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Selected phenolic compounds in fruits of wild growing *Cornus mas* L.

Jasna Bajić-Ljubičić[1], Zorica Popović[2,]*, Rada Matić[2] & Srdjan Bojović[2]

[1]Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Republic of Serbia;
[2]Institute for Biological Research, University of Belgrade, Bulevar despota Stefana 142, 11000 Belgrade, Republic of Serbia
E-mail: zoricajp@gmail.com

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The aim of the present study was to determine the content of six phenolic compounds, natural anti-oxidants, in fruits of wild growing *Cornus mas* L. Fruits were sampled from two different locations. The extracts were obtained from fresh fruits and subjected to LC-MS/MS analysis to identify and quantify the content of neochlorogenic acid and five derivatives of quercetin. All of the analyzed phenolic compounds were detected in total sample of *C. mas* fruits, and their occurrence and content were clearly locality-dependent. Out of analyzed compounds, the prevailing was neochlorogenic acid (5-O-CQ) in samples from Avala, whereas glucuronide (Q-3-O-GlcA) dominated in samples from Zlatar lake. These results showed that wild growing populations are rich source of natural antioxidants, especially those with proved pharmacological activity in humans, such as glucuronide. As indicated by data on traditional usages and ethnopharmacological knowledge, *C. mas* fruits collected in wild are valuable source of natural antioxidants and deserve attention in preservation of genetic and biological diversity.

Keywords: Cornelian cherry, Phenolic acid, Wild fruit, Quercetin derivatives, Antioxidants
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Plants particularly in Horticulture section are raw material and used by people for food, either as edible products, or for culinary ingredients, for medicinal use or ornamental and aesthetic purposes. They are genetically very diverse group and play a major role in modern society and economy. Fruits and vegetables are an important component of traditional food, but are also central to healthy diets of modern urban population[1-3].

Cornus mas L. (Cornaceae) is a fleshy-fruited medium to large shrub or a small tree, growing 2-6 m tall and the fruits ripen in the mid-late summer. Fruits are olive-shaped, mostly bright red and often consumed by birds. Fruits are also used in human nutrition, consumed fresh or processed into syrups, juices, jams and other traditional products in many regions of Europe and Asia. The species' area of distribution is from central and southern Europe to Asia Minor, but it can be also found outside of its natural range as ornamental or cultivated shrub or tree. It is usually found in thermophilous mixed deciduous broadleaved forests, and also in combinations with other sub-Mediterranean shurbs[4].

Ethnophramacological data on traditional usage of *Cornus* species from all over the world, according to the genus distribution, are well documented[5,6] and one the most traditionally used and cited genotypes/cultivars within the species is *C. mas*[4]. For different ethnomedicinal purposes, *C. mas* fruits are used fresh (unripen & ripen) or dried, as juice, infusion, decoction or fermented[7].

Phenolic acids (free phenolics) and flavonoids (polyphenolics) are well known for their strong free-radical scavenging activity and protective function in plant cell metabolism from oxidative damage, and total amount of polyphenolic compounds is the first indicator of potential antioxidant and antimicrobial properties of plant extracts or essential oils[8-11]. Important ecological role of these compounds is their participation in defense mechanisms of the plant[12]. Plants are continuously exposed to the constant complex environmental pressure, abiotic and biotic, and one of the adaptive responses of plants is the biosynthesis and expression of certain "protective" chemical compounds. For the economically and agriculturally important species, cultivation practices provide favorable conditions for growth and yield

Abbreviations: 5-O-CQ, neochlorogenic acid; Q-3-O-GlcA, quercetin-3-*O*-glucuronide; Q-3-O-Gal, quercetin-3-*O*-galactoside; Q-3-O-Glc, quercetin-3-*O*-glucoside; Q-3-O-Rut, quercetin-3-*O*-rutinoside; Q-3-O-Rha, quercetin-3-*O*-rhamnoside.

*Corresponding author

(irrigation, fertilization, protection from herbivores, parasites, diseases; the competitive intra- and inter-specific pressure is decreased by planned planting), thereby reducing the total pressure of the environmental factors. Because of these reasons, it is expected that natural populations of wild fruits should contain a higher concentrations of natural antioxidants. Also, the edible wild fruits gain more attention because they grow isolated of pollution and harmful effects of different agricultural measures.

Phytochemical characterization of the *Cornus mas* fruits documented the rich source of antioxidant chemical compounds, vitamins, polyphenols, anthocyanins, flavonoids, tannins, iridoids, carboxylic acids[13-18]. This study aimed to determine and quantify selected phenolic compounds in fruits of wild growing *Cornus mas*, neochlorogenic acid and five quercetin glycosides. Neochlorogenic acid is found to be more biologically active compared to its isomer chlorogenic acid[19], and quercetin is one of the most abundant flavonoids with evident antioxidant and health beneficial effects[20].

Methodology

Study site

Sampling of fruits was conducted at two localities. At Mt. Avala (44º41′25″ N, 20º 30′ 51″ E; 440 m a.s.l.), fruits were sampled from trees which grow within the sessile oak-hornbeam forest, on the acidic-brown soil. The prevailing climatic conditions are temperate continental, with mean yearly temperature 9.5 °C and mean yearly participation 631.7 mm. The dominating tree species in forest communities are sessile oak, Turkey oak, beech, and hornbeam. Forest communities on Mt. Avala are more or less degraded. At Lake Zlatar (43º 30′ 31″ N, 19º 50 19″ E; 900 m a.s.l.), sampled trees inhabited sessile oak-hornbeam forest. Locality is surrounded with the large mountain massifs, at equal distance from Adriatic Sea (Mediterranean climate) and Panonic Plane (continental climate). Climatic conditions for this location are temperate continental, with mean yearly temperature 9.3°C and mean yearly precipitation 789.5 mm. The entire area of the Mt. Zlatar and the lake surrounding is covered with large areas of forest vegetation, mostly spruce forests, and beech and oak-hornbeam forests.

Plant material

Fully ripened fruits of *C. mas* were collected in the fall of 2015, transported to the laboratory (in portable refrigerator). Sampling was conducted by choosing 10 mature, healthy individuals at each location. Each individual tree was mapped using the geographic positioning system (Garmin eTrex), and considered as one sample (precisely 2-3 berries from one tree were used for analysis). All berries were sampled from the outer part of the crown, same orientation at the branch, same exposition. Fresh berries were refrigerated over the night until further use.

Chemicals and reagents

Chemicals and standards of phenolic compounds (all of analytical grade) used in this study were purchased from Sigma-Aldrich Chem (Steinheim, Germany), Fluka Chemie GmbH (Buchs, Switzerland) or from Chroma Dex (Santa Ana, USA).

Preparation of extracts

Methanol was used as solvent for extract preparation; plant material (crushed fruits of *C. mas*) was diluted 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). Then the extracts were filtered (membrane filter 0.45 μm) and used for further analysis and determination of neochlorogenic acid (5-O-CQ) and five quercetin derivatives (Q-3-O-GlcA, Q-3-O-Gal, Q-3-O-Glc, Q-3-O-Rut, Q-3-O-Rha).

LC-MS/MS analysis of selected phenolic compounds

Determination of the selected compounds in the extracts of *C. mas* berries was performed using Agilent Technologies 1200 Series HPLC coupled with an Aligent Technologies 6410A QqQ mass spectrometer according to the procedure described by Orčić *et al.*[21]. Samples and standards were injected in volume of 5 μL. Separation was performed using a Zorbax Eclipse XDB-C18 (Agilent Technologies) column, 50 mm x 4.6 mm x 1.8 μm, held at 50 ºC. The mobile phase, consisting of 0.05 % aqueous formic acid (phase A) and methanol (phase B), was delivered at a flow rate of 1mL/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 six phenolic compounds were detected in negative mode, by using dynamic selected reaction monitoring with optimized compound-specific parameters (retention time, precursor ion, product ion, fragmentor voltage, collision voltage).

Preparation of a mixture of standard solutions

For preparing of basic solutions for each of analyzed phenolic compounds (10 mg/mL), the reference standards were dissolved in DMSO, and used for the preparation of the basic mixture with methanol:water (1:1), at a concentration of 100 μg/mL. Then, the series of working solutions was prepared by successive dilution of the basic mixture, and ranged from 15.5 ng/mL to 40000 ng/mL.

Data analysis

Reference datasets were used for reading the peak areas in Mass Hunter 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 (Origin Pro 8). The results are expressed as μg of a compound in 1 g of fresh berries (μg/g).

Statistical analysis

For the statistical analysis of obtained data the Statistica (10.0) program was used, i.e., one factor analysis of variance (ANOVA I): factor locality with two levels, locality 1 (Avala) and locality 2 (Lake Zlatar). Number of elements in pooled sample (pooled sample size) was 20 and results were expressed as average values ± standard deviations. Differences between data were tested by Kruskal-Wallis, and the significance of difference was determined at $p < 0.05$. Grouping average method (Eucledean distance) was applied for grouping of the studied individuals based on content of analyzed phenolic compounds. Quantities of phenolic compounds were correlated with altitude.

Results

All of the analyzed phenolic compounds were detected in total sample of berries of *C. mas*. The content of all compounds was clearly locality-dependent, i.e., showed significant differences between two groups of samples (Table 1). According to the total amount of analyzed phenolic compounds, samples from Zlatar lake had higher amounts than samples from Avala (total sum of analyzed compounds 2413.8 μg/g and 212.8 μg/g, respectively). Out of the six determined phenolic compounds, neochlorogenic acid dominated in samples from Avala (47.03 %), whereas glucuronide was the highest present compound in samples from Zlatar lake (69.19 %). The amounts of all examined compounds significantly differed, depending on the locality. Out of the five analyzed derivatives of quercetin, the majority was conjugated with glucuronic acid in all samples (39.59 % and 83.51 % in Avala and Lake Zlatar, respectively); the rest was conjugated with galactose (26.78 % and 7.35 %), rutinose (16.81% and 5.02 %) and glucose (16.81 % and 4.09 %) and the minor part was conjugated with rhamnose.

The prevailing Q-3-O-GlcA was present in high amount in samples from Lake Zlatar (151.82±138.10 μg/g), followed by 5-O-CQ (37.64±27.24 μg/g) and Q-3-O-Rut (9.12±7.50 μg/g), whereas Q-3-O-Gal, Q-3-O-Glc and Q-3-O-Rha were present in smaller amount (13.35±20.40 μg/g, 7.42±6.99 μg/g and 0.23±0.19 μg/g, respectively). In samples from Avala, the most abundant of analyzed compounds was 5-O-CQ (9.98±8.33 μg/g), followed by Q-3-O-GlcA (4.45±1.50 μg/g). Other analyzed compounds were present in smaller amounts: Q-3-O-Rut (1.89±1.03 μg/g), Q-3-O-Gal (3.01 ±2.30 μg/g), Q-3-O-Glc (1.89±1.28 μg/g) and Q-3-O-Rha (not detected) (Fig. 1).

Quantities of all analyzed phenolic compounds were correlated with the altitude (positions of each tree), and significant positive correlations were found for five compounds: neochlorogenic acid ($r = 0.70$, $p < 0.05$), querciturone ($r = 0.90$, $p < 0.05$), hyperoside ($r = 0.52$, $p < 0.05$), rutoside ($r = 0.70$, $p < 0.05$) and isoquercetin ($r = 0.59$, $p < 0.05$).

Table 1 — Content of six phenolic compounds in fruit extracts of *C. mas* (μg/g of fresh berries, means ± SD) with statistically significant differences between samples from different localities.

Locality	Quantities of analyzed phenolic compounds (μg/g)					
	5-O-CQ	Q-3-O-GlcA	Q-3-O-Gal	Q-3-O-Rut	Q-3-O-Glc	Q-3-O-Rha
Avala	$^{A}9.98^{a}$ (8.33)	4.45^{a} (1.50)	3.01^{a} (0.28)	1.89^{a} (1.03)	1.89^{a} (1.28)	0.00^{a} (0.00)
Lake Zlatar	37.64^{b} (27.74)	151.82^{b} (138.10)	13.55^{b} (0.40)	9.12^{b} (7.50)	7.42^{b} (6.99)	0.23^{b} (0.19)
	$^{B}F_{1,19}=9.15$*	$F_{1,19}=11.34$*	$F_{1,19}=2.53$	$F_{1,19}=9.09$*	$F_{1,19}=6.06$*	$^{C}H_{1,18}=17.53$*

One-factorial ANOVA (n=21); ^{A}, mean values (standard deviation); ^{a,b}, *F*-test indicator with numbers – degrees of freedom; ^{C}, Kruskal-Wallis test ($p<0.05$); * – statistically significant difference. 5-O-CQ, neochlorogenic acid; Q-3-O-GlcA, quercetin-3-*O*-glucuronide; Q-3-O-Gal, quercetin-3-*O*-galactoside; Q-3-O-Glc, quercetin-3-*O*-glucoside; Q-3-O-Rut, quercetin-3-*O*-rutinoside; Q-3-O-Rha, quercetin-3-*O*-rhamnoside.

The grouping of all analyzed individuals is shown on dendrogram graph (Fig. 2). According to the quantities of analyzed phenolic compounds, noticeable overlapping of individuals from two populations occurred (samples from Avala 1-10 and samples from Lake Zlatar 11-21, respectively).

Discussion

Strong ethnophramacological background of *Cornus mas*[7] contribute to continuous interest for exploring the chemical composition of its fruits and studying the biological activity of specific compounds. Phenolic acids (free phenolics) and flavonoids (polyphenolics) are among the most studied chemical constituents of *C. mas* fruits, due to their pronounced anti-oxidant and anti-radical potential. Numerous evidences on antimicrobial, antidiabetic and antiobesity, hypolipidemic, cytotoxic, hepatoprotective, renalprotective, neuroprotective, anti-inflammatory, antioxidant, antiplatelet, cardioprotective, antiglaucomic, reproductive organ protective, radio-protective and aldose reductase inhibitory activities of *C. mas* fruit extract are obtained from *in vitro* and *in vivo* trials[7] and some of predicted biological activities are still to be investigated in controlled pharmacological and medicinal studies. There are recent evidences of its cardiovascular, endocrine, immune, gastrointestinal and overall anti-oxidative and anti-microbial effect in humans[22]. Many natural compounds from medicinal plants showed significant biological activities in different model systems, but their bioavailability is crucial for expected effects in humans. Oral bioavailability of quercetin in humans is matter of its metabolism, and according to comprehensive data, mostly low and highly variable[23]. After consummation of quercetin rich diet, this compound undergoes a series of reactions responsible for biological effects. Main metabolites in human plasma, which can be orally absorbed, are quercetin-3-glucuronide, 3-methylquercetin-3glucuronide and quercetin-3sulfate[24]. Therefore, in-deep studies on quantities of selected biologically active compounds give a clearer picture on potential biological effects of recommended natural products.

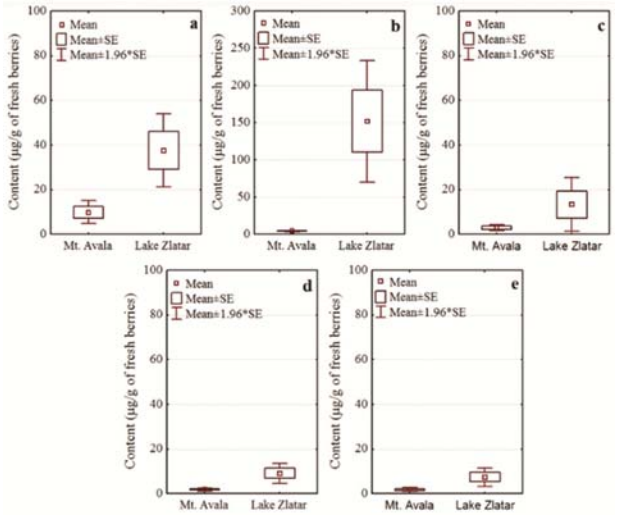

Fig. 1 — Content of quercetin glycosides in fruit extracts of *C. mas* (µg/g of fresh berries, means ± SE): a) 5-O-CQ, neochlorogenic acid; b) Q-3-O-Gal, quercetin-3-*O*-galactoside; c) Q-3-O-GlcA, quercetin-3-*O*-glucuronide; d) Q-3-O-Rut, quercetin-3-*O*-rutinoside; e) Q-3-O-Glc, quercetin-3-*O*-glucoside.

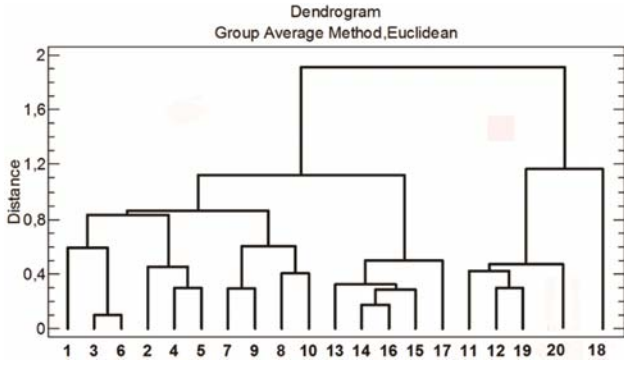

Fig. 2 — Dendrogram of six phenolic compounds based on Group Average Method (Euclidean distance) of 20 studied individuals of *C. mas* sampled from two locations: Avala (1-10) and Lake Zlatar (11-20). The numbers on the vertical axis refer to distance level, calculated on the basis on differences between individual contents of selected compounds.

The composition and quantity of individual phenolic compounds in fruits of cultivated species vary in a broad range, depending on various abiotic (geographic position, climate, soil, water and light availability, cultivation practice) and biotic (herbivore, microorganisms, competitive pressure) conditions; it depends on season, developmental stage and even on the position of the tree[12]. Therefore, concerning the quantification of individual chemical compounds in *C. mas* fruits, various data are provided. Some authors reported presence of chlorogenic acid[25], while another study evidenced three fold higher content of neochlorogenic acid[18]. Although the isomers, neochlorogenic acid seems to have the enhanced potency to reduce both glucose and cholesterol uptakes when compared to chlorogenic acid[19]. Quercetin was quantified from 120 mg/kg to 360 mg/kg per fresh weight of *C. mas* fruits, being the third most abundant flavonoid, after quercitin and rutin[23]. In study provided by Pawlovska *et al.*[14], examined quercetin was mostly

present in the form of Q-3-*O*-glucuronide, Q-3-*O*-rhamnoside, Q-3-*O*-xyloside and Q-3-*O*-rutinoside. Considering that there is not much data on the quantity of specific chemical compounds in the fruits of wild growing populations, our data could make a significant contribution to the perception of wild relatives of cultivated species as the important genetic resource. In our study, *C. mas* population located in Zlatar lake was pronouncedly rich in quercetin-3-*O*-glucuronide, which is a major quercetin metabolite that plays a protective role against oxidative modification of human plasma[26]. Also, identification and quantification of individual phenolic compounds in different natural populations of plant species indicates the degree of divergence between distant populations within taxa. Thus, the grouping of all analyzed individuals didn't show clear distinction between two populations, but overlapped five individuals from Lake Zlatar, which were more similar to the samples from Avala. This may indicate existence of different genotypes within the population from Lake Zlatar, or broader phenotypic plasticity of individuals. Moreover, bearing in mind different adaptive functions attributed to phenolics, especially flavonoids, it may point to specific dynamics of biosynthesis and expression of individual compounds influenced by local environmental factors. This is supported with evidence on significant positive correlations between quantities of analyzed phenolic compounds and position of individuals, specifically altitude[27,28].

For cultivated specimens, many of plant nutritional traits and the content of potentially pharmacologically active compounds can be predicted. However, different content and distribution of active compounds between distant populations growing in wild may be significant and impact the biological activity concerning the long-term using of particular plant parts. Analyzing of wild populations by quantifying selected active compounds may (1) provide more data to chemical fingerprinting of a species (2) point to populations rich in targeted chemical compounds and using them for cultivation (bioprospecting), (3) emphasize the value of products obtained from these non-standardized plant resources for the commercial benefit of local people and (4) raise awareness of local community for preservation of specific populations for biodiversity purposes.

Conclusion

Wild fruits are important, accessible and cheap source of vitamins, minerals, proteins, carbohydrates and fats, and have an important role in nutrition and economy of local people. Also, they are the reservoirs of valuable chemical compounds that are increasingly used in pharmacology. The characterization and quantification of individual compounds in natural populations provide the insight in specificity of chemical composition, which is the result of the specific genetic and environmental impacts. Consequently, the nutritional value, biological activities and marking of potential sources of active compounds (bioprospecting) can be transferred from the species to the population level, which emphasizes the need for conservation of natural populations.

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