Quali-quantitative analysis of flavonoids of *Cornus mas* L. (Cornaceae) fruits

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Abstract

The methanol extract obtained from the ripe fruits of *Cornus mas* L. (Cornaceae) have been phytochemically studied. On the basis of HPLC-PDA-MS/MS^n analyses eight compounds have been recognized as quercetin, kaempferol, and aromadendrin glycosilated derivatives. Three major compounds have been also isolated by Sephadex LH-20 column chromatography followed by HPLC and characterized by NMR spectroscopy. Moreover, LC-PDA-MS analyses of the red pigment revealed the presence of three anthocyanins. The quantitative analysis of all compounds was reported.

Keywords: *Cornus mas*, flavonoids, anthocyanins, HPLC, ESI-MS/MS

1. Introduction

Cornus mas L. (Cornaceae family) known as the European and Asiatic cornelian cherry is a species of dogwood native to Southern Europe and Southwest Asia. It is a medium to large deciduous shrub or small tree growing to 5-12 m tall, with dark brown branches and greenish twigs. The fruit is an oblong, red drupe 2 cm long and 1.5 cm in diameter, containing a single seed. The fruit is edible, but the unripe is astringent. The most common use of cornelian cherry fruits, is to produce different drinks, sweets, gels, and jams (Millspaugh, 1974). Extract from the fruits is also used in Europe for cosmetic purposes, replacing synthetic astringent substances, and are claimed to exert a favorable

action on the human complexion (Polinicencu, Popesci, & Nistor, 1980). As ornamental, cornelian cherry, with its brilliant leaves and abundant, attractive flowers, is employed with very interesting effect in parks and small gardens (Slimestad & Andersen, 1998).

Fruits and vegetables are a good source of natural antioxidants, which containing many different radical scavenger components provide protection against harmful-free radicals and so are associated with lower incidence and mortality rates of cancer and heart diseases in addition to a number of other health benefits (Wang, Cao, & Prior, 1996; Shui & Leong, 2006). Among natural compounds, phenolics and in particular flavonoids were found to be an important part of human diet and are considered as active principles in many medicinal plants. In addition, flavonoids with their subclass anthocyanins, contribute the yellow, orange, red, and blue color to flowers, fruits, and vegetables (Cooper-Driver, 2001), and could become important in the replacing of the synthetic pigments by the natural ones.

There are some investigations regarding the physical and chemical properties of cornelian cherry fruits, their antioxidant capacity, phenol, ascorbic acid, as well as anthocyanin contents (Tural & Koca, 2008; Vareed, Reddy, Schutzki, & Nair, 2006; Marinova, Ribarova, & Atanassova, 2005; Klimenko, 2004; Demir & Kalyoncu, 2003; Seeram, Schutzki, Chandra, & Nair, 2002; Didin, Kızılaslan, & Fenercioğlu, 2000; Guleryuz, Bolat, & Pirlak, 1998). Two old studies revealed that the berries of *C. mas* contain five anthocyanins, identified by paper chromatography, spectrophotometric, and peroxide oxidation analyses as delphinidin 3-galactoside, cyanidin 3-galactoside, cyanidin 3-rhamnosylgalactoside, pelargonidin 3-galactoside, and pelargonidin 3-rhamnosylgalactoside (Du & Francis, 1973a; Du & Francis, 1973b). The later work

(Seeram et al., 2002) showed that anthocyanins of cornelian cherry are the mixture of three compounds: delphinidin 3-*O*-galactoside, cyanidin 3-*O*-galactoside, and pelargonidin 3-*O*-galactoside. The last data (Tural & Koca, 2008) indicated as three main *C. mas* anthocyanins cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, and pelargonidin 3-*O*-glucoside. However, the survey of the literature revealed that the flavonoids content of the fruits has never been reported.

The objectives of this study were to identify individual flavonoids of the fruits, evaluate their profile, and compare the content of anthocyanins, in order to contribute to the knowledge the potential value of these fruits as food.

2. Materials and methods

2.1. Plant material

Cornus mas L. fruits were collected in Riparbella (PI), Italy, in September 2006. The specimen was further identified and authenticated by Dr. Fabiano Camangi, Scuola Superiore S. Anna di Studi Universitari e di Perfezionamento di Pisa, Pisa, Italy.

2.2. Reagents

Standard of quercetin 3-*O*-rutinoside was purchased from Merck (E. Merck, Darmstadt, Germany). Standards of cyanidin 3-*O*-galactoside and pelargonidin 3-*O*-glucoside were obtained from Extrasynthese (Extrasynthese, France). Standard of pelargonidin 3-*O*-rutinoside was kindly provided by Indena, Italy. HPLC grade acetonitrile (CH_3CN), formic (HCOOH) and acetic (CH_3COOH) acids were purchased from J.T. Baker (Baker

Mallinckrodt, Phillisburg, NJ, USA). HPLC grade water (18 mΩ) was prepared by a Mill-Ω^{50} purification system (Millipore Corp., Beddford, MA, USA).

2.3. General methods

An Avance Bruker 250 NMR spectrometer was used for NMR experiments; chemical shifts are expressed in δ (parts per million) referenced to the solvents peaks δ_H 3.34 and δ_C 49.0 for CD_3OD. Column chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden); HPLC separations were conducted on a Shimadzu LC-8A (Shimadzu Corp., Kyoto, Japan) series pumping system equipped with a Waters R401 refractive index detector and a Shimadzu injector with a Waters μ-Bondapak C_{18} column (7.8 × 300 mm, 10 μm, Waters, Milford, MA, USA). Thin-layer chromatography (TLC) was performed on precoated Kieselgel 60 F_{254} plates (E. Merck, Darmstad, Germany). HPLC-PDA-ESI-MS analyses were performed using a Surveyor LC pump, a Surveyor autosampler, coupled with a Surveyor PDA detector, and a LCQ Advantage ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with Xcalibur 3.1 software. Flavonoid analyses were performed using a 4.6 × 250 mm, 5.0 μm, X Terra C_{18} column (Waters, Milford, MA) and the eluent was a mixture of 0.1% acetonitrile solution of CH_3COOH (solvent A) and 0.1% aqueous solution of CH_3COOH (solvent B). The solvent gradient was as follows: 0-50 min, 8-45% (A). Elution was performed at a flow rate of 1.0 ml/min with a splitting system of 2:8 to MS detector (200 µl/min) and PDA detector (800 µl/min), respectively. Analyses were performed with an ESI interface in the negative mode. The ionization conditions were optimized, and the parameters were as follows: capillary temperature, 260 °C; capillary voltage, 18 V; tube lens offset, 5 V;

sheath gas flow rate, 60 arbitrary units; auxiliary gas flow rate, 6 arbitrary units; spray voltage, 4.5 kV; scan range of *m/z* 200-700. PDA data were recorded with 220-500 nm range with the preferential channels 254 and 324 nm as the detection wavelengths. Anthocyanin analyses were performed using a 4.6 × 250 mm, 4 μm, Synergi Polar-RP 80 A column (Phenomenex Corp., Torrance, CA) and the eluent was a mixture of 1% acetonitrile solution of HCOOH (solvent A) and 1% aqueous solution of HCOOH (solvent B). The solvent gradient was as follows 0-20 min, 8-25% (A). Elution was performed at a flow rate of 1 ml/min with the splitting system of 2:8 to MS detector, respectively. Analyses were conducted with an ESI interface in the positive mode. Cyanidin 3-*O*-galactoside was used to optimize the ioinization and the fragmentation conditions; parameters were as follows: capillary temperature, 280 °C; capillary voltage, 11 V; tube lens offset, 15 V; sheath gas flow rate, 60 arbitrary units; auxiliary gas flow rate, 6 arbitrary units; spray voltage, 3.5 kV; scan range of *m/z* 200-700. PDA data were recorded with 220-600 nm range with a preferential channel of 515 nm as the detection wavelength. In both cases N_2 was used as the sheath and auxiliary gas. The volumes of injections were 20 μl. HPLC-PDA quantitative analyses were performed using a Waters 600E multisolvent delivery system, a Waters 717plus autosampler, and a Waters 996 PDA detector (Waters, Milford, MA) equipped with Millenium[32] Chromatography Manager Software. The experimental conditions (solvent gradient, PDA channels, and columns) were the same as described above. The volume of injection was 25 μl. Quantitative determination was carried using calibration curves of standards. Quercetin 3-*O*-rutinoside, aromadendrin 7-*O*-glucoside, and cyanidin 3-*O*-galactoside were selected as the external standards of calibration for flavonols, dihydroflavonol, and anthocyanins,

136 respectively. Standard calibration curves were prepared in a concentration range 0.0005-
137 0.05 mg/ml with five different concentration levels (0.0005, 0.001, 0.005, 0.01, and 0.05
138 mg/ml) for quercetin 3-*O*-rutinoside and in a range of 0.001-0.05 mg/ml with four
139 concentration levels (0.001, 0.005, 0.01, and 0.05 mg/ml) for aromadendrin 7-*O*-
140 glucoside and cyanidin 3-*O*-galactoside. Triplicate injections were made for each level,
141 and a weight linear regression was generated. The calibration curves with the external
142 standards were obtained using concentration (mg/ml) with respect to the area obtained
143 from the integration of the PDA peaks at a wavelength of 254 nm for flavonoids, 285 nm
144 for dihydroflavonol, and 515 nm for anthocyanins. The relation between variables was
145 analyzed using linear simple correlation. For the linear regression of the external
146 standard, R^2 was 0.9993 for quercetin 3-*O*-rutinoside, 0.9996 for aromadendrin 7-*O*-
147 glucoside, and 0.9998 for cyanidin 3-*O*-galactoside, respectively. For the quantification
148 of the compounds, a GraphPad Software Prism 3.0 was used. The amount of the
149 compound was finally expressed in mg/10 g of fresh fruits.
150
151 *2.4. Extraction, isolation, and identification of flavonoids*
152 Lyophilized fruits of *C. mas* (280 g) were defatted at room temperature with *n*-hexane,
153 and extracted with MeOH by exhaustive maceration (5 × 500 ml) to yield 178 g of
154 residue, which was dissolved in water and partitioned firstly with EtOAc and then with *n*-
155 BuOH. The dried *n*-butanol extract (5 g) was subjected to fractionation on a Sephadex
156 LH-20 column, using MeOH as eluent at a flow rate of 0.8 ml/min. Fractions of 8 ml
157 were collected and grouped into ten (A-J) fractions by TLC analyses on silica 60 F_{254} gel-
158 coated glass sheets developed with *n*-BuOH-AcOH-H_2O (60:15:25) as the eluent.

Fractions G (55.9 mg), H (36.1 mg), I (21.3 mg), and J (31.4 mg) were separately purified by RP-HPLC on a 7.8 x 300 mm i.d., C_{18} μ-Bondapak column at a flow rate of 2.0 ml/min with MeOH-H_2O (45:55) for fraction G, MeOH-H_2O (35:65) for fraction H, MeOH-H_2O (3:7) for fraction I, and MeOH-H_2O (4:6) for fraction J, to afford compounds **1** (6.1 mg, t_R = 12 min) and **7** (2.5 mg, t_R = 30 min) from fraction H, **4** (12.0 mg, t_R = 20 min) from fraction G, and **7** from fraction I (1.5 mg, t_R = 16 min) and J (3.3 mg, t_R = 9 min), respectively. Compounds were identified by spectroscopic methods and HPLC-PDA-ESI-MS analysis with authentic standards (Fig. 1):

Aromadendrin 7-*O*-*β*-D-glucoside (**1**): yellow amorphous powder. Negative ESI-MS: *m/z* 449 $[M-H]^-$. ^1H and ^{13}C data are consistent with previously published data (Slimestad, Anderson, & Francis, 1994).

Quercetin 3-*O*-*β*-D-xyloside (**2**): ESI-MS: *m/z* 433 $[M-H]^-$. The compound was identified by HPLC-PDA-ESI-MS analysis (retention time, UV spectrum, and ESI-MS spectrometric data) and comparison with authentic standard.

Quercetin 3-*O*-*a*-L-rhamnoside (**3**): ESI-MS: *m/z* 447 $[M-H]^-$. The compound was identified by HPLC-PDA-ESI-MS analysis (retention time, UV spectrum, and ESI-MS spectrometric data) and comparison with authentic standard.

Quercetin 3-*O*-rutinoside (**4**): yellow amorphous powder. Negative ESI-MS: *m/z* 609 $[M-H]^-$. ^1H and ^{13}C data are consistent with previously published data (Rastrelli, Saturnino, Schettino, & Dini, 1995).

Quercetin 3-*O*-*β*-D-galactoside (**5**): ESI-MS: *m/z* 463 $[M-H]^-$. The compound was identified by HPLC-PDA-ESI-MS analysis (retention time, UV spectrum, and ESI-MS spectrometric data) and comparison with authentic standard.

Quercetin 3-*O*-*β*-D-glucoside (**6**): ESI-MS: *m/z* 463 $[M-H]^-$. The compound was identified by HPLC-PDA-ESI-MS analysis (retention time, UV spectrum, and ESI-MS spectrometric data) with authentic standard.

Quercetin 3-*O*-*β*-D-glucuronide (**7**): yellow amorphous powder. ESI-MS: *m/z* 477 $[M-H]^-$. ^1H and ^{13}C data are consistent with previously published data (Moon, Tsushida, Nakahara, & Terao, 2001).

Kaempferol 3-*O*-*β*-D-galactoside (**8**): ESI-MS: *m/z* 447 $[M-H]^-$. The compound was identified by HPLC-PDA-ESI-MS analysis (retention time, UV spectrum, and ESI-MS spectrometric data) and comparison with authentic standard.

2.5. Extraction and identification of anthocyanins

Fresh fruits (10 g) of *C. mas* were homogenized in 60 ml of 2% HCl methanol solution. The solution was filtered on Büchner funnel and the filtrate was used for HPLC analyses. Identifications were made by comparison of MS, PDA/UV, and retention data recorded for standard anthocyanins.

3. Results and discussion

The methanol extract of the fruits of *C. mas* was partitioned with *n*-hexane, EtOAc, and *n*-BuOH. The butanol extract was subjected to fractionation with an initial separation by Sephadex LH-20 column chromatography. Subsequent purification of the fractions by semipreparative HPLC led to the isolation of three compounds: aromadendrin 7-*O*-β-D-glucoside (**1**), quercetin 3-*O*-rutinoside (**4**), and quercetin 3-*O*-β-D-glucuronide (**7**) (Fig.

205 1). The structure of the isolated compounds was established by ^{1}H and ^{13}C NMR data and
206 confirmed by comparison with those reported in the literature. The presence of not
207 isolated compounds, quercetin 3-*O*-β-D-xyloside (**2**), quercetin 3-*O*-α-L-rahmnoside (**3**),
208 quercetin 3-*O*-β-D-galactoside (**5**), quercetin 3-*O*-β-D-glucoside (**6**), and kaempferol 3-*O*-
209 β-D-galactoside (**8**), was revealed by evaluation of the flavonoid profile throughout
210 HPLC-PDA-ESI-MS analyses. The components were identified by comparison of
211 obtained data (retention times, UV spectra, MS spectrometric data) with those of
212 authentic standards. The LC-MS Base Peak chromatogram of the methanol extract and
213 the chromatographic, spectroscopic, and spectrometric data, as well as, the quantitative
214 amounts of individual compounds are shown in Fig. 2 and Table 1, respectively. Results
215 obtained from quantitative analyses demonstrated the flavonoids content of 221.3 mg/10
216 of fruits. Methanol extract of cornelian cherries presented rich flavonoid glycosides
217 composition, that included eight compounds. Except for compound **1**, which belongs to
218 the dihydroflavonols, they were all *O*-flavonol glycosides with quercetin and kaempferol
219 as aglycones and oligosaccharide moieties as mono- or disaccharides linked at the 3-OH
220 position. Quercetin 3-*O*-β-D-glucuronide (**7**) was the major constituent (69.9 mg),
221 followed by kaempferol 3-*O*-β-D-galactoside (**8**) (41.3 mg).

222 The anthocyanin profile of cornelian cherry berries was carried out by means of HPLC-
223 PDA-ESI-MS analyses. The chromatogram of the anthocyanins extract, recorded at 515
224 nm, is shown in Fig. 3. All the compounds were identified by comparison of their HPLC
225 retention times, elution orders, ESI-MS spectrometric data, and photodiode array
226 PDA/UV-vis with anthocyanin standards (Table 2). Compound **9** was recognized as
227 cyanidin 3-*O*-galactoside, **10** as pelargonidin 3-*O*-glucoside, and **11** as pelargonidin 3-*O*-

228 rutinoside, respectively (Fig. 1). Our results of the anthocyanin qualitative composition
229 were not completely in agreement with those previously mentioned (Du & Francis,
230 1973a; Du & Francis, 1973b; Seeram et al., 2002; Tural & Koca, 2008). The total amount
231 of anthocyanins (Table 2) in *C. mas* fruits, determined on the cyanidin 3-*O*-galactoside
232 basis, was 11.7 mg/10 g of fresh fruits. This result is quite comparable with those
233 reported by Tural et al. (2008) and Pantelidis et al. (2007). Pelargonidin 3-*O*-glucoside
234 (**10**) was the predominant anthocyanin, followed by cyanidin 3-*O*-galactoside (**9**).
235 Pelargonidin 3-*O*-rutinoside was the least abundant one and present only in trace. The
236 differences in the composition of the fruits, could depend on the growing conditions, such
237 as soil, geographical and environmental conditions during the fruit development, degree
238 of maturity at harvested, and/or genetic differences.
239 Considering, that the epidemiological and experimental studies are correct in
240 suggesting that higher intake of phenolics from food are associated with reduced risk of
241 cancer, heart disease, and stroke, the immediate challenge is how to increase the level of
242 these beneficial phytochemicals in major food plants and find their new sources. The
243 fruits of cornelian cherry revealed the presence of considerable amounts of flavonoids.
244 Thus, results of the present study supported the antioxidant and nutraceutical potential of
245 this plant species.

246 **References**

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