Rendiconti Lincei. Scienze Fisiche e Naturali (2021) 32:407–416
https://doi.org/10.1007/s12210-021-00991-5

RESEARCH PAPER

Evaluation of the in vitro antibacterial activity of some essential oils and their blends against *Staphylococcus* spp. isolated from episodes of sheep mastitis

Filippo Fratini[1,2] · Margherita Giusti[3] · Simone Mancini[1] · Francesca Pisseri[3] · Basma Najar[4] · Luisa Pistelli[2,4]

Received: 19 February 2021 / Accepted: 16 April 2021 / Published online: 11 May 2021

Abstract
Staphylococcus aureus and coagulase-negative staphylococci are among the major causes of mastitis in sheep. The main goal of this research was to determine the in vitro antibacterial activity of several essential oils (EOs, *n* 30), then five of them were chosen and tested alone and in blends against staphylococci isolates. Five bacteria were isolated from episodes of ovine mastitis (two *S. aureus* and three *S. xylosus*). Biochemical and molecular methods were employed to identify the isolates and disk diffusion method was performed to determine their antimicrobial-resistance profile. The relative percentage of the main constituents in the tested essential oils and their blends was detected by GC-EIMS analysis. Antibacterial and bactericidal effectiveness of essential oils and blends were evaluated through minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). All of them showed sensitivity to the used antimicrobials. The EOs with the highest antibacterial activity were those belonging to the Lamiaceae family characterized by high concentrations of thymol, carvacrol and its precursor *p*-cymene, together with cinnamon EO, rich in cinnamaldehyde. In terms of both MIC and MBC values, the blend composed by *Thymus capitatus* EO 40%, *Cinnamomum zeylanicum* EO 20%, *Thymus serpyllum* EO 20% and *Satureja montana* EO 20% was found to be the most effective against all the isolates. Some essential oils appear to represent, at least in vitro, a valid tool against ovine mastitis pathogens. Some blends showed a remarkable effectiveness than the single oils, highlighting a synergistic effect in relation to the phytocomplex.

Keywords Cinnamon bark · Everlasting flowers · Winter savory · Thyme · Synergy

1 Introduction

Mastitis is one of the most important health problem in dairy sheep worldwide. Clinical mastitis incidence during lactation is usually less than 5%, but, in some cases, it can reach or overcome 30% (Contreras et al. 2007). *Staphylococcus*

Filippo Fratini and Margherita Giusti equally contributed to this work.

✉ Filippo Fratini
filippo.fratini@unipi.it

Margherita Giusti
anguilla3@virgilio.it

Simone Mancini
simone.mancini@unipi.it

Francesca Pisseri
info@francescapisseri.it

Basma Najar
basmanajar@hotmail.fr

Luisa Pistelli
luisa.pistelli@unipi.it

[1] Department of Veterinary Sciences, University of Pisa, Viale Delle Piagge 2, Pisa, Italy

[2] Interdepartmental Research Center "Nutraceuticals and Food for Health", University of Pisa, Via del Borghetto 80, Pisa, Italy

[3] Veterinary practitioner, Pisa, Italy

[4] Department of Pharmacy, University of Pisa, Via Bonanno 6, Pisa, Italy

aureus and coagulase-negative staphylococci (CoNS) are considered, respectively, as the main pathogens involved in clinical and subclinical mastitis in sheep (Bergonier et al. 2003; Mork et al. 2005; Contreras et al. 2007). The increasing concern about antimicrobial resistance among strains of these bacteria represents a great challenge for research, to find alternative, safe and innovative strategies, as the use of phytochemicals and essential oils (EOs) (Sharma et al. 2018).

The antimicrobial effects of EOs originated from medicinal and aromatic plants are relevant, since they may inhibit the growth of *Staphylococcus* strains or kill bacterial cells (Fratini et al. 2014; Tariq et al. 2019) both if used alone or in combination (Fratini et al. 2017; van Vuuren et al. 2019). The phytocomplex contained in a single EO, which consists of compounds of several different functional-group classes, can be used alone or in association with other phytocomplexes providing a multiplicity of pharmacological actions, related to the presence of active compounds that may perform various functions and others that modulate the effect (Pisseri et al. 2008; Marassi and Rossi 2015). *Thymus capitatus*, *Thymus serpyllum* and *Satureja montana* belong to the Lamiaceae family, which includes plants with a wide range of biological and pharmacological activities (Marin et al. 2018). Essential oils and extracts from the genus *Thymus* exhibited different biological properties such as antioxidant, antibacterial, antifungal, antiviral, antiparasitic, cytotoxic, carminative and spasmolytic (Paaver et al. 2008; Nabavi et al. 2015; Maissa and Walid 2015). EOs from various *Satureja* species as well have demonstrated antibacterial, antiviral, antiparasitic, antioxidant, anti-inflammatory, antinociceptive, hepatoprotective, antidiabetic and anticholesterolemic activities (Jafari et al. 2016; Caprioli et al. 2019). The antibacterial activity has been attributed to the presence of oxygenated monoterpenes, especially thymol and carvacrol in synergy with its precursor *p*-cymene (Jarić et al. 2015; Tepe and Cilkiz 2016). These compounds showed to be effective against *S. aureus* and CoNS (Hyldgaard et al. 2012; dos Santos Rodrigues et al. 2017; Gaio et al. 2017).

The genus *Cinnamomum* consists of over 250 aromatic trees and shrubs members of the Lauraceae family, distributed in warm temperate and tropical regions. The main components of *Cinnamomum zeylanicum* EO are monoterpenes, sesquiterpenes and their related derivatives, mostly cinnamaldehyde obtained from bark and eugenol from leaves (Barceloux 2009). This plant extract showed antimicrobial properties against *Staphylococcus* spp., also in association with antibiotics (Dal Pozzo et al. 2012; Mahadlek et al. 2012; Saleem et al. 2015).

Helychrysum italicum (Asteraceae), a Mediterranean aromatic shrub, EO exhibits a marked anti-inflammatory and antimicrobial properties against Gram-positive bacteria (Demir et al. 2009; Antunes Viegas et al. 2014; Djihane and Mihoub 2016), along with an elevated capacity to increase the antibiotics effectiveness against multidrug-resistant Gram-negative bacteria (Lorenzi et al. 2009). Water or steam distillation of the aerial parts produced a mixture of phytochemicals mainly rich in monoterpenes and sesquiterpenes, followed by β-diketones (Maksimovic et al. 2017).

The aim of this research was to screen by in vitro tests the antibacterial activity of some essential oils. The EOs were selected on the basis of the available scientific literature, but also taking into account their synergistic activity encountered during personal field experience. Then, the most effective five, were tested alone and in blends against staphylococci isolated from episodes of sheep mastitis.

2 Materials and methods

2.1 Farm description and sampling

Casorelle is a multifunctional agrozootechnical farm (43°49′43,5′′N—10°54′26,2′′E) where Assaf sheep are reared in which the animals fed mainly forage through olive agroforestry system. Health management is focused on prevention and use of natural medicines such as phytotherapy and homeopathy. Forty-five individual milk samples, one for each udder half were collected from symptomatic and asymptomatic subjects during the period from April 2016 to June 2016 Table 1.

2.2 Phenotypical and genotypical identification and antimicrobial-resistance profile of isolates

All isolates presumptively imputable to mastitis etiological agents, were submitted to phenotypical and genotypical characterization according to Windria et al. (2016). Subsequently, the antibiotic resistance profile of each isolate was determined by disk diffusion method (Cockerill III et al. 2012), performing three replicates. Nineteen antimicrobials were tested (Table 2): amoxicillin-clavulanic acid (30 µg, AMC), amikacin (30 µg, AMK), ampicillin (10 µg, AMP), amoxicillin (10 µg, AMX), ceftazidime (30 µg, CAZ), cephalothin (30 µg, CEF), ciprofloxacin (5 µg, CIP), cefotaxime (30 µg, CTX), doxycycline (30 µg, DOX), enrofloxacin (5 µg, ENR), gentamicin (10 µg, GEN), cephalexin (30 µg, LEX), neomycin (30 µg, NEO), piperacillin (100 µg, PIP), rifampin (30 µg, RIF), streptomycin (10 µg, STR), trimethoprim-sulfamethoxazole (1.25–23.75 µg, SXT), tetracycline (30 µg, TET) and tobramycin (10 µg, TOB). Antimicrobial disks were purchased from Oxoid Thermo Scientific (Milan, Italy).

The interpretation of the results was performed following EUCAST (2017) and CLSI (2013) guidelines.

Table 1 Characteristics of the essential oils used in screening tests

Order	Family	Specie	Parts of plant	Extraction method	Origin
Apiales	Apiaceae	*Anethum graveolens* L	Whole plant during flowering	Steam distillation	Italy
		Coriandrum sativum L	Seeds	Steam distillation	Ukraine
		Cuminum cyminum L	Seeds	Steam distillation	Egypt
		Pimpinella anisum L	Seeds	Steam distillation	France
Astrales	Asteraceae	*Chamaemelum nobile* All	Whole plant during flowering	Steam distillation	Italy
		Helichrysum italicum (Roth) G. Don	Whole plant during flowering	Steam distillation	Italy
Illiciales	Illiciaceae	*Illicium verum* Hook.f	Fruits/seeds	Steam distillation	Vietnam
Lamiales	Lamiaceae	*Lavandula officinalis* Chaix	Flowers	Steam distillation	Italy
		Ocimum basilicum L	Whole plant during flowering	Steam distillation	Egypt
		Origanum majorana L	Whole plant during flowering	Steam distillation	Egypt
		Origanum vulgare L	Whole plant during flowering	Steam distillation	Italy
		Rosmarinus officinalis L	Whole plant during flowering	Steam distillation	Italy
		Rosmarinus officinalis L. ct. CINEOLE	Whole plant during flowering	Steam distillation	Tunisia
		Satureja montana L	Whole plant during flowering	Steam distillation	Egypt
		Thymus capitatus (L.) Hoffmanns. & Link	Whole plant during flowering	Steam distillation	Spain
		Thymus serpyllum L	Whole plant during flowering	Steam distillation	Turkey
		Thymus vulgaris L. ct. THYMOL	Whole plant during flowering	Steam distillation	Italy
Laurales	Lauraceae	*Cinnamomum zeylanicum* Blume (60%)	Bark	Steam distillation (40% ethanol)	Germany
		Laurus nobilis L	Leaves	Steam distillation	Bosnia
		Litsea cubeba (Lour.) Pers	Fruits/leaves	STEAM distillation	China
Myrtales	Myrtaceae	*Leptospermum scoparium* J.R.Forst. & G.Forst	Flowers	Steam distillation	New Zealand
Pinales	Cupressaceae	*Juniperus communis* L	Berries	Steam distillation	Albania
	Pinaceae	*Picea abies* (L.) H. Karst	Tops	Steam distillation	France
		Pinus cembra L	Tops	Steam distillation	Austria
Rosales	Rosaceae	*Rosa*×*damascena* Mill	Flowers	Solvent	Turkey
Sapindales	Rutaceae	*Cistus ladanifer* L	Tops	Steam distillation	Spain
		Citrus×*bergamia* Risso & Poit	Peel	Cold pressing	Italy
		Citrus limon (L.) Osbeck	Peel	Cold pressing	Argentina
		Citrus medica L	Fruits	Cold pressing	Italy
Zingiberales	Zingiberaceae	*Elettaria cardamomum* (L.) Maton	Seeds	Steam distillation	Sri Lanka

2.3 Essential oils and blends

Thirty EOs were employed in the screening test (Table 1) provided by FLORA s.r.l. ® (Lorenzana, Pisa, Italy). EOs were selected on the basis of their antibacterial potential and their anti-inflammatory activities (i.e. everlasting flowers and rosemary). The inflammatory process in mammary gland enhances the development and progression of the bacterial infection; therefore, it is significantly advantageous to use a mixture that has both characteristics, antibacterial and anti-inflammatory, in the preparation of a therapeutic aid to be used in the clinical practice of ovine mastitis (Pisseri et al. 2008).

Basing on the EO antibacterial activities and the anti-inflammatory properties, five blends (A, B, C, D and E) were constituted with *Cinnamomum zeylanicum* (*Cz*), *Helichrysum italicum* (*Hi*), *Satureja montana* (*Sm*), *Thymus capitatus* (*Tc*) and *Thymus serpyllum* (*Ts*) EOs as following:

Blend A: *Sm* 30%; *Tc* 30%; *Hi* 25%; *Cz* 15%,
Blend B: *Sm* 30%; *Ts* 25%; *Tc* 25%; *Cz* 20%,
Blend C: *Sm* 34%; *Cz* 34%; *Tc* 22%; *Ts* 10%,
Blend D: *Tc* 50%; *Sm* 30%; *Hi* 20%,
Blend E: *Tc* 40%; *Cz* 20%; *Sm* 20%; *Ts* 20%.

2.4 Chemical characterization of essential oils and blends

The analysis of the five EOs chosen and their blends was performed by GC–EIMS (gas chromatography–electron impact mass spectrometry) in Table 3 according to the method previously described by Pistelli et al. (2017).

Table 2 Antimicrobial susceptibility test

Class	Drug	Abbreviation	*Sa*1	*Sa*2	*Sx*1	*Sx*2	*Sx*3
Penicillins	Amoxicillin	AML	S	S	R	R	R
	Amoxicillin–clavulanic acid	AMC	S	S	S	S	R
	Ampicillin	AMP	R	R	R	R	R
	Piperacillin	PRL	S	S	S	S	I
Cephalosporins	Cephalexin	CL	I	S	R	I	S
	Cephalothin	KF	S	S	S	S	S
	Cephotaxime	CTX	I	I	I	R	I
	Ceftazidime	CAZ	S	S	R	R	R
Fluoroquinolones	Ciprofloxacin	CIP	I	S	S	S	S
	Enrofloxacina	ENR	I	S	S	I	S
Aminoglycosides	Amikacin	AK	S	S	I	R	S
	Neomycin	N	I	S	R	S	S
	Streptomycin	S	I	I	R	I	S
	Gentamycin	CN	S	S	S	S	S
	Tobramycin	TOB	S	S	S	I	S
Tetracyclines	Doxycycline	DO	S	R	I	I	S
	Tetracycline	TE	S	R	R	R	S
Diaminopyrimidines + Sulfonamides	Trimethoprim-sulfamethoxazole	SXT	S	S	S	S	S
Rifamycins	Rifampicin	RD	S	S	S	I	S

Staphylococcus aureus isolates: *Sa*1 and *Sa*2. *Staphylococcus xylosus* isolates: *Sx*1, *Sx*2 and *Sx*3

Antimicrobial susceptibility following EUCAST (2017) and CLSI (2013) guidelines determine as sensible (S), intermediate (I) and resistant (R)

2.5 MIC and MBC determinations

For each EO alone and, subsequently, for each mixture, MIC and MBC determinations were performed by the two-fold serial microdilution method (Fratini et al. 2019) and expressed as w/v. Both assays were carried out in triplicate (Table 4).

3 Results and discussion

Only five individual milk samples out of forty-five (11.11%) were positive for imputable mastitis etiological agents. Isolates were phenotypically and genotypically identified as *Staphylococcus aureus* (named *Sa*1 and *Sa*2) and *Staphylococcus xylosus* (named *Sx*3, *Sx*4 and *Sx*5). Consistently with the existing literature on the frequency of the aetiological agents responsible for mastitis in sheep, our study led to the isolation of staphylococci specifically related to the species *S. aureus* and *S. xylosus* (Bergonier et al. 2003; Mork et al. 2005; Contreras et al. 2007). *S. xylosus* is one of the most frequently identified microorganisms in course of ovine mastitis (Vanderhaeghen et al. 2015; Cannas et al. 2019).

Antimicrobial-resistance profile of isolates is reported in Table 2. *S. aureus* isolates showed an overall antimicrobial susceptibility in contrast to Wendlandt et al. (2013). Nevertheless, some studies proved that many *S. aureus* strains responsible for mastitis in livestock are characterized by high susceptibility to antibiotics, especially in those farms where limited use of these substances is made (Antonios et al. 2015; Lisowska-Łysiak et al. 2018). The three isolates belonging to *S. xylosus* specie showed heterogeneous results in terms of susceptibility to antimicrobials. This extreme variability was predictable in wild-type microorganisms of environmental origin are generally more often subjected to high selective pressure by various molecules and are characterized by a greater probability of horizontal transmission of genetic material (Pyorala and Taponen 2009; Taponen et al. 2015).

The main aim of this work was to evaluate potential alternatives to antimicrobial treatments of ovine mastitis, identifying which essential oils or their mixtures could represent an effective therapeutic tool. Seven out of thirty EOs analysed in the screening step showed a strong in vitro antibacterial activity (data not shown). In brief, *Cinnamomum zeylanicum* (mode values between 1:512 v/v and 1:1024 v/v), *Leptospermum scoparium* (mode values between 1:256 v/v and 1:1024 v/v), *Thymus vulgaris* thymol CT (mode values between 1:128 v/v and 1:512 v/v), *Satureja montana* (mode values between 1:128 v/v and 1:256 v/v), *Origanum vulgare*, *Thymus serpyllum* and *Thymus capitatus* (mode values between 1:256 v/v and 1:512 v/v) were the most effective.

Table 3 The GC-EIMS analysis results of individual essential oils (EOs) and mixtures (relative abundance)

Compound	Class	LRI	Eos					Mixture of EOs				
			Cz	*Hi*	*Sm*	*Ts*	*Tc*	A	B	C	D	E
α-Thujene	mh	930	0.2	–	0.5	0.4	0.3	0.3	0.4	0.4	0.3	0.3
α-Pinene	mh	939	0.8	–	1.2	0.6	0.6	5.3	0.9	0.9	3.2	0.7
Camphene	mh	954	0.3	0.6	0.7	0.2	0.1	0.4	0.4	0.4	0.2	0.3
Benzaldehyde	Nt	960	0.2	–	–	–	–	–	–	–	–	–
Sabinene	mh	975	0.4	0.5	0.6	0.2	0.1	0.4	0.4	0.4	0.4	0.3
1-Octen-3-ol	Nt	979	–	–	0.6	0.2	0.2	0.2	0.4	0.4	0.3	0.3
Myrcene	mh	991	0.1	–	1.3	1.4	1.5	1.1	1.4	1.2	1.2	1.2
α-Phellandrene	mh	1003	1.0	–	0.3	0.1	0.2	0.2	0.3	0.4	0.2	0.3
δ-3-Carene	mh	1007	0.1	–	–	–	–	–	0.1	0.1	–	0.1
α-Terpinene	mh	1017	0.5	–	1.3	1.2	1.1	1.0	1.2	1.1	1.0	1.0
p-Cymene	mh	1025	2.0	1.1	13.6	6.3	8.3	9.2	10.6	10.8	10.0	9.7
β-Phellandrene	mh	1030	1.3	–	–	–	–	–	–	–	–	–
1,8-Cineol	om	1031	–	3.3	–	–	1.2	2.5	–	1.7	1.6	1.3
Limonene	mh	1033	2.0	–	2.0	1.6	0.8	–	1.8	–	–	–
(*Z*)-β-Ocimene	mh	1037	–	–	0.4	–	–	–	0.3	0.3	0.2	0.2
(*E*)-β-Ocimene	mh	1050	–	–	0.1	–	–	–	–	–	–	–
γ-Terpinene	mh	1060	–	–	6.4	1.8	4.7	–	–	–	–	–
p-Mentha-2.4(8)-diene	mh	1088	–	–		0.3		–	–	–	–	–
Terpinolene	mh	1089	–	–	0.3	–	0.2	0.3	0.3	0.3	0.3	0.3
Linalool	om	1097	5.8	0.8	1.7	9.1	1.7	1.7	4.1	2.9	1.3	3.0
α-Pinene oxide	om	1099	–	1.3	–	–	–	0.4	–	–	0.2	–
α-Campholenal	om	1126	–	0.3	–	–	–	–	–	–	–	–
trans-Pinocarveol	om	1139	–	0.5	–	–	–	0.1	–	–	–	–
cis-Verbenol	om	1141	–	1.5	–	–	–	–	–	–	–	–
trans-Verbenol	om	1145	–	–	–	–	–	0.3	–	–	0.2	–
Camphor	om	1146	–	0.2	0.3	–	–	0.1	0.1	0.1	–	–
Hexyl isobutyrate	nt	1152	–	1.2	–	–	–	–	–	–	–	–
2-Methylbutyl angelate	nt	1158	–	0.4	–	–	–	0.2	–	–	–	–
Borneol	om	1169	–	–	2.7	0.6	0.4	1.3	1.4	1.5	1.1	0.9
4-Terpineol	om	1177	0.5	–	1.5	1.0	1.2	1.0	1.1	1.0	0.9	0.9
α-Terpineol	om	1189	1.2	0.4	0.9	0.6	0.3	0.6	0.7	0.9	0.3	0.4
Dihydrocarvacrol	om	1194	–	–	–	–	–	0.2	0.2	–	0.2	0.2
Myrtinol	om	1196	–	0.3	–	–	–	–	–	–	–	–
Verbenone	om	1205	–	0.4	–	–	–	–	–	–	–	–
trans-Carveol	om	1217	–	0.5	–	–	–	–	–	–	–	–
γ-Terpineol	om	1218	–	–	0.4	–	–	–	–	–	–	–
(*Z*)-Cinnamaldehyde	nt	1219	3.1	–	–	–	–	0.2	–	0.2	–	0.1
Nerol	om	1230	–	0.2	–	–	–	–	–	–	–	–
cis-Sabinene hydrate	om	1240	–	–	0.3	0.6	0.2	0.2	0.3	0.2	0.2	0.2
Cuminaldehyde	om	1242	–	–	5.1	–	–	2.2	2.0	2.3	1.8	1.2
Carvone	om	1243	–	0.2	0.2	–	0.2	–	0.1	0.1	–	0.2
(*Z*)-3-Hexenyl isovalerate	nt	1245	–	0.3	–	–	–	–	–	–	–	–
trans-myrtanol	om	1261	–	–	–	–	–	–	–	–	–	–
Geranial	om	1267	–	–	–	–	0.2	–	–	–	–	–
(*E*)-cinnamaldehyde	nt	1270	55.3	–	–	–	–	5.0	6.1	10.4	–	5.8
hexyl angelate	nt	1286	–	0.8	–	–	–	0.6	–	–	–	–
isobornyl acetate	om	1286	–	–	0.2	–	–	–	–	–	–	–
Thymol	om	1290	–	–	7.0	2.4	0.5	3.0	3.3	3.0	2.8	2.1

Table 3 (continued)

Compound	Class	LRI	Eos					Mixture of EOs				
			Cz	*Hi*	*Sm*	*Ts*	*Tc*	A	B	C	D	E
Carvacrol	om	1299	–	–	44.5	69.3	70.5	39.9	52.3	46.7	54.7	59.2
neryl formate	om	1307	–	0.2	–	–	–	–	–	–	–	–
Eugenol	pp	1359	–	–	–	–	–	0.4	0.4	0.9	0.9	0.5
neryl acetate	om	1362	–	6.2	–	–	–	–	–	–	–	–
cyclosativene	sh	1371	–	–	–	–	–	0.1	–	–	–	–
geranyl acetate	om	1372	–	–	0.1	–	–	–	–	–	–	–
linalool isobutyrate	om	1374	–	–	–	–	–	1.2	–	–	–	–
α-copaene	sh	1377	1.7	–	–	–	–	1.0	0.4	0.6	0.8	0.3
Daucene	sh	1382	–	4.1	–	–	–	–	–	–	–	–
iso-italicene	sh	1402	–	6.0	–	–	–	1.1	–	–	0.9	–
cis-α-bergamotene	sh	1413	–	1.5	–	–	–	0.3	–	–	0.2	–
β-caryophyllene	sh	1419	8.7	0.2	2.6	1.1	2.7	2.6	2.6	3.4	2.2	2.7
trans-α-bergamotene	sh	1435	–	1.4	–	–	–	0.3	–	–	0.2	–
α-guaiene	sh	1444	–	–	0.2	0.3	–	–	–	–	–	–
8-decene-3,5-dione,4,6,9-trimethyl-	nt	1449	–	–	–	–	–	–	–	–	–	–
neryl propanoate	om	1455	–	1.0	–	–	–	0.2	–	–	0.3	–
α-humulene	sh	1455	3.8	–	0.1	–	–	0.4	0.4	0.6	–	0.5
allo-aromadendrene	sh	1460	–	–	–	–	–	0.1	–	–	–	–
dehydro-aromadendrene	sh	1465	–	–	–	–	–	-	–	0.1	–	–
α-acoradiene	sh	1466	–	0.6	–	–	–	0.1	–	–	–	–
β-acoradiene	sh	1466	–	0.9	0.1	–	–	0.1	–	–	–	–
γ-muurolene	sh	1480	–	2.6	–	–	–	–	–	–	–	–
ar-curcumene	sh	1481	0.1	–	–	–	–	3.4	–	–	–	–
γ-himachelene	sh	1483	–	–	–	–	–	0.6	–	–	0.1	-
β-selinene	sh	1490	–	–	0.2	0.2	–	1.1	0.1	0.1	3.4	0.1
viridiflorene	sh	1497	–	18.9	–	–	–	0.9	–	–	0.7	–
α-muurolene	sh	1500	–	–	–	–	–	0.2	–	–	0.1	–
β-bisabolene	sh	1506	–	0.4	0.7	–	0.4	0.5	0.3	0.4	0.5	0.3
α-bulnesene	sh	1510	–	1.9	–	–	–	–	–	–	–	–
Cubebol	os	1515	–	1.7	–	–	–	–	–	–	–	–
β-curcumene	sh	1516	–	–	0.2	–	–	–	–	–	–	–
α-cadinene	sh	1539	–	0.2	–	–	–	–	–	–	–	–
caryophyllene oxide	os	1583	0.9	–	0.7	0.1	1.3	1.8	0.5	0.7	1.6	0.7
Globulol	os	1585	–	1.1	–	–	–	–	–	–	–	–
humulene oxide II	os	1608	–	0.5	–	–	–	–	–	–	–	–
tetradecanal	nt	1613	0.3	–	–	–	–	–	–	–	–	–
cis-cadin-4-en-7-ol	os	1637	–	0.3	–	–	–	–	–	–	–	–
14-hydroxy-9-epi-(*E*)-caryophyllene	os	1670	–	–	–	–	–	0.2	–	–	–	–
(*Z*,*E*)-farnesol	os	1690	–	0.3	–	–	–	–	–	–	–	–
benzyl benzoate	nt	1760	2.3	–	–	–	–	–	0.1	0.4	–	0.3
dirm-8-en-7-one	os	1763	–	1.1	–	–	–	–	–	–	–	–
α-bisabolol acetate	os	1798	–	0.4	–	–	–	–	–	–	–	–
sesquilavandulyl acetate	os	1809	–	0.6	–	–	–	–	–	–	–	–
Class of compounds												
Monoterpene Hydrocarbons (mh)			8.9	26.8	28.7	14.1	17.9	18.2	18.1	16.3	17.0	14.4
Nom-terpene derivatives (nt)			61.2	5.4	0.6	0.2	0.2	6.2	6.6	11.4	0.3	6.5
Oxygenated Monoterpenes (om)			7.5	17.6	65.0	83.6	76.4	54.9	65.6	60.4	65.6	69.6
Oxygenated Sesquiterpenes (os)			0.9	5.7	0.7	0.1	1.3	2.0	0.5	0.7	1.6	0.7

Table 3 (continued)

Compound	Class	LRI	Eos					Mixture of EOs				
			Cz	*Hi*	*Sm*	*Ts*	*Tc*	A	B	C	D	E
Phenylpropanoids (pp)			4.1	–	–	–	–	0.4	0.4	0.9	0.9	0.5
Sesquiterpene Hydrocarbons (sh)			14.3	39.0	4.1	1.6	3.1	12.8	3.8	5.2	9.1	4.1
Total Identified			96.9	94.8	99.1	99.6	98.9	94.5	95.0	94.9	94.5	95.8

LRI linear retention indices

Single EO: *Cinnamomum zeylanicum* (*Cz*), *Helichrysum italicum* (*Hi*), *Satureja montana* (*Sm*), *Thymus capitatus* (*Tc*) and *Thymus serpyllum* (*Ts*)

Blends: A—*Sm* 30%, *Tc* 30%, *Hi* 25%, *Cz* 15%; B—*Sm* 30%, *Ts* 25%, *Tc* 25%, *Cz* 20%; C—*Sm* 34%, *Cz* 34%, *Tc* 22%, *Ts* 10%; D—*Tc* 50%, *Sm* 30%, *Hi* 20%; E—*Tc* 40%, *Cz* 20%, *Sm* 20%, *Ts* 20%

Table 4 MIC and MBC mode values of single essential oil and blends

Single EO—EOs blend	MIC mode (g/ml)					MBC mode (g/ml)				
	*Sa*1	*Sa*2	*Sx*1	*Sx*2	*Sx*3	*Sa*1	*Sa*2	*Sx*1	*Sx*2	*Sx*3
Cz	0.94	0.94	1.88	1.88	1.88	3.75	3.75	3.75	3.75	3.75
Hi	7.37	7.37	7.37	14.73	14.73	29.47	29.47	29.47	58.94	58.94
Sm	3.69	3.69	7.38	7.38	3.69	7.38	7.38	7.38	14.76	7.38
Ts	1.9	3.8	3.8	3.8	3.8	1.9	7.61	7.61	7.61	7.61
Tc	1.86	1.86	1.86	3.71	3.71	1.86	1.86	1.86	7.42	7.42
A	0.46	0.93	0.93	0.93	0.46	0.93	0.93	0.93	1.85	0.46
B	1.84	1.84	1.84	1.84	0.92	1.84	1.84	1.84	1.84	1.84
C	0.47	0.47	0.47	0.47	0.24	0.95	0.47	0.47	0.47	0.47
D	0.94	0.94	0.94	0.94	0.47	0.94	0.94	0.94	0.94	0.94
E	0.47	0.47	0.47	0.47	0.47	0.47	0.47	0.47	0.47	0.47

Staphylococcus aureus isolates: *Sa*1 and *Sa*2. *Staphylococcus xylosus* isolates: *Sx*1, *Sx*2 and *Sx*3

Single EO: *Cinnamomum zeylanicum* (*Cz*), *Helichrysum italicum* (*Hi*), *Satureja montana* (*Sm*), *Thymus capitatus* (*Tc*) and *Thymus serpyllum* (*Ts*)

Blends: A—*Sm* 30%, *Tc* 30%, *Hi* 25%, *Cz* 15%; B—*Sm* 30%, *Ts* 25%, *Tc* 25%, *Cz* 20%; C—*Sm* 34%, *Cz* 34%, *Tc* 22%, *Ts* 10%; D—*Tc* 50%, *Sm* 30%, *Hi* 20%; E—*Tc* 40%, *Cz* 20%, *Sm* 20%, *Ts* 20%

The corresponding MBC mode values for each of these EOs coincided with MIC mode values or settled one dilution step forward. These results are in accordance with the relevant literature, which states that essential oils obtained from Lamiaceae plants often show a marked antibacterial activity due to the presence of thymol, carvacrol and its precursor *p*-cymene (Hyldgaard et al. 2012; Rajput et al. 2018).

Likewise, the broad-spectrum antibacterial activity of Cinnamon bark essential oil is widely documented and has been attributed to the presence of cinnamaldehyde (Ananda Baskaran et al. 2009; Dal Pozzo et al. 2012; Friedman 2017).

Fewer studies have been conducted on the antibacterial activity of Manuka essential oil, which has remarkable antimicrobial properties referable to the presence of some bioactive compounds, such as leptospermone and iso-leptospermone (Lis-Balchin et al. 2000; Fratini et al. 2017).

A second group of essential oils used in the screening test demonstrated an intermediate level of antibacterial activity: *Helichrysum italicum* and *Origanum majorana* (mode values between 1:64 v/v and 1:128 v/v); *Cistus ladanifer*, *Litsea cubeba* and *Ocimum basilicum* (mode values between 1:32 v/v and 1:128 v/v); *Citrus medica, Cuminum cyminum, Lavandula officinalis, Rosa* × *damascene, Rosmarinus officinalis, Rosmarinus officinalis* ct cineole (mode values between 1:32 v/v and 1:64 v/v); *Citrus* × *bergamia*, *Citrus limon*, *Coriandrum sativum*, *Elettaria cardamomum*, *Juniperus communis* (mode values of 1:32 v/v). It is important to note that the MBC mode values for all the above mentioned EOs did not follow the same trend as the first group, in some cases deviating by two dilutions in comparison to MIC values. However, the wide variability in antimicrobial activity showed by this second group is reported in several studies (Chao et al. 2008; Giovannini et al. 2016; Imane et al. 2020; Mollova et al. 2020). The remaining EOs selected for initial screening test showed mild antibacterial activity against isolated staphylococci.

GC-EIMS determination of the five chosen EOs and their blends are reported in Table 3. The three Lamiaceae EOs

were characterised by the presence of *p*-cymene, γ-terpinene and carvacrol. These compounds were found, respectively, in *Satureja montana* EO at 13.6%, 6.4%, 44.5%, in *Thymus serpyllum* at 6.3%, 1.8%, 69.3% and in *Thymus capitatus* at 8.3%, 4.7% and 70.5%. Thymol was present in a higher percentage in *Satureja montana* EO (7.0%), together with cuminaldehyde (5.1%). Linalool, identified in *Cinnamomum zeylanicum* (5.8%) and *Thymus serpyllum* (9.1%), is a monoterpene also present in EOs obtained from lavender, basil, laurel, bergamot, jasmine, mandarin leaves, orange leaves, lemon leaves and many other plants; this compound interferes with the integrity and functionality of the bacterial cell membrane, causing alterations in membrane potential and consequent loss of cytoplasmic material (Silva et al. 2015; Greay and Hammer 2015).

The predominant compound in cinnamon-bark EO was the (*E*) isomer of cinnamaldehyde (55.3%) followed by β-cariophyllene (8.7%), a sesquiterpene which has demonstrated both a selective antibacterial activity against *S. aureus* and a marked antioxidant activity (Dorman and Deans 2000; Dahham et al. 2015). The main molecules identified in everlasting flowers EO were α-pinene (24.6%) and viridiflorene (18.9%). The former is a monoterpene with proven antibacterial activity (de Sousa Eduardo et al. 2018; Ložienė et al. 2018) while the latter is a sesquiterpene produced by the dehydration of viridiflorol whose biological activities are not yet well known, firstly isolated from *Majorana hortensis* EO (Taskinen 1974) and then also found in *Melaleuca alternifolia* EO (Swords and Hunter 1978).

Interestingly, we want to highlight that the mixtures' GC-EIMS showed different compounds percentages compared to the one expected by calculation based on the single EOs composition. These values differ significantly especially in the case of carvacrol (A: + 5.38%; B: + 4.00%; C: + 9.10%; D: + 6.08%; E: + 8.18%) and the *trans* isomer of cinnamaldehyde (A: − 3.32%; B: − 4.96%; C: − 8.35%; E: − 5.24%). This discrepancy in data could be ascribed to oxidative phenomena affecting the numerous carvacrol precursors present in some EOs and degradation of cinnammaldehyde to benzaldehyde, a non-volatile compound not detectable by gas chromatographic analysis (Wang et al. 2009). Major deviations could be, therefore, caused by chemical phenomena occurring between the various components of EOs. All these suggests emphasize that combination of different phytocomplexes could determine dynamic and mutable mixture, leading to reciprocal modifications through interactions of their several compounds.

MIC and MBC values of both single EOs and their blends are reported in Table 4. Whereas the behaviour of the two strains of *S. aureus* was similar with respect to the selected EOs and their mixtures. *S. xylosus* showed slight differences in terms of susceptibility. The five mixtures tested showed a strong synergistic activity among the essential oils. Comparing the MIC mode values obtained from individual oils and those obtained from the mixtures, it is evident that lower concentrations of each mixture were required to inhibit staphylococci isolates. Even if compared to *Cz* EO, which showed the highest antibacterial activity amongst the single EOs (MIC range from 0.94 g/ml to 1.88 g/ml), each of the blends reported a lower MIC range (A: MIC range from 0.46 g/ml to 0.93 g/ml; B: MIC range from 0.92 g/ml to 1.84 g/ml; C: MIC range from 0.24 g/ml to 0.47 g/ml; D: MIC range from 0.47 g/ml to 0.94 g/ml; E: MIC range equal to 0.47 g/ml).

MBC values of blends followed the same trend observed for the single EOs. The best results were obtained from mixture E with MIC and MBC mode values of 0.47 g/ml for each strain tested. Mixture B reported a MIC range between 0.92 g/ml and 1.84 g/ml proving to be the less effective of the mixtures, but still slightly more active than the best single EO used in the tests. *Cz*, *Sm*, *Tc* and *Ts* EOs showed a strong antibacterial activity against *S. aureus* strains, that was less marked for *Hi* EO. Mixtures B, C and D were more effective on *S. xylosus* strains, while mixtures A and E showed equal activity against both the staphylococci species.

4 Conclusions

Both individually tested EOs and their blends proved to have in vitro antibacterial properties, particularly remarkable for the latter. This is ascribable to the synergistic effect between different essential oils, allowing to reduce the EO percentage in the formulation of mixtures and limiting the risk of side effects associated with the use of these substances. Synergy should be more thoroughly investigated by determining FIC (fractional inhibitory concentration) and FBC (fractional bactericidal concentration) values at the same time as those of MIC and MBC concerning single EOs. On the other hand, this type of analysis turns out to be very laborious and expensive for the high amount of essential oil needed, especially using mixtures with more than three different EOs. On the basis of these in vitro positive results, it would therefore be advisable to assess the effectiveness and safety of these substances in vivo, so that essential oils and their appropriate mixtures may hereafter represent a real therapeutic option, as an alternative or complement to traditional antibiotic therapy.

Acknowledgements The authors want to thank Mario Rosario Rizzi (FLORA s.r.l.®) for the essential oil samples provided and Stefano Spinelli for the effective cooperation, both essential for carrying out the research.

Funding Open access funding provided by Università di Pisa within the CRUI-CARE Agreement.

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