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GC/MS ANALYSES FOR DETECTION AND IDETIFICATION OF ANTIOXIDANT CONSTITUENTS OF *ACHILLEA MILLEFOLIUM* L. ESSENTIAL OIL

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Abstract

The chemical composition, antioxidant properties of *Achillea millefolium* essential oil and its main compounds are reported. The essential oil was obtained from the aerial parts of the plant by hydrodistillation and analysed by GC/MS, with its contribution to the oil 20.17%. The *A. millefolium* oil showed the high radical scavenging activity (IC_{50}: 22.11 ± 0.06 mg/ml) and main antioxidant compound which were identified as thymol (61%) and carvacrol (23%). These results clearly show the antioxidant effects of the plant essential oil.

Introduction

Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods by free radicals. Antioxidants are known as molecules capable of inhibiting oxidation process in body so preventing of forming free radicals. Phenolic compounds may serve this purpose by reducing or donating hydrogen to other compounds, scavenging free radicals, and quenching singlet oxygen. In the search for sources of natural products, in the last years some medicinal plants have been extensively studied for their biological properties (Desmarchelier *et al.* 2000, Schinella *et al.* 2002, VanderJagt *et al.* 2002). Natural products with their diverse biological and pharmacological activities represent a gold mine for scientists searching for lead compounds for the treatment of health disorders and antioxidant activity. Numerous studies exhibited a strong relationship between total phenolic content and antioxidant activity in fruits, vegetables, and medicinal plants. Aromatic plants are potential natural sources of novel antibiotics and particular interest has focused on their essential oils as main sources of potent antimicrobial and antifungal compounds classified as terpenoids, flavonoids and phenolics (Sengul *et al.* 2009). Aromatic and medicinal plants are known to produce certain bioactive molecules which react with other organisms in the environment, inhibiting bacterial or fungal growth (Webster *et al.* 2008). The present study was performed to investigate components of essential oils *A.millefolium* and to evaluate the efficacy of chemically characterized *A. millefolium* essential oil as antioxidant agent.

Materials and Methods

The aerial parts of growing *A. millefolium* were collected during the growing period in 2013-2014 from the regions of Iran central. The plant was identified by Mr. Esmaili, and the voucher specimen was deposited at private herbarium of Dr. F. Esmaili (voucher no. 22). The *A. millefolium* aerial parts were ground and the resulting powder was subjected to hydrodistillation for 3 hrs in an all glass Clevenger-type apparatus according to the method recommended by the European Pharmacopoeia (1975). The obtained essential oils were dried over anhydrous sodium

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sulphate and after filtration, stored at + 4ºC until tested and analysed. The GC/MS analyses were executed on a Hewlett-Packard 5973N gas chromatograph equipped with a column HP-5MS (30 m length × 0.25 mm i.d., film thickness 0.25 lm) coupled with a Hewlett-Packard 5973N mass spectrometer. The column temperature was programmed at 50ºC as an initial temperature, holding for 6 min, with 3ºC increases per minute to the temperature of 240ºC, followed by a temperature enhancement of 15ºC per minute up to 300ºC, holding at the mentioned temperature for 3 min. Injector port temperature was 290ºC and helium used as carrier gas at a flow rate 1.5 ml/min. Ionization voltage of mass spectrometer in the EI-mode was equal to 70 eV and ionization source temperature was 250ºC. Linear retention indices for all components were determined by coinjection of the samples with a solution containing homologous series of C_8-C_{22} *n*-alkanes and comparing them and their mass spectra with those of authentic samples or with available library data of the GC/MS system (WILEY 2001 data software) and Adams libraries spectra (2007). The scavenging activity was estimated using the following equation: Scavenging effect (%) = [100× (Ac - AS/Ac)], where Ac is the absorbance of the control reaction (containing all reagents except the test sample) and AS is the absorbance of the tested sample (Hanato *et al.* 1988). The β-CLAMS method by the peroxides generated during the oxidation of linoleic acid at elevated temperature (Wettasinghe and Shahidi 1999). The antioxidant activity (AA) of the extracts was evaluated in term of β-carotene blanching using the following formula: AA (%) = [(A_0 - A_1)/A_0]*100. where A_0 is the absorbance of the control at 0 min, and A_1 is the absorbance of the sample at 120 min. The results are expressed as IC50 values (l g/ml). All samples were prepared and analyzed in triplicate. For screening of antioxidant compounds in *A. millefolium* essential oil, the TLC-bioautography method was carried out (Burits and Bucar 2000, Guleria *et al.* 2002). The diluted oil (1 : 20 in methanol) was spotted on silica gel sheets (silica gel 60 F254 TLC plates) and developed in *n*-hexane-ethyl acetate (9 : 1). Plates were sprayed with the methanolic solution of DPPH (0.2%). The active constituents were detected as yellow spots on a violet background. Only zones where their color turned from violet to yellow within the first 30 min (after spraying) were taken as positive results. For the isolation and identification of the active compounds in the essential oil, PTLC was performed using the conditions previously described (Guleria *et al.* 2002). The regions showing DPPH scavenging activity were scrapped off then, they were eluted with chloroform. All resulting constituents were analyzed by GC/MS and also tested for their antioxidant activities. All the experiments were performed in triplicate, being the results expressed as mean ± SEM of three independent experiments. The means were statistically compared using two-way ANOVA, with a Dunnett's multiple comparison test. The differences between the means were considered significant for values of $p < 0.001$.

Results and Discussion

The results obtained by GC/MS analysis of the essential oils of *Achillea millefolium* are shown in Table 1. GC/MS analysis resulted in the identification of 24 from *Achillea millefolium*. Despite, monoterpenes were dominant in oils, the abundance of the oxygen-containing monoterpenes or the monotrepene hydrocarbons varied. The major constituents of the EO from the aerial parts of *Achillea millefolium* were α-pinene (10.12%), camphene (4.23%), limonene (5%), borneol (5%), γ-terpinene (8%), carvone (5%), bornyl acetate (2.43%), thymol (15.32%) and carvacrol (20.43%). Carvacrol and thymol were the main component of the essential oils. These results are in agreement with previous data for the same species (Rustaiyan *et al.* 1999). Javidnia *et al.* (2004) reported carvacrol (25.1%), linalool (11.0%), 1, 8-cineol (10.3%), enerolidol (9.0%) and borneol (6.4%) as the main constituent of the *Achillea tenuifolia* essential oil. A previous report by Afsharypour *et al.* (1996) indicated the major constituent of the essential oil of *Achillea tenuifolia* was caryophyllene oxide and in other studies, borneol was the second most abundant

constituent of oil (Aghajani *et al.* 2000). Similar to previous studies (Esmaeili *et al.* 2005), 1, 8-cineole was found to be the major constituent of the oil *Achillea tenuifolia*, while others reported camphor as the major constituent of this oil (Kundakovic *et al.* 2007). It has been reported that the chemical compositions of the essential oil are highly influenced by climatic conditions and geographical factors (Burt 2004). The antioxidant activity of the essential oil was determined by

Table 1. Chemical composition of *Achillea millefolium* volatile oil constituents.

Compound	%	RI	Compound	%	RI
α-pinene	6.96	932	Carvacrol	19.05	1308
Camphene	3.65	950	β-caryophyllene	0.65	1418
Sabinene	7.09	975	γ- elemene	0.59	1430
α- phellandrene	2.12	1000	γ- muurolene	0.65	1476
Limonene	8.07	1020	β-selinene	0.43	1490
trans- limonene oxide	0.76	1140	β- guaiene	0.74	1496
Borneol	2.06	1160	α- selinene	1.00	1490
γ-terpinene	5.06	1195	calarene	0.67	1500
Carone	1.67	1200	Calacorene	0.75	1529
Carvone	2.76	1240	Ccaryophyllene oxide	0.43	1579
Bornyl acetate	0.65	1281			
Thymol	26.17	1300			
Total	91.98				

Table 2 Antioxidant potential of *Achillea millefolium* L. essential oils.

	A. millefolium	Trolox
DPPH scavenging activity (IC_{50} mg/ml)	24.01 ± 0.01^{b}	26.02 ± 0.04^{a}
β-carotene bleaching inhibition (IC_{50} (µl/ml)	2.01 ± 0.05^{b}	3.00 ± 0.0^{a}

Values are the mean of three replication ± Sd. Mean separation among treatments was done by DMRT at $p \leq 0.01$. Mean values followed by different letters are significantly different.

Table 3. Components identified and their antioxidant activity relative percentage constituents.

Compounds	%
Other component	16
Thymol	61
Carvacrol	23

the DPPH test system. Table 2 shows DPPH scavenging activity, expressed in IC50 (µg/ml), caused by of essential oil *Achillea millefolium* and its main constituents. The weakest radical scavenging activity (25 ± 0.1 µg/ml) was exhibited by the bornyl acetate, whereas the strongest activity (12.0 ± 0.1 µg/ml and) was exhibited by the thymol. The next highest activity (13.43 ± 0.0

µg/ml) was for the carvacrol. In this system, the carvone (17 ± 0.8 µg/ml), showed activity as strong as the synthetic antioxidant, and α-pinene (20 ± 0.1 µg/ml), limonene (20 ± 0.3 µg/ml) and camphene (20.01 ± 0.3 µg/ml) exhibited similar activity to *Achillea millefolium* essential oil (20.06 ± 0.0 µg/ml). Because of high antioxidant and free radical scavenging activities of *Achillea millefolium* essential oil, it was further investigated to identify its active constituents. Therefore, a preliminary screening was initially carried out using the dot-blot DPPH staining method on TLC. As the essential oil presented a significant antioxidant activity in the assays and bioautography test, it was subjected to the PTLC for isolation of the active compounds. Components identified and their antioxidant activity relative percentages have been showed in Table 3. The major compound found in the active band were thymol (85%) and carvacrol (25%). The antioxidant activities of these volatiles in cellular assays have not been previously reported and the results could be explained by the fact that in vitro tests do not take the physiological conditions of the cell, bioavailability of the antioxidant molecule, as well as general cellular metabolism into account. Carvacrol and thymol have been reported to contribute to the in vitro antioxidant activity of essential oil (Piccaglia *et al.* 1993, Burits and Bucar 2000). The antioxidant activity may be due to different mechanisms, such as prevention of chain initiation, decomposition of peroxides, prevention of continued hydrogen abstraction, free radical scavenging, reducing capacity, and binding of transition metal ion catalysts (Mao *et al.* 2006).

The essential oil of *Achillea millefolium* revealed antioxidant effects and these results support the traditional use of this plant in antimicrobial activity, relieving pain and inflammation.

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