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Chemical Composition and Biological Activity of the Volatile Extracts of *Achillea millefolium*

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In this study, flowering aerial parts of wild *Achillea millefolium* growing on the Mediterranean coast (Sardinia Island, Italy) and on the Atlantic coast (Portugal- Serra de Montemuro) were used as a matrix for supercritical extraction of volatile oil with CO_2 (SFE). The collected extracts were analyzed by GC-FID and GC-MS methods and their composition were compared with that of the essential oil isolated by hydrodistillation. A strong chemical variability in essential oils depending on the origin of the samples was observed. The results showed the presence of two type oils. The Italian volatile extracts (SFE and essential oil) are predominantly composed by α-asarone (25.6-33.3%, in the SFE extract and in the HD oil, respectively), β-bisabolene (27.3-16.6%) and α-pinene (10.0-17.0%); whereas the main components of the Portuguese extracts are *trans*-thujone (31.4-29.0%), *trans*-crhysanthenyl acetate (19.8-15.8%) and β-pinene (1.2-11.1%).
The minimal inhibitory concentration (MIC) and the minimal lethal concentration (MLC) were used to evaluate the antifungal activity of the oils against *Candida albicans*, *C. tropicalis*, *C. krusei*, *C. guillermondii*, *C. parapsilosis*, *Cryptococcus neoformans*, *Trichophyton rubrum, T. mentagrophytes*, *T. mentagrophytes* var. *interdigitale, T. verrucosum, Microsporum canis*, *M. gypseum*, *Epidermophyton floccosum*, *Aspergillus niger*, *A. fumigatus* and *A. flavus*. The oils showed the highest activity against dermatophyte strains, with MIC values ranging from 0.32-1.25 μL mL^{-1}.

Keywords: *Achillea millefolium* L., essential oil, supercritical CO_2, antifungal activity.

The genus *Achillea* (Asteraceae) consists of about 85 species found in the Northern hemisphere, mostly in Europe and Asia [1]. Generally yarrow species are used against digestive problems, liver and gallbladder conditions, menstrual irregularities, cramps, inflammation, fever; they have wound healing effect and increase urine flow [2].

Achillea is composed by a group of hardly distinguishable species and subspecies, and their essential oil composition can be used as an additional characteristic for their differentiation [3]. There is increasing evidence that intraspecific chemical diversity must be widespread and common in this genus [4-6]. Some studies proved that also differences even at the level of plant individuals inside a population may be significant [7-9].

Achillea millefolium L., a medicinal plant commonly known as yarrow, milfoil, nosebleed, thousand leaf, knights' milfoil, soldier's woundwort or military herb [10-12], is a very polymorphic species with white, yellowish or pink flowers [13]. The relative amounts of α- and β-pinene, sabinene, 1,8-cineole, borneol, caryophyllene, camphor and chamazulene in the oil may serve as distinguishing criteria [14,15]. The intraspecific variation is so high that in some cases chamazulene content may vary from 0 to 85% [8]. However, it could be related to misidentification of *A. millefolium* due to its difficult taxonomy.

The qualitative and quantitative composition of the essential oil is significantly influenced by several biotical and abiotical factors. During ontogenesis, major differences could be found before and after flower development. Compositions of essential oils distilled from different plant organs show characteristic variations: in several cases a proportional dominance of sesquiterpene components above the monoterpenes was found in the vegetative organs [2]. The content and composition of essential oils depends on many factors such as growing place, stage of development, etc. [16].

The chemical composition of *Achillea millefolium* L. essential oils was investigated in many countries: Hungary [8], Greece [17], Portugal [18], Iran [19, 20], Cuba [21], Serbia [22], Estonia [15], Norway [14], India [23, 24] and

Lithuania [25-27]. In most cases, essential oil composition was compared in flowers and herb, however, only a few articles addressed the significance of flower colour in essential oil composition [26, 27]. The aim of the present work was to characterize the volatile oils extract by SFE and hydrodistillation of aerial parts of *Achillea millefolium* L. growing in Sardinia Island and in Portugal, as well as their antifungal activity.

The study was carried out on samples harvested from different locations in Sardinia and in Portugal. Volatile oil of a pale yellow colour was recovered in the separator that worked at 15 bar and 10°C. *A. millefolium* essential oils yields were 0.7-0.9%, respectively for Portuguese and Italian plants. SFE extracts yields 0.7 %. The results concerning the qualitative and quantitative analysis of the volatile oils extract by SFE and hydrodistillation are presented in Table 1. The product of the supercritical CO_2 extraction seems to be qualitatively comparable with the traditional hydrodistillation; however, the proportion of the components is regularly different.

The volatile extracts obtained from the Italian *Achillea millefolium* were characterized by high content of α-asarone (25.6-33.3%, in the SFE extract and in the HD oil, respectively), β-bisabolene (27.3-16.6%) and α-pinene (10.0-17.0%). The chemical composition of Portuguese *Achillea millefolium* is quite different from those of the Italian one, *trans*-thujone (31.4-29.0%), *trans*-crhysanthenyl acetate (19.8-15.8%) and β-pinene (1.2-11.1%) were the main constituents in the extracts.

The results showed that the Sardinian and Portuguese *Achillea millefolium* belong to different chemotypes.

Comparing our results with *A. millefolium* oils earlier reported from other countries, it was evident that these oils are quite different from the others. Recently, Gudaityte *et al*. [27] investigated 14 natural habitats in Lithuania and according to the major components they found that the essential oils were distributed into six chemotypes: α-pinene β-pinene; β-caryophyllene 1,8-cineole α-phellandrene; β-pinene α-phellandrene chamazulene; β-myrcene; β-pinene camphor *trans*-nerolidol; β-pinene.

Agnihotri *et al*. [24] in plants collected in India identified only two chemotypes: borneol and 1,8-cineole. In addition, Figueiredo *et al*. [18], Orav *et al*. [15], and Rohloff *et al*. [14] reported β-caryophyllene, germacrene D, chamazulene, β-pinene, sabinene, and 1,8-cineole as main components of essential oils. It was also observed that sabinene dominated in Norwegian, β-pinene in Estonian, β-pinene and sabinene in Siberian. In Portugal, the single report of *A. millefolium* essential oil obtained from plants from the Botanical Garden of Lisbon points 1,8-cineole as the main compound [18]. However in the present work the essential oils obtained from wild plants indicate *trans*-thujone as the main compound.

Table 1: Retention index and chromatographic area percentages of compounds identified in *Achillea millefolium* essential oil extracted by SFE at 90 bar 40°C: Sadali (SFE-1); Serra de Montemuro (SFE-2); and by Hydrodistillation: Sadali (HD-1); Serra de Montemuro (HD-2).

RI	HD-1	SFE-1	HD-2	SFE-2	Compound
930	0.2	tr.	-	-	α-Thujene
938	17.2	10.0	2.9	tr.	α-Pinene
953	0.7	0.5	0.9	tr.	Camphene
976	3.9	2.5	5.0	2.1	Sabinene
980	2.1	1.2	11.1	1.2	β-Pinene
992	2.8	1.8	-	-	Myrcene
1019	0.4	-	0.4	-	α-Terpinene
1027	0.4	tr.	1.0	1.2	*o*-Cymene
1031	1.4	1.5	0.8	tr.	Limonene
1032	1.5	1.4	-	-	β-Phellandrene
1034	-	-	1.6	1.0	1,8-Cineole
1062	0.7	tr.	0.9	tr.	γ-Terpinene
1100	0.3	tr.	tr.	tr.	Linalool
1116	-	-	29.0	31.4	*trans*-Thujone
1123	-	-	0.5	tr.	*cis*-p-Menth-2-en1-ol
1127	-	-	tr.	1.0	Chrysanthenone
1147	-	-	9.7	3.3	Camphor
1167	-	-	1.6	tr.	Borneol
1179	1.4	tr.	1.5	tr.	Terpinen-4-ol
1191	tr.	-	0.5	tr.	α-Terpineol
1238	-	-	15.8	19.8	*trans*-Chysanthenyl acetate
1286	0.4	0.7	1.5	tr.	Bornyl acetate
1293	-	-	0.8	1.1	Thymol
1376	tr.	tr.	tr.	0.9	α-Copaene
1410	tr.	0.5	-	-	α-Cedrene
1419	0.3	1.1	2.6	5.7	β-Caryophyllene
1436	0.4	9.1	-	-	*trans*-α-Bergamotene
1454	0.9	1.0	tr.	tr.	α-Humulene
1459	0.4	0.8	0.6	2.0	*trans*-β-Farnesene
1474	1.6	4.4	-	-	10-epi-β-Acoradiene
1481	0.9	2.5	2.5	11.0	Germacrene D
1485	1.4	3.9	-	-	β-Selinene
1494	0.9	2.0	-	-	α-Selinene
1498	8.8	tr.	-	-	(*E*)-Methyl isoeugenol
1509	16.6	27.3	tr.	tr.	β-Bisabolene
1514	tr.	tr.	tr.	4.8	Cubebol
1523	tr.	tr.	1.1	2.3	δ-Cadinene
1565	-	-	0.7	2.6	*(E)*-Nerolidol
1575	tr.	tr.	tr.	0.8	Germacrene D-4-ol
1581	-	-	1.7	3.0	Caryophyllene oxide
1628	-	-	1.3	1.6	1-*epi*-Cubenol
1635	-	-	1.2	1.8	Caryophylla-4-(12),8(13)-dien-5-a-ol
1640	-	-	2.2	1.4	α-Muurolol
1681	33.3	25.6	-	-	α-Asarone

tr: traces

It is evident that there is great variation in the chemical composition and several chemotypes occur in nature. Consequently, further selection of yarrows is advised in order to obtain the most valuable breeds.

The minimal inhibitory concentration (MIC) and the minimal lethal concentration (MLC) were used to evaluate the antifungal activity of the oils against *Candida albicans*, *C. tropicalis*, *C. krusei*, *C. guillermondii*, *C. parapsilosis*, *Cryptococcus neoformans*, *Trichophyton rubrum*, *T. mentagrophytes*, *T. mentagrophytes var. interdigitale*, *T. verrucosum*, *Microsporum canis*, *M. gypseum*, *Epidermophyton floccosum*, *Aspergillus niger*, *A. fumigatus* and *A. flavus* (Table 2).

Table 2: Antifungal activity (MIC and MLC) of *Achillea millefolium* oil against *yeasts*, dermatophyte and *Aspergillus* strains.

Strains	Portugal		Italy		Fluconazole		Amphotericin B	
	MIC[(a)]	MLC[(a)]	MIC[(a)]	MLC[(a)]	MIC[(b)]	MLC[(b)]	MLC[(c)]	MLC[(c)]
Candida albicans ATCC 10231	2.5	2.5	2.5	2.5-5	1	>128	N.T	N.T
Candida tropicalis ATCC 13803	2.5	2.5	2.5	5	4	>128	N.T	N.T
Candida krusei H9	2.5	2.5	2.5	5	64	64-128	N.T	N.T
Candida guillermondii MAT23	1.25	1.25	1.25	2.5	8	8	N.T	N.T
Candida parapsilosis ATCC 90018	2.5	2.5	2.5	≥20	<1	<1	N.T	N.T
Cryptococcus neoformans CECT 1078	1.25	1.25	0.64	1.25	16	128	N.T	N.T
T. mentagrophytes FF7	0.64	0.64	0.32	0.64	16-32	32-64	N.T	N.T
Microsporum canis FF1	0.32	0.32	0.64	0.64-1.25	128	128	N.T	N.T
Trichophyton rubrum CECT 2794	0.32	0.64	0.32	1.25	16	64	N.T	N.T
M. gypseum CECT 2905	0.64	0.64	0.64	0.64-1.25	128	>128	N.T	N.T
Epidermophyton floccosum FF9	0.64	0.64	0.64	0.64	16	16	N.T	N.T
T. mentagrophytes var. *interdigitale* CECT 2958	0.64	1.25	0.64	1.25	128	≥128	N.T	N.T
T. verrucosum CECT 2992	1.25	1.25	0.64	1.25	>128	>128	N.T	N.T
Aspergillus niger ATCC16404	5	>20	1.25	>20	N.T	N.T	1-2	4
A. fumigatus ATCC 46645	2.5-5	>20	1.25	>20	N.T	N.T	2	4
A. flavus F44	10	>20	1.25	>20	N.T	N.T	2	8

[a] MIC and MLC were determined by a macrodilution method and expressed in µL/mL (V/V). [b] MIC and MLC were determined by a macrodilution method and expressed in µg/mL (W/V). [c] Not tested. Results were obtained from 3 independent experiments performed in duplicate

Antifungal activity of the essential oils of *A. millefolium* was signalized owing to data presented in Table 2. Activity was revealed against dermatophyte strains, with MIC values ranging from 0.32 to 1.25 $\mu L.mL^{-1}$. From our knowledge this is the first evaluation of antifungal activity of *A. millefolium* essential oils against the tested dermatophytes, yeasts and *Aspergillus* spp.

Experimental

Materials: Aerial parts of *Achillea millefolium* were collected from two different sites: Sadali (Sardinia, Italy) and Serra de Montemuro (Portugal). Vegetative material, air-dried at 40°C with forced ventilation for two days, was ground with a Malavasi mill (Bologna, Italy) taking care to avoid overheating. Vegetal material was subjected to two different extraction methodologies: hydrodistillation and supercritical fluid extraction.

Hydrodistillation: Hydrodistillation (HD) was performed for three hours in a circulatory Clevenger-type apparatus according to the procedure described in the European Pharmacopoeia [28].

SFE extraction: Supercritical CO_2 (purity 99% - Air Liquide Italia, Cagliari, Italy) extractions were performed in a laboratory apparatus equipped with a 400 cm^3 extraction vessel, E, operate in the single-pass mode by passing CO_2 through the fixed bed of charged vegetable particles. Waxes and essential oil were recovered in two separator vessels connected in series, S_1 and S_2, 300 and 200 cm^3, respectively. The cooling of the first separator was achieved by using a thermostated bath (Neslab, Model CC100II, accuracy of 0.1°C). The second separator allowed the discharge of the liquid product at desired time intervals. A high pressure diaphragm pump, P, (Lewa, Model EL 1) with a maximum capacity of 6 kg/h, pumped liquid CO_2 at the desiderate flow rate. Carbon dioxide was then heated to the desired extraction temperature in a thermosted oven (accuracy of 0.02°C). The extraction was carried out in a semibatch mode by charging the vegetable matter in the vessel, followed by a continuous flow solvent. Carbon dioxide flow was monitored by a calibrated rotameter (Sho-rate, Model 1355) located after the last separator. The CO_2 delivered during an extraction test was measured by a dry test meter. Temperatures and pressures along the extraction apparatus were measured by thermocouple and Bourdon-tube test gauges, respectively. Pressure was regulated by high pressure valves under manual control. Experiments were carried out at 90 bar and 40°C in the extraction section. In the first separator the temperature was set at -10°C and the pressure at the same value as the extraction section. The second separator was set at 15 bar and 10°C. Extractions were carried out in a semi batch mode: batch charging of vegetable matter and continuous flow solvent. About 180 g of material were charged in each run.

GC and GC/MS analysis: Analysis of the volatile extracts was carried out by gas chromatography (GC) and by gas chromatography-mass spectrometry (GC-MS) under experimental conditions as reported earlier [29].

Antifungal activity: Antifungal activity of the essential oils was evaluated against various fungal strains available from an earlier study [30]. A macrodilution broth method was used to determine the Minimal Inhibitory Concentrations (MIC) and Minimal Lethal Concentrations (MLC), according to Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) references documents M27-A3 [31], M27-S3 [32] and M38-A2 [33] for yeasts and filamentous fungi, respectively.

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