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Phenolic Compounds in Olive Leaf Extract as a Source of Useful Antioxidants

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Summary

Using electron paramagnetic resonance (EPR) spectroscopy, antioxidant properties of olive leaves extracts obtained of three autochthonous olive varieties grown in Istria, Buža, Istarska bjelica and Rosinjola have been investigated.

The highest content of total phenolic compounds ((57.6 ± 0.1) mg quercetin equivalents/g of dry extract) was obtained for extract of Buža. The analysis of chemical composition of extracts was performed by HPLC/DAD and LC/MS method. Futhermore, Buža has shown the highest antioxidant activity in scavenging DPPH radical (AA=80.0 ± 3.0%), followed by extracts of Rosinjola and Istarska bjelica. In DPPH radical scavenging assay final concentration of extracts in was 0.1 mg/mL. Antioxidant activity was in good correlation with total phenolics. It seems that these bioproducts could be useful in therapy of conditions related with oxidative stress. The results have indicated a high potential of application of olive leaf and olive leaf extract in foodstuff and food additive. Also, it can be used to improve the shelf life of foods and to develop functional foods.

Key words: Olea europaea L. leaves, HPLC, phenol compounds, antioxidative activity, EPR spectrometry

Introduction

Olive is of eastern Mediterranean origin and it spread widely around southern Europe, northern Africa, and the Iberian Peninsula (Connor, 2005). In the Istria region, the first historical records of olive growing date from the first century (Hugues, 1999). The favorable geographic position and Mediterranean climate encouraged long olive growing tradition in this area which presents the northeastern limit of olive growing. The morphological and molecular characterization of four autochthonous Istrian olive varieties: Buža, Buža puntoša, Istarska bjelica and Rosinjola has already been investigated (Poljuha et al., 1999). The leaves of olive trees have not yet been exploited industrially and they have been used only as animal feed (Delgado-Pertinez et al., 2000). For getting further benefit from olive trees, olive leaves may be important especially for Mediterranean countries.

The olive leaf (*Oleaceae*) has been widely used in folk medicine for several thousand years in European Mediterranean islands and countries. Leaves are taken orally for stomach and intestinal diseases and used as mouth cleanser, while decoctions of the dried leaf are taken orally for diarrhoea and to treat respiratory and urinary tract infections (Delgado-Pertinez et al., 2000; Bellakhdar et al., 1991). Previous investigations carried out on olive leaf extracts have demonstrated hypotensive, hypoglycaemic, hypouricaemic, antimicrobial and antioxidant activities (Benavente-Garcia et al., 2000). It is known that olive leaves contain triterpenes, flavonoids and chalcones (Dekanski et al, 2009.). The bitter compound oleuropein, the major constituent of the secoiridoid family in the olive (*Olea europaea* L.) trees, has been shown to be a potent antioxidant endowed with antiinfammatory properties (Mazziotti et al., 2007). Although the antioxidant activities of some single phenolic compounds in olive leaf are well known, antioxidant activities of its extract have not been clearly investigated (Lee et al., 2009).

Electron paramagnetic resonance (EPR) spectroscopy of spin probes is widely used in model membrane investigations and also in living systems (Mojović et al., 2005). Using DPPH (2,2-diphenyl-1-picrylhydrazyl) for evaluation of free radical-scavenging capacities of natural anti-oxidants was proposed by Blois more then half century ago. DPPH is a stable free radical and one of the most widely used substances as a primary standard in quantitative EPR spectrometry (Yordanov et al., 1996). Since reactive oxygen species (ROS) can react with biomolecules to form organic, hydrophilic radicals, we performed series of experiment to evaluate the ability of olive leaves preparations to remove such reactive species. The reduction of DPPH in the presence of an extracts is customary monitored by measuring the intensity of EPR signals.

The present study was carried out to evaluate the antioxidant activities of olive leaves (Fig. 1). The ability of olive leaf preparations obtained from three local varieties grown in Istria to remove organic hydrophobic radicals has been tested using DPPH. Also, the ability of olive leaves preparations to

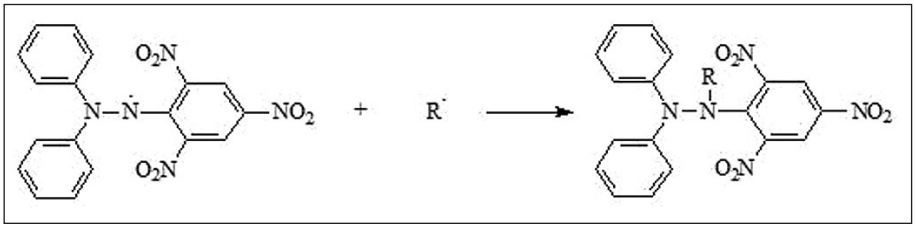

Figure 1. *Structure of DPPH and the mechanism of its reaction with radical*

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remove organic hydrophylic radicals has been tested using nitroxyl radical Tempone (2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-one), model compound for organic radicals stabile in aqueous solution. Furthermore, the objective of the study was to identify the main phenolic compounds present in an olive leaf extract and to point it as a potent natural resource of high antioxidant activity.

Materials and Methods

Sample preparation

The analyzed autochthonous olive varieties (Buža, Istarska bjelica and Rosinjola) are part of collection of the Institute of Agriculture and Tourism in Poreč, Croatia. Olive trees were grown in the same pedoclimatic conditions and cultivated with the same agrotechnical treatments. 5 kg of healthy olive leaves were collected from each tree at the same stage of ripening. Leaves were left to dry on pruned branches in the air, and under cover, for 2 months. The branches were then crushed and the leaf was coarsely separated by hand.

General chemical reagents

Quercetin and DPPH were obtained from Sigma chemical company, (St.Louis, MO, USA). Tempone was purchased from Molecular Probes (Junction City, OR, USA). All solvents used were of ACS grade, unless otherwise specified.

Preparation of the extracts

The samples were milled for further analysis. 1 g of ground plant material with 100 mL of pure methanol was extracted for 1 h, using an ultrasonic bath Branson model b-220 Smith-Kline Company (50/60 Hz, 125 W). The procedure of extraction was similar to that used by (Škerget et al., 2005) but applied extraction period was 1 hour shorter. The temperature of extraction was 40 °C at atmospheric pressure. The solvent was evaporated under vacuum using a rotary evaporator and the yield of extraction was determined in wt%. The extracts were stored in a dry and cool place.

HPLC analysis

The dry extracts (50 mg) were dissolved in 5 mL of methanol. The aliquots (2 mL) were mixed with 2 mL of H_2O and 1 mL of trifluoroacetic acid. The vials with reaction mixtures were sealed and heated in water bath for 3 h. After cooling, the samples were evaporated to dryness and then dissolved in 2 mL of methanol. The solutions were filtered through 0.45 μm pore size filter and injected in HPLC/DAD or LC/MS system.

The analysis of the hydrolyzed extracts was performed using an Agilent 1200 series HPLC with RR Zorbax Eclipse Plus C18 column (1.8 μm, 150 x 4.6 mm). Mobile phase A consisted of 0.2% formic acid in water, and mobile phase B consisted of acetonitrile. The injection volume was 5 μL, and elution at 0.95 mL/min with gradient program (0-20 min 5-16% B, 20-28 min 16-40% B, 28-32 min 40-70% B, 32-36 min 70-99% B, 36-45 min 99% B, 45-46 min 99-5% B). UV detection was carried out at 320 nm. Quantification was based on the measured integration area applying the calibration equation of quercetin in concentration range of 0.05-0.5 mg/mL. All experiments were performed five times and presented as means ± S.D. The content of the phenolic compounds was expressed as quercetin equivalents per g of dry extract. LC/MS analysis was performed on an Agilent MSD TOF coupled to an Agilent 1200 series HPLC, using the same column and gradient program as those for HPLC–DAD analysis. Mass spectra were acquired using an Agilent ESI-MSD TOF. Drying gas (N_2) flow was 12 L/min; nebulizer pressure was 45 psig; drying gas temperature was 350 °C. The parameters for ESI analysis were: capillary voltage, 4000 V; fragmentor, 140 V; skimmer, 60 V; Oct RF V 250 V, for positive and negative modes. The mass range was from 100 to 2000 *m*/*z*.

EPR measurements

DPPH radical scavenging assay

A final concentration of 0.6 mM methanol solution of DPPH in sample was 0.02 mM. Final concentration of extracts (previously dissolved in water) was 0.1 mg/mL. Sample with no extract served as a control. Each preparation was incubated for 20 minutes at room temperature (20 °C), and then measurements involving 4 minutes scanning time were conducted. The EPR spectra were recorded on Varian E104-A EPR spectrometer. EPR measurements were performed using parameter modulation amplitude, 1 G; modulation frequency, 100 kHz; microwave power, 10 mW; time constant, 0.032 s; field centre, 3370 G; scan range, 40G. All experiments were performed five times and presented as means ± S.D. The amplitude (A) of the middle peak in the EPR signal was measured, and antioxidative activity (AA) of each extract was calculated using Eq. 1:

$$AA = (A_{control} - A_{extract}) / A_{control} \quad /1/$$

Tempone radical scavenging assay

A final concentration of Tempone in the samples was 0.1 mM. Final concentration of extracts was 0.1 mg/mL. As previously explained sample with no extract served as a control. Each preparation was incubated for 20 minutes at room temperature (20 °C), and then measurements involving 4 minutes scanning time were conducted. EPR measurements were performed using parameters modulation amplitude, 1 G; modulation frequency, 100 kHz; microwave power, 10 mW; time constant, 0.032 s; field centre, 3380 G; scan range, 100G. All experiments were performed five times and presented as means ± S.D. The amplitude (A) of the middle peak in the EPR signal was measured, and antioxidative activity (AA) of each extract was calculated using Eq. 1.

Statistical Analysis

Statistical analysis was carried out using Statistica 6.0 (StatSoft Inc, Tulsa, OK, US). All experiments were performed at least in triplicate. Results are presented as mean values ± SD. Statistical significance was determined using Student's

Table 1. *Mean particle size and yield of dry extract*

Extracts	Mean particle size* (mm)	Extraction yield (%, w/w)
Istarska bjelica	0.15 ± 0.01	22.06 ± 0.11
Rosinjola	0.12 ± 0.01	26.93 ± 0.13
Buža	0.15 ± 0.01	31.83 ± 0.09

*Values are presented as means ± SD (from three separate experiments)

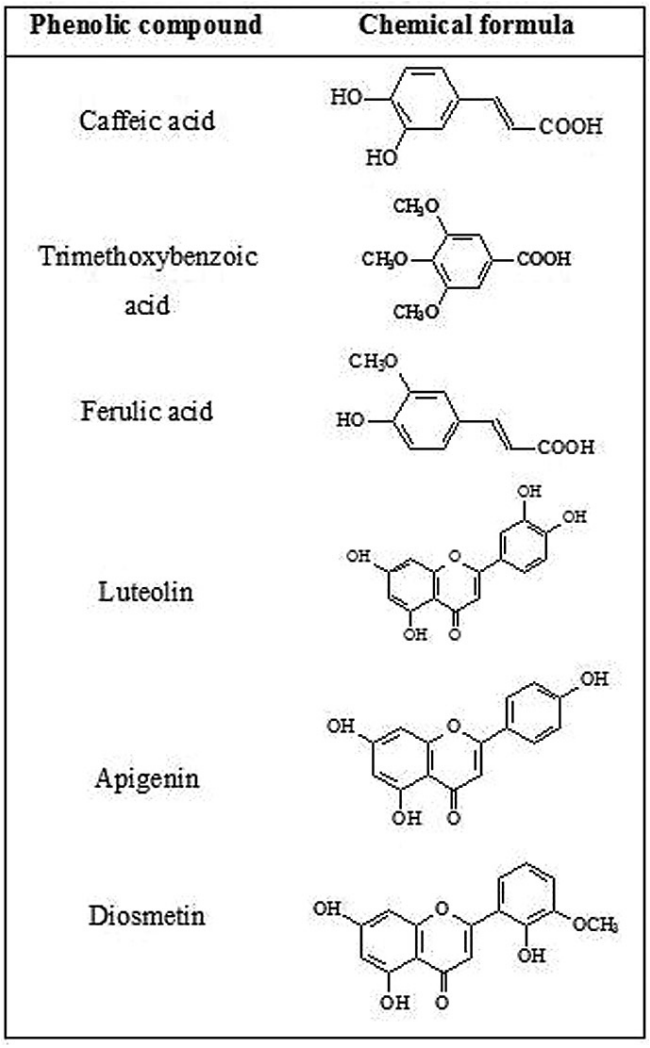

Figure 2. *Chemical structures of the identified compounds in olive leaf extract*

t test ($p<0.05$). Correlation coefficients (r) determining the relationship between AA and content of tannin derivates were calculated using Pearson correlation test. Significant levels were defined at $p\leq0.05$.

Results and Discussion

The extraction yields, (expressed in percentages % by mass) are given in Table 1.

The plant material was milled to a very fine particle size powder. The yield of the dry extract was from (22.06 ± 0.11)% for Istarska bjelica to (31.83 ± 0.09)% for Buža. The extraction yield of 34.70% was determined (Škerget et al., 2005), probably as a results of longer extraction period.

HPLC analysis of phenolic compounds present in olive leaf extract

The tentative* identification of the constituents of hydrolysates was performed by LC/MS and HPLC/DAD. Exact mass measurements of molecular and fragment ions of analyses were performed with a time-of-flight (TOF) mass spectrometer, in negative polarity mode. Six compounds are present: caffeic acid, trimethoxybenzoic acid, ferulic acid, luteolin, apigenin and diosmetin (Fig. 2).

All identified compounds exhibited quasi-molecular ion $[M-H]^-$ in the negative mode, as well as $[2M-H]^-$ ions for some of them, confirming molecular mass. Additionally, spectral data from a UV/vis photodiode array detector afforded definite structure determination. UV/vis maximums, which are characteristic for various classes of phenolic compound, were compared with the literature data (Marby et al., 1970). The results of HPLC/DAD and LC/MS analysis are summarized in Table 2 and Table 3.

The flavonoids, luteolin, apigenin and diosmetin are the main compounds in all hydrolysates. Traces of some phenolic acids are also observed. The highest content of total phenolic compounds ((57.6 ± 0.1) mg quercetin equivalents/g of dry extract) or (18.33 g/kg of dried leaves) was obtained for extract

Table 2. *HPLC/DAD data*

Peak	tR (min)	Compound	UV λ_{max} (nm)	Content**		
				Istarska bjelica	Rosinjola	Buža
1	12.6	Caffeic acid	242, 298, 324	1.1 ± 0.1*	0.9 ± 0.1	2.0 ± 0.1
2	16.5	Trimethoxybenzoic acid	234, 286, 314	3.2 ± 0.1	not detected	not detected
3	25.8	Ferulic acid	242, 298, 324	2.5 ± 0.1	2.6 ± 0.1	4.2 ± 0.1
4	27.9	Luteolin	254, 266, 292sh, 348	17.4 ± 0.2	29.4 ± 0.2	40.2 ± 0.3
5	29.5	Apigenin	266, 396sh, 336	7.1 ± 0.1	7.9 ± 0.1	5.9 ± 0.1
6	30.0	Diosmetin	252, 268, 292sh, 346	4.3 ± 0.1	7.4 ± 0.1	5.3 ± 0.1
Total phenolic compounds content**				35.6 ± 0.1	48.2 ± 0.2	57.6 ± 0.1

sh = shoulder

*Values are presented as means ± SD (from five separate experiments)

**(mg quercetin equivalents/g of dry extract)

*For determination of the structure without isolation of the pure compound or lack of characteristic standard compound for comparison, it is recommended to use term "tentative".

Table 3. LC/MS data

Peak	Ion species detected	Molecular mass	Molecular formula
1	M-H	180.0418	$C_9H_8O_4$
2	M-H	212.0677	$C_{10}H_{12}O_5$
3	M-H	194.0576	$C_{10}H_{10}O_4$
4	M-H, 2M-H	286.0472	$C_{15}H_{10}O_6$
5	M-H	270.0516	$C_{15}H_{10}O_5$
6	M-H	300.0628	$C_{16}H_{12}O_6$

of Buža. Olive tree leaves can be regarded as a particularly rich source of polyphenolic compounds. So, in investigation of Silva et al., (2006) total phenolic compounds expressed as tannic acid, presented values in the range of 11.7 to 40.1 g/kg of dry tissue. On the other hand, Mylonaki et al., (2008) found the maximum theoretical yield of total polyphenol content to be 250.2 ± 76.8 mg gallic acid equivalent/g of dry weight of olive leaf extract using optimal recovery techniques of polyphenols from olive leaves.

Antioxidant activity

Table 4 shows the activity for removal of DPPH and Tempone.

***Table 4**. Antioxidant activity of olive leaf extracts*

Extracts	AA*(DPPH) (%)	AA(Tempone) (%)
Istarska bjelica	64 ± 6	12 ± 2
Rosinjola	69 ± 2	22 ±1
Buža	80 ± 3	16 ± 0.5

AA=antioxidant activity against DPPH or Tempone
*Values are presented as means ± SD (from five separate experiments).
Antioxidative activities of ascorbate (0.2 mg/mL) were AA(DPPH)= 91 ± 4

From the results presented it could be concluded that all samples have shown to be remarkably good for quenching DPPH. Olive leaf extract of Buža has shown the highest activity (AA=80.0 ± 3.0%), followed by extracts of Rosinjola and Istarska bjelica.

Generally, all samples have expressed relatively low activity for removimg Tempone. Rosinjola extract has shown the highest activity, followed by Buža and Istarska bjelica extract.

One of the most important aspects of EPR is the possibility to determine the concentration of radical species, particularly in biological systems. For example in some experiment have been successfully employed the spin stabilization technique to detect and analyze the reactive *o*-semiquinone radicals of oleuropein (Tzika et al., 2008). Animal and in vitro studies suggest that olive oil phenols are effective antioxidants (Vissers et al., 2002). The results of investigation of relative abilities of olive leaf to scavenge the $ABTS^{\bullet+}$ radical cation have shown that the flavonoids and other phenolics expressed synergic behaviour when mixed, as it occurs in the extracts (Benavente-García et al., 2000). Furthermore, our research presents strong evidence of a free radical scavenging activity of olive leaves extracts.

Correlation between phenolic content and antioxidant activity

High positive correlation was obtained between yield of dry extract and total phenolics (r=0.996) and between AA(DPPH) and total phenolics (r=0.956). For the AA(Tempone) there was no correlation with the other investigated parameters.

The olive processing industry, which includes the production of olive oil and table olives, generates vast amounts of wastes. Thus, it has been estimated that the pruning of olives tress alone produces 25 kg of by-products (twigs and leaves) per tree annually (Mylonaki et al., 2008). Our research shows that olive tree leaves obtained of three autochthonous olive varieties grown in Istria can be regarded as a particularly rich source of polyphenolic compounds with strong antioxidant activity.

Conclusions

All investigated extracts possess high ability to scavenge DPPH radical and antioxidant activity was in good correlation with total phenolics. The results obtained pointed out to a high potential of application of olive leaf, considered traditionally as disposable byproducts. After adequate treatment they can, for example, be included as food additives and functional food materials with benefits for human or animal health. These characteristics indicate that selected phenolic compounds of olive extracts could be used as easily accessible, natural antioxidants in the diet and treatment of pathophysiological conditions related to oxidative stress.

References:

Benavente-García O., Castillo J., Lorente J., Ortuño A., Del Rio J.A. (2000) Antioxidant activity of phenolics extracted from *Olea europaea* L. leaves. *Food Chemistry*, 68 (4), 457-462.

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Science*, 181, 1199-1200.

Connor D. J. (2005) Adaptation of olive (*Olea europaea* L.) to water-limited environments. *Australian Journal of Agricultural Research*, 56 (11), 1181-1189.

Dekanski, D., Janićijević-Hudomal, S., Tadić, V., Marković, G., Arsić, I., Mitrović D. M. (2009), Phytochemical analysis and gastroprotective activity of an olive leaf extract. *Journal of Serbian Chemical Society*, 74 (4), 367-377.

Delgado-Pertinez M., Gomez-Cabrera A., Garrido A. (2000) Predicting the nutritive value of the olive leaf (*Olea europaea*): digestibility and chemistry composition and in vitro studies. *Animal Feed Science and Technology*, 87 (3-4), 187-201.

Hugues C. (1999) Olive Growing in Istria. Elaiografia Istriana. Ceres, Zagreb, Croatia (in Croatian).

Bellakhdar J, Claisse R, Fleurentin J, Younos C. (1991) Repertory of standard herbal drugs in the Moroccan Pharmacopoeia. *Journal of Ethnopharmacology*, 35 (2), 123-143.

Lee, O. -H., Lee, B. -Y., Lee, J., Lee, H. -B, Son, J. -Y., Park, C. -S., Shetty, K. , Kim Y. -C. (2009) Assessment of phenolics-enriched extract and fractions of olive leaves and their antioxidant activities. *Bioresource Technology*, 100 (23), 6107-6113.

Marby T. J., Markham K. R., Thomas M. B. (1970) The Systematic identification of Flavonoids. Springer–Verlag, New York, USA.

Mazziotti A., Mazziotti F., Pantusa M., Sportelli L., Sindona G. (2007) Pro-oxidant activity of oleuropein determined in vitro by electron spin resonance spin-trapping methodology. *Journal of Agricultural and Food Chemistry*, 54 (20), 7444-7449.

Mylonaki, S., Kiassos, E., Makris, D. P., Kefalas, P. (2008). Optimisation of the extraction of olive (Olea europaea) leaf phenolics using water/ethanol-based solvent systems and response surface methodology. *Analytical and Bioanalytical Chemistry*, 392, 977–985.

Mojović M, Spasojević I, Vuletić M, Vučinić Ž, Bačić G. (2005) EPR spin-probe and spin-trap study of free radicals produced by plant plasma membranes. *Journal of the Serbian Chemical Society*, 70 (2), 177-186.

Poljuha D., Sladonja B., Brkić Bubola K., Radulović M., Brščić K., Šetić E., Krapac M., Milotić A. (2008) A multidisciplinary approach to the characterisation of autochthonous Istrian olive (*Olea europaea* L.) varieties. *Food Technology and Biotechnology,* 46 (4), 347-354.

Silva S., Gomes L., Leitão F., Coelho A.V., Vilas Boas L. (2006) Phenolic Compounds and Antioxidant Activity of *Olea europaea* L. Fruits and Leaves. *Food Science and Technology International,* 12, 385–396.

Škerget M., Kotnik P., Hadolin M., Rižner Hraš A., Simonić M., Knez Ž. (2005) Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. *Food Chemistry,* 89 (2), 191-198.

Tzika D. E., Papadimitriou V., Sotiroudis G. T., Xenakis A. (2008) Oxidation of oleuropein studied by EPR and spectrophotometry. *European Journal of Lipid Science and Technology,* 110 (2), 149-157.

Vissers M. N., Zock P. L., Roodenburg A. J., Leenen R., Katan M.B. (2002) Olive oil phenols are absorbed in humans. *Journal of Nutrition,* 132, 409-417.

Yordanov N. D. (1996) Is our knowledge about the chemical and physical properties of DPPH enough to consider it as a primary standard for quantitative EPR spectrometry. *Applied Magnetic Resonance,* 10 (1-3), 339-350.