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Chemical Composition and Antibacterial Potential of *Artemisia arborescens* L. Essential Oil

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Abstract This study was undertaken to characterize the essential oil (EO) of *Artemisia arborescens* growing wild in Sicily. EO, extracted by steam distillation, was examined for its chemical composition and for its capability to inhibit some food-borne pathogen bacteria. A total of 43 compounds (13 monoterpene hydrocarbons, 14 oxygenated monoterpenes, 10 sesquiterpene hydrocarbons, three oxygenated sesquiterpenes and less amount of other three compounds), which account 93.73% of the total oil, were identified by gas chromatography and gas chromatography–mass spectrometry. Oxygenated monoterpenes (57.32%) constituted the main fraction, with β-thujone as the main compound (45.04%), followed by the sesquiterpene hydrocarbon chamazulene (22.71%). Undiluted EO showed a large inhibition spectrum against strains of *Listeria monocytogenes* (34 out of 44), whilst it was ineffective against enterobacteria and salmonellas. The minimum inhibition concentration (MIC) was evaluated for the two most sensitive strains (*L. monocytogenes* 186 and 7BO) at two cellular concentrations (10^6 and 10^7 CFU ml^{-1}). The lowest MIC (0.625 µl ml^{-1}, dilution of oil with acetone) was found for strain *L. monocytogenes* 186 at 10^6 CFU ml^{-1}.

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Introduction

In recent years, consumers have become particularly aware of the health concerns regarding food additives. "Natural" and "traditional" foods, processed without any added chemical preservative, are becoming more and more attractive [42]. When chemical preservatives are used, the low levels used to avoid health implications expose the food-makers to a risk of poor stability and microbial contamination of the final products, since several microorganisms (pathogens and spoilage agents) may acquire a resistance [47]. In particular, the emergence of pathogens, which are resistant to classical preservatives, has determined an urgent necessity for alternative antimicrobial agents.

Several compounds found in plants, which have long been used as natural agents for food preservation [38], are generally well accepted. Amongst these naturally occurring compounds, essential oils (EOs) and extracts of various species of edible and medicinal plants, herbs and spices are considered by the food industry because of their antimicrobial potential. The aptitude of EOs to inhibit the growth of certain microorganisms is of paramount importance, particularly, when it is expressed against food-borne pathogens. Several microorganisms, especially bacteria, are responsible for food-associated diseases. One of the major microbial threats to the food safety in the past two decades is represented by the Gram-positive

Listeria monocytogenes, which has been recognized as an emerging agent of food-borne diseases [17]. Listeriosis has a very low incidence, but with a high case fatality rate that can exceed 30% [13]. Recently, several European countries are experiencing an apparently increasing incidence of listeriosis, mainly amongst persons aged 65 years and older [20].

Many spices have been consumed daily by mankind for millennia; they are added to the food matrices mainly to improve their taste and flavour; thus, the consumers are quite used to their presence. For these reasons, EOs from spices represent the natural additives easy to employ in the common applications of food preservation. However, the discovery of new active natural components may generate innovative food preservation strategies. Many examples may be found for plant-derived substances useful as food additives, with a broad spectrum of aptitudes including antioxidant [7, 21] and antimicrobial activities [5, 27]. Those characteristics are due to a number of secondary metabolites that may be found in various parts of the plants, and a strong interest is currently addressed to their individuation and exploitation [8]. So far, the approach applied to the study of herbal ingredients has been generally addressed to the identification of specific molecules (or group of molecules), inside the plant or its utilized part, which are directly responsible of a given (antioxidant, antimicrobial, etc.) action, the so-termed "active principles". As a matter of fact, the biological effect traditionally attributed to a plant-derived material, including EOs, is often due to the occurrence of synergistic and/or antagonistic effects amongst its various components. For food applications, the effectiveness of a specific EO as natural antimicrobial additive is studied considering the EO itself as a whole ingredient, rather than a mixture of components.

Artemisia arborescens (Vaill.) L. is a perennial evergreen woody shrub belonging to the family Asteraceae. The genus *Artemisia* comprises more than 200 species, and some of them (*A. absinthium*, *A. herba-alba*, *A. annua* and *A. vulgaris*), which are traditionally used in many areas as medicines, seasoning items or basic ingredients for the manufacturing of liqueurs, have been targeted to many studies concerning their phytochemical features [31]. Many *Artemisia* species may be found in dry areas of the Northern hemisphere [28]; about 20 species have been detected in Italy, and only five of them grow wild in Sicily [40]. Amongst these, *A. arborescens* is a strongly smelling, morphologically variable species (or mixture of species), up to 150 cm height, erect, many-branched, tomentose and whitish, silvery and glabrous in the youngest parts, bearing at flowering time (June–July) many small yellow flowers. Some authors [39] reported that *A. arborescens* was spread by Moorish invaders and Templar Knights at the time of Crusades. At present, the species is largely diffused in the Mediterranean area (Italy and North-Africa) and also in the Pacific North America areas.

The scientific interest towards *A. arborescens* goes back to the past decade. The studies carried out on this species were mainly concerned about its phytochemical characteristics [1, 29, 30, 32, 33, 39], but other aspects have been also considered, such as its genomics [19], its antiviral [43], antioxidant [11], anti-mycoplasmal [3] and allelochemical properties [14]. However, to our knowledge, no studies have been carried out on the antibacterial properties of EOs of this species.

This study is part of a project aimed at characterizing the EO of *A. arborescens* growing wild in Sicily. Based on the above considerations, the objectives of this study were to determine the chemical composition of EO and to evaluate its potential against common food-borne pathogens.

Materials and Methods

Plant Material and EO Preparation

Aerial parts of *A. arborescens* L., at the vegetative stage, were collected, in January 2010, from morphologically homogeneous wild populations grown in the North-western area of Sicily (Italy). The fresh plant material was subjected to steam distillation (Estrattore Albrigi Luigi-Verona, Italy) for 3 h to achieve EO.

Gas Chromatography and Mass Spectrometry

EO was first subjected to capillary gas liquid chromatography (GC) analysis using a Clarus 500GC Perkin-Elmer apparatus equipped with a flame ionization detector (FID), a Hewlett-Packard HP-1 (cross-linked methyl silicone) capillary column (30 m long, 0.2 mm i.d. with 0.33 mm film thickness). The column temperature program was 60°C for 5 min, with 3°C increases per min up to 180°C, then 20°C increases per min up to 280°C, which was maintained for 10 min. The carrier gas was helium at a flow rate of 1 ml min^{-1}. The FID and injector port temperature were maintained at 250 and 220°C, respectively.

EO was also analysed by gas chromatography–mass spectrometry (GC–MS) in a Varian Saturn 2000 equipped with a Varian C.S VA-5MS capillary column (30 m long, 0.25 mm i.d. with 0.25 mm film thickness). The same working conditions used for GC and split mode injection (ratio 1:25) were employed. Mass spectra were taken over the *m*/*z* 28–400 range with an ionizing voltage of 70 eV. Kovats retention index (KI) was calculated using co-chromatographed standard hydrocarbons. The individual compounds were identified by MS, and their identity

was confirmed by comparison of their KIs, relative to C_8–C_{32} *n*-alkanes, and by comparing their mass spectra and retention times with those of pure substances or with data available in the NIST 98 library and in the literature [2].

Microbial Strains

The bacterial strains used as indicators (sensitive to EO) for the inhibition assays belong to the culture collection of the Department of Health Promotion Sciences "G. D'Alessandro" (Palermo, Italy) and represent some species generally associated with food-borne diseases. *L. monocytogenes, Enterobacter* spp. and *Salmonella enterica* strains were subcultured in Brain Heart Infusion (BHI) agar (Oxoid) and incubated overnight at 37°C.

Antibacterial Activity Screening

EO of *A. arborescens* was tested for antibacterial activity applying the paper disc diffusion method [26] with a few modifications. An agar base medium consisting of nutrient agar was overlaid with 7 ml of BHI soft agar (0.7% w/v), previously inoculated with approximately 10^7 CFU ml^{-1} of a given strain to be tested for sensitivity versus EO. Four sterile filter paper discs (Whatman No. 1) of 6 mm diameter were placed onto the surface of the double agar layer, at a distance of approximately 3 cm from one another. Two discs were used as control and soaked with 10 µl of sterile water (negative control), or streptomycin [(10% w/v), positive control], whilst the remaining two discs were both soaked with 10 µl of EO. Plates were incubated at 37°C for 24 h and the inhibitory activity was evaluated as positive if a definite clear area was detected around the paper disc.

Determination of Minimum Inhibitory Concentration

The antibacterial activity of EO was measured as minimum inhibitory concentration (MIC), which represents the most common expression of EO antibacterial performances [5]. MIC is defined as the lowest concentration of an active compound inhibiting visible growth of test organisms [25]. In brief, a given EO is serially diluted (dilution factor = 2) using an organic solvent and all dilutions are tested against the sensitive strains.

The strains showing the highest sensitivity to the screening assay were used for MIC calculation. The EO was diluted in acetone, added to BHI broth medium and tested employing the sensitive strains at two different final cell concentrations (10^7 and 10^6 CFU ml^{-1}), in order to evaluate the effect of the number of cells on the sensitivity of the species. Acetone alone was used as negative control.

Results

Chemical Characterization of EO

Steam distillation produced a dark blue EO with a yield of 0.33% (v/w). This preparation was analysed by GC and GC–MS in order to determine its chemical composition. The qualitative and quantitative composition determined by GC and GC–MS is given in Table 1, where the compounds are classified by phytochemical groups and listed in order of their elution on a methyl silicone HP-1 column. Forty-three compounds were identified, accounting for the 93.37% of total oil. The essential oil was dominated by the monoterpene fraction with 13 monoterpene hydrocarbons (8.32%) and 14 oxygenated monoterpenes (57.32%) identified. Amongst the monoterpene hydrocarbons only sabinene (2.33%), myrcene (2.03%) and γ-terpinene (1.29%) ranged percentages higher than 1%. In the oxygen-containing monoterpenes, β-thujone (45.04%) followed by camphor (6.78%) and terpinen-4-ol (2.16%) were the main compounds.

On the other hand, in the sesquiterpene fraction (27.13%), large amount of the sesquiterpene hydrocarbon, chamazulene (22.71%), and trace amount of three oxygenated sesquiterpenes (dehydro-sesquicineole, germacrene-D-4-ol and caryophyllene oxide) were found.

Inhibition of Bacterial Growth

The antibacterial activity of *A. arborescens* EO is shown in Table 2. The spectrum of inhibition was evaluated against enterobacteria, *Listeria monocytogenes* and salmonellas. The active extract was effective only against listerias: 34 out of 44 strains where inhibited in growth. The strains belonging to this species showed a different level of sensitivity to the treatment. *L. monocytogenes* ATCC 19114^T resulted positive to the test, but its inhibition was low; the width of the clear area was barely 8 mm, including diameter of paper disc (6 mm). The strains showing the most interesting results were *L. monocytogenes* 186 and 7BO, for which a clear zone of 12 mm diameter was detected. For the majority of sensitive strains, an average inhibition diameter of 10 mm was registered. Due to the strongest inhibition determined by EO against *L. monocytogenes* 186 and 7BO, these two strains were further characterized for their sensitivity in terms of MIC. *E. cloacae* 13A was also used as negative control since it was not inhibited by EO.

L. monocytogenes 186 was found to be the most sensitive strain at both concentrations tested. EO showed an MIC of 2.5 and 1.25 µl ml^{-1} against strain *L. monocytogenes* 7BO at 10^7 and 10^6 CFU ml^{-1}, respectively, whilst the MIC was calculated to be 1.25 and 0.625 µl ml^{-1}

Table 1 Chemical composition of EO of *A. arborescens* (Vaill.) L. genotype growing wild in Sicily

Chemical compounds	KI[a]	%[b]
Monoterpene hydrocarbons		8.32
cis-Salvene	856	t
α-Thujene	930	t
α-Pinene	939	0.70
Camphene	954	0.61
Sabinene	975	2.33
β-Pinene	979	t
Myrcene	990	2.03
α-Phellandrene	1002	0.34
α-Terpinene	1014	0.69
p-Cymene	1024	0.33
Limonene	1029	t
γ-Terpinene	1054	1.29
Terpinolene	1088	t
Oxygenated monoterpenes		57.32
1,8-Cineole	1031	t
cis-Sabinene hydrate	1070	1.65
linalol	1096	0.22
trans-Sabinene hydrate	1098	t
α-Thujone	1102	0.75
β-Thujone	1114	45.04
Menth-2-en-1-ol	1121	t
Camphor	1146	6.78
Borneol	1169	t
Terpinen-4-ol	1177	2.16
α-Terpineol	1188	t
Carvacrol	1299	t
Neryl isovalerate	1583	t
Geranyl isovalerate	1607	0.72
Sesquiterpene hydrocarbons		27.13
α-Copaene	1376	0.19
β-Bourbonene	1388	t
β-Caryophyllene	1419	0.89
α-Humulene	1454	t
Germacrene-D	1484	3.34
Bicyclogermacrene	1500	t
α-Farsene	1505	t
Calacorene	1516	t
δ-Cadinene	1523	t
Chamazulene	1731	22.71
Oxygenated sesquiterpenes		t
Dehydro-sesquicineole-	1471	t
Germacrene- D-4-ol	1575	t
Caryophyllene oxide	1583	t
Others		0.96
6-Methyl-5-hepten-2-one	985	t
Methyl-butyl-2-methyl hydrate	1100	0.96
Methyl eugenol	1403	t
Total		94.87

[a] *KI* Kovats retention index relative to C_8–C_{32} *n*-alkanes

[b] Percentage of each compound on the total oil (computed from the total GC peak area)

against strain *L. monocytogenes* 186 at 10^7 and 10^6 CFU ml^{-1}, respectively.

Discussion

Plant-derived EOs enjoy a "natural" status and, for this reason, are generally recognized as safe by consumers, which accept well their use for food preservation purposes. Due to their antimicrobial potential, EOs are considered with attention by the food scientists. Furthermore, also the interest of the food industries is on the increase, thanks to the consumer demand for effective natural products. However, it became evident that EOs cannot be employed inappropriately, because of their adverse consequences for humans, e.g. some EOs are characterized by cancer-causing effects [34]. Hence, the evaluation of their safety constitutes an important point for the future application of EOs. Studies on human voluntaries and sensory evaluation by panels of experts are needed in order to test the safety of the plant-derived molecules and their acceptability by consumers. These operations are time-consuming and difficult to realize; thus, before any other evaluation, it is fundamental to test the efficacy of the new natural compounds with supposed antibacterial properties at least against the major food pathogens.

A. arborescens is widespread in Sicily, where it grows spontaneously. The chemical analysis of EO of plants collected within Palermo province (North-western Sicily) revealed that this species elaborated an essential oil rich in *β*-thujone (45.04%) and chamazulene (22.71%). The main compound, thujone, is an oxygenated monoterpene commonly found in spice and medicinal plants such as sage (*Salvia officinalis* L.), clary sage (*Salvia sclarea* L.), wormwood (*Artemisia absinthium* L.) and tansy (*Tanacetum vulgaris* L.). Thujone is present in some alcoholic beverages, but the isomer *β*-thujone is claimed to be toxic for human consumption [16, 35]. Thus, the use of thujone undergoes several restrictions, although the average intake by consumers is about 100 times lower than the NOEL (no-observed-effect level) derived from a 14-week study in rats [16]. Chamazulene, the second most abundant compound in *A. arborescens* EO, is a sesquiterpene hydrocarbon, characteristic of several plants of Asteraceae family, such

Table 2 Inhibitory activity of *A. arborescens* EO

Bacterial species	Strain (inhibition)	Source of isolation
E. amnigenus	DHPS70B3(−)	Freeze-dried chicken
E. amnigenus	DHPS60A2(−)	Freeze-dried lamb
E. cloacae	DHPS24(−), DHPS25(−)	Milk powder
E. cloacae	DHPS13A(−)	Multi-cereal cream
E. cloacae	DHPS62A(−)	Freeze-dried chicken
E. cloacae	DHPS 32A(−)	Milk flour
E. cloacae	DHPS43B1(−)	Semolina
E. hormaechei subsp. *steigerwaltii*	DHPS1(−), DHPS2(−), DHPS6(−), DHPS7(−), DHPS8(−), DHPS11(−), DHPS13 (−), DHPS15(−), DHPS19(−), DHPS31(−)	Milk powder
E. sakazaki	DHPS2B(−)	Rice cream
E. sakazaki	DHPS23A(−)	Green rice cream
L. monocytogenes	ATCC 19114^{T}(+)	Animal tissue
L. monocytogenes	DHPS129(+), DHPS130(+), DHPS131(+), DHPS132(−), DHPS133(−), DHPS134(−), DHPS135 (+), DHPS136(+), DHPS137(+), DHPS138(+), DHPS139(+), DHPS140(+)	Human
L. monocytogenes	DHPS179(+)	Salmon
L. monocytogenes	DHPS180(+), DHPS182(+)	Ricotta cheese
L. monocytogenes	DHPS184(−)	Rice salad
L. monocytogenes	DHPS185(−)	Beef
L. monocytogenes	DHPS186(++)	Mozzarella salad
L. monocytogenes	DHPS187(+)	Roasted chicken
L. monocytogenes	DHPS188(+)	Green salad
L. monocytogenes	DHPS1BO (+), DHPS10BO(+)	Chopped meat
L. monocytogenes	DHPS2BO(+), DHPS 3BO(+)	Fresh salami
L. monocytogenes	DHPS4BO(+), DHPS5BO (+)	3-week ripened salami
L. monocytogenes	DHPS6BO(+), DHPS7BO(++), DHPS8BO(−)	4-week ripened salami
L. monocytogenes	DHPS11BO(+)	Meat factory surfaces
L. monocytogenes	DHPS12BO(+)	1-week ripened salami
L. monocytogenes	DHPS13BO(+), DHPS14BO(−), DHPS15BO(−), DHPS16BO(−), DHPS7BO(+), DHPS18BO(+), DHPS19BO(+), DHPS20BO(+), DHPS21BO(−)	Gorgonzola cheese
L. monocytogenes	DHPS22BO(+), DHPS23BO(+), DHPS24BO(+)	Taleggio cheese
Salmonella Abony	DHPS50398(−)	Human
Salmonella Agona	DHPS50361(−)	Human
Salmonella Blockley	DHPS50314(−)	Human
Salmonella Bredeney	DHPS50374(−)	Human
Salmonella Derby	DHPS50399(−)	Human
Salmonella Enteritidis	DHPS50339(−), DHPS50430(−), DHPS50371(−)	Human
Salmonella Hadar	DHPS50272(−)	Human
Salmonella Infantis	DHPS50356(−)	Human
Salmonella Muenchen	DHPS50393(−)	Human
Salmonella Napoli	DHPS50376(−)	Human
Salmonella Newport	DHPS50404(−)	Human
Salmonella Panama	DHPS50347(−)	Human
Salmonella Saintpaul	DHPS50415(−)	Human
Salmonella Thompson	DHPS50280(−)	Human
Salmonella Typhimurium	DHPS50414(−), DHPS 50384(−)	Human
Salmonella Typhimurium	DHPS50432(−)	Seafood
Salmonella Veneziana	DHPS50391(−)	Human

–, no inhibition; +, clear inhibition (8–10 mm diameter); ++, strong inhibition (>10 mm diameter)

The culture collections are as follows: *DHPS* Department of Health Promotion Sciences "G. D'Alessandro" (Palermo, Italy); *ATCC* American Type Culture Collection (Manassa, VA, USA)

as chamomile and achillea. Indeed, it is an artefact molecule obtained from the precursore prochamazulene contained in plants' tissues [18, 46] during steam distillation. Chamazulene possesses interesting anti-inflammatory properties [41], and for this reason it has a relevant industrial importance. Our results showed that EO of *A. arborescens* growing wild within Palermo province is characterized by high concentrations of both compounds.

So far, *A. arborescens* plants examined for these characters have been divided into two chemotypes, high producers of thujone or high producers of chamazulene. It was also supposed that the biochemical process for the generation of thujone is in competition with that for the production of chamazulene [39]. Thus, this aspect deserves a deeper investigation. However, previous studies carried out on the chemical characterization of *A. arborescens* EO reported a different number of compounds identified, from 21 to 82 [1, 32]. Although chamazulene followed by camphor dominated the EO of plants collected in different areas of South Italy [32], the main compounds recognized from this species collected in the Algerian maritime littoral were the same (chamazulene and β-thujone), with similar high concentrations, found in our study. Thus, a third *A. arborescens* chemotype, dealing with high concentration of both β-thujone and chamazulene, might be proposed, even though other studies are needed to support this hypothesis. It is well known that geographical origin, environmental factors and the stage of plant development, as well as day and night duration, can affect the qualitative/quantitative composition of the oils [24, 36]. Moreover, the results of our study, compared with those previously published by Lo Presti et al. [32], which included also plants collected in Sicily, highlight the observations that different chemotypes may cohabit in restricted geographical areas.

In view of the possible future food application of *A. arborescens* EO, as natural alternative to traditional chemical additives, the antimicrobial activity of this mixture of chemical compounds has been evaluated. The inhibitory spectrum was evaluated against bacterial species (Enterobacteria, *L. monocytogenes* and salmonellas) responsible for human outbreaks commonly associated with food matrices [9, 22, 44]. All Gram-negative species (*Salmonella* spp. and *Enterobacter* spp.) used as test organisms were insensitive to the EO assayed. However, interestingly enough inhibition was observed with several strains of the food-borne pathogen *L. monocytogenes*, the only Gram-positive species used in this study. Also in other studies Gram-positive bacteria were found to be more susceptible to EO and various solvent extracts than Gram-negative bacteria [4, 6, 10]. This effect is related to the presence of an outer membrane on Gram-negative bacteria, which provides a strong impermeable barrier [37]. *L. monocytogenes* if often reported to be susceptible to EOs [12].

The highest inhibition was obtained with strains *L. monocytogenes* 186 and 7BO, both of food origin (mozzarella salad and ripened salami, respectively). This species has a ubiquitous nature and it is characterized by hardiness. Unlike many pathogens, *L. monocytogenes* survives to the food-processing technologies that rely on acidic or salty conditions [23], and shows a strong ability to multiply, although slowly, at low temperatures; thus, it may be found even in properly refrigerated foods. For all these reasons, this bacterium constitutes a relevant risk for consumers' health and its control by means of natural strategies is desirable. Several components of *A. arborescens* EO, including caryophyllene, caryophyllene oxide, α-humulene and germacrene-D, have been reported as important components of various EOs with enormous potential to inhibit microbial pathogens [15, 45, 48].

The above strains *L. monocytogenes* 186 and 7BO were further characterized for their sensitivity in terms of MIC. The test was carried out at two cell concentrations to determine the effect of the inoculums on the sensitivity level. The results showed that *A. arborescens* EO tested was active against high concentrations (10^7 CFU ml^{-1}) of *L. monocytogenes* 186, although its effect diminished with the increment of cell number.

In conclusion, the results showed by our study indicate that EO of *A. arborescens* is effective against *L. monocytogenes*, one of the major agents of food-borne illnesses; thus, it might represent a natural preservative, alternative to the common chemical additives. Works are being prepared in order to better characterize the in situ efficacy of this EO using food matrices, its suitability in active packaging strategies, and to evaluate its organoleptic acceptability by consumers, as well as its safety.

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