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Antibiofilm activities and *in vitro* susceptibility testing of eucalyptus (*Eucalyptus camaldulensis*) essential oil (EO) against fish pathogen *Pseudomonas* species

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Abstract: Essential oils are concoctions of aromatic and volatile chemicals extracted from several plant species. These mixes have been used by society for a variety of reasons, and they play significant functions in nature. This study aimed to analyze the biological properties of essential oil extracted from *Eucalyptus camaldulensis* leaves, including its antipseudomonal and antibiofilm effects. Antibiotics have been heavily used both to treat bacterial infections and to stimulate fish growth, which has led to the emergence of germs that are resistant to the drugs. The study inoculums have been defined by the McFarland 0.5 standard and disk diffusion method has been used to analyze antimicrobial activity. The essential oils from *E. camaldulensis* possessed antibacterial activity against tested *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Pseudomonas putida* at 5-10 µg/disc. The antibacterial effect has been established to be dependent on the concentration. Our findings showed that *E. camaldulensis* essential oil has been a great source of antipseudomonal, and also exhibited inhibition of *Pseudomonas* species biofilm formation. Based on its antibacterial and antibiofilm potential, *E. camaldulensis* essential oil shows promise as a potential source of antibacterial agents. Therefore, the use of *E. camaldulensis* essential oil in applications may have the potential to be a natural antibacterial agent against pathogenic and spoiling microorganisms.

Keywords: Antibacterial activity, antibiofilm activity, essential oil, Eucalyptus, *Pseudomonas*

INTRODUCTION

Bacterial diseases which caused by various bacterial pathogens are the main cause of high mortalities and economic losses among fish farms (Austin and Austin, 2007; Algammal et al., 2020).

Pseudomonads are one of the most prevalent bacterial species naturally present in almost all aquatic environments. They only become pathogenic when the fish is exposed to unfavorable environmental conditions like poor sanitation and water quality. *Pseudomonas* infections in fish mostly result in ulcerative syndrome and hemorrhagic septicemia (Oh et al., 2019; Narvaez et al., 2021; Eissa et al., 2010).

To control the diseases in fish that are mainly infectious, a wide range of chemicals like antimicrobials have been widely used in fish farming (Mohamed et al., 2000). Intensive use and misuse of antimicrobial agents in the aquaculture industry cause antimicrobial resistance, which results in not only treatment failures but also limits sustainable food animal production and animal welfare (Schar et al., 2020).

The rise in bacterial resistance, which has become a major concern worldwide, has focused the attention of researchers on natural products that could have similar effects on bacteria and that they could use instead of conventional antibiotics. Essential oils (EO) derived from plants seem to be a potential alternative because of their anti-inflammatory, antimicrobial, and antioxidant properties (Yap, 2014; Wińska et al., 2019).

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Eucalyptus species are commonly used in traditional medical applications for their antimicrobial properties. Essential oils (Eos) derived from this plant have been widely examined (Asiaei et al., 2017; Aleksic Sabo and Knezevic, 2019).

Park et al. (2016) reported antimicrobial activity of the essential oil obtained from *Eucalyptus globulus* against seven fish pathogenic bacteria, and they claimed that this EO can be used in fish farms as an antimicrobial agent in cases of bacterial infections.

To respond to changing environmental conditions, one of the strategies for bacteria to adapt and survive is forming multicellular communities known as biofilms (Čabarkapa et al., 2019). Bacterial biofilms are aggregates of microorganisms attached to surfaces or to each other and embedded in a self-assembled matrix of extracellular polymeric substances (Vestby et al., 2020).

Bacteria can protect themselves from hosts' defenses and antibacterial agents through the formation of biofilms and they also tend to develop their resistance mechanisms in many ways, such as through physical, physiological, and gene-related factors. Planktonic forms of bacteria are much more susceptible to antimicrobial agents than the bacteria that are inside a biofilm. Antimicrobial resistance, caused by biofilm-forming bacterial pathogens, not only results in treatment failures in cases of bacterial fish diseases but also causes recurrent exposure of fish to infections (Sundell and Wiklund, 2011; Abebe, 2020; Dinçer et al., 2019).

Antimicrobial susceptibility testing (AST) is an important task for the microbiology laboratories that are commonly used to determine possible drug resistance and antimicrobial susceptibility against common pathogens (Jorgensen and Ferraro, 2009). Although a variety of methods exist, the Kirby-Bauer agar diffusion method is well documented, cost-effective, more accurate, fast screening, and a standardized method for determining antibiotic susceptibility (Liu et al., 2016; Nassar et al., 2019).

The aim of the present study is to determine the antibiofilm activities and antimicrobial susceptibilities of eucalyptus (*Eucalyptus camaldulensis*) EOs against the fish pathogen *Pseudomonas* species.

MATERIALS AND METHODS

Plant material and preparation of EO

Eucalyptus (*Eucalyptus camaldulensis*) fresh leaves used in the study were harvested from the trees growing wild in Sinop, Turkey (42°02'43.4" N, 35°02'27.9" E) during June. Samples were cleaned to remove any dust and impurities, then dried at room temperature before use (Insuan and Chahomchuen, 2020). For the complete extraction of the essential oil, a total of 500 g of the dried sample was crushed and exposed to hydro-distillation using a Clevenger's apparatus. The EOs evaporated together with water vapor and passed through the refrigerant before being collected into the condensation flask. After the liquid phase was removed, the essential oil was collected in a glass vial and stored at 4 °C until further testing and analysis (Ghalem and Mohamed, 2008; Mazumder et al., 2020).

Gas Chromatography-Mass Spectrometry Analysis

Analysis was carried out in Eskisehir Anadolu University Medicinal Plants, Drugs and Scientific Research Center (AUBİBAM). The EO was subjected to Gas chromatography (Hewlett Packard system, HP 5973) and Mass spectrometry (GC-MS 6890 GC system). 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 minutes, then programmed to 220 °C at a rate of 4 °C/min, kept constant at 220 °C for 10 min and then programmed to 240 °C at a rate of 2 °C/min for 40 minutes. Retention time (RT) was measured in minutes, and relative quantities of the described components were represented in percentages. (Sevindik et al., 2016).

Bacterial strains

P. aeruginosa (ATCC 9027), *P. fluorescens* (BC 7324), and *P. putida* (BC 1617) were obtained from the Microbiology Laboratory of the Department of Food Engineering at Atatürk University in Erzurum, Turkey, for use as test organisms (Cetin et al., 2011).

Antibacterial assay

Antimicrobial disc diffusion assays were performed for the screening of essential oils efficacy. After 24 h of bacterial culture at 37C in nutrient Broth, a diffusion test for Eucalyptus Essential Oil was performed. The bacterial cultures of different *Pseudomonas* species were visually adjusted to 0.5 McFarland standard with sterile saline. The bacterial suspensions were swabbed on the surface of Nutrient Agar plates and left to stand for 3 minutes before testing. Sterile Whatman No. 1 filter paper discs (6 mm, Biolife) were loaded with following concentration of EO (5 and 10 µl/disc) and placed on the surface of the freshly inoculated medium. As positive controls, Cefoperazone + Sulbactam (105 µg), Oxolinic acid (2µg), and Chloramphenicol (C30 µg) were employed. For 20 hours, the plates were incubated at 37 °C. The antibacterial activity was measured by measuring the diameter of the zones of

inhibition surrounding each of the disks (Bauer, 1966; Merghni et al. 2016; Andoğan et al., 2002).

Determination of biofilm inhibition

Using EEOs, a biofilm inhibition experiment was conducted against *P. aeruginosa* (ATCC 9027), *P. fluorescens* (BC 7324), and *P. putida* (BC 1617) in 96-well culture plates. The strains were kept at 37 °C for 24 hours while being cultured in 10 mL of tryptic soy broth that contained 1% glucose. The creation of dilutions equal to the 0.5 McFarland standard value came next. One hundred mi lliliters of eucalyptus oil, with final concentrations ranging from 7.8 to 125 µg/ml, 90 µl of growth medium (TSB with 1% glucose), and 10 µl of test bacterial dilutions were combined in each well of the plates.

While the negative control simply included growth media, the positive controls combined 10 µl of the bacterial dilutions with 190 µl of growth medium. The 96-well plate was incubated for 24 hours at 37 °C, then the unattached planktonic cells were washed out three times with distilled water to remove them. The remaining adherent sessile cells were then dyed with 200 µl of 0.4% crystal violet for 30 min, the excess dye was poured out, and the wells were then three times washed with distilled water. The leftover colored biofilm was dissolved in 200 µl of 70% ethanol and left to stand for 30 minutes. A microplate reader (Thermo Scientific Inc., Multiscan GO, Finland) was used to read the wells' optical density (OD) at 570 nm (Bai et al., 2019).

The following equation was used to compute biofilm inhibition:

Biofilm inhibition (%) = [(Control OD570nm – Test OD570nm) / Control OD570nm] x 100

RESULTS and DISCUSSION

The industries employ a variety of naturally occurring antimicrobials derived from plants and spices to minimize or eradicate harmful bacteria, improve the overall quality of products, and prolong the shelf life of goods. In the current study, the antibacterial capacity of 5 and 10 µl/disc of essential oil of *E. camaldulensis* was tested against *P. aeruginosa*, *P. fluorescens*, and *P. putida*.

The results obtained are shown in Table 1. On each of the studied bacteria, the EO inhibited it to a different extent. The zone of inhibition was 7–16 mm wide. The highest inhibition zone (16 mm) was observed against *P. putida* at a concentration of 10 µl/disc. The diameter of inhibition increased as the concentration of EO increased, indicating that the inhibition was dependent on dose concentration. *P. aeruginosa* was shown to be more resistant to the examined antibiotics and EEOs than the other two microorganisms.

In our previous study, the GC-MS analyses resulted with the identification of 18 components above 0.63%, representing 86.68% of the eucalyptus EO. The major constituents and amounts were detected as p-cymene (20.09%) and α-phellandrene (18.61%), respectively and antibacterial effect against *Aeromonas caviae* LipT51 also reported (Bektaş & Özdal, 2022).

EOs rich in carvacrol, p-cymene, and γ-terpinene showed strong inhibitory activity on the growth of all tested pathogenic bacteria (Anastasiou et al., 2019).

The primary compounds of EEO are terpenes and alcohol. As reported by Barbosa et al. (2016), D-limonene, 3-carene, myrcene, and α-pinene are terpenes that are linked to EEO's antibacterial effect. A relative increase in terpene content may lead to increased antibacterial activity. In actuality, it's also conceivable that substances with lower concentrations may work in a synergistic manner with other active substances.

Bioactive compounds and EOs derived from aromatic plants are alternative antibacterial agents that are thought to be safe and promising.

According to research by Chen et al. (2007) and Lu et al. (2022), biofilms provide difficulties for water treatment systems and have a specific influence on food industry. When compared to their planktonic counterparts, bacteria in biofilms can be up to 1000 times more resistant to antibiotic treatment (Simoes et al., 2009). *Pseudomonas* species, an opportunistic pathogen with a high degree of viability in environments such as water, air, soil, and food, are widely colonized (Osman et al., 2019).

Table 1. Antibacterial activity of essential oil of *E. camaldulensis* against *Pseudomonas* species

	Zones of inhibition (mm)		
	P. aeruginosa	*P. putida*	*P. fluorescens*
EO 5 µl/disc	7	10	12
EO 10 µl/disc	10	16	14
Cefoperazone + Sulbactam (105 µg)	16	27	30
Oxolinic acid (2µg)	11	13	15
Chloramphenicol (C30 µg)	13	20	16

P. fluorescens and *P. putida* biofilms were found to be suppressed by the anti-biofilm activity of EEO at low doses. Our findings show that *P. fluorescens* and *P. putida* are more vulnerable to EEO than *P. aeruginosa*. When EEO at concentrations of 7.81, 15.62, 31.25, 62.5, and 125 µg/mL were used, 0, 0, 0, 6, and 39.2% of *P. aeruginosa* biofilm formation was inhibited, respectively (Figure 1). Likewise, the same concentrations of EEO (7.81, 15.62, 31.25, 62.5, and 125 µg/mL) prevented 35, 41, 68, 99, and 99,8% of biofilm formation by *P. fluorescens*. For *P. putida* at the same concentrations, these values were measured as 46, 67, 98, 99, and 100.

Many studies have demonstrated the antibiofilm activity of EEOs against *Staphylococcus aureus* (Merghni et al., 2018), *Streptococcus mutans* (Goldbeck et al., 2014), *Actinobacillus pleuropneumoniae* (Rodrigues et al., 2022), *Listeria monocytogenes*, *P. aeruginosa*, *Escherichia coli*, *Pectobacterium carovotorum* (Caputo et al., 2020).

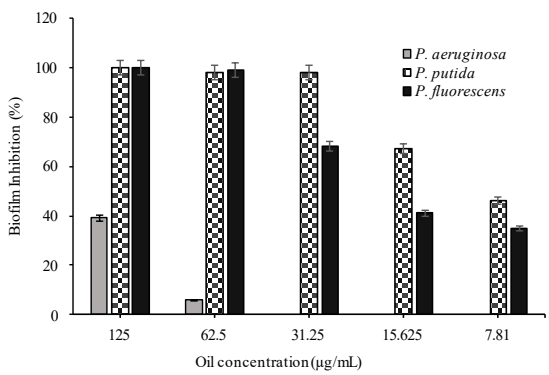

Figure 1. Inhibition of biofilm formation by *P. aeruginosa*, *P. fluorescens*, and *P. putida* using EEO.

CONCLUSIONS

According to these observations, Eucalyptus essential oil's antibacterial activity may thus point to its potential value as a microbiostatic, antiseptic, or hygienic agent, particularly against Gram-negative bacteria. The current study gives information that EEO have antibacterial and antibiofilm properties when it comes to *Pseudomonas* species. There is an urgent need to find effective solutions to battle Pseudomonads due to the growth of multidrug-resistant strains and the predominance of biofilm formation, and EOs have come to light as a promising solution. Numerous EOs have been demonstrated to be efficient antimicrobials and antibiofilm agents, enabling them to be employed either alone or in conjunction with well-established antibiotics in therapeutic formulations.

COMPLIANCE WITH ETHICAL STANDARDS

CONFLICT OF INTEREST

The authors declared no conflict of interest. All the authors read and approved the final manuscript.

ETHICAL APPROVAL

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CONSENT FOR PUBLICATION

Applicable.

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