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Achillea ligustica: composition and antimicrobial activity of essential oils from the leaves, flowers and some pure constituents*

Ammar Bader[1], Lirio Panizzi[2], Pier Luigi Cioni[3], Guido Flamini[3†]

[1] *Al-Zaytoonah Private University of Jordan, 11733 Amman, Jordan*
[2] *Dipartimento della Prevenzione e Biotossicologia presso Presidio Ospedaliero di Livorno, 57100 Livorno, Italy*
[3] *Dipartimento di Chimica Bioorganica e Biofarmacia, 56126 Pisa, Italy*

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Abstract: The composition of the essential oils obtained from the leaves and the flowers of *Achillea ligustica* (Asteraceae) growing in Sicily has been studied. The main constituents of the leaves were 4-terpineol (19.3%), carvone (8.9%), γ-terpinene (7.2%) and β-phellandrene (6.8%). 4-terpineol (12.0%), carvone (10.0%), and β-phellandrene (5.4%), along with linalool (20.4%) and cedrol (4.3%) were detected in the flower's oil. Furthermore, the antimicrobial activity of the essential oils and of some of the main constituents were assayed on bacteria and fungi.

Keywords: Achillea ligustica, Asteraceae, essential oil, antimicrobial activity, 4-terpineol, carvone, linalool

1 Introduction

Achillea ligustica All. (incl. *A. distans* Ten not W. et K., *A. sylvatica* Ten., *A. sicula* Rafin), Asteraceae, known in Italy as Millefoglio ligure or Camomilla selvatica, is a small shrub that grows in Italy on arid slopes between 0 and 800 m a.s.l., mainly along the Thyrrenian coast, from Liguria to Sicily [1].

Previous studies on the essential oil composition of the flowering aerial parts collected

† E-mail: flamini@farm.unipi.it

* In memory of Prof. Ivano Morelli (1940-2005)

in Greece [2] and North Italy [3] pointed out a chemical variability, probably due to the habitat. Very recently, [4] a study was published on the essential oil composition from flowering aerial parts of this species collected in eight different localities of Sardinia where the chemical composition was very different from that reported here. Another recent study of the aerial part of this species collected in Corsica also reported a different composition of the essential oil that contained camphor (21.3%), santolina alcohol (19.3%), artemisia ketone (5.9%), borneol (6.2%) and bornyl acetate (3.5%) as the main constituents [5]. The present paper reports the composition of the essential oils of leaves and flowers of *A. ligustica* collected in Sicily.

In Sicilian folk medicine, the fresh leaves of *A. ligustica* are used as an antimicrobial and haemostatic or, swallowed as pellets, against stomach-ache. An infusion is used in Sardinia for gastralgia and neuralgia. Ethnopharmacological studies report the use of the sap obtained from the fresh plant as an anthelmintic [6]; the infusion is also used as cataplasm against rheumatism and skin disorders [7].

This and other *Achillea* species [8–10] also show good antimicrobial activity. Therefore the *in vitro* efficacy of the flower and leaf essential oils and some of their pure constituents against bacteria and fungi were assayed. Antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Candida albicans, Fusarium oxysporum, Rhizoctonia solani, Penicillium commune* and *Aspergillus flavus* was found in this study.

2 Experimental procedures

Full flowering aerial tops of *Achillea ligustica* All. were collected at the end of April 2006 in Fantina (Messina), Sicily, Italy, on calcareous soil, at about 600 m altitude. The leaves and flowers were separately dried by the air and in the shade to a constant weight and the two samples (100 g each), coarsely ground, were hydrodistilled in a Clevenger-type apparatus for two hours.

The GC analyses were accomplished with a HP-5890 Series II instrument equipped with HP-WAX and HP-5 capillary columns (30 m × 0.25 mm, 0.25 μm film thickness), set to the following conditions: temperature program of 60 °C for 10 min, followed by an increase of 5 °C/min to 220 °C; injector and detector temperatures at 250 °C; carrier gas nitrogen (2 mL/min); detector dual FID; split ratio 1:30; injection of standards of 0.5 μL).

For both the columns, identification of the chemicals was performed by comparing their retention times with those of pure authentic samples and by means of their Linear Retention Indices (LRI) relative to the series of *n*-hydrocarbons. The relative proportions of the individual constituents, expressed as percentages, were obtained by FID peak-area normalisation (mean of three replicas).

GC/EIMS analyses were performed with a Varian CP3800 gas-chromatograph equipped with a DB-5 capillary column (both 30 m × 0.25 mm; coating thickness 0.25 μm) and a Varian Saturn 2000 ion trap mass detector. The analytical conditions included: injector and transfer line temperatures at 220 and 240 °C respectively; oven temperature was pro-

grammed from 60 °C to 240 °C at 3 °C/min; carrier gas helium at 1 mL/min; injection of 0.2 μL (10% hexane solution); split ratio 1:30. Identification of the constituents was based on comparison of the retention times with those of authentic samples, comparing their Linear Retention Indices relative to the series of n-hydrocarbons, and by computer matching against commercial (NIST 98 and ADAMS 95) and home-made library mass spectra built from pure substances and components of known essential oils and MS literature data [11–16]. Moreover, the molecular weights of all the identified substances were confirmed by GC/CIMS, using MeOH as CI gas.

The antimicrobial tests were performed against the yeast fungus *Candida albicans* and the bacteria *Hafnia alvei, Listeria monocytogenes, Bacillus cereus* (all isolated from pathological material), *Staphylococcus aureus* ATCC 13709, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 9336. The microorganisms obtained from pathological material were selected because they were among the main ones present in these isolates.

The bacteria were cultivated on Unipath Iso Sensitest (Unipath Oxoid) and the fungi on Sabouraud Dextrose Agar (Unipath Oxoid). The cultures were suspended in a sterile saline solution with reference to 0.5 and 2.0 values of the McFarland scale for bacteria and fungi, respectively. These values correspond to about 150×10^6 bacteria/ml and 20×10^6 yeast cells/ml. The standardized inocula were distributed on the surface of the culture media, using the Kirby-Bauer technique. The antimicrobial assays were performed using the agar disk diffusion technique. Stock solutions of the essential oils (1.0 mg/ml) or pure constituents (1.0 and 10.0 mg/ml) were prepared in DMSO. The tested substances were placed on 6 mm sterile paper disks imbibed with 10 μg of essential oil; the Petri dishes were incubated at $35 - 37$ °C for 48 hours. Final doses added to the discs were expressed as equivalent micrograms. Blank control assays with pure DMSO do not show any activity of the carrier at the tested doses (1-10 μl).

The three main constituents of the essential oils, carvone, 4-terpineol and linalool (Aldrich Italia) were tested at decreasing doses ($10 - 2$ μg) against *Candida albicans*; the Petri dishes were then incubated at 25 °C for 96 hours.

The activity was expressed as diameter of the growth inhibition zones (mm ± SD). All the experiments were performed in triplicate.

3 Results and Discussion

The composition of the essential oils of leaves and flowers of *Achillea ligustica* is reported in Table 1. Their average yields were 0.38% and 0.48% (w/w), respectively. The main constituents of the oil from leaves were 4-terpineol (19.3%), carvone (8.9%), γ-terpinene (7.2%) and β-phellandrene (6.8%), while in the flowers' oil, beside 4-terpineol (12.0%), carvone (10.0%), and β-phellandrene (5.4%), linalool (20.4%) and cedrol (4.3%) were also identified. There is a noteworthy difference in the percentages of linalool between the two oils (1.6 and 20.4%), as previously described by Tzakou *et al.* [2]. On the contrary, Maffei *et al.* [3] reported the presence of artemisia ketone as main constituent (43.92%).

Compounds	l.r.i.[a]	l.r.i.[b]	Leaves %	Flowers %	Identif.
santolina triene	910	1010	1.5	0.7	MS, RI[e]
α-thujene	932	1016	1.7	1.2	MS, RI, ST
α-pinene	941	1029	1.7	1.2	MS, RI, ST
α-fenchene	953	1054	– [c]	tr[d]	MS, RI
camphene	955	1071	tr	–	MS, RI, ST
benzaldehyde	963	1491	–	tr	MS, RI, ST
sabinene	978	1112	3.3	1.4	MS, RI, ST
β-pinene	981	1126	5.8	3.3	MS, RI, ST
2,3-dehydro-1,8-cineole	992	–	tr	tr	MS, RI
yomogi alcohol	997	1401	2.0	–	MS, RI
α-terpinene	1020	1183	4.5	2.3	MS, RI, ST
p-cymene	1028	1274	3.0	2.9	MS, RI, ST
limonene	1033	1198	1.0	1.0	MS, RI, ST
santolina alcohol	1035	1413	2.4	1.9	MS, RI
β-phellandrene	1035	1199	6.8	5.4	MS, RI, ST
1,8-cineole	1039	1204	0.9	0.4	MS, RI, ST
γ-terpinene	1064	1252	7.2	4.0	MS, RI, ST
cis-sabinene hydrate	1070	–	–	tr	MS, RI, ST
cis-linalool oxide	1075	–	–	tr	MS, RI
linalool	1101	1547	1.6	20.4	MS, RI, ST
trans-sabinene hydrate	1103	–	tr	tr	MS, RI, ST
α-thujone	1106	1428	2.1	2.7	MS, RI, ST
β-thujone	1106	1446	1.4	1.8	MS, RI, ST
myrcenol	1118	1588	1.6	1.2	MS, RI
fenchol	1123	1584	–	2.1	MS, RI
chrysanthenone	1125	–	tr	–	MS, RI
cis-*p*-menth-2-en-1-ol	1127	–	tr	tr	MS, RI
trans-*p*-menth-2-en-1-ol	1142	–	0.6	0.4	MS, RI
camphor	1145	1522	0.8	2.4	MS, RI, ST
cis-chrysanthenol	1164	–	tr	tr	MS, RI
borneol	1175	1796	0.9	2.5	MS, RI, ST
4-terpineol	1182	1607	19.3	12.0	MS, RI, ST
p-cymen-8-ol	1185	1838	0.3	0.2	MS, RI
α-terpineol	1193	1698	2.4	1.6	MS, RI, ST
methyl chavicol	1197	–	0.7	–	MS, RI, ST
verbenone	1208	1716	0.9	–	MS, RI, ST
carvone	1246	1741	8.9	10.0	MS, RI, ST
isobornyl acetate	1285	1582	0.7	1.3	MS, RI
thymol	1292	2187	-	0.7	MS, RI, ST
carvacrol	1298	2219	tr	tr	MS, RI, ST
α-terpinyl acetate	1350	–	tr	–	MS, RI, ST
β-caryophyllene	1419	1604	tr	tr	MS, RI, ST
germacrene D	1491	1691	2.9	–	MS, RI
bicyclogermacrene	1496	1493	1.0	–	MS, RI
δ-cadinene	1524	1731	tr	–	MS, RI, ST
caryophyllene oxide	1578	2071	1.0	–	MS, RI, ST
cedrol	1603	2143	1.7	4.3	MS, RI
β-eudesmol	1651	–	tr	–	MS, RI
Total identified			88.9	88.1	

[a] linear retention indexes (apolar column)
[b] linear retention indexes (polar column)
[c] not detected
[d] trace amounts < 0.1 [e] identification: MS=mass spectrometry, RI=retention index, ST=pure reference compound

Table 1 Composition and yields of the essential oils from leaves and flowers of *Achillea ligustica*.

Tuberoso *et al.* [4] reported high percentages of irregular terpenes, mainly santolina alcohol (6.7%-21.8%), santolina triene (0.8%-2.0%), artemisia ketone (0.3%-7.6%) and yomogi alcohol (0.1%-0.5%). Only the latter one has been identified in the present study (2.0%). Additional differences in the other main constituents include: borneol (3.4%-20.8% vs. 0.9%-2.5%, in Sardinian and present study, respectively), sabinol (2.1%-15.5% vs. 0%), sabinyl acetate (0.9%-17.6% vs. 0%), α-thujone (0.4%-25.8% vs. 2.1%-.27%), 4-terpineol (1.4%-6.1% vs. 12.0%-19.3%), carvone (0% vs. 8.9%-10.0%), and linalool (2.1%-10.4% vs. 1.6%-20.4%).

Both the essential oils from leaves and flowers showed an inhibitory activity on all the microorganisms tested. The effectiveness of the two essential oils was comparable, with growth inhibition zones of $10 - 13$ mm; *C. albicans* only was particularly susceptible, with an inhibition zone of 24 mm (Table 2). These results agree with those previously reported by Tuberoso *et al.* [4] with essential oils from Sardinia (Italy) at a dose of 20 μl but, possibly because of their different composition, the essential oils from Sicily seem to be equally effective at a dose of only 10 μg. Furthermore, they showed good antibacterial activity against *Hafnia alvei, Listeria monocytogenes* and *Bacillus cereus* (Table 2).

Microorganisms	Inhibition zones (mm ± SD)	
	Leaves	Flowers
Staphylococcus aureus	10.3 ± 1.53	10.7 ± 1.16
Pseudomonas aeruginosa	0	0
Escherichia coli	11.3 ± 1.53	11.3 ± 1.53
Hafnia alvei	13.0 ± 1.00	13.3 ± 1.53
Listeria monocytogenes	12.0 ± 1.00	11.7 ± 0.58
Bacillus cereus	11.2 ± 1.04	12.7 ± 1.16
Candida albicans	24.3 ± 1.53	23.7 ± 1.53

Table 2 Antimicrobial activity of the essential oil from leaves and flowers of *Achillea ligustica* (10 μg/plate); mean of three replication±SD.

Because of its sensitivity, the main constituents of the oils, carvone, 4-terpineol and linalool have been tested against *C. albicans* (Table 3). Carvone and 4-terpineol, at 10 μg, were more effective than the essential oils themselves, with inhibition zones of 30 and 26 mm, respectively; linalool, at the same dose, was less active (17 mm) than the two oils. At lower doses the activity of carvone and 4-terpineol were still high: at 4 μg the inhibition zone was still 12 mm for both the compounds. At the lowest dose (2 μg) 4-terpineol continued to exert its action (10 mm), while carvone was completely ineffective. Linalool, in spite of its lesser activity at higher doses, at 4 and 2 μg maintained an activity equivalent to that of 4-terpineol.

The effective doses for the three pure compounds (4 μg for carvone and 2 μg for 4-terpineol and linalool) were considerably lower than those found for some common synthetic antifungal drugs (Table 4). Consequently, the antifungal activity of the oil should be due in larger part to these constituents. Apart from the synergistic effect of

many effective compounds contained in the oil, the use of an essential oil as an antifungal drug may additionally prevent the resistance phenomena seen in many active synthetic principles.

Compounds	10	8	6	4	2	1
carvone	31.7 ± 2.08	24.3 ± 0.58	18.3 ± 0.58	12.0 ± 1.00	0	0
4-terpineol	25.7 ± 0.58	24.3 ± 1.53	17.7 ± 0.58	11.7 ± 0.58	10.0 ± 1.00	0
linalool	17.3 ± 0.58	14.0 ± 1.00	15.3 ± 0.58	11.3 ± 0.58	8.4 ± 0.53	0

Table 3 Antimycotic activity of carvone, 4-terpineol and linalool against *Candida albicans* at decreasing doses (μg); average growth inhibition zones diameter (mm) for three replicates with standard deviation.

Substances	Doses	Inhibition zones (mm)
5-fluorocytosine	10 μg	0
myconazole	50 μg	16.7 ± 0.58
econazole	50 μg	20.3 ± 0.58
nistatine	100 IU	15.0 ± 1.00
amphotericine	100 μg	18.5 ± 0.50
ketoconazole	50 μg	16.3 ± 0.58
clotrimazole	50 μg	13.2 ± 0.29

Table 4 Antimycotic activity of carvone, 4-terpineol and linalool against *Candida albicans* at decreasing doses (μg); average growth inhibition zones diameter (mm) for three replicates with standard deviation.

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