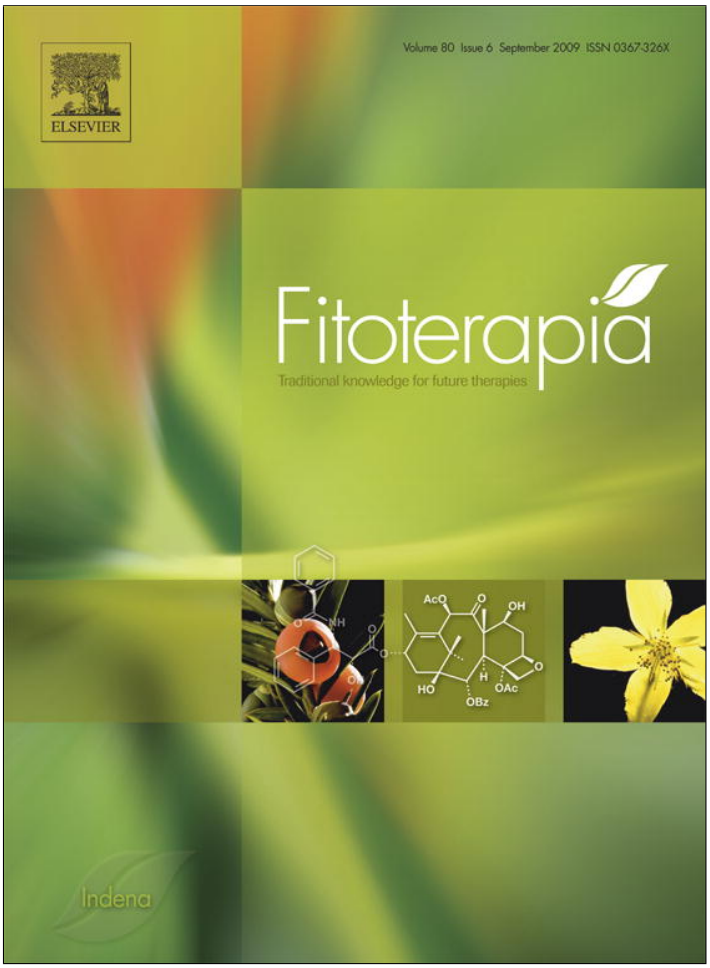

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Composition and biological activity of essential oil of *Achillea ligustica* All. (Asteraceae) naturalized in central Italy: Ideal candidate for anti-cariogenic formulations

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ABSTRACT

Essential oil from flowers (FL) and vegetative parts (VP) of *Achillea ligustica* (Asteraceae), naturalized after cultivation in central Italy, was investigated by GC–FID and GC–MS. The most abundant components were linalool, viridiflorol, β-pinene, 1,8-cineole and terpinen-4-ol. The antioxidant assays (DPPH and ABTS radical scavenging assays, and β-carotene bleaching test) demonstrated a moderate activity of essential oils. The antimicrobial activity was evaluated by the broth micro-dilution method on 6 microbial strains and showed to be quite strong against the cariogenic Gram-positive *Streptococcus mutans*, suggesting that this essential oil could be a valid candidate for anti-cariogenic formulations. Moderate cytotoxic activity was observed in assays on four tumour cell lines by MTT assay.

1. Introduction

Essential oils obtained from plants have a number of potential uses, including food additivation, as preservative from spoilage, and pharmaceuticals, owing to their notable antimicrobial [1] and antioxidant [2] properties. The above is in line with the increasing demand from consumers to limit the use of synthetic additives, since those chemicals have sometimes demonstrated to be a source of potential health hazard, owing to toxic impurities deriving from synthetic pathway. Essential oils are much more acceptable to the end consumers than synthetic substances, and they do not cause bacterial resistance, mainly because they are constituted by a wide spectrum of compounds. Evidences of the above are given, for example, by the increase of production and commerce of essential oil-containing mouthrinses, which were found to be effective in inhibiting the plaque flora, particularly the cariogenic Gram-positive *Streptococcus mutans* [3]. Caries are caused by a number of microbial species, among which *S. mutans* plays a leading role. Recently, it has been suggested that oral bacteria are also associated with many systemic diseases, like cardiovascular diseases and pneumonia. From the above, the importance of an effective mouthrinse, acceptable by the general public and without side effects, is evident. Uses of essential oils for dental care is traced back in the history of the medical uses of plants in the world since time immemorial. In fact, many people from several African tribes used to chew sticks of plant origin, or gum and

Abbreviations: FL, flowers;VP, vegetative parts, corresponding to stems and leaves;MIC, minimal inhibitory concentration;MLC, minimal lethal concentration;MTT, 3-(4,5-dimethylthiozol-2-yl)-2-5 diphenyl-tetrazolium bromide;DPPH, 2,2-diphenyl-1-picrylhydrazyl;ABTS, 2,2¢-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid);BHA, butylated hydroxyanisole;BHT, butylated hyroxytoluene.

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resins for dental hygiene and to prevent oral ailments [4]. Moreover, essential oils act as a good barrier against the primary oxidative process leading to loss of food quality, owing to their content in phenols, monoterpene hydrocarbons, monoterpene alcohols and benzene derivatives [2].

Among plants known and used for their essential oil, *Achillea ligustica* All. (Ligurian yarrow), belonging to the family of Asteraceae, is a perennial, herbaceous and aromatic plant occurring in the Mediterranean area. In Italy, this plant grows spontaneously in the Tyrrhenian region, Sicily and Sardinia [5]. In Italian folk medicine it was used as an antihelmintic and against rheumatism and skin disorders and inflammation [6]. Essential oils obtained from different species of yarrow have been employed in herbal, food and cosmetics applications. In particular, because of the specific aroma derived from the chemical profile of the volatile fraction, yarrow has been employed as a flavouring agent in the liqueur industry. In previous investigations the chemical composition of the volatile fraction of *A. ligustica* showed a strong variability, depending on geographical factors [7–11]. At the same time, some of these oils were also investigated for antimicrobial activity, and showed a different range of inhibition levels, depending on the different chemical composition of the oils [9–11].

In this study, we analyzed the essential oil composition from *A. ligustica* naturalized after cultivation in Marche (central Italy), in order to detect qualitative and quantitative differences with respect to naturally occurring populations, using separately extracted flowers and aerial parts essential oils. We also investigated a number of different biological activities (antioxidant, using three different methods; antimicrobial, against 5 bacteria and a yeast species commonly causing human health problems as oral diseases; antiproliferative, against three human and a murine cancer cell lines) of the two essential oils (obtained from FL and VP) from the above cited naturalized population.

2. Experimental

2.1. Plant material

A population naturalized after cultivation at the Botanical Garden in Camerino (central Italy, 600 m above sea level, GPS coordinates: N 43°08′02″ E 13°04′06″) was considered in this study. FL and VP were separately collected in May 2007 during the blooming period. The plant was botanically confirmed by F. Maggi using available literature [12]. A voucher specimen was deposited in the Herbarium Camerinensis, Dept. of Environmental Sciences, Sect. of Botany and Ecology, University of Camerino, Italy, under the accession code CAME 13420; it is also available at the following website: http://erbariitaliani.unipg.it. Plant material was air dried at room temperature protected from light for one week.

2.2. Reagents and standards

α-Pinene, camphene, benzaldehyde, sabinene, β-pinene, octanal, α-terpinene, p-cymene, 1,8-cineole, γ-terpinene, linalool, camphor, borneol, terpinen-4-ol, α-terpineol, myrtenal, myrtenol, trans-carveol, trans-myrtanol, thymol, eugenol, α-copaene, α-gurjunene, (E)-caryophyllene, α-humulene, caryophyllene oxide, globulol, guaiol, α-bisabolol, phytol, *n*-alkanes (from C8 to C30), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 2,2¢-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), butylated hydroxyanisole (BHA), butylated hyroxytoluene (BHT), and L-ascorbic acid were from Sigma-Aldrich-Fluka (Milan, Italy). 3-(4,5-Dimethylthiozol-2-yl) 2-5 diphenyl-tetrazolium bromide (MTT) was purchased from Sigma, St. Louis, MO. All compounds were of analytical grade standards. *n*-Hexane was analytical grade solvent from Carlo Erba (Milan, Italy); it was distilled by a Vigreux column before use. Na_2SO_4 was of analytical reagent grade from J.T. Baker (Deventer, Holland).

2.3. Isolation of the volatile oils

FL and VP essential oils were isolated by hydrodistillation in a Clevenger-type apparatus for 4 h, using *n*-hexane (10 ml) as collector solvent. After evaporation of the solvent under N_2 flow, the oil was dried over anhydrous sodium sulphate and stored in sealed vials protected from the light at −20 °C before analyses. Three oil samples for each collection were prepared and then analyzed by GC–FID and GC–MS. The percentage values of volatile components were mean of three determinations. The oil yields were calculated on a dry weight basis.

2.4. GC–FID and GC–MS analyses

GC–FID analysis of the volatile components was carried out using an Agilent 4890D instrument with an FID detector and a HP-5 capillary column (5% phenylmethylpolysiloxane, 25 m, 0.32 mm i.d.; 0.17 μm film thickness) (J & W Scientific, Folsom, CA), working with the following temperature program: 5 min at 60 °C, 4 °C/min up to 220 °C, then 11 °C/min up to 280 °C, held for 15 min, for a total run time of 65 min; injector and detector temperatures, 280 °C; carrier gas, helium (1.4 ml/min); injection volume of 1 μl, split ratio, 1:34.

GC–MS analysis was performed using an Agilent 6890N–5973N GC–MS system operating in the EI mode at 70 eV, using a HP-5MS (5% phenylmethylpolysiloxane, 30 m, 0.25 mm i.d., film thickness 0.1 μm) (J & W Scientific, Folsom, CA) capillary column which was programmed at 60 °C for 5 min, then ramp of 4 °C/min to 220 °C, then 11 °C/min up to 280 °C, held for 15 min, finally 11 °C/min up to 300 °C, held for 5 min, for a total run time of 72 min; carrier gas: helium; flow rate: 1.0 ml/min; injector and transfer line temperatures: 280 °C; injection volume: 2 μl; split ratio: 1:50; scan time: 75 min; acquisition mass range: 29–400 *m*/*z*.

2.5. Identification and quantification of volatile components

The identification of volatile components was based on computer matching with the WILEY275, NIST05, and ADAMS libraries, as well as by comparison of the mass spectra and retention indices (RI), with those reported in the literature [13]. In addition, a home-made library, constructed based on the analyses of reference oils and commercial available standards, was used as well. Retention indices (RI) relative to *n*-alkanes were also calculated, and were in good agreement with those reported by Adams [13]. Finally, whenever possible, components were identified by comparison of their

retention times, mass spectra, and retention indices related to *n*-alkanes with those of authentic standards available in the author's laboratory. Percentage compositions of the oil components were calculated as percentage at FID by peak area internal normalization. The percentage values were the mean of three determinations.

2.6. Antioxidant activity

2.6.1. Determination of total phenolic content

Total soluble phenols were determined according to the Folin–Ciocalteu method [14]. The total phenolic content was determined as gallic acid equivalents (GAE) per mg of extract.

2.6.2. DPPH radical scavenging assay

The antioxidant activities of the essential oils and control substances were measured in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH as reagent [15]. All determinations were performed in triplicate. The percent inhibition of the DPPH radical by the samples was calculated according to the following formula: % inhibition = ((AB − AA) / AB)) × 100], where AB is the absorption of blank sample, which contains essential oil (*t* 0 min), and AA is the absorption of the tested oil control solution (*t* 5 min). Sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against oil concentration. L-ascorbic acid was used as positive control.

2.6.3. ABTS radical scavenging activity assay

An improved ABTS decolorization assay was used, which has been applied for both lipophilic and hydrophilic compounds [16]. Ethanol and L-ascorbic acid were used as negative and positive control, respectively.

2.6.4. β-carotene bleaching test (βCBT)

The CBT was performed according to literature [17]. Negative (ethanol) and positive (BHA and BHT) controls were used in the assay.

2.7. Antimicrobial activity

2.7.1. Microorganisms and growth conditions

Staphylococcus aureus (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 13706) and *Candida albicans* (ATCC 14053) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). *S. mutans* (DSM 20523) was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen, GmbH (DSM, Braunschweig, Germany). Bacterial strains were cultivated on Müeller Hinton Broth (OXOID, Unipath Limited, Basingstoke, UK) while *C. albicans* was cultivated on Sabouraud liquid Medium (OXOID). Cells cultures were incubated at 37 °C for 24 h and then used. The cells suspension was adjusted with sterile saline solution to obtain a turbidity comparable to that of McFarland n. 0.5 standard (1.5×10^8 cells/ml).

2.7.2. Minimal inhibitory concentration (MIC)/minimal lethal concentration (MLC) assay

MIC and MLC were determined using a broth microdilution method with a 96-well micro-titer plate [18]. One series of 2-fold dilutions of each oil sample in acetone (ranging from 10 to 0.001 mg/ml) for each microbial strain was prepared using the same media as above. Each series was inoculated with 0.8 µl of each microbial strain (1.5×10^8 cells/ml). Determinations were carried out evaluating the microbial growth in the wells using a stereomicroscope (GSZ2, Ascania, Germany). Chloramphenicol for Gram-positive and Gram-negative bacteria, and amphotericin B for yeasts (both from Sigma-Aldrich, Buchs, Switzerland), were used as positive controls. Available standard β-pinene, 1,8-cineole, linalool and terpinen-4-ol (all from Sigma-Aldrich, Milan, Italy) were also tested under identical conditions to compare their activities with that of the investigated oils. All the experiments were conducted in triplicate. A negative control, inoculating acetone without oils, was also included.

2.8. Antiproliferative activity

2.8.1. Cell culture

T98G cells (ATCC CRL-1690, human glioblastoma multiforme cells) were cultured in Eagle's minimum essential medium (EMEM) with 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin, and supplemented with 10% heat inactivated foetal bovine serum (HI-FBS) (Sigma, St. Louis, MO). B16-F1 cells (ECACC, European Collection of Cell Cultures, 92101203, mouse melanoma cells) were cultured in Dulbecco's Modified Eagle's Medium with 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and supplemented with 10% HI-FBS. A431 cells (ECACC 85090402, human squamous carcinoma cells) were cultured in RPMI1640 medium with 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and supplemented with 10% HI-FBS. PC-3 cells (ECACC 90112714, human prostatic adenocarcinoma cells) were cultured in Coon's modified Ham's F12 medium with 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and supplemented with 7% HI-FB5. Cells were cultured in a humidified atmosphere at 37 °C in the presence of 5% CO_2.

2.8.2. MTT bioassay

The MTT assay was used as a relative measure of cell viability. Cell viability assays were carried out as described in literature [19]. Briefly, cells were seeded at the initial density of 2×10^4 cells/ml in 96-well micro-titer plates (Iwaki, Tokyo, Japan). After incubation for 24 h at 37 °C, cells were treated with different concentrations of FL and VP essential oils, ranging from 0.1 to 2 mg/ml, and incubated for 24, 48, and 72 h in a humidified atmosphere at 37 °C in the presence of 5% CO_2. 10 µl of 3-(4,5-dimethylthiozol-2-yl) 2-5 diphenyl-tetrazolium bromide (MTT) (5 mg/ml in phosphate-buffered saline, PBS) were added to each well and further incubated for 4 h at 37 °C. The supernatant was removed and replaced with 100 µl of DMSO. Optical density of each sample was measured with a microplate spectrophotometer reader Titertek Multiscan microElisa (Labsystems, Helsinki, Finland) at 540 nm. Cytotoxicity was expressed as the concentration of essential oil inhibiting cell growth by 50% (IC_{50}). Available standard β-pinene, sabinene, 1,8-cineole, linalool, terpinen-4-ol, α-humulene, (E)-caryophyllene, caryophyllene oxide, α-bisabolol were mixed and tested under identical conditions as the investigated oils.

Table 1
Chemical composition of *Achillea ligustica* All. essential oils.

Compounds [a]	RI [b]	Flowers	Stems/leaves	ID [c]
Hexanol	873	–	Tr	MS,RI
α-thujene	927	Tr	Tr	MS,RI
α-pinene	933	0.61	0.65	MS,RI,std
Camphene	948	Tr	Tr	MS,RI,std
Benzaldehyde	968	Tr	–	MS,RI,std
Sabinene	974	1.33	1.25	MS,RI,std
β-pinene	976	6.39	9.55	MS,RI,std
Yomogi alcohol	1004	0.45	0.91	MS,RI
Octanal	1009	–	Tr	MS,RI,std
α-terpinene	1018	1.04	1.87	MS,RI,std
p-cymene	1028	0.78	0.73	MS,RI,std
β-phellandrene	1031	0.22	0.40	MS,RI
1,8-cineole	1033	5.77	3.43	MS,RI,std
(Z)-β-ocimene	1044	–	0.11	MS,RI
γ-terpinene	1062	2.22	4.26	MS,RI,std
Artemisia ketone	1068	1.27	1.22	MS,RI
cis-sabinene hydrate	1071	0.27	1.51	MS,RI
Artemisia alcohol	1087	–	Tr	MS,RI
Terpinolene	1089	0.56	1.19	MS,RI
Trans-sabinene hydrate	1098	0.30	0.52	MS,RI
Linalool	1105	24.79	0.22	MS,RI,std
Nonanal	1109	–	Tr	MS,RI
Hotrienol	1110	0.64	–	MS,RI
2-methyl butyl isovalerate	1114	0.28	Tr	MS,RI
cis-ρ-menth-2-en-1-ol	1125	0.29	–	MS,RI
α-campholenal	1130	Tr	–	MS,RI
Veratrole	1137	0.38	–	MS,RI
trans-pinocarveol	1141	1.32	–	MS,RI
trans-ρ-menth-2-en-1-ol	1145	Tr	–	MS,RI
Camphor	1146	0.41	–	MS,RI,std
Pinocarvone	1164	1.64	1.44	MS,RI
Borneol	1168	0.57	0.29	MS,RI,std
δ-terpineol	1172	0.17	Tr	MS,RI
cis-pinocamphone	1175	–	0.13	MS,RI
terpinen-4-ol	1179	5.30	12.95	MS,RI,std
Artemisyl acetate	1181	0.14	Tr	MS,RI
α-terpineol	1192	1.94	1.62	MS,RI,std
Myrtenal	1195	0.19	0.48	MS,RI,std
Myrtenol	1197	0.47	0.70	MS,RI,std
trans-piperitol	1210	0.13	0.24	MS,RI
trans-carveol	1224	–	Tr	MS,RI,std
trans-myrtanol	1256	0.10	0.11	MS,RI,std
cis-chrysanthenyl acetate	1265	0.44	0.27	MS,RI
Bornyl acetate	1287	0.79	0.46	MS,RI
Thymol	1291	–	Tr	MS,RI,std
Geranyl formate	1296	Tr	Tr	MS,RI
trans-pinocarvyl acetate	1300	0.51	0.88	MS,RI
Myrtenyl acetate	1329	0.13	0.12	MS,RI
trans-carvyl acetate	1343	Tr	Tr	MS,RI
Eugenol	1361	–	Tr	MS,RI,std
Cyclosativene	1363	0.51	0.53	MS,RI
cis-carvyl acetate	1367	0.34	0.23	MS,RI
α-copaene	1373	0.27	0.40	MS,RI,std
β-bourbonene	1381	–	Tr	MS,RI
trans-myrtanol acetate	1386	0.51	–	MS,RI
(E)-β-damascenone	1386	–	0.17	MS,RI
β-elemene	1390	0.12	0.16	MS,RI
Italicene	1398	–	Tr	MS,RI
α-gurjunene	1405	0.13	0.13	MS,RI,std
(E)-caryophyllene	1415	1.01	1.57	MS,RI,std
α-humulene	1450	0.15	0.13	MS,RI,std
9-epi-(E)-caryophyllene	1458	0.81	0.99	MS,RI
cis-muurola-4(14),5-diene	1460	–	Tr	MS,RI
α-acoradiene	1465	–	Tr	MS,RI
(E)-β-farnesene	1465	Tr	–	MS,RI
trans-cadina-1(6),4-diene	1475	0.18	0.24	MS,RI
Germacrene D	1478	Tr	0.47	MS,RI
gamma-curcumene	1480	0.67	0.18	MS,RI
ar-curcumene	1483	0.51	0.83	MS,RI
Viridiflorene	1492	0.16	0.15	MS,RI
α-zingiberene	1495	0.28	Tr	MS,RI
α-muurolene	1498	Tr	0.13	MS,RI
β-curcumene	1512	0.10	0.13	MS,RI
δ-cadinene	1523	1.47	1.50	MS,RI
β-sesquifellandrene	1526	0.15	–	MS,RI
α-calacorene	1542	0.11	0.23	MS,RI
cis-dracunculifoliol	1545	0.18	0.11	MS,RI
Palustrol	1565	0.37	0.55	MS,RI
Spathulenol	1577	0.16	0.38	MS,RI
Caryophyllene oxide	1580	0.99	2.04	MS,RI,std
ar-turmerol	1583	0.19	0.32	MS,RI
Viridiflorol	1591	9.59	14.54	MS,RI
Globulol	1600	0.83	0.95	MS,RI,std
Guaiol	1601	0.72	1.25	MS,RI,std
Humulene epoxide II	1604	Tr	Tr	MS,RI
Isolongifolan-7-α-ol	1614	1.78	0.69	MS,RI
10-epi-γ-eudesmol	1625	0.92	0.22	MS,RI
1-epi-cubenol	1629	0.89	2.02	MS,RI
Zingiberenol	1635	1.52	0.79	MS,RI
3-thujopsanone	1656	0.59	1.56	MS,RI
Helifolenol A	1664	Tr	1.06	MS,RI
α-bisabolol	1685	0.52	1.26	MS,RI,std
11-αH-himachal-4-en-1-beta-ol	1691	2.44	1.58	MS,RI
(2Z,6Z)-farnesol	1697	0.42	0.43	MS,RI
Chamazulene	1727	0.23	0.17	MS,RI
(6S,7R)-bisabolone	1748	0.22	0.18	MS,RI
Xanthorrhizol	1757	–	0.10	MS,RI
Phytol	2112	–	0.83	MS,RI
Tricosane	2300	0.10	Tr	MS,RI,std
Pentacosane	2500	Tr	Tr	MS,RI,std
Total identified (%)		92.8	87.6	
Grouped compounds				
Monoterpenes hydrocarbons		13.32	20.13	
Oxygenated monoterpenes		49.15	27.98	
Alcohols		42.59	19.25	
Ketones		3.32	2.78	
Oxides		5.77	3.43	
Esters		2.97	2.05	
Aldehydes		0.27	0.48	
Sesquiterpenes hydrocarbons		7.09	8.21	
Oxygenated sesquiterpenes		22.33	30.20	
Alcohols		20.54	26.25	
Ketones		0.80	1.91	
Oxides		0.99	2.04	
Others		0.94	1.11	

RI, by comparison of RI with those reported from Adams [13]; std, by injection of an authentic sample. –, not detected. Tr, trace (<0.1%). FL, flowers; VP, vegetative parts (stems/leaves).

[a] Compounds are listed in order of their elution from a HP-5 column.

[b] RI, retention indices as determined on HP-5 column using homologous series of C8–C26 alkanes.

[c] Identification methods: MS, by comparison of the mass spectrum with those of the computer mass libraries and Adams [13].

2.9. Statistical analysis

Data represent the mean ± SD of 3 determinations. Continuous variables were examined by one-way ANOVA followed by Dunnett's multiple-comparison test. $P<0.05$ was used as the criterion for statistical significance. Unpaired *t*-tests were used within group test comparisons. The minimum level of significance considered was $P<0.01$.

3. Results and discussion

The essential oil composition of *A. ligustica* is given in Table 1. The GC–FID and GC–MS analysis led us to the identification of

one hundred and one components (85 in FL and 90 in VP, respectively), accounting for 87.6–92.8% of the total oils. This high number of constituents makes the oil obtained from the studied naturalized *Achillea* population qualitatively richer than the oils obtained from spontaneous populations studied in the past [7–11]. Moreover, 38 compounds of those identified in our research have been discovered for the first time in essential oil of *A. ligustica*, accounting for an analytical procedure possessing high accuracy (use of 3 different MS libraries for structure determination; 32 standards used in the analytical process) and/or for a special composition of essential oil deriving from the origin of the plants, which were naturalized after cultivation in the area where they were harvested for analysis. Of the 38 compounds newly identified in *A. ligustica*, flowers essential oil contained 26 (30.6% of total) of them, whereas oil from vegetative parts showed the presence of 33 (36.7%) new compounds. Of those 38 compounds, 12 are present only in trace amount (<0.1%), whereas 5 of them show concentration higher than 1% of essential oil in either one of the extracts (FL or VP).

The average yields of FL and VP oils were 0.91% and 0.18% (w/w), respectively. The most abundant components (>5%) of the oil from FL were linalool (24.79%), viridiflorol (9.59%), β-pinene (6.39%), 1,8-cineole (5.77%) and terpinen-4-ol (5.30%); in the VP the main components were viridiflorol (14.54%), terpinen-4-ol (12.95%) and β-pinene (9.55%), whilst linalool, the major components of the FL oil, was present only in scanting amounts (0.22%). The oxygenated monoterpenes represented the main fraction of the FL oil (49.15%), showing a concentration almost double with respect to the VP oil (27.98%); on the other hand, hydrocarbon monoterpenes showed an opposite trend (13.32 in FL and 20.13 in VP). This suggests that in flowers oxidative processes are stronger and/or more efficient than in stem and leaves. Oxygenated monoterpenes and sesquiterpenes showed a very similar concentration (27.98% and 30.20%, respectively) in the VP oil. In both cases, the alcohols represented the main constituents of these fractions: among the monoterpenes, linalool (24.8%) and terpinen-4-ol (5.30%) predominated in FL oil, and terpinen-4-ol (12.95%) the most abundant in VP oil; among the sesquiterpenes, the major component was viridiflorol both in FL (9.59%) and VP (14.54%) oils. We did not detect at all in oils the neurotoxic thujone, found in other investigated *A. ligustica* oils [8–11]; this makes the essential oil of our yarrow more suitable than the previous studied ones for use in the food industry. Hydrodistilled oils showed a strong blue color, due to the presence of chamazulene and other compounds of azulenic nature; this fact suggests the use of essential oil also as natural colorant for food. That use would bring about also antioxidant and antimicrobial properties to the coloured food. Our oils presented similar chemical profile (concerning the main volatiles) with respect to those obtained from populations growing in Greece and Sicily [8,11]. However, if we compare our results with those obtained studying other naturally occurring populations, we may notice both qualitative and quantitative differences [7,9,10]. These differences consist in lower amounts of artemisia ketone and camphane derivatives, and in the absence of santolina alcohol, sabinol, trans-sabinyl acetate, bornyl acetate and α-thujone in our data; they may be related to the different geographical origins of the samples.

The antioxidant activities of *A. ligustica* essential oils were evaluated using three methods, including two based on the free radical scavenging capacity, i.e. DPPH radical scavenging assay, ABTS radical scavenging assay, and β-carotene bleaching test. The results showed that essential oils had concentration-dependent antioxidant activity, whereas their potency was much poorer than that of L-ascorbic acid or BHA (Table 2). In βCBT, the mechanism of bleaching of β-carotene is a free radical mediated phenomenon resulting from the hydroperoxides of linoleic acid. β-Carotene undergoes rapid discoloration in the absence of an antioxidant. Table 2 shows that *A. ligustica* oils have considerable antioxidant activity by retaining β-carotene in the medium, which suggests that *A. ligustica* oils, due to its high content in oxygenated components (Table 1), can inhibit lipid peroxidation reactions by scavenging free radicals. In this study, the samples were tested at low concentration because of their strong hydrophobic property. Results, reported in Table 2, show that on all the three tests FL oil possesses higher efficacy than VP oil. This can be related to the higher level of oxygenated (alcohol) compounds in the former; in fact, it is well known that antioxidant properties are very often related to the presence of an easily oxydizable portion on the molecule. The presence of a hydroxyl group on a hydrocarbon chemical makes the compound much easier to be oxydized.

The antimicrobial activity of *A. ligustica* essential oils expressed as MIC and MLC is given in Table 3. The results showed that the essential oils were particularly active against the Gram-positive bacteria *S. mutans* and *B. subtilis*, and the Gram-negative *E. coli*, and moderately active against the yeast *C. albicans*. No remarkable inhibition activity was observed against *S. aureus* and *E. faecalis*. Quite important, VP oils resulted more active on Gram-positive bacteria than FL oils. *S. mutans* is commonly present in the human oral cavity, and is responsible for the plaque that can lead to dental caries. Furthermore, recently it has been shown that the oral bacteria are associated with many systemic diseases, such as pneumonia and cardiovascular diseases [20]. Hence, the reported efficacy against *S. mutans*, responsible for tooth decay, along with the lack of the toxic tujone and the pleasant blue colour, makes this essential oil a convincing candidate for the formulation of plant based toothpaste or mouthrinses, to be used as part of a daily oral hygiene regimen, alternatively to conventional oral products containing antimicrobial active ingredients such as chlorhexidine [3], that sometimes can lead to side effects, such as alteration of teeth colour and taste [21]. Efficacy of *A. ligustica* essential oils on the cariogenic

Table 2
Antioxidant activity of *Achillea ligustica* essential oil.

Essential oil samples	DPPH	ABTS	β-Carotene assay
	IC_{50}	IC_{50}	% of inhibition [a]
FL [b]	47.2 μg/ml (PL) [c]	35.4 μg/ml (PL)	27.6 ± 2.9
VP [d]	55.1 μg/ml (PL)	68.2 μg/ml (PL)	12.3 ± 3.1
L-ascorbic acid	6.0 μg/ml	0.25 μg/ml	–
BHT (100 ppm)	–	–	62.6 ± 4.8
BHA (100 ppm)	–	–	64.6 ± 6.2

[a] The oil concentration was 24.5 μg/ml of PL.
[b] FL, flowers.
[c] PL= polyphenols.
[d] VP, vegetative parts (stems/leaves).

Table 3
Antimicrobial activity of *Achillea ligustica* essential oils and some major oil components given as MIC and MLC[a] (expressed in μg/ml).

Oil samples	*S. aureus*		*S. mutans*		*B. subtilis*		*E. faecalis*		*E. coli*		*C. albicans*	
	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC
FL[b]	1250	2500	155	310	78	78	2500	5000	310	625	625	625
VP[c]	1250	2500	39	39	39	39	1250	5000	625	2500	625	625
Positive control[d]	5	500	10	78	10	19	10	19	5	78	1	5
Major oil components												
(β)-Pinene	2500	2500	625	1250	310	310	2500	2500	2500	2500	310	625
1,8-Cineole	2500	2500	155	1250	625	625	1250	2500	2500	2500	155	155
Linalool	1250	1250	310	310	310	625	1250	1250	625	625	155	310
Terpinen-4-ol	625	1250	310	625	310	625	1250	250	1250	1250	155	310

[a] Mean value, $n = 3$.
[b] FL, flowers.
[c] VP, vegetative parts (stems/leaves).
[d] Chloramphenicol for Gram-positive and Gram-negative bacteria, Amphotericin B for yeast; solvent control (acetone) was negative for all tested strains.

bacteria may be due to the oxygenated monoterpenes terpinen-4-ol and 1,8-cineole, and to the hydrocarbons α-terpinene and γ-terpinene, with the former being the most abundant. In fact, these components resulted the most prevalent in the oil of the Tea Tree (*Melaleuca alternifolia*, Myrtaceae) [22], which has been found to have a great inhibition power on the growth and adhesion of periodontopathic and cariogenic bacteria, and therefore useful in treating oral diseases [20,23]. The stronger activity against *S. mutans* detected for VP oil with respect to FL oil could be explained by its higher content in terpinen-4-ol (12.95% vs 5.30%, respectively), which resulted in the volatile compound most responsible for the biological effects of Tea Tree oil [22]. However, from comparison of antimicrobial values between oil samples and some pure components (Table 3), we can conclude that a synergistic effect of other minor components is to be taken into account. For example, MIC of oils, in the case of *B. subtilis*, is from 4 to 8 times higher than that of Chloramphenicol, whereas for the single components of oils, tested alone, that values range from 15 to 62 times; from those figures, the most active single component tested is from 2 to 4 times less effective than oils.

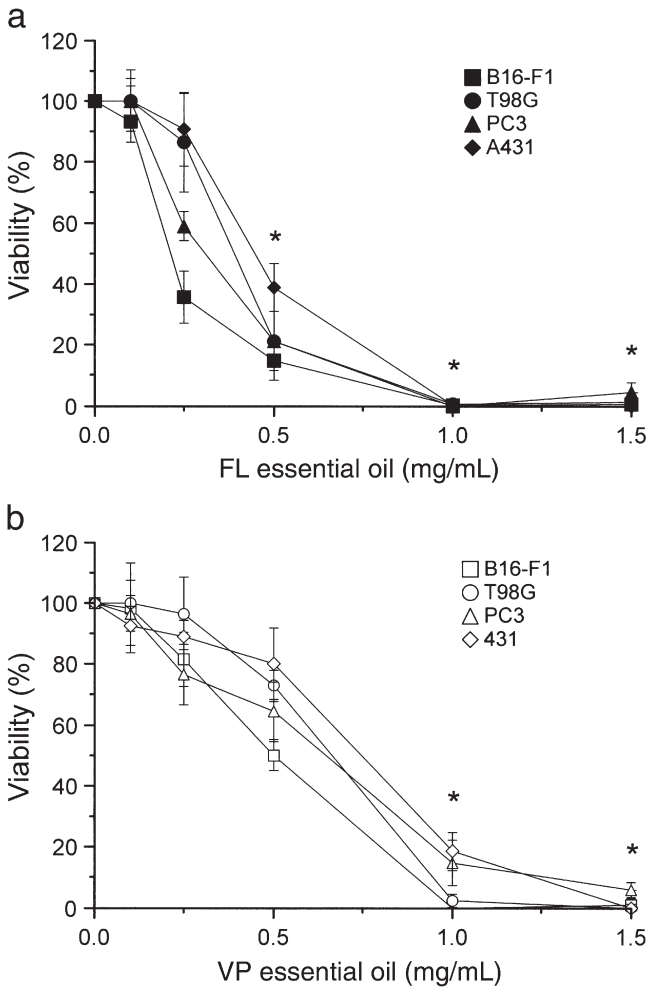

Fig. 1. B16-F1, T98G, PC3 and A431 cells were treated with different concentrations of FL (a) and VP (b) essential oils for 72 h. Cell viability was determined by MTT assay and is reported as the percentage of viable cells. The results represent the mean value (± SD) of three independent experiments; (a) and (b) $P<0.0001$, Dunnett's test; $^{*}P<0.01$ vs control without essential oil.

In this research, we decided also to test the antiproliferative activity of essential oil of *A. ligustica*, taking into account their evident antioxidant activity and the fact that in literature no data are available about this. We evaluated the antiproliferative effects of essential oils on a selection of tumour cell lines by MTT assay. Three human cell lines, a glioblastoma multiforme cell line (T98G), a squamous carcinoma cell lines (A431), a prostatic adenocarcinoma cell line (PC-3), and one murine cell line, a melanoma cell line (B16-F1), were treated with different concentrations of FL and VP essential oils and the effects on cell proliferation were recorded after 24, 48, and 72 h. As shown in Fig. 1, essential oils were active against all four tumour cell lines tested, with statistically significant higher activity of FL oil over VP oil ($P<0.01$). This result correlates well with the stronger antioxidant action of FL vs VP essential oil. The IC_{50} values, obtained after 72 h of incubation in presence of essential oils, were reported in Table 4. The data show that the highest

Table 4
Cytotoxic activity of *Achillea ligustica* essential oils given as IC_{50} value (mg/ml).

Oil samples	T98G	A431	PC3	B16-F1
FL[a]	0.376 ± 0.021	0.446 ± 0.039	0.294 ± 0.015	0.220 ± 0.022
VP[b]	0.663 ± 0.048*	0.847 ± 0.037*	0.593 ± 0.018**	0.459 ± 0.067*

Each value represents mean ± SD of three determinations; $^{*}P<0.01$ vs FL essential oil, $^{**}P<0.0001$ vs FL essential oil (unpaired *t*-test).
[a] FL, flowers (mg/ml).
[b] VP, vegetative parts (stems/leaves) (mg/ml).

activity was observed on B16-F1 cell line, with IC_{50} values of 0.220 ± 0.022 mg/ml and 0.459 ± 0.067 mg/ml for FL and VP oil, respectively. Lowest activity was obtained on A431, with IC_{50} values of 0.446 ± 0.039 mg/ml and 0.847 ± 0.037 mg/ml for FL and VP oil, respectively. The antiproliferative activity of *A. ligustica* essential oil could, of course, be due to single, specific components of the oils. In fact, it has been reported that terpinen-4-ol (5.30% in FL and 12.95% in VP oil, respectively) possesses some antiproliferative activity against human melanoma M14 cell line [24]. α-Bisabolol (0.52% in FL oil and 1.26% in VP oil) has been reported to be cytotoxic on human and rat glioma cells [25]. α-Humulene (0.15% in FL and 0.13% in VP oil, respectively) also exerts cytotoxic activity against several solid tumour cell lines as MCF-7, PC-3, A-549, DLD-1, M4BEU, and CT-26 [26]. β-Elemene (0.12% in FL oil and 0.16% in VP oil) has been reported to inhibit the proliferation of leukaemia cells (HL60 and K562) [27], glioma cells (SHG-44), and non-small-cell lung cancer cells (NSCLC) [28]. The above listed compounds, with demonstrated antiproliferative activity, together with the compounds present at the highest concentrations in FL and VP oils, were mixed in proper amount to reconstitute a sort of simplified essential oil, using commercially available compounds. Hence, (−)-linalool, (−)-β-pinene, (E)-caryophyllene, caryophyllene oxide, 1,8-cineole, terpinen-4-ol, α-humulene, α-bisabolol, and sabinene were mixed at the same percentage reported in Table 1 for FL and VP oils, and the solutions obtained were tested on the above cell lines. The cytotoxic activities resulted about eight-fold lower than those of the original essential oils (data not shown). This result is in relation with the fact that some other compounds, present in the extracted essential oil but not in "artificial" one, were active. Furthermore, minor components may potentiate, in a synergistic way, the action of compounds known to be active in this assay.

4. Conclusions

On the results of this work, it can be affirmed that *A. ligustica* presents several chemotypes, as a consequence of the strong variability in the essential oil compositions from different areas; this supports the hypothesis that the chemical compositions of *Achillea* species are complex and susceptible to variation [29]. The naturalized population investigated in this study resulted quite similar to those from Sicily and Greece in terms of concentration of the major volatile components, but resulted qualitatively richer, with a higher number of identified compounds. In fact, in our experiments 38 components were identified for the first time in *A. ligustica* essential oil, due to the high accuracy of our analytical procedure and to the origin of the plants, which were naturalized after cultivation in the area they were harvested for analysis. The essential oil showed an inhibition activity on some pathogen strains; in particular, it could be usefully employed in food, cosmetic and pharmaceutical applications, mainly toothpastes and mouthrinses, taking into account its anti-oral pathogen activity and the absence of the neurotoxic thujone. Our study confirmed also that *A. ligustica* essential oils possess antioxidant properties; assays on tumour cell lines showed a moderate cytotoxic activity on all tested cancer cells. Regarding the last two activities, essential oils show a very similar trend, with FL oil, richer in oxygenated hydrocarbons, being more active than VP oil.

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