

# Effect of the extraction solvent on the oleuropein content and antioxidant properties of olive leaf (cv. *Oblica*, *Lastovka* and *Levantinka*) extracts

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## Summary

In the last few decades numerous studies have proved that an olive leaf is a rich source of bioactive phenolic compounds, mainly oleuropein and its derivatives. The aim of this study was to investigate the influence of the extraction solvent on the phenolic and oleuropein content in the leaf extracts of Dalmatian autochthonic olive cultivars: *Oblica*, *Lastovka* and *Levantinka*. The antioxidant activity of leaf extracts was determined using FRAP method and by metal chelating activity evaluation. The recovery obtained using methanol and ethanol (50:50, v/v) was higher than by use of water solvents. The highest share of total phenols and oleuropein was detected in ethanolic extract of *Lastovka*, while almost two-fold lower amounts were obtained using water extracts, both hot water and room temperature water. The extremely significant correlation between the FRAP and oleuropein/phenolic content points out the importance of these compounds in the total reducing activity of the extracts. All tested extracts provided good chelating activity probably due to the high concentrations of oleuropein but also the presence of other compounds with catechol structure, which is the most important structural feature of strong chelating activity. According to the obtained results it can be concluded that the extraction of polyphenols from olive leaves, especially from *Lastovka* cultivar, could present an interesting means of increasing the value of this cheap plant material that often remains unused after the harvest.

**Keywords:** olive leaf, oleuropein, HPLC, reducing ability, chelating activity

## Introduction

Olive tree and vines are traditional and the most important crops in the Mediterranean countries (Ahmad-Qasem et al., 2013; Peralbo-Molina and Luque de Castro, 2013). In the last few decades, due to the perception of gastronomic, nutritional and health-related benefits of Mediterranean diet, the demand for olive oils and wines have increased significantly (Erel et al., 2013). The cultivation of olive trees and vines has been usually focused on obtaining single high-value product, oil or wine, but vast quantities of agricultural residues remain after the production.

More than 8 million ha of olive trees are cultivated worldwide, especially in the Mediterranean basin. After the harvesting of olive fruits or pruning of olive trees, by-products (mainly leaves and branches) are often used as animal feed or burned (Ahmad-Qasem et al., 2013). It has been estimated that one hectare of olive trees generates over 3 tons of pruning biomass. Olive leaves are also an industrial residue and their amount in the total weight of olives arriving to mills is around 10 % (Brahmi et al., 2012; Bilgin and Sahin, 2013; Peralbo-

Molina and Luque de Castro, 2013). On the other hand it is well known that those residues contain biologically active substances with the good antioxidant potential (Hayes et al., 2011; Alzweiri et al., 2013; Erel et al., 2013). Beside antioxidant properties, olive leaf extracts have been shown to possess other biological activities like antiviral, antibacterial, anti-inflammatory, they act as lipid stabilisers and they are used in treatment of different ailments like diabetes and cardiovascular diseases etc. (Hayes et al., 2011; Alzweiri and Al-Hiari, 2013; Peralbo-Molina and Luque de Castro, 2013).

The biological activity of olive leaves is attributed to the presence of numerous secondary metabolites. The main category of those bioactive compounds is secoiridoids that are present in Oleaceae and other few dicotyledonous plant families (Alagna et al., 2012). One of the most dominant secoiridoid found in olive leaves is oleuropein (Benavente-García et al., 2009; Silva et al., 2010; Sahin et al., 2012). Olive leaves also contain other bioactive phenolics like phenyl ethyl alcohols (tyrosol and hydroxytyrosol), simple phenols (caffeic acid, *p*-coumaric acid, vanillic acid, vanillin, etc.) and flavonoid-like

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compounds (verbascoside, luteolin, diosmetin, rutin, luteolin-7-glucoside, apigenin-7-glucoside and diosmetin-7-glucoside, etc.) (Benavente-Garcia et al., 2000; Vissers et al., 2004; Lee et al., 2009; Hayes et al., 2011; Ahmad-Qasem et al., 2013). All these compounds could be of interest to the pharmacological, nutraceutical, cosmetic, and food industries. Therefore, the rational use of olive tree waste materials could have perspective future (Peralbo-Molina and Luque de Castro, 2013).

Thereby, the aim of this study was to investigate the phenolic potential and antioxidant activity of olive leaf extracts obtained from autochthonous Dalmatian cultivars *Oblica*, *Lastovka* and *Levantinka*. The impact of the solvent on the extraction efficiency of oleuropein was also analysed, as well as the contribution of this dominant compound to antioxidant activity of extracts.

## Materials and methods

### General

All reagents and solvents used were of adequate analytical grade and were obtained from Sigma (Sigma-Aldrich GmbH, Steinheim, Germany), Kemika (Zagreb, Croatia) and Merck (Darmstadt, Germany). Oleuropein (90 %, HPLC, CAS 32619-42-4) was purchased from Extrasynthese (Genay, France). Spectrophotometric measurements were performed on a UV-Vis spectrometer Lambda EZ 201 (Perkin-Elmer, Waltham, Massachusetts, USA). The HPLC system used was HP 1090 Series II, equipped with diode array detector (DAD), binary gradient solvent delivery system and autoinjector (Agilent Technologies, Palo Alto, California, USA). The average particle size was determined by sieving using shaker Retsch AS 200 (Retsch-Allee, Haan, Germany), after sieving through 40-500 mesh screens (Fisher Scientific, BS410/ISO3310, Loughborough, UK).

### Plant material and extraction procedure

Plant material, olive leaf of *Oblica*, *Lastovka* and *Levantinka* cultivars were hand-picked from olive trees in Kaštela, Dalmatia, Croatia in February 2012. The plant material was dried at room temperature until constant weight and pulverized using high-speed grinder (2 minutes). The average particle size was  $234.6 \pm 178.1 \mu\text{m}$ . The sieving time was 30 minutes and the amplitude was 3 mm. The extraction was performed using different solvent systems: methanol/water mixture (50/50, v/v), ethanol/water mixture (50/50, v/v), room temperature (RT) water and hot (boiled) water. For olive leaf extract, 1 g of dried and ground leaf samples was weighed and 5 mL of solvent was added. The suspensions were stirred during 5 minutes (300 rpm),

filtered and centrifuged (10 minutes, 4000 rpm). Supernatants were collected and thus obtained extracts were used in further analysis.

### Determination of total phenols

The concentration of total phenolics was measured according to the Folin-Ciocalteu (FC) method (Amerine and Ough, 1980). This method is based on colorimetric oxidation/reduction reaction where Folin-Ciocalteu reagent is used as oxidising agent. The olive leaf extracts (0.25 mL) were transferred to a 25 mL volumetric flask, containing 15 mL of distilled water and 1.25 mL of Folin-Ciocalteu reagent. The solution was neutralized by adding 20% sodium carbonate (3.75 mL). The volume was made up with distilled water to 25 mL and after 2 hours the absorbance was measured at 765 nm (Generalić et al., 2012). The calibration curve was created using pure oleuropein concentration ranging from 25-500 mg/L with a regression coefficient of 0.9986. All measurements were carried out in triplicate and the amount of total phenols was expressed in g of oleuropein equivalents per litre of extract (g OLE/L).

### Separation, identification and quantification of oleuropein by HPLC

For the quantification of oleuropein using HPLC, the extracts were filtered through a  $0.45 \mu\text{m}$  syringe filter. The stationary phase was a Nucleosil 100-C18 (250×4.0 mm) with a particle size of  $5 \mu\text{m}$  (Macherey-Nagel, Germany). The content of oleuropein was determined according to the method proposed by the International Olive Oil Council (2009) with a slight modification due to used HPLC system with the binary pump. The flow rate was 1 mL/min and the absorbance changes were monitored at 280 nm. The mobile phases were (A) water/formic acid (99.5/0.5, v/v) and (B) methanol/acetonitrile (50/50, v/v). The elution program is given in Table 1.

**Table 1.** The HPLC program for gradient elution

Time (minute)	Eluent A (%)	Eluent B (%)
0	96	4
40	50	50
45	40	60
60	0	100
70	0	100
72	96	4
82	96	4

The oleuropein was identified by comparison of its retention time with the corresponding standard and by its UV-VIS spectra, and quantified from the area of the peak using external standard calibration curve.

### Reducing power (FRAP method)

The reducing activity of olive leaf extracts was measured as ferric reducing antioxidant power (FRAP) as described by Benzie and Strain (1996). In this method at low pH, ferric-tripyridyltriazine ( $\text{Fe}^{\text{III}}$ -TPTZ) complex is reduced to the ferrous ( $\text{Fe}^{\text{II}}$ ). The change in absorbance at 593 nm, therefore, is directly related to the combined or "total" reducing power of the electron-donating antioxidants present in the reaction mixture (Katalinić et al., 2010; Generalić et al., 2012). The calibration curve was made by the results obtained using pure oleuropein solution, testing, concentrations ranging from 25 to 1000 mg/L. Each determination was performed in triplicate. The results were expressed as means  $\pm$  standard deviation (SD) in g of oleuropein equivalents per litre of extract (g OLE/L).

### Metal chelating activity

The chelation of ferrous ions by olive leaf extracts was estimated by the method of Yen et al. (2000). Briefly, 1 mL of the adequately diluted sample was added to 3.7 mL of methanol and 50  $\mu\text{L}$  of 2 mM ferrous chloride solution. The reaction was initiated by the addition of 0.2 mL of 5 mM ferrozine. The solution was shaken and left to stand at RT for 10 minutes. The absorbance of the resulting solution was measured at 562 nm and the percentage of inhibition was calculated as  $[(A_0 - A_s)/A_s] \times 100$ , where  $A_0$  was the absorbance of the control and  $A_s$  was the absorbance of the sample. All determinations were performed in triplicate and the results are expressed as the mean value  $\pm$  SD.

### Statistical analysis

Statistical analysis was performed using GraphPad InStat3 (GraphPad Software, San Diego, USA)

software package. Pearson's correlation coefficient was used to determine the relation between the variables.

## Results and discussion

Olive leaves are cheap and unutilized material that often remains unused and its disposal often presents a serious economic and environmental problem. Since it is known that this material is a source of highly valuable and biologically active compounds, the identification of the most appropriate extraction method is an important step to increase the yield of such bioactive components from plant material (Brahmi et al., 2012). The main aim of the present work was to quantify the content of total phenols and oleuropein in olive leaf extracts obtained from autochthonous Dalmatian cultivars *Oblica*, *Lastovka* and *Levantinka*, and to explore their antioxidant potential. Different extraction solvents were used to obtain the most effective extracts with highest share of phenolics and oleuropein, because it is well known that the extraction rate may be improved by choosing the best combination of process variables, such as the type of solvent (Ahmad-Qasem et al., 2013). Also, the recovery of phenolics from plant materials is influenced by their solubility in the solvent used for the extraction process and the polarity of the solvent plays a key role in increasing phenolic solubility (Brahmi et al., 2012). For that purpose we used conventional extraction that has been carried out by the maceration using liquid solvents; methanol/water mixture (50/50, v/v), ethanol/water mixture (50/50, v/v), room temperature (RT) water and hot water.

In general, better extraction yield was obtained using alcoholic solvents, in contrast to water solvents. Table 2 shows the total phenol concentrations in olive leaves extracts, expressed as milligrams of oleuropein equivalents per 1 L of extract.

**Table 2.** The content of total phenols (TP) and antioxidant properties of olive leaf extracts determined as ferric reducing /antioxidant power (FRAP) and  $\text{Fe}^{2+}$  chelating activity (CA)

Extraction solvent	Cultivar	TP (g OLE/L)	FRAP (g OLE/L)	CA (%)
Methanol/Water 50/50, v/v	<i>Oblica</i>	17.3 $\pm$ 0.3	19.0 $\pm$ 0.2	79.5 $\pm$ 2.9
	<i>Lastovka</i>	21.4 $\pm$ 0.6	25.4 $\pm$ 0.6	74.2 $\pm$ 0.3
	<i>Levantinka</i>	18.2 $\pm$ 0.3	18.5 $\pm$ 0.5	80.3 $\pm$ 0.7
Etanol/Water 50/50, v/v	<i>Oblica</i>	16.6 $\pm$ 0.1	17.9 $\pm$ 0.8	83.2 $\pm$ 0.5
	<i>Lastovka</i>	21.7 $\pm$ 0.1	25.4 $\pm$ 1.5	81.8 $\pm$ 0.7
	<i>Levantinka</i>	17.9 $\pm$ 0.1	17.1 $\pm$ 1.4	84.2 $\pm$ 0.1
Hot water	<i>Oblica</i>	9.9 $\pm$ 0.1	9.5 $\pm$ 0.3	71.4 $\pm$ 0.1
	<i>Lastovka</i>	9.6 $\pm$ 0.3	9.6 $\pm$ 0.4	71.7 $\pm$ 0.6
	<i>Levantinka</i>	12.3 $\pm$ 0.2	10.5 $\pm$ 0.3	81.9 $\pm$ 1.2
Room temperature water	<i>Oblica</i>	8.1 $\pm$ 0.1	6.9 $\pm$ 0.3	82.6 $\pm$ 0.6
	<i>Lastovka</i>	9.2 $\pm$ 0.2	8.8 $\pm$ 0.1	78.4 $\pm$ 0.0
	<i>Levantinka</i>	9.0 $\pm$ 0.1	8.3 $\pm$ 0.2	75.8 $\pm$ 1.5

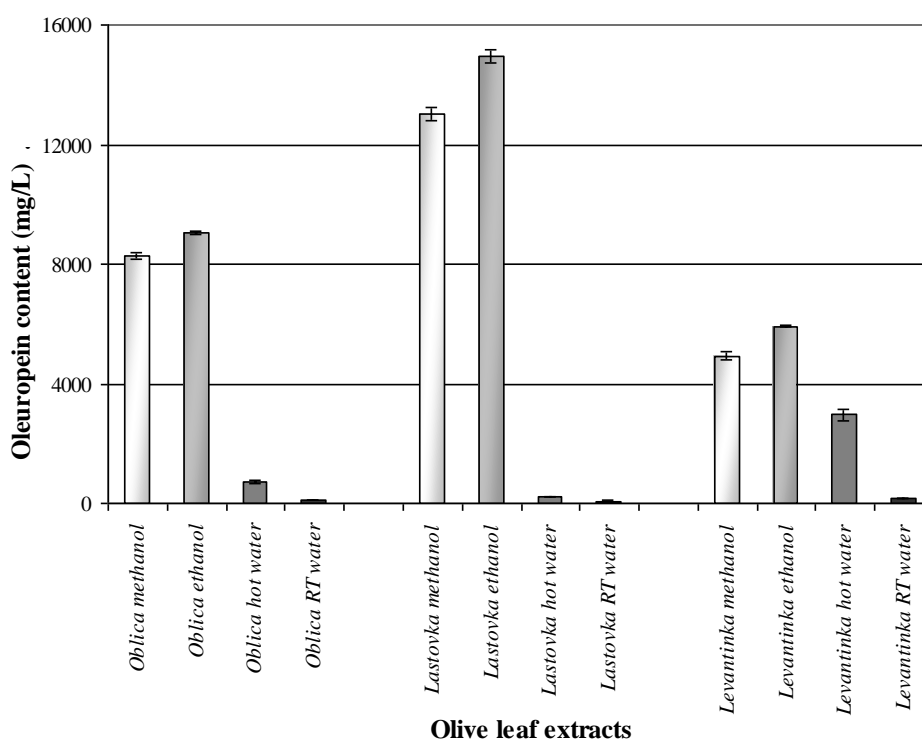
\*The activity of 10-fold diluted extracts

As shown in Table 2, the highest share of total phenols determined by the Folin-Ciocalteu assay for olive leaf extracts was detected in ethanolic extract of *Lastovka* ( $21.7 \pm 0.1$  g OLE/L) as well as its methanolic extract ( $21.4 \pm 0.6$  g OLE/L).

On the other hand, methanolic extracts of *Oblica* and *Levantinka* cultivars contain slightly higher amounts of phenolics ( $17.3 \pm 0.3$  g OLE/L and  $18.2 \pm 0.3$  g OLE/L, respectively), in comparison with their ethanolic extracts. It is well known that the use of high temperatures lead to a kinetic improvement, but it is limited by the fact that most of phenolic compounds are thermo labile. Thus, heat treatments could reduce both the phenolic content and

antioxidant capacity (Ahmad-Qasem et al., 2013). This state has been also confirmed by our study where almost two-fold lower results for total phenols were obtained for water extracts. The content of total phenols in room temperature water extracts ranged from 9.6 to 12.3 g OLE/L, while in hot water extracts ranged from 8.1 to 9.2 g OLE/L.

HPLC profiles of oleuropein present in the investigated olive leaf extracts are shown (Fig. 1). Oleuropein, bitter compound of olives, was discovered in 1908 by Bourquelot and Vintilesco, and its structure was specified as being that of a heterosidic ester of elenolic acid and dihydroxyphenylethanol (Benavente-Garcia et al., 2000).



**Fig. 1.** The concentration of oleuropein in olive leaf extracts of *Oblica*, *Lastovka* and *Levantinka* cultivars prepared using different solvents: 50 % methanol, 50 % ethanol, hot water and room temperature (RT) water extracts

Results of our study showed that the dominant phenolic in olive leaves extracts of *Oblica*, *Lastovka* and *Levantinka* cultivars obtained using different solvents, was oleuropein and its concentrations ranged from 65-14956 mg/L (Fig. 1). The highest content was found in ethanolic extract of *Lastovka*, while the lowest was present in room temperature water extract of the same cultivar. HPLC chromatogram of *Lastovka* ethanolic extract is shown in Fig. 2. Other extracts rich in oleuropein were methanolic extract of *Lastovka* (13022 mg/L), ethanolic extract of *Oblica* (9047

mg/L) and methanolic extract of *Oblica* (8280 mg/L). The concentration of oleuropein in water extracts ranged from 65 to 2963 mg/L, reaching the highest concentration in *Levantinka* hot water extract. Hot water extracts contained oleuropein in higher amounts which lead to the conclusion that the stability of oleuropein is not under the influence of high temperature treatment. The extremely significant linear correlation was found between the content of total phenolics and oleuropein ( $r = 0.9415$ ,  $p < 0.0001$ ) (Fig. 2).

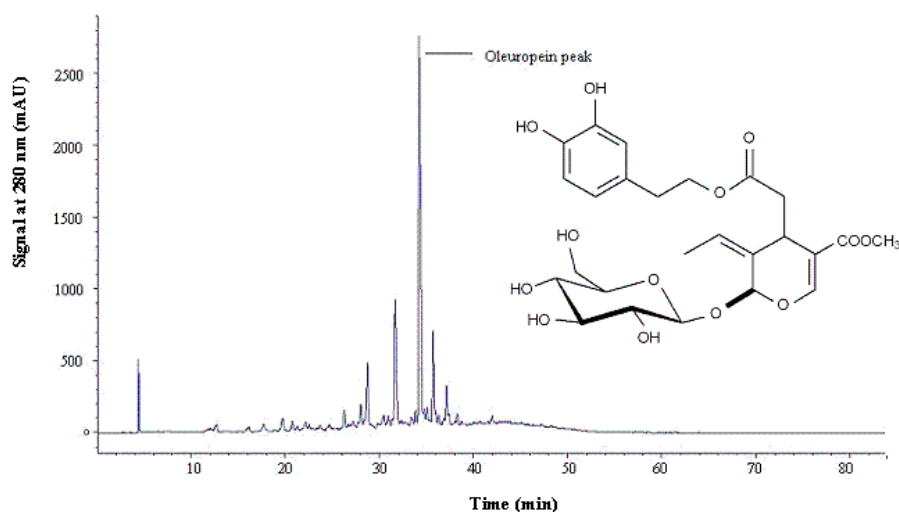


Fig. 2. HPLC chromatogram of *Lastovka* ethanolic extract with signed peak of oleuropein with its structure

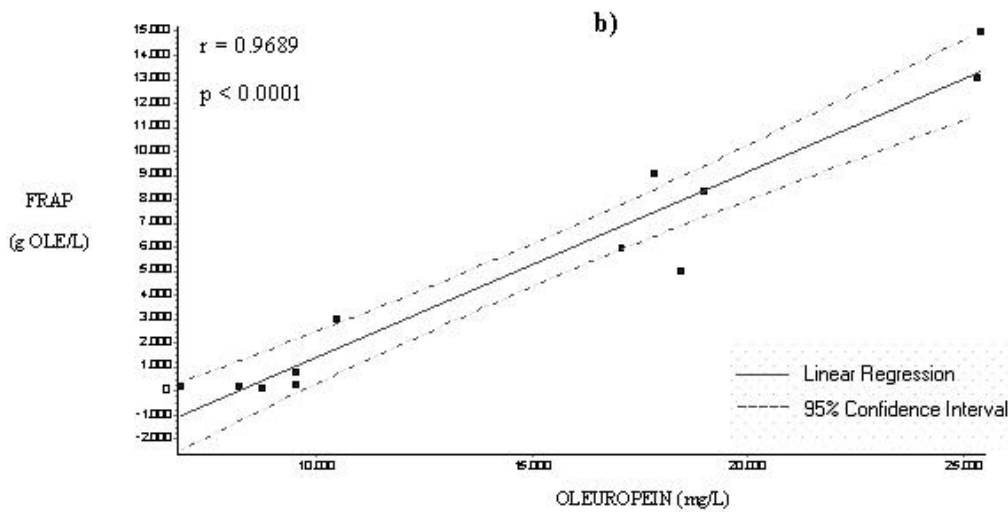
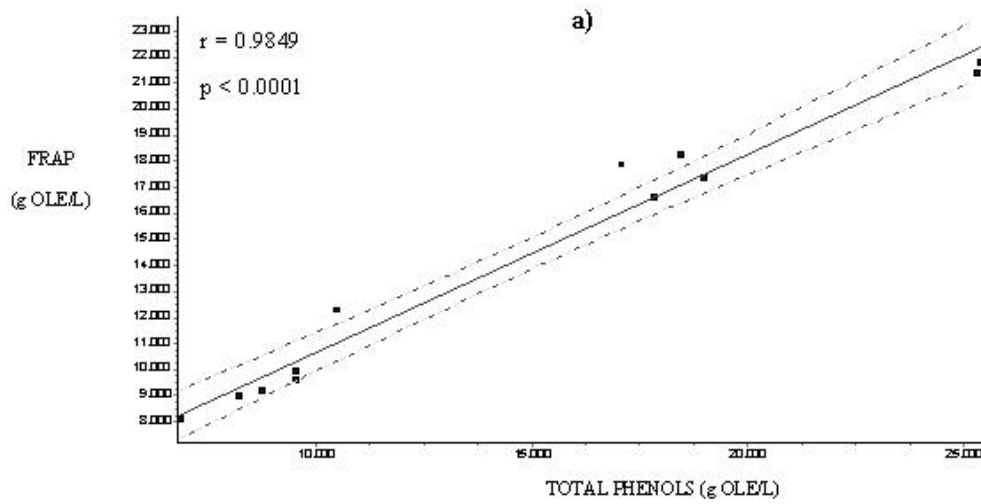
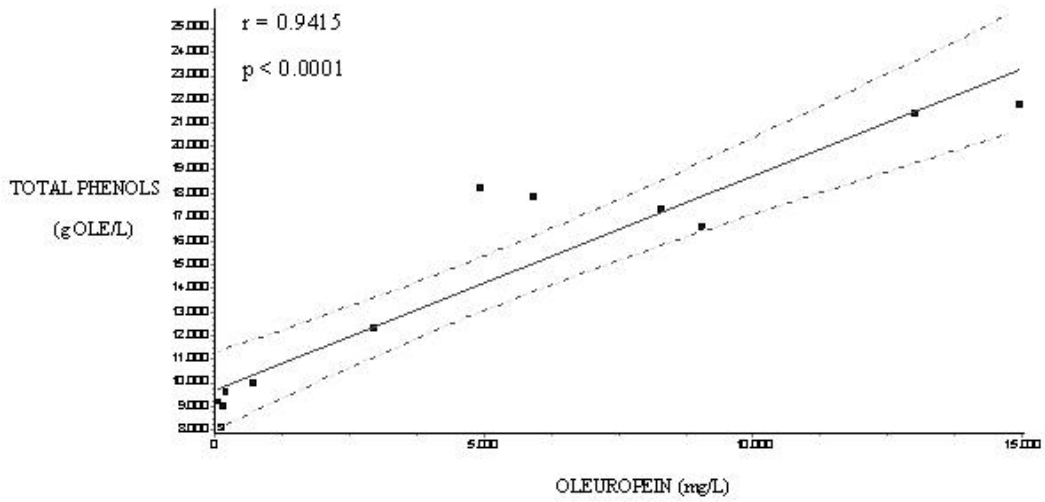
There are many methods available for the measurement of the antioxidant activity of different samples and most researchers use one or more of the available assays due to the different mechanism that occur in reaction systems (Hayes et al., 2011). Although, the antioxidant activity of some individual phenolics from olive leaves is well known, the activity of extracts obtained using different solvents, especially from Croatian cultivars, has not been clearly investigated.

In this study we used FRAP method for the determination of the reducing capacity of extracts, while the chelating method provided the information about the extracts components that are able to bond metal ions.

On the basis of the FRAP values, the strength of reducing activity was in order: methanolic extracts > ethanolic extracts > hot water extracts > room temperature water extracts. Again, the best reducing activity was obtained for *Lastovka* alcoholic extracts. The FRAP value of those extracts was 25.4 g OLE/L. The FRAP value for methanolic extracts of *Oblica* and *Levantinka* was 19.0 and 18.5 g OLE/L, respectively. FRAP for hot water extracts ranged from 9.5 to 10.5 g OLE/L, while for room temperature extracts it ranged from 6.9 to 8.8 g OLE/L. The different antioxidant activities of extracts can be attributed to the different extracting solvents, as the antioxidant activity depends on its type and polarity (Brahmi et al., 2012). Therefore, we can conclude that the high temperature does not affect the reducing capacity of the extract probably due to the resistance of oleuropein. The extremely significant linear correlation was found between the content of total phenolics and FRAP values ( $r = 0.9849$ ,

$p < 0.0001$ ), as well as between the content of oleuropein and FRAP ( $r = 0.9689$ ,  $p < 0.0001$ ). Those results are presented in Fig. 3. The correlation between the FRAP results and oleuropein content also pointed out the importance of this compound in the total reducing activity of olive leaf extracts.

The second method that has been used in antioxidant evaluation was chelating activity. This method is based on the ability of ferrozine to form complexes with ferrous ions, but in the presence of chelating agents (like phenolic compounds) the complex formation is disrupted (Ebrahimzadeh et al., 2008). According to the obtained results, all tested extracts provided good chelating activity that ranged from 71.4 to 84.2 % (Table 2), with average values in order: ethanolic extracts (83 %) > room temperature extracts (79 %) > methanolic extracts (78 %) > hot water extracts (75 %). It is interesting to see that extracts with high share of total phenols and oleuropein, like ethanolic and methanolic extracts of *Lastovka* provided lowest results for chelating activity, while among room temperature water extracts, *Oblica* extract which was the most indigent in phenolics, gave the best results. It has been established that the catechol structure of the compound is the most important structural feature of strong chelating activity (Shan et al., 2005). This structure is also present in oleuropein molecule and could be the reason for good activity of all extracts. Although oleuropein is found to be dominant phenolic compound in the olive leaf extracts, other phenolics that are present in the extracts are probably responsible for the observed better chelating properties of water extracts.



**Fig. 3.** The correlation between total phenolic and oleuropein content (a), total phenolic content and FRAP value (b) and oleuropein concentration and FRAP value (c)

## Conclusions

The extraction of polyphenols from olive leaves could present an interesting means of increasing the value of this by-product. Due to high content of total phenols and oleuropein, the important factors for antioxidant activity, olive leaves extracts (especially alcoholic extracts of *Lastovka* cultivar) have a strong potential to be used in pharmaceutical preparations.

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