Data Report

Phenolics, fatty acids, and biological potential of selected Croatian EVOOs

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Nutritional value of extra virgin olive oil is associated with its complex chemical composition. The aim of this study was to determine phenolic secoiridoids in Extra virgin olive oils (EVOOs) from autochthonous Croatian cultivars (*Drobnica*, *Krvavica*, *Lastovka*, and *Oblica*) by qNMR, to determine simple phenolics by UPLC, as well as to analyze the fatty acid profile, the antioxidant activity, and the oxidative stability of selected oils. This is the first study on chemical and biological characterization of selected autochthonous olives varieties. *Drobnica* EVOO contained the highest amount of total phenols and major secoiridoid derivatives (oleocanthal, oleacein, oleuropein aglycon, and ligstroside aglycon) compared to other oils. The antioxidant activity of *Drobnica* phenolics was very high by FRAP and copper-induced LDL oxidation assays, while the oxidative stability of *Drobnica* oil by Rancimat method was very long (23 h).

Practical applications: This study represents the contribution to the research of chemical and biological potential of monovarietal extra virgin olive oil from Croatia. EVOOs from selected Croatian autochthonous cultivars had very high phenolic content that is related to high inhibitory rate of copper-induced oxidation of human LDL as well as the long oxidative stability. *Drobnica* EVOO showed very long oxidative stability. EFSA approved health claim on olive oil polyphenols (EU, 432/2012) and selected Croatian cultivars, especially *Drobnica*, are of interest due to its high phenolic content and strong biological potential.

Keywords: EVOO / Fatty acid / Oxidative stability / Phenolics / qNMR

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1 Introduction

In the field of olive oil production, the potential of Croatian autochthonous cultivars are poorly investigated. At the present time, Extra virgin olive oils (EVOO) is considered as natural functional food rather than as a simple dressing, especially due to the health claim on olive oil polyhenols approved by European Food Safety Authority (EFSA) (EU, 432/2012). According to this claim, olive oil polyphenols contribute to the protection of blood lipids from oxidative stress. It is well known that phenolic content in EVOO is influenced by agronomic factors, fruit ripening stage during the harvest period, and oil processing method. According to archaeological findings, the beginnings of olive growing in Dalmatia date back to the late Bronze Age [1]. In spite of this long tradition of olive oil production in Dalmatia (Croatia),

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Abbreviations: EVOO, extra virgin olive oils; **UPLC,** ultra-performance liquid chromatographic; **FRAP,** ferric reducing/antioxidant power; **qNMR,** quantitative NMR

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Table 1. Major simple phenolics in EVOOs from *Drobnica*, *Krvavica*, *Lastovka,* and *Oblica* autochthonous cultivars

			Variety			
Phenolic compound concentration (mg/kg of oil)	RT (min)	UPLC-DAD (nm)	*Drobnica*	*Krvavica*	*Lastovka*	*Oblica*
Hydroxytyrosol	2.979	281	9.8 ± 0.2	11.3 ± 0.3	11.3 ± 0.5	9.3 ± 0.5
Tyrosol	3.653	266, 276	6.2 ± 0.1	5.1 ± 0.2	3.7 ± 0.1	7.1 ± 0.3
Vanillic acid	4.130	257, 289	2.9 ± 0.1	2.0 ± 0.0	–	1.5 ± 0.1
Vanillin	4.856	310	–	0.6 ± 0.0	0.6 ± 0.0	–
4-coumaric acid	5.014	226sh, 275, 327	4.2 ± 0.1	4.1 ± 0.1	2.6 ± 0.2	6.7 ± 0.3
2-coumaric acid	6.207	226sh, 275, 327	4.9 ± 0.1	2.6 ± 0.1	1.6 ± 0.1	2.5 ± 0.2
Luteolin	8.606	254, 266, 350sh, 347	18.2 ± 0.4	12.9 ± 0.4	18.0 ± 0.8	21.4 ± 1.0
Apigenin	9.905	340, 267	18.5 ± 0.3	7.5 ± 0.3	5.8 ± 0.2	6.8 ± 0.1

sh – shoulderpeak.
Quantification was performed by calibration curve using caffeic acid at 280 nm as an external standard. Results represent mean values of two independent experiments and expressed as a mean value ± standard deviation (mg/kg of oil).

according to available literature, there is no data on phenolics secoiridoids content in Croatian EVOOs.

Studies on the antioxidant activity of EVOO from *Masnjaca* and *Krvavica* cultivars [2] and on the correlation between phenolic compounds and the oxidative stability of EVOO from *Oblica*, *Lastovka*, *Levantinka*, *Mastrinka*, and *Drobnica* cultivars [3] were published recently. The objectives of this study was to analyse the content of phenolics secoiridois in selected Croatian EVOOs using quantitative 1^H NMR method published by Karkoula et al. [4] and to determine major simple phenolics by ultra-performance liquid chromatographic analysis (UPLC). In addition, the objectives of this study was to determine fatty acid composition of selected EVOOs by GC-MS, to evaluate the radical scavenging and the antioxidant activities of phenolic fractions from EVOOs by DPPH assay, Ferric reducing/antioxidant power (FRAP) assay and copper-induced oxidation of human LDL as well as to measure the oxidative stability of selected EVOOs by Rancimat method.

EVOO from four autochthonous Croatian cultivars were obtained from olive fruits in the orchards in Dalmatian region in 2014. Olive orchards were from the following regions: Island Korčula (*Lastovka*), Split region (*Oblica*, *Drobnica*), island Šolta (*Krvavica*). All olive fruits were processed immediately after harvesting and olive oils were collected at the olive mills where olives were processed with two-phase centrifugation systems. For all olive oils, the temperature of the malaxation was 26°C and the period of processing was 35 min. All oil samples were stored in dark glass bottles at the temperature between 18 and 20°C until analyses.

Extraction of phenolic fractions from EVOO samples was carried out following the method described by Pirisi et al. [5] and Rotondi et al. [6]. The total phenol content in extracted samples of phenolic fractions from EVOO was measured according to Amerine and Ough [7] and Singleton and Rossi [8]. Phenolics extract of olive oils were prepared for UPLC analysis according to IOOC procedure for the extraction of minor polar (BMP) compounds in olive oils (COI/T.20/Doc No. 29). The Agilent 1290 Infinity LC system (Agilent Technologies, Palo Alto, CA) equipped with ChemStation software and a diode array detection system was used for analysis of olive oil phenolic extracts. For NMR analysis, olive oil (5.0 g) was mixed with cyclohexane (20 mL) and acetonitrile (25 mL). The mixture was homogenized using a vortex mixer for 30 s and centrifuged at 4000 rpm for 5 min. A part of the acetonitrile phase

Table 2. ^{1}H-NMR analysis of major phenolics from selected EVOO samples

Variety	Oleocanthal	Oleacein	Oleuropein aglycon (monoaldehyde forms)	Oleuropien aglycon (dialdehyde and enolic forms)	Ligstroside aglycon (monoaldehyde forms **6a,6b**)	Ligstroside aglycon (dialdehyde and enolic forms)	Total phenolic secoiridoids
Oblica	154 ± 7	139 ± 7	28 ± 2	51 ± 4	9 ± 1	77 ± 5	458
Drobnica	182 ± 10	338 ± 15	139 ± 6	129 ± 8	52 ± 2	177 ± 9	1017
Lastovka	126 ± 6	142 ± 10	71 ± 3	328 ± 16	21 ± 2	171 ± 8	859
Krvavica	169 ± 4	52 ± 12	<1	<1	15 ± 2	<1	236

Results are expressed as a mean value ± standard deviation (mg/kg of oil).

Table 3. Fatty acid profile of extra virgin olive oils from *Drobnica*, *Krvavica*, *Lastovka,* and *Oblica* autochthonous cultivars

	Oil sample			
Fatty acid (%)	*Drobnica*	*Krvavica*	*Lastovka*	*Oblica*
Myristic ($C_{14:0}$)	0.03 ± 0.05	0.01 ± 0.10	0.05 ± 0.02	0.01 ± 0.10
Palmitic ($C_{16:0}$)	11.91 ± 0.04	11.94 ± 0.05	13.46 ± 0.05	12.03 ± 0.10
Palmitoleic ($C_{16:1}$, n-9)	1.88 ± 0.19	2.32 ± 0.05	3.18 ± 0.02	1.60 ± 0.12
Palmitoleic ($C_{16:1}$, n-7)	0.50 ± 0.00	0.52 ± 0.10	0.82 ± 0.10	0.96 ± 0.01
Heptadecanoic ($C_{17:0}$)	0.29 ± 0.01	0.05 ± 0.09	0.21 ± 0.03	0.12 ± 0.04
Heptadecenoic ($C_{17:1}$)	0.23 ± 0.04	0.10 ± 0.40	0.82 ± 0.10	0.12 ± 0.04
Stearic ($C_{18:0}$)	2.95 ± 0.03	2.90 ± 0.19	2.96 ± 0.09	1.92 ± 0.05
Oleic ($C_{18:1\ n\text{-}9}$)	70.91 ± 0.01	69.71 ± 0.03	67.41 ± 0.04	75.00 ± 0.10
Linoleic ($C_{18:2}$)	8.37 ± 0.09	10.55 ± 0.03	8.79 ± 0.03	6.65 ± 0.10
18:2 trans-cis	0.02 ± 0.00	n.d.	n.d.	n.d.
18:2 cis-trans	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.00
Linolenic ($C_{18:3}$)	0.88 ± 0.10	0.58 ± 0.10	0.84 ± 0.03	0.65 ± 0.10
Arachidic ($C_{20:0}$)	0.47 ± 0.02	0.34 ± 0.01	0.63 ± 0.02	0.25 ± 0.25
Gadoleic ($C_{20:1}$)	0.41 ± 0.04	0.11 ± 0.02	0.42 ± 0.10	0.14 ± 0.08
Behenic acid ($C_{22:0}$)	0.29 ± 0.04	0.11 ± 0.02	0.21 ± 0.24	0.14 ± 0.08
Lignoceric acid ($C_{24:0}$)	0.17 ± 0.06	0.16 ± 0.23	0.10 ± 0.10	0.12 ± 0.06
SFA	16.11	15.51	17.62	14.59
MUFA	73.91	72.76	72.04	77.82
PUFA	9.29	11.14	9.65	7.31

Results represent mean values ± SD of two independent experiments and are expressed as percentages of total fatty acid methyl esters (FAMEs).

(25 mL) was collected, mixed with 1.0 mL of a syringaldehyde solution (0.5 mg/mL) in acetonitrile and evaporated under reduced pressure using a rotary evaporator (Buchi, Switzerland). The residue of the above procedure was dissolved in $CDCl_3$ (750 μL) and an accurately measured volume of the solution (550 μL) was transferred to a 5 mm NMR tube.^{1}H-NMR spectra were recorded at 600 MHz (Bruker Avance600) and 400 MHz (Bruker DRX400). The spectra were phased corrected and accurate integration was performed manually for the peaks of interest using TOPSPIN as described in details by Karkoula et al. [4, 9] and Diamantakos et al. [10]. Analysis of fatty acids were done according to method described by Yang et al. [11]. The measurement of the DDPH (2,2′-diphenyl-1-picrylhydrazyl) radical-scavenging activity of olive oils phenolic fractions was described by Kulisic et al. [12]. FRAP method measures the total reducing capability of flavonoids as described by Generalic et al. [13]. Copper-induced oxidation of these LDL samples (0.1 μM) was triggered at 37°C by 2.5 μM $CuSO_4$ under aerated conditions as described in Kulisic et al. [14]. Olive oil samples (3 g) were tested for its oxidative stability at a temperature of 120°C ($\Delta t = 1.4$°C) with the constant air flow of 20 L/h using Rancimat 743 (Metrohm, Herisau, Switzerland) instrument. The concentration of synthetic antioxidants (BHA and BHT with purity grades above 99%) in the oil was 0.016% w/w. Conductivity was

Table 4. Total phenolics, antioxidant activity of phenolic fractions from *Drobnica*, *Krvavica*, *Lastovka,* and *Oblica* extra virgin olive oils by DPPH method, FRAP method, and copper-induced LDL oxidation method

Sample	Total polyphenols (TP) (mg/L GAE)	DPPH scavenging activity (%)	FRAP (c (Fe^{2+}) μmol/g)	LDL lag phase prolongation(min)
Drobnica	439.71 ± 39.42	39.96 ± 1.29	249 ± 1.20	127 ± 50
Krvavica	153.17 ± 43.23	40.63 ± 1.65	125 ± 1.54	55 ± 10
Lastovka	383.48 ± 25.46	72.37 ± 1.98	185 ± 1.22	84 ± 80
Oblica	236.85 ± 9.11	43.05 ± 1.24	99 ± 1.36	52 ± 60

Results represent mean values of three independent experiments ± standard deviation.

measured conductometrically as a function of the time. Statistical analysis was performed using GraphPad InStat3 (GraphPad Software, San Diego, USA). The relationship between the obtained parameters was described using Pearson correlation coefficient "r." Differences at $p < 0.05$ were considered to be statistically significant.

The highest content of total phenolics, expressed as mg/L GAE, was measured in *Drobnica* EVOO (439.71 ± 39.42), in comparison with *Lastovka* (383.48 ± 25.46), *Oblica* (236.85 ± 9.11), and *Krvavica* (153.17 ± 43.23). Results on total phenolics content in *Krvavica* olive oil are very similar to that of Šarolić et al. [2]. In addition, obtained data on total phenolics content in *Drobnica*, *Lastovka,* and *Oblica* EVOO are much higher than that reported by Zanetic et al. [3]. Obtained results are in line with value of total phenols from Italian, French, Spanish, and Tunisian olive oils [15].

The concentration of free hydroxytyrosol and tyrosol in EVOOs is very low because they are present in the oil in form of their esterified derivatives at much higher concentration (Table 1). Hydroxytyrosol is mainly present in the form of oleacein (3,4-DHPEA-EDA) and the monoaldehydic form of oleuropein aglycon (3,4-DHPEA-EA), while tyrosol is mainly present in the form of oleocanthal (*p*-HPEA-EDA), and the monoaldehydic form of ligstroside aglycon (*p*-HPEA-EA) [4]. In that sense, the use of simple and rapid method using quantitative NMR (qNMR) described by Karkoula et al. [4] is optimal choice to analyze four major secoiridoid derivatives of hydroxytyrosol and tyrosol in EVOO. *Drobnica* EVOO had the highest content of all identified phenolics in comparison with other EVOOs (Table 2).The content of oleacein in *Drobnica* EVOO is very high (338 ± 15 mg/kg of oil) compared with results for top 10 highest concentration Greek EVOOs of *Koroneiki* variety, which was found, among 19 varieties studied, as the variety with the highest concentration of oleocanthal and oleacein [4]. In addition, *Drobnica* EVOO has high concentration of oleacein and oleuropein aglycon in comparison with results presented in Karkoula et al. [4, 9] (Table 2). The content of essential fatty acids, linoleic an linolenic, was the following, respectively: 6.65–10.55%, 0.58–0.88% (Table 3).

Biological potential of selected Croatian EVOOs was evaluated by three antioxidative methods. The highest ability to reduce free DPPH radical was shown by phenolic fractions from *Lastovka* EVOO (72.37% ± 1.98) (Table 4). The highest antioxidant power measured by FRAP assay as well as the highest ability to prevent the oxidation of human LDL was achieved by *Drobnica* EVOO (Table 4). In present study, the accelerated test performed using Rancimat instrument was used in order to evaluate the oxidative stability of selected EVOOs. Obtained results show that *Drobnica* oil had the longest induction time (23.04 ± 0.24) under standard conditions (the temperature of 120°C, the air flow of 20 L/h) (Table 5). Other tested olive oils had significantly lower oxidative stability expressed through their induction times (9.88–11.52 h). Generally, induction periods for all tested EVOOs are very good in comparison with the induction period of olive oil measured by Rancimat method at 120°C reported by Laubli and Brutell [16] and by Farhoosh and Moosavi [17]. Presented results are not in line with conclusion of Angerosa and Basti [18] that the fatty acid composition, particularly high level of oleic acid is important factor for the oxidative stability of olive oil. Results presented by this study show that the content of phenolics compounds and also the presence of particularly phenolic compound such as secoiridoid derivatives are crucial factor influencing the olive oil oxidative stability. *Drobnica* oil had the highest content of four secoiridoid derivatives (oleocanthal, oleacein, oleuropein aglycon, and ligstroside aglycon) in comparison with other tested oil and shows the longest induction time.

Table 5. Oxidative stability (induction time) of extra virgin olive oils from *Drobnica*, *Krvavica*, *Lastovka,* and *Oblica* autochthonous cultivars

Sample	Induction time (h)
Drobnica	23.04 ± 0.24
Krvavica	9.88 ± 0.27
Lastovka	11.15 ± 0.61
Oblica	11.52 ± 0.52

Results represent mean values of three independent experiments ± standard deviation.

Obtained results represent the first report on phenolics secoiridoids profile done by ^{1}H-NMR technique from four Croatian autochthonous cultivars: *Drobnica*, *Lastovka*, *Krvavica,* and *Oblica*. In line with well-known olive cultivars such as *Koroneiki*, Croatian autochthonous olive cultivar *Drobnica,* showed very high amount of total phenolics as well as very high content of oleacein, and possess high biological potential and very long oxidative stability. Results of present study proved that EVOOs with high content of phenolics have very long oxidative stability (23 h).

The authors have declared no conflict of interest.

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