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Effect of Olive (*Olea europaea* L.) Variety on Leaf Biophenolic Profile

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Summary

Olive leaves are rich in valuable biophenolic compounds, characterised by high antioxidant activity, antimicrobial properties and beneficial effects on human health. Leaf biophenols are essential for metabolic adaptation of olive to various abiotic or biotic stressors and maintenance of homeostasis. The aim of this study was to determine the influence of olive variety ('Buža', 'Karbonaca' and 'Oblica') on the leaf biophenols concentrations. The experiment was conducted as a completely randomised design in 3 replicates. Olive leaves were collected in three sampling periods, starting from harvest in October 2017 until pruning in March 2018. The variety 'Buža' differed significantly from the other cultivars with its highest content of oleuropein (5239.88 mg 100 g^{-1} DW) and total biophenols (5943.25 mg 100 g^{-1} DW). The highest levels of 4-hydroxybenzoic acid (3.92 mg 100 g^{-1} DW), luteolin (48.17 mg 100 g^{-1} DW) and apigenin (7.55 mg 100 g^{-1} DW) were recorded in 'Karbonaca' samples. Tyrosol concentrations were not significantly different between 'Karbonaca' (4.79 mg 100 g^{-1} DW) and 'Oblica' (4.96 mg 100 g^{-1} DW) cultivars, however both differed from 'Buža' with the highest tyrosol concentration (6.67 mg 100 g^{-1} DW). The obtained results showed significant differences in the content of important biophenols between the selected olive varieties. Accordingly, the highest concentration of oleuropein, the most important secoiridoid in olive leaves, in cultivar 'Buža' could strongly determine their metabolic response to different stressors.

Key words

Buža, Karbonaca, Oblica, oleuropein, verbascoside

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Introduction

Biophenolic compounds are bioactive products ubiquitously synthesized throughout the plant kingdom (Boudet, 2007). They are known to exhibit a wide range of protective physiological properties and are utilized in various plant survival and defense mechanisms. Biophenols have long been recognized for their antioxidant, antimicrobial as well as anti-inflammatory activities (Abaza et al., 2015).

Olive (*Olea europaea* L.) is one of the most prominent crops cultivated throughout the Mediterranean basin, ensuring livelihood and sustenance since ancient times. Biophenols are found in all parts of *O. europaea*, but their nature and concentration vary greatly among different tissues (Serrilli et al., 2008). Recent studies have focused on recovering biophenolic fractions from rich natural matrices such as vegetative "by-products" of cultivation process that until recently had no significant practical value. Thus, researchers approached olive leaves as rich source of valuable biophenols, uncovering their biotechnological, nutraceutical and pharmaceutical potential. For instance, olive leaves have been proposed as a rich source of oleuropein from which a strong antioxidant hydroxytyrosol can be more easily synthesized compared to the conventional synthetic processes. Hydroxytyrosol can be useful not only for laboratory applications but also as a natural food preserving agent whose regular consumption provides antioxidant and anti-inflammatory benefits to humans (Martínez et al., 2018). Moreover, the encapsulation of olive leaf extracts increases the solubility of phenolic compounds and can be used for oil or food enrichment (Farag et al., 2007). Olive leaf extracts were found to be protective against cardiovascular diseases due to their effects on adrenaline, arrhythmia and muscle spasms (Khayyal et al., 2002; Somova et al., 2004). Biophenolic compounds from these extracts are also known to alleviate repercussions of some metabolic disorders, exerting for instance, high anti-diabetic properties by increasing peripheral glucose utilization and improving glucose-stimulated insulin release (Sato et al., 2007). Furthermore, biophenols originating from olive leaf extracts are shown to be especially effective against certain bacterial genera which exhibit major antibiotic resistance such as *Klebsiella* and *Pseudomonas* whilst also having antimicrobial effects against *Escherichia coli* and *Candida albicans* (Neu, 1992). In terms of their molecular properties, biophenolic compounds present in olive leaves are numerous and diverse (Abaza et al., 2015) exhibiting antioxidant (Lee et al., 2010; Herrero et al., 2011; Peralbo-Molina, 2013), antiproliferative (Han et al., 2009) and lipid-lowering (Kontogianni et al., 2012) activities. Therefore, in order to procure abundant source of these biophenols, evaluation of their concentrations in leaves of different olive cultivars is justifiable.

Olive cultivation has traditionally focused on the agronomic properties, however the increasing number of scientific evidence supporting the beneficial effects of these compounds on human health makes this functional quality of olive leaves a new point of interest. As sustainable by-products of cultivation, olive leaves can be easily obtained. Simple biophenols and biophenolic acids, flavonoids, lignans and secoiridoids, as major components of olive leaves may occur in various amounts depending on the cultivar (Fiorentino et al., 2003, Pasković et al., 2020; Lukić et al., 2020), geographic origin (Bilgin and Sahin, 2013), nutritional status (Pasković et al., 2019) and exposure to abiotic or biotic stressors (Fernández-Escobar, 2019). Cultivars usually vary in their capability to cope with their environment and their ability to synthesize protective metabolites under adverse conditions can determine their future growth and productivity. However, even under suitable growing conditions, synthesis of major olive biophenols seems to be regulated differently between different olive genotypes, regardless of them sharing similar biochemical pathways. Therefore, genetic predisposition of cultivars may be the predominant factor accounting for the quantitative differences of biophenols in olive leaves. These differences can be exploited in an effort to obtain sources with higher concentrations of certain compounds that may be of crucial importance for sustainable olive cultivation, as well as to human health. Thus, this work deals with the quantification of biophenolic components present in olive leaves of three indigenous *Olea europaea* cultivars, cultivated in the Croatian province of Istria. The specific aim of this study was to investigate the inter-cultivar biophenolic specificity in leaves of each indigenous cultivar, in order to evaluate their particular potential as a source of these valuable compounds.

Materials and Methods

Olive Leaves Sampling

Leaves were collected in three sampling periods. The first sampling was conducted promptly after harvest in October 2017, the second in January and the third in March 2018, after pruning at the olive orchard near Vodnjan (44°57'4.4"/ 13°50'41"/ 109m), in the province of Istria, Croatia. Leaves were collected from three native olive cultivars: 'Buža', 'Karbonaca' and 'Oblica'. Olive orchard is planted on *Terra rossa* soil and located in the climate area classified as Cfa, according to Köppen (Šegota and Filipčić, 2003).

Olive trees were 15 years old at the time of leaf sampling, and were cultivated in identical habitat with no visible symptoms of adverse pest or pathogen presence. Standard fertilization practice was applied on a yearly basis. Well developed, healthy trees were selected for sampling. Experiment was set as completely random design in 3 repetitions and each sample comprised of leaves collected from the middle portion of one year old olive shoots, taken equally from all four orientation sides.

After sampling, leaves were taken to the laboratory and washed in 1% acetic acid dissolved in deionized water of the highest purity (type I), then double rinsed in deionized water. Deionized water was obtained from Siemens UltraClear device (Siemens AG, München, Germany). Upon rinsing, leaves were separated, adequately labeled and set to dry at 35°C until constant mass (UF-260 Universal Oven, Memmert GmbH, Büchenbach, Germany) (Pasković et al., 2020).

Extraction of Biophenols

After reaching constant mass, leaves were finely milled using centrifugal mill (Retsch Ultra Centrifugal Mill ZM 200, Düsseldorf, Germany). Around 500 mg of each milled sample was weighed (Radwag AS 310.X2, Radom, Poland) inside glass vials. Using 20 mL of 80% methanol (v/v MeOH, Merck, Germany), extracts were made in an ultrasonic bath (frequency 35 kHz, power 125

140/560W with nominal output power of 400 W, Sonorex Digitec, Bandelin electronic, Berlin Germany) for 20 min. Approximately 15 mL of extract aliquot was centrifuged for 7 min at 4000 rpm (Centric 350, Domel, Železniki, Slovenia) and the supernatant was filtered through a 0.45 µm-pore cellulose acetate syringe filter.

High Performance Liquid Chromatography

Biophenols were determined by high-performance liquid chromatography (HPLC) using a Thermo Ultimate 3000 System, comprised of a degasser, a binary pump, an autosampler, a column oven, and an UV/Vis detector capable of simultaneous measurement at 4 different wavelengths (Thermo Fisher Scientific, Waltham, MA, USA). The separation of biophenols was performed using a Lichrospher 100 RP-18 (250 mm × 4 mm, 5 µm) analytical column with a pre-column Lichrospher 100 (4 mm × 4 mm, 5 µm), both supplied by Agilent Technologies (Santa Clara, CA, USA). The analyses were performed under conditions similar to a procedure described and published in our previous work (Pasković et al., 2020). The analyses were performed at a constant temperature of 25 °C. The mobile phase consisted of (A) 0.2% phosphoric acid and (B) MeOH: AcN (1: 1). The chromatographic conditions were as follows: 10% B 0–0.5 min; 10%–16.5% B 0.5–25 min; 16.5%–30% B 25–80 min; 30%–100% B 80–95 min; 100% B 95–100 min; 100%–10% B 100–102 min; and 10% B 102–105 min, followed by equilibration time for 10 min. The flow rate was 0.8 mL min^{-1}. UV/Vis detection range was set at 250 nm for 4-hydroxybenzoic acid, luteolin-7-*O*-glucoside and oleuropein. Wavelength of 280 nm was used to detect hydroxytyrosol, tyrosol, vanillin, apigenin-7-*O*-glucoside and catechin. Caffeic acid, ferulic acid, verbascoside and apigenin were detected at 305 nm whereas for luteolin and rutin 370 nm wavelength was used. Identification was performed by comparing retention times of the target compounds in the sample extracts with the retention times of pure standards and quantified using corresponding calibration curves, following the procedure from our previous work (Lukić et al., 2020).

Statistical Analysis

Experiment was conducted as completely randomized design in three repetitions. One-way analysis of variance (ANOVA) was performed by taking data from cultivars as the main factor regardless of the sampling period, using Statistica® v.12.0 software (Tibco Software Inc., Palo Alto, CA, USA). Comparisons of means were based on a Tukey's test at $P \leq 0.05$.

Results and Discussion

Considerable phytochemical differences were found in the three analyzed cultivars. Table 1 shows the contents of various simple biophenols, biophenolic acids and oleuropein as a major secoiridoid. Unlike the other simple biophenols, no significant differences were recorded in hydroxytyrosol contents among the three cultivars, with concentrations averaging 37.14 mg per 100 g of dry weight (DW). In general, concentrations of hydroxytyrosol increase at the expense of oleuropein after its degradation during hydrolysis. For this reason, hydroxytyrosol can account for more than 80% of the total biophenol content in hydrolyzed olive leaf extracts (Quirantes-Piné et al., 2013; Fki et al., 2020).

Table 1. Concentrations of simple phenols, phenolic acids, oleuropein and total phenols of three olive cultivars, expressed in mg 100 g^{-1} of dry weight (DW)

Source of variation	Simple phenols			Phenolic acids				Secoiridoids	Total phenols
	Hydroxytyrosol	Tyrosol	Vanillin	4-hydroxybenzoic acid	Caffeic acid	Ferulic acid	Verbascoside	Oleuropein	
	mg/100 g DW			mg/100 g DW				mg/100 g DW	mg/100 g DW
Buža	33.77 ± 4.53	6.67 ± 0.86^{a}	1.63 ± 0.14^{a}	1.03 ± 0.18^{b}	1.07 ± 0.06^{b}	0.66 ± 0.09^{b}	204.51 ± 82.39	5239.88 ± 631.54^{a}	5943.25 ± 700.15^{a}
Karbonaca	38.52 ± 6.89	4.79 ± 0.85^{b}	0.92 ± 0.14^{b}	3.92 ± 0.43^{a}	1.84 ± 0.35^{ab}	1.68 ± 0.29^{a}	204.84 ± 73.41	1685.24 ± 594.05^{c}	2247.92 ± 695.59^{c}
Oblica	39.15 ± 3.12	4.96 ± 1.04^{b}	1.18 ± 0.21^{ab}	1.14 ± 0.30^{b}	2.22 ± 0.34^{a}	0.95 ± 0.1^{b}	224.94 ± 68.34	3068.24 ± 505.39^{b}	3785.85 ± 546.38^{b}
P-value	n.s.	*	**	***	*	***	n.s.	**	***

Results are expressed as means ± standard errors. Different superscript lowercase letters in a column represent statistically significant differences between mean values for each main effect at $P < 0.05$ obtained by a one-way ANOVA and Tukey's test. Significance: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$

In addition, during the table olive production process, the conversion of oleuropein into hydrolyzed products such as hydroxytyrosol and tyrosol increases, resulting with a decrease in non-hydrolyzed oleuropein forms (Charoenprasert and Mitchell, 2012). Due to such relationship between hydroxytyrosol and oleuropein, hydrolyzed olive leaf extracts provide a rich source of hydroxytyrosol, whereas unprocessed olive leaves provide an abundant source of oleuropein. Hydroxytyrosol concentrations are generally fairly variable in leaves of different cultivars, which can be confirmed from our previous results (Pasković et al., 2020). Here, the compared cultivars showed moderate hydroxytyrosol contents without significant differences in between. However, the shown similarity in leaf hydroxytyrosol concentrations between the analyzed cultivars was not analogous to the status of other simple biophenols such as tyrosol and vanillin (Table 1). The cultivar 'Buža' had significantly higher levels of leaf tyrosol (6.67 mg 100 g^{-1} DW) and vanillin (1.63 mg 100 g^{-1} DW) compared to the other two cultivars. In a previous study (Pasković et al., 2020) reported cultivar-dependent variations of tyrosol and vanillin concentrations in leaves of other cultivars grown at the northern parts of the Mediterranean region. In that instance 'Drobnica' and 'Istarska bjelica' had the highest tyrosol levels (8.81 and 7.8 mg 100 g^{-1} DW respectively). Based on this, and on the data shown in Table 1, leaves of 'Buža' cultivar are able to secure moderate tyrosol contents.

Biophenolic acids are among the most widely distributed plant non-flavonoid biophenolic compounds present in the free, conjugated soluble and insoluble-bound forms (Varelis et al., 2019). 'Karbonaca' cultivar had the highest levels of 4-hydroxybenzoic (3.92 mg 100 g^{-1} DW) and ferulic acids (1.68 mg 100 g^{-1} DW), whereas 'Oblica' showed highest concentrations of caffeic acid (2.22 mg 100 g^{-1} DW). In vitro study on antioxidant activities of individual biophenols in olive leaf extracts proved caffeic acid as a strong antioxidant, markedly surpassing oleuropein in its specificity towards scavenging nitrites (Lee and Lee, 2010).

The glycoside of caffeic acid and hydroxytyrosol, known as verbascoside, plays a crucial role in quenching free radicals and inhibiting lipid peroxidation (Mechri et al., 2019). According to Laguerre et al. (2009), verbascoside is found in higher concentrations in mature leaves, whereas younger leaves have higher contents of oleuropein. The relationship between verbascoside and oleuropein content of olive leaves was studied by the same authors, who reported that a bioconversion of oleuropein into verbascoside isomers occured during leaf maturation. This was also confirmed in our recent comprehensive study of biophenolic variability in relation to cultivar and leaf sampling period (Lukić et al., 2020), in which verbascoside was found to be the most accurate differentiatior of leaf sampling period, albeit in different indigenous cultivars than those presented here. In the present study, no significant differences in leaf verbascoside contents were found among the analyzed cultivars, averaging 211.43 mg 100 g^{-1} DW, although they differed significantly in oleuropein content (Table 1). Here, the correlation coefficient between verbascoside and oleuropein content showed a moderate positive association between the two compounds among the investigated cultivars (r = 0.51, $P < 0.001$). With regards to cultivars, the relationship between the two compounds was perhaps best described by Soler-Rivas et al. (2000), who showed a general tendency of small-fruit cultivars to have high oleuropein and low verbascoside contents, while the opposite was characteristic of large-fruited cultivars. Finally, verbascoside contents in leaves of all three analyzed cultivars reached lower concentrations compared to other cultivars from our previous reports (Pasković et al., 2020).

Oleuropein is the most abundant bioactive component in olive leaves (Hassen et al., 2015) and its concentrations can be significantly affected by the genetic background of the cultivar, with marked differences in its accumulation within the leaves of different cultivars (Ranalli et al., 2006). These differences were significant in the results presented in Table 1. Leaves of cultivar 'Buža' accumulated significantly highest content of oleuropein (5239.88 mg 100 g^{-1} DW), followed by leaves of cultivars 'Oblica' and 'Karbonaca' (3068.24 and 1685.24 mg 100 g^{-1} DW, respectively). These cultivar-based differences were in agreement with findings from other studies (Ranalli et al., 2006; Petridis and Therios, 2012, Pasković et al., 2020) and confirmed that olive leaves are oleuropein-rich sources with significant differences among cultivars. Since oleuropein is the most abundant compound in olive leaves, its concentrations usually accurately reflect the concentrations of total biophenols. Accordingly, the changes in the concentrations of total biophenols between the analyzed cultivars, corresponded to those of oleuropein concentrations (Table 1), with 'Buža' having significantly highest (5943.25 mg 100 g^{-1} DW) and 'Karbonaca' the lowest (2247.92 mg 100 g^{-1} DW) concentrations of total biophenols.

Besides secoiridoids, various flavonoids are also present in olive leaves (Laguerre et al., 2009), either in aglycone form (apigenin and luteolin) or in glycosylated form (apigenin-7-*O*-glucoside and luteolin-7-*O*-glucoside). Flavonoids possess a catechol function conjugated to unsaturated carbonyl and this structural configuration confers stability to radicals, resulting in greater superoxide scavenging ability compared to non-flavonoids (Lee and Lee, 2010). Rutin is one such flavonoid that exhibits high superoxide scavenging capacity. In fact, its ability to quench superoxide radicals exceeds that of oleuropein (Lee and Lee, 2010). Significantly highest levels of rutin were recorded in leaves of cultivar 'Buža' (41.99 mg 100 g^{-1} DW, Table 2). Leaves of the latter cultivar also exhibited highest concentrations of apigenin-7-*O*-glucoside (33.68 mg 100 g^{-1} DW) and luteolin-7-*O*-glucoside (43.38 mg 100 g^{-1} DW). On the other hand, 'Karbonaca' showed significantly higher levels of apigenin (7.55 mg 100 g^{-1} DW) and luteolin (48.17 mg 100 g^{-1} DW) compared to the other two cultivars (Table 2). Flavonoids in general have many interesting pharmacological activities and, depending on the extraction procedure, a strong nutraceutical potential as well.

Table 2. Concentrations of flavonoids in leaves of three olive cultivars, expressed in mg 100 g^{-1} of dry weight (DW)

Source of variation	Flavonoids					
	Apigenin	Apigenin-7-*O*-glucoside	Catechin	Luteolin	Luteolin-7-*O*-glucoside	Rutin
	mg/100 g DW					
Buža	2.49 ± 1.21^{b}	33.68 ± 0.24^{a}	17.33 ± 1.58^{a}	14.50 ± 2.04^{b}	43.38 ± 7.25^{a}	41.99 ± 1.82^{a}
Karbonaca	7.55 ± 2.69^{a}	10.56 ± 0.79^{c}	3.97 ± 2.98^{b}	48.17 ± 4.15^{a}	21.92 ± 2.32^{c}	37.15 ± 4.2a^{b}
Oblica	1.86 ± 1.51^{b}	25.59 ± 0.38^{b}	26.24 ± 7.37^{a}	17.96 ± 2.39^{b}	35.41 ± 8.73^{b}	31.87 ± 2.68^{b}
p-value	***	***	***	***	***	*

Results are expressed as means ± standard errors. Different superscript lowercase letters in a column represent statistically significant differences between mean values for each main effect at $P < 0.05$ obtained by a one-way ANOVA and Tukey's test. Significance: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$

Conclusion

Pruned leaves, as byproducts of olive cultivation, can represent a rich source of beneficial biophenols that could be harnessed as valuable nutraceuticals. In our study significant phytochemical variation was observed among the leaves of different cultivars analyzed. The cultivar 'Buža' differed from the other indigenous cultivars by having the highest concentrations of total biophenols, oleuropein and tyrosol, as well as flavonoids, such as rutin and glycosylated forms of apigenin and luteolin. On the other hand, leaves of cultivar 'Karbonaca' appeared to generate higher amounts of 4-hydroxybenzoic and ferulic acid along with non-glycosylated apigenin and luteolin flavonoids. Although cultivar variations in other important compounds such as hydroxytyrosol and verbascoside occur widely, they were not recorded in this study. The observed differences among cultivars can be utilized to obtain sources with higher concentrations of certain compounds that may be of crucial importance for both sustainable olive cultivation, as well as to human health. Olive cultivation has been traditionally focused on the agronomic traits, but with the increase in scientific evidence supporting the positive impact of leaf biophenols on human health, olive leaves are becoming a matter of interest for their functional features.

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