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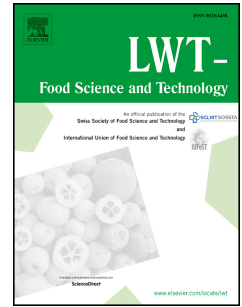
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Antibacterial activity of Tuscan *Artemisia annua* essential oil and its major components against some foodborne pathogens

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

3  
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12  
13 **Abstract**

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

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

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

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

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

29

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

31

## 32 1. Introduction

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

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

47 Recently, the attention of researchers for this plant has also been focused on the antimicrobial  
48 activity of the essential oil, rich in mono- and sesquiterpenes, with a strong activity against some  
49 Gram-negative and Gram-positive bacteria and fungi (Bilia et al., 2014). A great variability in the  
50 essential oil content is reported in literature, attributed to geographical source, harvesting season,  
51 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.

## 78 2. Materials and methods

### 79 2.1. Chemical compounds

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

82

### 83 2.2. Extraction of the essential oil

84 The flowering aerial parts of *Artemisia annua* were collected at the end of October 2011 in Sesto  
85 Fiorentino, Tuscany, Italy. The plant was identified and the voucher specimen is deposited at the  
86 Phytolab, Department of Chemistry, under the authentication number AA 10/2011. The fresh plant  
87 material (1kg) was coarsely cut and hydrodistilled in a Clevenger-like apparatus for 2 h. The oils  
88 were dried over anhydrous  $\text{Na}_2\text{SO}_4$ , frozen, and stored in sealed vials at 4 °C before GC analysis.  
89 The oil was conserved at -22 °C for the GC-MS and microbiological assays.

90

### 91 2.3. GC/MS analysis of the essential oil

92 Gas chromatographic (GC) analyses were accomplished with an HP-5890 series II instrument  
93 equipped with an HP-5 capillary column (30  $\mu\text{m} \times 0.25$  mm, 0.25  $\mu\text{m}$  film thickness), working with  
94 the following temperature program: 60°C for 10 min, ramp of 5°C/min to 220°C; injector and  
95 detector temperatures, 250°C; carrier gas, nitrogen (2 mL/min); detector, dual flame ionization  
96 detection (FID); split ratio, 1 : 30; injection, 0.5  $\mu\text{L}$ . The identification of the components was  
97 performed, for both columns, by comparison of their retention times with those of pure authentic  
98 samples and by means of their linear retention indices (LRI) relative to the series of -hydrocarbons.  
99 Gas chromatography-electron impact mass spectrometry (GC-EIMS) analyses were performed with  
100 a Varian CP 3800 gas chromatograph (Varian, Inc. Palo Alto, CA) equipped with a DB-5 capillary  
101 column (Agilent Technologies Hewlett-Packard, Waldbronn, Germany; 30 m  $\times$  0.25 mm, coating  
102 thickness 0.25  $\mu\text{m}$ ) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions were as  
103 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<sup>-1</sup>  
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.

#### 130 2.4.1. Antimicrobial disk susceptibility tests

131 The antibacterial activity of *Artemisia annua* essential oil and its three constituents (artemisia  
132 ketone, 1,8-cineole and camphor) was tested using disk diffusion method according to the standard  
133 procedure of the Clinical and Laboratory Standards Institute (CLSI, 2012). Standard 6 mm paper  
134 disks (International PBI srl) were placed on the surface of agar inoculated using spread plate  
135 technique. Then, paper disks were individually impregnated with 20  $\mu\text{L}$  of the antimicrobial test  
136 solution. This test was performed as screening, and for this reason we used only one concentration.  
137 Standard antibiotic disks were used as positive controls, amoxicillin (10  $\mu\text{g}/\text{mL}$ ) for *E. coli*, *S.*  
138 *Enteritidis*, *S. Typhi* and *L. monocytogenes* and tetracycline (40  $\mu\text{g}/\text{mL}$ ) for *Y. enterocolitica*.  
139 The Petri dishes were kept at 37 °C and incubated 24 h. After incubation, all plates were observed  
140 for zones of growth inhibition, and the diameters in millimeters of these zones were measured. Each  
141 assay was performed in triplicate, and the results were expressed as mean  $\pm$  SD.

#### 142 2.4.2. Broth microdilution assay (Minimal Bactericidal Concentration - MBC)

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

150

### 151 3. Results and discussion

#### 152 3.1. Composition of the essential oil

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

156 seven compounds, representing 95.3 mL/100 mL of the composition of the volatile oil, were  
157 identified. The predominant constituents (91%) were represented by monoterpenes (12.6%  
158 monoterpene hydrocarbons and 78.4% oxygenated ones). The main compounds were artemisia  
159 ketone (24%), camphor (17.7%) and 1,8-cineole (16.1%). Sesquiterpenes hydrocarbons represented  
160 only 4.1% of the total essential oil constituents, and germacrene D (1.5%) and  $\beta$ -caryophyllene  
161 (1.6%) were the main ones. Most of these components are present in many other essential oils such  
162 as rosemary, sage, and mint (Burt, 2004 and Mimica-Dukić et al., 2003), generally regarded as safe  
163 (GRAS) herbs (Duke, 2001).

164

### 165 3.2 Antimicrobial Activity

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

169 The antimicrobial disk susceptibility test was selected as a preliminary procedure for screening the  
170 antibacterial efficacy, and the results, with two positive controls (amoxicillin and tetracycline), are  
171 reported in Table 2. The most interesting results were obtained with the essential oil *in toto*; in fact,  
172 all bacteria tested were clearly sensitive to the oil, demonstrated by the presence of large inhibition  
173 zones. A notable result is related to the two strains of *Y. enterocolitica* that were more sensitive to  
174 oil *in toto* than the related positive control (1.5 cm vs 0.9 cm). In general, the inhibition zones of the  
175 three components tested had a smaller diameter than those obtained with the oil *in toto*. 1,8-cineole  
176 showed an inhibition zone bigger than oil *in toto*, but only against *S. Typhi* CIP 6062 (1.32 cm vs  
177 1.25 cm).

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

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

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

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

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

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

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

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

#### 212 4. Conclusion

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

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

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283

ACCEPTED MANUSCRIPT

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

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

\*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 ( $\pm$  SD).

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

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

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