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Scientific paper

Effects of Extraction Period on Bioactive Compounds Extracted from *Olea Europaea* (var. Domat) Leaves by Ultrasound-Assisted Extraction

Dua Danjolli-Hashani[1,2,*] and Şebnem Selen-Işbilir[2]

[1] *Heimerer College, Health Sciences of Diagnostic Profile: Laboratory Technician, 10000, Pristina, Kosovo*

[2] *Trakya University, Faculty of Science, Department of Chemistry, 22030, Edirne, Turkey*

** Corresponding author: E-mail: dua_danjolli@hotmail.com*
Phone: +383 49 850662

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Abstract

In this study, polyphenols from olive leaves (Domat var.) were compared by shaking water bath and ultrasound-assisted extraction to compare the polyphenol contents and antioxidant activity in olive leaves. The effects of the extraction time on the antioxidant activity of olive leaves will be analyzed depending not only on the extraction method but also on the extraction time, due to the extraction yield, antioxidant activity, as well as the type of polyphenols recovered.
Objective: To obtain high antioxidant results and to determine the phenolic compounds contained in olive leaf (Domat var.) by LC-MS/MS after ultrasonic water bath and shaking water bath extraction comparison in a shorter time instead of 2 h.
Conclusions: The phenolic compounds contained in olive leaf by ultrasound-assisted extraction were higher than water bath extraction. We said that there is no significant difference between the extraction time and TPC and TFC. There was also no relationship between extraction time and DPPH and ABTS EC50 values ($p < 0.05$), which means that 15 min of sonication can be performed instead of 120 min.

Keywords: Ultrasound-assisted extraction; Domat olive tree leaves; antioxidant activity; phenolic compounds; LC-MS/MS

1. Introduction

Antioxidants are endogenous or exogenous substances that protect metabolism against the harmful effects of free radicals. They are also used in the food industry, cosmetics, and pharmaceutical industries to prevent lipid peroxidation. In these sectors, it is preferred to use natural antioxidants found in herbal sources instead of the synthetic antioxidants. The antioxidant properties of plants are due to their contents phytocomponents, especially polyphenolic compounds, carotenoids, and ascorbic acid. Polyphenolic compounds including mainly phenolic acids, flavanones, flavones, anthocyanins, isoflavones, tannins which are the secondary metabolites of plants. It is known that olive tree (*Olea europaea* L.) leaf, which is rich in phenolic compounds,[1–3] has antioxidant properties[4] as well as antimicrobial,[5–6] antihypertensive[7–8] and cholesterol-lowering[9] properties. The positive effects and antioxidant properties of olive tree leaf on health are related to its phenolic components such as oleuropein, verbascoside, rutin, hydroxytyrosol, luteolin-7-glucoside, and tyrosol. Besides there are studies on the pharmacological effects of oleuropein, which is the major phenolic component of olive tree leaf.[10–11]

Olive tree is an economically important tree, especially in Mediterranean countries. Although the minor part of leaves is traditionally drunk as tea, the others are by-product of table olive and olive oil industries, or natural waste as fallen leaf. Therefore, the phenolic-rich extract could be obtained from olive leaves, and this extract can be evaluated in food additive as antioxidant, functional food production or drug candidate search studies because of their rich bioactive components and inexpensiveness. There is an increasing effort of some industries to obtain bioactive compounds from natural products through extraction and purification for food additive manufacturing.[12–14] The first and most important step in obtaining the phytocomponents of natural products such as plant leaves is an efficient extraction process. Especially oleuropein is extracted from olive leaves, fruits, and olive seeds.[15] To extraction of phenolic compounds from plants the organic solvents are used most and, the extraction procedures are

divided into two groups as traditional and modern methods. Traditional methods such as maceration, percolation, infusion and soxhlet extraction are inexpensive, but have very solvent-used, longer extraction time. Recently, modern techniques such as supercritical fluid extraction, microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE) are replacing these traditional methods.[16–17] These alternative techniques are green extraction processes, and both reduce the use of solvents and speed up the extraction procedure.

Polyphenolic compounds are secondary metabolites of plants with various biological effects[18]. Due to the variety and structural differences of phenolic compounds, it is difficult to develop an optimum extraction procedure. In recent years, techniques such as UAE, MAE, supercritical and accelerated extraction have been used to obtain biologically active extracts from plants[19–20]. These techniques have specific benefits such as environmental friendliness, cost, and time savings. In addition, the fact that some of them offer high temperature or high-pressure operation which significantly shortens the extraction time.[21–22] In recent years, studies on ultrasonic applications have been increasing due to its use in food processing and preservation.[23] The efficiency of ultrasound-assisted extraction is that it allows the intracellular material to pass into the solvent by disrupting the plant cell wall.

Various parameters such as extraction method, time, temperature, solvent type and solvent-sample ratio affect the yield of the bioactive compounds obtained. Since olive tree leaf is known to be effective as antioxidants,[4] it was used as a model in this study. In the study, the olive tree (*Olea europaea* L.) leaves belong to Domat variety were extracted both by shaking in a water bath, which is a traditional extraction method, and ultrasound-assisted extraction method at the same conditions as a modern extraction method. Domat variety orginates from Akhisar district of Manisa province (Turkey) and, has large fruits. It is the best green olive variety grown in Turkey. Firstly the two extraction methods were compared in terms of antioxidant activity and polyphenolic compounds content. In the other part of study the UAE conditions were constant, namely, extraction solvent, temperature and solid-solvent ratio, and the ultrasound treatment was performed in intervals 15 min, 30 min, 60 min and 120 min. Although there are few studies about the UAE of polyphenols from other varieties of olive leaves (Serrana variety from Spain and Tavsan Yuregi variety from Turkey),[24–25] this is the first report on time effect in UAE for antioxidant compound recovery from Domat variety of olive leaves.

2. Experimental

2. 1. Materials

All the reagents and solvents used in the experiments were of analytical grade. Olive tree leaf which is Domat variety (2019 November) was obtained from Olive Research Institute in Izmir-Turkey. Olive tree leaves were dried at room conditions in airy environment without direct sunlight and then ground in a Waring blender.

2. 2. Extraction Procedure

In the extraction with shaking water bath (WBE), samples are shaken with a 75% (v/v) ethanol solvent at 30 °C and 125 rpm with a sample:solvent ratio of 1:10 (w/v) during 2 h. Ultrasound- assisted extraction (UAE) was carried out in an ultrasonic bath (Daihan WiseClean, Korea, 230 V, 296 Watt and 50 Hz) at 30 °C for 2 h with 75% (v/v) ethanol solvent at a sample:solvent ratio of 1:10 (w/v). In the second part of the study, samples were sonicated for 15, 30, 60 and 120 min under the same conditions. After the extraction procedures, the samples were filtered through filter paper and their solvents were evaporated at 35 °C in the evaporator. The obtained crude extracts were used in all experiments.

2. 3. Determination of Total Phenolic Content

The determination of total phenolic content was performed according to the method of Singleton and Rossi [26] using Folin-Ciocalteau reagent (FCR). Gallic acid solutions in the concentration range of 50–500 μg / mL were used as the standard phenolic substance. The total phenolic content of the samples was calculated using the equation ($y = 0.0011x - 0.0372$, $R^2 = 0.996$) obtained from the concentration-absorbance graph.

2. 4. Determination of Total Flavonoid Content

It was determined according to Zhishen, Mengcheng and Jianming.[27] Rutin solution was used as standard flavonoid in the concentration range of 25–200 μg / mL. Total flavonoid contents of the samples were calculated from the equation of the standard graph ($y = 0.0012 + 0.0206$, $R^2 = 0.9996$).

2. 5. DPPH Radical Scavenging Assay

The free radical scavenging capacity of the samples was determined using the DPPH radical scavenging method. According to the method of Blois,[28] 0.1 mM DPPH solution was added to the sample or standard solutions (BHA, butylatedhydroxy anisole) which were prepared at different concentrations (100–1000 μg / mL). The samples were kept in the dark for 30 min at room conditions, and absorbances at 517 nm were measured. The control was prepared using ethanol or water instead of the sample. Free radical scavenging efficiencies of the standards and samples were calculated as % inhibition using Equation 2.1. $A_{control}$ is the absorbance of the con-

trol tube and A_{sample} is the absorbance of the sample or standard compound.

$$\% I = [(A_{Control} - A_{Sample}) / A_{Control}] \times 100 \quad (1)$$

2. 6. ABTS Cationic Radical Scavenging Assay

In the ABTS radical scavenging method, ABTS solution and sodium persulphate solution were mixed at a ratio of 1: 0.5 and kept for 16 h and then ABTS radical ($ABTS^{\bullet +}$) was formed. The absorbance of the prepared solution was used after diluting with ethanol to give an absorbance of 0.70 at 734 nm. The samples (100–1000 μg/mL) to which ABTS radical solution was added were kept in the dark for 30 min and their absorbance were measured at 734 nm.[29] Radical scavenging capacities were calculated using the formula in Equation 2.1.

Free radical scavenging results of olive leaf extracts were given as EC50 value. EC50 (effective concentration) value is defined as the amount of extract required to remove half of the radical in the environment and is calculated by plotting inhibition (%) against extract concentrations graph. The EC50 value is the most used parameter to evaluate antioxidant activity.

2. 7. Identification of Phenolic Content with LC-MS/MS

LC-MS/MS analysis was used for identification of phenolic compounds quantitatively. Phenolic content of olive leaf extracts was compared to standard thirty-three organic compounds. The chromatographic separation was performed on a C8 (150 mm × 3 mm, 3.5 μm) reversed phase analytical column (Agilent Zorbax SB-C8). The mobile phase A consisted of 5 mM ammonium acetate and ultrapure water. The mobile phase B consisted of 5 mM acetonitrile: methanol (1:1, v/v) and 0.1 % acetic acid. The injection volume of sample was 5 μL. It was runned at 0.7 mL/min flow. For the mass spectrometry analysis, it was carried out using the Agilent Technologies 6460 Triple Quad LC/MS system. The ionizations were detected by ESI. The other parameters were ion spray (IS) voltage 3500 V; 10 L/min for nebulizing gas flow; nitrogen as nebulizer gas and source temperature 375 °C. The multiple reaction monitoring (MRM) mode was used to quantify the analyzes.

2. 8. Statistical Analysis

In the experiments, each sample was analyzes two times in parallel, and each experiment was carried out in dublicate (n=2). Data were expressed as means ± standard deviation. Pearson correlation test was used to determine the correlation between the antioxidant properties and extraction time. The value of $p < 0.05$ was considered to be statistically significant. The statistical analysis was done using SPSS 21 programme.

3. Results and Discussion

Due to the biological activities of polyphenolic compounds, they are extract from plant materials using various extraction methods. The ultrasound-assisted extraction is also used as a cheap and simple extraction technique that provides high extraction yield and quality, especially in the extraction of phenolic compounds from various natural and waste herbal sources.[30–31] The extraction yield, total phenolic and flavonoid contents of extracts obtained from ultrasound-assisted and shaking water bath extraction techniques are presented in Table 1. The extraction yields (gram extract/100 g dried leaves) were 18.7% for WBE and 16.5% for UAE. Although UAE has a slightly lower extraction yield than WBE, total phenolic amount of UAE was twice that of WBE (Table 1). The extract obtained by the ultrasound-assisted extraction showed higher antioxidant activity due to its high phenolic and flavonoid contents. In addition, the low EC50 value indicates high antioxidant capacity. The EC50 values of extracts obtained from UAE are lower than these of other extraction method in both of free radical scavenging assays (Table 1). According to Table 1, the difference was found between the two methods in the free radical activities, the phenolic and flavonoid contents of the extracts.

Phenolic contents of olive leaf extract were compared to standard thirty-three organic compounds and thirteen of them were identified in both extracts at quantifiable levels. The flavonol quercetin, isorhamnetin, luteolin, five phenolic acids (chlorogenic, protocatechuic, 2,5-dihydroxybenzoic, caffeic and gallic acid) and as well as glycosides oleuropein, verbascoside and rutin were determined higher amount at olive leaf extract obtained by UAE (Table 2) and (Figure 1). As seen in Table 1, in the determination of total phenolic and flavonoid contents by spectrophotometric method, the contents of ultrasonic extract were already determined higher than the water bath

Table 1. Effect of UAE and WBE methods on antioxidant properties of olive leaf extract.

Extraction Conditions	Extraction Method	Extract yield (%)	TPC (GAE μg/mg)	TFC (Rutin μg/mg)	DPPH EC50 assay mg/mL	ABTS assay EC50 mg/mL
75% EtOH, 2h, 30 °C	UAE	16.5	111.9 ± 0.018	534.44 ± 0.045	138.1	245.9
	WBE	18.7	56.9 ± 0.0013	528.11 ± 0.072	284.1	650.0

Table 2. Content of individual phenolics of olive leaf extracts by LC-MS/MS

Phenolic compound	UAE (ppb)	WBE (ppb)
Oleuropein	34090.33	30715.84
Chlorogenic Acid	2621.13	2395.64
Protocatechuic Acid	1088.58	710.85
Verbascoside	515.37	178.54
Rutin	417.76	325.78
Quercetin	224.19	20.80
Isorhamnetin	197.97	39.02
2,5-dihyrdoxybenzoic acid	121.96	88.76
Luteolin	111.37	76.36
Caffeic acid	88.35	76.12
Gallic acid	20.88	5.58
Salicyclic acid	9.95	15.94
Apigenin	6.52	10.47

extract. This indicates that the high antioxidant capacity in the olive leaf extract obtained UAE is due to its high phenolic composition in comparison with WBE.

Researchers who interested in natural products have been studying on extraction process to compare the efficiency of the UAE method with others by using various plant sources. Similar to our study, it was observed that the extracts obtained from ultrasonic application were more efficient in other studies performed to compare traditional and ultrasound-assisted extraction techniques for the extraction of bioactive components. It has been reported that UAE technique from the leaves of lemon scented tea tree (*Leptospermum petersonii*) is more efficient in terms of total phenolics and antioxidant capacity compared to traditional shaking water bath extraction.[17] Ali and Kumar (2015) used two extraction methods (UAE and Soxhlet extraction) for the extraction of bioactive compound from the pomegranate peel. They reported that UAE extract showed higher antioxidant activity and had a higher amount of chlorogenic acid.[30] In another study, *Bauhinia purpurea* leaf was extracted by Soxhlet, ultrasonication and maceration extraction methods, and it was reported that UAE was better method for the extraction of antioxidant and antibacterial substances compared to others.[32] Similarly, Wang et al. (2011) compared ultrasound extraction, high-pressure liquid extraction, and Soxhlet extraction for antioxidants from *C. sulcate* fruit and peel and suggested that UAE is the ideal method for the extraction of antioxidants.[31]

In our study, similarly other studies in the literature, the extract from UAE showed better results compared to its extract by obtained WBE for Domat variety of olive leaf. Since extraction conditions have an effect on the extraction of phenolic and biocomponents, it was aimed to investigate the effect of extraction duration on extraction yield by keeping other conditions same in the continuation of the study. If the extraction time is shortened without changing antioxidant capacity of the extract significantly, time and energy will be saved that is important for manufacturing cost industrially. Therefore, in the present study, olive leaf

Table 3. Effect of ultrasonication period on antioxidant properties of olive leaf extract

Extract	Yield (%)	TPC (μg GAE/mg)	TFC (μg Rutin/mg)	EC50 mg/mL DPPH assay	EC50 mg/mL ABTS assay
UAE-15	14.2[a]	99.7 ± 0.12[a]	421.3 ± 0.10[a]	130.3[a]	411.3[a]
UAE-30	15.4[a]	97.5 ± 0.03[a]	410.1 ± 0.04[a]	137.6[a]	376.9[a]
UAE-60	19.1[a]	102.7 ± 0.04[a]	418.7 ± 0.02[a]	127.6[a]	409.2[a]
UAE-120	19.4[a]	104.5 ± 0.018[a]	530.2 ± 0.045[a]	122.8[a]	406.7[a]

SD = standard deviation (n=2)
Different letters of upper index within the column indicate significant differences at $p<0.05$ level.

Table 4. Linear correlations between the analyzed parameters

	Time	TPC (GAE)	TFC (Rutin)	DPPH assay (EC50 value)	ABTS assay (EC50 value)
Time	1	0.865	0.911	–0.789	–0.904
		0.135	0.089	0.211	0.096
TPC (GAE)		1	0.763	–0.972*	0.334
			0.237	0.028	
TFC (Rutin)			1	–0.777	–0.982*
				0.223	0.018
DPPH assay (EC50 value)				1	0.656
					0.344
ABTS assay (EC50 value)					1

*Correlation is significant at the 0.05 level (2-tailed).

was extracted by ultrasound-assisted extraction at a different interval of time (15 min, 30 min, 60 min, 120 min) at 30 °C using ethanol (75%) as the solvent. According to results (Table 3), it was seen that the maximum extraction yield was obtained by 120 min of sonication in comparison with 15 min, 30 min and 60 min intervals of sonication. The sonication interval was increased the extraction yield (%), however, there are not significantly differences between the sonication time and both total phenolic and flavonoid contents ($p < 0.05$).

According to Table 4, it can be inferred that extraction time values are not significantly correlated with TPC, TFC, DPPH, ABTS, but DPPH values are significantly correlated with TPC, and ABTS values are significantly correlated with TFC. We said that there is no significant difference between the extraction time and TPC and TFC which responsible antioxidant activity. There was also no relationship between extraction time and DPPH and ABTS EC50 values ($p < 0.05$), which means that 15 min of sonication can be done instead of 120 min. In recent studies, the extraction time has been reported at different timings. The most efficient extraction time of the total polyphenol contained in soybeans extract with solid-liquid extraction has been reported as 120 min.[33] It has been shown that the pepper pulp extract obtained by ultrasonic bath and shaking water bath methods was obtained with the 20 min extraction process in terms of the highest total phenolic substance and antioxidant activity which is similar with our results.[34] Altemimi et al. was reported the optimum extraction process time of spinach leaves with ultrasound-assisted extraction method was reported as 30 min.[35]

In other studies, about comparing microwave-assisted extraction and ultrasound-assisted extraction methods, the extraction times for optimum antioxidant activity were reported as 30.5 for *Prunella vulgaris* L.extract.[36] Similarly, the extraction time was reported as 5–30 min by keeping temperature and power constant as the optimum extraction conditions of Nettle and Chokeberries extracts by comparing microwave-assisted extraction and ultrasound-assisted extraction methods.[37–38] According to the reports of other researchers, Wang et al. also indicated that the ultrasound-assisted extraction method is a convenient and economical method for the extraction of total phenolic compounds for *Inula helenium* sample.[39]

The olive tree leave is a source of valuable active compounds such as oleuropein, verbascoside and rutin, which has benefit in health promoting potencial, and also have an importance in agricultural as industrial waste. Therefore, the extraction process for biophenols obtained from olive leaf is important and the researchers focused on optimum extraction conditions. Nowadays, ultrasound-assisted extraction methods have been studied mostly because of its advantages.

There are few studies about olive leaf extraction by ultrasound-assisted extraction method related to Serrana variety from Spain[25] and Tavsan Yuregi variety from Turkey.[24] In this study, we studied another variety of olive leaf which is Domat variety from Manisa/Turkey. Ahmad-Qasem et. al. has addressed the ultrasound-assisted extraction of Serrana var. olive leaf bioactive compounds and evaluated the influence of some process parameters such as the electric amplitude, the emitter surface and temperature. Also, they reported that olive leaf extracts were similar content of bioactive compounds, such as oleuropein, verbascoside and luteolin-7-O-glucoside in comparing the conventional technique with ultrasound-assisted extraction method. In their results, the extraction time of conventional technique reduced from 24 h reduced to 15 min with ultrasound-assisted extraction method.[25] In another study by Sahin and Samli for ultrasound-assisted extraction of olive leaves (Tavsan Yuregi variety) with response surface methodology, were reported the optimum extraction conditions such as 0.5 g sample to 10 mL solvent ratio, 50% ethanol and 60 min of extraction time[24]. As stated above, different extraction process times have been reported so far regarding to antioxidant activity, total phenolic and flavonoid contents of different plants, so the results of our study may suggest the best extraction time for olive leaf (Domat var.) as 15 min with ultrasound-assisted extraction. Comparing to the conventional extraction process, ultrasound-assisted extraction can use for reducing the long extraction time, and to decrease the risk of biocompound degradation.

4. Conclusions

This study aimed to analyse whether high antioxidant results of olive leaf extracts in a shorter time instead of two hours by ultrasound-assisted extraction, and to determine the phenolic compounds contained in olive leaf (Domat var.) by LC-MS/MS. So, choosing the right extraction method is important for obtaining extracts with various pharmacological activities expected from plants and also it is important to minimize or avoid degradation of biocompounds and to select the best working conditions. For the extraction of antioxidant compounds from Domat variety olive leaves, the UAE technique gave better results than the extraction with shaking water bath technique. When the effect of time on ultrasound-assisted extraction is examined, it can be said that the extraction time of 15 minutes instead of 2 hours is sufficient, according to the results obtained from the antioxidant activity trials of the extracts and their extract yields. The results show that the UAE method provides time and energy saving, and can be used in biocomponent recovery, which is faster and more effective than traditional methods. This technique can also find application in the development of industrial extraction processes. In order to contribute to the industrial field, extraction-time optimization or other extraction parameters can be also investigated by using different herbal materials with biological activity.

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Povzetek

V tej raziskavi smo primerjali učinkotiost ekstrakcije polifenolov iz oljčnih listov (Domat) s pomočjo stresane vodne kopeli in ultrazvoka ter rezultate primerjali z vsebnostjo polifenolov in antioksidativno aktivnostjo oljčnih listov. Učinkovitost ekstrakcije na antioksidativno aktivnost oljčnih listov smo preverjali preko izkoristka ekstrakcije, antioksidativne aktivnosti in vrste pridobljenih polifenolov, pri čemer smo poleg same metode spreminjali tudi trajanje ekstrakcije.

Cilj: doseči visoke antioksidativne vrednosti in določiti vrste fenolnih spojin v oljčnih listih (Domat) z LC-MS/MS pri uporabi ekstrakcije z ultrazvokom ali stresano vodno kopelijo v prej kot 2 urah

Zaključki: Ugotovili smo, da z ekstrakcijo z ultrazvokom dosežemo višjo koncentracijo fenolnih komponent kot pri ekstrakciji s stresano vodno kopeljo. Obenem pa smo ugotovili, da pri tem tipu ekstrakcije čas ekstrakcije ne vpliva na celotno vsebnost fenolov in flavonoidov, kot tudi ne na antioksidativne vrednosti določene z DPPH in ABTS EC 50 ($p < 0.05$), kar pomeni, zadostuje že 15 minut namesto 120 min ultrazvočne ekstrakcije.