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Effects of the bath and probe ultrasound treatment on the antioxidant activity of phenolic extract from oregano (*Origanumvulgare*L.) leaves

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Abstract

Ultrasound assisted extraction (UAE) has attracted growing interest, due to its great advantages for the extraction of bioactive compounds from plant species. Ultrasound efficiency is affected by various factors such as extraction temperature, extraction time, intensity of ultrasound waves, and type of ultrasound system. Therefore, this study aimed to investigate the process conditions of two ultrasonic systems (bath and probe) to determine the highest extraction efficiency of phenolic and flavonoid compounds in oregano leaves and the best ultrasound extraction conditions. The effects of different combinations of the ultrasonic variables include bath (40-60 °C; 30-60 minutes) and probe (40-60°C; 5-25 minutes and ultrasound amplitude: 20- 40%) were studied using ethanol-water (50:50v/v) solvent. The antioxidant activities of the extracts were then evaluated by the 2, 2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and reducing power tests.The best conditions for extraction of phenolic compounds and flavonoids were obtained using ultrasonic probe system at 50 ° C for 15 minutes and amplitude of 40%. The maximum total phenol and flavonoid were 473.22 ± 25.9 µg of gallic acid equivalent and 46± 1.24 µg of quercetin equivalent per mg of dry extract, respectively. The results showed that the UAE method had a considerable effect on the extraction of bioactive compounds from oregano leaves, and also the probe system had a higher efficiency than the bath system.

Keywords: Ultrasonic bath, Ultrasonic probe, Oregano leaf, Phenolic Content, Antioxidant activity.

Introduction

Antioxidants are compounds that inhibit or delay the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidation chain reaction. In recent years, many herbs especially vegetables and medicinal plants, have been identified as important sources of natural antioxidants.The extracts of some of these plants can be used as an alternative to the food preservatives due to their antioxidant and antibacterial activity (Diem Do *et al.*, 2014). Among chemical constituents of these plants, phenolic metabolites are considered a good sources of natural antioxidants in the diet. Phenolic compounds comprise phenolic acids (hydroxybenzoic and hydroxycinnamic acids), polyphenols (hydrolyzable and condensed tannins), flavonoids, and lignin. Antioxidant activity of plant phenolic compounds is mainly due to their reducing properties and chemical structure, which can play an important role in neutralizing the free radicals, chelating transition metals, and quenching the singlet and triplet oxygen through the destruction of peroxides (Chun *et al.*, 2005).

Aromatic plants of *Lamiaceae* family such as oregano, rosemary, thyme and mint have shown strong antioxidant activity due to the high content of phenolic antioxidants. Meanwhile, oregano is known as a native plant of Mediterranean regions, Irano-Siberian and Euro-Siberian. It has long been used in traditional medicine to treat the various diseases such as digestive disorders, menstrual problems, spasms, colds and severe coughs (Zhang *et al.*, 2014; Vazirian *et al.*, 2015). The antioxidant and antibacterial activity of this plant is very strong that can explain its use in traditional medicine (Zhang *et al.*, 2014; Lemhadri *et al.* 2004; Li *et al.* 2016).The antibacterial activity of oregano is due to its high content of essential oil, and soluble phenolic compounds are responsible for its

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antioxidant activity and consequently the positive therapeutic effects (Zhang *et al.*, 2014; Li *et al.* 2016).Oregano extract is a good source of flavonoids, such as naringenin and caffeic acid derivatives that rosmarinic acid is the most abundant among the compounds (Zhang *et al.*, 2014; Vazirian *et al.*, 2015).

Phenolic compounds from the plant sources are traditionally extracted using solvent extraction or steam distillation techniques. Traditional methods have many disadvantages such as high solvent consumption, long extraction time, and lower extraction yield (Dey and Rathod 2013). Therefore, the implementation of novel extraction techniques such as ultrasound assisted extraction, supercritical fluid extraction, subcritical water extraction, and microwave assisted extraction, has been promoted. The ultrasound method has gained higher consideration because of its high efficiency, short time, low solvent consumption, cheap, and easy utilization. The mechanical effects of ultrasound during the cavitation phenomenon lead to more solvent penetration into the cells, and it can improve the mass transfer. Therefore, efficient cellular degradation and effective mass transfer are two major factors that increase ultrasound extraction (Cheok *et al.* 2013; Huang *et al.* 2009). Many factors such as temperature, time, and intensity of waves, type and composition of solvent, the nature of plant material, and the ratio of solvent to sample affect the ultrasound mechanism.

Many studies have been investigated the effects of different conditions to improve the extraction of compounds by using ultrasound, for example, extraction of phenolic compounds from red grape extract (Morelli and Prado, 2012), extraction of melatonin from *Oryza sativa* (Setyaningsih *et al.*, 2015), and extraction of polyphenols from *Zizyphuslotu* (Hammi *et al.*, 2015). Different studies have also been carried out to investigate the phenolic content and antioxidant activity of oregano leaves and plants in which extraction of plant extracts was performed by different solvents in traditional ways (Zhang *et al.*, 2014; Chishti *et al.*, 2014; Chun *et al.*, 2005). So far, the effect of different conditions (temperature, time and the amplitude of ultrasound) on the content of phenolic compounds and antioxidant activity of oregano has not been investigated. The objective of this study was to determine the best conditions for extraction of oregano phenolic compounds and flavonoids by examining the different conditions such as extraction temperature, extraction time and the type of ultrasound system (bath and probe) in comparison with traditional solvent extraction method.

Materials and Methods

Oregano leaves (*Origanum vulgare*) were purchased from a medicinal herb farm (Arak, Iran) in February 2017.The dried samples were sieved through No. 67, and were packed tightly in two-layer polyethylene bags and kept at -18°C in order to avoid moisture absorption (Rezaie *et al.* 2015).

Chemicals and Reagents

All chemicals used in this study were purchased from Merck Chemicals Co. (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA) company. The chemicals were of an analytical grade.

Preparation of Extracts

To extract the antioxidant compounds of Oregano in each treatment, 10 g of sample was placed in 100 ml of ethanol: water (1: 1 v/v) and mixed well. Solvent extraction was done as described by Maghsoudlou *et al.* (2016). In this method, for the best extraction of antioxidant compounds, the mixture was shacked at ambient temperature and kept in dark place for 24 hours. Also, the ultrasound treatment was done as described by Altemimi *et al.* (2015).In the bath method, Elma Ultrasonic (S30H, Germany) was used with a frequency of 37 kHz, a power of 280 W and an internal diameter of 50× 106× 198 cm. The mixture of sample was subjected to ultrasonic bath at 40, 50 and 60°C for 30, 45 and 60 min. In this method, temperature was controlled and maintained through the circulation of water. In the probe method, the ultrasound cell disruptor (KS-250F, China, Ningbo Zhejiang) was used at

frequency 20 kHz (amplitude 20% and 40%) and power 250W. Probe ultrasound treatments were performed at 40, 50, 60°C for 5, 15 and 25 min, the temperature elevation was controlled during sonication by mixing water and ice. The suspension obtained by extraction of the ultrasound and solvent method was filtered with Whatman No. 1 (Whatman International Ltd, Maidstone, United Kingdom) filter paper. Afterwards solvents evaporated by rotary evaporator (BUCHI, Labortechnik AG, Flawil, Switzerland). Finally, the extracts were kept at -18°C till further experiments (Maghsoudlou *et al.*, 2016).

Measurement of Total Phenolic Content

Total phenol was measured as described by Sfahlan *et al.* (2009) with a slight modification by using a spectrophotometer. Briefly, 2.5 ml of Folin-Ciocalteu reagent was diluted ten times with distilled water, 2 ml of sodium carbonate 7.5% and 50 µl of the extract was mixed well. After heating at 45 °C for 15 min, absorption of the mixture was measured using a spectrophotometer (PG Instrument, Ltd.) at 765 nm. The total phenol content of all samples was expressed as gallic acid equivalents per g of extract using the following linear equation based on the calibration curve:

$$Y= 0.0083\,X +0.018 \qquad R^2= 0.094 \qquad (1)$$

Where Y is absorbance at 765 nm and X is concentrations of gallic acid equivalents (mg/g) (Sfahlan *et al.*, 2009).

Determination of Total Flavonoid Content

Evaluation of total flavonoid content was performed by aluminum chloride method as described by Nabavi *et al.*, (2012). Briefly, 0.5mL of each plant extract in methanol was separately mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1Mpotassium acetate and 2.8 mL of distilled water, and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with a double beam Perkin Elmer UV/ Visible spectrophotometer (PG Instrument, Ltd.). The calibration curve was plotted by preparing pure quercetin solutions at concentrations 12.5- 100 mg/ml in methanol. Total flavonoid content was calculated as quercetin per g of extract using the following linear equation based on the calibration curve:

$$Y= 0.0060X+ 0.0024 \qquad R^2= 0.98 \qquad (2)$$

Where Y is absorbance at 415 nm and X is concentrations of flavonoids compounds (mg quercetin /g extract).

Measurement of the extract antioxidant activity
DPPH radical scavenging activity

The stable 2, 2'-diphenyl-1-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts (Ebrahimzadeh *et al.* 2010). Different concentrations of each extract were added, at an equal volume, to methanolic solution of DPPH (100 µM). The samples were kept at room temperature in the darkness and after 30 min the absorbance of each sample was measured at 517 nm and the percentage of scavenging activity was calculated from equation 3. The experiment was done in triplicate. IC_{50} values denoted the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

$$\%Inh = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \qquad (3)$$

Reducing power

The ability of extracts to reduce iron (ш) was evaluated using the method of Altemimi *et al.* (2015). Extracts (2.5 ml) were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide ($K_3Fe\,(CN)_6$; 10 g/L) and incubated for 30 min at 50 °C. Thereafter 2.5 ml of trichloroacetic acid (10% w/v) was added to the solution and the solution was then centrifuged for 10 min. Finally, 2.5 ml of supernatant was combined with 2.5 ml of distilled water and 0.5 ml $FeCl_3$ (1 g/L). The absorbance of samples was measured at 700 nm. Higher absorbance values indicated higher reducing power.

Statistical Analysis

Each experiment was carried out at least in Triplicate. Statistical analysis of data was performed using Microsoft Excel. Analysis of

variance was done using the SPSS21 program with a confidence level of a 0.05, to find any significant difference between treatments. Duncan multiple range test was used for mean separation at $P<0.05$ where treatment effect was significant.

Results and Discussion

The effect of ultrasound variables on total phenolic content, flavonoids, and antioxidant activity

The results of comparisons between means of total phenolic and flavonoid compounds (Table 1), DPPH radical scavenging (Table 2) and reducing power (Table 3) tests in different treatments showed that the effect of ultrasound extraction type as well as extraction conditions (temperature, time and amplitude of ultrasound) were significantly different ($P<0.05$).

The highest yield of oregano phenolic compounds was observed 473.32± 25.9 µg GAE/mg of dry extract in probe system at 50°C, for 15 min and amplitude 40%, followed by probe extract (at 50°C for 5 min at amplitude 40%) and bath extract (at 50°C for 45 min) with the 443.24± 5.87 and 430.81± 6.94 µg/ ml of phenolic contents of dry extracts, respectively.

Among the phenolic compounds, flavonoids are considered as stronger antioxidants. These are derived from diphenylpropanes and a 6-membered heterocyclic ring containing oxygen. Increasing the number of hydroxyl groups has a direct relation with an antioxidant strength of flavonoids (Koda *et al.*, 2008). Therefore, measurement of these compounds is very important to determine the antioxidant properties.

Radical scavenging and reducing power tests are widely used to evaluate hydrogen-donor compounds that inhibit free radicals. Results showed that, antioxidant activity was dose-dependentin in all extracts. By increasing the concentration of phenolic compounds and flavonoids, the probability of hydrogen donation to free radicals followed by the inhibitory strength of the extract was increased due to the increase in the number of hydroxyl groups in the reaction medium (Maghsoudlou *et al.* 2016). So that, by increasing concentration to 40 µg/ ml, DPPH scavenging activity was increased and reached its highest level (79%- 86% in different samples). In all treatments, the obtained extracts at 40 µg/ml concentration showed the highest DPPH radical scavenging activity and then by increasing the concentration, radical scavenging activity of the extracts remained stable or decreased slightly (Table 2).

IC_{50} value of the ultrasound in different treatments and control samples is depicted in Table 1.The IC_{50} values are the concentration of an antioxidant extract that can inhibit 50% of free radicals or have absorption of 0.5 at 700nm. Therefore, the lower IC_{50} values in the samples showed higher antioxidant activity.

Probe treatment at 50°C for 15 min and 40% ultrasound amplitude showed the lowest IC_{50} of DPPH radical scavenging and reducing power (14.21± 0.17 and 102.91± 4.19 µg/ml, respectively), so the highest antioxidant activity was obtained in these conditions, followed by probe treatment at 50°C for 5 min, amplitude 40% and bath treatment at 50°C for 45 min respectively.

Effect of temperature

Temperature is one of the most important factors in the extraction of antioxidant compounds by ultrasound. Generally, in both types of ultrasonic bath and probe systems, at each experiment time, by increasing extraction temperature up to 50°C, the phenolic and flavonoid content, and antioxidant activity of the oregano leaf extracts increased and then at higher temperatures (60°C) reduced. Increasing the efficiency of phenolic and flavonoid compounds up to 50°C can be attributed to the positive effect of temperature increase on improving the extraction efficiency due to the rupture of plant tissue links, increasing the solubility of compounds, increasing the velocity of solvent propagation and mass transfer, and reducing the solvent viscosity and surface tension. On the other hand, at higher temperatures of the ultrasound, the solvent vapor pressure rises as a result of lower surface tension and it causes of entering more solvent vapors to a large number of cavitation bubbles, which are less dispersed and reduced the

intensity of cavitation. Therefore, in this study, the sonochemistry effects probably decreased due to the collapse of cavitation bubbles at 60°C (Capelo Martinez *et al.*, 2009; Deyand Rathod, 2013; Medina-Torres *et al.*, 2017).

Table 1. Phenolic and flavonoid compounds and IC_{50} of oregano leaf extracts antioxidant activity test of different treatments

Extraction treatment		Total phenol (µg gallic acid/mg of dry extract)	Flavonoid (µg quercetin/mg of dry extract)	IC_{50} of DPPH (µg /ml)	IC_{50} of Reducing power (µg /ml)
Probe, 40 °C	5 min, 40%*	394.3± 8.71^{d}	28.5± 1.278^{e}	16.41± 0.27^{bcd}	131.8± 2.58^{def}
	5 min, 20%	310.90± 7.28^{h}	23.18± 0.71^{jk}	20.09± 1.09^{h}	151.5±3.73^{k}
	15 min, 40%	409.35± 14.27^{cd}	31.78± 0.82^{d}	15.19± 0.54^{ab}	124.55±1.65^{c}
	15min, 20%	335.80± 16.58^{g}	25.16±1.27^{hi}	19.64± 1.14^{h}	144.09± 1.92^{ij}
	25min, 40%	389.03± 15.27^{de}	27.09± 1.10^{efg}	17.24± 0.40^{cde}	135.79± 1.62^{fg}
	25 min, 20%	309.44± 12.40^{h}	23.5± 0.46^{j}	19.75± 1.36^{h}	151.32± 3.91^{k}
Probe, 50 °C	5 min, 40%	443.24± 5.87^{b}	39.64± 0.86^{b}	15.35± 0.45^{ab}	118.02± 1.68^{b}
	5 min, 20%	352.16± 7.27^{fg}	25.89± 1.15^{fgh}	18.75± 0.79^{efgh}	140.8± 3.28^{ghij}
	15 min, 40%	473.32± 25.90^{a}	46± 1.24^{a}	14.21± 0.17^{a}	102.91± 4.19^{a}
	15min, 20%	369.72± 3.88^{ef}	26.16± 0.47^{fgh}	17.90± 0.81^{def}	137.51± 2.33^{gh}
	25min, 40%	408.10± 21.62^{d}	28.81± 0.46^{e}	16.28± 0.11^{bc}	130.74± 2.16^{def}
	25 min, 20%	334.30± 12.27^{g}	23.86± 0.50^{ij}	19.79± 0.92^{h}	144.36± 2.58^{ij}
Probe, 60 °C	5 min, 40%	405.23± 14.96^{d}	28.5± 1.02^{e}	16.44± 0.15^{bcd}	129.88± 4.7^{d}
	5 min, 20%	386.89± 13.69^{de}	26.62± 1.24^{fgh}	17.29± 1.17^{cdef}	135.34± 1.07^{efg}
	15 min, 40%	368.79± 8.56^{ef}	26.67± 0.63^{fgh}	17.93± 1.29^{defg}	137.43± 1.71^{gh}
	15min, 20%	352.28± 11.34^{fg}	25.21± 1.09^{hi}	17.97± 0.59^{defg}	139.45± 3.69^{ghi}
	25min, 40%	356.61± 7.42^{fg}	25.63± 1.26^{fgh}	17.93± 1.14^{defg}	139.89± 2.17^{ghij}
	25 min, 20%	308.48± 9.85^{h}	21.83± 0.63^{k}	19.87± 0.66^{h}	153.45± 4.81^{k}
Bath, 40 °C	30 min	305.4± 12.44^{h}	21.72± 1.27^{k}	19.56± 0.42^{gh}	150.74± 4.23^{k}
	45 min	387.62± 15.36^{de}	27.35± 1.47^{ef}	17.26± 1.25^{cdef}	135.94± 2.21^{fg}
	60 min	335.76± 15.60^{g}	25.21± 0.95^{hi}	18.78± 1.57^{efgh}	145.21± 1.09^{j}
Bath, 50 °C	30 min	353.53± 8.26^{fg}	25.68± 0.95^{fgh}	18.75± 0.75^{efgh}	141.92± 3.31^{hij}
	45 min	430.81± 6.94^{bc}	35.89± 0.47^{c}	15.39± 0.15^{ab}	116.95± 3.54^{b}
	60 min	408.06± 15.45^{d}	28.76± 0.80^{e}	16.56± 0.88^{bcd}	130.2±2.77^{de}
Bath, 60 °C	30 min	355.07± 7.67fg	25.47± 1.06^{ghi}	18.91± 0.69^{fgh}	139.28± 1.48^{ghi}
	45 min	409.80± 8.32^{cd}	32.61± 0.36^{d}	16.19± 0.81^{bc}	123.95± 1.62^{c}
	60 min	353.08± 5.80^{fg}	25.42± 0.36^{ghi}	18.75± 0.63^{efgh}	139.83± 4.32^{ghi}
Control (SSE:ambient temp.,24h)		268.03± 8.58^{i}	17.51± 0.65^{i}	22.50± 0.84^{i}	177.81± 3.14^{l}

Data expressed as means ± standard deviations (n=3). Values with different letters within column indicate significance difference at $P < 0.05$. SSE: Shaker Solvent Extraction.

* %: amplitude of ultrasound waves

The best extraction temperature of oregano bioactive compounds is 50°C which is higher than the optimal temperature reported by Altemimi *et al.* (2015). This researchers announced 40°C as an optimal temperature by investigating the different temperature of ultrasound at 30- 50°C on the recovery rate of spinach extract polyphenols. They explained that the higher temperature of ultrasound can degrade some of the phenolic compounds that are dispersed in the extraction environment and had less thermal stability (Altemimi *et al.*, 2015). Most likely, the reason of the difference in the optimum temperature of the present study with the mentioned researchers is the presence of more heat stable phenolic compounds in oregano.

Table 2. DPPH radical scavenging activity (%) of different concentrations of Oregano leaf extract (µg /ml) in various treatments

Extract concentration / treatment		10	20	40	100	500	1000
Probe, 40 °C	5 min, 40%*	33.14 ±1.21 ^{bcd}	58.27± 2.71^{bc}	82.61± 0.28^{ab}	81.28± 1.11^{bc}	77.77± 1.15^{bcd}	75.39± 1.47^{abc}
	5 min, 20%	25.42± 1.88^{i}	49.37± 2.91^{fgh}	79.46± 1.40^{fg}	78.92±2.21^{b}	75.98±1.72^{def}	72.36±0.82^{def}
	15min, 40%	33.55± 0.97^{bc}	65.26±2.58^{a}	82.05± 0.44^{bcd}	80.86±2.37^{bc}	77.786±1.08^{bcd}	74.81±0.92^{bcd}
	15min, 20%	28.24± 2.89^{efgh}	48.27± 2.89^{gh}	82.10± 1.42^{bcd}	80.39± 2.27^{bc}	77.75± 0.54^{bcd}	73.13± 2.23^{cdef}
	25min, 40%	31.98± 3.02^{cdef}	55.03± 2.19^{cdef}	82.55± 1.46^{bcd}	80.36± 2.24^{bc}	76.21± 0.72^{cde}	74.61± 1.08^{bcd}
	25min, 20%	25.72± 2.70^{hi}	50.44±3.30^{efg}	80.20± 1.16^{def}	78.717± 2.10^{b}	75.66± 1.48^{def}	72.00± 1.71^{def}
Probe, 50 °C	5 min, 40%	36.31± 0.98^{ab}	61.40± 2.36^{ab}	82.61± 0.45^{ab}	81.85± 1.41^{bc}	78.99± 0.64^{a}	75.24± 1.88^{bcd}
	5 min, 20%	27.86± 1.41^{ghi}	52.22± 2.51^{def}	82.01± 0.79^{bcd}	81.21± 1.66^{bc}	77.00± 0.87^{bcde}	74.51± 2.53^{bed}
	15min, 40%	40.59±.1.66^{a}	65.51± 1.21^{a}	84.39± 1.80^{a}	82.56± 0.99^{a}	79.02± 0.9^{a}	76.21± 1.22^{ab}
	15min, 20%	28.10± 1.73^{efgh}	55.80±2.01^{bcde}	82.30± 1.31^{bcd}	81.59± 1.92^{bc}	76.86± 1.13^{bcde}	74.22± 1.86^{bcd}
	25min, 40%	33.72± 2.50^{bc}	58.39± 2.09^{bc}	82.03± 0.96^{bcd}	81.77± 1.56^{bc}	78.66± 2.28^{ab}	74.62± 1.20^{bcd}
	25min, 20%	25.86± 3.055^{h}	50.03± 1.50^{efg}	79.80± 1.48^{efg}	78.98± 1.14^{b}	75.51± 1.08^{def}	71.84± 2.08^{ef}
Probe, 60 °C	5 min, 40%	33.84± 3.66^{bc}	57.34± 3.02^{bcd}	81.40± 1.4^{cdrf}	80.83± 1.41^{bc}	77.73± 0.49^{bcd}	76.81± 1.39^{a}
	5 min, 20%	31.92± 3.36^{cdef}	55.06± 4.74^{cdef}	81.72± 1.00^{cde}	80.42± 2.18^{bc}	77.00± 1.02^{bcde}	73.93± 1.42^{bcd}
	15min, 40%	30.17± 1.40^{cdef}	53.74± 6.63^{cdef}	81.52± 1.18^{cdef}	80.70± 1.66^{bc}	1.02± 1.06^{bcde}	73.56± 1.48^{cde}
	15min, 20%	29.51± 0.83^{cdef}	53.98± 3.21^{cdef}	80.99± 1.21^{def}	80.02± 1.59^{bc}	76.87± 0.33^{cde}	73.46± 2.27^{cde}
	25min, 40%	30.44± 5.37^{cdef}	53.41± 0.83^{cdef}	80.71± 1.21^{cdef}	80.71± 1.60^{bc}	76.80± 2.50^{bcde}	73.73± 2.10^{cde}
	25min, 20%	25.45± 1.74^{i}	50.07± 1.34^{efg}	80.31± 1.63^{cdef}	80.31± 1.63^{bc}	75.73± 0.52^{def}	72.47±0.78^{def}
Bath, 40 °C	30 min	26.09± 3.59^{gh}	50.60± 2.11^{efg}	79.87± 0.97^{efg}	79.34± 1.89^{bc}	75.07± 2.21^{ef}	72.28± 1.33^{def}
	45 min	32.07± 4.03^{bcde}	55.12± 3.80^{cdef}	81.51± 0.35^{cdef}	79.81± 1.55^{bc}	76.88± 1.02^{bcde}	75.03± 2.62^{bcd}
	60 min	26.12± 3.45^{hi}	54.13± 4.12^{cdef}	79.18± 0.48^{g}	79.43± 1.43^{bc}	75.83± 1.63^{def}	72.67± 1.02^{def}
Bath, 50 °C	30 min	27.70± 2.46^{ghi}	52.38± 3.72^{def}	80.87± 0.56^{def}	80.37± 1.30^{bc}	74.65± 0.88^{ef}	72.38± 3.04^{def}
	45 min	36.40± 2.19^{ab}	61.07± 1.26^{ab}	83.96± 0.34^{ab}	81.73± 0.14^{bc}	78.53± 1.09^{abc}	75.21± 1.63^{bcd}
	60 min	32.78± 1.07^{bcde}	57.95± 3.82^{bcd}	82.67± 1.14^{ab}	81.46±0.90^{bc}	78.65±0.88^{ab}	75.31±1.02^{bcd}
Bath, 60 °C	30 min	27.99± 0.31^{efgh}	51.40± 3.14^{efg}	81.81± 1.26^{cde}	80.83± 2.08^{bc}	76.11± 0.78^{de}	72.78± 2.13^{cdef}
	45 min	33.50± 1.84^{bc}	59.26± 2.80^{bc}	81.91± 0.73^{cde}	80.87± 1.00^{bc}	78.60± 0.80^{abc}	75.44± 0.84^{abc}
	60 min	28.63± 0.39^{defg}	51.39± 3.03^{efg}	81.49± 0.18^{cdef}	79.85± 1.77^{bc}	75.80± 1.61^{def}	73.58± 2.35^{cde}
Control (SSE:ambient temp.,24 h)		20.93± 2.41^{j}	45.77± 2.44^{h}	76.79± 0.66^{h}	75.38± 0.53^{c}	73.70± 0.46^{f}	70.35± 1.14^{f}

Data expressed as means ± standard deviations (n=3). Values with different letters within column indicate significance difference at $P < 0.05$. SSE: Shaker Solvent Extraction.

* %: amplitude of ultrasound waves

In the other study, Moralli and Prado (2012) studied the extraction optimization for antioxidant phenolic compounds in red grape jam using ultrasound and reported that 50°C was the best extraction temperature of these compounds with the highest antioxidant activity, which shows the same results to the present study.

Effect of time

It was also observed that the phenolic and flavonoid content, and antioxidant activity of the ultrasonic probe was influenced by the exposure time, so that the phenolic content and antioxidant activity of the extracts increased at a constant temperature of 50°C and 40°C with increasing the time from 5 to 15 min. This phenomenon can be explained by the fact that more time is needed to release bioactive compounds from plant tissues and also increasing the variety of extracted compounds. However, the phenolic content of extracts decreased by increasing the extraction time from 15 to 25 min. Also, at the constant temperature of 60°C, with increasing extraction time we observed a decrease in the amount of phenolic compounds. The findings of Veggi *et al.* (2013) and Hammi *et al.* (2015) confirm these results. They investigated the extraction of Jatoba (*Jatoba bark*) polyphenols by using ultrasound at 50°C. They obtained the higher phenolic content during the first 15 minutes of the extraction process, due to the effect of phenolic concentration gradient between the solvent and the plant material.

At the beginning of the extraction, high phenolic content is located in contact with the solvent on the particle surface and accelerated the release of these compounds. However, after this time, the release of these compounds reduced the concentration gradient, as well as residual polyphenols which are located in the inner and deep parts of the particles are less accessible to the solvent. The reduction process of poly phenolic compounds at 60°C can be attributed to the further degradation of the obtained compounds at high temperature during the time (Cheok *et al.* 2013).

In the bath system, the same as the probe system, the phenolic and flavonoid content increased by increasing the extraction time from 30 to 45 min, and then decreased at 60 min. The difference is that in the bath system, the extraction of polyphenols takes a little more time than ultrasonic probes, which is probably due to lower power, lack of uniformity in the distribution of ultrasound energy, and the loss of produced energy in the fluid environment and the glass wall container and therefore leads to the less cavitation in the sample suspension.

Effect of amplitude

Based on Table 1, amplitude played an important role in intensification of extraction during the extraction of phenolic compounds and flavonoids by probe system. So that, in different combinations of temperature and time, the extraction efficiency of phenolic compounds and antioxidant activity in the upper range of the ultrasound (40%) was higher than the lower range (20%). In a similar study, Carrera *et al.* (2012) reported that the efficiency of tannin anthocyanin extraction using the amplitude (20 and 50%), increased the number of cavities thus improved the extraction process. Increasing the amplitude of ultrasound waves will increase the number of contraction and expansion cycles and the pressure range, resulting in more burst of cavitation bubbles. Hence, the higher ranges increase the effects of sonochemistry (Mason *et al.*, 2002; Cheok *et al.* 2013; Mediana-Torres *et al.*, 2017).

In this investigation, the traditional solvent extraction at ambient temperature for 24 hours as a control had the lowest total phenolic compounds (268.03± 8.58 μg GAE/ mg of dry extract) and the highest IC_{50} in DPPH radical scavenging and reducing power tests. Therefore, ultrasound technique was successful to extract the oregano phenolic compounds in all treatments. This can be attributed to the cavitation of the ultrasound technique, which imposes mechanical effects on the plant cell wall.

Table 3. Reducing power of different concentrations of Oregano leaf extract (µg /ml) in various treatments

treatment	Extract concentration	80	100	200	300	500	1000
Probe, 40 °C	5 min, 40%*	0.420± 0.00 ^{b}	0.448± 0.02^{cde}	0.690± 0.01^{def}	1.165± 1.00^{cde}	1.599± 0.01^{de}	2.647± 0.00^{ef}
	5 min, 20%	0.303± 0.012^{e}	0.388± 0.01^{kl}	0.602± 0.01^{j}	1.006±1.01^{i}	1.445±0.03^{h}	2.551±0.1^{efg}
	15min, 40%	0.445± 0.02^{ab}	0.464±0.02^{bc}	0.740± 0.01^{c}	1.225±0.00^{cd}	1.839±0.11^{c}	2.912±0.11^{c}
	15min, 20%	0.355± 0.03^{d}	0.401± 0.01^{ijkl}	0.639± 0.00^{i}	1.112± 0.00^{fgh}	1.498± 0.01^{gh}	2.582± 0.02^{efg}
	25min, 40%	0.392± 0.01^{c}	0.427± 0.00^{efgh}	0.677± 0.01^{efg}	1.131± 0.01^{fgh}	1.568± 0.01^{def}	2.639± 0.01^{efg}
	25min, 20%	0.303± 0.01^{e}	0.384±0.02^{kl}	0.606± 0.01^{j}	1.041± 0.04^{hi}	1.464± 0.00^{gh}	2.562± 0.09^{efg}
Probe, 50 °C	5 min, 40%	0.444± 0.01^{ab}	0.485± 0.00^{b}	0.785± 0.01^{b}	1.439± 0.01^{b}	2.001± 0.01^{b}	3.054± 0.01^{b}
	5 min, 20%	0.371± 0.00^{cd}	0.412± 0.01^{hijk}	0.653± 0.02^{ghi}	1.189± 0.09^{cde}	1.537± 0.01^{efg}	2.615± 0.00^{efg}
	15min, 40%	0.460±.0.01^{a}	0.512± 0.02^{a}	0.852± 0.04^{a}	1.568± 0.00^{a}	2.140± 0.01^{a}	3.222± 0.05^{a}
	15min, 20%	0.395± 0.01^{c}	0.415± 0.00^{ghij}	0.675± 0.01^{efgh}	1.151± 0.02^{def}	1.572± 0.00^{def}	2.639± 0.02^{efg}
	25min, 40%	0.424± 0.00^{b}	0.445± 0.00^{def}	0.702± 0.02^{de}	1.169± 0.01^{cde}	1.594± 0.01^{de}	2.651± 0.02^{ef}
	25min, 20%	0.364± 0.01^{d}	0.396± 0.00^{jkl}	0.642± 0.02^{hi}	1.113± 0.00^{fgh}	1.492± 0.00^{gh}	2.600± 0.01^{efg}
Probe, 60 °C	5 min, 40%	0.420± 0.01^{b}	0.455± 0.02^{cd}	0.700± 0.02^{de}	1.174± 0.10^{cde}	1.613± 0.02^{de}	2.653± 0.02^{ef}
	5 min, 20%	0.395± 0.02^{c}	0.431± 0.00^{defg}	0.677± 0.00^{efg}	1.128± 0.01^{fgh}	1.572± 0.01^{def}	2.641± 0.01^{ef}
	15min, 40%	0.394± 0.01^{c}	0.422± 0.01^{ghij}	0.668± 0.01^{fghi}	1.149± 0.02^{def}	1.575± 0.01^{def}	2.637± 0.01^{efg}
	15min, 20%	0.374± 0.01^{cd}	0.418± 0.01^{ghij}	0.658± 0.01^{fghi}	1.133± 0.00^{fgh}	1.534± 0.01^{fgh}	2.609± 0.01^{efg}
	25min, 40%	0.375± 0.01^{cd}	0.418± 0.01^{ghij}	0.654± 0.02^{ghi}	1.133± 0.01^{fgh}	1.534± 0.02^{fgh}	2.613± 0.02^{efg}
	25min, 20%	0.303± 0.01^{e}	0.375± 0.01^{l}	0.602± 0.02^{j}	1.066± 0.01^{ghi}	1.467± 0.00^{gh}	2.563± 0.02^{efg}
Bath, 40 °C	30 min	0.306± 0.01e	0.387± 0.011^{kl}	0.608± 0.02^{j}	1.048± 0.00^{ghi}	1.463± 0.01^{gh}	2.494± 0.01^{g}
	45 min	0.391± 0.00^{c}	0.424± 0.01^{fghi}	0.679± 0.02^{efg}	1.128± 0.01^{fgh}	1.57± 0.02^{def}	2.639± 0.02^{efg}
	60 min	0.305± 0.01^{e}	0.394± 0.01^{jkl}	0.638± 0.00^{i}	1.106± 0.01^{fgh}	1.465± 0.02^{gh}	2.529± 0.07^{fg}
Bath, 50 °C	30 min	0.374± 0.01^{cd}	0.409± 0.02^{hijk}	0.647± 0.01^{ghi}	1.137± 0.00^{efg}	1.540± 0.03^{efg}	2.629± 0.01^{efg}
	45 min	0.442± 0.01^{ab}	0.489± 0.02^{bc}	0.793± 0.02^{b}	1.426± 0.03^{b}	2.019± 0.04^{b}	3.062± 0.18^{b}
	60 min	0.420± 0.01^{b}	0.446± 0.02^{cde}	0.706± 0.01^{d}	1.174±0.10^{cde}	1.628±0.12^{d}	2.690±0.14^{e}
Bath, 60 °C	30 min	0.377± 0.01^{cd}	0.416± 0.01^{ghij}	0.660± 0.00^{fghi}	1.181± 0.08^{cde}	1.539± 0.01^{efg}	2.608± 0.02^{efg}
	45 min	0.445± 0.01^{ab}	0.464± 0.02^{bc}	0.745± 0.00^{c}	1.254± 0.12^{c}	1.820± 0.17^{c}	2.849± 0.01^{d}
	60 min	0.374± 0.01^{cd}	0.417± 0.01^{ghij}	0.656± 0.02^{ghi}	1.135± 0.00^{fgh}	1.543± 0.02^{efg}	2.614± 0.01^{efg}

Control (SSE:ambient temp.,24 h)	0.202 ± 0.02^{d}	0.285 ± 0.01^{m}	0.558 ± 0.02^{k}	0.782 ± 0.02^{j}	1.249 ± 0.13^{i}	1.948 ± 0.09^{h}

Data expressed as means ± standard deviations (n=3). Values with different letters within column indicate significance difference at $P < 0.05$. SSE: Shaker Solvent Extraction.

[*] %: amplitude of ultrasound waves

On the other hand, ultrasound accelerates the hydration and swelling process, causing to grow the plant cell wall cavities, increasing the mass transfer of soluble compounds from plant to solvent, and thus improving the efficiency of phenolic compounds extraction. Probably, the degradation of plant cells increases the solvent penetration rate into the plant tissue after the collapse of cavitation bubbles. Altemimi *et al.* (2015) and Han *et al.*, (2011) reported the same results which confirmed that ultrasound power can play an important role during the dissolution of biologically active compounds of plant tissues. Ultrasound treatments also produced higher polyphenol content in shorter time and thus reduced energy consumption (Veggi *et al.*, 2013).

In this study, the obtained content of oregano extracts phenolic compounds was the highest in ultrasonic probe treatment (50°C, 5 min and amplitude 40%) and its lowest content was unsteady from 268.03 to 473.32 µg GAE/mg of dry extract in traditional solvent extraction, which was higher than the total content of phenol (220 µg GAE/ mg of dry extract) reported by Sahin *et al.* (2004). This diversity of the content and even types of the compounds can be attributed to the difference in geographical area of plant growth due to natural cross-pollination. However, many references were reported rosmarinic acid as the most significant phenolic compound of oregano (Chun *et al.*, 2005).

Based on the results of phenolic and flavonoid compounds, it can be expressed that ultrasonic probe was more efficient than ultrasonic bath due to higher power as well as the power to concentrate energy production on the target sample if other conditions, such as temperature and extraction time, were more favorable than ultrasonic bath. However, this system showed a lower efficiency at the lower ultrasound amplitude (20%), as well as unfavorable time and temperature conditions due to degradation of dispersed bioactive compounds. Therefore, efficient extraction of phenolic compounds with an ultrasound system requires the selection of optimal conditions

Correlation between Amounts of Phenolic Compounds and Flavonoids with Antioxidant Activity

There was a significant negative linear correlation between the polyphenol content with IC_{50} of the DPPH radical scavenging and the reducing power, and between the flavonoids with IC_{50} of the DPPH radical scavenging and IC_{50} of the reducing power, meanwhile there was a positive significant correlation between IC_{50} of DPPH radical scavenging and reducing power tests (Table 4).The negativity of this equation means that as the polyphenol content increases, IC_{50} decreases and as a result, antioxidant activity increases (Maghsoudlou *et al.*, 2016).

Conclusions

In all conditions, ultrasound treatments have been able to increase the extraction efficiency of oregano leaves phenolic and flavonoid compounds compared to solvent extraction. The best conditions for extraction of phenolic and flavonoid compounds, and antioxidant activity were obtained by probe system at 50 °C for 15 min and ultrasound amplitude 40%. In addition, oregano leaf extract indicated high antioxidant activity based on DPPH free radical scavenging and reducing power tests. Therefore, oregano leaf extract can be used as good sources of natural antioxidants to improve the oxidative stability of food. Extraction of oregano antioxidants by ultrasound significantly reduced the extraction time from 24 h in solvent extraction to 15 and 45 min in ultrasonic probe and bath, respectively.

.Table 4. Line equations and correlation coefficients between the amount of phenolic and flavonoid compounds with antioxidant activity of extracts

Methods	r (Pearson's correlation coefficients)	R^2	Line equation
(A) Total phenolic compounds (µg gallic acid/mg of dry extract)			
IC_{50} of DPPH (µg/ml)	- 0.973	0.94	y = - 0.0386x + 32.114
IC_{50} of reducing power (µg /ml)	- 0.967	0.93	y = - 0.2862x + 242.96
(B) Total flavonoids (µg quercetin/mg of dry extract)			
IC_{50} of DPPH (µg /ml)	- 0.883	0.78	y = - 0.2906x + 25.875
IC_{50} of reducing power (µg /ml)	- 0.935	0.87	y = - 0.2975x + 200.69
(C) Relationship between antioxidant activity assays			
IC_{50} of DPPH and Reducing power (µg /ml)	0.960	0.92	y = 0.1287x+ 0.188

The results of this study confirm the effectiveness of the ultrasound method for extracting the polyphenols with high antioxidant activity.

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اثر فرآیندهای حمام و پروب فراصوت بر فعالیت آنتی‌اکسیدانی عصاره فنولی برگ‌های پونه کوهی (*Origanum vulgar* L.)

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چکیده

استخراج به کمک فراصوت به دلیل مزایای بسیار آن در استخراج ترکیبات زیست‌فعال از نمونه‌های گیاهی، مورد توجه زیادی قرار گرفته است. کارایی اولتراسوند تحت تاثیر عوامل مختلفی نظیر دمای استخراج، زمان استخراج، شدت امواج و نوع سیستم فراصوت متفاوت است. بنابراین، این مطالعه با هدف بررسی شرایط فرآیند دو نوع سیستم فراصوت(حمام و پروب) برای تعیین بالاترین راندمان استخراج ترکیبات فنولی و فلاونوئیدی برگ پونه کوهی و بهترین شرایط استخراج انجام شد. اثر ترکیب‌های متفاوت متغیرهای اولتراسوند شامل حمام (دما، 60-40 درجه سانتی‌گراد ؛ زمان، 60-30 دقیقه) و پروب (دما، 60-40 درجه سانتی‌گراد ؛ زمان، 25-5 دقیقه؛ دامنه اولتراسوند، 40-20 درصد) با استفاده از حلال اتانول- آب (50:50) موردررسی قرار گرفت. سپس فعالیت آنتی‌اکسیدانی عصاره‌ها توسط مهار رادیکال DPPH و قدرت احیاکنندگی ارزیابی شد. بهترین شرایط برای استخراج ترکیبات فنولی و فلاونوئیدی با استفاده از سیستم پروب فراصوت در دمای50°C به مدت 15 دقیقه و دامنه 40% به‌دست آمد. حداکثر فنول و فلانوئید به‌ترتیب معادل25/9 ±473/32 میکروگرم گالیک اسید در میلی‌گرم عصاره خشک و 1/24 ±46 میکروگرم کوئرستین در میلی‌گرم عصاره خشک بود. نتایج نشان داد روش فراصوت اثر قابل توجهی بر استخراج ترکیبات زیست‌فعال از برگ‌های پونه کوهی دارد، همچنین سیستم پروب کارایی بالاترینسبت به سیستم حمام داشت.

واژه‌های کلیدی: حمام فراصوت، پروب فراصوت، برگ پونه کوهی، ترکیبات فنولیک، فعالیت آنتی‌اکسیدانی

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