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; Dönmmez, 2004). Few data about the composition of leaves were reported, e.g. five flavonoids were found in *Crataegus davisi* (hyperoside, vitexin 2’- rhamnoside, vitexin 4’- rhamnoside, rutin, quercetin) together with chlorogenic acid and crataequinone B (Sozer et al., 2006). Belkhir et al.
tetrachloride (CCl₂) was recently evaluated in a rat model with acute myocardial injury and enhancing antioxidant defense (Çaliskan et al., 2012). High total phenolic content of 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: protocatechuic (108-128 mg/100g), p-hydroxy benzoic (141-468 mg/100g), 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 cardioprotective 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 (butylhydroquinone, 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 (CCl₄) (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 cardiototoxicity 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 salicylic 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 laurocerasus), 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:

\[
\text{DPPH scavenging effect (\%)} = \frac{[(A_0 - A) \times 100]}{A_0}
\]

where \(A_0\) is absorbance of the blank, and \(A\), 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 x 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<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. Crataegus extract.

Our data are in good agreement with other reported results (Roșu 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.

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

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

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 \(\mu g\) of each molecule/ml extract: Retention time \((t_R\) min), UV absorptions \(\lambda_{max}\) nm and peak areas at 280 and 340 nm, MS data \([\text{M-H}]^+\) expressed in \(m/z\)

<table>
<thead>
<tr>
<th>Peak Nr.</th>
<th>(t_R) (min)</th>
<th>(\lambda_{max}) (nm)</th>
<th>([\text{M-H}]^+) (m/z)</th>
<th>Identified phenolics</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.6</td>
<td>280</td>
<td>171, 153</td>
<td>Gallic acid</td>
<td>Phenolic acid</td>
</tr>
<tr>
<td>2</td>
<td>10.1</td>
<td>270, 300</td>
<td>155</td>
<td>Protocatequic acid</td>
<td>Phenolic acid</td>
</tr>
<tr>
<td>3</td>
<td>11.5</td>
<td>330</td>
<td>155</td>
<td>Gentisic acid</td>
<td>Phenolic acid</td>
</tr>
<tr>
<td>4</td>
<td>12.1</td>
<td>250, 340</td>
<td>355, 163</td>
<td>Chlorogenic acid</td>
<td>Phenolic acid</td>
</tr>
<tr>
<td>5</td>
<td>12.7</td>
<td>280</td>
<td>291</td>
<td>Catechin</td>
<td>Flavan 3 ol</td>
</tr>
<tr>
<td>6</td>
<td>14.0</td>
<td>250, 340</td>
<td>181, 163</td>
<td>Caffeic acid</td>
<td>Phenolic acid</td>
</tr>
<tr>
<td>7</td>
<td>15.7</td>
<td>250, 360</td>
<td>611, 475</td>
<td>Rutin</td>
<td>Glycosidic flavonoid</td>
</tr>
<tr>
<td>8</td>
<td>16.5</td>
<td>250, 370</td>
<td>303, 284</td>
<td>Elagic acid</td>
<td>Phenolic acid</td>
</tr>
<tr>
<td>9</td>
<td>17.1</td>
<td>240, 340</td>
<td>225</td>
<td>Sinapic acid</td>
<td>Phenolic acid</td>
</tr>
<tr>
<td>10</td>
<td>17.3</td>
<td>240, 330</td>
<td>195</td>
<td>Ferulic acid</td>
<td>Phenolic acid</td>
</tr>
<tr>
<td>11</td>
<td>19.5</td>
<td>250, 360</td>
<td>319</td>
<td>Myricetin</td>
<td>Flavonol</td>
</tr>
<tr>
<td>12</td>
<td>21.0</td>
<td>280, 330</td>
<td>595</td>
<td>Tilirosid</td>
<td>Glycosidic flavonoid</td>
</tr>
<tr>
<td>13</td>
<td>22.1</td>
<td>260, 370</td>
<td>303</td>
<td>Quercetin</td>
<td>Flavonol</td>
</tr>
<tr>
<td>14</td>
<td>23.1</td>
<td>280</td>
<td>149</td>
<td>Trans-cinnamic acid</td>
<td>Phenolic acid</td>
</tr>
<tr>
<td>15</td>
<td>23.4</td>
<td>260,370</td>
<td>287</td>
<td>Kaempherol</td>
<td>Flavonol</td>
</tr>
</tbody>
</table>
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. 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, $\lambda_{max} = 282, 340$ nm) and FA (right, $t_R=10.010$ min, $\lambda_{max} = 280$ nm).

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

Fig. 3. D. UV spectra corresponding to the peaks F4 (left, $t_R=14.366$ min, $\lambda_{max} = 280, 340$ nm) and F5 (right, $t_R=14.867$ min, $\lambda_{max} = 280, 360$ nm).
Fig. 3. E. UV spectra corresponding to the peaks F7 (left, t<sub>R</sub>=16.401 min, λ<sub>max</sub> = 280, 360 nm) and F8 (right, t<sub>R</sub>=17.437 min, λ<sub>max</sub> = 280, 360 nm)

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

<table>
<thead>
<tr>
<th>t&lt;sub&gt;R&lt;/sub&gt; (min)</th>
<th>UV λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>Area 280min (mAU)</th>
<th>Area 340min (mAU)</th>
<th>Total flavonoids (mg/100 ml)</th>
<th>[M+H]&lt;sup&gt;+&lt;/sup&gt; (m/z)</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.117</td>
<td>270, 360</td>
<td>14980.6</td>
<td>22540.2</td>
<td>59.95</td>
<td>578, 476, 453.2</td>
<td>Vitexin 2’-O-rhamnoside</td>
</tr>
<tr>
<td>15.631</td>
<td>260, 360</td>
<td>1487.9</td>
<td>1868.7</td>
<td>4.97</td>
<td>579.1; 475.1, 453</td>
<td>Vitexin 4’-O-rhamnoside</td>
</tr>
<tr>
<td>Sum</td>
<td>16468.5</td>
<td>24408.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tab. 4. LC-MS data of <i>Corus mas</i> leaf extract: Retention times (t<sub>R</sub>- 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<sup>-1</sup>) were eliminated from calculation of total phenolics.

<table>
<thead>
<tr>
<th>t&lt;sub&gt;R&lt;/sub&gt; (min)</th>
<th>UV λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>Peak area (280 nm)</th>
<th>Peak area (340 nm)</th>
<th>Total phenolics (mg/100ml)</th>
<th>Total flavonoids (mg/100ml)</th>
<th>[M-H]&lt;sup&gt;-&lt;/sup&gt; (m/z)</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.690</td>
<td>282; 330</td>
<td>4192.3</td>
<td>2127.8</td>
<td>13.92</td>
<td>-</td>
<td>329.7, 233.7, 181, 163, 156</td>
<td>F1 – p-coumaric acid derivative</td>
</tr>
<tr>
<td>10.010</td>
<td>265</td>
<td>3643.2</td>
<td>-</td>
<td>12.13</td>
<td>-</td>
<td>352, 181, 163</td>
<td>FA1 – Acid 5-caffeoyl quinic (Chlorogenic acid)</td>
</tr>
<tr>
<td>11.2</td>
<td>270</td>
<td>4109.2</td>
<td>-</td>
<td>13.68</td>
<td>-</td>
<td>291, 207, 181</td>
<td>FA2 – Epicatechin</td>
</tr>
<tr>
<td>11.537</td>
<td>282; 330</td>
<td>2627.5</td>
<td>1587.8</td>
<td>8.75</td>
<td>-</td>
<td>359, 181, 163, 156</td>
<td>F2 – Caffeic acid hexoside</td>
</tr>
<tr>
<td>11.778</td>
<td>265</td>
<td>3569.3</td>
<td>-</td>
<td>11.88</td>
<td>-</td>
<td>352, 181, 163</td>
<td>FA3 – Caffeic acid derivative</td>
</tr>
<tr>
<td>13.524</td>
<td>280; 340</td>
<td>1378.2</td>
<td>359.1</td>
<td>4.55</td>
<td>-</td>
<td>225.1, 209.1, 166.0, 149, 135.0, 130</td>
<td>F3 – Caffeic acid hexoside I</td>
</tr>
<tr>
<td>14.366</td>
<td>280; 340</td>
<td>1288.8</td>
<td>612.1</td>
<td>-</td>
<td>1.63</td>
<td>236.6, 181, 166.0, 149, 135.0, 130</td>
<td>F4 – Caffeic acid hexoside II</td>
</tr>
<tr>
<td>14.867</td>
<td>260; 360</td>
<td>1003.6</td>
<td>777.3</td>
<td>-</td>
<td>2.07</td>
<td>610.8, 478.8, 352.2, 302.9, 229.1, 202.1</td>
<td>F5 – Quercetin-3-O-galactoside, 7-O-rhamnoside</td>
</tr>
<tr>
<td>16.081</td>
<td>260; 365</td>
<td>711.7</td>
<td>299.1</td>
<td>-</td>
<td>0.80</td>
<td>463, 294.4, 283.6, 252.0</td>
<td>F6 –isorhamnetin 7-rhamnoside</td>
</tr>
<tr>
<td>16.401</td>
<td>260360</td>
<td>1632.4</td>
<td>2148.1</td>
<td>-</td>
<td>5.71</td>
<td>478.8, 302.6, 283.5, 217.9, 166.2</td>
<td>F7 – Quercetin 3-glucuronide</td>
</tr>
<tr>
<td>17.437</td>
<td>260 360</td>
<td>1019.9</td>
<td>1112.5</td>
<td>-</td>
<td>2.96</td>
<td>462.9, 352.1, 207.0, 164.0, 130.1</td>
<td>F8 – Kaempherol 3-glucuronide</td>
</tr>
<tr>
<td>Sum</td>
<td></td>
<td>31421.6</td>
<td>4949.1</td>
<td>64.9</td>
<td>13.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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 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 flavanol 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-gluconuride, respectively. The different fragmentation of F6 indicated a possible assignment as Isorhamnetin 7-rhamnoside, while F8 as Kaempherol 3-gluconuride.

These data are in agreement with previous results reported for the leaves of *Crataegus davisci*, 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 analysis eight compounds have been identified as quercetin, kaempferol, and aromadendrin glycosylated 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 *Crataegus 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 kaempferol 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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**References**


Kalyoncu IH, Ersoy N, Yilmaz M (2009). Physico-chemical and nutritional properties of cornelian cherry fruits (*Cornus mas* L.)
Öztürk N, Tunçel M (2011). Assessment of phenolic acid content
Meriçli A H, Ergezen K (1994). Flavonoids of
Pantelidis GE, Vasilakakis M, Manganaris GA, Diamantidis G
capacity of cornelian cherry (Cornus mas L) - Comparison
between permanganate reducing antioxidant capacity and
Rop O, Mlcek J, Kramarova D, Jurikova T (2010). Selected
cultivars of cornelian cherry (Cornus mas L.) as a new food
source for human nutrition. Afri J Biotechnol 9(8):1205-
1210.
Rosu AM, Olteanu Z, Truta E, Ciornea E, Mânzu C, Zamfirache
MM (2011). Nutritional value of Rosa spp. L. and Cornus mas
L. fruits, as affected by storage conditions. Anal Sci Univ
Dietary polyphenols and the prevention of diseases. Crit Rev
Food Sci Nutr 45:287-306
Simirgiotis MJ (2013). Antioxidant capacity and HPLC-DAD-
MS profiling of Chilean Peumo (Cryptocarya alba) fruits and
comparison with German Peumo (Crataegus monogyna) from
Southern Chile. Molecules 18:2061-2080.
Singleton VL (1999). Analysis of total phenols and other
oxidation substrates and antioxidants by means of Folin-
Complementary advanced techniques applied for plant and
Stef DS, Iosif G, Ioan-Trasca T, Stef L, Pop C, Harmanescu M,
extracts for the antioxidant capacity and total phenols. J Food
properties of cornelian cherry fruits (Cornus mas L.) grown in
Verpoorte R (2009). Medicinal plants: A renewable resource for
novel leads and drugs. p 1-5. In: Ramawat K.G., Herbal Drugs-
Ethnomedicine to Modern Medicine. Springer-Verlag, Berlin
Heidelberg.
Wichtl M (2004). Herbal drugs and phytopharmaceuticals. 3rd
Yadav NP, Dixit VK (2008). Recent approaches in herbal drug
Preliminary characterisation of cornelian cherry (Cornus mas
L.) genotypes for their physico-chemical properties. Food
Chem 114(2):408-412.

Kashyap CP, Arya V, Thakur N (2012) Ethnomedicinal and
phytopharmacological potential of Crataegus oxyacantha
Linn. - A review, Asian Pacific J Tropical Biomed 2(2):S1194-
S1199.
analysis of plant phenolic compounds. Molecules 18:2328-
2375.
Martioli L, Cangi F, Ghiara C, Burico M, Maiedicchi A, Bianchi E,
fingerprinting for the characterization of commercial botanical dietary supplements. Metabolomics
Pharmazie 55:326-327.
Melioglu G, Merichi F, Meriçi AH (1999). Flavonoids of
tanacetifolia (Lam.) Pers. (Rosaceae) an endemic species from
Nichita C, Neago G, Vulturescu V, Pirvu L, Badea N, Albulescu
R, Giurginca M (2009). Evaluation of scavenger properties of
some flavonoidic vegetal extracts obtained from Crataegus
monogyna Jacq. Key Engineer Mater 415:41-44.
Öztürk N, Tunçel M (2011). Assessment of phenolic acid content
and in vitro antiradical characteristics of hawthorn. J Med
Pantelidis GE, Vasilakakis M, Manganaris GA, Diamantidis G
(2007). Antioxidant capacity, phenol, anthocyanin and
ascorbic acid contents in raspberries, blackberries, red currants,
Pawloska AM, Camangi F, Braca A (2010). Quali-quantitative
analysis of flavonoids of Cornus mas L. (Cornaceae) fruits.
Comparative determinations of antioxidant free radical
scavenging polyphenols in certain natural products by HPLC
methods and UV-Vis spectrophotometry. Rev Chimie