PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 46:e07779, 2026
DOI: 11590/1678-5150-PVB-7779

Original Article
Livestock Diseases

Antibacterial activity *in vitro* of essential oils from Brazilian plants against caseous lymphadenitis-related *Corynebacterium pseudotuberculosis*[1]

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ABSTRACT.- Lima PR, Silva WMB, Felix SF, Garcez Neto JR, Pereira RCA, Silva BF, Sousa NP, Policarpo WA, Faccioli-Martins PY, Carneiro VA, Costa RA, Morais SM. **Antibacterial activity *in vitro* of essential oils from Brazilian plants against caseous lymphadenitis-related *Corynebacterium pseudotuberculosis*.** *Pesquisa Veterinária Brasileira 46:e07779, 2026.* Universidade Estadual do Ceará, Av. Dr. Silas Munguba 1700, Campus do Itaperi, Fortaleza, CE 60714-903, Brazil. E-mail: selenemaiademorais@gmail.com

Caseous lymphadenitis (CLA) is a chronic infectious disease caused by *Corynebacterium pseudotuberculosis*, resulting in significant economic losses in sheep and goat farming. Considering limited treatment options, the use of natural compounds has emerged as a promising alternative. This study aimed to evaluate the antibacterial potential of essential oils from *Lippia origanoides* (EOLO) and *Ocimum micranthum* (EOOM) against *C. pseudotuberculosis* strains isolated from small ruminants with clinical CLA. Thirty isolates were genotypically characterized using quadruplex polymerase chain reaction (PCR) (16S rRNA, *rpoB*, *pld*, *narG* genes). The essential oils were extracted by hydrodistillation, and their chemical compositions were analyzed by gas chromatography-mass spectrometry (GC-MS). Antibacterial activity was determined by disk diffusion and broth microdilution assays. Minimum inhibitory concentration resulting in complete growth inhibition (MIC_{100}) and minimum bactericidal concentrations leading to complete bacterial eradication (MBC_{100}) were determined. Susceptibility to penicillin and tetracycline was assessed by disk diffusion. All strains were confirmed as biovar *ovis* (16S rRNA$^+$, *rpoB*$^+$, *pld*$^+$, *narG*$^-$). EOLO presented thymol (66.23%) as the major compound, while eugenol (38.62%) was the principal constituent in EOOM. EOLO exhibited large inhibition zone diameters (IZD), ranging from 40.67 mm to 98.33 mm, indicating strong activity. EOOM also demonstrated strong inhibition, with IZD values between 18.67 mm and 31.67 mm. All isolates were classified as "I" for penicillin (susceptible, increased exposure) and as susceptible ("S") to tetracycline. EOLO showed MIC_{100} values ranging from 15 to 250 µg/mL, while EOOM ranged from 62.5 to 125 µg/mL. MBC_{100} values ranged from 125 to 1,000 µg/mL. This study seems to be the first report of the *in vitro* antibacterial activity of EOLO and EOOM against *C. pseudotuberculosis*, highlighting their potential as effective, natural alternatives for the treatment and control of CLA in small ruminants.

INDEX TERMS: Bactericidal activity, *Lippia origanoides*, *Ocimum micranthum*, essential oil, small ruminants.

[1] Received on November 3, 2025.
Accepted for publication on January 12, 2026.
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RESUMO.- [**Atividade antibacteriana *in vitro* de óleos essenciais de plantas brasileiras contra cepas de *Corynebacterium pseudotuberculosis* relacionadas à linfadenite caseosa.**] A linfadenite caseosa (LC) é uma doença infecciosa crônica causada pela bactéria *Corynebacterium pseudotuberculosis* que provoca perdas econômicas significativas na ovinocaprinocultura. Considerando que as opções de tratamento são limitadas, o uso de compostos naturais representa uma alternativa promissora. O presente estudo teve como objetivo avaliar o potencial antibacteriano dos óleos essenciais de *Lippia origanoides* (EOLO) e *Ocimum micranthum* (EOOM) contra cepas de *C. pseudotuberculosis* isoladas de pequenos ruminantes com LC. Trinta isolados foram caracterizados genotipicamente usando reação em cadeia da polimerase (PCR) quadruplex (genes 16S rRNA, *rpoB*, *pld*, *narG*). Os óleos essenciais foram extraídos por hidrodestilação e suas composições químicas foram analisadas por cromatografia gasosa e espectrometria de massas (GC-MS). A atividade antibacteriana foi determinada por ensaios de disco-difusão e microdiluição em caldo. As concentrações inibitórias mínimas capazes de promover inibição completa do crescimento (CIM_{100}) e as concentrações bactericidas mínimas responsáveis pela erradicação bacterina completa (CBM_{100}) foram determinadas. A suscetibilidade à penicilina e à tetraciclina foi avaliada por difusão em disco. Todas as cepas foram confirmadas como biovar *ovis* (16S rRNA$^+$, *rpoB*$^+$, *pld*$^+$, *narG*$^-$). O EOLO apresentou o timol (66,23%) como o composto majoritário, enquanto o eugenol (38,62%) foi o principal constituinte do EOOM. O EOLO apresentou diâmetro de zona de inibição (DZI) variando de 40,67 mm a 98,33 mm, indicando forte atividade antibacteriana. O EOOM também demonstrou forte capacidade de inibição, com valores de DZI entre 18,67 mm e 31,67 mm. Todos os isolados apresentaram classificação "I" para penicilina (susceptível, com aumento da exposição) e foram classificados como sensíveis ("S") à tetraciclina. A CIM_{100} do EOLO variou de 15 a 250 µg/mL, enquanto a do EOOM variou de 62,5 a 125 µg/mL. Os valores de CBM_{100} variaram de 125 a 1.000 µg/mL. Este estudo parece ser o primeiro relato da atividade antibacteriana *in vitro* de EOLO e EOOM contra *C. pseudotuberculosis*, destacando o potencial desses óleos como alternativa naturais e eficazes para o tratamento e controle de LC em pequenos ruminantes.

TERMOS DE INDEXAÇÃO: Atividade bactericida, *Lippia origanoides*, *Ocimum micranthum*, óleo essencial, pequenos ruminantes.

INTRODUCTION

Caseous lymphadenitis (CLA) is a chronic infectious disease caused by Gram-positive *Corynebacterium pseudotuberculosis*. CLA results in economic losses in sheep and goat farming worldwide, with marked geographic differences in host prevalence. The biovar *ovis* infects sheep and goats, while biovar *equi* infects cattle, horses, camels, and buffaloes. This etiological agent is also associated with other diseases of veterinary importance, such as ulcerative lymphangitis, mastitis, and ulcerative dermatitis, and can occasionally cause chronic infections in humans (Dorella et al. 2006, Schlicher et al. 2021, Pinho et al. 2021, Torky et al. 2023).

CLA is characterized by abscess formation in peripheral lymph nodes and visceral organs, most commonly in goats and sheep (Faccioli-Martins et al. 2014, Silva et al. 2017, Farias et al. 2018b, El Damaty et al. 2023). The recommended conventional treatment for advanced abscesses involves drainage followed by chemical cauterization with 10% iodine tincture. However, this approach risks environmental contamination through spontaneous abscess rupture or improper handling of highly contaminated materials (Santiago et al. 2013, Farias et al. 2018a). Treating closed abscesses with antibiotics remains challenging due to limited drug penetration into the purulent content.

Natural products, such as essential oils from plants of the order Lamiales, offer an alternative for combating pathogenic bacteria. Lamiales includes more than 20 families, 1,000 genera, and 20,000 species, with Lamiaceae, Verbenaceae, Plantaginaceae, Scrophulariaceae, and Acanthaceae as major families (Pongkitwitoon et al. 2024). Among these, *Lippia origanoides* and *Ocimum micranthum* Willd. are native to tropical and subtropical regions of the Americas. Both species are rich in bioactive compounds such as thymol, 1,8-cineole, and eugenol, with documented antimicrobial, antiproliferative, and anti-inflammatory effects (Jaramillo et al. 2014, Caamal-Herrera et al. 2016, Chen et al. 2021, Santos Filho et al. 2023). However, their efficacy against *C. pseudotuberculosis* remains relatively unexplored.

Given the lack of effective treatments for *C. pseudotuberculosis* infections in sheep and goats, this study aimed to investigate the antibacterial potential of essential oils from *L. origanoides* (EOLO) and *O. micranthum* (EOOM) against clinical CLA isolates of *C. pseudotuberculosis* from small ruminants in Northeast Brazil.

MATERIALS AND METHODS

Ethical approval. Not applicable. This study used bacterial strains obtained from an existing institutional collection, and no experiments involving animals or human subjects were conducted.

Strains' origin and cultivation conditions. A total of 30 strains of *Corynebacterium pseudotuberculosis* (Table 1) selected from the "Coleção de Microrganismos Patogênicos a Caprinos e Ovinos" (Collection of Microorganisms Pathogenic to Goats and Sheep – CMPCO) at "Embrapa Caprinos e Ovinos" (Brazilian Agricultural Research Corporation Goats and Sheep), Sobral, Ceará, Brazil, were used. These strains (Table 1) were previously isolated from abscesses of goats and sheep with CLA from 2014 to 2021. Access activity to the genetic heritage of the samples registered in the National Genetic Heritage and Associated Traditional Knowledge Management System (SisGen) under registration number A875E13. All the strains were preserved at -70 °C in brain heart infusion (BHI) broth with 0.1% Tween 80® (BHI-T) and 20% glycerol. An aliquot of 10 µL was cultivated on blood agar with incubation at 37 °C for 72 h. Then, it was replicated in BHI 0.1% Tween (BHI-T) with incubation at 37 °C for 24 h to be used in the tests.

Genotypic characterization of bacterial isolates. DNA was extracted using the PureLink® Genomic DNA Mini Kit (Thermo Fisher Scientific), following the manufacturer's protocol. The extracted DNA was quantified using a NanoDrop® spectrophotometer (Loccus) at "Embrapa Caprinos e Ovinos" in Sobral/CE, Brazil. Species and biovar identification was performed by quadruplex polymerase chain reaction (PCR), targeting the 16S rRNA, *rpo*B, *pld*, and *nar*G genes (Almeida et al. 2017). Quadruplex PCR was carried out in a final volume of 50 µL, containing 20 ng of genomic DNA, 1 µM of each primer (Table 2), 0.25 mM dNTPs, four units of Taq DNA polymerase (Platinum™ II Taq Hot-Start DNA Polymerase, Invitrogen), and 1X

of its supplied buffer with $MgCl_2$. Amplification was performed in a thermal cycler (T100™ Thermal Cycler, Bio-Rad) under the following conditions: initial denaturation at 95 °C for 4 min; followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1.5 min. PCR products were analyzed by electrophoresis on a 1.5% agarose gel in 1x TBE (Tris-borate-EDTA) buffer. The gel was stained with ethidium bromide and visualized under ultraviolet light. *C. pseudotuberculosis* strains 1002 (biovar *ovis*) and 258 (biovar *equi*) were used as positive controls.

Plant material. *Lippia* origanoides was collected in the Serra da Gadelha region, Iguatu/CE, Brazil, in April 2024, while *Ocimum* micranthum was collected from the Medicinal Plant Garden at "Embrapa Agroindústria Tropical", Fortaleza/CE, Brazil, in 2022. Botanical identification was performed at the Prisco Bezerra Herbarium, "Universidade Federal do Ceará" (UFCE), where voucher specimens were deposited under accession numbers EAC 63412 and EAC 5685, respectively.

Extraction of essential oils. The essential oils from the leaves of *L. origanoides* (EOLO) and the aerial parts (leaves and inflorescences) of *O. micranthum* (EOOM) were extracted by hydrodistillation using a Clevenger-type apparatus. Six hundred grams of plant material were placed in a 1 L round-bottom flask with distilled

Table 1. Strains identification, year of isolation, host species (breed), and city/state of origin

Number	ID*	Year	Species host	Breed	City/state of origin**
1	BRM 029971	2014	*Ovis aries*	Morada Nova	Sobral/CE
2	BRM 044303	2014	*Capra hircus*	Saanen	Sobral/CE
3	BRM 044367	2016	*Ovis aries*	Morada Nova	Sobral/CE
4	BRM 044370	2016	*Ovis aries*	Morada Nova	Sobral/CE
5	BRM 044373	2016	*Capra hircus*	Anglo Nubiana	Sobral/CE
6	BRM 044376	2016	*Capra hircus*	Moxotó	Sobral/CE
7	BRM 044377	2016	*Capra hircus*	Saanen	Sobral/CE
8	BRM 044378	2016	*Capra hircus*	Anglo Nubiana	Sobral/CE
9	BRM 044379	2016	*Ovis aries*	Santa Inês	Sobral/CE
10	BRM 044397	2016	*Ovis aries*	Santa Inês	Sobral/CE
11	BRM 044399	2016	*Capra hircus*	Saanen	Sobral/CE
12	BRM 044402	2016	*Capra hircus*	Anglo Nubiana	Sobral/CE
13	BRM 044409	2016	*Capra hircus*	Saanen	Sobral/CE
14	BRM 044414	2016	*Ovis aries*	Somalis Brasileira	Sobral/CE
15	BRM 050762	2017	*Ovis aries*	Morada Nova	Sobral/CE
16	BRM 050763	2017	*Ovis aries*	Somalis Brasileira	Sobral/CE
17	BRM 050764	2017	*Capra hircus*	Canindé	Sobral/CE
18	BRM 050765	2017	*Ovis aries*	Santa Inês	Sobral/CE
19	BRM 050767	2017	*Capra hircus*	Moxotó	Sobral/CE
20	BRM 050773	2017	*Capra hircus*	Anglo Nubiana	Sobral/CE
21	BRM 050911	2017	*Capra hircus*	Saanen	Teresina/PI
22	BRM 064776	2021	*Capra hircus*	Saanen	Sertânia/PE
23	BRM 064777	2021	*Capra hircus*	Saanen	Ouro Velho/PB
24	BRM 064784	2021	*Capra hircus*	Saanen	Santo André/PB
25	BRM 064785	2021	*Ovis aries*	Santa Inês	Pintadas/BA
26	BRM 064787	2021	*Ovis aries*	Santa Inês	Pintadas/BA
27	BRM 064788	2021	*Ovis aries*	Santa Inês	Pintadas/BA
28	BRM 064790	2021	*Ovis aries*	Santa Inês	Pintadas/BA
29	BRM 064791	2021	*Capra hircus*	Parda Alpina	Quixabeira/BA
30	BRM 064792	2021	*Capra hircus*	Saanen	Quixabeira/BA

*BRM = identifier code for the microorganism record in Embrapa's microbial collection. **CE = Ceará, PI = Piauí, PE = Pernambuco, PB = Paraíba, BA = Bahia.

Table 2. Oligonucleotide primers used in this study

Gene	Primer	Sequence (5'–3')	Amplicon size (bp)
16S rRNA	Forward	ACCGCACTTTAGTGTGTGTG	816
	Reverse	TCTCTACGCCGATCTTGTAT	
rpoB	Forward	CGTATGAACATCGGCCAGGT	446
	Reverse	TCCATTTCGCCGAAGCGCTG	
Pld	Forward	ATAAGCGTAAGCAGGGAGCA	203
	Reverse	ATCAGCGGTGATTGTCTTCCAGG	
narG	Forward	ACCCGTACTTGCACTCTTTC	612
	Reverse	AGTCAGTACTTCCGCAGGTC	

Source: Almeida et al. (2017).

water, ensuring complete submersion. The mixture was heated until distillation began. The essential oil was collected, dried over anhydrous sodium sulphate, and stored under refrigeration until analysis. The distillation process lasted approximately 3 h after the onset of steam condensation (Teles et al. 2012). The yields of EOLO and EOOM were 2.53% and 0.2% w/w, respectively.

Chemical analysis of the essential oils. The chemical composition of the essential oils was determined by gas chromatography-mass spectrometry (GC-MS). Identification of the constituents was based on comparisons of retention times and mass fragmentation patterns with data from the National Institute of Standards and Technology (NIST) library and literature spectral database. Quantification was performed by calculating the relative area under each chromatographic peak using 10 mg of the essential oil previously diluted in 10 mL of chloroform. GC-MS analysis was conducted using a Shimadzu QP-2010 instrument equipped with an Rtx-5MS column (5% diphenyl/95% dimethylpolysiloxane; 30 m × 0.25 mm i.d. × 0.25 µm film thickness); Helium was used as the carrier gas at a constant linear velocity (24.2 mL/min). The injector temperature was set to 250 °C and operated in split mode (1:100), with the detector also maintained at 250 °C. The oven temperature program was as follows: from 35 °C to 180 °C at 4 °C/min, then from 180 °C to 280 °C at 17 °C/min, followed by a 10-minute hold at 280 °C. Mass spectra were obtained by electron impact ionization at 70 eV. One microliter (1 µL) of the sample was injected. Compound identification was confirmed by comparing relative retention times and fragmentation patterns with those of known compounds in the NIST database and published literature (Adams 2017).

Antibacterial activity: disk diffusion. As an initial screening of the antibacterial activity of EOLO and EOOM, the disk diffusion assay was performed according to Santos et al. (2021), with slight modifications. The bacterial strains were grown in BHI-T broth at 37 °C for 24 h, and their concentrations were adjusted to approximately 2 x 10^8 CFU/mL using 0.85% saline, based on turbidity equivalent to the 0.5 McFarland standard. Subsequently, the bacterial suspension was spread onto the surface of BHI agar plates. After a 5-minute absorption period, sterile paper disks impregnated with 10 µL of pure essential oils were placed on the agar surface. The plates were incubated at 37 °C for 48 h. Three independent assays were conducted, and the diameters of the inhibition zones were measured in millimeters. Results were expressed as the mean ± standard deviation of the triplicate measurements.

Antibiotic susceptibility testing by disk diffusion. Antimicrobial susceptibility to penicillin and tetracycline was evaluated by the disk diffusion method using 30 *Corynebacterium pseudotuberculosis* isolates, in accordance with the BrCAST-EUCAST Table of Clinical Breakpoints (BrCAST 2025). Bacterial suspensions were adjusted to a 0.5 McFarland standard and inoculated onto Mueller-Hinton agar supplemented with 5% defibrinated horse blood and 20 mg/L β-NAD (MH-F). Disks containing benzylpenicillin (1 U) and tetracycline (30 µg) were applied, and plates were incubated at 35 ± 1 °C under 5% CO_2 for 18–24 h; isolates exhibiting insufficient growth were re-incubated and read after 40–44 h. Inhibition zone diameters were measured and interpreted according to the BrCAST-EUCAST breakpoints for *Corynebacterium* spp. The selection of antibiotics was based on their relevance in veterinary practice, as they are among the antimicrobials authorized and commonly used in small ruminants in Brazil by the "Ministério da Agricultura, Pecuária e Abastecimento" (Ministry of Agriculture, Livestock and Food Supply – MAPA), where antibiotic therapy is used as an adjunct to abscess drainage in the management of caseous lymphadenitis.

Antibacterial activity: microdilution test — minimum inhibitory concentration (MIC_{100}) and minimum bactericidal concentration (MBC_{100}). The broth microdilution method was used to determine MIC_{100} and MBC_{100} in 96-well polystyrene microplates, as previously described by Santos et al. (2021), with modifications. Essential oils were individually diluted in BHI broth supplemented with 1% Tween 80 (Sigma-Aldrich) and tested at concentrations ranging from 3 to 8,000 µg/mL. Bacterial suspensions were adjusted in fresh BHI broth to an optical density of 0.08–0.10 at 600 nm and further diluted to a final concentration of 1 × 10^6 CFU/mL. Microplates were prepared by dispensing 100 µL of each test solution and 100 µL of bacterial inoculum per well and were incubated at 37 °C for 48 h. Growth control (bacterial suspension + BHI), sterility control (BHI only), and turbidity control (test compound + BHI without inoculum) were included. After incubation, MIC_{100} was defined as the lowest concentration showing complete inhibition of visible bacterial growth. For MBC_{100} determination, 10 µL aliquots from wells without visible growth were plated onto BHI agar and incubated at 37 °C for 48 h; MBC_{100} was defined as the lowest concentration at which no bacterial colonies were detected. All assays were performed in triplicate. In addition, the ratio between MBC_{100} and MIC_{100} (MBC_{100}/MIC_{100}) was calculated for each isolate to classify the antibacterial effect of the essential oils. Ratios ≤ 4 were considered indicative of bactericidal activity, whereas ratios > 4 were interpreted as bacteriostatic, according to previously established criteria (Khalfaoui et al. 2021, Makade et al. 2024, Olatunya et al. 2024).

RESULTS

All 30 strains from CMPCO at "Embrapa Caprinos e Ovinos" (Sobral/CE, Brazil) were genotypically confirmed as *Corynebacterium pseudotuberculosis* biovar *ovis*, based on detection of 16S rRNA (+), *rpo*B (+), *pld* (+) and *nar*G (-) genes (Fig. 1).

The GC-MS analysis (Table 3) identified 18 compounds in EOLO, primarily classified as oxygenated monoterpenes and hydrocarbon monoterpenes, accounting for 99.9% of the total composition. The major constituent was thymol (66.23%), followed by eucalyptol (12.33%) and o-cymene (6.01%), characterizing the oil as a thymol chemotype. In EOOM, 11 compounds were identified, predominantly sesquiterpenes and phenylpropanoids, representing 98.88% of the total oil content. The most abundant component was eugenol (38.62%), followed by β-caryophyllene (17.09%) and elemicin (14.65%), indicating a chemical profile consistent with the eugenol chemotype.

All strains were sensitive to EOLO and EOOM in the initial screening using the disk diffusion method. For EOLO, the inhibition zones ranged from 40.67 to 98.33 mm (Table 4), while for EOOM, they ranged from 18.67 to 31.67 mm (Table 5).

Disk diffusion assays using commercial antibiotics showed that all 30 isolates were classified as "I" for penicillin (susceptible, increased exposure), with inhibition zone diameters ranging from 15 to 30 mm. In contrast, all isolates were classified as susceptible ("S") to tetracycline, with inhibition zones ranging from 24 to 45 mm (Table 6).

EOLO showed minimum inhibitory concentrations (MIC_{100}) of 15 µg/mL, 31.2 µg/mL, 62.5 µg/mL, 125 µg/mL and 250 µg/mL, which inhibited 3.3% (n = 1), 36.7% (n = 11), 10% (n = 3), 26.7% (n = 8), and 23.3% (n = 7) of the tested strains (n = 30), respectively. As for the minimum bactericidal concentration (MBC_{100}), values of 31.2 µg/mL, 62.5 µg/mL,

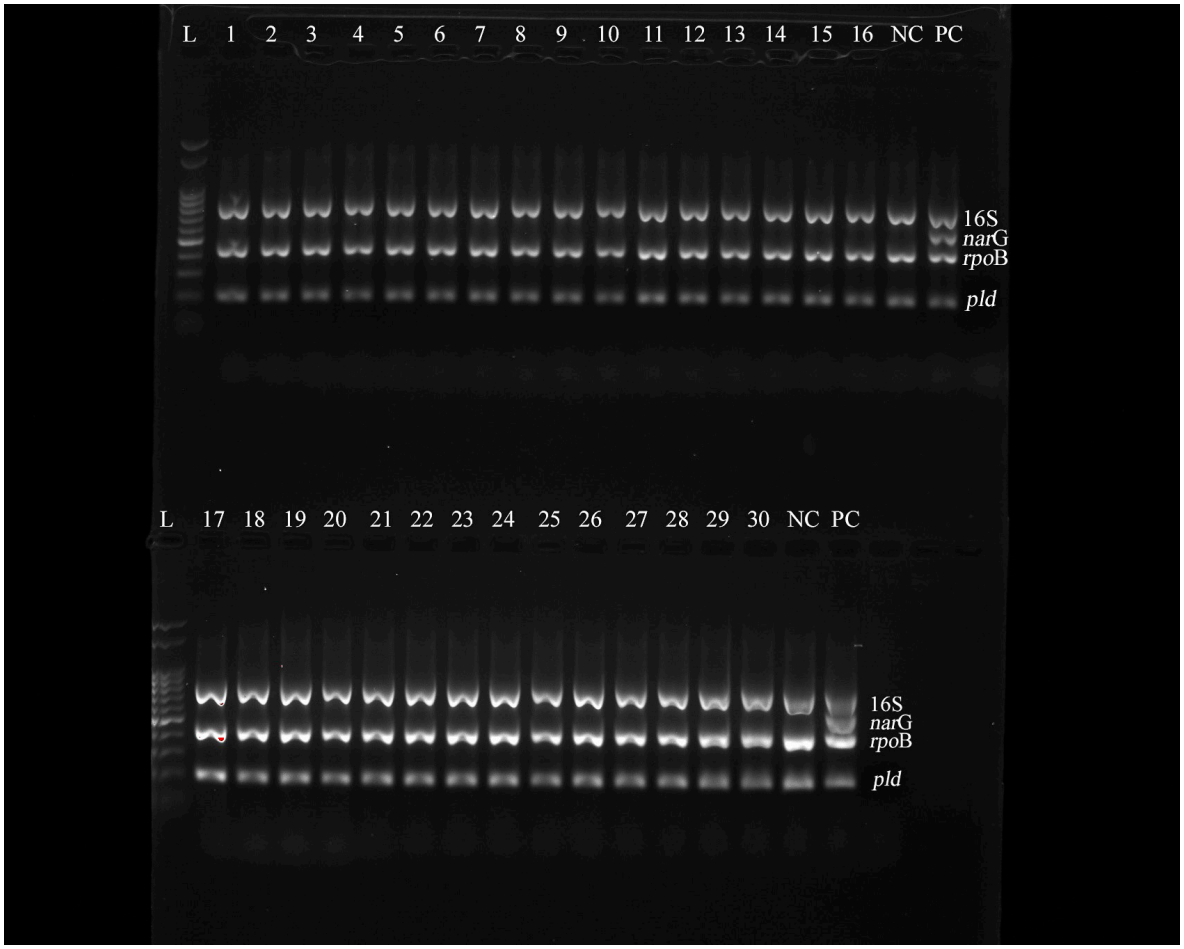

Fig. 1. Electrophoresis on 1.5% agarose gel showing amplicons of 16S rRNA, *rpoB*, *pld* and *narG* genes. Quadruplex PCR for *Corynebacterium pseudotuberculosis* species and biovar identification. L = 100 pb ladder (Ludwig Biotecnologia Ltda); Lanes 1–30 = 30 *C. pseudotuberculosis* strains listed in Table 1; NC = *C. pseudotuberculosis* biovar *ovis* (strain 1002); PC = *C. pseudotuberculosis* biovar *equi* (strain 258).

Table 3. Chemical composition of essential oils from Brazilian plants

Lippia origanoides EO			*Ocimum micranthum* EO		
Constituent	RI	Yield (%)	Constituent	RI	Yield (%)
α-Pinene	1014	0.70	Eucalyptol	1036	2.22
3-Carene	1016	0.16	Linalol	1103	1.22
β-Pinene	1020	0.15	Eugenol	1344	38.62
β -Myrcene	1020	0.63	β–Elemene	1374	6.67
α-Terpinene	1021	0.33	Methyl-eugenol	1387	2.17
O-cimene	1029	6.01	β-caryophyllene	1397	17.09
D-Limonene	1031	0.37	Humulene	1426	3.13
Eucalyptol	1033	12.33	β-Selinene	1453	2.28
ϒ-Terpinene	1060	1.72	γ-Elemene	1462	9.46
cis-4-Thujanol	1062	0,20	β-Bisabolene	1472	1.37
Terpinen-4-ol	1137	1.08	Elemycin	1512	14.65
α-Terpineol	1143	2.39			
Thymol ester	1231	5.00			
Thymol	1267	66.23			
Thymol acetate	1421	1.24			
α-Copaene	1421	0.31			
Caryophyllene	1494	0.67			
δ-Cadinene	1524	0.29			

EO = Essential oil; RI = retention indices of compounds were estimated by linear regression using Kovat´s index from the NIST library and the retention times of the main compounds, and by comparison with mass spectra (Adams 2017).

125 µg/mL, 250 µg/mL, 500 µg/mL, and 1,000 µg/mL were observed against 6.7% (n = 2), 10% (n = 3), 16.7% (n = 5), 33.3% (n = 10), 30% (n = 9), and 3.3% (n = 1) of the strains, respectively. A bactericidal effect of EOLO (MBC_{100}/MIC_{100}) was observed in 93.3% (n = 28) of the isolates tested, according to the criteria established by Limboo & Singh (2024), Makade et al. (2024), and Siugzdaite et al. (2025) (Table 4).

For EOOM, MIC_{100} values of 62.5 µg/mL and 125 µg/mL were observed for 11.1% (n = 2) and 88.9% (n = 16) of the tested strains (n = 18), respectively. MBC_{100} values of 125 µg/

Table 4. Inhibition zone diameter (IZD), minimum inhibitory concentration (MIC_{100}) and minimum bactericidal concentration (MBC_{100}) of *Lippia origanoides* essential oil against *Corynebacterium pseudotuberculosis* strains

Lippia origanoides									
Strains	IZD (mm)	MIC_{100} (µg/mL)	MBC_{100} (µg/mL)	MBC_{100}/MIC_{100}	Strains	IZD (mm)	MIC_{100} (µg/mL)	MBC_{100} (µg/mL)	MBC_{100}/MIC_{100}
BRM050762	82.33 ± 11.8	250	500	2	BRM064792	67.67 ± 4.9	250	500	2
BRM050763	98.33 ± 8.9	250	250	1	BRM029971	78.67 ± 3.6	62.5	250	4
BRM050764	71.67 ± 14.4	31.2	125	4	BRM044303	80.00 ± 0.7	125	250	2
BRM050765	93.33 ± 2.2	250	500	2	BRM044367	81.67 ± 8.2	125	500	4
BRM050767	92.33 ± 0.4	125	500	4	BRM044370	68.00 ± 1.3	62.5	250	4
BRM050773	86.00 ± 4.7	31.2	125	4	BRM044373	71.33 ± 4.4	125	250	2
BRM050911	90.33 ± 3.6	125	500	4	BRM044376	40.67 ± 0.9	125	500	4
BRM064776	93.00 ± 3.3	31.2	125	4	BRM044377	80.67 ± 6.2	250	500	2
BRM064777	87.67 ± 4.9	31.2	125	4	BRM044378	82.67 ± 5.1	31.2	62.5	2
BRM064784	82.33 ± 0.9	31.2	62.5	2	BRM044379	77.67 ± 0.9	250	1,000	4
BRM064785	71.00 ± 10.7	62.5	250	4	BRM044397	86.33 ± 4.2	125	250	2
BRM064787	68.67 ± 4.2	125	250	2	BRM044399	81.33 ± 7.1	15	31.2	2
BRM064788	88.67 ± 5.1	31.2	250	8	BRM044402	87.00 ± 6.7	31.2	31.2	1
BRM064790	77.67 ± 1.8	31.2	250	8	BRM044409	85.67 ± 1.1	31.2	62.5	2
BRM064791	72.00 ± 4.0	250	500	2	BRM044414	80.00 ± 3.3	31.2	125	4

Results are expressed as means of three independent assays; ND = not defined.

Table 5. Inhibition zone diameter (IZD), minimum inhibitory concentration (MIC_{100}) and minimum bactericidal concentration (MBC_{100}) of *Ocimum micranthum* essential oil against *Corynebacterium pseudotuberculosis* strains

Ocimum micranthum									
Strains	IZD (mm)	MIC_{100} (µg/mL)	MBC_{100} (µg/mL)	MBC_{100}/MIC_{100}	Strains	IZD (mm)	MIC_{100} (µg/mL)	MBC_{100} (µg/mL)	MBC_{100}/MIC_{100}
BRM050762	21.67 ± 2.4	125	500	4	BRM064792	24.00 ± 3.3	125	500	4
BRM050763	28.67 ± 2.9	125	250	2	BRM029971	28.00 ± 4.0	62.5	500	8
BRM050764	ND	ND	ND	-	BRM044303	27.33 ± 4.9	125	500	4
BRM050765	27.33 ± 2.2	125	250	2	BRM044367	23.33 ± 3.8	125	500	4
BRM050767	27.00 ± 2.0	125	500	4	BRM044370	20.33 ± 1.1	125	250	2
BRM050773	ND	ND	ND	-	BRM044373	25.67 ± 2.2	125	500	4
BRM050911	30.33 ± 5.6	62.5	500	8	BRM044376	28.00 ± 7.3	125	500	4
BRM064776	ND	ND	ND	-	BRM044377	29.67 ± 2.9	125	500	4
BRM064777	ND	ND	ND	-	BRM044378	ND	ND	ND	-
BRM064784	ND	ND	ND	-	BRM044379	30.00 ± 3.3	125	500	4
BRM064785	31.67 ± 6.4	125	125	1	BRM044397	29.00 ± 0.7	125	500	4
BRM064787	18.67 ± 0.9	125	250	2	BRM044399	ND	ND	ND	-
BRM064788	ND	ND	ND	-	BRM044402	ND	ND	ND	-
BRM064790	ND	ND	ND	`-	BRM044409	ND	ND	ND	-
BRM064791	21.33 ± 4.4	125	500	4	BRM044414	ND	ND	ND	-

Results are expressed as means of three independent assays; ND = not defined.

Table 6. Disk diffusion susceptibility of *Corynebacterium pseudotuberculosis* isolates to penicillin and tetracycline according to BrCAST-EUCAST (2025)

Antibiotic	Disk content	IZD values (mm)		Interpretation	Isolates, n (%)
		Minimum	Maximum		
Penicillin	1 U	15	30	I (Susceptible, increased exposure)	30 (100)
Tetracycline	30 µg	24	45	S (Susceptible)	30 (100)

IZD = inhibition zone diameters; Interpretation of IZD was performed according to the BrCAST-EUCAST Clinical Breakpoint Table (BrCAST 2025).

mL, 250 µg/mL, and 500 µg/mL were found in 5.6% (n = 1), 22.2% (n = 4), and 72.2% (n = 13) of the strains, respectively. EOOM showed a bactericidal effect (MBC_{100}/MIC_{100}) in 88.9% (n = 16) of the tested strains (Table 5).

DISCUSSION

All isolates evaluated in this study were confirmed as *Corynebacterium pseudotuberculosis* biovar *ovis* (Fig. 1), ensuring the etiological homogeneity required for subsequent antibacterial analyses. Based on this confirmation, the discussion focuses on the *in vitro* antibacterial activity of the essential oils and the relationship between their chemical composition and the observed biological effects.

Given the refractoriness of caseous lymphadenitis to antibiotic therapy (Santos et al. 2024), essential oils have emerged as promising alternatives due to their antimicrobial potential. In this context, the chemical characterization of EOLO and EOOM allows the establishment of a relationship between their major constituents and the activity observed against *C. pseudotuberculosis*. In the present study, GC-MS analysis identified thymol as the major compound in EOLO, representing 66.23% of the total composition. This result is consistent with the findings of Leal et al. (2019), who reported 55.53% of thymol in samples of the same species. Similarly, Zapata-Zapata et al. (2023) observed a high concentration of thymol (49.4%) in EOLO collected in Ceará, Brazil.

Regarding EOOM, eugenol was identified as the main constituent, accounting for 38.62% of the oil composition. This finding is consistent with Freitas et al. (2018), who classified *Ocimum micranthum* as belonging to the eugenol chemotype and reported significant levels of this compound. Sacchetti et al. (2004) likewise identified eugenol as the major component (46.55%) in EOOM from the Amazon region. Previous studies have also demonstrated the presence of eugenol, β-elemene, and β-caryophyllene in the chemical composition of *O. micranthum* leaf essential oil (Barbosa et al. 2021). In addition, Silva et al. (1998) described differences in the composition of essential oils obtained from leaves and inflorescences, reporting predominance of eugenol (44.8%), β-elemene (10.3%), and caryophyllene (14.7%) in leaves, and elemicin (32.9%) and eugenol (14.0%) in inflorescences. The elevated elemicin content observed in the present study is therefore likely associated with the inclusion of floral structures in the plant material.

The predominance of thymol in EOLO and of eugenol in EOOM can be attributed to genetic and environmental factors that influence the biosynthesis of secondary metabolites. According to Leal et al. (2019), variations in essential oils composition may arise from genetic differences, climatic conditions, soil characteristics, and the developmental stage of the plant. These factors affect the expression of genes involved in volatile compound biosynthesis, leading to distinct chemical profiles, or chemotypes, even within the same species.

The chemical composition of both oils evaluated in this study supports their investigation against C. pseudotuberculosis, as EOLO and EOOM have attracted interest as rich sources of bioactive compounds, including thymol, eucalyptol, caryophyllene, phenolic compounds, cymene, limonene, monoterpenes, and sesquiterpenes, all of which exhibit recognized antibacterial activity (Ribeiro et al. 2021, Dharsono et al. 2022). In the present study, EOLO (66.23% thymol) and EOOM (38.62% eugenol) demonstrated in vitro activity against all tested strains (Table 4 and 5).

Regarding the interpretation of inhibition zone diameters (IZD) obtained by disk diffusion, it is important to emphasize that no standardized guidelines are currently available for essential oils (EUCAST 2024, BrCAST 2025). According to Hulankova (2024), EOs producing inhibition zones ≤ 8 mm are considered ineffective, those with diameters ≥ 15 mm are regarded as very effective, and diameters ≥ 20 mm are classified as extremely effective. In the present study, EOLO exhibited high IZD values across all tested strains, with an overall mean exceeding 75 mm. These findings indicate a high level of bacterial susceptibility to EOLO and highlight its excellent diffusion capacity in BHI agar. Moreover, the low standard deviation observed in several cases reflects a consistent response among strains, suggesting a uniform antibacterial effect.

Although the inhibition zones produced by EOOM were smaller than those observed for EOLO, all values remained within the range indicative of antimicrobial activity. Notably, no inhibition halo was smaller than 15 mm, reinforcing that EOOM also exhibits relevant antimicrobial potential, albeit less pronounced than that of EOLO. Furthermore, the inhibition zone diameters reported in the present study exceed those described by Awadalla et al. (2022) and Poole et al. (2022) for other essential oils tested against Corynebacterium species, indicating superior antimicrobial efficacy.

Considering the susceptibility profile observed for the commercial antibiotics tested, the role of natural compounds requires careful interpretation. Antimicrobial susceptibility of the isolates was assessed against penicillin and tetracycline, two of the main antimicrobials used in sheep and goat production and authorized for veterinary use in Brazil by the "Ministério da Agricultura, Pecuária e Abastecimento" (Brasil 2025), and interpreted according to the clinical breakpoints recommended by the BrCAST-EUCAST guidelines (2025). The classification of isolates as "I" for penicillin (susceptible, increased exposure) and as susceptible ("S") to tetracycline is consistent with the conventional therapeutic management of caseous lymphadenitis, in which abscess drainage represents the primary control measure. At the same time, antimicrobial therapy plays a complementary role with variable effectiveness (Santos et al. 2021). Previous reports have highlighted the limited and inconsistent response of *C. pseudotuberculosis* to conventional antibiotics, particularly β-lactams, whereas the activity of tetracycline may vary according to strain and geographic origin (El Damaty et al. 2023). In this context, natural products should not be interpreted as direct substitutes for antibiotics, but rather as complementary agents acting on multiple bacterial targets. Therefore, direct quantitative comparison between inhibition zones produced by antibiotics and those produced by essential oils should be interpreted with caution, given the distinct physicochemical properties of essential oils, especially their hydrophobicity and volatility, which affect diffusion behavior in agar-based assays (Hulankova 2024). The consistent *in vitro* antibacterial activity observed for the essential oils evaluated in the present study, even against isolates showing reduced susceptibility to penicillin, supports their potential as natural candidates for further exploration as adjuvant strategies in the control of caseous lymphadenitis (Abdulkarim & Issa 2025).

Furthermore, both EOLO and EOOM exhibited effective MIC_{100} values against C. pseudotuberculosis, ranging from 15 to 250 µg/mL for EOLO and from 62.5 to 125 µg/mL for EOOM. These values are comparable to those reported by Awadalla et al. (2022) against Corynebacterium stationis and notably lower than those described by Poole et al. (2022), underscoring the promising performance of these natural compounds in combating this pathogen.

Although no previous studies have investigated the antibacterial activity of EOLO and EOOM against C. pseudotuberculosis strains, it is noteworthy that the activity of these oils against other Gram-positive and Gram-negative bacterial species has already been documented (Caamal-Herrera et al. 2018, Silva et al. 2022). Thus, the present findings contribute to the growing body of evidence supporting the antibacterial potential of these essential oils.

Regarding MIC_{100} values, there is currently no consensus on standardized criteria for evaluating the antimicrobial activity of natural products or defining acceptable MIC_{100} thresholds (Pérez Zamora et al. 2018). Nevertheless, some authors propose that essential oils with MIC_{100} values between 50 and 500 µg/mL exhibit strong antimicrobial activity, those between 600 and 1,500 µg/mL indicate moderate activity, and values above 1,500 µg/mL reflect low activity (Assane et al. 2020, Furlani et al. 2021, Gallani et al. 2020). According to this classification, EOLO and EOOM can be considered strong antibacterial agents against C. pseudotuberculosis (Table 4 and Table 5).

The MBC_{100}/MIC_{100} ratio is commonly used to differentiate bactericidal (≤ 4) from bacteriostatic (> 4) antimicrobial effects (Khalfaoui et al. 2021, Makade et al. 2024, Olatunya et al. 2024). In the present study, both EOLO and EOOM were categorized as bactericidal, as their MBC_{100}/MIC_{100} ratios were ≤ 4. Specifically, MBC_{100} values for EOLO (125–1,000 µg/mL) resulted in ratios ≤ 4 for most strains, indicating a bactericidal effect against C. pseudotuberculosis. These findings are consistent with those of Abdulkarim & Issa (2025), who reported rapid elimination of several bacterial species, including C. pseudotuberculosis, by essential oils.

In isolates of *C. pseudotuberculosis* associated with caseous lymphadenitis, previous studies have reported 100% resistance to bacitracin and florfenicol and high resistance rates to penicillin and erythromycin (92.6% each), with all isolates classified as multidrug-resistant (MDR), highlighting the therapeutic challenges posed by commercial antibiotics in some veterinary settings (El Damaty et al. 2023). In this context, recent evidence suggests that essential oils may represent an alternative or adjunct approach. However, their efficacy is highly dependent on both the oil composition and the target pathogen. For example, peppermint essential oil failed to completely inhibit the growth of *C. pseudotuberculosis* under the tested conditions, illustrating this variability (Issa 2024). Against this background, the present study is particularly encouraging, as the EOLO and EOOM demonstrated consistent *in vitro* antibacterial activity against *C. pseudotuberculosis*, with MIC_{100} values within effective ranges and evidence of bactericidal behavior in part of the isolate panel, supporting their potential for further investigation, particularly in contexts where resistance to conventional antimicrobials has been documented.

Previous studies have explored natural products as alternative antimicrobial agents against *C. pseudotuberculosis*, primarily using disk diffusion, MIC_{100} and MBC_{100} assays. Propolis extracts have shown *in vitro* antibacterial activity against *C. pseudotuberculosis*, with MIC_{100} and MBC_{100} values varying according to extract type and concentrations, confirming the susceptibility of this pathogen to natural compounds (Santos et al. 2021). Similarly, medicinal plant extracts have produced measurable inhibition zones and MIC_{100} values consistent with antibacterial effects, although with substantial variability among plant species and experimental conditions (Naeem et al. 2024). More recently, lavender and mint essential oils exhibited antibacterial activity against *C. pseudotuberculosis*, as evidenced by inhibition halos and MIC_{100} and MBC values within moderate ranges, indicating oil-dependent efficacy (Abdulkarim & Issa 2025). In this context, the EOLO and EOOM evaluated in the present study stand out by exhibiting consistent *in vitro* antibacterial activity across all tested isolates, with MIC_{100} and MBC_{100} values equal to or lower than those reported for other natural products.

Considering the growing concern over bacterial resistance, the use of natural compounds represents a strategic approach. Recent studies have demonstrated that oils such as rosemary, cinnamon, and citronella also exhibit significant antibacterial activity against various Corynebacterium species, as demonstrated by disk diffusion and MIC_{100} assays (Awadalla et al. 2022, Reffit et al. 2022). These findings reinforce the potential of the essential oils evaluated in the present study as adjuvants or alternative strategies for the control of CLA in small ruminants.

The antimicrobial activity observed can be largely attributed to the major constituents of the analyzed essential oils. Thymol, present in EOLO, and eugenol, the main component of EOOM, are phenolic compounds known for their ability to disrupt bacterial membranes. These compounds induce alterations in membrane permeability, promote ion leakage, and cause loss of intracellular contents, ultimately leading to cell lysis (Issa 2024, Li et al. 2024). The hydrophobic nature of EOs facilitates their interaction with bacterial membranes, resulting in structural and functional changes, enzyme inhibition, and disruption of ion channels, which collectively contribute to bacterial cell death (Guimarães et al. 2019). Proposed antibacterial mechanisms of thymol include disruption of membrane and cell wall integrity, inhibition of cell division, interference with nutrient uptake and metal ion transport, and impairment of nucleotide biosynthesis, DNA repair, and transcriptional processes (Yin et al. 2022).

Overall, the results of this study demonstrate that EOLO and EOOM exhibit significant antibacterial activity against C. pseudotuberculosis strains, as evidenced by large inhibition zones and MIC_{100} and MBC_{100} values consistent with a bactericidal effect. These findings are particularly relevant given the limited number of studies evaluating the efficacy of essential oils against this pathogen and provide novel insights into the potential use of plant-derived antimicrobials in the control of caseous lymphadenitis.

CONCLUSION

The treatment of caseous lymphadenitis (CLA) with conventional antimicrobials remains challenging, mainly due to limited drug penetration into purulent abscesses and the associated risk of bacterial resistance. In this context, essential oils from *Lippia origanoides* (EOLO) and *Ocimum micranthum* (EOOM) emerge as promising alternatives, as they demonstrate strong *in vitro* antibacterial activity against *Corynebacterium pseudotuberculosis*. These findings support

the potential use of EOLO and EOOM in future studies aimed at preventing pathogen dissemination and reducing disease recurrence following lesion removal.

Acknowledgments.- This study was supported by the funding "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES), Brazil, Finance Code 001, "Empresa Brasileira de Pesquisa Agropecuária" (Embrapa) (20.20.03.002.00.00). "Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico" (FUNCAP) scholarship (BP5-0197-00148.01.00/22), "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq) productivity scholarship (312597/2023-1). The authors would like to thank the "Centro Universitário INTA" (UNINTA), "Universidade Estadual do Ceará" (UECE), and "Embrapa Caprinos e Ovinos" for their support in carrying out the experiments.

Conflict of interest statement.- The authors declare that they have no conflicts of interest.

Credit author statement.- Patricia R. Lima: Conceptualization, Methodology, Formal analysis, Investigation (microbiological and molecular biology), Data curation, Visualization, Validation, Project administration, Writing – original draft & review. Wildson M.B. da Silva: Essential oil extraction (EOLO), GC-MS analysis (EOLO and EOOM), Software. José R. Garcez Neto: Essential oil extraction (EOLO), GC-MS analysis (EOLO and EOOM). Stênio F. Felix: Plant material collection (Lippia origanoides). Rita C.A. Pereira: Plant material collection (Ocimum micranthum), Essential oil extraction (EOOM). Benise F. Silva: Investigation (microbiological). Nirlir Pl. Sousa: Investigation (molecular biology). Wanderléia A. Policarpo: Investigation (disk diffusion assay with commercial antibiotics). Patrícia Y. Faccioli-Martins: Conceptualization, Methodology, Formal analysis, Investigation (microbiological and molecular biology), Data curation, Supervision, Writing – original draft & review, Resources & funding. Victor A. Carneiro: Conceptualization, Resources & funding. Renata A. Costa: Conceptualization, Methodology, Formal analysis, Investigation (microbiological and molecular biology), Data curation, Supervision, Writing – original draft & review. Selene M. Morais: Conceptualization, Methodology, Formal analysis, Investigation (essential oil extraction, GC-MS analysis, microbiological validation), Data curation, Supervision, Writing – original draft & review, Resources & funding.

Data availability statement.- The data is available in the article. This study is part of the doctoral research of the first author.

Editor-in-Chief.- Fabiano José Ferreira de Sant'Ana.

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