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Antibacterial activity of essential oils, their blends and mixtures of their main constituents against some strains supporting livestock mastitis

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ABSTRACT

Ten of the most known and used commercial essential oils (*Cinnamomum zeylanicum* L., *Citrus bergamia* Risso, *Eucalyptus globulus* Labill., *Foeniculum vulgare* Mill., *Origanum majorana* L., *Origanum vulgare* L., *Rosmarinus officinalis* L., *Satureja montana* L., *Thymus vulgaris* L. ct. carvacrol, *Thymus vulgaris* L. ct. thymol) were tested against six bacteria strains *Staphylococcus aureus*, *Staphylococcus chromogenes*, *Staphylococcus sciuri*, *Staphylococcus warneri*, *Staphylococcus xylosus* and *Escherichia coli*, responsible for mastitis in animals. The best results were achieved by *S. montana*, *T. vulgaris* ct. thymol and *O. vulgare*. Two binary mixtures of essential oils (EOs) were prepared of *S. montana* and *T. vulgaris* ct. thymol (ST) and of *S. montana* and *O. vulgare* (SO). The ST mixture exhibited the best inhibitory activity against all the tested bacterial strains. Two artificial mixtures of carvacrol/thymol (AB) and carvacrol/thymol/*p*-cymene (CD) were prepared and tested against all of the bacterial strains used. The results exhibited a general reduction of the inhibitory activity of mixture AB, although not reaching the inhibition of the ST and SO mixtures. However the mixture CD presented an apparent strong inhibition against *S. aureus* and *S. sciuri*. The EO mixtures and the mixture CD represent promising phytotherapic approaches against bacteria strains responsible for environmental mastitis.

1. Introduction

Mastitis is defined as an inflammatory reaction of the mammary gland induced when pathogenic microorganisms in the udder produce toxins that are harmful to the mammary gland [1]. As a result of the inflammation, milk composition is altered with a decrease of caseins/lactose synthesis and fat quality [2,3]. Mastitis can be clinical or subclinical and represents a relevant damage for the breeders because of milk waste, loss of udder functionality and sometimes death of the animal. The clinic forms of mastitis can be hyper acute, acute and chronic. The first two forms are mainly caused by *Staphylococcus aureus* and occasionally by *Pseudomonas aeruginosa* and *Pasteurella spp.* (*Mannheimia*), while *Mycoplasma agalactiae*, *Streptococcus agalactiae*, and *Staphylococcus epidermidis* are often involved in the chronic

Abbreviations: EO, essential oil.

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form [4]. *Staphylococcus spp.* is the main causative agent of bovine mastitis, with higher prevalence in cases of clinical and subclinical manifestations [5]. In the environmental mastitis *Staphylococcus spp.* and *Escherichia coli* are the main pathogens responsible for the inflammation [6] and, together with coagulase-negative strains, are the most frequent pathogens, particularly such as *S. epidermidis*, *Staphylococcus simulans*, *Staphylococcus hyicus*, *Staphylococcus sciuri* and *Staphylococcus xylosus* in ovine mastitis [7]. Clinical mastitis leads to a significant decrease of the quality, milk and cheese production [8]. Worldwide, economic losses due to this infection have been estimated at $35 billion [9]. The most common treatment of mastitis is based on intramammary infusion of antibacterial agents. A large number of commercial antibiotics cause drug resistance, super infections and alteration of enteric microbiota, and negative repercussions due to an increase of the chemoresistance of certain bacterial strains [10]. Moreover an overuse or an untargeted utilisation of antibiotics can lead to serious consequences for public health. In fact the current guidelines of WHO recommend to limit the antibiotic utilisation in livestock, especially in organic farms [11]. Therefore, it is necessary to develop alternative natural and safe methods for controlling infections. Medicinal and aromatic plants (MAPs) are well known to have antibacterial activity against different pathogenic agents. Alternative treatments to bovine mastitis were carried out with natural compounds from plants giving interesting results for new phytotherapic approaches [6,12].

Essential oils (EOs) and their constituent's antiseptic properties are well known and many scientific investigations were performed to test their antimicrobial activity in the last twenty years [13–16]. Today the use of EOs and herbs to protecting livestock from infections mainly in organic farms is becoming a common practice [17].

The aim of the present work was to test the antimicrobial activities of ten EOs (*Cinnamomum zeylanicum* L., *Citrus bergamia* Risso, *Eucalyptus globulus* Labill., *Foeniculum vulgare* Mill., *Origanum vulgare* L., *Origanum majorana* L., *Rosmarinus officinalis* L., *Satureja montana* L., *Thymus vulgaris* L. ct. carvacrol and *T. vulgaris* L. ct. thymol), two selected mixtures of EOs, and two artificial mixtures of their main constituents (thymol, carvacrol and *p*-cymene) against the bacterial strains involved in the pathogenesis of mastitis. The EOs, chosen on the basis on the antimicrobial activity reported in the literature [18–22] and their availability on the market, were tested against *S. aureus*, *Staphylococcus chromogenes*, *Staphylococcus warneri*, *S. xylosus*, *S. sciuri* and *E. coli*.

The chemical characterization of the tested EOs was performed by GC–MS to establish a relationship between their composition and their activity. The analysis of EO composition is essential to confirm the presence and concentration of the active compounds whose antimicrobial effect is well known from the literature against the target bacteria [23].

2. Experimental sections

2.1. Chemicals

The linear alkane hydrocarbons (C_9–C_{32}) and the standard volatile compounds used were commercial substances purchased from FLUKA (Sigma-Aldrich, St Louis, MO) or isolated substances with 98–99% pure grade. The stock and working solutions were prepared using *n*-hexane HPLC grade (Carlo Erba, Milano, IT).

2.2. Essential oils

The essential oils (EOs) tested *C. zeylanicum L.* (*Cz*), *C. bergamia* Risso (*Cb*), *E. globulus* Labill. (*Eg*), *F. vulgare* Mill. (*Fv*), *O. majorana* L. (*Om*), *O. vulgare* L. (*Ov*), *R. officinalis* L. (*Ro*), *S. montana* L. (*Sm*), *T. vulgaris* L. ct. carvacrol (*Tvc*) and *T. vulgaris* L.ct. thymol (*Tvt*), were purchased directly from the market (FLORA®, Pisa, Italy) in June 2011.

2.3. Gas chromatography–mass spectrometry (GC–MS)

GC/EIMS (Gas chromatography/Electron impact mass spectrometry) analyses were performed with a Varian CP-3800 gas chromatograph equipped with a DB-5 capillary column (30 m × 0.25 mm; coating thickness 0.25 μm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions: injector and transfer line temperatures 220 and 240 °C respectively; oven temperature programmed from 60 °C to 240 °C at 3 °C/min; carrier gas helium, at 1 ml/min; injection of 0.5 μl (1% hexane solution); and split ratio of 1:30. Identifications of the constituents were based on comparison of retention times with those of authentic samples, comparing their retention indices relative to the series of *n*-hydrocarbons and on computer matching against commercial mass spectral libraries (NIST 98 and ADAMS) [24] as well as a homemade library, built up from pure substances or known oils and MS literature data [25].

2.4. Quantitative analysis of carvacrol, p-cymene and thymol

Quantification of the main components (carvacrol, *p*-cymene and thymol) present in EOs of *Sm* and *Tvt* was performed using a suitable internal standard (IS) added to the volatile oils [15,23]. *n*-Nonanol (10 mg/ml in *n*-hexane) was chosen as IS and eluted at 12.82 min under the conditions of the GC–MS analysis. The standard calibration curves relative to the thymol, carvacrol and *p*-cymene were determined by gas-chromatographic injection of five different concentrations of pure compounds and an accurate concentration of the IS solution. The mass percent of composition of the main components was determined by the injection of 1 ml of a solution obtained by mixing a volume of EOs diluted at 250 mg/ml in *n*-hexane with the same volume of internal standard solution at 10 mg/ml. The results are shown in Table 1, with the relative calibration curve equation and the correlation coefficient (R^2) of the regression line of standard. The relative regression line was calculated from five points.

2.5. Preparation of tested mixtures

The EO mixtures were prepared maintaining the same concentration of the single main component present in original EO. The mixture called ST was obtained by mixing 100 μl of a solution S (10 μl of *Sm* EO in 90 μl of dimethylsulfoxide (DMSO) and 100 μl of a solution T (10 μl of *Tvt* EO in 90 μl of DMSO) to give 200 μl of the total mixture,

Table 1
Presence of thymol, carvacrol and *p*-cymene in pure EOs of *S. montana* (*Sm*), and *T. vulgaris* ct. thymol (*Tvt*). Data are expressed as mg/100 mg EO.

$Y = 0.8573x + 0.0353$; $R^2 = 0.99971$; detection limit (δ) (mg/ml) 0.007		
Components	*Sm*	*Tvt*
Thymol	2.29	50.18
Carvacrol	43.06	4.00
p-Cymene	9.52	16.19

from which 20 μl were used for biological test. The mixture called SO was obtained in a similar way: by mixing 100 μl of a solution S (10 μl of *Sm* EO in 90 μl of DMSO) and 100 μl of a solution O (10 μl of *Ov* EO in 90 μl of DMSO) to give 200 μl of the total mixture, from which 20 μl were used for biological test.

The mixture AB with two pure compounds, was prepared as follows: the solution A was prepared by mixing 50.18 mg of thymol, 4.00 mg of carvacrol and 45.82 mg of DMSO for a total amount of 100 mg as in *Tvt* EO, while the solution B was obtained by mixing 2.29 mg of thymol, 43.06 mg of carvacrol and 54.65 mg of DMSO for a total amount of 100 mg as in *Sm* EO. Then 10 μl of A were diluted with 90 μl of DMSO and 10 μl of (B) were diluted with 90 μl of DMSO, respectively. Successively the two solutions were mixed together to obtain 200 μl of total mixture AB, and 20 μl of this mixture were used to distribute on filter paper discs.

The pure *p*-cymene is also tested and was prepared by mixing 10 μl of pure compound diluted to 100 μl with DMSO. The mixture CD with three pure compounds was prepared as follows: solution C was obtained by mixing 50.18 mg of thymol, 4.00 mg of carvacrol, 9.52 mg of *p*-cymene and 36.30 mg of DMSO for a total amount of 100 mg as in *Tvt* EO. The solution D contained 2.29 mg of thymol, 43.06 mg of carvacrol, 16.19 mg of *p*-cymene, and 38.46 mg of DMSO, for a total amount of 100 mg as in *Sm* EO. Ten microliters of C were diluted with 90 μl of DMSO and 10 μl of D were diluted with 90 μl of DMSO. The two solutions were mixed to obtain 200 μl of total mixture, and 20 μl of this mixture were used to distribute on filter paper discs. All the EOs and the mixtures were dried with sodium sulphate and stored at −20 °C until the analysis.

2.6. Antibacterial tests

Ten EOs, two binary EO mixtures (ST and SO) as well as two artificial mixtures of their main constituents AB and CD were tested for antimicrobial activity against the following bacterial strains: *S. aureus* (ATCC 6538), *Escherichia coli* (ATCC 25422) belonging to the American Type of Culture Collection (ATCC), *S. chromogenes*, *S. warneri*, *S. xylosus* and *S. sciuri*. All the bacterial strains used, except those already above indicated as ATCC (American Type of Culture Collection) are wild strains isolated by episodes of clinical or subclinical sheep/goat mastitis.

Bacterial strains stored at −80 °C in glycerol suspension were sowed on tryptic soy agar (TSA) and incubated overnight at 37 °C. Subsequently one colony from these cultures was inoculated in brain heart infusion broth (BHI) and incubated at 37 °C for 24 h with shaking in order to obtain freshly cultured microbial suspensions. The antibacterial activity of EOs, their mixtures and reference solutions were carried out using the paper disc diffusion method according to the Kirby–Bauer method [26].

Before the assay, the microbial suspensions were adjusted to $1 * 10^7$ CFU/ml corresponding to 0.5 McFarland and spread in Mueller–Hinton agar plates (MH) employing a sterile cotton swab. After a few minutes filter paper discs of 6 mm diameter were placed on the surface of inoculated plates and impregnated with 10 μl of each essential oil and reference solutions 1:10 diluted in DMSO. Furthermore filter paper discs of 6 mm diameter were placed on the surface of inoculated plates and impregnated with 20 μl respectively of (ST and SO) solution, AB and ABC mixtures prepared as above described. Negative controls were prepared using a filter paper disc impregnated only with 10 μl of DMSO. Positive controls were prepared using paper discs impregnated with tetracycline (30 μg), ciprofloxacin (5 μg), and gentamycin (10 μg). Triplicate plates for each oil/substance were used. Antimicrobial activity was evaluated by measuring the diameter (in mm) of the growth inhibition zones. All the EOs were stored at room temperature in the dark and were subjected to microbial analysis for quality control before their employment in the tests. Dilutions of each oil carried out in peptone water were spread onto agar plate count (APC) and these were enumerated after incubation at 30 °C for 72 h.

3. Results and discussion

The aim of this study was to find EOs with a good antibacterial activity with particular attention to the efficacy on the gram-positive bacterium *S. aureus*, one of the most common causes of clinical and subclinical infections [4,5]. Ten commercial EOs were chosen and analysed by GC–MS and showed a quite different composition of the volatile compounds (Table 2). The percentage of identified compounds ranged between 76.60% of *Cz* to 99.8% of *Cb*. 1,8-Cineole (84.88%) was the highest compound identified in *Eg*, while carvacrol was the main compound found in *Ov*, *Sm* and *Tvc* with a relative concentration of 65.94, 47.10 and 39.83%, respectively. Furthermore *Cz*, *Tvt* and *Fv* contained high amounts of eugenol (64.77%), thymol (52.61%) and (E)-anethole (54.51%), respectively.

All the analysed EOs were tested for the in vitro antibacterial activity against six bacteria strains responsible of mastitis infection in cows and sheep (*S. aureus*, *S. chromogenes*, *S. warneri*, *S. xylosus*, *S. sciuri*, *E. coli*) using the Kirby–Bauer method. Their inhibition zone is shown in Table 3. EOs from *Sm* and *Tvt*, characterized by the presence of thymol and carvacrol as main constituents, resulted to be the most active against all the tested strains. In fact, the antimicrobial activity of *S. montana*, *T. vulgaris* and *O. vulgare* against different *Staphylococcus spp.* was already well documented [27,28]. *Sm* EO confirmed its strong activity against the *S. aureus* strain (inhibition zone of 21.7 mm) as reported in the literature [18,21]. *Sm* EO exhibited also a good efficacy against the other strains with inhibition area of 11.3 mm (*S. chromogenes*), 12.0 mm (*S. warneri*), 12.7 mm (*S. xylosus*), and 11.7 mm (*S. sciuri*), respectively. Moreover *Sm* EO was the only one with an inhibition zone of 13.3 mm against *E. coli*,

Table 2
Relative percentage of the main constituents of essential oils detected by GC–MS analysis. *Cinnamomum zeylanicum* L. (*Cz*), *Citrus bergamia* Risso (*Cb*), *Eucalyptus globulus* Labill. (*Eg*), *Foeniculum vulgare* Mill. (*Fv*), *Origanum majorana* L. (*Om*), *Origanum vulgare* L. (*Ov*), *Rosmarinus officinalis* L. (*Ro*), *Satureja montana* L. (*Sm*), *Thymus vulgaris* L. ct. carvacrol (*Tvc*) and *T. vulgaris* L.ct. thymol (*Tvt*).

Compound	LRI[a]	*Cb*	*Cz*	*Eg*	*Fv*	*Om*	*Ov*	*Ro*	*Sm*	*Tvc*	*Tvt*
(*E*)-2-Hexenal	860	0.37		0.19					0.04	0.04	0.13
1-Hexanol	875		0.11								
Heptanal	899				0.13						
Santolina triene	910							0.31			
α-Thujene	931		0.07		0.06	0.84	0.84	0.83	0.29	0.37	0.11
α-Pinene	939	1.41	0.62	5.57	9.09	0.72	0.98	24.33	0.55	0.74	0.85
α-Fenchene	951						0.14				
α-Thujone	951								0.07		
Camphene	953		0.25		0.38			7.27	0.29	0.47	0.28
Thuja-2,4(10)-diene	959							0.25			
Benzaldehyde	965		0.12								
n-Heptanol	969			0.14	0.13			0.10	0.07	0.02	0.06
Sabinene	976	1.43			0.24	3.22					
1-Octen-3-ol	978									0.72	0.54
β-Pinene	980	7.49	0.23	0.47	0.95	0.67	0.43	3.75	0.91		
Myrcene	991	1.06	0.16	0.65	1.06	1.55	2.2	2.43	0.96	0.97	0.66
3-Octanone	984							0.42		0.06	0.07
3-Octanol	993					0.07	0.05	0.10	0.05	0.06	
α-Phellandrene	1005		0.3	0.14	1.63	0.18	0.28	2.32	0.14	0.14	0.09
δ-Carene	1011		0.06					0.08	0.05	0.09	0.07
α-Terpinene	1018	0.21	0.05			4.73	2.05	1.17	1.17	1.31	0.82
p-Cymene	1026	0.48	0.98	5.29	2.79	4.17	9.33	1.42	8.96	15.22	15.25
Limonene	1031	34.86			2.83	2.13	0.65				0.41
1,8-Cineole	1033		0.14	84.88	0.03	0.09	0.82	20.29	1.01	1.02	0.66
(*Z*)-β-Ocimene	1040			0.13	0.13				0.16		
(*E*)-β-Ocimene	1050	0.25		0.02		0.05	0.08		0.08	0.03	
γ-Terpinene	1062	8.24		0.78	0.34	7.90	5.25	1.62	6.06	5.04	2.92
cis-Sabinene hydrate	1070				0.07	3.16	0.29	0.09	0.50	0.66	0.10
Terpinolene	1088	0.44	0.06			1.53	0.26	1.25	0.17	0.22	0.19
Fenchone	1087				17.40						
Linalool	1097	7.32						0.95	3.11		
trans-Sabinene hydrate	1098					12.83	1.78			3.76	3.76
Mentha-2-en-1-ol<*cis*-p>	1121					0.87					
Chrysantenone	1128							0.31			
trans-Pinocarveol	1139			0.36						0.05	
cis-Pinene hydrate	1144					0.63					
Camphor	1146				0.51	0.18	0.05	8.33	0.74	0.32	0.51
Borneol	1169					0.18	0.27	2.90	2.05	1.06	1.56
cis-Pinocamphone	1175								0.69		
4-Terpineol	1177		0.08	0.06	0.12	17.61	0.9	1.56	0.90	0.79	2.41
iso-Verbanol	1180					0.23					
α-Terpineol 13,44	1189	0.14	0.29	0.17		2.73	0.2	1.70	0.41		0.39
neoiso-Verbanol	1190					0.23					
Verbenone	1205					0.33		2.53			
Thymol methyl ether	1235									0.66	1.72
Neral	1240	0.20									
Cumin aldehyde	1242		0.79								
Carvone	1248					0.62			1.90		
p-Anisaldehyde	1250				1.77						
Geraniol	1253		0.29			2.73		0.31	0.41	0.08	0.39
Linalyl acetate	1257	32.17				3.22			1.19		
trans-Myrtanol	1258									0.14	
Geranial	1270	0.32								0.17	
(*E*)-Cuminaldehyde	1274		0.79								
Isobornyl acetate	1285					0.21	0.11	2.20	0.39		
(*E*)-Anethole	1285				54.51						
Safrole	1287		1.36								
Thymol	1290					0.17	0.88		2.57	17.98	52.61
Carvacrol	1299					20.84	65.94		47.10	39.83	0.23
α-Terpinyl acetate	1350	0.34		0.28							
Eugenol	1359		64.77				0.06				
Neryl acetate	1365	0.58									
Carvacrol acetate	1371								0.27	0.18	0.19
α-Copaene	1376		0.65			0.05					
Geranyl acetate	1383	0.45									
β-Bourbonene	1384								0.24	0.06	0.14
β-Caryophyllene	1418	0.50	2.46		0.15	1.72	3.72	2.11	3.60	3.31	6.77

Table 2 (*continued*)

Compound	LRI[a]	*Cb*	*Cz*	*Eg*	*Fv*	*Om*	*Ov*	*Ro*	*Sm*	*Tvc*	*Tvt*
β-Gurujene	1434								0.14	0.04	0.40
trans-α-Bergamoptene	1437	0.45		0.35					0.77		
Cinnamyl acetate	1449		1.12								
α-Humulene	1454		0.47				0.13	1.82	1.58	0.13	0.20
γ-Muurolene	1477								0.44		
ar-Curcumene	1480								0.33		
Valencene	1491								0.69		
α-Muurolene	1499					1.36			0.23		
β-Bisabolene	1509	0.73				0.11	0.35		1.53		
trans-γ-Cadinene	1513								0.78	0.28	0.67
δ-Cadinene	1524									0.43	1.02
Spathulenol	1578					0.21			0.20	0.05	
Caryophyllene oxide	1583	0.39	0.17			0.21	0.4	0.12	0.22	0.71	
Globulol	1585			0.20							
Humulene epoxide II	1608		0.21					0.08	0.22		
tau-cadinol	1640										
Benzylbenzoate	1765		2.74								
		99.83	76.60	99.70	94.32	98.28	98.44	92.95	94.09	97.21	96.18

[a] Linear Retention Index on a DB-5 column.

one of the main etiological agents of environmental mastitis [6].

The second effective EO was *Tvt*, since it showed inhibition's zones quite similar to *Sm* EO, with the exception of the lower inhibition against *S. aureus* (13.3 mm). Any activity was observed against *E. coli*. The third EO to be considered in this study for its general antibacterial activity was the *Ov* EO, with the inhibition area slightly lower than *Tvt* EO. The chemical composition of these three EOs (*Sm*, *Tvt* and *Ov*) exhibited high amounts of two phenolic compounds well known for their antibacterial activity, i.e. thymol and carvacrol [6,23].

According to these results, only these EOs with good inhibitory activity against the majority of the tested bacteria were considered for further studies. Therefore we decided to combine *Sm* EO with *Tvt* or *Ov* EO_S in order to obtain two binary mixtures for improving their efficacy. The EO mixtures were prepared (see Experimental sections) by mixing EOs of *Sm* with *Tvt* (called ST) and EOs of *Sm* with *Ov* (called SO) and tested in the same conditions against all the selected bacterial strains.

ST showed a stronger and broader activity than the two EOs tested individually with inhibition area of 27.7 mm (*S. aureus*), 23.7 mm (*S. chromogenes*), 20.0 mm (*S. warneri*), 18.0 mm (*S. xylosus*), 16.3 mm (*S. sciuri*), and 18.0 mm (*E. coli*), respectively (Table 4). On the other hand the SO mixture exhibited an overall lower antibacterial activity than ST, because the inhibition zones did not reach 15 mm, with the exception of *S. aureus* (17.3 mm). Thus the efficacy of ST mixture can be attributed to a synergistic effect of its volatile compounds.

Taking into account these results we carried out further experiments to better understand the role of the *Sm* and *Tvt* EOs' main constituents. Since thymol, carvacrol and *p*-cymene were the most abundant components in these selected EOs, a quantitative analysis of these compounds was performed (Table 1). Pure thymol and carvacrol, separately, were not tested for their antibacterial activity in this work since many data are available in the literature against the selected bacteria [14,28–30]. Then an artificial mixture (solution AB) of these pure compounds was prepared maintaining the same concentration of the mother EOs (*Sm* and *Tvt* respectively, see Experimental sections) and tested as before. The majority of the bacterial strains (*S. aureus*, *S. warneri*, *S. xylosus*, *S. sciuri*) showed about 50% reduction of activity in comparison with the mixture ST (Table 4).

Table 3
Antibacterial activity: zone of inhibition of the selected EOs tested according to the Kirby–Bauer method against the selected bacterial strains. Values are expressed as means of 3 repeats + RSD%.

	Zone of inhibition											
	S. aureus ATCC 6538		*S. chromogenes*		*S. warneri*		*S. xylosus*		*S. sciuri*		*E. coli ATCC 25422*	
EOs	mm	RSD%	mm	RSD%	mm	RSD%	mm	RSD%	mm	RSD%	mm	RSD%
C. bergamia	6.0	0.00	6.0	16.67	5.7	10.19	0.0	0.00	6.0	0.00	0.0	0.00
C. zeylanicum	0.0	–	9.0	0.00	7.0	14.29	8.0	0.00	6.7	8.66	0.0	0.00
E. globulus	0.0	0.00	6.0	0.00	11.3	5.09	7.0	0.00	11.0	0.00	0.0	0.00
F. vulgare	0.0	0.00	0.0	0.00	8.0	0.00	8.0	0.00	0.0	0.00	0.0	0.00
O. majorana	0.0	0.00	0.00	0.00	7.0	0.00	6.7	8.66	0.0	0.00	0.0	0.00
O. vulgare	11.0	0.00	9.30	0.00	10.0	0.00	9.7	5.97	10.0	0.00	0.0	0.00
R. officinalis	6.3	9.12	0.0	0.00	0.0	0.00	8.0	0.00	0.0	0.00	0.0	0.00
S. montana	21.7	2.66	11.3	5.09	12.0	0.00	12.7	9.12	11.7	9.90	13.3	4.33
T. vulgaris ct. carvacrol	13.7	4.22	8.79	6.66	0.0	0.00	7.0	0.00	0.0	0.00	0.0	0.00
T vulgaris ct. thymol	13.3	4.33	12.3	4.68	14.7	3.94	12.0	0.00	10.5	6.73	0.0	0.00

Table 4
Zone of inhibition of the EO mixture (ST, *Sm* + *Tvt*, 1:1) and (SO, *Sm* + *Ov*, 1:1) and the artificial standard solution AB (artificial mixture of carvacrol + thymol) and solution CD (artificial mixture of *p*-cymene + thymol + carvacrol) respectively, tested with the Kirby–Bauer method against the selected bacteria strains. Values are expressed as means of 3 repeats + RSD%.

	Zone of inhibition strains [a]											
	S. aureus ATCC 6538		*S. chromogenes*		*S. warneri*		*S. xylosus*		*S. sciuri*		*E. coli ATCC 25422*	
	mm	RSD%	mm	RSD%	mm	RSD%	mm	RSD%	mm	RSD%	mm	RSD%
ST	27.7	2.09	23.7	2.44	20.0	0.00	18.0	0.00	16.3	3.53	18.0	0.00
SO	17.3	1.42	12.3	1.48	9.3	1.07	8.6	1.43	12.3	1.42	13.3	1.42
AB	14.0	0.00	18.3	3.15	11.0	0.00	9.70	5.97	8.0	0.00	13.3	4.33
CD	29.3	1.42	21.6	1.42	23.6	1.42	20.0	1.41	31.3	1.42	21.6	1.42

[a] Values are expressed as means of 3 repeats + RSD%.

The decrease of efficacy of AB in comparison with ST can be attributed to the absence of a synergism of thymol and carvacrol mixed together. Therefore other constituents present in ST might provide an increase of the antibacterial activity, then the synergism of *p*-cymene was also considered. Pure *p*-cymene showed good results only against *S. aureus* and *S. warneri* (20.0 mm and 22.3 mm respectively, data not shown).

A CD solution was prepared with thymol, carvacrol and *p*-cymene taking into account their amount present in each *Tvt* and *Sm* EO (see Experimental sections). Good results were obtained with CD mixture against all the tested bacteria showing inhibition areas very similar to that observed with the application of ST mixture. The highest value was exhibited against *S. sciuri* (31.3 mm) followed by *S. aureus* (29.3 mm). It is remarkable to note that these data are analogous to the alones of the antibiotics employed as positive control (data not shown).

The obtained results evidenced the efficacy of a synergistic effect due to the association of some EOs or mixtures of their main constituents against bacterial strains supporting mastitis. This paper gives a further contribution to previous literature, where thymol and carvacrol were considered as main constituents responsible for antimicrobial activity. Lis-Balchin and Deans [31] studied six different combinations of three EO mixtures for their possible synergistic activity against 13 food bacteria. Their results showed no efficacy due to a misleading dilution of the EO constituents.

Our results highlighted that the concentration of the active components in artificial mixtures has to be maintained as in the mother EOs. However the best results were obtained with the artificial mixture of pure constituents where also *p*-cymene was added (CD).

4. Conclusions

Mastitis in the livestock represents worldwide the most expensive health-related problem for the dairy industry [32,33]. The wide use of antibiotics leads to the emergence of resistant bacteria [10] and increases the amount of antibiotic residues in milk [5], justifying the study of alternative treatments, represented by the utilization of plant derived compounds or extracts like EOs [6,28].

The binary ST mixture showed a stronger inhibitory activity than the two EOs alone, against all the five *Staphylococcus* strains tested and against *E. coli* as well. This activity is not only due to the presence of thymol and carvacrol in the EO mixture, but other main constituents can provide a synergistic effect to the observed activity. This leads us to suggest that artificial mixtures might be prepared by adding other main constituents present in the EOs in order to increase of biological activity or verify the contribution of such pure compounds to the final activity (synergistic or antagonistic effect).

The EO mixtures as well as the mixture of their pure main constituents may contribute to develop a preventive herbal treatment against environmental mastitis to reduce their incidence and decrease the application of antibiotics during the first development phase of disease in animals.

Conflict of interest

The authors have declared that there is no conflict of interest.

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