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Essential Oils of Thymus Vulgaris and Melaleuca Alternifolia as Control Agents Against Klebsiella Spp.: An in Vitro Disk-Diffusion Evaluation

Saulo Abreu Almeida da Silva[1]; *José Geraldo de Vargas Junior*[1], *Pedro Pierro Mendonça2*, *Priscilla Cortizo Costas Pierro*[1], *Raphael Pires Bolzan*[1], *Aldino Neto Venancio*[2], *Mario Ferreira Conceição Santos*[1]; *Luciano Menini*[2]
✉mariosantos408@gmail.com

1. *Department of Chemistry and Physics, Alegre Campus – CCA, Federal University of Espírito Santo.*
2. *Alegre Campus, Federal Institute of Education, Science, and Technology of Espírito Santo.*

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Abstract: Aquaculture is the fastest-growing sector in various parts of the world. However, production intensification can lead to several issues, including microbial diseases. One of the most significant concerns in aquaculture production is *Klebsiella* spp., due to its high infectivity, rapid multiplication, and antibiotic resistance. Consequently, the search for alternatives to conventional treatments is essential. This study aimed to evaluate the effect of essential oils from *Thymus vulgaris* and *Melaleuca alternifolia* as alternative treatments against *Klebsiella* spp. in *in vitro* assays. The essential oil profiles were determined using gas chromatography with a flame ionization detector (GC-FID) and a mass spectrometry detector (GC-MS). The major compounds of Thymus vulgaris essential oil were carvacrol, thymol, and p-cymene. The predominant compounds in *Melaleuca alternifolia* essential oil were terpinen-4-ol, γ-terpinene, and α-terpinene. Both oils demonstrated antibacterial potential in controlling *Klebsiella* spp.; *Thymus vulgaris* essential oil performed better, achieving larger inhibition zones at lower concentrations. Five Klebsiella spp. Isolates from fish lesions were tested. Oxytetracycline (terramycin) was used as a positive control; $p < 0.05$ (Tukey's test).
Keywords: Antibiotics, Health, *Thymus vulgaris*, *Myrtaceae, Lamiaceae*, *Melaleuca alternifolia*.

Óleos Essenciais de Thymus Vulgaris e Melaleuca Alternifolia como Agentes de Controle contra Klebsiella Spp.: Uma Avaliação in Vitro por Difusão em Disco

Resumo: A aquicultura é o setor de crescimento mais rápido em diversas partes do mundo. No entanto, a intensificação da produção pode levar a vários problemas, incluindo doenças microbianas. Uma das maiores preocupações na produção aquícola é a Klebsiella spp., devido à sua alta infectividade, rápida multiplicação e resistência a antibióticos. Consequentemente, a busca por alternativas aos tratamentos convencionais é essencial. Este estudo teve como objetivo avaliar o efeito dos óleos essenciais de Thymus vulgaris e Melaleuca alternifolia como tratamentos alternativos contra Klebsiella spp. em ensaios in vitro. Os perfis dos óleos essenciais foram determinados por cromatografia gasosa com detector de ionização de chama (CG-DIC) e espectrometria de massas (CG-EM). Os principais compostos do óleo essencial de Thymus vulgaris foram carvacrol, timol e p-cimeno. Os compostos predominantes no óleo essencial de Melaleuca alternifolia foram terpinen-4-ol, γ-terpineno e α-terpineno. Ambos os óleos demonstraram potencial antibacteriano no controle de Klebsiella spp.; o óleo essencial de Thymus vulgaris apresentou melhor desempenho, atingindo zonas de inibição maiores em concentrações mais baixas. Cinco isolados de Klebsiella spp. provenientes de lesões em peixes foram testados. A oxitetraciclina (terramicina) foi utilizada como controle positivo; $p < 0,05$ (teste de Tukey).
Palavras-chave: Antibióticos, Saúde, *Thymus vulgaris, Myrtaceae, Lamiaceae, Melaleuca alternifolia*.

Aceites Esenciales de Thymus Vulgaris y Melaleuca Alternifolia como Agentes de Control Contra Klebsiella Spp.: Una Evaluación in Vitro Mediante Difusión en Disco

Resumen: La acuicultura es el sector de mayor crecimiento en diversas partes del mundo. Sin embargo, la intensificación de la producción puede generar varios problemas, incluidas las enfermedades microbianas. Una de las preocupaciones más importantes en la producción acuícola es Klebsiella spp., debido a su alta infectividad, rápida multiplicación y resistencia a los antibióticos. Por consiguiente, la búsqueda de alternativas a los tratamientos convencionales es esencial. Este estudio tuvo como objetivo evaluar el efecto de los aceites esenciales de Thymus vulgaris y Melaleuca alternifolia como tratamientos alternativos contra Klebsiella spp. en ensayos in vitro. Los perfiles de los aceites esenciales se determinaron mediante cromatografía de gases con detector de ionización de llama (GC-FID) y espectrometría de masas (GC-MS). Los principales compuestos del aceite esencial de Thymus vulgaris fueron carvacrol, timol y p-cimeno. Los compuestos predominantes en el aceite esencial de Melaleuca alternifolia fueron terpinen-4-ol, γ-terpineno y α-terpineno. Ambos aceites demostraron potencial antibacteriano para controlar Klebsiella spp.; el aceite esencial de Thymus vulgaris tuvo un mejor desempeño, logrando zonas de inhibición más grandes a concentraciones más bajas. Se analizaron cinco aislamientos de Klebsiella spp. provenientes de lesiones en peces. Se utilizó oxitetraciclina (terramicina) como control positivo; $p < 0,05$ (prueba de Tukey).
Palabras clave: Antibiotics, Health, *Thymus vulgaris, Myrtaceae, Lamiaceae, Melaleuca alternifólia.*

INTRODUCTION

The intensification of aquaculture production in captivity can lead to high concentrations of organic matter, which may compromise fish well-being and health. This condition promotes bacterial growth, posing significant risks to fish production (SOUZA et al., 2022). One of the most significant concerns in this context is the genus *Klebsiella,* an opportunistic pathogen that can cause severe infections in fish, leading to high mortality rates and significant economic losses. This bacterium is known for its resistance to multiple antibiotics, making its control and treatment increasingly challenging. This issue is exacerbated when producers or others indiscriminately use antibiotics, resulting in more resistant strains and compounding public health and environmental problems (MORGADO; FONSECA; VICENTE, 2022; VANECI-SILVA *et al.*, 2022).

In this scenario, the search for natural and sustainable alternatives to reduce disease incidence in aquaculture is essential. One promising approach is the use of essential oils, which are naturally derived compounds extracted from plants and may possess antimicrobial, antifungal, and antioxidant properties. These oils have been studied for their ability to inhibit the growth of various pathogenic bacteria, offering a potential solution to the sanitary challenges faced in fish farming (MORAIS, 2009; TARIQ *et al.*, 2019).

Essential oils are substances obtained from plant material and are a complex mixture of bioactive compounds that act synergistically to exert antimicrobial effects. Among the most

studied essential oils are oregano, thyme, eucalyptus, and clove oils, each with a distinct spectrum of antimicrobial activity. These oils have demonstrated efficacy against many bacterial pathogens, including some that can affect human and animal health (CUNHA *et al.*, 2018; RADULOVIC *et al.*, 2013; TARIQ *et al.*, 2019).

These effects may contribute to the overall resistance of fish to infections, reducing the frequency of diseases and the need for chemical interventions. Beyond their antimicrobial properties, essential oils offer additional benefits, including improving fish intestinal health and enhancing immune responses (SOUZA *et al.*, 2020).

Recent studies have explored the use of these oils in aquaculture, investigating their efficacy against specific pathogens, such as *Klebsiella* spp, and their impact on fish health, including growth, immune function, and stress resistance. These studies are crucial for understanding the potential of essential oils as alternatives to conventional antibiotics in aquaculture (PITONDO-SILVA, 2022). Thus, this study aimed to test the essential oils of *Thymus vulgaris* and *Melaleuca alternifolia* against *Klebsiella* spp. in *in vitro* assays.

MATERIALS AND METHODS

The analyses were conducted at the Microbiology Laboratory of the Federal Institute of Espírito Santo (IFES), Alegre Campus, ES, located at BR-482 Highway (Cachoeiro-Alegre, Km 72 - Rive, Alegre - ES, 29500-000).

Antimicrobial activity with essential oils

Klebsiella spp., strains were reactivated in BHI medium (Brain Heart Infusion, Himedia, India) and cultured until reaching a concentration of 10^8 CFU mL^{-1}. The number of cells per mL was quantified using a standard growth curve obtained by UV/VIS spectrophotometry (AGILENT CARY60 UV-VIS) against a blank containing TSB broth at 600 nm. Five *Klebsiella* spp. Isolates were obtained from fish lesions using sterile swabs; growth, isolation, subculture, and identification were performed at the Clinical Analysis Laboratory (LABCENTER®) following CLSI (2008) and NCCLS (2003) methodologies. The inoculum was standardized by comparison with the 0.5 McFarland turbidity standard (~1.5×10^8 CFU mL^{-1}).

Disk diffusion assay

The evaluation was performed using the disk diffusion method (NCCLS, 2000; CLSI, 2008). Mueller Hinton Agar (MHA) was inoculated with a bacterial suspension containing 10^8 CFU mL^{-1} and poured into sterile Petri dishes (140 mm diameter). After inoculation, sterile filter paper disks (6 mm diameter) impregnated with essential oils from *Thymus vulgaris* (thyme) and *Melaleuca alternifolia* were placed on the plates using absolute ethanol as a solvent. Following a 24-hour incubation period at 37°C, the inhibition halos around the disks were measured (SAPKOTA *et al.*, 2012; MAZUMDER *et al.*, 2006).

The tests were conducted in triplicate for each bacterial isolate. The essential oils of *Thymus vulgaris* and *Melaleuca alternifolia* were obtained from a specialized supplier. Each sterile filter paper disk (6 mm) was impregnated with 10 mg of essential oil dissolved in absolute ethanol. A solvent-control disk (absolute ethanol only, same volume) was included in each plate; no inhibition halo was observed for the solvent control. As the positive control, a commercial oxytetracycline (terramycin) disk was used.

Chromatographic characterization of essential oils

The constituents of the essential oils were analyzed by gas chromatography with a flame ionization detector (GC/FID) (Shimadzu GC-2010 Plus). The chromatographic conditions included a fused-silica capillary column (30 m x 0.25 mm) with an RTX-5MS stationary phase (0.25 µm film thickness). Nitrogen (for GC/FID analysis) or helium (for GC/MS analysis) was used as the carrier gas with a flow rate of 3.0 mL min^{-1}. The oven temperature program started at 60°C (held for 1 minute), then increased by 5°C per minute until reaching 220°C, where it was held for 10 minutes. The injector and detector temperatures were set to 240°C, with a split ratio of 1:30. A 1.0 µL volume of a 3% (v/v) essential oil solution in 96% ethanol was injected (DE SOUZA *et al.*, 2017). For GC-MS analyses, helium was used as the carrier gas at 3.0 mL min^{-1}, with the same column and temperature program as described above.

The ionization mode was electron impact at 70 eV; the ion source temperature was set to 200°C; the mass scan range was m/z 40–500. The identification of components was performed using retention indices (LTPRI) (ADAMS, 2017), calculated using the Van Den Dool and Kratz equation (1963). The retention index (RI) for each compound was compared with literature

values obtained from the NIST library (National Institute of Standards and Technology, NIST, 2011) and Adams (2017).

$$\text{LTPRI} = 100n + 100\left[\frac{t_{R(i)} - t_{R(n)}}{t_{R(n+1)} - (t_{R(n)})}\right] \qquad (1)$$

Where:

i = compound of interest;

n = number of carbon atoms in the hydrocarbon with a retention time immediately preceding that of compound

i; **tR(i)** is the adjusted retention time of compound

i; **tR(n)** is the adjusted retention time of

n; **tR(n+1)** is the adjusted retention time of the hydrocarbon with a retention time immediately following that of compound **i**.

Statistical Analysis

The tabulated data were analyzed descriptively, and the Shapiro-Wilks test assessed normality. If normality was confirmed, a one-way ANOVA was performed, followed by Tukey's post-hoc test with a significance level of 5% ($p < 0.05$).

RESULTS AND DISCUSSION

Chromatographic Characterization of Essential Oils

The chromatographic profile of thyme essential oil is presented in Figure 1 and Table 1, with 11 compounds identified. For the essential oil of Melaleuca (Figure 2, Table 1), 12 compounds were identified.

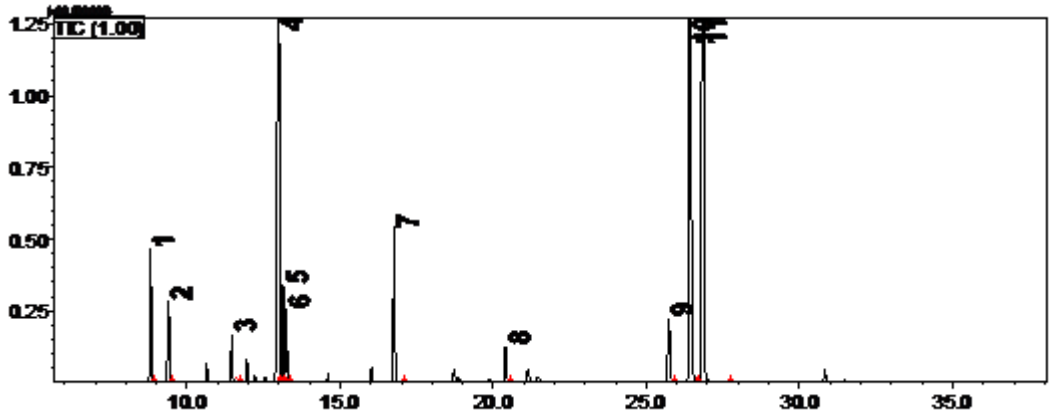

Figure 1. Chromatographic profile of *Thymus vulgaris* essential oil
Source: Authors

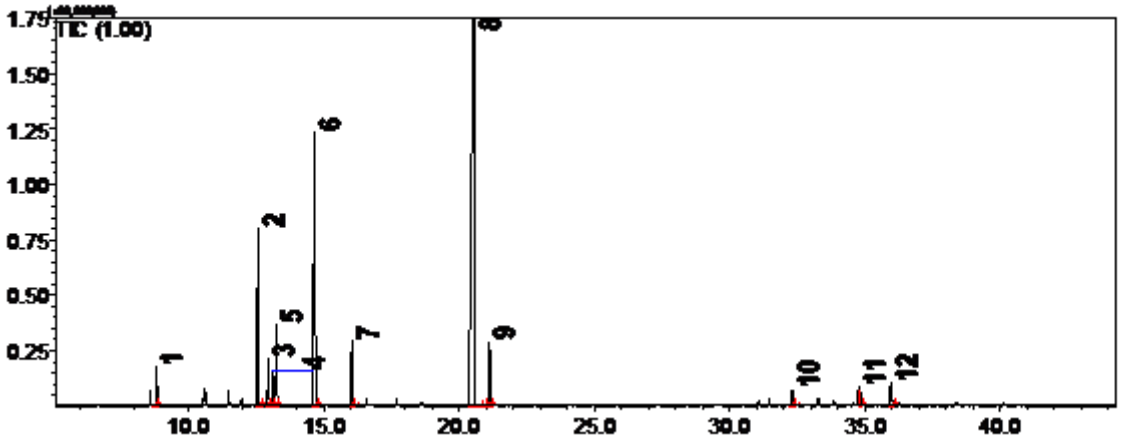

Figure 2. Chromatographic profile of *Melaleuca alternifolia* essential oil
Source: Authors

Table 1. Characterization of *Thymus vulgaris* and *Melaleuca alternifolia*[a] essential oils by LTPRI and GC-MS

Peak	IR[b]	IR[c]	Compound	Area%[d]	
				Thyme	*Melaleuca*
1	929	932	α-Pineno	4.03	1.83
2	943	946	Canfeno	3.05	-
3	992	988	β-Mirceno	1.53	-
4	1016	1014	α-Terpineno	-	9.57
5	1024	1022	o-Cimeno	-	2.46
6	1026	1020	*p*-Cimeno	22.74	-
7	1028	1025	β-Feladreno	-	1.87
8	1029	1024	Limoneno	3.07	-
9	1031	1026	Eucaliptol	2.26	4,16
10	1062	1054	γ-Terpineno	-	17.96
11	1092	1086	α-Terpinoleno	-	3.58
12	1108	1095	Linalol	5.81	-
13	1189	1174	Terpinen-4-ol	1.51	50.73
16	1322	1289	Timol	25.77	-
17	1333	1298	Carvacrol	26.82	-
18	1463	1439	Aromadreno	-	1.2
19	1526	-	Biciclοelemeno	-	1.12
20	1556	1522	δ-Cadineno	-	1.73

[a]Compounds identified by LTPRI and GC-MS using an Rtx®-5MS column. b Calculated using a mixture of saturated n-alkanes (C7 to C40). (IR) Retention indices. c Literature indices based on ADAMS (2017). d Relative area based on the chromatograms in Figures 1 and 2, identifying only compounds with relative areas >1%.
Source: Authors

Evaluation of the Efficacy of Essential Oils in Antimicrobial Activity

The antimicrobial activity of the essential oils was assessed using the disk diffusion method, which demonstrated inhibition zones (Figure 3, Table 2). Both essential oils tested exhibited antimicrobial activity against *Klebsiella* spp.

Thyme essential oil showed inhibition zones at lower concentrations than melaleuca essential oil. At a concentration of 8.00 mg, thyme oil displayed superior antimicrobial activity, while melaleuca oil showed optimal activity at 14.00 mg.

The disk diffusion test revealed that concentrations of 7.00 mg and 8.00 mg for thyme essential oil and concentrations above 11.00 mg for melaleuca essential oil produced inhibition zones comparable to the positive control. These results indicate that both oils have inhibitory effects on *Klebsiella* spp.

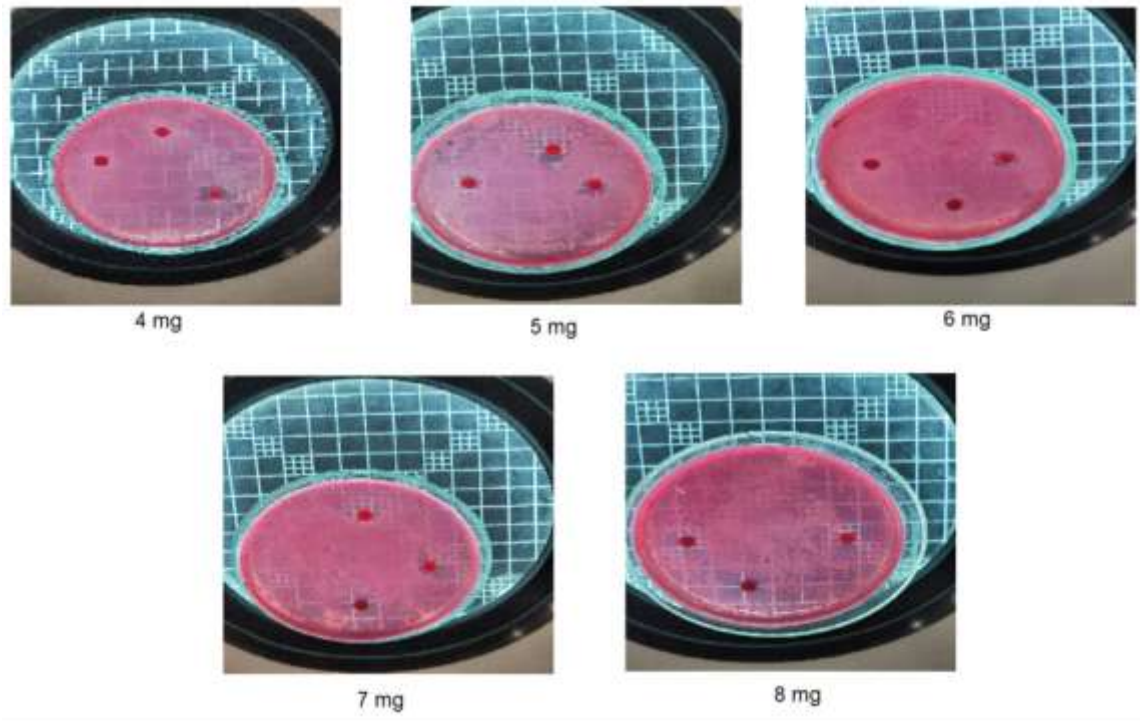

Figure 3. Disk diffusion test for thyme essential oil concentrations.
Source: Authors

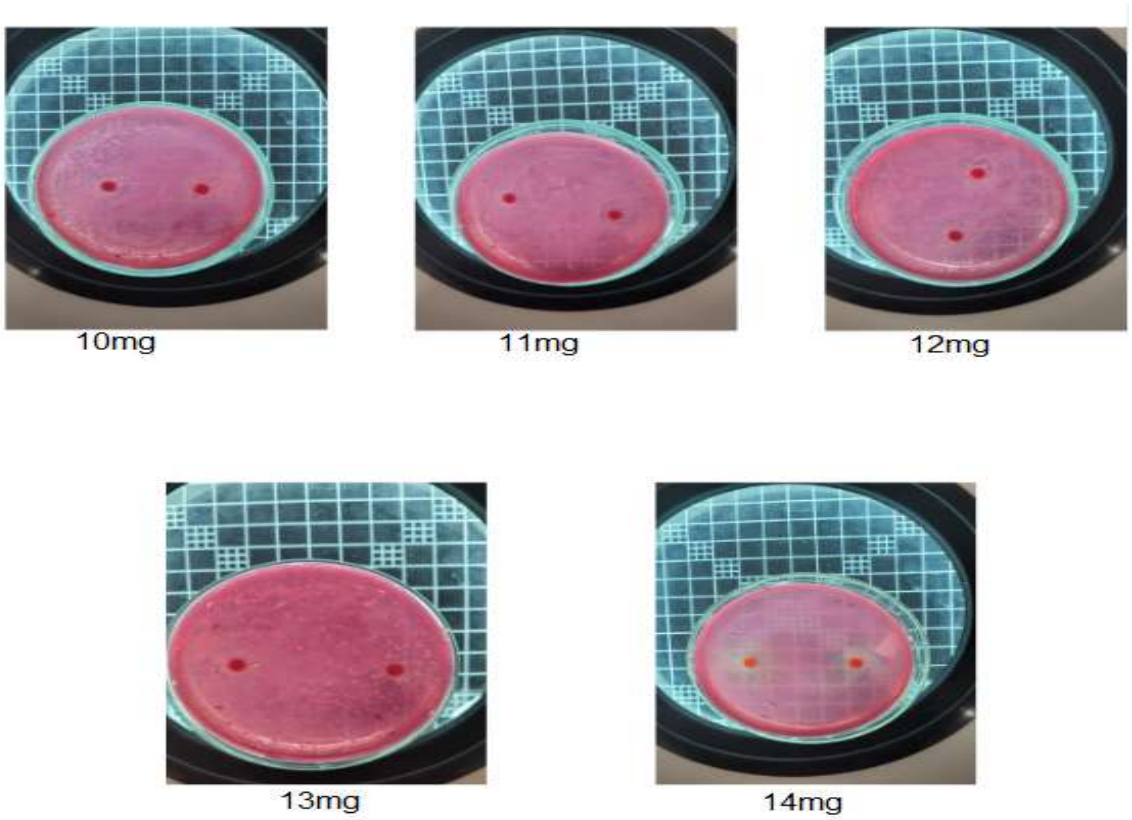

Figure 4. Disk Diffusion Test for Concentrations of Melaleuca Essential Oil
Source: Authors

Table 2. Inhibition zone diameters of Thyme and Melaleuca Essential Oils.

Average Inhibition Zone Diameter ± Standard Deviation (mm)		
Antibiotics	**Concentration (mg)**	**Isolated Microbial Strain**
		Gram-negative bacteria
		***Klebsiella* spp.**
Thymus vulgaris	4.00	8.8 ± 1.4c
	5.00	10.6 ± 2.0bc
	6.00	9.7 ± 0.7c
	7.00	11.5 ± 2.2abc
	8.00	14.2 ± 1.8ab
Melaleuca alternifolia	10.00	9.1 ± 2.5c
	11.00	11.4 ± 0.5abc
	12.00	11.6 ±1.9abc
	13.00	12.0 ± 1.9abc
	14.00	14.8 ± 3.0a
Positive control (oxytetracycline / terramycin)[a]	-	15 ± 0.0a

Means ± standard deviation. Means followed by the same letter do not differ from each other (Tukey test, $p < 0.05$)
Source: Authors

The antibacterial activity of essential oils can be attributed to the synergy of their compounds or to isolated components. For example, the major compound in thyme essential oil, carvacrol, exhibits antibacterial, antifungal, anticonvulsant, and cytotoxic activities against cancer cell lines (MORO *et al.*, 2017). Thymol, also found in thyme essential oil, can cause structural and functional changes in bacterial membranes, leading to leakage of vital intracellular constituents and metabolic imbalance. Consequently, thymol disrupts cellular homeostasis by increasing bacterial cell membrane fluidity and permeability (HAJIBONABI *et al.*, 2023).

In Melaleuca essential oil, terpinen-4-ol and γ-terpinene are the major components. Terpinen-4-ol significantly alters the phospholipid levels in bacterial membranes, disrupts their

surfaces, interrupts genetic material synthesis, and breaks bacterial cell connections by coagulating cytoplasmic membrane components (NOURBAKHSH *et al.*, 2022).

These results are consistent with previously published data on the antibacterial activity of both oils against *Klebsiella* spp. For *Thymus vulgaris*, Boruga *et al.* (2014) evaluated the essential oil by disk diffusion against *Klebsiella pneumoniae* (ATCC 13882) and reported dose-dependent inhibition zones at 5-20 uL per disk, with thymol (47.59%) and p-cymene (8.41%) as major components, a chemotype comparable to the one studied here (thymol 25.77%, carvacrol 26.82%, p-cymene 22.74%). In a recent study, Dawood *et al.* (2026) screened 33 essential oils against multidrug-resistant *K. pneumoniae* isolates and found that thyme essential oil produced inhibition zones up to 26 mm, attributing this activity primarily to phenolic compounds such as carvacrol and thymol. For *M. alternifolia*, Borotova *et al.* (2022) reported inhibition zones of 6.00-9.33 mm against Gram-negative bacteria by disk diffusion, consistent with the zones observed here at lower doses (9.1 +/- 2.5 mm at 10 mg). At higher doses (14.8 +/- 3.0 mm at 14 mg), the results approach values reported by Lima *et al.* (2024), who found that *M. alternifolia* essential oil produced inhibition zones of 7.05-23.35 mm against *Klebsiella* spp. isolated from bovine mastitis, with a minimum concentration of 50% required for complete inhibition. The superior antibacterial activity of *T. vulgaris* compared to *M. alternifolia* against *Klebsiella* spp. observed here is consistent with the general finding that phenolic-rich essential oils exhibit stronger antibacterial effects against Gram-negative bacteria than terpene-alcohol-rich oils (TARIQ *et al.*, 2019).

It should be noted that the disk diffusion method presents inherent limitations when applied to essential oils. This technique was originally developed for water-soluble antibiotics, and its reliability decreases for complex hydrophobic mixtures such as essential oils. In agar medium, water-soluble essential oil components diffuse readily, whereas hydrophobic terpenes tend to remain near the disk surface or evaporate during incubation, resulting in inhibition zones that may not fully reflect the oil's actual antimicrobial potency (SALVETOVA *et al.*, 2024). Furthermore, disk diffusion cannot differentiate between bactericidal and bacteriostatic effects, nor can it be used to determine minimum inhibitory concentration (MIC) values, because the amount of antimicrobial agent diffusing into the agar cannot be precisely quantified (BRNAWI *et al.*, 2023). Future studies using broth microdilution methods (CLSI M07) are therefore necessary to provide quantitative MIC and MBC values and a more rigorous assessment of the antibacterial potential of these oils against *Klebsiella* spp.

CONCLUSION

In vitro tests revealed that the essential oils of Thymus vulgaris and Melaleuca alternifolia have potential applications for controlling Klebsiella spp. bacteria at specific concentrations. The essential oil of Thymus vulgaris exhibited larger inhibition zones at lower concentrations than Melaleuca alternifolia. At a concentration of 8.00 mg, thyme oil showed the largest inhibition zone, whereas melaleuca essential oil achieved its maximum inhibition zone at 14.00 mg. Both oils demonstrated potential as components of antibacterial agents for controlling Klebsiella spp. under the in vitro conditions evaluated. Future studies should determine minimum inhibitory concentrations (MIC/MBC) by broth microdilution (CLSI M07), assess cytotoxicity in non-target aquatic species, evaluate stability under aquaculture water conditions, and conduct in vivo challenge tests in fish models.

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