

Original Article

In vitro study reveals antimicrobial activity of essential oils against *Klebsiella pneumoniae* isolates from Nile tilapia

Estudo *in vitro* revela atividade antimicrobiana de óleos essenciais contra cepas de *Klebsiella pneumoniae* isoladas de tilápia-do-Nilo

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Abstract

Recent studies have shown a growing presence of *Klebsiella pneumoniae* in aquatic environments, raising concerns for fish production due to its resistance to many antimicrobials. This bacterium is commonly associated with urinary tract infections, pneumonia, and septicemia in humans. Controlling bacterial infectious diseases in fish is a significant challenge in aquaculture. In Brazil, only florfenicol and oxytetracycline are approved for use in this sector. Therefore, in this study, we assessed the *in vitro* antibacterial impact and hemolytic activity of 16 essential oils (EOs) and two frequently used antimicrobial agents in aquaculture (florfenicol and oxytetracycline) against seven strains of *K. pneumoniae* isolated from Nile tilapia. The EOs were characterized chemically using gas chromatography with a flame ionization detector and verified with mass spectrometry. The microdilution technique using 96-well plates was employed to evaluate the minimum inhibitory concentration (MIC), ranging from 0.078125 to 20 µg mL⁻¹ for essential oils (EOs) and from 0.0075 to 64 µg mL⁻¹ for antimicrobials. To determine the minimum bactericidal concentration (MBC), 10 µL aliquots from wells that did not show growth in the MIC tests were cultured on Petri dishes containing Muller Hinton Agar. The hemolytic activity of the EOs was evaluated using an erythrocyte lysis assay. The primary components of *Thymus vulgaris* were thymol (53.3%) and p-cymene (20.8%), while those of *Melaleuca alternifolia* were 1-terpinen-4-ol (40.5%), γ-terpinene (19.7%), and α-terpinene (1.6%). Significant hemolytic activity was only observed in *M. alternifolia* tests (>33% lysis). *T. vulgaris* EO exhibited the highest activity against *K. pneumoniae* strains, while *M. alternifolia* EO demonstrated MIC and MBC against all strains. EOs from *Corymbia citriodora*, *Artemisia vulgaris*, *Citrus bergamia*, *Cananga odorata*, *Boswellia carterii*, and *Pelargonium peltatum* also yielded positive results. All strains were resistant to the tested antimicrobial agents. Based on these findings, *T. vulgaris* was the most promising EO for treating *K. pneumoniae* in fish.

Keywords: antimicrobial activity, erythrocyte lysis assay, aquatic animal health management, bacterial disease, *Klebsiella pneumoniae*.

Resumo

Estudos recentes têm mostrado uma crescente presença de *Klebsiella pneumoniae* em ambientes aquáticos, levantando preocupações para a produção de peixes devido à sua resistência a muitos antimicrobianos. Essa bactéria é comumente associada a infecções do trato urinário, pneumonia e sepse em humanos. Controlar doenças infecciosas bacterianas em peixes é um grande desafio na aquicultura. No Brasil, apenas florfenicol e oxitetraciclina são aprovados para uso nesse setor. Portanto, neste estudo, avaliamos o impacto antibacteriano *in vitro* e a atividade hemolítica de 16 óleos essenciais (OEs) e dois agentes antimicrobianos frequentemente usados na aquicultura (florfenicol e oxitetraciclina) contra sete cepas de *K. pneumoniae* isoladas de tilápias-do-Nilo. Os OEs foram caracterizados quimicamente usando cromatografia gasosa com detector de ionização por chama e verificados com espectrometria de massa. A técnica de microdiluição usando placas de 96 poços foi empregada para avaliar a concentração inibitória mínima (MIC), variando de para óleos essenciais (OEs) 0,078125 a 20 µg mL⁻¹ e de 0,0075 a 64 µg mL⁻¹ para antimicrobianos. Para determinar a concentração bactericida mínima (MBC), alíquotas de 10 µL dos poços que não mostraram crescimento nos testes de MIC foram cultivadas em placas de Petri contendo Ágar Muller Hinton. A atividade hemolítica dos OEs foi avaliada usando um ensaio de lise de eritrócitos. Os principais componentes de *Thymus vulgaris* foram timol (53,3%) e p-cimeno (20,8%), enquanto os de *Melaleuca alternifolia* foram 1-terpinen-4-ol (40,5%), γ-terpineno (19,7%) e α-terpineno (1,6%). A atividade hemolítica significativa foi observada apenas nos testes de *M. alternifolia* (>33% de lise). O OE de *T. vulgaris* exibiu a maior atividade contra

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cepas de *K. pneumoniae*, enquanto o OE de *M. alternifolia* demonstrou MIC e MBC contra todas as cepas. Os OEs de *Corymbia citriodora*, *Artemisia vulgaris*, *Citrus bergamia*, *Cananga odorata*, *Boswellia carterii* e *Pelargonium peltatum* também apresentaram resultados positivos. Todas as cepas foram resistentes aos agentes antimicrobianos testados. Com base nesses achados, *T. vulgaris* foi o OE mais promissor para tratar *K. pneumoniae* em peixes.

Palavras-chave: atividade antimicrobiana, ensaio de lise eritrocitária, gestão da saúde de animais aquáticos, doença bacteriana, *Klebsiella pneumoniae*.

1. Introduction

The intensification of aquaculture practices has led to a heightened incidence of bacterial diseases, which significantly impair the growth of this industry (Haenen et al., 2023). The prevalence of these diseases has led to the regular use of veterinary products for therapeutic measures. This approach is designed to inhibit the proliferation of pathogens across the entire system. When combined with unfavorable factors, intensive aquaculture systems foster host infections (Mishra et al., 2023).

The incidence of pathogenic outbreaks affecting farmed fish, particularly bacterial infections, continues to rise (Wright et al., 2023). This increase is largely due to the placement of net cages in rivers, river tributaries, or dams, which exposes them to contamination from nearby domestic and industrial waste. Consequently, these fish become reservoirs for potentially harmful bacteria (Morais et al., 2024; Radwan et al., 2023).

Klebsiella pneumoniae, a rising pathogen in fish, frequently displays resistance to numerous antimicrobials, especially beta-lactam antimicrobials, owing to the prevalent existence of beta-lactamase enzymes. In humans, this bacterium is typically linked with urinary tract infections, pneumonia, and septicemia (Azevedo et al., 2019). Recently, this particular species has been recognized as a contributor to disease outbreaks among aquatic organisms in several countries, including Brazil: Brazil (ornamental Nishikigoi carp, *Cyprinus carpio*), India (the Japanese threadfin bream, *Nemipterus japonicus*), and farmed Indian major carp, *Labeo rohita*, and the United States of America (California sea lions, *Zalophus californianus*) (Vaneci-Silva et al., 2022).

Legislation dictates the types of antimicrobials used to manage bacterial disease outbreaks in aquaculture. In Brazil, only florfenicol (FFC) and oxytetracycline (OTC) are sanctioned for use in this sector (SINDAN, 2022). As a result, numerous fish farmers use antimicrobials that are authorized for use in other animal species only. This practice substantially contributes to the rise and proliferation of bacterial resistance, environmental contamination, and food safety issues (Preena et al., 2020).

The escalating issue of bacterial resistance in both humans and animals, coupled with the accumulation of antimicrobial residues in fish and the environment, has led to an increased promotion and dissemination of the use of natural products (Serwecińska, 2020). Essential oils (EOs) derived from plants are increasingly used for disease prevention and treatment in fish farming (Alves et al., 2020; Dawood et al., 2021). EOs have demonstrated promising results in various fish species, exhibiting antimicrobial properties (Bandeira Junior et al., 2018; Klüga et al., 2021; Bektaş et al., 2023; Alnahass et al., 2023). These oils contain

components that target bacterial cell walls, causing lysis and inhibiting protein and DNA synthesis, while also interfering with the pathogen's signaling mechanisms. Additionally, they are recognized as a primary source of antioxidants, with their spices and herbs exhibiting preservative properties that indicate the presence of antioxidant and antimicrobial compounds (Aldosary et al., 2021; Nourbakhsh et al., 2022).

Considering the limited therapeutic options available, this study aimed to assess the *in vitro* antimicrobial activity and hemolytic potential of 16 EOs against *K. pneumoniae* isolated from Nile tilapia, and to juxtapose their efficacy with those of two antimicrobials sanctioned for use in Brazilian aquaculture. The broth microdilution method and erythrocyte lysis assay were utilized to evaluate antimicrobial activity and hemolytic potential, respectively. Furthermore, the chemical composition of the EOs was ascertained using gas chromatography with flame ionization detection.

2. Material and Methods

2.1. Bacterial strains

The study utilized seven *K. pneumoniae* strains named KP01D, KP02D, KP03D, KP04D, KP05D, KP06D, and KP07D. All strains were isolated from juvenile Nile tilapia (*Oreochromis niloticus*) in Brazil, causing bacteriosis and mass mortality in these fish. The strains were previously identified by conventional biochemical tests and 16S rRNA sequencing (Vaneci-Silva et al., 2022).

2.2. Essential oils

The EOs of *Artemisia* (*Artemisia vulgaris*), bergamot (*Citrus bergamia*), cedar (*Cedrela fissilis*), citronella (*Cymbopogon winterianus*), copaiba (*Copaifera langsdorffii*), Eucalyptus (*Eucalyptus*), lemon eucalyptus (*Corymbia citriodora*), ginger (*Zingiber officinale*), geranium (*Pelargonium peltatum*), peppermint (*Mentha × piperita*), basil (*Ocimum basilicum*), tea tree (*Melaleuca alternifolia*), frankincense (*Boswellia carterii*), petitgrain (*Citrus aurantium*), thyme (*Thymus vulgaris*), and ylang-ylang (*Cananga odorata*) were supplied by Phytoterapica® (São Paulo, SP, Brazil). The antimicrobials florfenicol (FFC) and oxytetracycline (OTC) were obtained from Sigma-Aldrich® (São Paulo, SP, Brazil).

2.3. Chromatographic profiles

The chemical properties of the EOs were analyzed using gas chromatography with flame ionization detection (GC-FID) on an Agilent HP 780A system (Agilent Technologies Inc., USA). The chemical composition of the 16 essential oils

was confirmed through GC/mass spectrometry, adhering to the method outlined in the reference (Baldin et al., 2013).

2.4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The antimicrobial activity test was performed using the microdilution method, as described by (Assane et al., 2019). The essential oils (EOs) were dissolved in dimethyl sulfoxide (DMSO) and diluted with sterile distilled water to prepare 16% (v/v) stock solutions. The highest final concentration of solvent used in the tests was 2% (v/v) for the DMSO solutions, resulting in an antimicrobial stock solution of 400 µg mL⁻¹. The methodology outlined by The Clinical and Laboratory Standard Institute (CLSI, 2020) was followed for the antimicrobials. They were dissolved in 95% ethanol (FFC) and 100% methanol (OTC) to create antimicrobial stock solutions.

The bacteria were cultured on Muller-Hinton agar (BD, Franklin Lakes, USA) and incubated at 37 °C for 24 hours. Following this incubation period, 4-5 colonies were relocated to a physiological saline solution (0.85% NaCl), with the density adjusted to 1×10⁸ CFU mL⁻¹ using a spectrophotometer (OD_{625 nm}), according to the protocols stipulated by the McFarland scale. Subsequently, an aliquot of the inoculum was plated to confirm the stipulated concentration in 1×10⁸ CFU mL⁻¹.

Upon preparation of the stock antimicrobial solution, 20 µL of each solution was dispensed into the first well of a 96-well plate (U-bottom) already containing 180 µL of culture medium. Subsequently, serial dilutions were conducted at a 2-fold factor, resulting in a concentration range of 0.078125 - 20 µg mL⁻¹ for EOs and 0.0075 - 64 µg mL⁻¹ for FFC and OTC.

Each test plate was allocated to a line for quality control, inoculated with *Escherichia coli* ATCC® 25922, along with a sterility control for the culture medium and the DMSO gradient under identical conditions.

Following the incubation period (37 °C for 24 h), the plates were examined by adding 20 µL of triphenyl tetrazolium chloride (TTC) (Sigma-Aldrich®) to all wells and incubating for 15 minutes. A red hue signified bacterial growth. The MIC was then identified as the concentration corresponding to the well with the lowest EO dilution that inhibited bacterial growth. For the MBC, 10 µL was transferred to Petri dishes filled with Muller-Hinton agar (BD, USA) and incubated under the prior conditions. The lowest concentration of the EO that prevented bacterial growth in the inoculation region was considered the MBC (CLSI, 2020). Depending on the presence or absence of growth, the bacterial activity was categorized as bactericidal or bacteriostatic. All MIC and MBC tests were performed in triplicate to ensure data reliability.

2.5. Analysis of EO hemolytic activity

The hemolytic activity of EOs was assessed by incubating erythrocyte suspensions derived from healthy fish, with varying concentrations (0 to 4%) in phosphate-buffered saline (PBS) containing serially diluted EOs (0.2-100 µm). These suspensions were incubated for an hour in a growth incubator maintained at 28 °C. Following incubation, the

suspensions were centrifuged at 800 g for 5 minutes, and the absorbance of the supernatant aliquots (100 µL) was determined at 540 nm using a microplate reader (Assane et al., 2021). The positive control comprised erythrocytes incubated under identical conditions with Triton X-100, while the negative control involved the same volumes supplemented with PBS. The hemolysis percentage was computed based on the absorbance values of the test and control samples using the following Equation 1:

$$\text{Percentage of hemolysis} = \frac{(\text{Absorbance of the test sample} - \text{Absorbance of the negative control})}{(\text{Absorbance of the positive control} - \text{Absorbance of the negative control})} \times 100 \quad (1)$$

3. Results

3.1. Chromatography

The chromatographic profile verified the identity of all compounds present in the essential oils (EOs) used in this research. The chromatographic analysis confirmed the identity and concentration of their significant compounds, revealing a diverse range of profiles among the tested oils.

The essential oil of *Thymus vulgaris* stood out due to its predominant components: thymol (59.3%) and p-cymene (43.8%) (Figure 1). The oil from *Melaleuca alternifolia* was primarily composed of 1-terpinen-4-ol (47.5%), γ-terpinene (19.7%), and α-terpinene (10%) (Figure 2). Other analyzed oils included *A. vulgaris* with eucalyptol (12.1%) and limonene (14.2%); *C. bergamia* with linalyl acetate (33.1%) and linalool (10.7%); eucalyptus oil with 1.8-cineole (9.3%) and limonene (73%); *P. peltatum* with citronellol (47.0%), citronellyl formate (10.6%), and geraniol (10.1%); *B. carterii* with α-pinene (65.1%), p-cymene (6.8%), and β-pinene (4.9%); and *C. odorata* with germacrene D (24.9%), β-caryophyllene (19.8%), and β-farnesene (10.2%).

3.2. Antimicrobial activity

Among the 16 essential oils tested, eight demonstrated significant in vitro activity against *Klebsiella pneumoniae* strains. The oil of *Thymus vulgaris* exhibited the highest antibacterial potency, with minimum inhibitory concentrations (MIC) as low as 1.25 µg/mL. This result aligns with previous studies highlighting the antimicrobial properties of thymol, due to its ability to disrupt bacterial cell membranes and metabolic processes.

The essential oil of *Thymus vulgaris* exhibited a minimum inhibitory concentration (MIC) ranging from 1.25 to 2.5 µg/mL. Similarly, the oil from *Melaleuca alternifolia*, which is rich in 1-terpinen-4-ol and γ-terpinene, showed significant antibacterial activity, aligning with its established role in treating bacterial infections, with an MIC of 10 µg/mL against all strains. Additionally, the essential oils from *Artemisia vulgaris*, *Boswellia carterii*, *Cedrus odorata*, *Citrus bergamia*, *Corymbia citriodora*, and *Pelargonium peltatum* all demonstrated MIC values of 20 µg/mL.

The quality control MIC test for *K. pneumoniae* strains revealed no activity of antimicrobials FFC and OTC against these bacteria (Table 1). It is essential to highlight that FFC and OTC, when tested against the standard strain of *E. coli*,

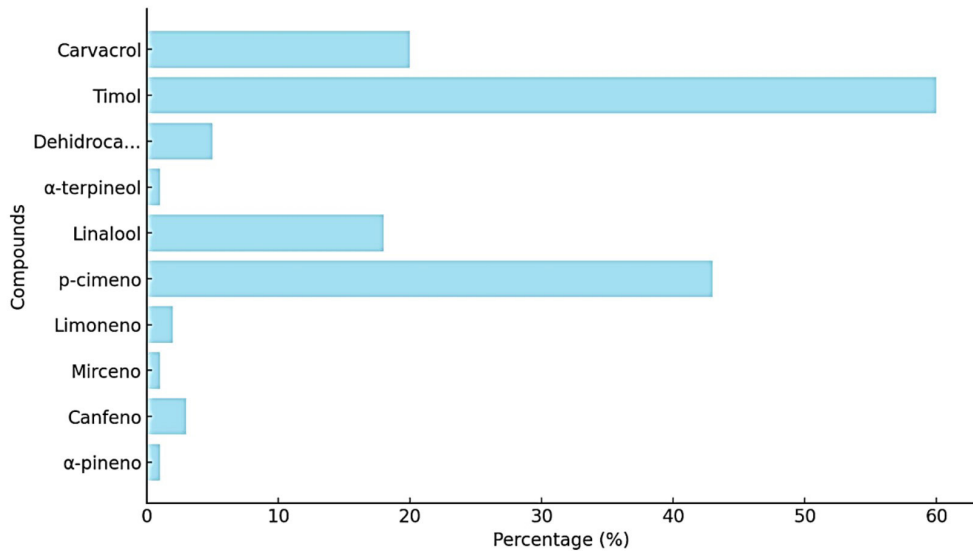


Figure 1. Chemical composition of thyme (*Thymus vulgaris*) essential oil, analyzed using gas chromatography with flame ionization detection (GC-FID) on an Agilent HP 780A system.

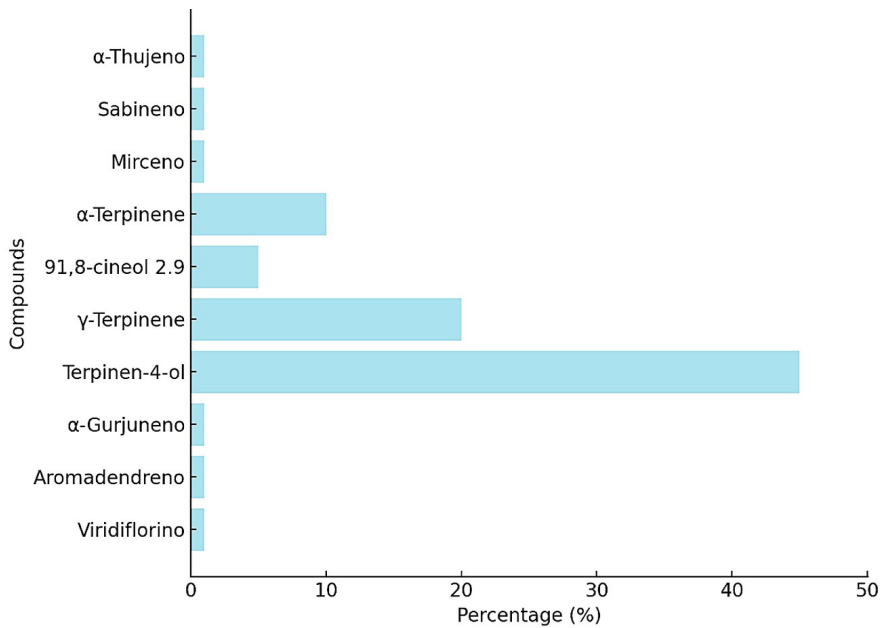


Figure 2. Chemical composition of Melaleuca (*M. alternifolia*) essential oil, analyzed using gas chromatography with flame ionization detection (GC-FID) on an Agilent HP 780A system.

exhibited a MIC of 0.128 $\mu\text{g mL}^{-1}$. This result confirms the sensitivity of the standard strain to the antimicrobials tested in this study.

The minimum bactericidal concentration (MBC) results for the EOs against *K. pneumoniae* strains (KP01D to KP07D) indicated consistent bactericidal activity across the eight oils tested. For most EOs, the MBC was 20 $\mu\text{g/mL}$ against all *K. pneumoniae* strains. Notably, *Melaleuca alternifolia* exhibited particularly potent bactericidal activity, with an

MBC of 10 $\mu\text{g/mL}$, underscoring its effectiveness. In contrast, *Thymus vulgaris* showed an MBC of 1.25 $\mu\text{g/mL}$ for all strains, indicating strong antimicrobial efficacy. These findings underscore the diverse bactericidal strengths among the EOs, with *T. vulgaris* and *M. alternifolia* emerging as the most effective against the tested *K. pneumoniae* strains. For the control strain, tested against FFC and OTC, only bacteriostatic activity was observed, as bacterial growth was present post-plating.

Table 1. Evaluation of the antimicrobial activity of essential oils against *K. pneumoniae* from Nile tilapia's kidney, spleen, and liver and a standard strain *Escherichia coli* ATCC® 25922TM, including MIC and MBC values.

ANTIMICROBIAL ACTIVITY (µG ML ⁻¹)										
<i>A. vulgaris</i>			<i>C. bergamia</i>		<i>C. citriodora</i>		<i>P. peltatum</i>		<i>M. alternifolia</i>	
CEPA	MIC	MBC	MIC	MBC	MIC	MCB	MIC	MBC	MIC	MBC
KP01D	20	20	20	20	20	20	20	20	10	10
KP02D	20	20	20	20	20	20	20	20	10	10
KP03D	20	20	20	20	20	20	20	20	10	10
KP04D	20	20	20	20	20	20	20	20	10	10
KP05D	20	20	20	20	20	20	20	20	10	10
KP06D	20	20	20	20	20	20	20	20	10	10
KP07D	20	20	20	20	20	20	20	20	10	10
<i>B. carterii</i>			<i>T. vulgaris</i>		<i>C. odorata</i>		FFC		OTC	
CEPA	MIC	MBC	MIC	MBC	MIC	MCB	MIC	MBC	MIC	MBC
KP01D	20	20	1,25	1,25	20	20	>64	-	>64	-
KP02D	20	20	1,25	1,25	20	20	>64	-	>64	-
KP03D	20	20	2,5	2,5	20	20	>64	-	>64	-
KP04D	20	20	1,25	1,25	20	20	>64	-	>64	-
KP05D	20	20	1,25	1,25	20	20	>64	-	>64	-
KP06D	20	20	2,5	2,5	20	20	>64	-	>64	-
KP07D	20	20	1,25	1,25	20	20	>64	-	>64	-
ATCC®	-	-	-	-	-	-	0.128	0.128	0.128	0.128

MIC = Minimum inhibitory concentration; MBC = Minimum bactericidal concentration; FFC = Florfenicol; OTC = Oxytetracycline.

Table 2. Haemolytic activity of essential oils against Nile-tilapia red blood cells.

CONCENTRATION (% V/V) OF EOS AND % OF HEMOLYSIS OBTAINED										
IDENTIFICATION	4	2	1	0.5	0.25	0.125	0.0625	0.03125	0.015625	0.007813
<i>A. vulgaris</i>	0.694111	0.005261	0.001718	0.001718	0.001718	0.001718	0.001718	0.001718	0.001718	0.001718
<i>B. carteri</i>	0.385089	0.381224	0.332914	0.332914	0.026627	0.0131	0.004404	0.004404	0.004404	0.004404
<i>C. odorata</i>	0.119833	0.03131	0.024023	0.017469	0.016009	0.006903	0.006173	0.005084	0.005084	0.005084
<i>C. bergamia</i>	0.4333	0.317954	0.260782	0.24674	0.044132	0.003009	0.002006	0	0	0
<i>E. globulus</i>	0.664463	0.554318	0.466423	0.08431	0.068991	0.055583	0.020523	0.009891	0.001099	0.000733
<i>M. alternifolia</i>	66.69838	33.30161	2.473834	1.427212	0.570884	0.190294	0	0	0	0
<i>P. graveolens</i>	0.631553	0.554082	0.543155	0.531329	0.530723	0.091123	0.052771	0.015079	0.002788	0
<i>T. vulgaris</i>	0.905093	0.312784	0.296755	0.292587	0.26332	0.251472	0.165655	0.069388	0.012158	0

3.3. Hemolytic activity

Most of the essential oils tested did not induce lysis of fish erythrocytes, suggesting an absence of hemolytic activity. The essential oil of *T. vulgaris*, affected only 0.25% and 0.26% of the erythrocytes, respectively. In contrast, the oil from *M. alternifolia*, despite showing positive MIC results against bacteria, demonstrated a hemolytic compromise exceeding 33%, which may be concerning (Table 2).

The EOs from *A. vulgaris*, *B. carterii*, *C. odorata*, *C. bergamia*, eucalyptus, and *P. peltatum*, all with erythrocyte lysis, with values below 1% at both tested concentrations.

4. Discussion

The effectiveness of eight essential oils (EOs) against *K. pneumoniae*, an emerging pathogen in aquaculture, was evaluated to determine their potential as antimicrobial agents. The increasing prevalence of *K. pneumoniae* in aquatic environments has become a significant concern for fish production (Whitaker et al., 2018; Vaneci-Silva et al., 2022). In response to this growing threat, research is being conducted to explore various strategies to mitigate bacterial infections, with plant-derived EOs showing

promising potential. However, despite the increasing prominence of studies on herbal therapeutics in recent years, there continues to be a notable gap in the scientific literature regarding the efficacy of EOs against emerging pathogens in fish.

The EO from *T. vulgaris* (TVEO) demonstrated significant *in vitro* potential for controlling *K. pneumoniae* in Nile tilapia (*O. niloticus*) among the 16 EOs tested. The Minimum Inhibitory Concentration (MIC) of TVEO against *K. pneumoniae* was 1.25–2.5 µg mL⁻¹ in this study, indicating the highest bacterial inhibition power. This finding corroborates previous studies that highlighted the potential of EO against common bacteria and parasites in fish (Cunha et al., 2018; Pathirana et al., 2019; Bandeira Junior et al., 2019; Klüga et al., 2021). The high efficacy of TVEO against parasites and bacteria can be attributed to its primary compounds: the terpenoid thymol, its phenolic isomer carvacrol, and clusters of flavonoids and flavonoid glycosides (Pisoschi et al., 2018). These compounds' lipophilicity appears crucial for inflicting cellular damage on bacteria.

The efficacy of terpenoids, particularly thymol and its phenolic isomer carvacrol, flavonoids, and flavonoid glycosides, in exerting antibacterial effects has been extensively documented in the literature. Thymol, a naturally occurring monoterpenic phenol, has been recognized for its potent antimicrobial properties (Zielińska-Błajet and Feder-Kubis, 2020). Similarly, carvacrol, another phenolic compound found in essential oils, has exhibited significant antibacterial activity against many pathogens (Nostro and Papalia, 2012).

These compounds are known to act directly on bacterial cell membranes by interacting with the lipid bilayers due to their lipophilic nature (Mosaddad et al., 2023). This disruption leads to increased membrane permeability, leakage of cellular contents, and eventual cell death (Joshi et al., 2009). Additionally, flavonoids and glycosides, abundant in many plant-derived essential oils, have also been shown to possess antimicrobial properties (Mondal and Rahaman, 2020). These compounds exert their antibacterial effects through various mechanisms, including inhibition of bacterial enzymes, disruption of membrane integrity, and interference with microbial cell signaling pathways (Biharee et al., 2020).

Furthermore, the synergistic interactions among the different compounds present in essential oils contribute to their overall antimicrobial efficacy. Studies have demonstrated that terpenoids, phenolic compounds, and flavonoids more excellent antimicrobial activity than individual components alone (Cáceres et al., 2020). Another critical point is that thymol, carvacrol, and flavonoid combinations possess anti-inflammatory, antioxidant, and immunomodulatory properties (Mondal and Rahaman, 2020; Gherairia et al., 2022). These multifaceted bioactivities make them attractive candidates for therapeutic interventions against bacterial infections.

Studies have emphasized the potency of EOs in combating bacteria resistant to antimicrobials, including *K. pneumoniae*. Research (Sakkas et al., 2016) has demonstrated the efficacy of EOs, at concentrations ranging from 0.5% to 0.75% v/v, against microorganisms

isolated from hospital patients. These findings reinforce the potent antimicrobial properties of EOs, particularly in combating bacteria resistant to traditional antimicrobial agents such as *K. pneumoniae*.

These findings echo those of previous research, reaffirming the broad-spectrum antimicrobial activity of EOs against a range of bacterial pathogens. Notably, it has been demonstrated that EOs exhibit efficacy against multidrug-resistant strains (Yamaguchi, 2022).

While *in vitro* studies provide valuable insights into the antimicrobial activity of EOs, translating these results into clinical practice requires rigorous *in vivo* validation. The present study emphasizes the need for conducting *in vivo* studies to corroborate the efficacy of EOs. Such studies are essential for elucidating the pharmacokinetic and pharmacodynamic profiles of EOs and assessing their safety and efficacy *in vivo* (Porter et al., 2020; Balta et al., 2021). Additionally, *in vivo* studies involving the application of EO in animal models provide valuable data on dosage optimization and potential adverse effects.

The essential oils of *Artemisia*, *Bergamot*, *Eucalyptus*, *Geranium*, *Frankincense*, and *Ylang-ylang* demonstrated promising results against the tested strains. The extensive diversity of medicinal plants and the multiplicity of components in their composition often exhibit a synergistic interaction between molecules (Yap et al., 2014).

Despite the recent increase in the use of EOs, studies need to evaluate their cytotoxicity in aquatic animals and their overall effectiveness. Studies such as Nogueira Sobrinho et al. (2016) have reported a low hemolytic impact of EOs in assessing the hemolytic action of *Vernonia scopioides* EO and found minimal percentages of hemolysis, ranging from 0.9% to 1.08% v/v, indicating minor damage to host blood cells. Similarly, in a previous study, only 5.1% of hemolysis was observed when evaluating the hemolytic activity of *Ocimum sanctum* on human blood cells (Amber et al., 2010). The findings suggest that EOs, particularly TVEO, exhibit significant *in vitro* inhibitory effects against *K. pneumoniae*. This suggests their potential as a viable therapeutic alternative, which could contribute to reducing or eliminating the indiscriminate use of synthetic antimicrobials.

In conclusion, it is crucial to highlight that the only two antimicrobials authorized for treating fish in Brazil (FFC and OTC) proved ineffective against the eight *K. pneumoniae* strains tested in our study. These strains, previously identified by our research team as causing outbreaks and mass mortality among juvenile Nile tilapia, underscore the escalating concern of antimicrobial resistance in *K. pneumoniae* across various sectors, including aquaculture, where Nile tilapia (*O. niloticus*) holds significant commercial value. Given these therapeutic limitations, addressing the challenge of antimicrobial resistance demands exploring effective, safe, and promising alternative therapies, as evidenced by the efficacy of essential oils demonstrated in this study.

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