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Araştırma Makalesi/*Research Article (Original Paper)*

Effect of Solvent Variation on Polyphenolic Profile and Total Phenolic Content of Olive Leaf Extract

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Abstract: Olive tree is one of the oldest plants, evergreen and of which fruits, leaves and trunk can be used for many different purposes. Olive has become a nutritional source widely used for especially in the Mediterranean diet and due to its extensive metabolic effects attracting everyone's attention recently. Olive and olive leaves are rich in polyphenolic compounds. Oleuropein is the most available and most active polyphenolic compound. Hydroxytyrosol, tyrosol and verbascoside are other important active compounds. Oleuropein has antioxidant, antidiabetic and antimicrobial effects. This study was investigated the effects of solvent variation on the amount of polyphenolic compounds and total phenolic content in olive leaves. The olive leaves were extracted using water, methanol/water and methanol solvents. The content of olive leaves extract was investigated by chromatographic analysis. Also total phenolic content was determined using Folin-Ciocalteu method by spectrophotometric method. The chromatographic analysis showed that methanolic solvents more effectively extracted oleuropein from olive leaf. However, when water was used as a solvent, hydroxytyrosol, tyrosol and verbascoside were extracted better from olive leaf. When solvents were compared in respect to their total phenolic content; the order from highest to lower was respectively methanol, methanol/water, and water. Consequently, due to their high hydrophilic character; polyphenolic compounds can more efficiently be extracted in methanolic solvents with high polarity.

Keywords: Extraction, HPLC, Oleuropein, Olive, Total phenolic content

Çözücü Farkının Zeytin Yaprağı Ekstraktı'nın Polifenolik Profiline ve Toplam Fenolik Madde Miktarına Etkisi

Özet: Zeytin ağaçları her dem yeşil, pek çok farklı amaç için meyveleri, yaprakları ve gövdesi kullanılabilen en eski bitkilerinden birisidir. Zeytin, özellikle Akdeniz diyetinde ve son zamanlarda da herkesin ilgisini çeken geniş metabolik etkileriyle çok kullanılan bir besin kaynağı haline gelmiştir. Zeytin ve yaprağı polifenolik bileşikler bakımından zengindir. Polifenolik bileşikler arasında da oleuropein en bol bulunan ve en aktif olanıdır. Diğer önemli aktif bileşikler ise hidroksitirozol, tirozol ve verbaskozittir. Oleuropein antioksidan, antidiyabetik ve antimikrobiyal etkilere sahiptir. Bu çalışma; çözücü farkının zeytin yaprağındaki polifenolik bileşiklerin miktarlarına ve toplam fenolik madde miktarına etkisinin araştırılması amacıyla planlanmıştır. Saf su, metanol/su ve metanol solventleri kullanılarak zeytin yaprağı ekstrakte edilmiştir. Elde edilen ekstraktta, içerik tayini kromatografik analiz kullanılarak araştırılmıştır. Ayrıca toplam fenolik madde miktarı Folin-Ciocalteu metodu ile spektrofotometrik yöntem kullanılarak tespit edilmiştir. Kromatografik analizler sonucunda metanolik solventlerin oleuropeini zeytin yaprağından daha etkin bir şekilde ekstrakte ettiği bulunmuştur. Solvent olarak su kullanıldığında ise hidroksitirozol, tirozol ve verbaskozitin daha iyi ekstrakte edildiği tespit edilmiştir. Ekstrakte edilen toplam fenolik madde miktarı bakımından solventler karşılaştırıldığında sırasıyla metanol, metanol/su ve suda en yüksek miktarlar bulunmuştur. Sonuç olarak, yüksek hidrofilik karakterlerinden dolayı polifenolik bileşiklerin, polaritesi fazla olan metanolik solventlerde daha verimli ekstrakte edilebileceği belirlenmiştir.

Anahtar kelimeler: Ekstraksiyon, HPLC, Oleuropein, Zeytin, Toplam fenolik madde

Introduction

Olive belongs to the family Oleaceae and its binomial name is *Olea europaea* L. Olive trees are one of the oldest plants in the world, evergreen, having potential of living more than a thousand years, history of which extends to the ancient times. Table olive and oil are the most commonly used products in daily life in

Mediterranean diet. All parts of olive tree ranging from its roots to leaves are evaluated in different areas. Olive has small white flowers, prominent green-gray upper surface, lower surface leaves, blue-silvery in color. Olive has a specific bitterish and somewhat acrid smell and taste. It was revealed that the source of the bitter taste of olive is created by a compound named oleuropein and it is involved in its fruit, leave and bark (Bourquelot and Vintilesco 1908). Olive has rich biophenolic content with high concentration ranging between 1-3% (Servili et al. 2004). The most abundant and the most active phenolic compound in olive leaves is oleuropein substance. Oleuropein has common characteristics with tannin (Cruess and Alsberg 1934). It is a natural bioactive compound from secoiridoids group. Oleuropein belongs to the group of secoiridoids and abundant in the families of Oleaceae, Gentianaceae, Cornaceae and in many other families. Secoiridoids belonging to the family of Oleacea usually originate from oleoside type of glycosides. They are a combination of elenolic acid and glycosidic residue. Oleuropein is an ester of 2-(3,4-dihydroxyphenyl) ethanol, i.e. hydroxytyrosol and has an oleositic sceleton. This structure is common in secoiridoids glycosides of oleaceae family and it forms sugar parts, insoluble in oil as an aglycone (Soler-Rivaset al. 2000). Antioxidant, antidiabetic, antimicrobial and hypolipidemic effects of oleuropein are some well-known metabolic effects (Omar 2010). A result of the hydrolysis of oleuropein are formed elenolic acid and hydroxytyrosol [2-(3,4-dihydroxyphenyl) ethanol] as being other active metabolites (Granados-Principal et al. 2010) (Figure 1). Verbascoside, luteolin-7-glucoside, apigenin 7-glucoside and tyrosol are other important active phenolic compounds included in olive leaves (Lee-Huang et al. 2003). Although phenolic compounds such as tyrosol and especially hydroxytyrosol are minor substances included in olive leaves and oil; they have significant metabolic effects (Di Benedetto et al. 2007; Jemai et al. 2009).

Phenolic compound types and levels are different in leaves, fruit and stones of olive (Ryan et al. 2002). There are significant changes at phenolic compounds in leaves and fruits during ripening period (Briante et al. 2002). The development of olive is divided into three phases. The developmental phases of olive fruit and leaf are similar. Thus, the changes in the amount of metabolite in fruit and leaves represent similarity. These stages are; “growth phase” in which oleuropein formation and accumulation take place; “green maturation phase” in which reduction in the level of chlorophyll and oleuropein takes place; and finally “black maturation phase” which is characterized by appearance of anthocyanins and in which reduction in the level of oleuropein goes on. As well as reduction in the level of oleuropein, the levels of other phenolic compounds such as verbascoside are increasing (Amiot et al. 1989).

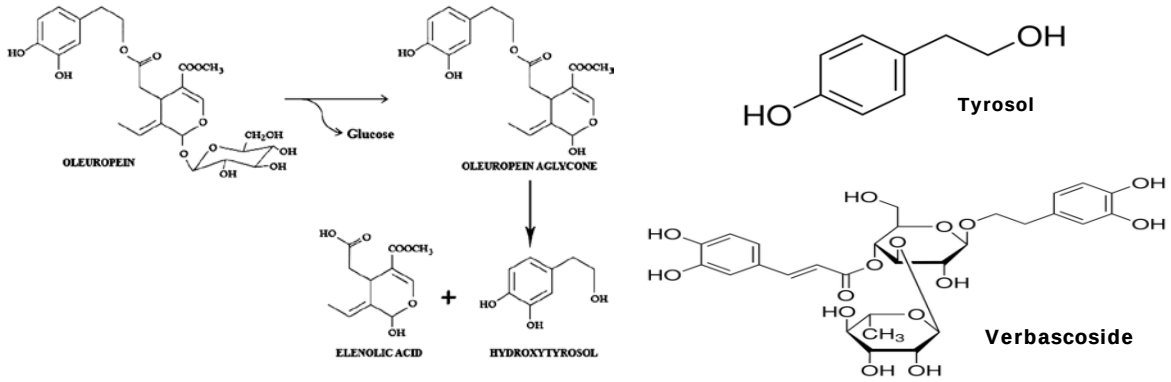


Figure 1. Hydrolysis of Oleuropein (Granados-Principal et al. 2010).

In recent years, revealing scientifically the effectiveness of some foods for prevention and treatment of diseases in natural ways led to increase the importance of nutrition support for protection of human health. Therefore, nutraceutical and therapeutic usages of functional foods began gradually increasing. Olive and its various products used for many different purposes in especially Mediterranean countries are prophylactically important.

The main aim of the study is to obtain optimum amount of oleuropein using different concentrations of traditional solvents and compare it in terms of total polyphenolic compound. In order to accomplish these task, effects of solvent variation on the amount of extracted oleuropein, hydroxytyrosol, tyrosol and verbascoside polyphenolic compounds and total phenolic content in olive leaves were investigated. Findings of this study might be useful in the field of pharmacognosy, ethnobotany, and biochemistry.

Materials and Methods

Plant Material

The olive (*Olea europaea* L.) leaves, the plant material of which effects would be investigated, was collected in Antalya in August 2013 and dried in outdoor under the shadow. They were recorded to Yuzuncu Yıl University herbarium with the code of VANF-164715. The olive leaves were ground with a plant mill and prepared for extraction process.

Olive Leaf Extraction

The effect of solvent variation on extraction was investigated by using chromatographic analysis. For determination of phenolic compound in olive leaf extract, 1 g powdered olive leaf was added into 50 mL distilled water. It was sealed with parafilm and covered with aluminum foil to prevent from light. The process of extraction was carried out on a magnetic hot plate (Wisd WiseStir MSH-20D) for 6 hours at 50 °C and 750 rpm. Afterwards, it was centrifuged (Hettich Universal 320r) for 5 minutes at +4 ºC and 3500 rpm in falcon tube, by being separated from the filtrate solid particles by a buncher funnel. The resulting supernatant was removed from small particles by being filtered by a 45 µm PTFE syringe filter. The final extract was transferred to vials for content analysis (Figure 2). The same processes were performed for extraction in the solvents of methanol/water (MeOH/water) (1:3 v/v) and methanol (MeOH) as well. Extraction was studied over 2 samples and 2 recurrences so that validation could be provided.

The reason why water and methanol were preferred in the study is that water is a general and widely used polar solvent and methanol is a good polar solvent for separation of polyphenolic compounds through acting plant cells. High temperature and long extraction time decreases the efficiency of polyphenolic compounds (Dai and Mumper 2010). That's why 50 °C of temperature was selected and temperature-originated deterioration of phenolic compounds was attempted to be prevented during their extraction process. Furthermore, since polyphenolic compounds are easily oxidized, as an added precaution both the extraction was limited to 6 hours and condensation process such as lyophilization or evaporation was not performed. Extracting oleuropein effectively from olive leaves is highly related with critical conditions that appropriate solvent and temperature.

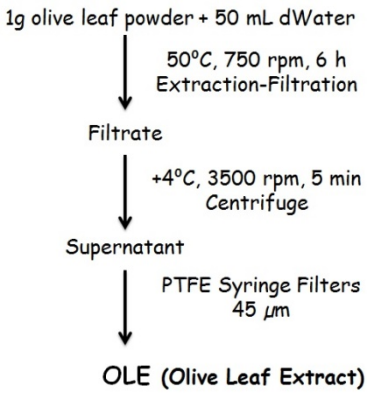


Figure 2. The schema of olive leaf extraction.

Determination of Extract Content by HPLC

Oleuropein, and also hydroxytyrosol, tyrosol and verbascoside were performed quantitatively against external standards in extracted olive leaves. Zorbax SB, 5 µm, 150 x 4.6 mm C18 column was used for separation process through HPLC. The mobile phase was 80% distilled water and 20% acetonitrile, both acidified to pH 3 with 0.1M orthophosphoric acid. The mobile phase used, was envisaged for the separation of phenolic compounds and performing quantitation easier. Waters 1525 binary HPLC pump and waters 2487 dual absorbance detector were used for content analysis. Flow rate was adjusted to 1 ml/min and injection volume was adjusted to 20 µL. Measurement was made adjusting chromatogram to 280 nm wavelength for determination and assignment of polyphenolic compounds.

Determination of Total Phenolic Content

Total phenolic content determination was performed modifying Folin-Ciocalteu method (Slinkard and Singleton 1977). Gallic acid, soluble in methanol as standard, was prepared in seven different concentrations in the range between 12.5 µg/mL - 800 µg/mL. Each standard and sample (50 µL) was added to appropriately labeled tubes and then 2.5 mL distillated water was added over it. Folin-Ciocalteu reagent (0.2 N - 250 µL) was added to tubes and then incubated for 3 minutes at room temperature. Then, the tubes were vortexed by adding 750 µL of 10% Na_2CO_3 (w/v) solution and allowed to incubate at room temperature for 2 hours. Distillated water was used as a blank. Absorbance reading was made at 765 nm through Boeco S-22 UV-Vis spectrophotometer. Total phenolic content in the samples was calculated using a standard curve ($y = 0.0009x + 0.0342$, $r^2 = 0.9875$) obtained by gallic acid and was expressed as mg gallic acid equivalent (GAE). Analyses were duplicated. All chemicals are HPLC grade and supplied from Sigma and Merck, Inc.

Statistical analysis

Data were expressed as mean ($\overline{X}$) and standard deviation (±SD). The significance between the groups was analyzed using one-way ANOVA, followed by the Tukey test. Statistical significant was accepted when $p \leq 0.05$.

Results and Discussion

The findings of the study, by which effect of olive leaf with many metabolic effects, on the amount of the polyphenolic compound of solvent variation and the total phenolic content was intended to reveal, are given below in tables and figures.

Chromatographic Analysis

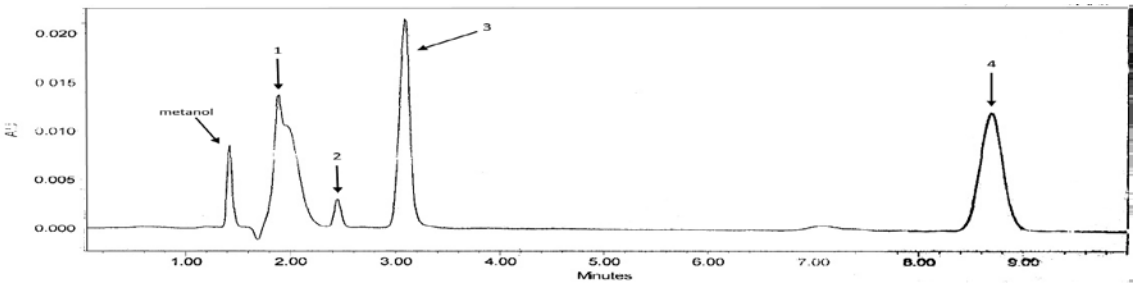


Figure 3. Chromatogram at 280 nm wawelength of the external standarts 1. Hydroxytyrosol; 2. Tyrosol; 3. Verbascoside; 4. Oleuropein

Table 1. 280 nm in wawelength information of the external standards

Phenolic Compound	Retention Time (min)	Calibration Range (ppm)	R^2	Unified Equivalent of Standards
Oleuropein	8.36	200-1600	0.987	y=4.78e+0.003x-1.32e+0.006
Verbascoside	2.90	60-480	0.999	y=7.51e+0.003x-5.03e+0.004
Hydroxytyrosol	1.82	5-40	0.999	y=1.41e+0.004x+7.56e+0.003
Tyrosol	2.25	1-64	0.985	y=1.17e+0.004x-4.46e+0.003

Table 2. Retention time at 280 nm wawelength of phenolic compounds in OLE

Phenolic Compound	Retention Time (min)		
	Water	MeOH/Water	MeOH
Oleuropein	8.725	8.706	8.656
Verbascoside	3.063	3.051	3.051
Hydroxytyrosol	1.898	1.900	1.899
Tyrosol	2.476	2.483	2.444

The chromatogram peaks with 280 nm wavelength, in the highest concentrations of the standards, have been seen in Figure 3. The retention times of the standards are given in Table 1; the retention times of the phenolic compounds in the extraction are given in Table 2. Many chromatographic peaks were determined in the study. Some of these peaks point out phenolic compounds; some of them point out substances of non-phenolic compound.

Table 3. Amount at 280 nm wavelength of phenolic compounds in OLE

Phenolic Compound	**Amount (g 100g^{-1} dry matter)**		
	Water	MeOH/Water	MeOH
Oleuropein	1.899±0.011	2.129±0.018	3.456±0.096
Verbascoside	1.280±0.008	0.509±0.003	0.309±0.001
Hydroxytyrosol	0.219±0.0007	0.141±0.0007	0.371±0.001
Tyrosol	0.184±0.0006	0.045±0.0001	0.055±0.0001

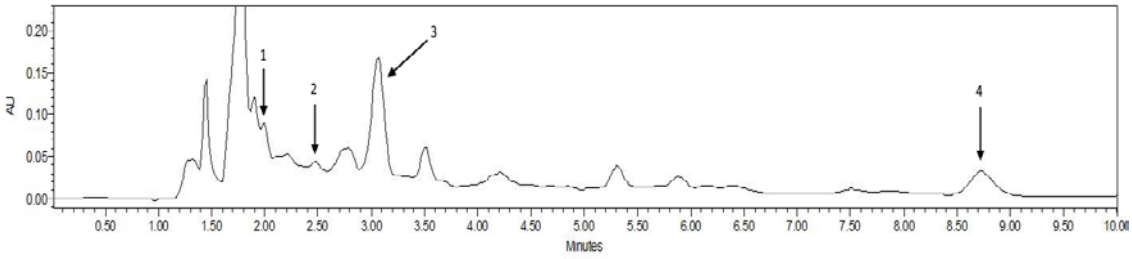


Figure 4. OLE aqueous extraction chromatogram 1. Hydroxytyrosol; 2. Tyrosol; 3. Verbascoside; 4. Oleuropein.

The polyphenolic profile of olive leaf as a result of aqueous extraction was given in Figure 4; amounts of phenolic compounds were given in Table 3. Pereira et al. (2007) boiled olive leaves for 30 min and then lyophilized the extract. They determined the amount of oleuropein and verbascoside as 26471.4±1760.2 mg/kg (2.647 g 100 g^{-1}) and; 966.1±18.1 mg/kg (0.0966 g 100 g^{-1}), respectively on the basis of lyophilized dry matter rediluted by water. Lee-Huang et al. (2003) determined the amount of oleuropein and verbascoside as 12.8% (w/w) and 0.38% (w/w) respectively, after extracted for 12 hours at 80 °C and concentrated by lyophilization. That the amount of oleuropein obtained in the present study (1.899±0.011 g/100g) is less than literature findings is due to not being performed of condensing/enrichment process after extraction.

Extraction in dynamic mode is performed in general when water is used as extraction solvent. Extraction process in static mode is performed in the selection of organic solvents such as methanol (Kubatova et al. 2001; Ibanezet al. 2003). In the present study, dynamic mode and organic solvent/water mixture was applied, combining these two methods. Furthermore, efficiency of extraction was increased, decreasing dielectric constant by means of methanol as well as water used as a solvent (Curren and King 2001). Without condensing/enrichment process, the amount of oleuropein was determined as 2.129±0.018 g 100g^{-1} in consequence of the resulting extraction (Figure 5). Bouaziz and Sayadi (2005) reported that MeOH/water (4:1 v/v) solvent mixture mainly including methanol more efficiently extracts oleuropein from olive leaves. Similarly, Jemai et al. (2008) obtained a rich extract from oleuropein condensing the extract through evaporation and re-extracting the extract 3 times with ethyl acetate after using the ratio of MeOH/water (4:1 v/v). They determined oleuropein as 4.32 g 100g^{-1} dry weight. In order to extract apolar polyphenols and to be removed from pigment and lipids following MeOH/water extraction, the extract re-treated with polar aprotic solvent such as ethyl acetate or nonpolar solvent such as hexane (Somova et al. 2003; Bouaziz and Sayadi 2005). Oleuropein was determined as 3.456±0.096 g 100g^{-1} in the extraction performed by using only methanol (Figure 6). The most active oleuropein amount was obtained in methanol as a result of using three different solvents in the extraction studies. Salta et al. (2007) macerated olive leaves for 3 days with methanol at room temperature and then filtered the obtain extract. Subsequently, the extract was evaporated two times and washed with various solvents. Salta et al. (2007) determined amounts of oleuropein, tyrosol and hydroxytyrosol in the extract as 1.68 g 100g^{-1}, 0.0341 g 100g^{-1} and 0.0138 g 100g^{-1}, respectively. The amount of polyphenolic compounds was lower than expected in Salta's study. The reason of that may be oxidization of polyphenolic compounds during being washed with various solvents and more than one evaporation processes thus subjecting to loss of amount. In addition, to this Salta's study polyphenolic compounds may not be diffused effectively to the solvent because maceration is a static process.

Based on these results, this increase is considered to be important according to the literature data regarding amount of verbascoside, hydroxytyrosol and tyrosol. That may be resulted from collection of the olive leaves, used as material in the study, in August in "green maturation stage" which is the second stage of polyphenolic profiles. Additionally, soil composition and climatic factors might be effective on that. Furthermore, it was determined comparing amounts of polyphenolic compounds obtained in this study with those of solvents that hydroxytyrosol, tyrosol and verbascoside are extracted better in water. Oleuropein was extracted in the methanolic solvent more effectively. Figure 7 illustrates phenolic compound overlay at the chromatogram.

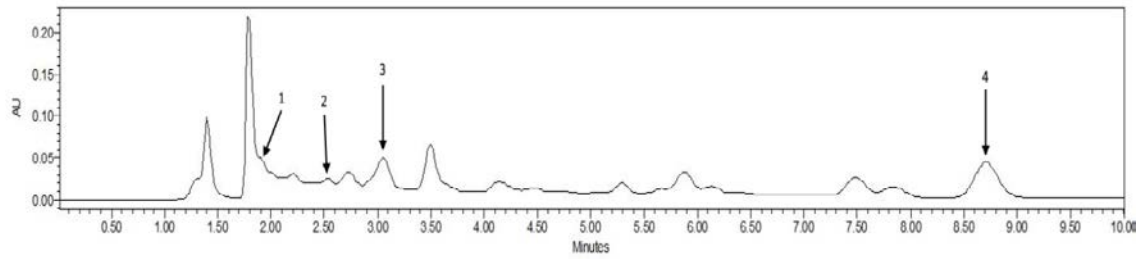


Figure 5. OLE MeOH/water extraction chromatogram 1. Hydroxytyrosol; 2. Tyrosol; 3. Verbascoside; 4. Oleuropein

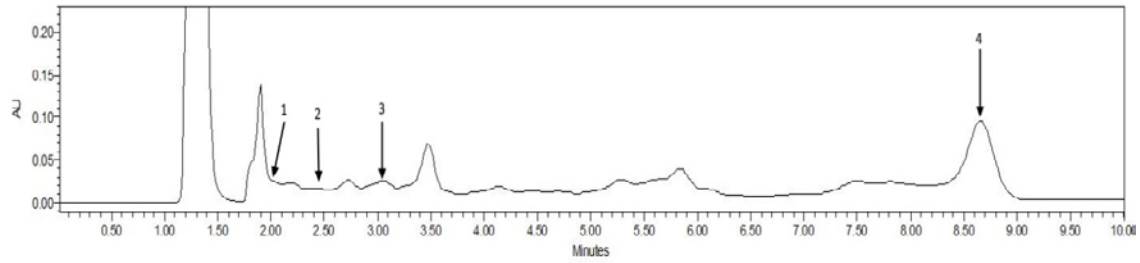


Figure 6. OLE MeOH extraction chromatogram 1. Hydroxytyrosol; 2. Tyrosol; 3. Verbascoside; 4. Oleuropein

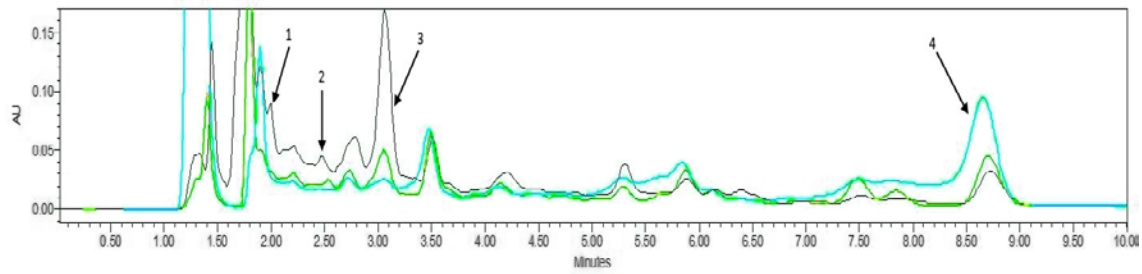


Figure 7. Chromatogram overlay of OLE solvent variation extraction 1. Hydroxytyrosol; 2. Tyrosol; 3. Verbascoside; 4. Oleuropein

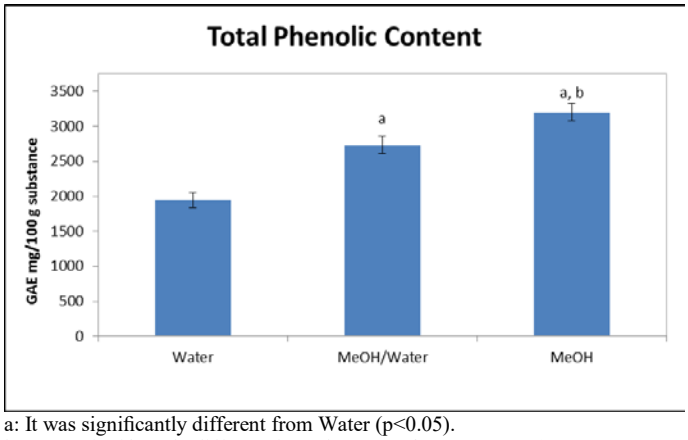
Total Phenolic Content Analysis: The effect of solvent variation on total phenolic content (TPC) of olive leaf extract (OLE) has been shown in Figure 8 and Table 4. Total phenolic contents were calculated 1946.1±105.6, 2729.4±121.4 and 3201.7±120.8 GAE mg $100g^{-1}$ matter in MeOH/water and MeOH solvents, respectively. Solvent variation and extraction methods both increase the amount of TCP and strengthen its antioxidant properties. Taamalli et al. (2012) compared the profile of phenolic compounds in olive leaf according to different extraction methods by using HPLC-ESI-TOF-MS. Besides, they expressed amounts of TPC in the resulting extracts on the basis of total peak area. They reported that microwave-assisted extraction method has the highest TPC as compared to other methods and this was followed by conventional solvent extraction method (24 hours maceration in solvents of MeOH/water (4:1 v/v)). They also reported that the amount of oleuropein was found to be highest likewise these two methods (Taamalli et al. 2012). Silva et al. (2006) removed pigment and lipids from different olive culture leaves, extracting 3 times with hexane at first. Afterwards, they extracted polyphenolic compounds by means of MeOH/water (4:1 v/v). They reported that TPC, as determined by the Folin-Denis method, was found between 11.7-40.1 g/kg (1.170 to 4.010 mg $100g^{-1}$) tannic acid equivalent at different olive culture.

It was seen in the present study and the literatures that hydroxytyrosol and tyrosol are trace phenolic compounds as compared to oleuropein. Hydroxytyrosol is a metabolite formed as a result of hydrolysis of oleuropein. After extraction, the extract, rich in hydroxytyrosol, resulted through hydrolysis of the oleuropein was compared with oleuropein and its various activities were investigated in some studies. Bouallagui et al. (2011) extracted olive leaves with MeOH/water (4:1 v/v) mixture overnight under agitation in the dark. After filtering extract solution they cleavaged oleuropein in filtrate at 100 °C by using acid hydrolysis. Then hydroxytyrosol-rich extract was obtained. TPC determination performed on the hydroxytyrosol-rich extract, was expressed as 15.55 mg GAE/g extract (Bouallagui et al. 2011). Similarly, Jemai et al. (2009) hydrolyzed OLE by using acid hydrolysis in order to obtained hydroxytyrosol-rich extract.

Table 4. Total phenolic contents of the water, MeOH/Water and MeOH extracts of OLE.

Analysis	**Water**	**MeOH/Water**	**MeOH**
	$\overline{X}$±SD	$\overline{X}$±SD	$\overline{X}$±SD
TPC (GAE mg/100 g substance)	1946.1±105.6	2729.4±121.4[a]	3201.7±120.8[a,b]

a: It was significantly different from Water ($p<0.05$).
b: It was significantly different from the MeOH/Water ($p<0.05$).



a: It was significantly different from Water ($p<0.05$).
b: It was significantly different from the MeOH/Water ($p<0.05$).
Figure 8. Total phenolic content of OLE solvent variation

Conclusion

High amount of oleuropein existence in methanolic extract obtained in the HPLC findings, might also yield higher TPC in methanolic extract as well. Resulting of high efficiency extraction in content cause increasing of synergistic effects of phenolic compounds contained in the extracts. Due to optimum acquirement of phenolic compounds and high extraction efficiency in the samples, conventional hydroalcoholic extraction offers a wide variety of phenolic compounds. Because of that polyphenols contain more than one -OH group, they have high hydrophilic character and they can be extracted much better with high polarity solvents such as methanol.

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