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RESEARCH ARTICLE

Antibacterial activity of eucalyptus (*Eucalyptus camaldulensis*) essential oil against fish pathogen bacterium, *Aeromonas caviae*

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ABSTRACT

The intensive use of antibiotics in aquaculture has resulted in increased resistance among fish pathogens, and this situation has led researchers to investigate the antibacterial properties of natural resources. The present study focused on an essential oil isolated from the leaves of *Eucalyptus camaldulensis* as a potential antibacterial that could be used against *Aeromonas caviae.* Eighteen compounds were identified in the essential oil, representing 86.68 % of the total oil. The components were found to be p-cymene (20.09%), β-phellandrene (18.61%), α-phellandrene (7.50%), α-terpineol (6.02%), terpinen-4-ol (5.50%), Crypton (5.36%), spathulenol (4.26%), linalool (3.56%), 1,8-cineole (2.77%), farnesol (2.31%) Cumin aldehyde (2.13%), limonen (2.12%), α-thujene (1.94%), fellendral (1.13%), γ-terpinene (1.10%), sabinene (0.97%), α-pinene (0.68%) and α -terpinen (0.63%). The antibacterial efficiency of essential oils against *Aeromonas caviae* was determined using Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values, ranging from 200 µg/ml to 400 µg/ml respectively. Our findings revealed the potential of essential oils isolated from Eucalyptus (*Eucalyptus camaldulensis*) as a natural antibacterial agent that could efficiently contribute to the control of *Aeromonas caviae* infection in fish.

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Introduction

Fish diseases, especially those caused by infectious organisms like bacteria which are responsible for the majority of infectious diseases, often result in serious economic losses in aquaculture facilities (Miller & Mitchell, 2009; Scott et al., 2011). The disease poses a major threat to aquaculture in many

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ways, such as losses caused by dead fish, decreased growth and the cost of treatment (Alfred et al., 2020).

Aeromonas spp. can cause diseases in fish with a high mortality rate (Bektas et al., 2007; Martínez-Murcia et al., 2008; Xue et al., 2022). Among the 31 species of *Aeromonas*, *A. hydrophila*, *A. sobria, A. caviae, A. allosaccharophila, A. encheleia* and *A. veronii* are classified as motile aeromonads (Chen et al., 2016; Stratev & Odeyemi, 2017).

Although, motile *Aeromonas* septicaemia (MAS) caused by motile aeromonads mostly associated with *A. hydrophila*. *Aeromonas sobria* and *Aeromonas caviae* are the other two most common species of the genus that cause disease. These opportunistic pathogens cause haemorrhagic septicaemia and ulcerative conditions in a variety of aquatic and terrestrial animals (Austin & Austin, 1993; Baumgartner et al., 2017).

Antimicrobials have a wide range of usage in preventing and treating the disease in intensive fish farming. These intensive applications of antimicrobials can cause the development of antimicrobial-resistant strains (Miller & Harbottle, 2018; Saengsitthisak et al., 2020). Xue et al. (2022) isolated *A. caviae* WH21406 from diseased largemouth bass (*Micropterus salmoides*) and pathogenicity of the strain was confirmed with the histopathological observation and virulent gene analysis. They also reported resistance of the strain against florfenicol, neomycin sulfate, compound sulfamethoxazole, doxycycline and tetracyclines. On the other hand, antibiotic-resistance genes may be passed to humans with food chain and this may complicate the treatment of some diseases in humans (Harikrishnan et al., 2009).

The mentioned side effects associated with synthetic antimicrobials have led scientists to develop alternative substances that can be used in case of a bacterial disease. (Sharma et al., 2012). Being a natural product, use of essential oils (EOs) which have antibacterial activity and low toxicity levels in the prevention and treatment of bacterial disease, makes them beneficial alternative against commonly used antibiotics in aquaculture (Da Cunha et al., 2018: Wińska et al., 2019).

Effective use of EOs in case of a bacterial infection in fish can be a promising strategy for reducing the consumption of antimicrobials in aquaculture (Klūga et al., 2021).

Eucalyptus is widely used in the pulp and paper industry, but its seeds and leaves also have the potential to produce essential oils. Being secondary plant metabolites, EOs have an important role in decreasing bacterial virulence and also weaken the mechanisms of bacterial invasion (Kartiko et al., 2021; Kouki et al., 2022).

Arial parts of *Eucalyptus* species are mostly used for their antibacterial, antifungal, analgesic and anti-inflammatory properties *Eucalyptus* essential oils exhibit high antimicrobial activity against a wide range of bacteria (Ghalem & Mohamed, 2008; Mulyaningsih et.al., 2011; Clavijo-Romero et al., 2019).

The objectives of the present study were to determine the chemical composition of the essential oils obtained from leaves of eucalyptus (*Eucalyptus camaldulensis*) and to determine the antimicrobial activity of this essential oil against fish pathogenic bacteria, *Aeromonas caviae.*

Material and Methods

Plant Materials and Extraction of the Essential Oil

Plant materials were collected from the leaves of Eucalyptus tree, growing wild in Sinop (Türkiye) region. Samples were cleaned and dried at the ambient temperature, protected from direct light.

About 500 g of fresh leaves were crushed and subjected to hydro-distillation using a Clevenger's apparatus. Briefly, crushed eucalyptus leaves were completely immersed in water and heated to boiling. The essential oil (EOs) evaporated together with water vapour passed through the refrigerant and collected into the condensation flask, after liquid phase removed the essential oil was collected in a glass vial (Ghalem & Mohamed, 2008; Mazumder et al., 2020).

Test Organism

The bacterium, *Aeromonas caviae* LipT51 (GenBank ID: MN818567.1), isolated from Ilica Hot Springs (Erzurum, Turkey), was obtained from the Microbiology Laboratory, Department of Biology, Atatürk University, Erzurum, Turkey. The bacterial isolate was identified by both 16S rRNA gene sequencing and classic identification tests (Gurkok & Ozdal, 2021). The bacterial strain was maintained in Nutrient Broth containing 20% glycerol at −86°C throughout the study and used as stock cultures.

GC and GC/MS Analyses

Hewlett Packard system, HP 5973 Mass Selective Detector System and GC–MS 6890 GC system were used in analyses. Agilent HP innowax column (60 m in length, inner diameter of 0.25 mm, film thickness of 0.25 µm) was used. As a carrier gas helium was used. The injection temperature was 250°C and the oven temperature was kept at 60°C for 10 minute and programmed to 220°C at a rate of 4°C/min and kept constant at 220°C for 10 min and programmed to 240°C at a rate of 2°C/min for 40 min. Relative amounts of the characterized components were expressed in percentages and the retention time (RT) was recorded in minutes (Sevindik et al., 2016). GC-MS analyses were conducted in Eskisehir Anadolu University Medicinal Plants, Drugs and Scientific Research Center (AUBİBAM).

Determination Of Antibacterial Concentration

The Minimum Inhibitory Concentration (MIC) of the Eucalyptus oil was measured by Broth Dilution Method (Rath & Priyadarshanee, 2017). Shortly, varying volumes of the oil were mixed with NB (Nutrient Broth containing 0.5% of Tween 20, v/v) to give a concentration of 50-1000 µg/ml by serial dilution method. A total of seven different concentrations used in the study. One hundred microliters of overnight grown culture (5×10^5) were inoculated into the tubes containing various concentrations of eucalyptus oil. The tubes with a volume of 4 ml were incubated with shaking at 37°C for 24 h and the lowest concentration inhibiting bacterial growth (no turbidity) was determined as MIC of the particular oil against the specific test organism. The optical density (OD600) was measured for MIC analysis at two different times: t_0, before incubation, and t_{24}, following incubation for 24 hours. To determine MBC, a 20-µl solution from the final three tubes that did not demonstrate bacterial growth was inoculated on NA plates and incubated overnight at 37°C.

Results and Discussion

The isolate was identified based on 16S rDNA sequence analyses and its morphological and biochemical characteristics. *A. caviae* LipT51 was characterized as a Gram-negative, non-spore forming, rod-shaped, catalase, oxidase, and protease-positive, urease-negative bacterium, forming cream-colored, bright, and round colonies (Gurkok & Ozdal, 2021).

GC-MS analyses of the essential oil of eucalyptus (*Eucalyptus camaldulensis*) revealed p-cymene and β-phellandrene as the major compounds with the highest peaks (Figure 1).

Although, components of essential oils obtained from plants differ relating to many factors such as region, climate, harvesting season, soil, relative humidity, day length and sampling methods (Sartorelli et al., 2007; Heikal, 2017), the major compounds of EO from *Eucalyptus* species were reported as 1,8-cineole (eucalyptol), p-cymene, terpinen-4-ol, β-pinene and γ-terpinene, (Sebei et al., 2015; Sabo & Knezevic, 2019; Almas et al., 2021).

Eighteen compounds were identified in the essential oil representing 86.68% of the total oil. The essential oil composition presented in Table 1.

Similar to our results p-cymene and β-phellandrene were reported as the major component in *Eucalyptus camaldulensis* (Dagne et al., 2000; Grbović et al., 2010). p-Cymene and β-phellandrene are monoterpenes, belong to the terpenes which are the major components of essential oils of various plant species (De Oliveira et al., 2015; Noriega, 2020). To date, various biological activities of these compounds have been reported. (Wang & Liu, 2010; Marchese et al., 2017).

Anastasiou et al. (2019), tested the EOs of twelve Mediterranean plants against certain bacterial fish pathogens like as *Aeromonas*, *Vibrio*, *Edwardsiella* and *Photobacterium* species. They have reported that compounds such as carvacrol, p-cymene and γ-terpinene showed a strong inhibitory activity on growth all of this fish pathogenic bacteria. They were also determined that these EOs had the highest total antioxidant capacity.

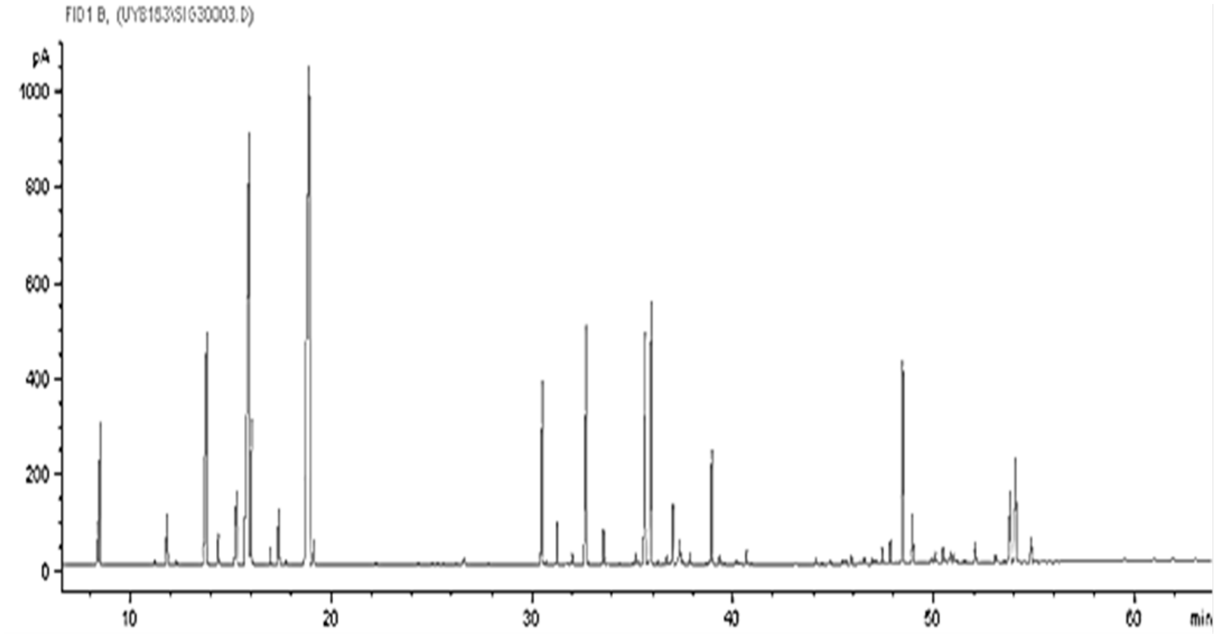

Figure 1. Gas chromatogram of the essential oil from *Eucalyptus camaldulensis*

Table 1. Essential oil composition of *Eucalyptus camaldulensis*

RT (min)	Component	Quantity (%)
8.33	p-cymene	20.09
8.46	β-phellandrene	18.61
11.80	α-phellandrene	7.50
13.78	α-terpineol	6.02
14.35	terpinen-4-ol	5.50
15.25	Crypton	5.36
15.89	Spathulenol	4.26
16.01	linalool	3.56
17.42	1,8-cineole	2.77
18.92	Farnesol	2.31
30.54	Cumin aldehyde	2.13
32.74	limonen	2.12
35.67	α-thujene	1.94
35.98	Fellendral	1.13
37.05	γ-terpinene	1.10
38.98	sabinene	0.97
48.50	α-pinene	0.68
54.12	α-terpinen	0.63
Total		86.68

Note: RT: Retention time

In our study, *Eucalyptus* oil presented antibacterial activity against *Aeromonas caviae*. The antibacterial efficiency of essential oils was detected by MIC and MBC values, which were observed at 200 µg/ml and 400 µg/ml respectively. The comparative MIC and MBC values of the eucalyptus essential oils are presented in Table 2.

Eucalyptus essential oils contain antibacterial active ingredients such as volatile terpenes and aromatic compounds (Barbosa et al., 2016; Gadhoumi et al., 2022). On the other hand, eucalyptus essential oil can cause cell wall and membrane dysfunction. He et al. (2022) determined by SEM that *S. putrefaciens* and *S. aureus* cells treated with eucalyptus oil showed abnormal and rough surface. This can lead to significant loss of intracellular ATP, pH disturbance and intracytoplasmic changes (Sabo & Knezevic, 2019; Limam et al., 2020).

Insuan & Chahomchuen (2020) reported the high antibacterial activity of essential oils of *Eucalyptus citriodora* against *Staphylococcus intermedius* and *Pseudomonas aeruginosa* as determined by the MIC and MBC values, and they suggested that *E. citriodora* oil affected the cell wall and cell membrane.

Bogavac et al. (2019) reported the MIC values of *Eucalyptus globulus* essential oil against *S. aureus* 2, *E. coli* 1, and *P. mirabilis* as 6.2, 12.5 and 25 µL/ml, respectively. The essential oil extracted from the fresh leaves of *E. grandis* showed MIC 0.31, 1.25 and 1.25 mg/ml and MBC 0.63, 2.5 and 1.25 mg/ml against bacteria such as *Klebsiella pneumonia*, *S. aureus* and *Moraxella catarrhalis*, respectively (Sewanu et al., 2015). The minimum inhibitory concentration (MIC) of leaf oil (*E. globulus*) was 0.72 µl/ml for *S. epidermidis*, 0.74 µl/ml for *S. aureus*, 1.5 µl/ml for *P. aeruginosa* and 2.75 µl/ml for *Klebsiella pneumoniae* (Bachheti, 2015).

Knezevic et al. (2016) reported the major components of essential oils obtained from *E. camaldulensis* as spatulenol, cryptone, p-cimene, 1,8-cineole, terpinen-4-ol and β-pinene and they have detected MICs values of essential oils against *Acinetobacter baumannii* wound isolates in a range of 0.5 to 2 µl/ml according to these findings they claimed that the polar terpene compounds and spathulenol are at least partially responsible for the antibacterial activity.

Table 2. Comparative MIC and MBC values of eucalyptus essential oils against fish pathogenic bacteria

Bacteria	*Eucalyptus*	MIC	MBC	References
Aeromonas hydrophila	*E. camaldulensis*	125 µL/mL	500 µL/mL	Debbarma et al., 2013
Lactococcus garvieae	*E. globulus*	31.24 µg/mL	125 µg/mL	Park et al., 2016
Edwardsiella tarda	*E. globulus*	7.81 µg/mL	125 µg/mL	Park et al., 2016
Vibrio harveyi	*E. globulus*	7.81 µg/mL	62.50 µg/mL	Park et al., 2016
Vibrio ichthyoenteri	*E. globulus*	125 µg/mL	250 µg/mL	Park et al., 2016
Photobacterium damselae	*E. globulus*	31.24 µg/mL	125 µg/mL	Park et al., 2016
Citrobacter freundii	*E. globulus*	0.36 µg/mL	0.36 mg/mL	Damjanović-Vratnica et al., 2011
A. caviae	*E. camaldulensis*	200 µg/mL	400 µg/mL	Present study

There are so many studies reporting variable results for MIC and MBC values of essential oils against Aeromonas strains. Chagas et al. (2020) reported MIC values ranging between 1.25 to 10 and MBC values 1.667 to 10 µg/mL of the essential oils against *Aeromonas* spp. isolates. In a study which aimed to evaluate in vitro efficacy of 13 essential oils and 16 compounds against four *Aeromonas salmonicida* subsp. *salmonicida* strains, researchers observed that MIC and MBC values of studied EOs and compounds, varied slightly among the strains (Hayatgheib et al., 2020). Monteiro et al. (2021) determined the antimicrobial activity of *Lippia sidoides*, *Ocimum gratissimum* and *Zingiber officinale*'s essential oils against *Aeromonas* spp. isolates and they have reported MIC and MBC values ranging from 625 to 5,000 µg/mL.

These differences among the studies may be due to many factors such as the extraction method and purity of the essential oil, the difference in the bacterial species studied, the inoculum volume and the difference in the medium used.

Conclusion

Bacteria concerned with fish disease are one of the limiting factors that have caused major production problems in aquaculture facilities. In case of a bacterial infection, antibiotics are the most applied chemicals for inhibiting the pathogen growth and also to control the disease. However intensive and unregulated use of antibiotics has led to antimicrobial resistant fish pathogens. Increased antimicrobial resistance (AMR) is a global concern, because microorganisms, causing disease are becoming resistant to antimicrobials which are used to control them.

Since the antibiotic resistant bacteria limited the use of conventional treatment with antibiotics, has led the researchers to seek alternative natural resources with plant origin that can replace antibiotics. A literature based survey has demonstrated that the use of essential oil extract for the prevention and treatment of infectious diseases, could substitute synthetic antibacterial.

The antimicrobial effects of EOs, mainly p-cymene and β-phellandrene being major components obtained from Eucalyptus (*Eucalyptus camaldulensis*) reported in the present study demonstrate that these components have an antimicrobial activity against one of a fish pathogen bacteria, *Aeromonas caviae.*

Compliance With Ethical Standards

Authors' Contributions

SB: Designed the study, wrote the first draft of the manuscript.
MÖ: Performed laboratory experiments.
Both authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

Data Availability

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

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