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Antimicrobial Activity of Essential Oils of *Lamiaceae* Aromatic Spices Towards Sheep mastitis-Causing *Staphylococcus aureus* and *Staphylococcus epidermidis*

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Abstract: Mastitis in ewes is responsible for massive economic losses. Antibiotics are routinely used for mastitis control but its excessive use leads to development of antibiotic resistance with deleterious outcomes both for animal and public health. Essential oils (EOs) show antibacterial proprieties and no resistance has been reported after prolonged exposure; however their efficacy depends on their chemical composition. In this study EOs chemical composition from four autochthonous aromatic herbs, from Alentejo region, southern Portugal, *Calamintha nepeta* subsp. *nepeta*, *Lavandula stoechas* subsp. *luisieri*, *Rosmarinus officinalis* and *Thymus mastichina* was accessed. EOs of *R. officinalis* showed predominance in monoterpene hydrocarbons (63 %) whereas EOs of *T. mastichina*, *L. luisieri* and *C. nepeta* were rich in oxygenated monoterpenes (71-95 %). The antimicrobial activity of selected EOs was investigated towards *Staphylococcus aureus* (n= 24) and *Staphylococcus epidermidis* isolates (n= 24) from ovine mastitic milk origin. Results of disk diffusion assay revealed that *C. nepeta*, *L. luisieri* and *T. mastichina* EOs are highly active against both *S. aureus* and *S. epidermidis* strains, whereas *R. officinalis* EO is highly active against *S. aureus* strains but inactive against several *S. epidermidis* isolates. EOs concentration causing bacterial growth inhibition ranged from 500 to 4,000 µg mL^{-1} in liquid microassays.

Key words: Essential oils, antimicrobial activity, ovine mastitis, *Staphylococcus aureus*, *Staphylococcus epidermidis*.

Introduction

Sheep milk production in Alentejo region, southern of Portugal, is mostly intended for the manufacture of artisanal cheeses, which constitute a heritage to the region and add value to the production of this raw material. For cheese production, the milk must hold certain physicochemical, biochemical and microbial features only found in milk from healthy mammary glands.

Mastitis in ewes is responsible for significant economic losses due to the death and early culling of animals [1], poor lamb growth and survival [2], lower milk yield [3] and lower milk quality [4]. As subclinical mastitis is asymptomatic, milk from affected animals enters cheese production resulting in lower cheese production [3,5] and worse quality [6].

Species of *Staphylococci* are the main etiological agents of ruminant mastitis. Coagulase negative *Staphylococci* showed to be the most prevalent agents of clinical and subclinical mastitis in ewes in Alentejo, with special relevance to *Sta-*

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phylococcus epidermidis [7]. In the same study, also *Staphylococcus aureus* was isolated from cases of both clinical and subclinical mastitis. This fact is particularly important as a public health concern, as *S. aureus* is an important enterotoxin producer [8].

Antibiotics are routinely used for mastitis control [9]. Nevertheless this practice is responsible for selection pressure for antibiotic-resistant strains, the main factor in development of antibiotic resistance [10]. Antimicrobial resistance genes have been detected in mastitis pathogens isolated from sheep [11] and the presence of these resistant strains in dairy products may lead to antibiotic resistance genes transfer to human intestinal indigenous microbiota [12].

Nowadays consumer demands regarding food safety requirements restrict the food presence of compounds used in animals' treatment. Therefore is appropriate to search for alternative substances that do not endanger consumer safety and contribute to the improvement of animal health.

Natural products as essential oils (EOs) and herbal extracts are currently used in medicine and food industry for their antimicrobial activities [13]. EOs are classified as *GRAS (generally recognized as safe)*, show antibacterial proprieties and resistance has not been reported after prolonged exposure. Previous investigation of some EO antimicrobial activity against *Staphylococcus* spp. isolated from bovine mastitis showed that they may be considered as alternative candidates in mastitis therapy [14].

The aim of this study was to investigate the antimicrobial properties of EOs of four Lamiaceae aromatic herbs, autochthonous from Portugal and used in Mediterranean diet, namely *Calamintha nepeta* subsp. *nepeta* (L.) Savi, *Lavandula stoechas* subsp. *luisieri* (Rozeira) Rozeira, *Rosmarinus officinalis* L. and *Thymus mastichina* L., towards important etiological agents of ovine mastitis, *S. aureus* and *S. epidermidis*.

Materials and methhod

Essential oils preparation

For the preparation of plant essential oils, leaves of *Calamintha nepeta* subsp. *nepeta* (*calamint*) and *Lavandula stoechas* subsp. *luisieri* (*lavender*) were collected in spring at Évora region, Alentejo. All plants were identified and voucher specimens were kept at the Herbarium of the University of Évora. After harvested, manual weeding of plants was done in order to obtain only the good parts of plants and eliminate the parts that could affect oil purity or interfere in oil pharmacological activity. The aerial parts of plants were air-dried in a dark room for 3 days. Essential oils of leaves from the plants studied were obtained by hydrodistillation for 3 h in a modified Clevenger-type apparatus, according to the European Pharmacopoeia method [15]. After extraction, the oils were stored at -20ºC.

Essential oils of *Rosmarinus officinalis* (rosemary) and *Thymus mastichina* (thyme) were provided by *ERVITAS CATITAS*, a local company that produce EOs from cultivated plants by biological agricultural practices.

Essential oils chemical characterization

Chemical characterization of EOs was performed by gas chromatography with an ionization flame detector (GC-FID)**.** Analysis was completed on HP-5890 SERIES II equipped with 30 m x 0.25mm i.d. x 0.25 µm Supelcowax™10 fused-silica polar capillary column (Supelco, Milford, USA) and ChemStation HP software, version A.04.02. The experiments were conducted under the following conditions: oven temperature programme, 70ºC (3 min), 70º - 220ºC (3ºC/min) and 220ºC (5 min); carrier gas flow rate 0.6 mL He/min; injector and detector temperatures 250ºC, injection volume 0.2 µL and split ratio 50:1.

The different components of EOs were identified by their retention indices, calculated using linear interpolation relative to retention times of C8 - C22 of n-alkanes, were compared with those of individual standards and data from the literature [16–18]. The quantification of compounds was performed using relative percentage abundance on the basis of their GC peak areas without correction factors, and percentage was determined with the mean value of three injections of each sample.

Bacterial isolates

The antimicrobial screening of essential oils was

performed by agar disk diffusion method against bacterial isolates from mastitic sheep milk origin, *Staphylococcus aureus* (n = 22) and *Staphylococcus epidermidis* (n = 22), with different profiles of antibiotic susceptibility, two *S. aureus* reference strains, ATCC 25923 and ATCC 29213, and two *S. epidermidis* reference strains, ATCC RP62A and ATCC 12228. Microbial isolates used in this study are deposited on the biobank of the Laboratory of Microbiology, Department of Veterinary Medicine, University of Évora and were isolated during a sheep mastitis survey [7].

Minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), determined using the broth micro-dilution method, were assayed against eight randomly chosen isolates of each two *Staphylococcus* species.

Bacterial inoculum were prepared by growing cells in blood agar (Blood Agar Base Nº 2, CM 0271 with 5 % sheep blood, Oxoid, England) for 24 h at 37°C. A cell suspension was prepared in MHB (Mueller-Hinton broth, CM 0405, Oxoid, England) to a final concentration of 10^8 CFU mL^{-1} for the disk diffusion assay and 10^6 CFU mL^{-1} for the broth dilution assay [19,20].

Agar disk diffusion assay

Agar disk diffusion assay was performed in accordance with CLSI Performance Standards for Antimicrobial Disk Susceptibility Tests [19]. For the evaluation of antimicrobial activity, MHA (Mueller-Hinton agar, CM0337, Oxoid, England) plates were inoculated with a sterile swab in three different directions, so that the inoculum was well distributed throughout the medium surface. Then three paper sterile disks were placed on each plate and 5 µL of EO was added. The assay was performed in triplicate for each EO. The plates were then incubated at 37°C for 24 hours, after which the diameter of the inhibition zone was measured. EOs were classified as highly active if the diameter of inhibition zone was greater than 8 mm, moderately active if the diameter was between 6 and 8 mm and inactive if the diameter was less than 6 mm.

Broth microdilution assay

To determine minimal inhibitory concentration (MIC) of EOs, broth microdilution assay was performed according to CLSI methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically [20]. EOs were diluted in MHB containing 5 %, v/v DMSO (dimethyl sulfoxide, D-8418, Sigma, USA) and 2.5 % v/v Tween 80 (polyoxyethylene-sorbitan monooleate, P-1754, Sigma, USA). Fifty microliters of each dilution were placed into 96-well plates and 50 µL of a bacterial suspension were added and the inhibitory action was assessed for the concentrations ranging 125-4,000 µg mL^{-1}. For growth control (positive control) 50 µL of MHB containing DMSO (5 %, v/v) and Tween (2.5 %, v/v) with 50 µL of a bacterial suspension was used and 100 µL of MHB with DMSO (5 %, v/v) and Tween (2.5 %, v/v) comprised the sterility control (negative control). All experiments were performed in triplicate. The plates were incubated at 37°C for 16 to 20 hours and bacterial growth was detected by the unaided eye observation. MIC was defined as the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the microdilution wells.

To determine the minimum bactericidal concentration (MBC), all cultures showing no turbidity were sub-cultured. Ten microliters from wells without bacterial growth were inoculated in the surface of MHA plates, in triplicate, and incubated at 37°C for 24 hours. The MBC was defined as the lowest concentration of EO that did not show bacterial growth [14].

Results and discussion

The chemical composition of crude essential oils is summarized in Table 1, reporting their main volatile components and respective percentage content. GC-FID analyses of each EO allowed the identification and quantification of its components. The composition of the EOs tested was very different among them, especially in what regards to their major components. EOs *C. nepeta*, L. *luisieri* and *T. mastichina* were rich in oxygenated monoterpenes whereas *R. officinalis* showed predominance of hydrocarbon monoterpenes.

The main components of EOs were isopulegol (43.0 %), isopulegone (15.6 %) and 1,8-cineole (14.4 %) for *C. nepeta*; 1,8-cineole (18.8 %) and

Table 1. Chemical composition of studied essential oils

Components	RI_{cal} [a]	RI	% Area			
			Calamintha nepeta	*Lavandula luisieri*	*Rosmarinus officinalis*	*Thymus mastichina*
α-Pinene	1027	1029 [c]	0.5	1.2	31.9	4.4
Camphene	1063	1073 [b]	0.1	-	8.1	-
β-Pinene	1119	1116 [b]	1.0	4.5	4.0	10.8
β-Myrcene	1157	1161 [b]	0.6	-	4.7	1.1
Limonene	1202	1205 [b]	1.4	-	11.2	1.7
1,8-Cineole	1215	1215 [b]	14.4	18.8	17.6	55.9
γ-Terpinene	1253	1251 [b]	-	-	1.8	1.2
p-Cymene	1265	1273 [b]	0.2	-	0.9	0.4
α-Terpinolene	1286	1287 [d]	-	-	-	1.2
Fenchone	1404	1401 [c]	-	1.9	-	-
Menthone	1463	1461 [d]	5.4	-	-	-
Isomenthone	1487	1488 [e]	6.3	-	-	-
Camphor	1514	1515 [b]	-	1.1	7.4	0.1
Linalol	1540	1542 [b]	-	4.1	-	3.9
Isopulegol	1560	1557 [e]	43.0	-	-	-
Isopulegone	1575	1578 [e]	15.6	-	-	-
trans-Necrodyl acetate	1590	1590 [c]	-	15.6	-	-
E-Caryophyllene	1593	1594 [e]	0.4	6.0	2.3	0.5
Terpinene-4-ol	1600	1599 [c]	1.2	3.6	1.0	0.2
Lavandulyl acetate	1602	1602 [c]	-	3.3	-	-
cis-Necrodyl acetate	1612	1611 [c]	-	1.2	-	-
allo-Aromadendrene	1636	1637 [b]	-	-	-	1.4
Isoborneol	1636	1665 [b]	-	10.0	1.3	-
Pulegone	1643	1640 [d]	7.0	-	-	-
trans-necrodol	1655	1657 [c]	-	10.1	-	-
Lavandulol	1670	1668 [c]	-	11.0	-	-
α-Terpinyl acetate	1682	1692 [b]	-	0.6	-	4.9
α-Terpineol	1700	1694 [c]	1.3	2.6	0.8	4.3
Borneol	1702	1696 [c]	-	1.2	3.8	1.4
Verbenone	1704	1698 [b]	-	-	1.8	-
Caryophyllene oxide	1970	1969 [b]	-	0.6	-	-
Viridiflorol	2071	2068 [c]	-	1.5	-	-
Total identified			98.4	98.9	98.6	93.4
Groups of components						
Monoterpene hydrocarbons			3.8	5.7	62.6	20.8
Oxygenated monoterpenes			94.2	85.1	31.9	70.7
Sesquiterpene hydrocarbon			0.4	6.0	2.3	1.9
Oxygenated sesquiterpenes				2.1	1.8	

[a] Retention indices relative to C8–C22 *n*-alkanes on the Supelcowax™10 column.

[b,c,d] Retention indices reported by literature: [b] Cavaleiro et al. (2004); [c] Zuzarte et al. (2012); [d] Mortram (2007); [e] Marongiu et al. (2010)

trans-α-necrodyl acetate (15.6 %), lavandulol (11.0 %) and *trans*-necrodol (10.1 %) for *L. luisieri*; α-pinene (31.9 %), 1,8-cineole (17.6 %), limonene (11.2 %) for *R. officinalis* and 1,8-cineole (55.9 %) and β-pinene (10.8 %) for *T. mastichina.*

According to disk diffusion assay, lavender and thyme EOs showed better inhibition activity for *S. aureus* isolates while calamint and lavender EOs exhibited greater activity for *S. epidermidis* isolates (Fig. 1 and 2).

Thyme EO evidenced to be highly active against all *S. aureus* isolates. The less active EO against this species isolates was rosemary EO, still it was strongly active against 16 isolates. Meanwhile, EOs of calamint and lavender, the most active against isolates of *S. epidermidis*, were both highly active against 23 isolates. Rosemary oil was poorly active against *S. epidermidis* isolates, being inactive against 18 isolates.

Results of broth dilution assay are summarized in Table 2 together with the inhibition zone on disk diffusion assay corresponding to each EO. The MIC value for the different EOs ranged from 500 μg mL^{-1} to 4,000 μg mL^{-1} (Table 2).

Overall, isolates of *S. epidermidis* showed to be more resistance to EOs than the isolates of *S. aureus*. Nine combinations of *S. epidermidis* isolate/ EO showed no inhibition, as bacteria were not inhibited with 4,000 μg mL^{-1} EO, while for *S. aureus* isolates, this happened for only four of the isolate/ EO cases.

Calamint displayed MIC values for *S. aureus* ranging from 500 to 4,000 μg mL^{-1} and for *S. epidermidis* from 2,000 to 4,000 μg mL^{-1}. However, by disk diffusion assay, this EO was highly

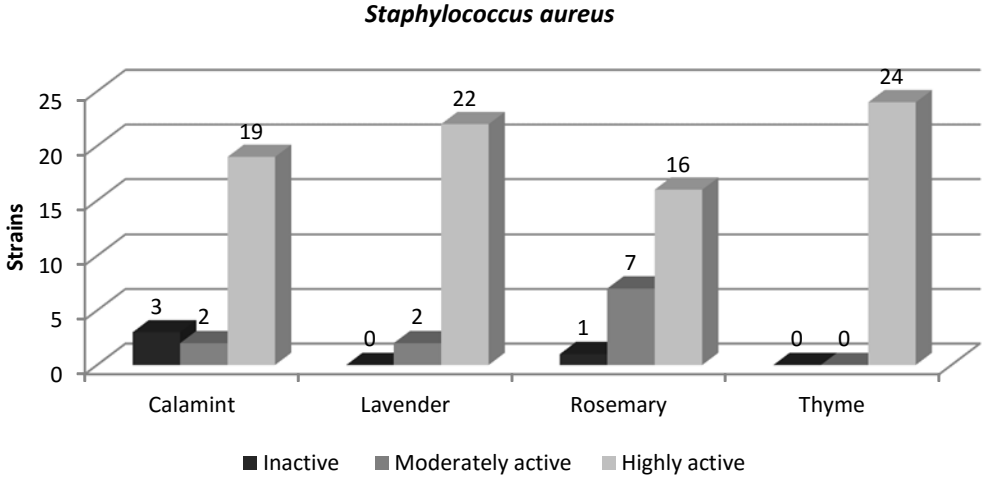

Figure 1. EOs disk diffusion assay antimicrobial activity against *S. aureus*

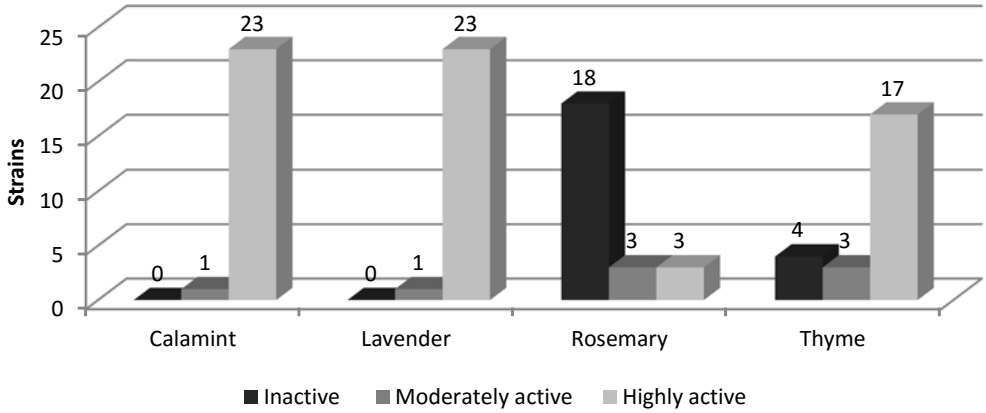

Figure 2. EOs disk diffusion assay antimicrobial activity against *S. epidermidis*

Table 2. Antimicrobial activity of essential oils of *C. nepeta*, *L. luisieri*, *R. officinalis* and *T. mastichina*

		Calamintha nepeta		*Lavandula luisieri*		*Rosmarinus officinalis*		*Thymus mastichina*	
		MIC (μg/mL)	**Inhibition zone (mm)**	**MIC (μg/mL)**	**Inhibition zone (mm)**	**MIC (μg/mL)**	**Inhibition zone (mm)**	**MIC (μg/mL)**	**Inhibition zone (mm)**
Staphylococcus aureus	201 E	4,000	11.2 ± 1.7	2,000	22.5 ± 2.7	4,000	8.3 ± 0.5	4,000	9.5 ± 0.6
	268 D1	2,000	13.8 ± 3.1	500	17.0 ± 2.4	500	7.5 ± 0.6	500	10.3 ± 0.5
	271 E	4,000	8.2 ± 0.4	>4,000	14.3 ± 0.8	4,000	7.5 ± 0.6	>4,000	9.0 ± 0.5
	286 D	500	11.3 ± 1.3	2,000	21.8 ± 3.3	2,000	7.8 ± 0.5	4,000	9.5 ± 0.6
	290 D	500	15.5 ± 1.9	2,000	21.8 ± 1.7	500	8.8 ± 0.5	500	11.8 ± 2.2
	335 E	500	19.3 ± 2.9	500	25.0 ± 2.6	500	9.5 ± 0.6	500	10.3 ± 0.5
	336 E2	2,000	8.8 ± 0.8	1,000	16.8 ± 3.8	4,000	9.3 ± 1.0	4,000	10.5 ± 0.6
	354 D	4,000	n.i.	>4,000	14.2 ± 1.2	4,000	10 ± 1.0	>4,000	11.0 ± 0.8
Staphylococcus epidermidis	170 D	4,000	13.3 ± 1.0	4,000	21.8 ± 2.1	4,000	n.i.	>4,000	10.0 ± 0.8
	186 D	4,000	14.5 ± 1.3	2,000	24.0 ± 2.2	1,000	n.i.	4,000	11.0 ± 1.0
	186 E	2,000	15.8 ± 3.3	2,000	23.3 ± 2.5	2,000	n.i.	4,000	11.5 ± 1.0
	213 E	2,000	15.5 ± 1.0	1,000	24.8 ± 3.6	500	n.i.	4,000	n.i.
	239 D3	4,000	9.8 ± 0.8	>4,000	33.0 ± 4.1	>4,000	n.i.	>4,000	9.8 ± 0.5
	242 E	4,000	8.2 ± 0.8	1,000	13.2 ± 1.5	1,000	n.i.	4,000	13.8 ± 1.3
	243 D	4,000	18.3 ± 0.5	>4,000	26.3 ± 1.5	>4,000	n.i.	>4,000	12.0 ± 0.8
	301 D	4,000	7.2 ± 0.8	>4,000	13.3 ± 2.2	4,000	n.i.	>4,000	9.0 ± 1.2

n.i.: no inhibition; n.d.; not determin

active against 19 *S. aureus* isolates and for 23 *S. epidermidis* isolates, exhibiting high antimicrobial potential for these Gram-positive microorganisms, some of them very resistant to commercial antimicrobial compounds.

Lavander MIC for *S. aureus* was between 500 and >4,000 µg mL^{-1}. This EO, though, showed excellent results on the disk diffusion assay displaying high activity towards 22 isolates, with zone inhibition areas ranging from 14.2 to 25 mm. For *S. epidermidis* isolates, Lavender MIC value ranged from 1,000 to >4,000 µg mL^{-1}, yet, on disk diffusion was highly active for 23 isolates, revealing zone inhibition areas between 13.2 and 33 mm. Other authors also report good antimicrobial activity for this EO. A study of Lai *et al.* [21], in which different organic extracts from Portuguese plants were evaluated for their antimicrobial potential, highlights *L. luisieri* extracts MIC values ranging between 15 and 250 µg mL^{-1} for *S. aureus* and 62 and 250 µg mL^{-1} for *S. epidermidis*. Arantes *et al*. [22] reported the high activity of *L. luisieri* EO against a wide spectrum of microorganisms, such as Gram-positive and Gram-negative bacteria, with MIC values ranging between 150 and 1,000 µg mL^{-1}.

Rosemary inhibited bacterial growth at concentrations between 500 and 4,000 µg mL^{-1} or higher, depending on the species, *S. aureus* or *S. epidermidis*. Also in disk diffusion assay the results were not very satisfying, as it was inactive for 18 *S. epidermidis* isolates. Likewise, Nascimento *et al.* [23] reported no antimicrobial properties in a disk diffusion test for rosemary EO, and Dal Pozzo *et al.* [14] stated that this EO showed no activity against *Staphylococcus* spp.. However, in other studies, rosemary EO was effective against *Staphylococcus* spp. [11,24,25].

Thyme MIC results were between 500 and >4,000 µg mL^{-1} for *S. aureus*, but, again, as to disk diffusion test it was highly effective for the totality of *S. aureus* isolates and for 17 of *S. epidermidis* isolates. Opposing to these results, Nascimento *et al*. [23] reported that *Thymus vulgaris* EO shows no antimicrobial properties in a disk diffusion assay, but according to Mohsenzadeh [26], the same thyme species EO has good activity against *S. aureus*, with a MIC of 10 to 100 µg mL^{-1}. Although the species of thyme used in this study was not the same, rendering to Faleiro *et al.* [27], the antimicrobial activity of EOs from different *Thymus* species collected in Portugal are comparable to other studies results on thyme EOs from mediterranean species [28].

Figure 3 to 6 underline the association between the results of the two tests. According to these results, there is no evidence of correlation between the zone of inhibition in the disk diffusion method

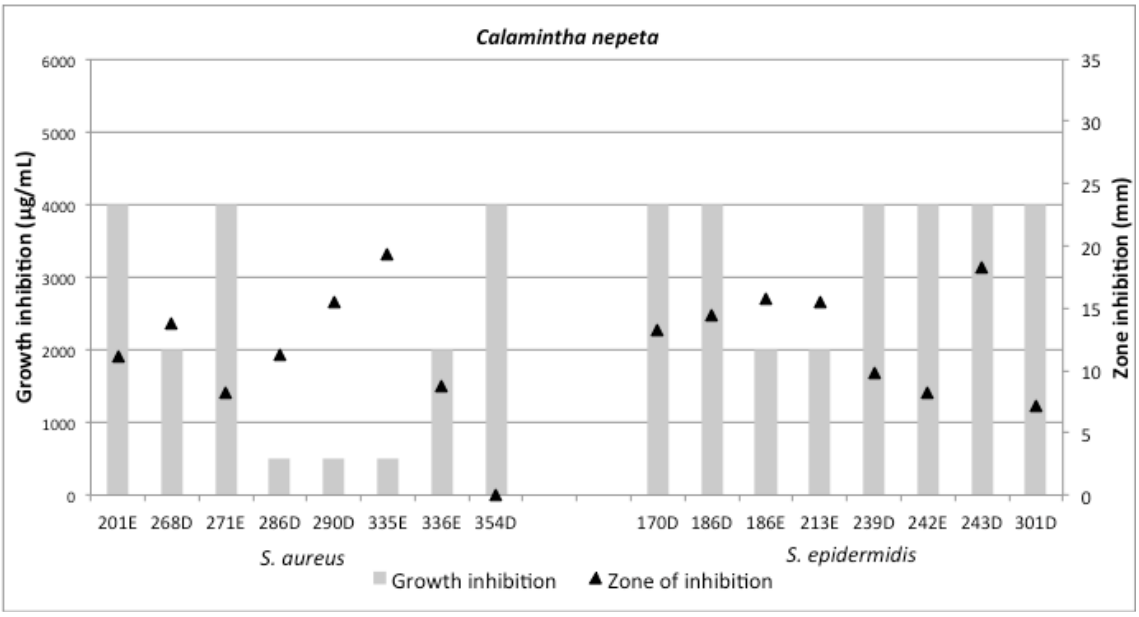

Figure 3. Calamint antimicrobial activity according to microdilution assay and disk diffusion method

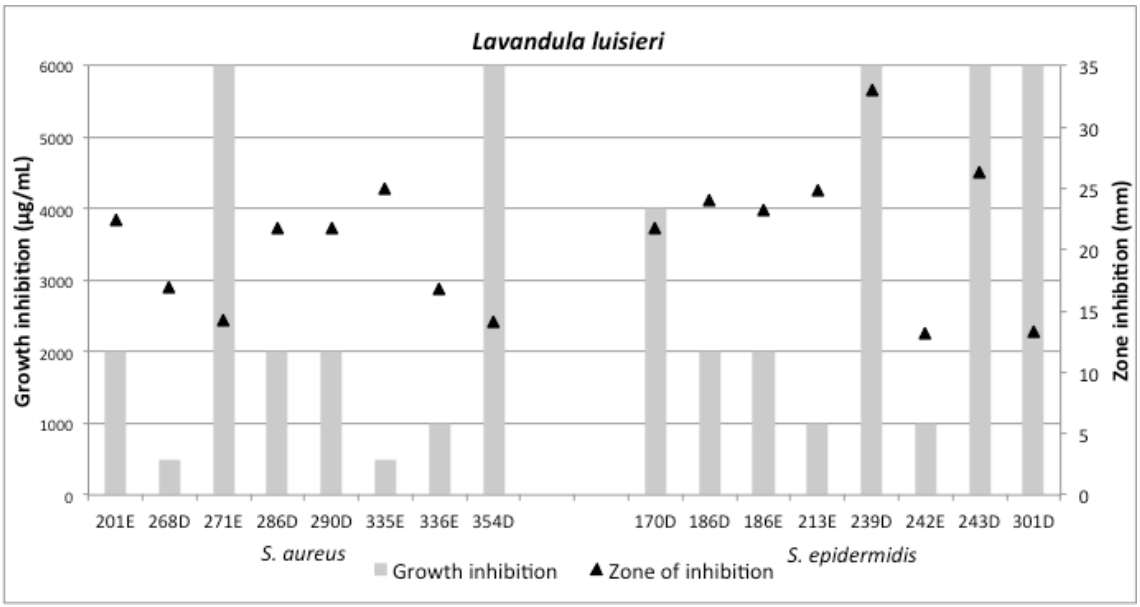

Figure 4. Lavender antimicrobial activity according to microdilution assay and disk diffusion method

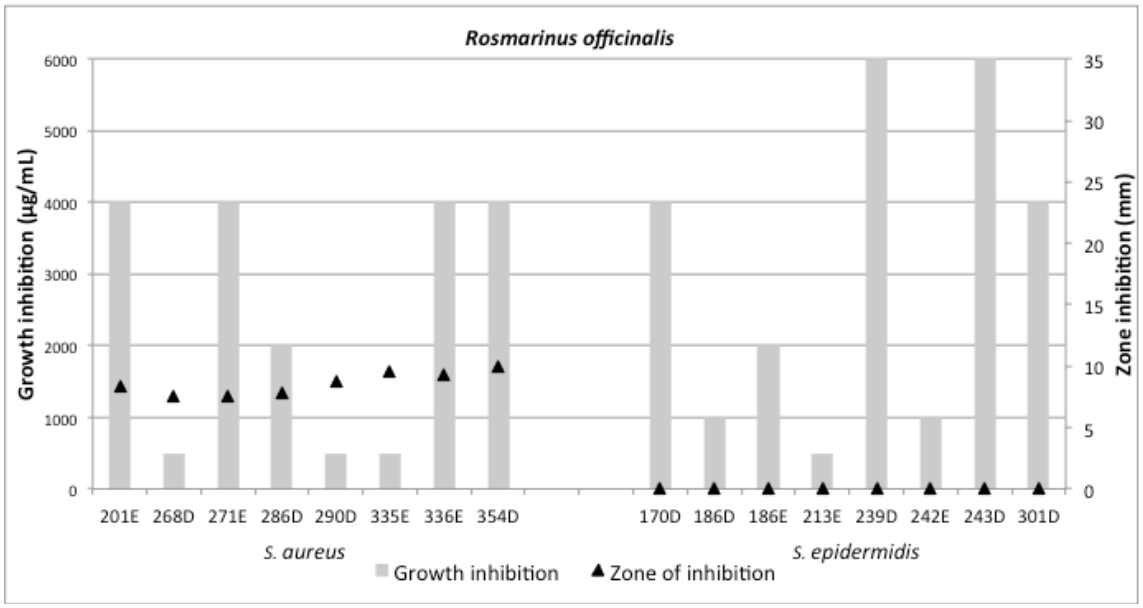

Figure 5. Rosemary antimicrobial activity according to microdilution assay and disk diffusion method

and the concentration of EO that inhibits bacterial growth (MIC).

In microdilution trial EOs showed low antibacterial activity comparatively to disk diffusion antimicrobial assays, probably due to their low solubility in liquid culture medium when added at high concentrations, which may facilitate their volatilization and resulting in loss of efficacy. Microdilution assay is widely used, as it is possible to work with very small volumes. However, this advantage can be the source of EOs inactivity towards bacteria, given that the small volume of EO used may volatilize before coming into contact with the bacteria [29,30]. In order to reduce the volatilization of the essential oils, the microtitre plates should be covered during the incubation.

There are many factors that may influence the results, as the EO composition, which depends

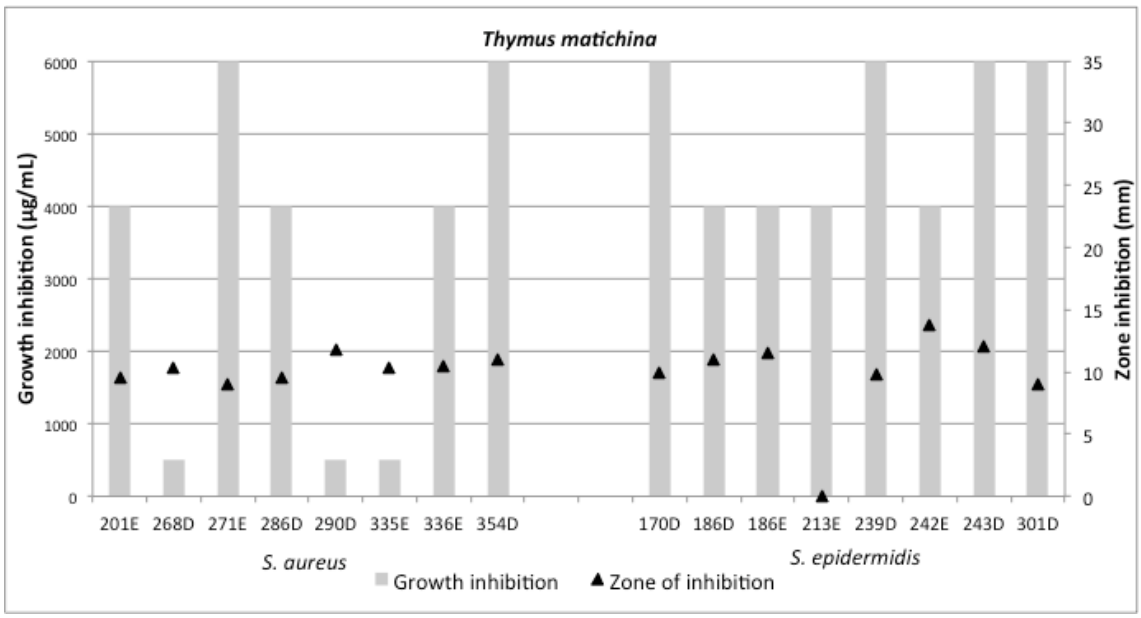

Figure 6. Thyme antimicrobial activity according to microdilution assay and disk diffusion method

on the plant growth conditions, development stage, extraction technique, fresh or dried plant material, among others [27,31] and the methodology followed for testing antimicrobial activity. Although the results of the microdilution tests indicate weak EOs antimicrobial activity towards these microorganisms, according to disk diffusion assay, the four EOs were highly active against most isolates. Some authors state that the microdilution method is not appropriate to test EOs [29,30]. Though in some studies using inhibition zones method and MIC assays, other researchers reported remarkable antimicrobial activity of some lamiaceae EOs against staphylococci [11,32].

For all cultures showing no turbidity the determination of the MBC was assessed. After incubation, bacterial growth was detected for the whole isolates, so the effect the studied EOs was bacteriostatic rather than bactericidal for the assessed field isolates.

Bacterial species used in this study are responsible for causing mastitis in sheep. These bacteria may have a relevant degree of resistance to antibiotics [33], so with this study we intended to seek for a different solution for the fight against sheep mastitis. We believe that the utilization of EOs from aromatic herbs growing wild in the south of Portugal and used in Mediterranean diet may be considered for mastitis control.

Teat seal is the post-milking teat disinfection with antiseptic solutions, which is a common and excellent practice in dairy cattle [34]. However it is a practice rarely used in sheep milk production. The use of EOs instead of antibiotics or antiseptic solutions for this type of disinfection presents a much smaller risk for public health. The EOs used in this work were extracted from Mediterranean plants often used as condiment in human cooking so the use of these EOs as antiseptics for teat sealing seems to be a safer alternative. To increase the antimicrobial effect of these EOs the combination of two or more of these oils could be an option. Therefore further studies are needed to evaluate the antimicrobial effect and usefulness of these EO combinations as well as *in vivo* studies to evaluate their effectiveness.

Conclusions

Disk diffusion assay showed that the four EOs are highly active against most isolates, although microdilution tests indicated weak EOs antimicrobial activity towards tested microorganisms. Considering the problems associated with antibiotics use for mastitis control, we believe that the utilization of aromatic herbs EOs may be considered for this purpose, but further studies are needed to evaluate its usefulness as well as *in vivo* studies to evaluate their effectiveness.

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