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Antibacterial activity of Tuscan *Artemisia annua* essential oil and its major components against some foodborne pathogens

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Abstract

Recently, the attention of researchers regarding *Artemisia annua* has been focused on the antimicrobial activity of the essential oil. This oil, rich in mono- and sesquiterpenes, has a strong activity against some Gram-negative and Gram-positive bacteria.

The aim of our study is to further contribute to the knowledge of the antibacterial activity of an *Artemisia annua* essential oil collected in Tuscany and its three most represented compounds (artemisia ketone, 1,8-cineole and camphor).

The essential oil obtained by hydrodistillation gave a yield of 0.37% (w/w fresh plant material). The composition of the essential oil extracted from flowering aerial parts was determined by gas chromatography (GC/FID and GC/MS).

The essential oil and compounds were tested for activity against *Escherichia coli* O157, *Salmonella* Enteritidis, *Salmonella* Typhi, *Yersinia enterocolitica* and *Listeria monocytogenes,* all of which have great significance in foodborne infections.

The antibacterial activity was tested using disk diffusion method and broth microdilution assay. The microorganisms tested were all sensitive to the *A. annua* essential oil *in toto* and to all its components, which often have lower activity than oil *in toto*.

Keywords: *Artemisia annua;* antimicrobial activity; foodborne pathogens; essential oil

1. Introduction

Throughout history, humankind has rightfully used extracts and essential oils of plants as natural food preservatives, antiseptics, and, sometimes, true therapeutic remedies, based simply on empirical experience. Although the pharmaceutical industry records thousands of molecules of new syntheses every year, scientific attention towards plants has not disappeared. Plants containing polyphenols are the most investigated herbal drugs for their antimicrobial and antioxidant activities (Quideau et al., 2011 and Vlase et al., 2014). However, in popular tradition, aromatic plants have often been used with success in the therapeutic field, such as the species *Artemisia annua* L., a very interesting plant because of its numerous constituents (Bilia et al., 2006, and Bilia et al., 2014).

This species, belonging to the Asteraceae family, is native to China. Cultivated for centuries in China and Vietnam for diverse medicinal uses, it is now naturalised in many other countries, such as Australia, Argentina, Brazil, Bulgaria, France, Hungary, Italy, Spain, Romania, the United States, and the former Yugoslavia. During the past few decades, the plant has become important in therapy because it is the unique source of artemisinin, a sesquiterpene lactone which represents one of the most important drugs in the treatment of malaria (Isacchi et al., 2011 and Isacchi et al., 2012).

Recently, the attention of researchers for this plant has also been focused on the antimicrobial activity of the essential oil, rich in mono- and sesquiterpenes, with a strong activity against some Gram-negative and Gram-positive bacteria and fungi (Bilia et al., 2014). A great variability in the essential oil content is reported in literature, attributed to geographical source, harvesting season, climate, drying process, and the part of the plant that is distilled, which are significant factors

52 influencing the chemical composition and relative proportions of the individual components in the
53 essential oil of *Artemisia annua* (Viuda-Martos et al., 2010). However, the activity does not seem to
54 be strongly related to the different chemical profiles (Bilia et al., 2014).
55 Plant extracts, and in particular essential oils, are now reported as potent natural antimicrobial
56 agents, in commercial fields as well as in clinical application, with interesting applications in
57 cosmetics and food, as recently described for *Rosmarinus officinalis* (Albu et al., 2004), *Eucalyptus*
58 *globolus* (Tyagi et al., 2014), *Satureja horvatii* (Bukvički et al., 2014), and *Coriandrum sativum*
59 (Michalczyk et al., 2012). The use of pre-cooked or prepared dishes for large communities presents
60 an ongoing risk for foodborne infections. Epidemiological data has confirmed that *Salmonella* is the
61 most frequently reported cause of foodborne outbreaks in the EU. In 2012, a total of 91,034
62 confirmed cases of human salmonellosis were reported, and the notification rate for confirmed cases
63 was 22.2 per 100,000 population. As in previous years, *S.* Enteritidis was the most frequently
64 reported serovar (41.3%) (EFSA, 2014).
65 A total of 5,671 confirmed verocytotoxigenic *Escherichia coli* infections were reported in 2012 in
66 the EU. Of those cases in which the serogroup was known, most were caused by serogroup O157,
67 followed by O26 and O91. There was an increasing European Union trend of confirmed human
68 verocytotoxigenic *Escherichia coli* infections in 2008–2012 (EFSA, 2014).
69 In 2012, 92 outbreaks caused by *Listeria*, *Shigella*, *Brucella*, *Francisella*, *Yersinia* and *Vibrio*
70 *parahaemolyticus* were reported, representing 1.7% of all outbreaks reported in the EU. Five of the
71 strong-evidence outbreaks were caused by *Listeria monocytogenes* (1,642 cases) (EFSA, 2014).
72 The aim of the present study is to further contribute to the knowledge of the antibacterial activity of
73 an *Artemisia annua* essential oil collected in Tuscany and its three most represented compounds,
74 namely artemisia ketone, 1,8-cineole and camphor. The microorganisms used in this study have
75 great significance in determining the occurrence of foodborne infections and, with the exception of
76 *S.* Enteritidis, had never been tested before for their susceptibility towards the *Artemisia annua*
77 essential oil.

2. Materials and methods

2.1. Chemical compounds

Artemisia ketone, camphor and 1,8-cineole were from Sigma–Aldrich Co. LLC. Purity by GC were ≥97.0%, ≥95.0% and 99%, respectively.

2.2. Extraction of the essential oil

The flowering aerial parts of *Artemisia annua* were collected at the end of October 2011 in Sesto Fiorentino, Tuscany, Italy. The plant was identified and the voucher specimen is deposited at the Phytolab, Department of Chemistry, under the authentication number AA 10/2011. The fresh plant material (1kg) was coarsely cut and hydrodistilled in a Clevenger-like apparatus for 2 h. The oils were dried over anhydrous Na_2SO_4, frozen, and stored in sealed vials at 4 °C before GC analysis. The oil was conserved at -22 °C for the GC-MS and microbiological assays.

2.3. GC/MS analysis of the essential oil

Gas chromatographic (GC) analyses were accomplished with an HP-5890 series II instrument equipped with an HP-5 capillary column (30 μm × 0.25 mm, 0.25 μm film thickness), working with the following temperature program: 60°C for 10 min, ramp of 5°C/min to 220°C; injector and detector temperatures, 250°C; carrier gas, nitrogen (2 mL/min); detector, dual flame ionization detection (FID); split ratio, 1 : 30; injection, 0.5 μL. The identification of the components was performed, for both columns, by comparison of their retention times with those of pure authentic samples and by means of their linear retention indices (LRI) relative to the series of -hydrocarbons. Gas chromatography-electron impact mass spectrometry (GC-EIMS) analyses were performed with a Varian CP 3800 gas chromatograph (Varian, Inc. Palo Alto, CA) equipped with a DB-5 capillary column (Agilent Technologies Hewlett-Packard, Waldbronn, Germany; 30 m × 0.25 mm, coating thickness 0.25 mm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions were as follows: injector and transfer line temperature at 250 and 240 °C, respectively, oven temperature

104 being programmed from 60 to 240 °C at 3 °C/min, carrier gas, helium at 1 mL/min, split less
105 injector. Identification of the constituents was based on comparison of the retention times with
106 those of the authentic samples, comparing their LRI relative to the series of n-hydrocarbons and on
107 computer matching against commercial and homemade library mass spectra built from pure
108 substances and components of known samples and MS literature data. Moreover, the molecular
109 weights of all the identified substances were confirmed by gas chromatography-chemical ionization
110 mass spectrometry (GC-CIMS), using methanol as chemical ionization gas. The GC-EIMS and GC-
111 CIMS analyses were performed with the same apparatus and analytical conditions. Only the CI-
112 mode was activated, and MeOH vapour as reagent gas was added. All procedures were according to
113 a previous publication (Flamini et al., 2004).
114
115 2.4. Microorganisms
116 Seven bacterial strains were tested: three were acquired from the American Type Culture
117 Collection: *Escherichia coli O157* (ATCC 35150), *Salmonella* Enteritidis (ATCC 13311),
118 *Salmonella* Typhi (ATCC 19430); one from CIP (Collection de l'Institut Pasteur): *Salmonella* Typhi
119 (CIP 6062); and three from the collection of the Department of Health Sciences of the University of
120 Florence: *Yersinia enterocolitica* (YeDHS11, isolated from cheese), *Yersinia enterocolitica*
121 (YeDHS17, isolated from unpasteurized milk), *Listeria monocytogenes* (LmDHS01, isolated from
122 cheese). The stock cultures were preserved in screw capped tubes (volume 15 ml) containing Muller
123 Hinton Agar slant (MHA-Oxoid Limited) at 4 °C and subcultured every two months. The cultures
124 were prepared by inoculating a loopful of each microorganism in 5 mL of Muller Hinton Broth
125 (MHB-Oxoid Limited). Broths were incubated at 37 °C for 24 hours. The suspension for each
126 microorganism was diluted with physiological solution (NaCl 0.85%) to obtain about 10^8 CFU mL^{-1}
127 evaluated by biophotometer (Eppendorf BioPhotometer) (OD 0.200 nm). To confirm this
128 concentration, 1 mL of the solution was poured in Petri dishes, and 20 mL of melted MHA cooled
129 to 45 °C was added. The plates were incubated at 37 °C for 24 h.

2.4.1. Antimicrobial disk susceptibility tests

The antibacterial activity of *Artemisia annua* essential oil and its three constituents (artemisia ketone, 1,8-cineole and camphor) was tested using disk diffusion method according to the standard procedure of the Clinical and Laboratory Standards Institute (CLSI, 2012). Standard 6 mm paper disks (International PBI srl) were placed on the surface of agar inoculated using spread plate technique. Then, paper disks were individually impregnated with 20 µL of the antimicrobial test solution. This test was performed as screening, and for this reason we used only one concentration. Standard antibiotic disks were used as positive controls, amoxicillin (10 µg/mL) for *E. coli*, *S.* Enteritidis, *S.* Typhi and *L. monocytogenes* and tetracycline (40 µg/mL) for *Y. enterocolitica*.

The Petri dishes were kept at 37 °C and incubated 24 h. After incubation, all plates were observed for zones of growth inhibition, and the diameters in millimeters of these zones were measured. Each assay was performed in triplicate, and the results were expressed as mean ± SD.

2.4.2. Broth microdilution assay (Minimal Bactericidal Concentration - MBC)

The MBC were determined by broth microdilution assay. In the wells of the microplate, scalar amounts of the oil and the other components were added. In each well 20 µL of MHB (Muller Hinton Broth-Oxoid Limited) with 0.5% Tween 80 and 20 µL of bacterial suspension (ca. 1×10^5 CFU mL^{-1}) were added. Therefore, serial dilutions from 600 to 70 µg/mL were obtained. After incubation (37 °C for 24h), an aliquot (60 µL) of each well was inoculated into plates containing MHA (Muller Hinton agar-Oxoid Limited). Plates were incubated for 24 h at 37 °C. MBC values have been calculated considering the plates where there was no growth.

3. Results and discussion

3.1. Composition of the essential oil

The essential oil obtained by hydrodistillation gave a yield of 0.37% (w/w fresh plant material). The composition of the essential oil extracted from flowering aerial parts was determined by gas chromatography (GC/FID and GC/MS). The GC essential oil profile is reported in Table 1. Twenty-

seven compounds, representing 95.3 mL/100 mL of the composition of the volatile oil, were identified. The predominant constituents (91%) were represented by monoterpenes (12.6% monoterpene hydrocarbons and 78.4% oxygenated ones). The main compounds were artemisia ketone (24%), camphor (17.7%) and 1,8-cineole (16.1%). Sesquiterpenes hydrocarbons represented only 4.1% of the total essential oil constituents, and germacrene D (1.5%) and β-caryophyllene (1.6%) were the main ones. Most of these components are present in many other essential oils such as rosemary, sage, and mint (Burt, 2004 and Mimica-Dukić et al., 2003), generally regarded as safe (GRAS) herbs (Duke, 2001).

3.2 Antimicrobial Activity

The antibacterial activity of *Artemisia annua* essential oil and its main constituents, the objects of this study, were tested qualitatively and quantitatively by determining the inhibition zones and MBC.

The antimicrobial disk susceptibility test was selected as a preliminary procedure for screening the antibacterial efficacy, and the results, with two positive controls (amoxicillin and tetracycline), are reported in Table 2. The most interesting results were obtained with the essential oil *in toto*; in fact, all bacteria tested were clearly sensitive to the oil, demonstrated by the presence of large inhibition zones. A notable result is related to the two strains of *Y. enterocolitica* that were more sensitive to oil *in toto* than the related positive control (1.5 cm *vs* 0.9 cm). In general, the inhibition zones of the three components tested had a smaller diameter than those obtained with the oil *in toto*. 1,8-cineole showed an inhibition zone bigger than oil in toto, but only against *S.* Typhi CIP 6062 (1.32 cm *vs* 1.25 cm).

The hydrophobic nature of most essential oils and plant extract components prevents their uniform diffusion through the agar medium; therefore, we performed MBC to have a complete view of the antibacterial activity of the compounds tested (Bilia et al., 2014). The hydrophobic properties are, in any case, decisive in order to destroy the microorganisms. The essential oils penetrate cell

membranes of bacteria and cause cell dysfunction, increasing permeability and removing ions and other cell contents (Massiha et al., 2013).

As also observed with the disk diffusion method, the oil *in toto* was more efficient with MBC values in the range of 0.18 - 23.5 mg/mL (Table 3). It is interesting to note that the microorganisms tested were all sensitive to the activity of the *A. annua* essential oil *in toto* and to all its components, confirming the results by disk diffusion method. The most sensitive microorganisms were *Salmonella* Enteritidis (ATCC 13311) and *Yersinia enterocolitica* (YeDHS17) with MBC of 0.18 mg/mL. However, we have to point out that there is variability between different strains of the same species. This is demonstrated by the different sensitivities of the two strains of *Yersinia* tested, which gave different results. The activities of the three components are often lower than oil *in toto*. Camphor showed microbicide activity with MBC generally lower than the other components, while it had negative results by disk diffusion method against most of the bacteria tested.

Most of our findings were consistent with those reported in literature (Radulović et al., 2013 and Bilia et al., 2014), while few data refer to the activity against *S.* Enteritidis and *E. coli*.

It should also be emphasized that the antimicrobial properties of Artemisia essential oil and its major constituents tested in this study against *Y. enterocolitica*, *S.* Typhi and *L. monocytogenes* have been screened for the first time.

The higher efficacy (ca. 10 times more) of the essential oil against *Yersinia enterocolitica* (YeDHS17) and *Salmonella* Enteritidis (ATCC 13311) is remarkable when compared to the pure compounds. For the other tests, both the essential oil and the isolated main constituents display a similar activity against the microorganisms.

Our results, like those of other authors, indicate low antimicrobial activity of all single tested components. Therefore, it is possible to assume that the antimicrobial activity can be determined by synergism and/or antagonism phenomena between the different components of the oil. It is not excluded that other non-tested components, reported in Table 1, may contribute to the increased activity of the essential oil *in toto* (Radulovic et al., 2013).

Further studies on the activity of this oil and its components, even minor, are therefore desirable, considering the current interest in natural preservatives as opposed to synthetic ones. In fact, Western society is experiencing a trend of 'green' consumerism, desiring products with minimal environmental impact (Burt, 2004).

4. Conclusion

Both the oil and the pure compounds have good efficacy in inhibiting microorganism growth, and can be considered potential alternatives to synthetic antimicrobials, although detailed studies regarding their mode of action and efficacy must be carried out before widespread application.

The chemical composition of the oil has led to the classification of this product as a safe one. In addition, numerous constituents have potential antioxidant properties which can confer additional value to the oil in the improvement of safety and shelf life of food, if the oil is added as a preservative.

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Table 1: Composition (%) and principal classes (%) of *Artemisia annua* essential oil.

Constituents	**LRI***	**Content %**
santolina triene	909	1.3
α-pinene	941	1.1
Camphene	956	2.5
Sabinene	978	1.9
β-pinene	982	0.8
Myrcene	992	4.6
yomogi alcohol	999	1.7
α-terpinene	1020	0.4
1,8-cineole	1035	16.1
artemisia ketone	1065	24.0
cis-sabinene hydrate	1071	0.2
artemisia alcohol	1084	7.4
Dehydrosabinaketone	1123	0.3
trans-pinocarveol	1141	0.4
Camphor	1148	17.7
β-pinene oxide	1159	5.4
Pinocarvone	1166	0.6
Borneol	1168	0.8
4-terpineol	1180	1.7
α-terpineol	1192	1.3
Myrtenol	1195	0.8
hexyl isovalerate	1243	0.2
α-copaene	1377	0.3
β-caryophyllene	1419	1.6
(E)-β-farnesene	1460	0.4
germacrene D	1482	1.5
Bicyclogermacrene	1495	0.3
Principal classes		
monoterpene hydrocarbons		12.6
oxygenated monoterpenes		78.4
sesquiterpene hydrocarbons		4.1
oxygenated sesquiterpenes		0.0
non-terpenoidic derivatives		0.2
Total identified		**95.3**

*LRI: linear retention indices relative to the series of *n*-hydrocarbon.

Table 2. Antimicrobial activity of *Artemisia annua* essential oil, artemisia ketone, 1,8-cineole and camphor using disk diffusion method. Diameters of inhibition zones in centimetres (± SD).

Bacterial strain	***Artemisia annua* (olio *in toto*)**	**Artemisia ketone**	**1,8-cineole**	**Camphor**	**Tetracycline (40 µg/mL)**	**Amoxicillin (10 µg/mL)**
Escherichia coli O157 (ATCC 35150)	1.27 ± 0.31	0.75 ± 0.07	1.03 ± 0.25	0	-	1.86 ± 0.09
Salmonella Enteritidis (ATCC 13311)	2.33 ± 0.29	1.43 ± 0.11	1.45 ± 0.07	0.75 ± 0.07	-	2.72 ± 0.16
Salmonella Typhi (ATCC 19430)	1.27 ± 0.15	0.80 ± 0.02	1.20 ± 0.14	0	-	2.42 ± 0.16
Salmonella Typhi (CIP 6062)	1.25 ± 0.21	0	1.32 ± 0.11	0.85 ± 0.07	-	2.65 ± 0.10
Yersinia enterocolitica (YeDHS11)	1.50 ± 0.10	0.90 ± 0.14	1.35 ± 0.07	0	0.92 ± 0.13	-
Yersinia enterocolitica (YeDHS17)	1.50 ± 0.02	0.95 ± 0.07	1.25 ± 0.07	0	0.92 ± 0.08	-
Listeria monocytogenes (LmDHS01)	1.60 ± 0.96	0.72 ± 0.04	0.70 ± 0.02	0	-	3.20 ± 0.12

Table 3. Antimicrobial activity of *Artemisia annua* essential oil, artemisia ketone, 1,8-cineole and camphor using Minimum Bactericidal Concentrations.

Bacterial strain	***Artemisia annua* (olio *in toto*)** (mg/mL)	**Artemisia ketone** (mg/mL)	**1,8-cineole** (mg/mL)	**Camphor** (mg/mL)
Escherichia coli O157 (ATCC 35150)	17.6	23.2	24.6	20.5
Salmonella Enteritidis (ATCC 13311)	0.18	11.6	12.3	15.4
Salmonella Typhi (ATCC 19430)	11.8	23.2	18.4	20.5
Salmonella Typhi (CIP 6062)	17.6	17.4	18.4	15.4
Yersinia enterocolitica (YeDHS11)	23.5	23.2	24.6	25.6
Yersinia enterocolitica (YeDHS17)	0.18	11.6	12.3	15.4
Listeria monocytogenes (LmDHS01)	17.6	23.2	24.6	25.6

Highlights

Antibacterial activity of *Artemisia annua* essential oil and several of its compounds.

Antimicrobial activity against foodborne pathogens.

The microorganisms tested were all sensitive to *A. annua*.

The most sensitive microorganisms were *S.* Enteritidis and *Y. enterocolitica*.