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Menba Kastamonu Üniversitesi Su Ürünleri Fakültesi Dergisi 2023; 9(1): 25-33 Araştırma Makalesi/Research Article

Determination of Antibacterial and Antibiofilm Activities for Laurel (*Laurus nobilis* L.) Essential Oil Against the Fish Pathogen *Pseudomonas* Species

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Abstract

The essential oil derived from *Laurus nobilis* exhibits a high concentration of bioactive components, imparting various therapeutic characteristics. This study aimed to analyze the chemical composition of the essential oil extracted from *Laurus nobilis* leaves as well as its biological properties, including its antipseudomonal and antibiofilm actions. Gas chromatography-mass spectrometry (GC-MS) analysis revealed that 1,8-cineole (%48.43) and α-terpinyl acetate (14.78) were the major compounds present in the essential oil (EO). While, the minimum inhibitory concentration (MIC) values of *Laurus nobilis* essential oils (LEO) against *P. fluorescens* and *P. putida* were determined as 31.25 µg/mL, it was 62.5 µg/mL for *P. aeruginosa*. LEO, at a MIC level of 31,25µg/mL, exhibited significant inhibition of *Pseudomonas* species biofilm formation except for *P. aeruginosa*. Based on its demonstrated antibacterial and antibiofilm potential, LEO holds promise as a prospective source of antibacterial agents.

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INTRODUCTION

Aquaculture, a rapidly growing sector of agriculture globally, faces various challenges that impede its production growth. Among these challenges, fish diseases caused by a diverse range of infectious organisms pose a significant concern, leading to substantial economic losses for farmers (Francis-Floyd, 2005; Alfred et al., 2020; Assefa and Abunna, 2018). Bacterial infections pose a significant threat to both wild and aquaculture-reared fish. In the aquatic environment, bacteria are commonly present as part of the normal flora, and they typically do not cause disease unless fish are exposed to unfavorable environmental conditions and inadequate husbandry techniques (Tendencia and Lavilla-Pitogo, 2004).

Pseudomoniasis, also known as *Pseudomonas* septicemia or *Pseudomonas* infection, is a bacterial disease caused by various species within the genus *Pseudomonas*. Several *Pseudomonas* species including *P. anguilliseptica, P. baetica, P. chlororaphis, P. fluorescens, P. luteola, P. plecoglossicida, P. pseudoalcaligenes, P. aeruginosa* and *P. putida* have been reported to be pathogenic that cause ulcerative syndrome and hemorrhagic septicemia in various fish species (Austin and Austin, 2012; Algammal et.al., 2020; Eissa, 2010; Bektas and Ayik, 2009).

When a bacterial disease is encountered in farms, one of the effective treatments is the use of antimicrobial agents for years. However, intensive and misuse of antibiotics for preventing and treating bacterial infection have led to the emergence of bacterial resistance, resulting in treatment failures, jeopardizing food safety, and posing environmental concerns (Romero et al., 2012; WHO, 2020). The increase in antimicrobial resistance not only poses challenges in the treatment of bacterial infections in fish but also constitutes a substantial and worldwide public health risk (Sabo and Knezevic, 2019; Insuan and Chahomchuen, 2020). The escalating occurrence of antibiotic resistance has directed researchers attention towards exploring alternative avenues for the treatment of infections (Da Cunha et al., 2018).

Biofilms, consisting of multicellular matrices, facilitate bacterial adhesion to the external environment and represent a contributing factor to microbial drug resistance. Biofilm cells exhibit distinct characteristics compared to planktonic cells, including differences in morphology, physiology, and gene expression. Consequently, it is crucial to invest efforts in identifying novel compounds capable of combating drug-resistant microbes and biofilms to prevent the emergence of resistance (Liu et al., 2022; Lim et al., 2022).

EOs obtained from various aromatic plants contain a diverse array of secondary metabolites and exhibit significant antimicrobial activity by inhibiting bacterial growth. Due to their antimicrobial properties, EOs have the potential to serve as an alternative to antibiotics for the management of infectious fish diseases (Kunová et al., 2021; Gholipourkanani et al., 2019; Nazzaro et al., 2013; Bektas and Ozdal, 2022).

Most EOs are composed of terpenes, terpenoids, and other aromatic and aliphatic constituents with low molecular weights. Not only the natural monoterpenes but also their synthetic forms have various pharmacological properties such as antifungal, antibacterial, antioxidant, anticancer, antibiofilm, antiarrhythmic, anti-aggregating, local anesthetic, antinociceptive, anti-inflammatory, antihistaminic, and anti-spasmodic activities (Loza-Tavera, 1999; Kozioł et al., 2014; Swamy et al., 2016).

The laurel (*Laurus nobilis* L.), an evergreen plant species endemic to Southern Europe and the Mediterranean, is a member of the Lauraceae family. Bay leaves derived from this plant are utilized as a spice in Mediterranean cuisine owing to their aromatic and pleasing scent. Additionally, they have been traditionally employed in medicinal practices for treating diverse infections and find applications in the cosmetics industry (Ramos et al., 2012; Fidan et al., 2019).

The present study was conducted to determine the chemical composition of the EOs of laurel and to evaluate the antibacterial and antibiofilm activities against the fish pathogen *Pseudomonas* species: *P. aeruginosa*, *P. fluorescens*, *and P. putida*.

MATERIAL and METHODS

Plant material

The leaves and fruits of laurel were collected from the trees growing wild in Sinop Region, Turkey (42°02'43.4"N 35°02'27.9"E) during June in 2015. Samples were cleaned and dried in the shade at room temperature.

Extraction of essential oil

About 500 g of dried plant material, comprising leaves and fruits, was crushed and subjected to hydro-distillation using a Clevenger's apparatus. Crushed samples were immersed in water and heated to boiling. The EOs evaporated together with water vapor and passed through the refrigerant before being collected into the condensation flask. Following the separation of the liquid phase, the EOs were collected in a glass vial (Ghalem and Mohamed, 2008; Mazumder et al., 2020).

GC and GC/MS analyses

Analyses were conducted in Eskisehir Anadolu University Medicinal Plants, Drugs and Scientific Research Center (AUBİBAM). Briefly, 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 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. Relative amounts of the characterized components were expressed in percentages, and the retention time (RT) was recorded in minutes (Sevindik et al., 2016).

Microbial Strains

The EOs and their components were tested against fish pathogenic *Pseudomonas* species; *P. aeruginosa* (ATCC 9027), *P. fluorescens* (BC 7324), *and P. putida* (BC 1617). All laboratory stock cultures of the microorganisms were obtained from the Microbiology Laboratory, Department of Food Engineering, Faculty of Agriculture, Atatürk University, Erzurum, Turkey. Bacteria were identified by morphological and biochemical tests, including the assessment of colony morphology, Gram staining, oxidase and catalase activities, indole, citrate utilization, methyl red, gelatin hydrolysis, fermentation of mannitol, sucrose, lactose, fructose, glucose, and urease, and growth at different temperature conditions. Throughout the investigation, bacterial cultures were stored frozen at -86 °C in nutrient broth (NB) containing 20% (v/v) glycerol and utilized as stock cultures.

Determination of antibacterial concentration

To determine the minimum inhibitory concentration (MIC) of LEO, the Broth Dilution Method was utilized (Rath and Priyadarshanee, 2017). This involved mixing varying amounts of the oil with NB, which included a 0.5% dilution of Tween 20, to create a range of concentrations from 7.8-125 µg/ml using a serial dilution approach. The study tested a total of five different concentrations. The experiment involved inoculating 100 µl of overnight culture with approximately $5x10^5$ bacterial cells into wells that contained 100 µl of laurel oil at varying concentrations. The 96-well plate, which contained a total of 200 µl, was then subjected to shaking and incubation for 24 hours at 37 ℃. The point at which bacterial growth was inhibited and no turbidity was observed was recognized as the MIC of the laurel oil against the particular strain of bacteria being tested. Following 24 h incubation, the optical density (OD_{600}) was measured for MIC analysis. In order to calculate the Minimum Bactericidal Concentration (MBC), a 20-µl solution obtained from the final three test wells, which did not exhibit bacterial growth, was inoculated on nutrient agar (NA) plates and incubated overnight at 37 °C.

Antibiofilm effects

Biofilm inhibition assay was tested against *P. aeruginosa* (ATCC 9027), *P. fluorescens* (BC 7324) and *P. putida* (BC 1617) using laurel oil in 96-well culture plates. The bacterial strains were incubated in 10 mL of Tryptic soy broth (TSB) that contained 1% glucose and were maintained at 37 °C for a period of 24 h. This followed by the preparation of dilutions equivalent

to 0.5 McFarland standard value. In each well of plates, 90 µL of growth medium (TSB with 1% glucose), 100 µL of laurel oil (final concentrations of 7.8-125 µg/ml), and 10 µL of test bacterial dilutions were mixed. In the positive controls, 10 µL of the bacterial dilutions were mixed with 190 µL of growth medium, while negative control contained only growth medium. After 24 h-incubation at 37 °C, the 96-well plate was washed with distilled water three times to remove unattached planktonic cells and the remaining adherent sessile cells were dyed with 200 µL of 0.4% crystal violet for 30 min, then the excess dye was poured and the wells were washed three-times with distilled water. The remaining dyed biofilm was suspended in 200 µL of 70% ethanol for 30 min at room temperature. OD of the wells were read at 570 nm by microplate reader (Thermo Scientific Inc., Multiscan GO, Finland) (Bai et al., 2019). Biofilm inhibition was calculated by the following formula.

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

RESULTS AND DISCUSSION

All the strains demonstrated the characteristic phenotypic traits commonly observed in fluorescent pseudomonads, including the production of a water-soluble yellow-green or yellow-brown pigment known as pyoverdine. While all the strains tested in this study were Gram-negative, motile, rod-shaped, oxidase, catalase, citrate, mannitol, fructose, and glucose positive, they were indole, methyl red, sucrose, lactose, and urease negative. *P. aeruginosa* is differentiated from the other strains by its ability to grow at 42 °C and is also recognized by its characteristic colony morphology. *P. fluorescens* and *P. aeruginosa* differ from *P. putida* in their ability to hydrolyze gelatin.

Chemical Composition of the laurel

The GC/MS analysis resulted in the identification of 15 components in LEO, which collectively accounted for 87.04% of the total composition of the analyzed EOs. Analysis of the LEO revealed 1,8-cineole or eucalyptol (%48.43) and α-terpinyl acetate (14.78) as the major compounds with the highest peaks (Figure 1).

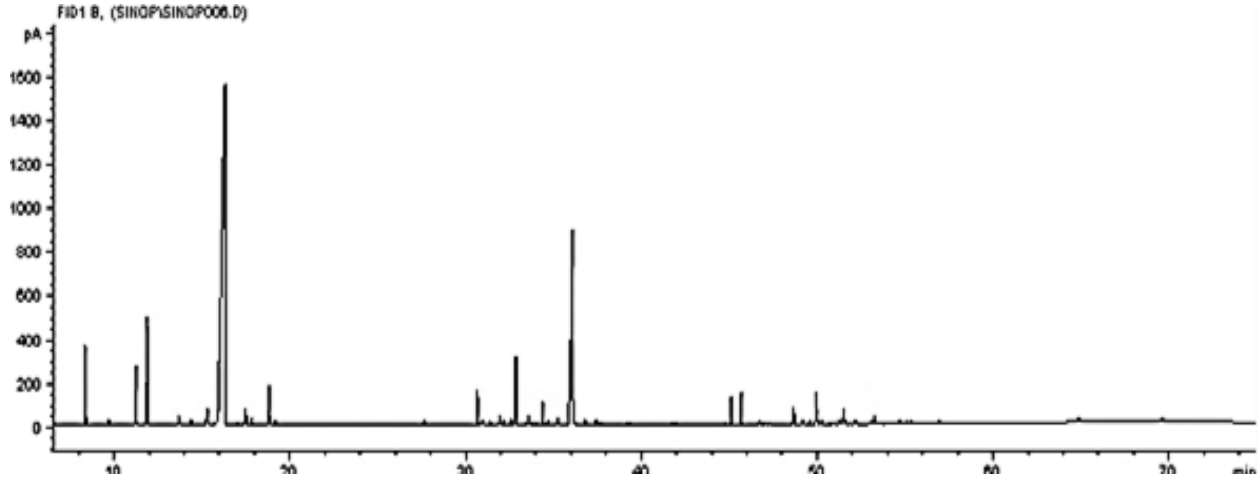

Figure 1. Gas chromatogram of the essential oil from laurel

The retention time and percentage composition of the identified compounds of laurel EOs are presented in Table 1.

Table 1. Essential oil composition of laurel

RT (min)	Component	Quantity (%)
16.35	1,8-cineole	48.43
36.03	α-terpinyl acetate	14.78
11.95	sabinene	5.68
32.84	terpinen-4-ol	2.95
11.31	β-pinen	2.73
8.40	α-pinene	2.59
18.88	p-cymene	1.67
15.36	limonen	1.34
30.63	linalool	1.23
45.10	caryophyllene oxide	1.20
45.63	methyl eugenol	1.18
49.91	eugenol	1.15
36.06	α-terpineol	0.82
48.62	Spathulenol	0.66
51.48	β-eudesmol	0.63
Total		87.04

RT: Retention time

The present study demonstrated that the EO from laurel involved eucalyptol and α-terpinyl acetate as the primary constituents. Similarly, Ramos et al. (2012) reported the major components of bay laurel as eucalyptol (27.2%), α-terpinenyl acetate (10.2%), linalool (8.4%), methyleugenol (5.4%), sabinene (4.0%) and carvacrol (3.2%). Bay laurel EOs were reported to contain 1,8-cineole (60.72%), α -terpinene (12.53%), sabinene (12.12%), and α -pinene (6.11%) as major constituents (Dadalioglu et al., 2004). Taban et al. (2018) reported the main chemical components in EOs obtained with different extraction methods from laurel as eucalyptol (34.4–50.0%), α-terpinenyl acetate (14.9–18.8%), terpinene-4-ol (4.7–6.0%), and sabinene (4.9–5.9%).

While the major components of laurel oil identified in this study were comparable to findings from various other studies, variations in the quantities of these essential oils were observed. These differences are likely influenced by several factors, including diverse seasons, regions, cultivation conditions, stages of maturity, harvesting methods, and extraction techniques (Arumugam et al., 2016; Swamy et al., 2016).

1,8-Cineole, referred to as eucalyptol, is a bicyclic terpenoid present in essential oils derived from diverse plant species and is known to possess antimicrobial properties (Mączka et al., 2021; Moo et al., 2021). 1.8-cineole was reported as the major component in the essential oil of laurel from Morocco (Derwich et al., 2009). Mazumder et al. (2020) reported significant antibacterial activities of 1,8-cineole, obtained from *Eucalyptus maculate* as a major compound, together with the crude EOs, against the fish pathogens *Aeromonas hydrophila and A. jandae*.

In this study, another main component of laurel oil was determined to be α-Terpinyl acetate (α-TA) with a ratio of 14.78%. α-Terpinyl acetate which could be found in some EOs bearing plants, is an organic, volatile monoterpene ester. Vaičiulytė et al. (2021) reported that α-TA EO has high antimicrobial activity against fungi but lower activity against bacteria.

The antibacterial activity of Laurel essential oil

In order to assess the antimicrobial efficacy of LEO against the tested *Pseudomonas* pathogens, the MIC and MBC values were determined. The findings revealed diverse effects of the essential oil on the bacterial strains under investigation (Table 2). Based on their characteristics as bactericides (close to to 1) or bacteriostatics (greater than 4), antibiotics are categorized using the MBC/MIC ratio. Table 2 displays MBC values that were higher than MIC. All of the isolates had an MBC/MIC ratio of 2. The MBC/MIC ratio of LEO was equivalent to 2.0, which clearly suggests that LEO had a bactericidal impact on Pseudomonas species.

Through our investigation, it was discovered that LEO possess diverse anti-pseudomonal properties as evaluated for antibacterial activity. Notably, LEO exhibited greater efficacy against *P. fluorescens* and *P. putida* compared to *P. aeruginosa*, suggesting that *P. aeruginosa* exhibited higher resistance to LEO when compared to the other strains.

Table 2. Antibacterial parameters (MIC and MBC μg/mL) of the essential oil of the *L. nobilis*

Bacteria	MIC	MBC	MBC/MIC
P. aeruginosa	62.5	125	2
P. putida	31.25	6.25	2
P. fluorescens	31.25	62.5	2

Consistent with the findings of this study, Řebíčková et al. (2020) observed limited inhibitory effects of LEO against *P. aeruginosa*, which could potentially be attributed to the notable lipid-degrading capability of *P. aeruginosa* (Ozdal et al., 2017).

Goudjil et al. (2015) reported variable bacteriostatic properties of the LEO against different strains. MIC values for *Salmonella enterica* and *Klebsiella pneumoniae* were reported as 0.2 and 0.11 mg/mL, respectively.

LEO was tested for its antimicrobial activity against four different bacterial species, including Gram-positive bacteria (*S. aureus* and *B. subtilis*) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*). Antibacterial activity against all tested pathogens varied from 1250 to 2250 μg/mL (Santoyo et al., 2006). Gram-positive bacteria tend to exhibit higher susceptibility to EOs compared to Gram-negative bacteria, primarily due to the presence of an additional outer membrane in Gram-negative bacteria, which provides an enhanced protective barrier for the cytoplasmic membrane against antimicrobial substances such as EOs (Caputo et al., 2017). In the investigation conducted by Ertürk (2006), MIC of *L. nobilis* ethanolic extract against the tested bacteria, including *B. subtilis*, *Staphylococcus aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa*, was determined to be 5 mg/mL using agar dilution methods (Ertürk, 2006).

Dadalioğlu et al. (2004) showed that LEO (5-80 μL/mL) exhibited antibacterial activity against *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhimurium*, and *S. aureus*. Unlikely, Jacobo et al. (2022) also reported that the MIC and MBC of LEO was 12.36 mg/mL for *S. aureus*, *E. coli*, and *Enterococcus faecalis*. Snuossi et al. (2016) reported varying antibacterial activities of LEO against *A. hydrophila, Staphylococcus spp., Vibrio alginolyticus, Enterobacter cloacae, K. ornithinolytica, K. oxytoca and Serratia odorifera*. MIC values for the bacteria, have been reported ranging from 0.05 to 0.39 mg/mL.

Antimicrobial effect of an EO is attributed to its chemical makeup, particularly the functional groups present in its main constituents such as alcohols, phenols, and aldehydes, as well as the synergistic interactions among these compounds. Previous investigations have demonstrated the antimicrobial activity of hydrocarbon and oxygenated monoterpenes, including 1,8-cineole, linalool, -terpineol, and terpinen-4-ol (Caputo et al., 2017; Badawy et al., 2019). The -OH groups of bioactive chemicals positioned at the meta and ortho locations are responsible for the antibacterial properties of essential oils. The cytoplasmic membrane of bacterial cells can interact with these -OH groups, causing cell death and disintegration (Shahbazi, 2019).

Antibiofilm Activity of the Essential Oil

Biofilms pose challenges in water treatment systems and particularly impact various sectors of the food industry, such as brewing, dairy processing, fresh produce, poultry processing, and meat processing, as evidenced by studies conducted by Chen et al. (2007) and Lu et al. (2022). Bacteria in biofilms can be up to 1000 times more resistant to antibiotics than their planktonic counterparts. (Simoes et al., 2009).

Pseudomonas is an opportunistic pathogen with a high level of vitality that is widely distributed in the water, air, soil, and food supply (Osman et al., 2019). Species such as *P. aeruginosa*, *P. fluorescens*, and *P. putida* are known capable of forming biofilms (Iseppi et al., 2020).

P. fluorescens and *P. putida* biofilms were found to be suppressed by the anti-biofilm activity of LEO at sub-MIC doses. Our findings show that *P. fluorescens* and *P. putida* are more vulnerable to LEO than *P. aeruginosa*. When LEO at concentrations of 7.81, 15.62, 31.25, 62.5, and 125 μg/mL were used, 0, 0, 0, 28, and 45% of *P. aeruginosa* biofilm formation was inhibited, respectively (Figure 2). Likewise, the same concentrations of LEO prevented 30, 54, 100, 100, and 100% of biofilm formation by *P. fluorescens*. For *P. putida* at the same concentrations, these values were measured as 24, 77, 100, 100 and 100. As seen in Figure 2, the addition of LEO at the MIC level (31.25 μg/mL) considerably reduced the development of *Pseudomonas* species biofilms except for *P. aeruginosa*.

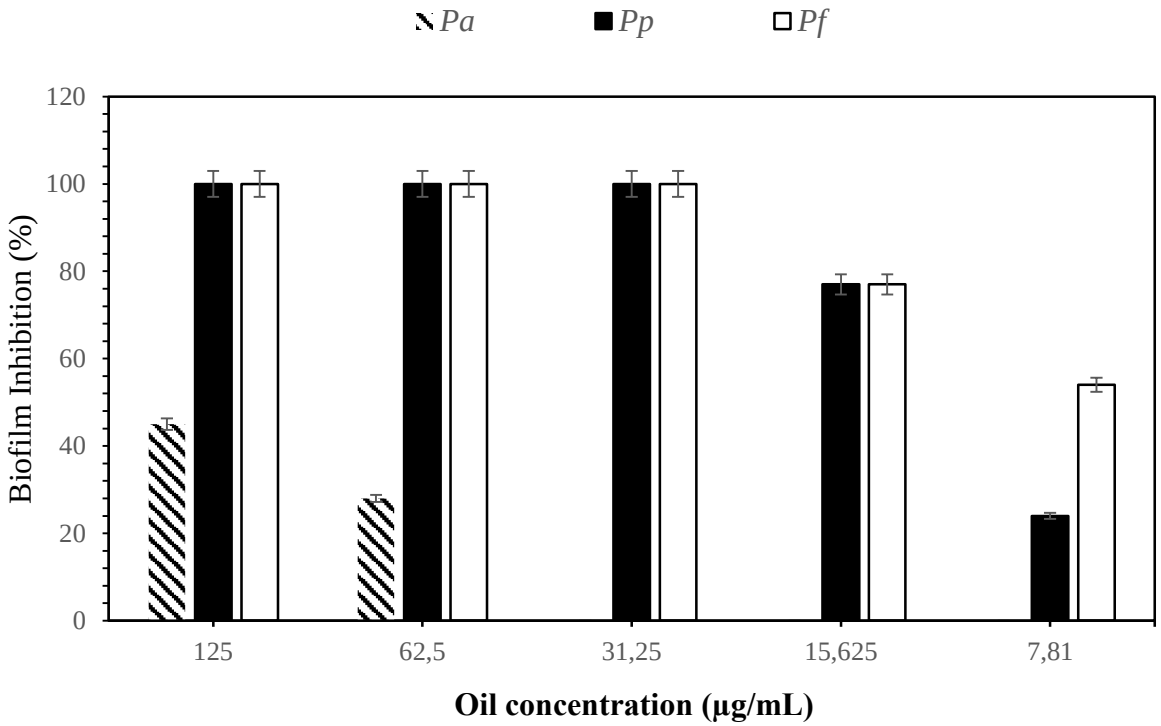

Figure 2. Inhibition of biofilm formation by *P. aeruginosa*, *P. fluorescens*, and *P. putida* using LEO.

The efficacy of LEO in eliminating *S. epidermidis* CIP 444 biofilms was investigated in a prior study, revealing biofilm inhibition rates ranging from 8.5% to 33.75% (Chmit et al., 2014). Merghni et al. (2016) reported that LEO, at a concentration of 1/16 x MIC (0.24 to 1.95 mg/mL), exhibited a biofilm inhibition rate exceeding 70% against *S. aureus* strains. Also, LEO at concentrations starting at 2 MICs inhibited the initial adhesion of *Candida albicans* (Peixoto et al., 2017).

In conclusion, *L. nobilis* EO exhibits antipseudomonal activity and affects the formation of biofilms in *Pseudomonas* species, possibly by interfering with cell wall biosynthesis and membrane ionic permeability, facilitated by the presence of identified monoterpenes and sesquiterpenes.

CONCLUSION

The present study provides evidence that LEO possess both antibacterial and antibiofilm activities against *Pseudomonas* species. Given the emergence of multidrug-resistant strains and the prevalence of biofilm formation, there is a pressing need to identify effective alternatives to combat Pseudomonads, and EOs have emerged as a potential option. Several EOs have been shown to be effective antimicrobials and antibiofilm agents, allowing them to be used in therapeutic formulations either alone or in combination with already established antibiotics. However, further study is necessary to gain a better understanding of the interactions of the biofilm formation phases with the EOs and their components independently. Also, additional acute investigations on volatility and solubility should be conducted in order to boost the antibacterial potential of EOs as a pharmaceutical product.

COMPLIANCE WITH ETHICAL STANDARDS

a) Authors' Contributions

SB & MÖ: Designed the study.

SB & SG: Wrote the first draft of the manuscript.

MÖ, SG & SB: Performed laboratory experiments

(All authors read and approved the final manuscript).

b) 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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