data of a mixture of 15 phenolic derivatives (phenolic acids and flavonoids) containing 6.66 µg of each molecule/ml extract: Retention time (t_R- min), UV absorptions (λ_{max}, nm) and peak areas at 280 and 340 nm, MS data $[M-H]^+$, expressed in m/z

Peak Nr.	tR (min)	λmax (nm)	[M-H]+ (m/z)	Identified phenolics	Category
1	5.6	280	171, 153	Gallic acid	Phenolic acid
2	10.1	270, 300	155	Protocatequic acid	Phenolic acid
3	11.5	330	155	Gentisic acid	Phenolic acid
4	12.1	250, 340	355, 163	Chlorogenic acid	Phenolic acid
5	12.7	280	291	Catechin	Flavan 3 ol
6	14.0	250, 340	181, 163	Caffeic acid	Phenolic acid
7	15.7	250, 360	611, 475	Rutin	Glycosidic flavonoid
8	16.5	250, 370	303, 284	Elagic acid	Phenolic acid
9	17.1	240, 340	225	Sinapic acid	Phenolic acid
10	17.3	240, 330	195	Ferulic acid	Phenolic acid
11	19.5	250, 360	319	Myricetin	Flavonol
12	21.0	280, 330	595	Tilirosid	Glycosidic flavonoid
13	22.1	260, 370	303	Quercetin	Flavonol
14	23.1	280	149	Trans-cinnamic acid	Phenolic acid
15	23.4	260,370	287	Kaempherol	Flavonol

separation was done by DAD detection at 280 and 340 nm, and indicates the differences in molar absorptions specific to phenolic acids compared to flavonoids. According to their specific absorptions in UV (250-280 nm and/or 340-370 nm) the increase of peak intensity for flavonoids (peaks 7, 11, 12, 13 and 15) is obvious. These data were used to determine the molar absorptions of molecules at 280 and 340 nm, under specific separation conditions.

The molecular mass specific to each molecule and a main fragment resulted from MS data $[M-H]^+$, expressed in m/z were represented as Tab. 2. These data were useful to identify and characterize the LC-ESI(+)MS data obtained in *Crataegus monogyna* and *Cornus mas* extracts.

HPLC-DAD-ESI (+)MS analysis of Crataegus monogyna and Cornus mas extracts

The data obtained from the aqueous extract of *Crataegus monogyna* leaf, namely the HPLC-DAD chromatograms, registered at 280 and 340 nm (A), the UV spectra specific to the major peaks separated (B) and the MS spectra corresponding to these two peaks (C) were demonstrated in Fig. 2.

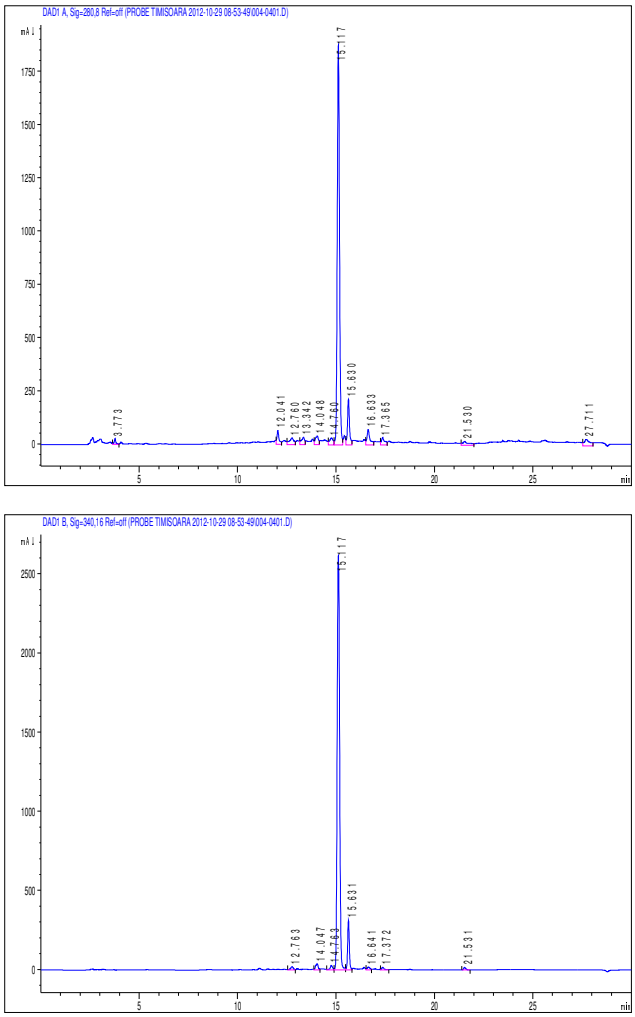

Fig. 2. A. HPLC-DAD chromatogram of the *Crategus* extract, registered at 280 nm (left) and 340 nm (right)

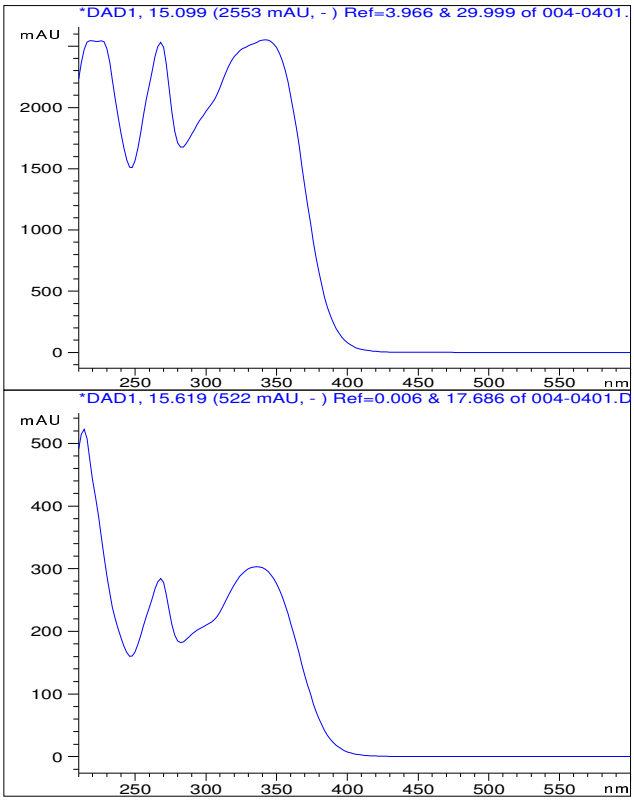

Fig. 2. B. UV spectra corresponding to the peaks with t_R=15.117 min (left) and 15.631 min (right)

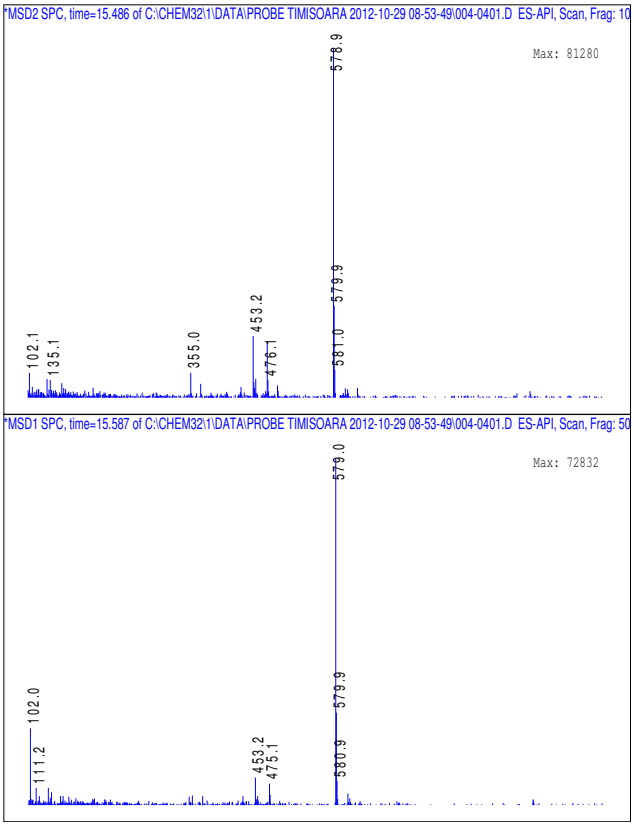

Fig. 2. C. MS spectra corresponding to the peaks found at t_R=15.117 min (left) and 15.631 min (right). Main fragments: m/z= 578.9, 476.1, 453.2 (left); m/z= 579.0, 475.1, 453.2 (right)

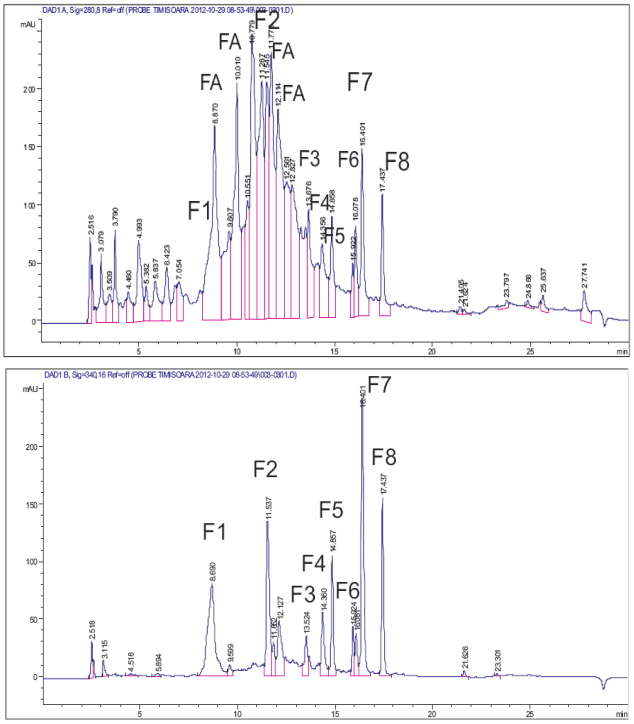

Fig. 3. A. HPLC-DAD chromatograms of the *Cornus mas* extract, at 280 nm (left) and 340 nm (right)

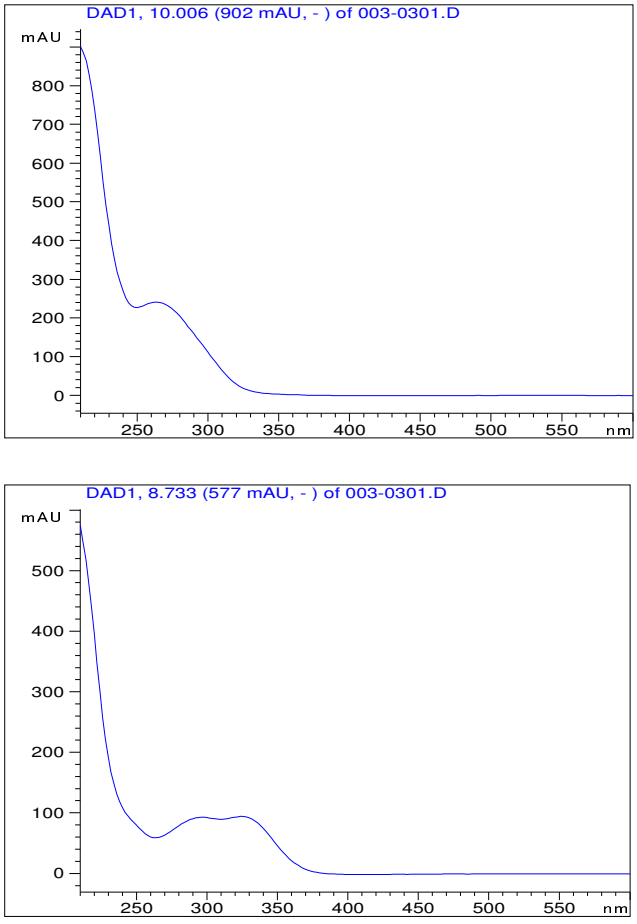

Fig. 3. B. UV spectra corresponding to the peak F1 (left, t_R=8.690 min λmax = 282, 340 nm) and FA (right, t_R=10.010 min, λmax = 280 nm)

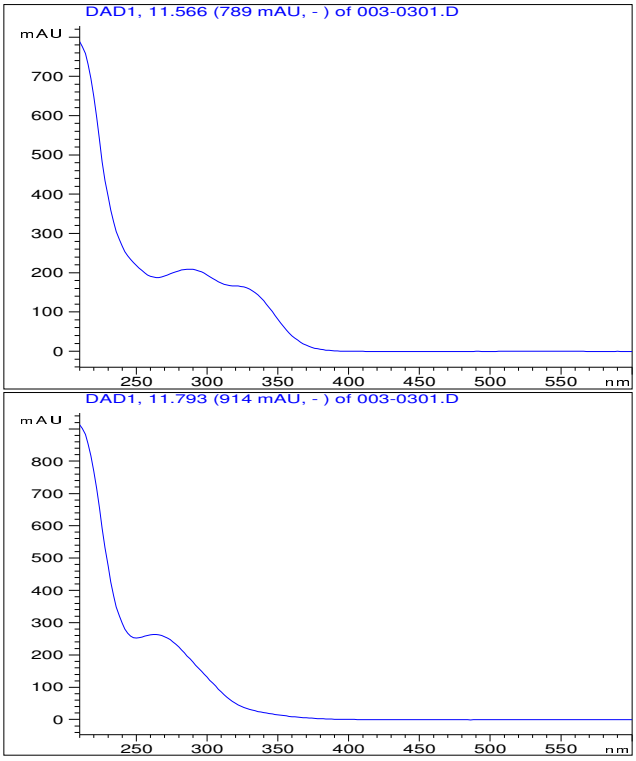

Fig. 3. C. UV spectra corresponding to peaks F2 (t_R=11.537 min) (left, max = 280, 330 nm) and FA (right, t_R=11.778 min, max = 280 nm)

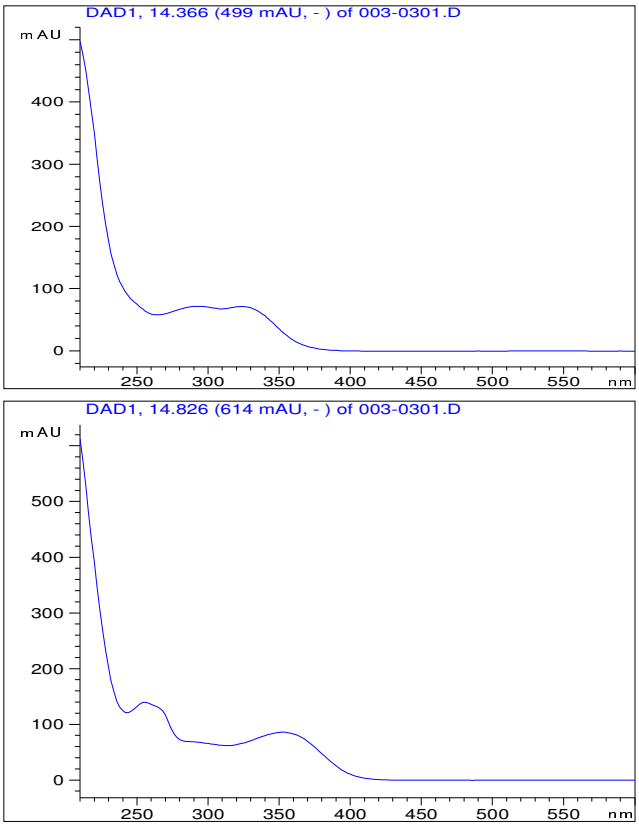

Fig. 3. D. UV spectra corresponding to the peaks F4 (left, t_R=14.366 min, λmax = 280, 340 nm) and F5 (right, t_R=14.867 min, λmax = 280, 360 nm)

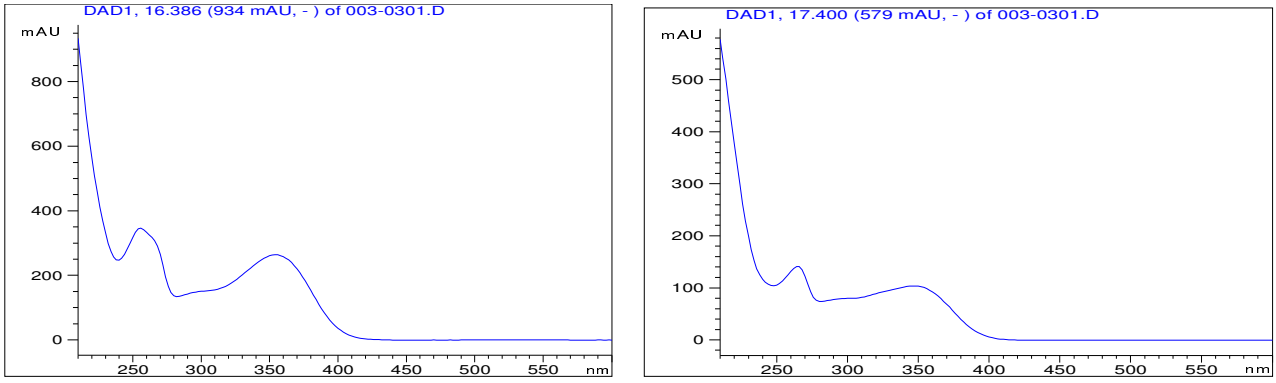

Fig. 3. E. UV spectra corresponding to the peaks F7 (left, t_R=16.401 min, max = 280, 360 nm) and F8 (right, t_R=17.437 min, λmax = 280, 360 nm)

Tab. 3. LC-MS data of *Crategus leaf* extract: Retention times (tR- min), UV max. absorptions (nm), peak areas at 280 and 340 nm (mAU), concentrations of flavonoids MS data (m/z). According to the tR, absorption spectra and m/z values. Total phenolics and flavonoid concentrations were calculated by comparison with calibration curves obtained with rutin

t_R (min)	UV λ_{max} (nm)	Area $_{280nm}$ (mAU)	Area $_{340nm}$ (mAU)	Total flavonoids (mg/100 ml)	$[M+H]^+$ (m/z)	Identification
15.117	270, 360	14980.6	22540.2	59.95	578, 476, 453.2	Vitexin 2'-O-rhamnoside
15.631	260 360	1487.9	1868.7	4.97	579.1; 475.1, 453	Vitexin 4'-O-rhamnoside
	Sum	16468.5	24408.9	64.92		

Tab. 4. LC-MS data of *Cornus mas* leaf extract: Retention times (t_R- min), UV absorptions (nm), peak areas at 280 and 340 nm (mAU), concentrations of total phenolics and flavonoids, as calculated by comparison with calibration curves obtained with gallic acid and rutin, respectively. The identifications were based on MS spectra and fragments m/z values. Minor peaks (representing 40 mg ml^{-1}) were eliminated from calculation of total phenolics.

t_R (min)	UV λ_{max} (nm)	Peak area (280 nm)	Peak area (340 nm)	Total phenolics (mg/100ml)	Total flavonoids (mg/100ml)	$[M-H]^+$ (m/z)	Identification
8.690	282; 330	4192.3	2127,8	13.92	-	329.7, 223.7, 181, 163.0, 156	F1 – p-coumaric acid derivative
10.010	265	3643.2	-	12.13	-	352, 181, 163	FA1 – Acid 5-caffeoyl quinic (Chlorogenic acid)
11.2	270	4109.2	-	13.68	-	291, 207, 181	FA2 - Epi-Catechin
11.537	282; 330	2627.5	1587.8	8.75	-	359, 181, 163, 156	F2 - o-coumaric acid hexoside
11.778	265	3569.3	-	11.88	-	352, 181, 163	FA3 – Caffeic acid derivative
13.524	280; 340	1378.2	359.1	4.55	-	225.1, 209.1, 166.0, 149, 135.0, 130	F3 – Caffeic acid hexoside I
14.366	280; 340	1288.8	612.1	-	1.63	236.6, 181, 166.0, 149, 135.0, 130.0	F4 – Caffeic acid hexoside II
14.867	260; 360	1003.6	777.3	-	2.07	610.8, 478.8, 352.2, 302.9, 229.1, 202.1	F5 – Quercetin-3-O-galactoside, 7-O-rhamnoside
16.081	260; 365	711.7	299.1	-	0.80	463, 294.4, 283.6, 252.0	F6 – Isorhamnetin 7-rhamnoside
16.401	260360	1632.4	2148.1	-	5.71	478.8, 302.6, 283.5, 217.9, 166.2	F7 - Quercetin 3-glucuronide
17.437	260 360	1019.9	1112.5	-	2.96	462.9, 352.1, 207.0, 164.0, 130.1	F8- Kaempherol 3-glucuronide
	Sum	31421.6	4949.1	64.9	13.2		

One can notice only 2 molecules at higher concentrations, the one with t_R=15.117 min being the major component, and a second one at t_R=15.631 min. Comparing the UV spectra of the two molecules, the absorption intensity was higher at 340 nm, around 1.5 times than the maxima from 270 nm, indicating the flavonoid structure. The MS spectra highlight for the major peak a molecular ion of m/z=579.0 and two main fragments with m/z= 476.1 and 453.2, while for the second peak, a molecular ion of 578.9 nm and two main fragments with m/z= 475.1 and 453.2. The strong similarity between their molecular mass and fragmentation suggested that both molecules are isomers of vitexin rhamnoside, namely the first one vitexin 2'-O-rhamnoside and the second one, vitexin 4'-O-rhamnoside. The ratios of peak areas, as presented in Tab. 3, were 12:1 and the total content, as determined by calibration with rutin was 64.92 mg flavonoids/100 ml extract.

The data obtained from the aqueous extract of *Cornus mas* leaf, namely the HPLC-DAD chromatograms, registered at 280 and 340 nm (A) and the UV spectra (Fig. 3B-E) specific to the major flavonoid peaks F1, F2, F4, F5, F7, F8 recorded at 340 nm (Fig. 3A) and phenolic derivative peaks FA recorded at 280 nm (Fig. 3A), at 10.010 min and 11.778 min.

In agreement with the previous data obtained with pure standards (Fig. 1 and Tab. 2), a complex mix of peaks is noticed clearly at 280 nm, which are not well separated, especially in the time range from 10 to 15 min, where mixes of phenolic and flavonoid derivatives absorb strongly. A clear image which can discriminate the flavonoids from phenolics was presented in Fig. 3A right, where the F1-F8 molecules can be easier identified than in the left chromatogram.

According to UV spectra and maxima identified in the two regions (280 and 340 nm), one can discriminate three types of molecules: three phenolic acid (FA) molecules with absorption at 270-280 nm (t_R= 10.010, 11.537 and 11.778 min) and other molecules named F1-F8 with absorptions at 260-280 and 330-365 nm, but with different fingerprints for F1, F2, F4 (characteristic to hydroxycinnamic acid derivatives) and for F5, F7, F8, respectively, typical for flavonol glycosides (Fig. 3B-E). Based on these data, we made first assignments on the identification of flavonoids or phenolic derivatives.

More precise data, namely details about the HPLC-ESI(+) MS analysis (retention time, UV maxima, peak areas and quantification of phenolics and flavonoids concentrations, as well as identification of these molecules are presented in Tab. 4. Based on the previous spectral data from HPLC-ESI(+) MS analysis, the molecules were classified in three categories: FA1-FA3 (molecules which absorb only at 260-280 nm), F1-F4 (phenolic molecules which absorb at 260 and 340 nm) and F5-F8 (flavonoid glycosides), as mentioned in Tab. 4.

For the first two groups of molecules FA1-FA3 and F1-F4, based on the individual peak areas and using a calibration curve with pure gallic acid (at different concentrations, from 10 to 150 mg/100 ml) the total phenolic concentration was calculated, reaching a value of 64.9 mg/100 ml, other non-identified peaks (representing 40 mg ml^{-1}) being eliminated from the calculation of total phenolics (Tab. 4). Meanwhile for the group of flavonoid derivatives, using a calibration curve with pure rutin (at different concentrations, from 1 to 50 mg/100 ml) the total concentration was calculated to be 13.17 mg/100 ml. Finally the extract of *Cornus mas* had a total quantity of 78.0 mg phenolic derivatives and flavonoids, a value which was close to the concentration of total phenolics determined by Folin method, considering that non-identified peaks were eliminated (Tab. 1). This value was close to the flavonoids concentrations found in the *Crataegus* extract (64.92 mg/100 ml) (Tab. 3) and may explain a strong antioxidant capacity of *Cornus mas*, due to its phenolics composition and content.

Regarding the MS-based identification of each molecule, consisting in the fragmentation model, possible formulas of phenolic or flavonoid derivatives were assigned. Compounds F1 and F2 were assigned to be a glycoside of p-coumaric acid, FA1, chlorogenic acid and FA2, epi-catechin. Compounds FA3, Fe3 and F4 may correspond to caffeic acid derivatives while F5 and F7 to quercetin derivatives, Quercetin-3-O-galactoside, 7-O-rhamnoside and Quercetin-3-glucuronide, respectively. The different fragmentation of F6 indicated a possible assignment as Isorhamnetin 7-rhamnoside, while F8- as Kaempherol 3-glucuronide.

These data are in agreement with previous results reported for the leaves of *Crataegus davisii,* where five flavonoids (hyperoside, vitexin 2″-rhamnoside, vitexin 4′-rhamnoside, rutin, quercetin) together with chlorogenic acid and crataequinone B were isolated. The flavonoid content of the leaves was found as 1.09 % and the antioxidant activity expressed by IC_{50} value was found as 1.57 mg/ml by DPPH method (Sozer *et al*., 2006).

In *Cornus mas*, on the basis of HPLC-PDA-MS/MS^n analysis eight compounds have been identified as quercetin, kaempferol, and aromadendrin glycosilated derivatives. Three major compounds have been also isolated by Sephadex LH-20 column chromatography followed by HPLC and characterised by NMR spectroscopy. Moreover, LC-PDA-MS analysis of the red pigment revealed the presence of three anthocyanins, their quantitative analysis being reported recently (Pawlowska *et al*., 2010).

Out of these peaks, the major percentage is represented by caffeic acid derivatives (among phenolic derivatives) and Quercetin glycosides (among flavonoid derivatives), in agreement with the flavonoid biosynthesis pathways *via* p-coumaroyl-caffeoyl derivatives converted to eriodictyol, dihydroquercetin, quercetin and its glycosides (KEGG database, 2013).

Finally, we consider that *Cornus mas* leaves are a rich source of mixed hydrophilic phenolic derivatives and flavonoids, compared to *Crataegus monogyna* leaves, which have few and major flavonoids, namely two vitexin isomers. Parallel studies are comparing the composition of these antioxidant aqueous extracts and their cardiotonic activity in vitro, in order to establish possible structure - activity relationships.

Conclusion

The data presented in this study demonstrate how HPLC-DAD-ESI (+)MS analysis may be useful to fingerprint and quantify phenolic and flavonoid molecules in aqueous leaf extracts of *Crataegus monogyna* and *Cornus*

mas, both plants being known to contain hydrophilic antioxidants with cardiotonic activity. While *Crateagus monogyna* extract contained mainly vitexin isomers (2' and 4' –O-rhamnoside), *Cornus mas* extract was very complex, containing a mixture of phenolic derivatives and flavonoid glycosides. The spectral patterns combined with m/z values of molecular ions and specific fragmentations allowed the identification of different categories of phenolics and flavonoids in *Cornus mas*, mainly epi-catechin, derivatives of coumaric, caffeic acid, and, respectively, quercetin kaempherol and derivatives.

HPLC-DAD-ESI (+)MS analysis may accurately determine each molecule content, based on peak area and parallel calibration with gallic acid for phenolic derivatives and rutin for flavonoids. Phenolics concentration reached values of 104 mg/100 ml and flavonoids of 13 mg/100 ml in *Cornus mas*, while 65 mg flavonoids in *Crataegus monogyna*, in a significant positive correlation the total phenol content, as determined by Folin method (105 mg/100 ml in *Cornus mas* and around three times less in *Crataegus monogyna* extract). Meanwhile, the antioxidant activity was only 1.5-1.7 increased in *Cornus mas*, suggesting a higher stability and potential of flavonoids to show scavenging potential against DPPH and to act as antioxidants in hydrophilic environment.

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