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

Filippo Fratini1,2 · Margherita Giusti3 · Simone Mancini1 · Francesca Pisseri3 · Basma Najar4 · Luisa Pistelli2,4

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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...
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 spasmyloytic (Paaer 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 Viega et al. 2014; Djihane and Mihoub 2016), along with an elevated capacity to increase the antibiotics effectiveness against multidrug-resistant Gram-negative bacteria (Lorenti 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), cephalosporins (30 µg, CAZ), ciprofloxacin (5 µg, CIP), cefotaxime (30 µg, CTX), doxycycline (30 µg, DOX), enrofloxacin (5 µg, ENR), gentamicin (10 µg, GEN), cephalaxin (30 µg, LEX), neomycin (30 µg, NEO), pipercillin (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.
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 flow- ers 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).
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 Sa1 and Sa2) and Staphylococcus xylosus (named Sx1, Sx2 and Sx3). 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), Leptosperrnum 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 |
|---------------------------|-------|------|------|----------------|
|                           |       |      |      | A   | B   | C   | D   | E   |
|                           |       |      |      |     |     |     |     |     |
| α-Thujene                 | mh    | 930  | 0.2  | 0.5 | 0.4 | 0.3 | 0.3 | 0.3 |
| α-Pinene                  | mh    | 939  | 0.8  | 1.2 | 0.6 | 0.6 | 0.3 | 3.2 |
| Camphene                  | mh    | 954  | 0.3  | 0.6 | 0.7 | 0.2 | 0.1 | 0.4 |
| Sabinine                  | nh    | 975  | 0.4  | 0.5 | 0.6 | 0.2 | 0.1 | 0.4 |
| 1-Octen-3-ol              | nh    | 979  | 0.6  | 0.2 | 0.2 | 0.2 | 0.2 | 0.3 |
| Myrcene                   | mh    | 991  | 0.1  | 1.3 | 1.4 | 1.5 | 1.1 | 1.2 |
| α-Phellandrene            | mh    | 1003 | 1.0  | 0.3 | 0.1 | 0.2 | 0.2 | 0.3 |
| δ-3-Carene                | mh    | 1007 | 0.1  | –   | –   | –   | 0.1 | 0.1 |
| α-Terpine                                                      |      |     |     |     |     |     |     |     |
| p-Cymene                  | mh    | 1025 | 2.0  | 1.1 | 13.6| 6.3 | 8.3 | 9.2 |
| β-Phellandrene            | mh    | 1030 | 1.3  | 0.3 | 1.2 | 1.4 | 1.1 | 1.0 |
| 1,8-Cineol                | om    | 1031 | 3.3  | –   | 1.2 | 2.5 | 1.7 | 1.6 |
| Limonene                  | mh    | 1033 | 2.0  | 2.0 | 1.6 | 0.8 | 1.8 | –   |
| (Z)-β-Ocimene             | nh    | 1037 | –    | 0.4 | –   | –   | 0.3 | 0.3 |
| (E)-β-Ocimene             | nh    | 1050 | –    | 0.1 | –   | –   | –   | –   |
| γ-Terpine                 | mh    | 1060 | –    | 6.4 | 1.8 | 4.7 | –   | –   |
| p-Mentha-2,4(8)-diene     | nh    | 1088 | –    | 0.3 | –   | –   | –   | –   |
| Terpinolene               | mh    | 1089 | –    | 0.3 | 0.2 | 0.3 | 0.3 | 0.3 |
| Linalool                  | nt    | 1097 | 5.8  | 0.8 | 1.7 | 9.1 | 1.7 | 1.7 |
| α-Pinene oxide            | om    | 1099 | –    | 1.3 | –   | –   | 0.4 | –   |
| 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 | –   |
| Camphor                   | om    | 1146 | 0.2  | –   | 0.3 | –   | 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 |
| 4-Terpineol               | om    | 1177 | 0.5  | 1.5 | 1.0 | 1.2 | 1.0 | 1.1 |
| α-Terpine                 | om    | 1189 | 1.2  | 0.4 | 0.9 | 0.6 | 0.3 | 0.6 |
| Dihydrocarvacrol          | om    | 1194 | –    | –   | –   | –   | 0.2 | 0.2 |
| Myrtenol                  | om    | 1196 | 0.3  | –   | –   | –   | –   | –   |
| Verbenone                 | om    | 1205 | 0.4  | –   | –   | –   | –   | –   |
| trans-Verbenol            | om    | 1217 | 0.5  | –   | –   | –   | –   | –   |
| γ-Terpine                 | om    | 1218 | 0.4  | –   | –   | –   | –   | –   |
| (Z)-Cinnamaldehyde        | nt    | 1219 | 3.1  | –   | –   | –   | 0.2 | 0.2 |
| Nerol                     | om    | 1230 | 0.2  | –   | –   | –   | –   | –   |
| cis-Sabinene hydrate      | om    | 1240 | 0.3  | 0.6 | 0.2 | 0.2 | 0.3 | 0.2 |
| Cuminumaldehyde           | om    | 1242 | 5.1  | –   | 2.2 | 2.0 | 2.3 | 1.8 |
| Carvone                   | om    | 1243 | 0.2  | 0.2 | 0.2 | –   | 0.1 | 0.1 |
| (Z)-3-Hexenyl isovalerate | nt    | 1245 | 0.3  | –   | –   | –   | –   | –   |
| trans-myrtenol            | om    | 1261 | –    | –   | –   | –   | –   | –   |
| Geranial                  | om    | 1267 | 0.2  | –   | –   | –   | –   | –   |
| (E)-cinnamaldehyde        | nt    | 1270 | 55.3 | –   | –   | –   | 5.0 | 6.1 |
| 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 |
Table 3 (continued)

| Compound                  | Class       | LRI  | Eos | Cz | Hi | Sm | Tc | Ts | Mixture of EOs |
|---------------------------|-------------|------|-----|----|----|----|----|----|----------------|
|                           |             |      |     |    |    |    |    |    | 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 |
| Dauccene                  | 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-guaiane                 | 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-muuroleene              | 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 | –   | –   | –   | –   | –   | –   | –   | –   | –   |
| Benzy1 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 | –   | –   | –   | –   | –   | –   | –   | –   | –   |
| Sesquialvandulyl acetate  | os          | 1809 | 0.6 | –   | –   | –   | –   | –   | –   | –   | –   | –   | –   |

Class of compounds

Monoterpe 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
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 leptospermine and iso-leptospermine (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 × damascena, 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

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**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) |
|---------------------|-----------------|-----------------|
|                     | Sa1  | Sa2  | Sx1  | Sx2  | Sx3  | Sa1  | Sa2  | Sx1  | Sx2  | Sx3  |
| 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 | 0.47 | 0.47 | 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: Sa1 and Sa2. *Staphylococcus xylosus* isolates: Sx1, Sx2 and Sx3

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%
were characterised by the presence of $p$-cymene, $\gamma$-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 monoterpenic moiety 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; Grey and Hammer 2015).

The predominant compound in cinnamon-bark EO was the (E) isomer of cinnamaldehyde (55.3%) followed by $\beta$-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 $\alpha$-pinene (24.6%) and viridiflorene (18.9%). The former is a monoterpenic moiety with proven antibacterial activity (de Sousa Eduardo et al. 2018; Ložienė et al. 2018) while the latter is a sesquiterpenic moiety produced by the dehydration of viridiflorol whose biological activities are not yet well known, firstly isolated from *Majo-

rana 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 cinnamaldehyde 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 suggest emphasize that combination of different phyto complexes 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.

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