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RESEARCH ARTICLE

Chemical composition and antioxidant activity of Tunisian *Origanum glandulosum* Desf. essential oil and volatile oil obtain by supercritical CO_2 extraction

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Abstract

Dried flowers and leaves of *Origanum glandulosum* Desf. were submitted to hydrodistillation (HD) and supercritical fluid extraction with CO_2 (SFE). The essential oils isolated by HD and volatile oils obtained by SFE were analysed by GC and GC/MS. Total phenolics content and antioxidant effectiveness were performed. The main components of the essential oils from Bargou and Nefza were: *p*-cymene (40.4% and 39%), thymol (38.7% and 34.4%) and γ-terpinene (12.3% and 19.2%), respectively. The major components obtain by SFE in the volatile oil, from Bargou and Nefza, were: *p*-cymene (32.3% and 36.2%), thymol (41% and 40%) and γ-terpinene (20.3% and 13.3%). Total phenolic content, expressed in gallic acid equivalent (GAE) g kg^{-1} dry weight, varied from 12 to 27 g kg^{-1} dw, and the ability to scavenge the DPPH radicals, expressed by IC_{50} ranged from 44 to143 mg L^{-1}.

Introduction

Volatile oils are used in the pharmaceutical, food and fragrance industries. Several procedures of extraction of volatile oils are used, steam distillation, solvent extraction, supercritical fluid extraction (SFE) and hydrodistillation. The latter is a traditional technique and it is usually applied. However, it has some disadvantages like the chemical alteration of essential oils and the heat sensitive compounds can easily destroy. For consequent, the quality of the essential oils extracted is extremely impaired (Illes et al., 2000). It is important that the natural proportion of the components is maintained during extraction of the volatile oils from plants (Anistese et al., 1997). Today, SFE become an acceptable technique applied in extraction of natural products. The use of supercritical fluids, especially carbon dioxide in extraction of volatile oils of plant has increased during two last decades due to the expected advantages of the supercritical extraction process (Pourmortazavi and Hajinirsadeghi, 2007). In fact, SFE can be used at low temperature. Therefore, it can be conserved the thermally labile compounds (Bruno et al., 1993; Dron et al., 1997). The CO_2 remains the most commonly used fluid for SFE application because of its critical constants (T_c =31.1°C and P_c = 72.8 atm), low cost, non toxic and non flammable properties and it can be recycled or reused thus minimizes waste generation (Bruno et al., 1993; Lang and Wai, 2001).

Origanum glandulosum Desf. has to be considered synonymous with *O. vulgare* L. subsp. *glandulosum* (Ietswaart, 1980). It is endemic plant from Algerian and Tunisian species (Alapetite, 1981). Some of the principal's applications are against whooping cough, cough, fever, bronchitis as well as to relieve rheumatic pains (Ruberto et al., 2002; Belhattab et al., 2005). Moreover, *Origanum glandulosum* Desf. has antioxidant (Ruberto et al., 2002; Mechergui et al., 2010), antifungal (Belhattab et al., 2005), antimicrobial (Bendahou et al., 2008) and insecticidal activities (Khalfi et al., 2008)

and at long last as spices (Kokkni, 1997).

The aims of our study were: (i) to determine the chemical composition of volatile oils obtained from wild Tunisian species of oregano dried leaves and flowers collected from two different regions: Nefza and Bargou extracted by hydrodistillation (HD) and supercritical fluid extraction (SFE); (ii) to investigate the antioxidant properties of volatile oils using the DPPH free radical method and total phenols assay; (iii) to conclude the influence of extraction processes of the antioxidant capacity.

Materials and methods

Plant material

Two samples of *O. glandulosum*, growing wild, were collected during the flowering season, from Nefza (389 m, 36°879' N, 009°098' E) and Bargou (681 m, 36°118' N, 009°518' E). The flowers and leaves were air dried at room temperature, in a shady place and protected from direct light. Then, flowers and leaves are crushed with liquid N_2 to avoid the loss and thermal degradation of the volatile oil and kept at -20°C in dark bottle until used. The Laboratory of Botany, National Institute of Agriculture of Tunisia, confirmed the species identification and a voucher specimen of the seeds of these wild populations was deposited in the Tunisian National Bank of Genes at numbers 23211 and 23212 from Nefza and Bargou respectively.

Extraction of the essential oils and volatile oils obtained by supercritical CO_2 extraction

Some quantity (about 30 g) of powdered dry flowers and leaves of each population was submitted to hydrodistillation for 4 h using a Clevenger-type apparatus according to the method given in the European Pharmacopoeia (Council of Europe, 2007).

Another quantity (60 g) was subjected to supercritical CO_2 extraction. Supercritical CO_2 extractions were carried out with the flow apparatus previous describe in the work of Reis-Vasco et al., (1999). Each experiment was carried out with oregano milled dry flowers and leaves at a supercritical CO_2 flow rate of 1.32 kg h^{-1} CO_2 for 150 min. The CO_2 (99.995% purity) used in the studies was supplied by Air Liquide (Portugal). The total volume of the extraction vessel was filled with plant material in the middle and glass beads and filters at the top and the base. Extractions were carried out at temperature and pressure of 40 °C and 90 bar, respectively. The experimental conditions tested are obtained from the previous experience in the field of SFE, being the best conditions of pressure in the range of 90-100 bar and temperature from 40 to 50 °C (Reis-Vasco et al., 1999; Grosso et al., 2009). For the fractionation steps, pressures are 80 bar and 20 bar for the first and the second separators, respectively and the temperature is -15 °C for the both. The quantity of oil collected in the first separator was determined by weight.

The essential oils and volatile oils obtained by hydrodistillation and supercritical CO_2 extraction, respectively, were stored after their collecting at +4 °C until use.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Gas chromatographic analyses were performed using a Perkin Elmer Autosystem XL gas chromatograph (Perkin Elmer, Shelton, CT, USA) equipped with flame ionisation detector (FID), a data handling system and a vaporising injector port into a column, DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific Inc., Rancho Cordova, CA, USA). The oven temperature was programmed at 45-175 °C, at 3 °C min^{-1}, then subsequently at 15 °C min^{-1} up to 300 °C, and then held isothermal for 10 min; injector and detector temperatures were 280 °C and 300 °C, respectively; the carrier gas, hydrogen, was adjusted to a linear velocity of 30 cm s^{-1}. The samples were injected using the split sampling technique, ratio 1:50. The volume of injection was 0.1 µL of a pentane-oil solution. The percentage composition of the oils was computed by the normalisation method from the GC peak areas, without using correction factors.

The GC-MS unit consisted of a Carlo Erba 6000 Vega gas chromatograph, equipped with a DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 µm; J&W Scientific Inc.), interfaced with a Finnigan MAT 800 Ion Trap Detector (ITD; software version 4.1). The oven temperature was programmed to 45-175 °C, at 3 °C min^{-1}, subsequently at 15 °C min^{-1} up to 300 °C, and then held isothermal for 10 min. The temperature conditions of the transfer line and ion trap were 280 °C and 220 °C, respectively. The linear velocity of the carrier gas, helium, was adjusted to 30 cm s^{-1} and the split ratio to 1:40. Furthermore, the ionisation energy was 70 eV, the ionisation current, 60 µA, the scan range, 40-300 u and the scan time, 1 s.

The identity of the components was assigned by comparison of their retention indices, relative to C_9-C_{16} n-alkanes, with GC-MS corresponding data of reference oils components, laboratory-synthesised components and commercially available standards from a home-made library, constructed based on the analyses of reference oils, laboratory-synthesised components and commercial available standards.

Free radical scavenging ability by the use of a stable 2,2-Diphenyl-1-picrylhydrazil (DPPH) radical

The antioxidant activities were determined using DPPH as a free radical, according to a modified version of method described by Brand-Williams et al., (1995). The inhibition of DPPH radicals, corresponding to the scavenging capacity

of each tested sample, was evaluated using four different concentrations of each volatile oil. The colorimetric of the solution changes, from deep-violet to light-yellow when $DPPH^{\bullet}$ is reduced. The decrease in absorbance was determined at 517 nm on an ATI Unicam UV- visible spectrophotometer at 0 min and every 5 min until the reaction reached a plateau (90 min). The Percentage of discoloration at 517 nm, corresponding to the inhibition of the DPPH radicals, was calculated according to the following equation:

$$\% \text{ inhibition} = [(A_0 - A_f)/A_0] \times 100$$

where A_0 and A_f represent the absorbance of the reaction mixtures measured at t = 0 and after 90 min, respectively. Furthermore, using equation of a calibration curve correlating the $DPPH^{\bullet}$ absorption with concentration, the percentage of inhibition for this $DPPH^{\bullet}$ solution, which is proportional to power scavenging of the sample, was calculated. The results were corrected for dilution. In order to evaluate the sensitivity of the DPPH assay, different concentrations of ascorbic acid (Panreac; Barcelone, Spain), standard, were used.

Total phenols assay

The total phenols in the volatile oils were determined by the Folin-Ciocalteu method described by Dorman et al. with some modifications (Dorman et al., 2003). Quantification was done with respect to the standard curve of gallic acid. The results were expressed as gallic acid equivalent (GAE) g kg^{-1} dry weight (dw). Each assay was performed five times (n = 5).

Results and discussion

The results of extraction yields using supercritical fluid extraction (SFE) and hydrodistillation (HD) in percentage of dry weight, the extraction time (min) and the colour of oils are presented in Table 1. The SFE oils showed an orange coloration being the odour of the volatile oils considered not distinguishable from that of the starting oregano flowers and leaves. Whereas, the essential oils obtained by hydrodistillation showed a deep yellow coloration and a sensible odour difference compared to the starting material. The aroma of SFE oils was more intense and more pleasant.

The hydrodistillation, presents superior yield: 4.3% and 3.5% (v/w) for the both populations Bargou and Nefza essential oils respectively, compared to the supercritical CO_2 extraction (2.2% and 2.1%). This report in the oil yields was also observed in *O. glandulosum* from Algeria (4.5% and 2.74% for HD and SFE, respectively) (Bendahou et al., 2007).

Composition of the essential oils and SFE volatile oils of flowers and leaves of *Origanum glandulosum* Desf. are reported in Table 2. The analysis allowed identifying 39 compounds consisting 100% of Bargou SFE oil and 99.5% of Nefza SFE oil. To essential oils 38 compounds were identified, representing 99.8% and 100% of the Bargou and Nefza samples, respectively.

The main compounds of SFE oils of Bargou and Nefza samples were thymol (41% and 40%, respectively), followed by *p*-cymene (32.3% and 36.2%), γ-terpinene (20.3% and 13.3%) and carvacrol (2.3% and 2.2%). Despite the hydrodistillation, the *p*-cymene (40.4% and 39%), thymol (38.7% and 34.4%), γ-terpinene (12.3% and 19.2%) and carvacrol (2.6% for the both) are the major components in *O. glandulosum* essential oils from Bargou and Nefza respectively.

Melegari et al., (1995), Ruberto et al., (2002), Belhattab et al., (2005), Hazzit et al.,(2006), Sari et al., (2006), Sahraoui et al., (2007), Bendahou et al.,(2008), and Berrehal et al., (2010), have reported the chemical composition of essential oils of *O. glandulosum* collected from different regions of Algeria. The main components were thymol and/or carvacrol, *p*-cymene and γ-terpinene representing more than 90 % of the total composition of the oils. Moreover, Bendahou et al., (2007) have reported the chemical composition of SFE oils of *O. glandulosum* from Algeria. They showed that thymol, *p*-cymene, γ-terpinene and carvacrol are the main compounds in those oils.

Essential oils and SFE oils possessed a very similar composition in all samples. But, the Nefza SFE sample has a compound, trans-α-Bergamotene at 1.3%. This compound may be was decomposed by hydrodistillation and it existed in trace. Moreover, SFE samples have the compound thymoquinone in trace which can't be found in hydrodistillation samples.

Aghel et al., (2002) have mentioned the similitude in composition between SFE and hydrodistillation oils from *Mentha Piperita* L. cultivated in Iran. Khajeh et al., (2005) showed that compositions of the oils obtained by SFE and hydrodistillation from *Ferula assa-foetida* are not qualitatively different, like our authentication in this study. Nevertheless, Bendahou et al., (2007) showed that the *O. glandulosum* essential oils obtained by hydrodistillation contain more constituents than those by SFE at 15°C, 66.7 bar and 1.23 g CO_2 min^{-1} of temperature, pressure and flow of CO_2 respectively, and the oxygenated monoteropenes were higher in all oils compared to the monoterpenes hydrocarbons. However, in this study, *O. glandulosum* oils are characterized by dominance of monoterpenes hydrocarbons (54.5 - 61.9%) compared to the oxygenated monoterpenes (37.0 - 43.3%) and there is a similitude in composition between SFE and hydrodistillation.

On the basis of the fact that the phenolic compounds and terpenes are known for their properties to trap the free radicals (Yanishlieva et al., 1991; Jukie et al., 1991) and considering the chemical composition of volatile and essential oils of oregano are rich in polyphenols, specially in thymol, the characterisation of the samples oils were performed.

DPPH radical scavenging capacity (IC_{50}) and total phenolics content (g GAE kg^{-1} dw) from *O. glandulosum* essential

oils (HD) and SFE oils of Nefza and Bargou populations, are summarised in Table 3.

The amount of total phenolics, measured by Folin-Ciocalteu method ranged from 12.38 ± 0.78 to 27.79 ± 0.63 g GAE/kg dry weight (dw) (Table 3). All samples have very high levels of phenolics. While, the highest level was found in essential oils of crushed vegetable plants and it is higher in Bargou sample (27.79 ± 0.63 g GAE/kg dw) than Nefza (22.65 ± 0.9 g GAE/kg dw).

The antioxidant activity of *Origanum glandulosum* volatile oils is low compared to reference compound, ascorbic acid (IC_{50} = 5 mg/L). The volatile oils extracted by supercritical fluid extraction presented the highest scavenging activity (Table 3). Indeed, the IC_{50} of SFE oils of Bargou and Nefza samples are 44.02 and 60.31 mg/L, respectively.

Table 1. Origins, methods of extraction, extraction time, yields (average ± standard deviation), and colours of volatile oils of *Origanum glandulosum* Desf.

Locality of collection	Method of extraction	Extraction time (min)	Oil yield (v/ w %)	Colour of oils
Bargou	hydrodistillation	240	4. 3 ± 0.2	Yellow
Bargou	SFE	150	2.2 ± 0.1	Orange
Nefza	hydrodistillation	240	3.5 ± 0.2	Yellow
Nefza	SFE	150	2.1 ± 0.1	Orange

Table 2. Composition of the essential oils and SFE oils of flowers and leaves of *Origanum glandulosum* Desf.

Components	RI	Bg_SFE	Nf_SFE	Nf_HD	Bg_HD
α-Thujene	924	0.5	0.7	0.9	0.8
α-Pinene	930	0.9	1.0	1.1	1.3
Camphene	938	t	0.1	0.1	0.1
1-Octen-3-ol	961	0.1	0.1	0.1	0.1
β-Pinene	963	0.1	0.1	0.1	0.1
β-Myrcene	975	t	0.8	0.1	0.7
α-Phellandrene	995	t	0.1	1	0.1
δ-3-Carene	1000	t	t	t	0.1
α-Terpinene	1002	1.2	1.6	1.3	1.1
p-cymene	1003	32.3	36.2	39.0	40.4
α-Phellandrene	1005	0.1	0.2	0.1	0.1
Limonene	1009	0.1	0.3	t	0.3
cis-β-Ocimene	1017	t	t	t	t
trans-β-Ocimene	1027	t	t	t	t
γ-Terpinene	1035	20.3	13.3	19.2	12.3
n-Octanol	1045	t	02	t	t
p -cymenene	1050	t	t	t	t
Terpinolene	1064	t	0.1	t	t
Linalool	1074	t	0.2	t	t
Borneol	1134	t	t	t	t
Terpinen-4-ol	1148	t	t	t	0.1
n-Nonanol	1151	t	t	t	t
α-Terpineol	1159	t	t	t	t
Thymoquinone	1210	t	t		
Carvacrol, methyl ether	1224	t	t	t	t
Thymol formate	1271	t	t	t	t
Thymol	1275	41.0	40.0	34.4	38.7
Carvacrol	1286	2.3	2.2	2.6	2.6
trans-β-Caryophyllene	1414	0.8	0.5	0.7	0.4
trans-α-Bergamotene	1434	t	1.3	t	t
α-Humulene	1447	t	t	t	t
trans-β-Farnesene	1455	t	t	t	t
ar-Curcumene	1475	t	t	t	t
β-Selinene	1476	t	t	t	t
trans-α-Zingiberene	1492	t	t	t	t
β-Bisabolene	1500	t	t	t	t
α-Sesquiphellandrene	1508	0.1	0.3	t	0.3

3-cis-Hexenyl benzoate	1533	t	t	t	t
β-Caryophyllene oxide	1561	0.2	0.2	0.3	0.2
% Identification		**100.0**	**99.5**	**100.0**	**99.8**
Grouped Components					
Monoterpene hydrocarbons		55.5	54.5	61.9	57.4
Oxygen-containing monoterpenes		43.3	42.4	37.0	41.4
Sesquiterpene hydrocarbons		0.9	2.1	0.7	0.7
Oxygen-containing sesquiterpenes		0.2	0.2	0.3	0.2
Others		0.1	0.3	0.1	0.1

RI = Retention index relative to C8-C16 n - alkanes on the DB-1 column, t = trace (< 0.05 %).
Bg_SFE: oil from Bargou obtained by SFE
Nf_SFE: oil from Nefza obtained by SFE
Nf_HD: oil Nefza from hydrodistillation
Bg_HD: oil Bargou from hydrodistillation

Table 3. DPPH radical scavenging capacity (IC_{50}) and total phenolics content (g GAE kg^{-1}dw) (average ± standard deviation) from *O. glandulosum* essential oils (HD) and SFE oils of Nefza and Bargou populations.

Collection site of *O. glandulosum* plants	**IC_{50} (mg L^{-1})**	**Total phenols (g GAE kg^{-1} dw)**
Bg SFE	44.02 ± 1.13	13.98 ± 0. 48
Bg HD	105. 29 ± 2.12	27.79 ± 0. 63
Nf SFE	60.31 ± 1.59	12.38 ± 0. 78
Nf HD	142. 86 ± 2.14	22.65 ± 0. 90

Conclusion

The supercritical fluid extraction of *Origanum glandulosum* Desf. was studied and the results were compared with essential oil composition obtained by hydrodistillation. The chemical composition data reveals that the volatile oils obtained using the two methods contain the same main components in both populations, Nefza and Bargou. However, their concentrations are different. The SFE method gives a better selectivity for the compound of interest (thymol) and offers many important advantages over hydrodistillation. Extraction time of SFE was lower that needed by hydrodistillation. Also, SFE is considered as the suitable process for obtaining *O. glandulosum* oil with high quality without heating in comparison by the hydrodistillation. All studied oregano samples have very high levels of phenolics compounds and they have a good DPPH radical scavenging capacity. Therefore, they have relatively high antioxidant activities. The phenolic compounds were major contributors to antioxidant activity and also that they contribute significantly to the total antioxidant capacity.

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