Fitoterapia 96 (2014) 1–7

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

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

Filippo Fratini [a,e], Sergio Casella [b], Michele Leonardi [b], Francesca Pisseri [c], Valentina Virginia Ebani [a,e], Laura Pistelli [d,e,*], Luisa Pistelli [b,e]

[a] Dipartimento di Scienze Veterinarie, University of Pisa, Viale delle Piagge 2, 56124 Pisa, Italy
[b] Dipartimento di Farmacia, University of Pisa, Via Bonanno 6, 56126 Pisa, Italy
[c] Scuola CIMI-Koinè, Via Ugo Bassi 2, Roma, Italy
[d] Dipartimento di Scienze Agrarie, Alimentari e Agro-ambientali, University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy
[e] Centro Interdipartimentale di Ricerca "Nutraceutica e Alimentazione per la Salute University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy

ARTICLE INFO

Article history:
Received 29 January 2014
Accepted in revised form 31 March 2014
Available online 13 April 2014

Keywords:
Mastitis infection
GC–MS analysis
Staphylococcus sp.
Carvacrol
Thymol
p-Cymene

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.

* Corresponding author at: Dipartimento di Scienze Agrarie, Alimentari e Agro-ambientali, University of Pisa, Italy. Tel.: +39 0502216536; fax: +39 0502216532.
E-mail addresses: filippo.fratini@virgilio.it (F. Fratini), sergio.casella@ymail.com (S. Casella), mikileonardi@gmail.com (M. Leonardi), francesca.pisseri@vet.unipi.it (F. Pisseri), vebani@vet.unipi.it (V.V. Ebani), laura.pistelli@unipi.it (L. Pistelli), luisa.pistelli@farm.unipi.it (L. Pistelli).

http://dx.doi.org/10.1016/j.fitote.2014.04.003
0367-326X/

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,