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Antioxidant and Antifungal Activities of Essential Oils of *Origanum vulgare* ssp. *gracile* Flowers and Leaves from Iran

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HIGHLIGHTS

- Remarkable antioxidant capacity was observed in *Origanum vulgare* ssp. *gracile* Essential Oil (EO).
- *Aspergillus flavus* was significantly more susceptible to EO than *A. niger*.
- The flowers EO had significantly higher antifungal activity comparing to leaves EO.
- *O. vulgare* ssp. *gracile* EO could be recommended as an antifungal preservative in food industries.

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Acronyms and abbreviations
EOs=Essential Oils
DPPH=2,2- diphenyl-picrylhydrazyl
MIC=Minimum Inhibitory Concentration
MFC=Minimum Fungicidal Concentration
BHT=Butylated Hydroxy Toluene
GC/MS=Gas Chromatography/Mass Spectrometry
PDA=Potato Dextrose Agar
ABTS=2,2'-azino-bis-3-ethyl-benzo thiazoline-6-sulphonic acid
RSA=Radical Scavenging Activity

ABSTRACT

Background: Herbal Essential Oils (EOs) are natural, volatile, and complex compounds that are defined by a strong odor. This study was conducted to investigate and compare antioxidant and antifungal properties of EO extracted from flowers and leaves of *Origanum vulgare* ssp. *gracile* from Iran.

Methods: EOs of leaves and flowers of *O. vulgare* ssp. *gracile* were extracted using a modified Clevenger apparatus and hydro-distillation method. *In vitro* antioxidant properties of the EOs were evaluated by 2,2- diphenyl-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethyl benzo thiazoline-6-sulphonic acid, $ABTS^{+}$) assays and their antifungal properties were assessed against *Aspergillus flavus* and *A. niger* by agar disk diffusion and micro well dilution methods. The EOs exhibited both fungistatic and fungicidal activity against tested fungal species. Statistical analysis of data was performed using SPSS, Inc, Chicago, IL software (v.16.0).

Results: Minimum Fungicidal Concentration (MFC) values for *A. niger* and *A. flavus* were 200 and 100 µg/ml, respectively; however, the MFC values for the mentioned fungi were 400 and 200, respectively. The flower EOs had significantly (p<0.05) higher inhibitory activities against both fungal species comparing to leave EOs. Also, it was found that *A. flavus* was significantly (p<0.05) more susceptible to EOs than *A. niger*. Remarkable antioxidant capacity was observed in both EOs, but it was significantly (p<0.05) lower than Butylated Hydroxy Toluene (BHT) as a synthetic antioxidant.

Conclusion: Although, antioxidant as well as antifungal efficacy of flowers EO was significantly higher than the leaves EO, both leaves and flowers EOs could be recommended as an antifungal preservative in food industries for their application in hurdle systems.

Introduction

Herbal Essential Oils (EOs) are natural, volatile, and complex compounds that are defined by a strong odor. They are formed by aromatic plants and attracted interest as potential preservatives to extend shelf life of food products (Abdollahzadeh et al., 2014; Azizkhani et al.,

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2013; Schnitzler et al., 2008). Most of their biological properties are due to their secondary metabolites such as flavonoids, alkaloids, and terpenoids. Many factors such as genotype, environmental and climate conditions, geographic location, cultivation and harvest time, growing season, method of distillation, and storage condition can affect the composition of secondary metabolites in medicinal plants EOs that cause different antimicrobial, antioxidant, and other biological properties (Alboofetileh et al., 2014; Aminzare et al., 2016; Azhdarzadeh and Hojjati, 2016; Dashipour et al., 2015; Figueiredo et al., 2008; Khorasany et al., 2016; Milos et al., 2000; Tounsi et al., 2011; Verma et al., 2010). Moreover, the EOs extracted from different parts of plants (such as flowers or leaves) have different components and properties (Entezari et al., 2009; Moradi et al., 2014).

Origanum vulgare L., popularly known as oregano is an annual and shrubby herb belongs to Lamiaceae family that is indigenous to the Mediterranean, Euro-Siberian and Irano-Turanian regions. A total of 38 *Origanum* species and 6 subspecies are recognized in the world that usually grow on stony mountain areas with 0-4000 m altitude (Aligiannis et al., 2001; Couto et al., 2015; Moradi et al., 2014; Şahin et al., 2004). Aerial parts of this plant are aromatic, rich in EO and have been traditionally used as spices and remedy for its spasmodic, diuretic, antimicrobial, stomachic, anti-neuralgic, expectorant, sedative, stimulant, and carminative characteristics (Daferera et al., 2003; Dundar et al., 2008; Şahin et al., 2004; Sokovic et al., 2002). In addition to its traditional and domestic uses, nowadays it is used in pharmaceutical and cosmetic industries for its biological properties and in food products and alcoholic beverages as a flavoring agent (Aligiannis et al., 2001; Novak et al., 2000). The chemical composition of different species of *Origanum* EOs previously has been investigated and the major components were phenolic monoterpenoides mainly carvacrol and occasionally thymol (Figiel et al., 2010; Mechergui et al., 2010; Teixeira et al., 2013; Vale-Silva et al., 2012). Biological activities of *Origanum* species mainly depend on carvacrol. Antioxidant and antifungal activities of *Origanum* species are mainly due to this component as an oxygenated monoterpene (Baydar et al., 2004; Chami et al., 2004; Dundar et al., 2008; Ipek et al., 2005; Nostro et al., 2004; Sokmen et al., 2004).

O. vulgare ssp. *gracile* is one of the most widely distributed subspecies growing in the Iran, Afghanistan, North of Iraq, East of Turkey, Northwest of Pakistan, South, and Center of Russia. In Iran, *O. vulgare* contains three subspecies including, ssp. *viride*, ssp. *vulgare*, and ssp. *gracile*. The distribution areas of this subspecies in Iran are mainly Kurdistan, Mazandaran, Gilan, and Azerbaijan provinces. Some studies have confirmed antibacterial, antifungal, and antioxidant activities of different *Origanum* species (Bakkali et al., 2008; Couto et al., 2015; Esen et al., 2007; Mitchell et al., 2010; Moradi et al., 2014). But, to our knowledge there is not any published document about antioxidant and antifungal activities of *O. vulgare* ssp. *gracile* EO. Therefore, the present study was conducted to evaluate antioxidant and antifungal properties of EOs extracted from flowers and leaves of *O. vulgare* ssp. *gracile* collected from Iran.

Materials and methods

Plant based material

The leaves and flowers of *O. vulgare* ssp. *gracile* were harvested at flowering stage in mid-July 2011 from wild grown plants in the Saral area, Zardavan district, Kurdistan province, Iran, and authenticated at Agriculture and Natural Resources Center of Kurdistan province, Iran.

Isolation and analysis of EOs

EOs of leaves and flowers of *O. vulgare* ssp. *gracile* were extracted using a modified Clevenger apparatus and hydro-distillation method. Plant material was added to volumetric flask containing 2 L distilled water and coupled to the altered Clevenger device (KOL, behr, Germany) and extracted for 2.5 h at 100 °C. The extracted oils were dried over anhydrous sodium sulfate and stored at 4 °C before use. Chemical composition of the EOs was determined by a varian 3400 Gas Chromatography/Mass Spectrometry (GC/MS) system (Moradi et al., 2014).

Evaluation of antifungal activity

The fungi strains used in this study were *Aspergillus niger* (ATCC 20466) and *A. flavus* (PTCC 5006), which were obtained from the culture collection of the Department of Food Hygiene, Faculty of Veterinary Medicine and Department of Plant Protection, Faculty of Agriculture, Urmia University, Urmia, Iran. Then, the molds were prepared in Potato Dextrose Agar (PDA) plates (Merck Darmstadt, Germany) for 5-7 days at 28 °C.

For preparation of fungal suspensions, at first, the fungal colonies were washed slowly from the surface of PDA plates with a saline solution containing 0.5% (v/v) tween 80. It was shaken vigorously to dislodge the spores in solution and then centrifuged. The supernatant was used as a culture suspension for inoculation. The spores were counted by Neubauer slides and suspensions were adjusted with a sterile saline solution containing 0.5% (v/v) tween 80 to a concentration of approximately 10^6 spore/ml. Dilutions of the inoculum were cultured on PDA medium to verify the validity of each inoculum.

For disk diffusion assay, the EOs were dissolved in the methanol to a final concentration of 1, 2.5, 5, and 10 mg/ml and subsequently sterilized by filtration via 0.45

µm Millipore filters. Then 100 µl of each fungal suspension (10^6 spore/ml) was spread on a PDA plate. The blank disks (6 mm in diameter, Padtan Teb, Iran) were impregnated with 10 µl of each EO concentrations (10, 25, 50, and 100 µg/disk) and placed on the inoculated PDA plates. Negative controls were prepared using methanol without EO. The inoculated plates were incubated at 28 °C for 72 h. Finally, antifungal activities of different concentrations of EOs were evaluated by measuring the diameter of the inhibition zone around the tested microorganisms using a caliper according to Şahin et al. (2004).

Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) values of EOs were determined against *A. niger* and *A. flavus* using a broth micro well dilution method (Akhondzadeh et al., 2014; Tian et al., 2014). Serial two-fold dilutions of EOs were made to obtain a concentration range from 250 to 8000 µg/ml. Aliquots of 160 µl of PD broth (Merck Darmstadt, Germany), 20 µl of spore suspension (10^6 spore/ml) were dispensed into the 96-well micro plates. Then amounts of 20 µl of each concentration of EOs was added to each well to reach a final volume of 200 µl per well. The final concentration of spore suspensions and EOs were approximately 10^5 spore/ml and 25 to 800 µg/ml, respectively. Positive controls (180 µl of PD broth+20 µl of inoculums) and negative controls (180 µl of the uninoculated PD broth+20 µl of EOs) were considered in the last wells. After incubation period of 72 h at 28 °C, the lowest concentrations without any visible growth were defined as the concentrations that completely inhibited fungal growth (MIC values). The MFC values were determined by serial sub-culturing of 5 µl of clear wells (wells without any visible growth) contents upon PDA plates and further incubation for 72 h at 28 °C. The lowest concentrations with no visible growth on PDA plates were defined as MFC values.

Antioxidant activity

The hydrogen atoms or electrons donation ability of EOs were measured by bleaching of purple colored methanolic solution of 2,2- diphenyl-picrylhydrazyl (DPPH). Fifty µl of various concentrations of EOs in methanol (1, 2.5, 5, and 10 mg/ml) was added to 2 ml of methanolic solution of DPPH (24 µg/ml). The absorbance was read against a blank at 517 nm using a spectrophotometer (LKB Novaspec II; Pharmacia, Uppsala, Sweden), after 1 h incubation period at room temperature and Radical Scavenging Activity (RSA) of EOs was calculated using following equation:

$$RSA\ (\%)=(A_{blank}-A_{sample}/A_{blank})\times 100$$

A_{sample} is the absorbance of the test compound (EOs) and A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound). Various concentrations of Butylated Hyroxy Toluene (BHT) as a reference synthetic antioxidant were used as positive controls (Aminzare et al., 2015).

The total antioxidant activity of EOs was measured by 2,2'-azino-bis-3-ethyl benzo thiazoline-6-sulphonic acid ($ABTS^+$) radical cation de-colorization assay (Chun et al., 2005). ABTS solution (7 mmol/L) and potassium persulphate solution (2.45 mmol/L) in distilled water were separately prepared and reacted together to produce ABTS radicals (1:1). The mixture was kept in the dark at room temperature for 16 h. $ABTS^+$ solution was diluted with phosphate buffer saline to an absorbance of 0.70 at 734 nm and 30 °C, in the moment of use. Aliquots of 2 ml $ABTS^+$ solution was added to glass test tubes containing 200 µl of various concentrations (1, 2.5, 5, and 10 mg/ml) of each EO in methanol and tubes were mixed by vortex mixer for 30 s. Tubes were incubated for 6 min at room temperature and then read at 734 nm and the percentage of inhibition was calculated by following equation:

$$Inhibition\ (\%)=[(A_{Control}-A_{Extract})/A_{Control}]\times 100$$

Statistical analysis

All experiments of this study were repeated thrice. Statistical analysis of data was performed using SPSS, Inc, Chicago, IL software (v.16.0). Tukey's test was used to compare differences among mean values ($p<0.05$).

Results

The MIC values of flowers EO for both *A. niger* and *A. flavus* species was 100 µg/ml, while MFC values for *A. niger* and *A. flavus* were 200 and 100 µg/ml, respectively. Regarding leaves EO, MIC values for *A. niger* and *A. flavus* were 200 and 100, respectively; however the MFC values for the mentioned fungi were 400 and 200, respectively. As indicated in Table 1, the flower EOs had significantly ($p<0.05$) higher inhibitory activities against both fungal species comparing to leave EOs. Also, it was found that *A. flavus* was significantly ($p<0.05$) more susceptible to EOs than *A. niger*.

As illustrated in Fig. 1 and Fig. 2, remarkable antioxidant capacity was observed in both leaves and flowers EOs, but it was lower than BHT. Scavenging ability rates of DPPH radicals for BHT, flowers EOs, and leaves EOs at highest concentration (10 mg/ml) were 88.7, 83.4, and 79.8%, respectively without any significant difference ($p>0.05$). The results of ABTS assay for BHT, flowers EOs, and leaves EOs at highest concentration (10 mg/ml) showed meaningful difference ($p<0.05$) and the inhibitory rates were 95.4, 89.6, and 78.6%, respectively.

Table 1: Values of diameter of inhibition zone (mm) obtained for evaluate antifungal effects of EO concentrations using agar disk diffusion method

Fungi species		EO concentrations (µg/disk)			
		10	25	50	100
A. niger	flowers	10.00±0.33 [a]	12.33±0.67 [b]	16.67±1.2 [b]	25.67±1.2 [b]
	leaves	7.67±1.2 [b]	10.00±0.2 [c]	13.2±0.67 [c]	21.80±2.1 [b]
A. flavus	flowers	11.33±0.67 [a]	15.67±1.2 [a]	22.33±0.88 [a]	35.2±1.53 [a]
	leaves	9.67±0.67 [ab]	12.67±0.33 [b]	16.50±0.33 [b]	25.40±2.1 [b]

Values followed by the same letter within the same column are not significantly different (p>0.05)

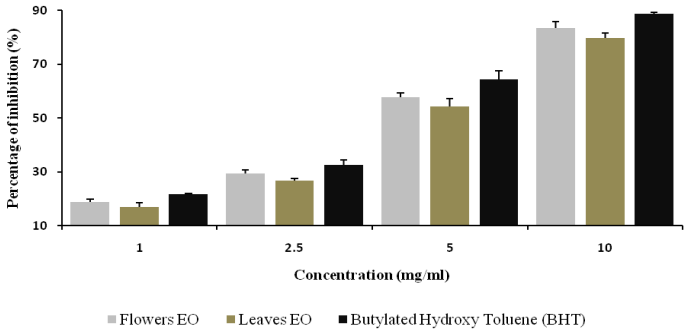

Fig. 1: DPPH results of flowers and leaves EOs of *O. vulgare* ssp. *gracile*

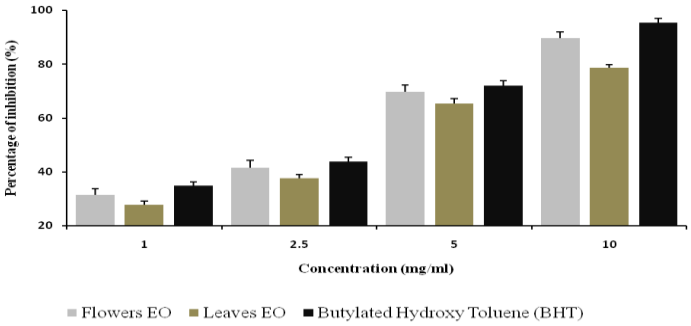

Fig. 2: ABTS results of flowers and leaves EOs of *O. vulgare* ssp. *gracile*

Discussion

In the present study, the disk diffusion results of flowers and leaves EOs against fungal strains showed a close agreement with MIC results and revealed that both EOs were more effective against *A. flavus* than *A. niger*. Also, the inhibitory effect of flowers EO was higher than leaves EO against tested fungal strains. Although this is the first study focusing on antifungal and antioxidant properties of *O. vulgare* ssp. *gracile* worldwide, but findings in this study can support reports of former researchers about antimicrobial potency of other subspecies of *O. vulgare* (Chun et al., 2005; Couto et al., 2015; Esen et al., 2007; Moradi et al., 2014; Viuda-Martos et al., 2010). Şahin et al. (2004) reported that 125 µg/ml of *O. vulgare* ssp. *vulgare* inhibited *A. flavus* growth using MIC agar dilution assay which was more sensitive than 15 other tested species. According to Adam et al. (1998), *O. vulgare* subsp. *hirtum* exhibited the highest antifungal properties among the four examined EOs. The high concentration of carvacrol may be responsible for considerable antifungal activities of the EOs in the current research, once other researchers have detected carvacrol and thymol as the major components caused antifungal activities of *O. vulgare* EO (Figiel et al., 2010; Mechergui et al., 2010; Teixeira et al., 2013; Vale-Silva et al., 2012). Moradi et al. (2014) reported that the main components of both leaves and flowers of *O. vulgare* ssp. *gracile* EOs were carvacrol (46.5% as well as 60.6%),

γ-terpinene (13.91% and 16.64%), α-terpinene (1.23 and 2.39), and ρ-cymene (13.54% and 7.21%), respectively. In comparison, probably high concentrations of carvacrol, γ-terpinene and α-terpinene (Moradi et al., 2014) are responsible for higher inhibitory effect of flowers than leaves of *O. vulgare* against tested fungal strains. Several studies have indicated that the chemical compositions of the EOs extracted from various parts of plants are different (Arcila-Lozano et al., 2004; Entezari et al., 2009; Kovacevic et al., 2007; Proestos et al., 2005). For examples, Satou et al. (2011) showed that predominant component in the EO obtained from the leaves of *Abies firma*, a native Japanese herb belonging to Pinaceae family, is α-pinene, while α-pinene was present in higher amounts in the EOs of *A. veitchii* (leaves and shoots), *A. sachalinensis* (shoots) and *A. mariesii* (leaves and shoots). Also, Santos et al. (2013) found that the important compounds of the flowers EO of *Aloysia gratissima* were germacrene B, E-caryophyllene, bulnesol as well as guaiol, while trans-pinocamphone, trans-pinocarveyl, acetate, and guaiol were the main compounds in the leaves.

In the present study, we found that *gracile* flowers and leaves EOs followed a dose dependent pattern for scavenging of DPPH radicals and also ABTS assay. Although both EOs had a notable antifungal abilities, especially in higher concentrations, but it was lower than BHT. Authors did not find any previous study about antioxidant activity of *O. vulgare* ssp. *gracile* in the literatures, but findings of this study are in agreement with the results of previous studies that reported remarkable antioxidant activities of other *Origanum* species. Şahin et al. (2004) found that thymol and carvacrol are the main effective ingredients in methanolic extract *O. vulgare* ssp. *vulgare* and behaved as a strong free radical scavenger. Vazirian et al. (2015) demonstrated that the EO of *O. vulgare* ssp. *vulgare* showed stronger antioxidant activity (IC_{50}=2.5 µg/ml in DPPH method) than the standard antioxidants (BHT).

Conclusion

In this study, considerable antioxidant and antifungal properties of the EOs extracted from flowers and leaves of a unique species of *Oregano* (*O. vulgare* ssp. *gracile*) were revealed for the first time. Although, antioxidant and antifungal efficacy of flowers EO was significantly higher than the leaves EO, both leaves and flowers EOs could be recommended as an antifungal preservative in food industries. Additional studies are needed to investigate the efficacy of *O. vulgare* ssp. *gracile* EOs or their components individually in food models to determine the mode of action as well as their application in hurdle systems due to their antioxidant and antifungal potency.

Conflicts of interest

The authors declare no conflict of interest.

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