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Antioxidant and Antimicrobial Activities of Essential Oils Obtained From Oregano (Origanum Vulgare Ssp. Hirtum) by Using Different Extraction Methods

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Annotation: In this study, antioxidant and antimicrobial activities of essential oils obtained from oregano (Origanum vulgare ssp. hirtum) were determined by using solvent-free microwave extraction (SFME), supercritical fluid extraction, and conventional hydrodistillation (CH) methods. The inhibitory effects on the 2,20 -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical of essential oils obtained from oregano by using SFME and CH were similar. However, essential oil extracted by CH showed greater (2.69 mmol=mL of oil) Trolox equivalent antioxidant capacity (TEAC) than oregano oils obtained by SFME ($P < .05$). The difference between percentage inhibition and TEAC values most probably is due to the fact that undiluted and diluted samples are used in the percentage inhibition assay and the TEAC assay, respectively. TEAC values of oregano essential oils obtained by SFME at different microwave power levels were found to be similar and ranged from 0.72 to 0.84 mmol=mL of oil. Essential oils obtained by CH and SFME at different microwave powers inhibited the survival of Listeria monocytogenes, Salmonella typhimurium, and Escherichia coli O157:H7, whereas survival of Staphylococcus aureus was not influenced. In addition, oregano oil obtained by SFME at 40% power level did not show any inhibitory effect on E. coli O157:H7.

Keywords: Escherichia coli O157:H7, hydrodistillation, Listeria monocytogenes, Salmonella typhimurium, solvent-free microwave extraction, supercritical fluid extraction, Trolox equivalent antioxidant capacity value.

INTRODUCTION

In recent years, there has been growing interest in bioactive compounds that show antioxidant activity against radicals such as superoxide anion, hydroxyl radicals, lipid peroxyl radicals, hydroperoxyl radicals, etc.1–3 A tendency toward the use of natural antioxidants in foods has grown gradually. So far many studies have been conducted to search antioxidant properties of many aromatic plants and spices.4–7 Plant foods and their essential oils are one of the main groups of foods that have a potential antioxidant effect. Essential oils are natural volatile compounds with a strong odor formed by aromatic plants as secondary metabolites. Since the middle ages, essential oils have been widely used for antimicrobial, medicinal, and cosmetic applications, especially nowadays in pharmaceutical, sanitary, cosmetic, agricultural, and food industries.7 Among them, the genus Origanum (Family Labiatae), which is an annual, perennial, and shrubby herb native to Mediterranean, Euro-Siberian, and Irano-Siberian regions, has attracted the most attention.8 In these regions oregano has been used for many years as a medicinal plant with health-aiding properties like its powerful antibacterial and antifungal properties. Studies have shown that antifungal and antibacterial properties of oregano are mainly based

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on carvacrol and thymol, which are the major components of its essential oil.9 The antimicrobial activity of essential oils has taken on great importance as an alternative for synthetic antimicrobials because they are a part of the human diet and their biodegradability suggests low toxic residue problems.10 Antimicrobial activities of spices, their active components, and their essential oils against the growth of pathogenic bacteria and other microorganisms, leading to spoilage of foods, have been studied intensively in recent years. It is well known that sage, anise, daphne, mustard, black pepper, thyme, cumin, mint, garlic, and cinnamon have inhibitory effects on the survival of bacteria such as Bacillus cereus, Bacillus subtilis, Clostridium botulinum, Escherichia coli, Staphylococcus aureus, and Listeria monocytogenes and several fungi.11,12 There are more than 1,340 plants with defined antimicrobial compounds, and over 30,000 components have been isolated from phenol group-containing plant oil compounds and used in the food industry. However, commercially useful characterizations of preservative properties are available for only a few essential oils.13 Therefore, there is a need for more studies on the antioxidant and antimicrobial properties of essential oils in order to evaluate their possible uses in food systems. It is well known that the extraction procedure has a great impact on the quality of the final product such as extraction efficiency, retention of volatile compounds, and bioactive components.14 Essential oils are most commonly produced commercially by steam distillation or hydrodistillation methods. Environmentally friendly methods such as supercritical fluid extraction (SFE) and microwave extraction have been developed recently. The other reason for the development of new extraction methods is to protect beneficial components of volatile oils from being decomposed or oxidized. In the present study, the aim was to investigate antioxidant activity against linoleic acid peroxidation, 2,20 - azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, and antimicrobial activity of essential oils obtained from oregano by using solvent-free microwave extraction (SFME) at different power levels, SFE, and conventional hydrodistillation (CH) methods.

MATERIALS AND METHODS

Materials and chemicals

Oregano (Origanum vulgare ssp. hirtum) was kindly donated by Ku¨tas¸ (Ku¨tas¸ Tarım U¨ ru¨nleri Dıs Tic. San. A.S., Izmir, Turkey). DPPH (catalog number D9132), linoleic acid (catalog number L1376), phosphate buffer tampon (catalog number P4417), hemoglobin (catalog number H2500), $FeCl_2$ (catalog number 372870), and methanol were purchased from Sigma-Aldrich (Munich, Germany). ABTS diammonium salt (catalog number 11557) was obtained from Sigma-Aldrich Fluka (Munich). Ammonium thiocyanate was purchased from Merck (Darmstadt, Germany). Pure cultures of E. coli O157:H7, L. monocytogenes Scott-A, Salmonella typhimurium NRRL E 4463, and S. aureus 6538P were obtained from the Microbiology Laboratory, Food Engineering Department, Ege University, Izmir, Turkey and used as pathogen cultures for the determination of antimicrobial activity.

CH

An herb:water ratio of 1:10 was used for CH by using a Clevenger apparatus. The process was continued until no more essential oil was obtained. The experiments were conducted twice. The essential oil was collected in ambercolored vials, dehydrated with anhydrous sodium sulfate, capped under nitrogen, and kept at 48C until analysis.

SFME

The experimental setup for the SFME was a Clevenger apparatus placed in a microwave oven (White-Westinghouse, Pittsburgh, PA, USA) operating at 2,450 MHz. The maximum power of the oven was 622 W, which was measured by the IMPI 2 L test.15 Extractions were carried out at four power levels: 100% (622 W), 80% (498 W), 60% (373 W), and 40% (249 W). Fifty grams of dried O. vulgare ssp. hirtum was soaked in 700 mL of distilled water at room temperature (258C) for 1 hour to hydrate the external layers of the herb. At the end of the soaking period excess water was drained off. The moistened herb was placed in a flat-bottom flask, and SFME was performed at the given power level. The extraction process was performed until no more essential oil was obtained. For each condition,

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experiments were replicated twice. The essential oil was collected in amber-colored vials, dehydrated with anhydrous sodium sulfate, capped under nitrogen, and kept at 48C until analysis.

SFE

SFE was performed by using an ISCO (Lincoln, NE, USA) supercritical fluid extractor (model SFX5100) at 408C temperature and 105 bar pressure for 30 minutes. Stainless steel cartridges having a capacity of 10 mL were filled with 0.3 g of sample. The CO2 flow rate was 2 g=mL. The extract was trapped in a tube containing n-hexane, placed in dry ice to provide condensation. After extraction, the extract was transferred to amber colored vials, n-hexane was evaporated under nitrogen, and the vials were kept at 48C until analysis. Because it was not possible to work with greater amounts of sample in SFE, in order to reach the desired volume for the analysis of antioxidant activity and antimicrobial activity, essential oils were collected from replicated (at least 10) SFE procedures. Therefore, antioxidant and antimicrobial activity analyses were performed as two parallels.

Antioxidant activity

ABTS decolorization assay. ABTS radical scavenging activities of the samples were determined by the method of Re et al. 16 In brief, ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 hours before use. For the determination of antioxidant activity, the ABTS radical solution was diluted with ethanol to an absorbance of 0.70 (0.02) at 734 nm. After addition of 1.0 mL of diluted ABTS radical solution to 10 mL of the sample, the absorbance was read (Cary 50 Scan UV-Visible spectrophotometer, Varian Australia Pty Ltd., Mulgrave, VIC, Australia) 5 minutes after initial mixing. Percentage inhibition was calculated by using the following equation: Inhibition (%) ¼ (1

[Asample=AABTS solution]) · 100 where Asample is the absorbance reading obtained for the sample and AABTS solution is the absorbance reading obtained for the ABTS solution. DPPH radical scavenging activity. The free radical scavenging activity was determined using DPPH,17 which is a stable free radical that has an unpaired valence electron at one atom of a nitrogen bridge. In this experiment 50 mL of each extract was added to 950 mL of a 0.030 mg=mL methanol solution of DPPH. Then, the mixture was shaken vigorously and left in darkness for 5 minutes. Finally, the absorbance of the mixture was measured against methanol (blank) at 515 nm by using a spectrophotometer (Cary 50 Scan UV-Visible) The DPPH scavenging activity was expressed as the inhibition of free radical DPPH:

Inhibition (%)=$(1-[A_{sample}/A_{blank}])*100$

where A_{sample} is the absorbance of the sample and A_{blank} is the absorbance of the DPPH.

Inhibition of linoleic acid peroxidation.

Inhibition effect of the samples on linoleic acid peroxidation was determined by the method of Kuo et al. 18 Ten microliters of sample, 0.37 mL of 0.05 M phosphate buffer (pH 7.0) containing 0.05% Tween 20, and 4 mM linoleic acid were mixed in a test tube. This mixture was equilibrated at 378C for 3 minutes. The peroxidation of linoleic acid in the mixture was initiated by adding 20 mL of 0.035% hemoglobin prepared in water. Then the mixture was incubated at 378C in a shaking water bath at 100 rpm for 10 minutes. The reaction was stopped by addition of 5 mL of 0.6% HCl prepared in ethanol. The hydroperoxide formed was quantified according to the ferric thiocyanate method. Absorbance readings were taken at 480 nm with a spectrophotometer (Cary 50 Scan UVVisible). Antioxidant activity of the sample was calculated according to the following equation:

Inhibition (%)= $\{1-([A_s-A_0]/[A_{100}-A_0])\}*100$

where A_0 is the absorbance obtained for the reaction mixture that does not contain hemoglobin, A_{100} is the absorbance obtained for reaction mixture that does not contain sample, and A_s is the absorbance obtained for the reaction mixture.

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Trolox equivalent antioxidant capacity. The Trolox equivalent antioxidant capacity (TEAC) assay based on the reaction of ABTS radical with Trolox was performed in order to compare radical scavenging activity of a sample with that of Trolox. In brief, this method is based on the ability of antioxidant to quench the ABTS radical cation relative to that of Trolox, a water-soluble vitamin E analog.19 The antioxidant activities of the samples were estimated within the range of the dose–response curve of Trolox and expressed as the TEAC. The latter is defined as the concentration of Trolox having the antioxidant capacity equivalent to a 1.0 mmol=L solution of the substance under investigation. In this study, the TEAC values were expressed as mmol of TEAC=mL of sample.

Antimicrobial activity

Gram-positive and Gram-negative bacterial species used in this study were kindly obtained from the culture collection of the Microbiology Laboratory, Food Engineering Department, Ege University. The bacterial species include L. monocytogenes Scott-A, S. aureus 6538P, E. coli O157:H7, and S. typhimurium NRRLE 4463. Tryptone soya broth (catalog number CM 129, Oxoid, Basingstoke, United Kingdom) was used as the medium for the development of the strains of pathogenic cultures, whereas plate count agar (catalog number CM 325, Oxoid) was used for the enumeration. Inocula used in antimicrobial assay were obtained from cultures grown on Tryptone soya broth at 358C for 24 hours. Essential oils were sterilized by filtration through Millipore (Bedford, MA, USA) filters (pore size, 0.45 mm). Antimicrobial tests were then carried out by the disc diffusion method using 100 mL of suspension containing 108 colony-forming units of pathogenic bacteria=mL spread on nutrient agar (catalog number CM0003, Oxoid). The sterilized paper discs (6 mm in diameter) were impregnated aseptically with 10 mL of essential oil placed on the inoculated agar. Three discs were placed on each Petri plate. Sterilized water was used as a control. Plates were kept at ambient temperature for 1 hour and then incubated at 378C for 24 hours. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms.20 Each assay was performed in parallels and triplicates.

Statistical analysis

The data were expressed as mean SD values. Statistical analysis was performed using SPSS for Windows version 10.0 (SPSS, Inc., Chicago, IL, USA). Analysis of variance (one-way) was conducted, and Tukey's HSD multiple range test was used to determine significant differences at $P < .05$.

RESULTS AND DISCUSSION

The maximum extraction levels of essential oils obtained from oregano following SFME were 0.054, 0.053, 0.052, and 0.049 mL of oil=g of oregano for 100%, 80%, 60%, and 40% microwave power levels, respectively. However, the yield from CH was 0.048 mL of oil=g of oregano, which was lower than those of SFME extracts obtained at different microwave power levels ($P < .05$). The highest yield was obtained with SFE (0.055 mL of oil=g). The time needed for obtaining the highest oil yield was 3 hours for CH, but this period for SFME was decreased up to 35 minutes and was related to the microwave power level. The time to reach the highest oil yield for SFE was 30 minutes. Therefore, the extraction time was reduced by about 80% and 83% by using SFME and SFE, respectively.

Antioxidant activity

Several methods have been introduced for determination of total antioxidant activity of foods and=or pure compounds. These methods are based on the generation of a different radical and inhibition extent of the scavenging by antioxidant compounds that are hydrogen or electron donors.16

Against ABTS radical. Generation of the ABTS radical cation forms the basis of one of the spectrophotometric methods that have been applied to the measurement of the ESSENTIAL OIL OF OREGANO 647 total antioxidant activity.15 Inhibition effects of oregano essential oils obtained by SFME at different power levels and CH on ABTS radical cation oxidation are shown in Figure 1. The inhibition effect on ABTS radical cation of oregano essential oils obtained by SFME at different power levels ranged between 99.09% and 94.77%.

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The inhibition effect of oregano oil obtained by CH was similar (97.45%), whereas that of the extract obtained by SFE was quite low (30.45%). The reason for this may be due to the difference in the composition of essential oil. A part of this project21 was published by Bayramoglu et al.,22 and they reported that the composition of the essential oils obtained by both SFME and CH methods was virtually the same. The main components of O. vulgare ssp. hirtum essential oil were determined to be thymol (650–750 mg=mL), followed by p-cymene (60– 85 mg=mL), carvacrol (40–60 mg=mL), g-terpinene (35– 50 mg=mL), b-mrycene (approximately 15 mg=mL), and a-terpinene (10–15 mg=mL). Oxygenated compounds were the main components of essential oil (80–85%), followed by monoterpene hydrocarbons (13–16%) and sesquiterpenes (1.0–1.6%). However, oxygenated compounds, monoterpene hydrocarbons, and sesquiterpenes in the extract of O. vulgare ssp. hirtum obtained by the SFE method were 73.16%, 24.57%, and 0.75%, respectively. p-Cymene was not determined in the SFE extract. There was no significant difference between the inhibition effects of oregano oils obtained by SFME and CH on ABTS radical oxidation. In contrast, TEAC values of oregano oils obtained by SFME and CH were significantly different ($P < .05$) (Table 1). Oregano oil extracted by CH showed higher TEAC value (2.69 mmol of Trolox=mL of oil) than oregano oils obtained by SFME ($P < .05$). TEAC values of oregano oils obtained by SFME at different microwave power levels were found to be similar and ranged from 0.72 to 0.84 mmol of Trolox=mL of oil. Puertas-Mejia et al. 23 found that total antioxidant activity of O. vulgare L. essential oil isolated by hydrodistillation against ABTS radical was 25.1 mmol of Trolox=kg of oil.

Inhibition of linoleic acid peroxidation. Apart from ABTS and DPPH radical scavenging effects, inhibition of linoleic acid peroxidation is an indicator that the sample is an effective inhibitor of lipid peroxidation. Results showed that oregano oil obtained by SFME at 100% power level as a weaker inhibitor of linoleic acid peroxidation (32.85%) than those of obtained by SFME at 80%, 60%, or 40% microwave power and CH ($P < .05$). Antioxidant capacities of SFME extracts obtained by 80%, 60%, and 40% of microwave power were 89.93%, 74.62%, and 85.57%, respectively (Table 3). Antioxidant capacity of CH extract was found to be 90.43% (Table 3). SFE extracts of oregano showed the strongest inhibition of linoleic acid peroxidation (by 99.73%) ($P < .05$). Nakibog˘lu et al. 30 studied the antioxidant activity of Sideritis sipylea Boiss and Origanum sipleum L., which are endemic to Turkey. They found that extracts of S. sipylea and O. sipleum were able to reduce the formation of peroxides. Total antioxidant capacities of methanol-soluble and ethanol-soluble extracts of S. sipylea and tert-butylated hydroxyanisole were found to be similar and significantly higher than the other extracts. Sxahin et al. 8 reported that essential oil (2 mg=mL) of oregano obtained by water distillation for 3 hours inhibited linoleic acid peroxidation by 36%; the researchers concluded that antioxidant activity might be improved at higher concentrations, which were not used in the study. Our results were not in agreement with the study of Sxahin et al. 8 This difference may be due to not only the concentrations used in both study but also the different chemical composition of the samples used in the studies. It is well known that essential oils are a heterogeneous group of complete mixtures of organic substances, the quality and quantity of which vary with growth stages, ecological conditions, and other plant factors. They found that thymol and carvacrol contents of oregano essential oil were 0.8% and 0.6%, respectively.8 However, we found that thymol and carvacrol contents of CH extracts of oregano were about 75% and 4.8%, respectively.21 Kulisic et al.28 reported that oregano oil possessed remarkable antioxidant properties. The antioxidant effect was due to the presence of thymol and carvacrol, but a possible synergistic effect among oxygencontaining compounds could be suggested too. Similarly, Martinez-Rocha et al.31 demonstrated the variety of antioxidant compounds present in oregano and the reason why oregano is considered one of the best antioxidant species.

Conclusion

In this study, antioxidant and antimicrobial activities of essential oils obtained from oregano (Origanum vulgare ssp. hirtum) were determined by using solvent-free microwave extraction (SFME), supercritical fluid extraction, and conventional hydrodistillation (CH) methods. The inhibitory effects on the 2,20 -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical of essential oils obtained from oregano by using SFME and CH were similar. However, essential oil extracted by CH showed greater

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(2.69 mmol=mL of oil) Trolox equivalent antioxidant capacity (TEAC) than oregano oils obtained by SFME ($P < .05$). The difference between percentage inhibition and TEAC values most probably is due to the fact that undiluted and diluted samples are used in the percentage inhibition assay and the TEAC assay, respectively. TEAC values of oregano essential oils obtained by SFME at different microwave power levels were found to be similar and ranged from 0.72 to 0.84 mmol=mL of oil. Essential oils obtained by CH and SFME at different microwave powers inhibited the survival of Listeria monocytogenes, Salmonella typhimurium, and Escherichia coli O157:H7, whereas survival of Staphylococcus aureus was not influenced. In addition, oregano oil obtained by SFME at 40% power level did not show any inhibitory effect on E. coli O157:H7.

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