



ORIGINAL ARTICLE

Evaluation of the antimicrobial efficacy of
Minthostachys verticillata essential oil and limonene
against *Streptococcus uberis* strains isolated from
bovine mastitis



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Received 7 October 2015; accepted 18 April 2016

Available online 9 September 2016

KEYWORDS

Streptococcus uberis;
Minthostachys verticillata;
Essential oil;
Limonene;
Bovine mastitis;
Antibacterial activity

Abstract Bovine mastitis is a disease that causes great economic losses per year, being *Streptococcus uberis* the main environmental pathogen involved. The aim of the present study was to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of *Minthostachys verticillata* essential oil and limonene for *S. uberis* strains isolated from bovine mastitis. In addition, the effect of MIC on biofilm formation was analyzed. MIC values for the essential oil ranged from 14.3 to 114.5 mg/ml (1.56–12.5% v/v) and MBC between 114.5 and 229 mg/ml (12.5–25% v/v). MICs for limonene ranged from 3.3 to 52.5 mg/ml (0.39–6.25% v/v) and MBC was 210 mg/ml (25% v/v). Both compounds showed antibacterial activity and affected the biofilm formation of most of the strains tested. In conclusion, these compounds could be used as an alternative and/or complementary therapy for bovine mastitis caused by *S. uberis*.

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PALABRAS CLAVE

Streptococcus uberis;
Minthostachys verticillata;
Aceite esencial;
Limoneno;
Mastitis bovina;
Actividad antibacteriana

Evaluación de la eficacia antimicrobiana del aceite esencial de *Minthostachys verticillata* y limoneno contra cepas de *Streptococcus uberis* aislados de mastitis bovina

Resumen La mastitis bovina es una enfermedad que causa grandes pérdidas económicas por año, *Streptococcus uberis* es el principal patógeno ambiental involucrado. El objetivo del presente estudio fue determinar la concentración inhibitoria mínima (CIM) y la concentración bactericida mínima (CBM) del aceite esencial de *Minthostachys verticillata* y del limoneno sobre cepas de *S. uberis* aisladas de mastitis bovina. Además, se analizó el efecto del aceite esencial y el limoneno en la CIM determinada en caso sobre la formación de *biofilm* de estas cepas. Los valores de CIM del aceite esencial oscilaron entre 14,3 y 114,5 mg/ml (1,56%-12,5% v/v) y los de CBM entre 114,5 y 229 mg/ml (12,5%-25% v/v). Las CIM del limoneno oscilaron entre 3,3 y 52,5 mg/ml (0,39% - 6,25% v/v) y la CBM fue de 210 mg/ml (25% v/v). Ambos compuestos mostraron actividad antibacteriana y afectaron la formación de *biofilm* de la mayoría de las cepas. En conclusión, estos compuestos podrían ser utilizados como terapia alternativa o complementaria para la mastitis bovina causada por *S. uberis*.

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Introduction

Mastitis is a worldwide disease of dairy cattle that is caused by a wide variety of organisms that affect milk quality and yield, resulting in major economic losses. In many countries it is the most costly disease in dairy milk production¹⁸. *Streptococcus uberis* is an important pathogen implicated in bovine mastitis, which is predominantly associated with subclinical and clinical intramammary infections in both lactating and non-lactating cows. This species is particularly problematic due to the fact that it is ubiquitous in the dairy environment. A potential virulence factor, possibly linked to the ability of *S. uberis* to adhere to cells, would be the formation of biofilm³³. It is important to forestall the formation of biofilm in order to treat and prevent intramammary infections.

The ineffectiveness of the different procedures to reduce the rate of new infections has directed research studies toward the search for alternative control methods^{21,27}. Within this context, the search for new effective natural prototypes for the treatment of bovine mastitis does not compromise the milk quality that is important for a better quality of dairy farming and food production. Alternative treatments with medicinal plants may be a safe, efficient and a low-cost option for treating bovine mastitis²³. Essential oils classified as GRAS (Generally Regarded As Safe), show antibacterial properties and resistance has not been reported after prolonged exposure. Therefore, the investigation of their antimicrobial activity against bacterial agents of mastitis is justifiable⁹.

Minthostachys verticillata (Griseb) Epling (Lamiaceae), commonly referred to as "peperina", is an ethnobotanical aromatic herb with various uses and properties. This species is distributed in South American countries such as Colombia, Venezuela, Brazil, Ecuador, Peru, Bolivia, and in the northwest and central regions of Argentina^{24,29}. According to folk traditional medicine it is used as a digestive, sedative, antispasmodic, stimulant,

and also to alleviate respiratory illnesses, bronchitis, and asthma¹⁰.

Moreover, numerous *in vitro* studies have described the antiviral, antibacterial and antifungal properties of *M. verticillata* essential oil (EO)^{2,11,14,20,26}.

In addition, the lack of toxic effect of *M. verticillata* EO and its main compounds, both *in vitro* as *in vivo*, has been demonstrated^{5,7,12,13,32}. In a previous assay, we demonstrated that EO obtained from this species and from limonene, one of its main compounds, showed antimicrobial activity against the major bovine mastitis pathogens such as *Staphylococcus aureus*, *S. uberis*, *Escherichia coli* and coagulase negative *Staphylococcus* (CNS) by the disk diffusion method⁶.

The aim of the present work was to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of *M. verticillata* EO and limonene for *S. uberis* strains isolated from bovine mastitis. In addition, the effect of MIC on biofilm formation was analyzed.

Materials and methods

Plant material

Green leaves and thin stems from *M. verticillata* were collected in Villa Larca city, province of San Luis, Argentina in April, 2013. The voucher specimens were deposited in the herbarium of Universidad Nacional de Río Cuarto (Río Cuarto city, province of Córdoba, Argentina).

Essential oil extraction

Essential oil was obtained from the aerial parts of the plant, composed of leaves and parts of the stem. To prepare the EO, 60 grams of ground material were hydrodistilled using a Clevenger-type apparatus for 3 h. The oil was separated

from the aqueous phase, dried over anhydrous Na_2SO_4 and stored in the dark at -20°C until use³⁰.

The pure compound limonene was purchased from Sigma Aldrich (St. Louis, USA) as (*R*)-(+)-Limonene.

Identification and quantification of essential oil compounds by gas chromatography–mass spectrometry (GC/MS). To perform this analysis, a gas chromatograph *Clarus 600*, PerkinElmer (Shelton, Connecticut, USA) serial number 664N9100105 fitted with a DB5 capillary column (60 m, 0.25 mm ID, 0.25 μm particle), was used. Carrier gas: helium (49.6 psi). Oven temperature program: initial temperature 60°C (2 min), ramp: $5^\circ\text{C}/\text{min}$, final temperature 240°C (10 min). Injector temperature was 300°C . The sample was injected in split mode (20 ml/min). The chromatogram was obtained in "scan" mode, since $m/z = 30$ to $m/z = 450$ (scan time: 0.2 s, inter-scan time: 0.1 s, solvent delay: 5 min). The identification of the compounds present in the EO sample was performed by comparison of retention times and mass spectrum of the components found with the mass spectrum of the program library NIST MS Search 2.0. Under the same chromatographic conditions, the pure compounds (standard grade) were injected to verify the identity of the major components in the sample. Quantification of components present in the oil sample was carried out by measuring the area under each peak of the chromatogram^{4,34}.

Microorganisms

Fifteen *S. uberis* strains isolated from cows with mastitis from the central dairy region of Argentina were used in this study. The strains were previously identified according to Jayarao et al.¹⁵ and additionally confirmed by RFLP analysis of the 16S rRNA gene according to Khan et al.¹⁶ All strains were maintained at -20°C as stock strains in tryptic soy broth (TSB) (Britania S.A., Argentina) with glycerol until use.

MIC and MBC assay

The microdilution method was employed to determine the MIC and the MCB as recommended by the National Committee for Clinical Laboratory Standards⁸. For inoculum preparation, each strain was grown in 3 ml of TSB for 24 h at 37°C . The microorganisms to be tested were prepared by dilutions from cultures grown on TSB (1×10^8 colony forming units (CFU/ml), resulting in a bacterial suspension equivalent to 1×10^6 CFU/ml. EO and limonene were twofold serially diluted volume to volume in dimethylsulfoxide (DMSO) (Sintorgan, Buenos Aires, Argentina) and phosphate buffer saline pH 7.4 (PBS) (1:8) to facilitate solubility in the culture medium, and serial dilutions were performed, resulting in concentrations from 229 (25% v/v) to 0.45 mg/ml (0.049% v/v) and 210 (25% v/v) to 0.41 mg/ml (0.049% v/v) for EO and limonene, respectively.

The antimicrobial activity of limonene naturally present in the EO was also evaluated. A volume of $75 \mu\text{l}$ of each diluted agent and an equal volume of the standardized culture of each strain (1×10^6 CFU/ml) to be tested were added aseptically into the microplates, which were incubated at 37°C for 24 h. In addition, wells containing each microorganism culture in TSB without the tested agents

were measured as positive control, DMSO as vehicle control and only TSB as negative control. The MIC was determined as the lowest concentration of EO or limonene inhibiting visible growth. Absorbance was read at 560 nm using a spectrophotometer (Labsystem Multiskan MS, Thermo, Vantaa, Finland). The percentage of inhibition for the MIC of each agent was calculated using the formula described by Aiemsaard et al.¹:

$$[1 - (\text{OD}_{560} \text{ sample} / \text{OD}_{560} \text{ control}) \times 100\%]$$

All experiments were repeated twice using different microplates on each occasion.

To determine the MBC, volumes of $100 \mu\text{l}$ from wells without visible bacterial growth after 24 h of incubation were inoculated onto the surface of tryptic soy agar (TSA) (Britania S.A.) and incubated at 37°C for 24 h. The MBC was determined as the lowest agent concentration that killed greater than 99.9% of the initial bacterial population, which was indicated by no visible bacterial growth on the TSA plate surfaces. The positive growth of each microorganism culture in TSB without the tested agents served as a positive control and the growth was demonstrated only by TSB. In addition, oil diluent was used as vehicle control.

Antibacterial activity of essential oil and limonene against produced *S. uberis* biofilms

Seven strains, previously assayed for biofilm formation^{28,31} and considered to be strong formers according to Stepanovic et al.³¹ were used in this study. After aerobically incubation at 37°C for 24 h in order to produce biofilm, the medium was gently removed and the wells were washed three times with PBS pH 7.4. The MICs of the EO and limonene were then added separately to the biofilms and incubated for a further 24 h at 37°C . After biofilm formation, the medium was aspirated and non-adherent cells were removed by washing the biofilms twice with PBS. The adherent bacteria were stained with $100 \mu\text{l}$ of 0.1% of crystal violet for 15 min at room temperature. After rinsing with $200 \mu\text{l}$ of distilled water, the dye bound to the cells was extracted with $200 \mu\text{l}$ of 99% ethanol for 20 min. The extracted dye was then quantified by measuring absorbance at 560 nm. A series of biofilm oil-free wells and medium alone were also included to serve as positive and negative controls, respectively.

The percentage of biofilm inhibition for each agent was calculated using the formula described by Aiemsaard et al.¹

Statistical analysis

All the tests were performed in duplicate. The data obtained by microdilution assays were evaluated to a one-way analysis of variance (ANOVA) and the Tukey's multiple comparison tests using the program GraphPad Prism version 5.00.288 (San Diego, USA, 2007). MIC values (OD) were expressed as mean \pm standard deviation (SD). *P* values <0.05 were considered significant.

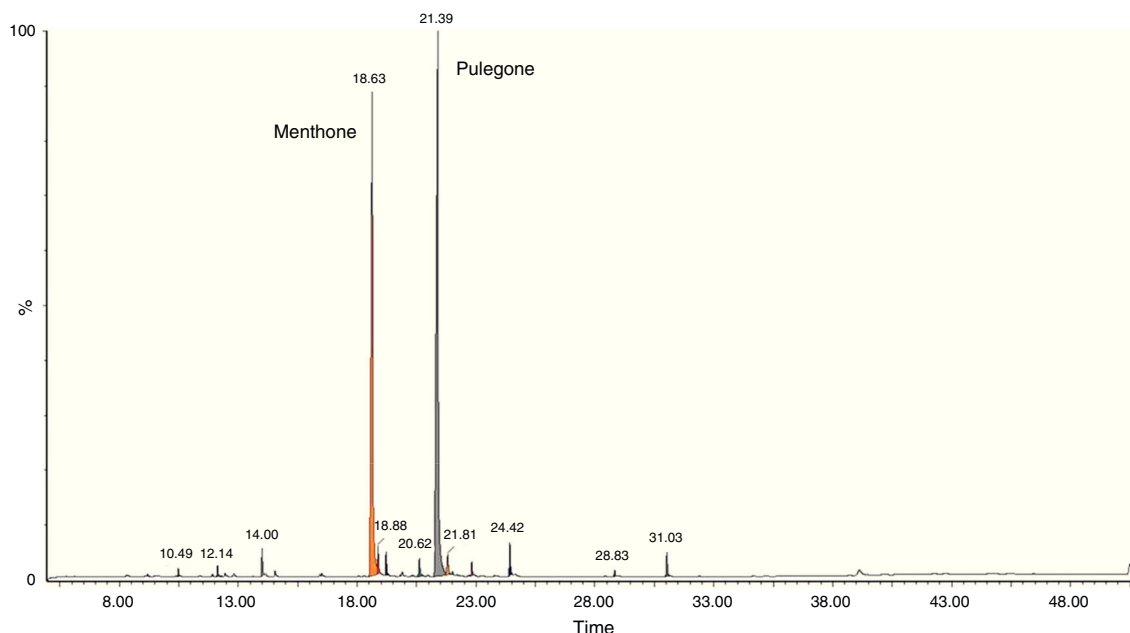


Figure 1 Chromatographic profile of the essential oil from the species *Minthostachys verticillata* collected from Villa Larca, San Luis, Argentina, obtained by GC–MS. The area represented by the peaks corresponds to the proportions in which each component is in the mixture. Retention times of each peak are observed.

Results and discussion

The aim of this study was to determine the antimicrobial efficacy of *M. verticillata* EO and limonene on *S. uberis* strains isolated from bovine mastitis.

The yield of *M. verticillata* EO was 4.8% w/v. GC/MS analysis revealed that the main components of the oil were pulegone (51.7%) and menthone (37.8%). Other compounds present were *cis*-menthone (1.4%), piperitone (1.4%) and limonene (1.2%). (Fig. 1, Table 1). This work has demonstrated that *M. verticillata* EO is mainly composed of pulegone and menthone, in agreement with previous studies^{3–5,11,12,34} where the composition of *M. verticillata* EO was reported.

The antimicrobial activity of *M. verticillata* EO and limonene against *S. uberis* strains isolated from bovine mastitis has not been previously reported. This is the first study that demonstrated significant antimicrobial activity of both EO and limonene against this bacterium. MIC values for EO ranged from 14.3 to 114.5 mg/ml (1.56–12.5% v/v) and MBC values, between 114.5 and 229 mg/ml (12.5–25% v/v). MICs for limonene ranged from 3.3 to 52.5 mg/ml (0.39–6.25% v/v) and MBC was 210 mg/ml (25% v/v). The MIC values were corroborated by absorbance measurement. Table 2 shows MIC and MBC values (% v/v and OD) of EO and limonene against the seven *S. uberis* strains that were more sensitive to these compounds. Our results demonstrated that EO as limonene showed good antibacterial activity. The bacterial strains tested have shown susceptibility to both agents.

Limonene showed inhibitory effects against the strains tested; these results indicated that limonene could be one of the compounds responsible for the antibacterial effect showed by EO. However the whole essential oil

Table 1 Chemical composition of essential oil obtained from *Minthostachys verticillata*

Identified compound	<i>M. verticillata</i> essential oil	
	Retention times (min)	Relative percentage (%)
δ -carene	10.486	0.29
β -pinene	12.136	0.40
Limonene	14.002	1.2
Menthone	18.634	37.8
<i>cis</i> -menthone	18.879	1.4
Isopulegone	19.219	1.15
Cyclohexanone, 2-isopropyl-2.5-dimethyl-	20.620	1.14
Pulegone	21.390	51.7
Piperitone	21.810	1.4
Bicyclo[2.2.1]heptan-2-ol, 2-allyl-1,7,7-trimethyl-	22.820	0.63
Pipieritenone	24.421	1.56
γ -elemene	28.833	0.27
Spathulenol	31.029	1.19
Total	-	100

demonstrated to be a more potent bacterial agent compared to limonene¹⁹.

The EO and limonene were also tested against *S. uberis* strains considered to be strong biofilm formers. Both agents were able to reduce biofilm production. The percentages of

Table 2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of *Mintostachys verticillata* essential oil and limonene against *Streptococcus uberis* strains

<i>S. uberis</i> strains	Positive control Growth (OD)	Vehicle control DMSO (OD)	Negative control growth (OD)	MIC EO (%v/v)	MIC EO (OD)	% Inhibition EO	MBC EO (%v/v)	MIC limonene (% v/v)	MIC limonene (OD)	% Inhibition limonene	MBC limonene (%v/v)
8	0.61 ± 0.06	0.64 ± 0.05	0.06 ± 0.02	6.25	0.07 ± 0.02 ^{***}	89.02 ± 3.93	25	3.12	0.06 ± 0.01 ^{***}	89.67 ± 1.39	25
44	0.49 ± 0.12	0.51 ± 0.04	0.06 ± 0.02	12.50	0.05 ± 0.02 ^{***}	90.71 ± 0.44	12.5	0.78	0.05 ± 0.01 ^{***}	89.70 ± 1.59	25
52	0.56 ± 0.09	0.61 ± 0.04	0.06 ± 0.02	6.25	0.05 ± 0.02 ^{***}	90.90 ± 0.50	12.5	1.56	0.07 ± 0.01 ^{***}	87.59 ± 0.63	25
56	0.57 ± 0.09	0.59 ± 0.02	0.06 ± 0.02	1.56	0.06 ± 0.02 ^{***}	89.74 ± 4.09	12.5	0.39	0.06 ± 0.01 ^{***}	88.51 ± 1.11	25
118	0.69 ± 0.04	0.67 ± 0.01	0.06 ± 0.02	3.12	0.09 ± 0.02 ^{***}	86.23 ± 0.83	12.5	0.78	0.05 ± 0.01 ^{***}	92.68 ± 1.13	25
148	0.53 ± 0.06	0.51 ± 0.02	0.06 ± 0.02	6.25	0.08 ± 0.01 ^{***}	79.34 ± 8.41	12.5	0.78	0.06 ± 0.01 ^{***}	89.34 ± 1.47	25
214	0.61 ± 0.12	0.62 ± 0.01	0.06 ± 0.02	12.50	0.06 ± 0.02 ^{***}	90.74 ± 0.11	12.5	6.25	0.07 ± 0.01 ^{***}	88.77 ± 1.96	25

Positive control growth, each strain growth with Trypticase Soy Broth; Negative control growth, Trypticase Soy Broth alone; DMSO, dimethylsulfoxide; MIC, minimum inhibitory concentration; EO, *Mintostachys verticillata* essential oil; OD, optical density; MBC, minimum bactericidal concentration.

^{***} $p < 0.0001$ respect to positive control (ANOVA and Tukey's test).

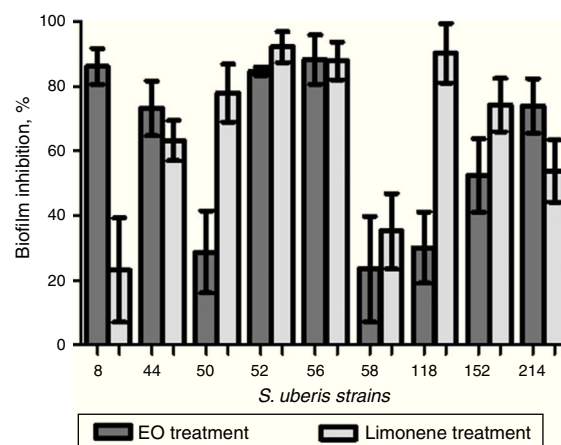


Figure 2 Effect of minimum inhibitory concentrations (MICs) of essential oils and limonene against biofilm of *S. uberis*.

inhibition of EO MIC were from 88.25 ± 7.62 to 23.50 ± 16.26 and of limonene were from 92.18 ± 4.78 to 23.20 ± 16.05 . Fig. 2 shows the effectiveness of EO and limonene against *S. uberis* biofilm.

Different studies have evaluated the efficacy of plant essential oils for improving milk quality in dairy cattle. The literature reports numerous studies referring to the antibacterial activity of essential oils against other pathogens isolated from bovine mastitis such as *Staphylococcus* spp., *S. aureus*, *Streptococcus agalactiae*, *Bacillus cereus*, *E. coli*^{1,9,25}. However, few studies demonstrated the antibacterial activity of essential oils against *S. uberis*. Lambrecht Gonçalves et al.¹⁷, evaluated the antibacterial activity of the essential oils of *Cymbopogon citratus* (DC.) Stapf., *Elionurus* sp. and *Tagetes minuta* L., against bacteria isolated from bovine milk. These authors obtained MIC values of 0.9%, 0.15% and 0.75% v/v, respectively, for the three vegetal species against *S. uberis*, which were similar to those obtained with limonene in our study.

Mullen et al.²² evaluated the antibacterial activity of Phyto-Mast (Bovinity Health LLC, Narvon, PA), an herbal intramammary product, against 3 mastitis-causing pathogens: *S. aureus*, *S. chromogenes*, and *S. uberis* by a modified protocol for broth dilution *in vitro*. The presence of the essential oil of *Thymus vulgaris* (thyme) in the formula may account for antibacterial action. The results showed that thyme essential oil had consistent antibacterial activity against the 3 mastitis-causing organisms tested.

The effect of EO and limonene against biofilm formation in *S. uberis* has not been previously reported. However, different studies showed the inhibition of *S. aureus* biofilm formation by essential oils. Aiensaard et al.¹ determined the effects of lemongrass oil in inhibiting biofilm formation of *S. aureus* isolated from bovine mastitis and they found that, it had $44.9 \pm 7.4\%$, inhibition on *S. aureus* biofilm formation at concentrations of 0.025% v/v of lemongrass oil.

It is important to note that the toxic effect of *M. verticillata* EO and its main compounds has been evaluated *in vitro*^{5,7,12,32} and *in vivo*^{5,7,12,13,32}. These authors have found no toxic effects of *M. verticillata* EO and its main compounds.

In conclusion, this study demonstrated that *M. verticillata* EO, as well as limonene, one of its main components showed antimicrobial efficacy against *S. uberis* strains presented as alternatives to be evaluated *in vivo* for the treatment of intramammary infections caused by this agent in cattle. However, further studies are needed to determine the mode of action of EO and limonene.

These results reinforce the importance of compounds isolated from plants and their influence on the elimination of pathogenic microorganisms, reaffirming the role of natural products.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina (CONICET) and PICT 2268/13. Mic. Ivana Dalila Montironi has Fellowship from CONICET. Dr. Laura Noelia Cariddi and Dr. Elina Beatriz Reinoso are Members of the Research Career of CONICET.

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