Short Communication

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First evidence and quantification of quercetin derivatives in dogberries (*Cornus sanguinea* L.)

Cornus sanguinea L. bir tür kıvılcık kuersetin türevlerinin ilk bulgusu ve miktarının

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Abstract

Aim: *C. sanguinea* L. is a widespread European shrubby species. It is a potential source of biologically active compounds, especially antioxidants, as indicated by the dogberries' black color. The aim of the present study was to determine the content of several quercetin derivatives in the dogberries and to evaluate phytogeographical variability of these compounds.

Materials and methods: The dogberries were collected in the middle of September at two natural habitats of this species: Mt. Avala and Lake Zlatar, Serbia. The extract obtained from fresh fruits was subjected to LC-MS/MS analysis to identify and quantify the content of five quercetin derivatives: quercetin-3-O-glucuronide (Q-3-O-GlcA), quercetin-3-O-galactoside (Q-3-O-Gal), quercetin-3-O-rutinoside (Q-3-O-Rut), quercetin-3-O-glucoside (Q-3-O-Glc) and quercetin-3-O-rhamnoside (Q-3-O-Rha).

Results: All of examined quercetin derivatives were detected in extract of fresh dogberries and their contents were in the following order: Q-3-O-GlcA > Q-3-O-Gal > Q-3-O-Rut > Q-3-O-Glc > Q-3-O-Rha. The average amount of Q-3-O-Rut varied significantly and depended on the geographic origin of the fruit sample.

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Conclusions: These results indicate that dogberries could be a potential source of natural antioxidants, and encourage further investigation of this species considering that it has not yet been exploited in either nutrition or as a source of important pharmacological compounds.

Keywords: Common dogwood; Berry extract; Flavonols; Chemical variability.

Özet

Amaç: *C. sanguinea* L, yaygın bir avrupa ağacı türüdür. Dogberries'in siyah rengiyle belirtilen biyolojik olarak aktif bileşiklerin, özellikle de antioksidanların potansiyel bir kaynağıdır. Bu çalışmanın amacı, dogberries çeşitli quercetin türevlerinin içeriğini belirlemek ve bu bileşiklerin fitocoğrafik değişkenliklerini değerlendirmektir.

Gereç ve yöntemler: Köpek yavruları, Eylül ortasında bu türün iki doğal yaşam alanlarında toplandı: Mt. Avala ve Lake Zlatar, Sırbistan. Yeni meyvelerden elde edilen ekstre beş quercetin türevinin içeriğini belirlemek ve nicelleştirmek için LC-MS/MS analizine tabi tutuldu: quercetin-3-O-glukuronid (Q-3-O-GlcA), quercetin-3-O-galaktosid (Q-3-O-Gal), kuersetin-3-O-rutinosid (Q-3-O-Rut), kersetin-3-O-glukozid (Q-3-O-Glc) ve kersetin-3-O-Ramnosid (Q-3-O-Rha).

Tartışma: Kontrol edilen tüm quercetin türevleri taze dogberries ekstraktında tespit edildi ve içerikleri aşağıdaki sırada oldu: Q-3-O-GlcA > Q-3-O-Gal > Q-3-O-Rut > Q-3-O-Glc > Q-3-O-Rha'dır. Ortalama Q-3-O-Rut miktarı, önemli ölçüde değişir ve meyve örneğinin coğrafik kökenine bağlıdır.

Sonuç: Bu sonuçlar dogberries'in doğal antioksidanların potansiyel bir kaynağı olabileceğini ve beslenmede veya

önemli farmakolojik bileşiklerin kaynağı olarak henüz kullanılmadığını göz önüne alarak bu türün daha fazla araştırılmasını teşvik ettiğini göstermektedir.

Anahtar Kelimeler: Ortak köpek ağacı; çilek özütü; flavonoller; kimyasal değişkenlik.

Introduction

Cornus sanguinea L. (Cornaceae) is a fleshy-fruited shrub, 1–5 m in height, with a growth form similar to a small tree. It is widely distributed in the temperate regions of Europe, from the Mediterranean basin to England; it occurs on base-rich soils, in plentiful light conditions and prefers the temperature regime and soil moisture of a continental climate [1]. The blooming period is from May to June; the plant is insect-pollinated. Immature fruits are red, and when fully ripened, black; fruit ripening usually occurs in September. Leaves are rich in total phenolics and tannins [2] and the fruit pulp is rich in lipids (24.9%); it has relatively high protein content (6.4%), and the fiber fraction and soluble carbohydrates account for 10.3% and 53.8%, respectively [3].

Cornus sanguinea berries are not toxic, but their tart flavor makes them unpalatable in the raw form. This flavor can be improved by cooking, and so the plant could be potentially palatable, especially considering its seeds are rich in proteins and oils. Cooking decreases tannin content; if the sugar added, the sugar/acid ratio increases, which both contribute to better taste of tart berries [4]. Dogwood is used in the treatment of malaria, as it is rich in bitter alkaloids [5]. It is also part of homeopathic and naturopathic medicines, indicated for mainly transitory syndromes of numerous subacute inflammatory and necrotic ailments. Ethnopharmacological importance of *C. sanguinea* berries is documented by several studies, i.e. for their nutraceutical properties [6], as a remedy for diarrhea and stomachaches [7] and for treatment of sore eyes, throat and gastrointestinal disorders [8].

Fleshy-fruited plants, namely berries, are rich in phytochemicals with antioxidant properties, and many of their health-beneficial effects are attributed to their high content of polyphenolic compounds, especially flavonoids [9]. Flavonols, a class of flavonoids, and especially glycosides of quercetin and kaempferol, exhibit strong free-radical scavenging activity and protect plant cell metabolism from oxidative damage; they protect animal cell metabolism from oxidative damage and consequently may have an important role

in human health [10]. Quercetin is one of the most abundant flavonoids present in many fruits and vegetables, mainly in the leaves and external tissues of fleshy fruits (epicarp), as aglycones and glycosides. Quercetin glycosides contain a sugar group at the 3-position; the most frequently found sugar is glucose, followed by galactose and rhamnose [11].

Phytochemical characterization of the members of the genus *Cornus* has been mainly based on the relative distribution of total polyphenols [12], anthocyanins [13], flavonoids [14], iridoids [15] and carboxylic acids [16]. In addition to the phytochemical studies of certain species of the genus *Cornus*, data on the biological activities of fruit and leaf extracts, their antioxidant and cytotoxic properties have been reported [17].

Since the focus of phytochemical and pharmacological research has been directed toward species with edible fruits, data about the biologically active compounds in *C. sanguinea* are scarce. In several previous studies, an antioxidative activity of the leaves' and fruits' extracts has been reported [18, 19]. This study aimed to determine and quantify certain natural antioxidants in dogberries.

Materials and methods

Chemicals and reagents

All chemicals, including standards of phenolic compounds, were purchased from Sigma-Aldrich Chem (Steinheim, Germany), Fluka Chemie GmbH (Buchs, Switzerland) or from ChromaDex (Santa Ana, USA), and were of analytical grade.

Plant material

Plant material was collected in September 2015, at two localities: Mt. Avala (44°41'25" N, 20° 30' 51" E; 440 m a.s.l.) and Lake Zlatar (43° 30' 31" N, 19° 50' 19" E; 900 m a.s.l.). Each population was represented with 10 plants (Mt. Avala, n=10 and Lake Zlatar, n=10). Fully mature fruits were dark-purple, almost black and easily detachable from the stem. From each tree, five berries were collected (pooled sample). Fresh berries were refrigerated immediately after harvesting and over the night until further use. Samples of branches, leaves and fruits have been deposited in the Institute for Biological Research, Belgrade.

Extract preparations

For the determination of five quercetin derivatives: quercetin-3-*O*-glucuronide (Q-3-*O*-GlcA), quercetin-3-*O*-galactoside (Q-3-*O*-Gal), quercetin-3-*O*-rutinoside (Q-3-*O*-Rut), quercetin-3-*O*-glucoside (Q-3-*O*-Glc) and quercetin-3-*O*-rhamnoside (Q-3-*O*-Rha), a methanol extract prepared from the crushed fruits of *C. sanguinea* was used. Extract was obtained by diluting the plant material in methanol 70% (v/v) at a ratio 1:10; 0.5 g of fresh berries (without seeds)

were extracted with 5 mL of methanol 70% (20 h of shaking at 150 rpm/min, at 20°C). Then the extracts were filtered through membrane filter (0.45 µm) and used for analysis.

LC-MS/MS analysis of selected flavonols

Determination of the selected compounds in the extracts of dogberries was conducted according to the Orčić et al. [20], using Agilent Technologies 1200 Series HPLC

Table 1: Optimized dynamic MRM parameters.

Compound	Precursor m/z	Product m/z	V _{fragmentor} (V _F)	V _{collision} (V _C)	tR
Quercetin-3- <i>O</i> -glucuronide	477	300	145	20	1.97
Quercetin-3- <i>O</i> -galactoside	463	300	200	30	2.03
Quercetin-3- <i>O</i> -rutinoside	609	300	135	42	2.06
Quercetin-3- <i>O</i> -glucoside	463	300	210	30	2.11
Quercetin-3- <i>O</i> -rhamnoside	447	300	190	27	2.61

Precursor ion; Product ion; V_{fragmentor}, fragmentor voltage (cone voltage); V_{collision}, collision voltage; tR, retention time.

Figure 1: Representative multiple reaction monitoring (MRM) chromatograms for (A) standard mix of five compounds (all present in the concentration of 2.5 µg/mL): 1, Q-3-*O*-GlcA 3,0 min; 2, Q-3-*O*-Gal 3,08 min; 3, Q-3-*O*-Rut 3,12 min; 4, Q-3-*O*-Glc 3,13 min; 5, Q-3-*O*-Rha 3,69 min. (B) Sample 048 (from Mt. Avala) [99 mg fresh sample/mL 70% MeOH, diluted 20× in mobile phase (5 mg/mL)], (C) Sample 125 (from Lake Zlatar) [97.4 mg fresh sample/mL 70% MeOH, diluted 20× in mobile phase (5 mg/g)]. Retention times: Q-3-*O*-GlcA (3,01 min); Q-3-*O*-Gal (3,08 min); Q-3-*O*-Rut (3,12 min); Q-3-*O*-Glu (3,13 min); Q-3-*O*-Rha (3,70).

coupled with an Agilent Technologies 6410A QqQ mass spectrometer with electrospray ion source. Injection volume was 5 μL of samples/standards. Separation was performed using a Zorbax Eclipse XDB-C18 (Agilent Technologies) column, 50 mm $\times$ 4.6 mm $\times$ 1.8 μm , held at 50°C. The mobile phase, consisting of 0.05% aqueous formic acid (phase A) and methanol (phase B), was delivered at a flow rate of 1 mL/min in gradient mode (0 min 30% B, 6 min 70% B, 9 min 100% B, 12 min 100% B, post time 3 min; total 15 min). The eluted compounds were ionized with ion source parameters as following: nebulization gas pressure 40 psi, drying gas flow 9L/min and temperature 350°C, capillary voltage 4000 V. All compounds were detected in negative mode, using dynamic selected reaction monitoring with optimized compound-specific parameters (Table 1).

Preparation of a mixture of standard solutions

Basic solutions of individual phenolic compounds (10 mg/mL) were prepared by dissolving the reference

standard in DMSO, and were used for the preparation of the basic mixture with methanol:water (1:1), at a concentration of 100 $\mu\text{g/mL}$. The series of working solutions was prepared by successive dilution of the basic mixture, and ranged from 15.5 ng/mL to 40,000 ng/mL.

Data analysis

Peak areas were read in MassHunter Workstation Software-Qualitative analysis, version B.04.00 (Agilent Technologies). The concentrations of standard compounds in extracts were determined from the peak areas using the equation for linear regression obtained from the calibration curves (OriginPro 8), and expressed as μg of a compound in 1 g of fresh berries ($\mu\text{g/g}$).

Statistical analysis

Statistica (10.0) program was used for data analyses: one factor analysis of variance (ANOVA I), factor locality with two levels, locality 1 (Avala, $n=10$) and locality 2 (Lake Zlatar, $n=10$). For comparison of the localities by

Figure 2: Content of quercetin glycosides in fruit extracts of *C. sanguinea* ($\mu\text{g/g}$ of fresh berries, means $\pm$ SE): (A) Q-3-O-GlcA, (B) Q-3-O-Gal, (C) Q-3-O-Rut, (D) Q-3-O-Glc.

Table 2: Content of five quercetin glycosides in fruit extracts of *C. sanguinea* (µg/g of fresh berries).

Locality	Q-3-O-GlcA	Q-3-O-Gal	Q-3-O-Rut	Q-3-O-Glc	Q-3-O-Rha
Avala (n = 10)	868.29 (311.21) ^a	151.71 (166.86)	20.72 (18.77)	197.91 (164.70)	0.15 (0.03) ^b
Lake Zlatar (n = 10)	1091.82 (383.02) ^a	333.08 (327.71)	54.56 (31.25)	390.27 (311.62)	0.25 (0.09) ^b
	t = − 1.43 ^c	t = − 1.56 ^c	t = − 2.93 ^{c,e}	t = − 1.73 ^c	w = 65.5 ^d

n, sample size (number of plants); ^amean ± standard deviation; ^bmediane (minimum-maximum); ^cunpaired t-test (df = 18) applied; ^dMann-Whitney U test applied; ^estatistically significant differences (p < 0.05).

compounds, a parametric test, un-paired t-test [for normally distributed data (χ^2 test) with equality of variance in standard deviation (F-test, $p < 0.05$)], and Mann-Whitney U-test (when data did not show normality even after log transformation) were used.

Results and discussion

All of the examined glycosides from the group of quercetin derivatives were detected in the extracts. According to the results obtained by chromatography (chromatogram presented in Figure 1), dogwood berries stand out for their high content of quercetin derivatives (total amount of five analyzed derivatives in Avala and Lake Zlatar 1240 µg/g and 1830 µg/g, respectively). The prevailing Q-3-O-GlcA (Figure 2A) was present with 868.29 ± 311.21 µg/g and 1090.82 ± 383.02 µg/g in samples from Avala and Lake Zlatar, respectively; Q-3-O-Glc (Figure 2B) and Q-3-O-Gal (Figure 2D) were present in high amounts, whereas Q-3-O-Rut (Figure 2C) was detected in smaller amounts, with only traces of Q-3-O-Rha (Table 2). The amounts of Q-3-O-rutoside were significantly different, depending on the locality. Out of the five determined quercetin derivatives, the majority was conjugated with glucuronic acid (70% and 60% of the total analyzed quercetin glycosides in Avala and Lake Zlatar, respectively); the rest were conjugated with glucose (16% and 21.4% in Avala and Lake Zlatar, respectively), galactose (12.2% and 18.3% in Avala and Lake Zlatar, respectively) and the minor part was conjugated with rutinose and rhamnose. In the fruits of most frequently studied representative of the Cornaceae family, *C. mas*, quercetin is mostly present in the form of Q-3-O-glucuronide, Q-3-O-rhamnoside, Q-3-O-xyloside and Q-3-O-rutinoside [13]. In comparison to other berries rich in quercetin, dogberries far exceed the published content of individual quercetin derivatives, i.e. elderberry (total quercetin derivatives in cultivated plants 495.2 mg/kg of fresh weight, in wild grown 432.3 mg/kg, dominantly as rutinoside), chokeberry (263.2 mg/kg, highly present galactoside, glucoside and rutinoside), wild-growing blackberry (232.9 mg/kg, most abundant glucuronide) [9].

Quercetin-3-O-glucuronide, the most abundant glycoside in dogberries, is a major quercetin metabolite that plays a protective role against oxidative modification of human plasma, which is why the consumption of berries rich in this compound is advocated [10].

Conclusion

In this work it is shown that the fruits of dogwood contain high concentrations of natural antioxidants in the form of quercetin derivatives. The amounts of quercetin-3-O-glucuronide, which displays powerful antioxidant activity in human plasma, exceed the amounts previously reported in other species that are regarded as natural sources of quercetin-based compounds. Considering the species has not been widely studied phytochemically, these results point to the need to determine its complete phytochemical profile. The natural antioxidant content in dogberry fruit promises considerable benefits from the cultivation and exploitation of this widespread and ecologically undemanding species.

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