OKU Fen Bilimleri Enstitüsü Dergisi
8(5): 2111-2132, 2025

OKU Journal of The Institute of Science and Technology, 8(5): 2111-2132, 2025

Osmaniye Korkut Ata Üniversitesi Fen Bilimleri Enstitüsü Dergisi

Osmaniye Korkut Ata University Journal of The Institute of Science and Technology

Exploring the Antibacterial Potential of *Laurus nobilis* Essential Oil and the Synergistic Interaction with 1,8-Cineole and Gentamicin Against Methicillin-Resistant *Staphylococcus aureus*

Demet HANÇER AYDEMİR[1*]

[1]Süleyman Demirel University, Isparta Vocational School of Health Services, Medical Services and Techniques Department, 32260, Isparta, Türkiye

[1]https://orcid.org/ 0000-0003-0174-8769
*Corresponding author: demetaydemir@sdu.edu.tr

Research Article

Article History:
Received: 27.12.2024
Accepted: 20.05.2025
Published Online:15.12.2025

Keywords:
Methicillin-resistant
Staphylococcus aureus
Laurus nobilis
1,8-cineole
Antibacterial activity
Synergism
Membrane permeability

ABSTRACT

This study aims to evaluate the *in vitro* antibacterial activity of *Laurus nobilis* essential oil (LnEO) and investigate the synergistic interaction between 1,8-cineole and gentamicin against methicillin-resistant *Staphylococcus aureus* (MRSA). The chemical composition of LnEO was analyzed using gas chromatography-mass spectrometry (GC-MS). Its antibacterial activity was assessed via the disk diffusion method, while the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the broth macro-dilution method. The mechanism of action of LnEO was tested using the cell membrane permeability test. The interaction between 1,8-cineole and gentamicin was evaluated using the checkerboard method. A total of 45 volatile compounds, accounting for 100% of the essential oil composition, were identified, with 1,8-cineole (51.43%) being the predominant component. LnEO showed no effect on MRSA growth at 1:40 and 1:20 dilution ratios. However, inhibition zones of 14.2 mm, 11.8 mm, and 8.7 mm were observed at dilution ratios of 1:1, 1:5, and 1:10, respectively, indicating that MRSA is susceptible to LnEO at these concentrations. The MIC and MBC values were established as 1:4 and 1:2, respectively. In the time-kill assay, LnEO at 2xMIC caused a progressive decrease in bacterial viability, with no viable bacteria detected at 90 min. At 1xMIC, a 2.4 log10 reduction in viable cell count was observed after 6 h. LnEO at 1xMIC and 1/2xMIC concentrations caused loss of membrane integrity, evidenced by increased crystal violet uptake. Furthermore, the combination of gentamicin and 1,8-cineole exhibited a synergistic effect against MRSA (FICI $\leq$ 0.5). These findings suggest that LnEO and its major component, 1,8-cineole, hold potential for developing effective strategies against MRSA infections. The results also indicate that combining synthetic antimicrobials with natural bioactive compounds at lower doses could reduce potential toxicity and mitigate resistance development, offering a promising alternative to high-dose monotherapy.

Laurus nobilis Esansiyel Yağının Antibakteriyel Potansiyelinin ve 1,8-Sineol ile Gentamisin Arasındaki Sinerjik Etkileşimin Metisiline Dirençli *Staphylococcus aureus*'a Karşı İncelenmesi

Araştırma Makalesi

Makale Tarihçesi:
Geliş tarihi: 27.12.2024
Kabul tarihi:20.05.2025
Online Yayınlanma:15.12.2025

Anahtar Kelimeler:

ÖZ

Bu çalışmanın amacı, *Laurus nobilis* esansiyel yağının (LnEO) *in vitro* antibakteriyel aktivitesini değerlendirmek ve metisiline dirençli *Staphylococcus aureus*'a (MRSA) karşı 1,8-sineol ile gentamisin arasındaki sinerjik etkileşimi araştırmaktır. LnEO'nun kimyasal bileşimi gaz kromatografisi-kütle spektrometresi (GC-MS) kullanılarak analiz edildi. Antibakteriyel aktivitesi disk difüzyon yöntemi ile değerlendirilirken,

Metisiline dirençli *Staphylococcus aureus*
Laurus nobilis
1,8-sineol
Antibakteriyal aktivite
Sinerji
Membran geçirgenliği

minimum inhibitör konsantrasyonu (MİK) ve minimum bakterisidal konsantrasyon (MBK) broth makro dilüsyon yöntemi kullanılarak belirlendi. LnEO'un etki meknanizması ise hücre zarı geçirgenliği testi kullanılarak test edildi. 1,8-sineol ile gentamisin arasındaki etkileşim dama tahtası yöntemi kullanılarak değerlendirildi. Esansiyel yağ bileşiminin %100'ünü oluşturan toplam 45 uçucu bileşik tanımlandı ve 1,8-sineol (%51,43) baskın bileşendi. LnEO, 1:40 ve 1:20 seyreltme oranlarında MRSA'nın büyümesi üzerinde hiçbir etki göstermedi. Bununla birlikte, 1:1, 1:5 ve 1:10 seyreltme oranlarında sırasıyla 14,2 mm, 11,8 mm ve 8,7 mm'lik inhibisyon zonları gözlenmesi, MRSA'nın bu konsantrasyonlarda LnEO'ya duyarlı olduğunu göstermektedir. MİK ve MBK değerleri sırasıyla 1:4 ve 1:2 olarak belirlendi. Zaman-öldürme testinde, 2xMİK konsantrasyonunda LnEO, bakteriyel canlılıkta kademeli bir azalmaya yol açtı ve 90. dakikada canlı bakteri tespit edilmedi. 1xMİK konsantrasyonunda ise 6 saat sonunda canlı hücre sayısında 2.4 log10 azalma kaydedildi. LnEO 1xMİK ve 1/2xMİK konsantrasyonda hücre zarının bütünlüğünün kaybolmasına neden oldu; bu, kristal viyole alımının artmasıyla kanıtlandı. Ayrıca, gentamisin ve 1,8-sineol kombinasyonu, MRSA'ya karşı sinerjik bir etki gösterdi (FICI ≤ 0,5). Bu bulgular LnEO ve onun ana bileşeni olan 1,8-sineolün MRSA enfeksiyonlarına karşı etkili stratejiler geliştirme potansiyeli taşıdığını göstermektedir.Sonuçlar ayrıca sentetik antimikrobiyalleri doğal biyoaktif bileşiklerle düşük dozlarda birleştirmenin potansiyel toksisiteyi azaltabileceğini ve direnç gelişimini hafifletebileceğini, yüksek doz monoterapiye umut verici bir alternatif sunabileceğini göstermektedir.

To Cite: Hançer Aydemir D. Exploring the Antibacterial Potential of *Laurus nobilis* Essential Oil and the Synergistic Interaction with 1,8-Cineole and Gentamicin Against Methicillin-Resistant *Staphylococcus aureus*. Osmaniye Korkut Ata Üniversitesi Fen Bilimleri Enstitüsü Dergisi 2025; 8(5): 2111-2132.

1. Introduction

Staphylococcus aureus (*S. aureus*) causes nosocomial and community-associated infections. It causes various human diseases, including skin infections, sepsis, osteomyelitis, pneumonia, bacteremia, and endocarditis (Ibrahim et al., 2023). Since *S. aureus* is very resistant to environmental conditions, it is widespread both in nature and in the throat and nasal cavities of humans, acne, and abscessed wounds (Gulbandilar et al., 2012). It is spread by direct contact with an infected person, handling a contaminated object or consuming food, and inhaling infected droplets dispersed through sneezing or coughing. In particular, food contamination with enterotoxin-producing *S. aureus* causes staphylococcal food poisoning (Çakıcı et al., 2015). With the therapeutic use of penicillin antibiotics in 1941, the number of cases decreased. Still, after a while, the emergence of beta-lactamase-producing strains led to the emergence of penicillin-resistant *S. aureus* bacteria. In this case, methicillin antibiotics started to be used in the treatment, but methicillin-resistant *S. aureus* (MRSA) strains emerged after a while. Later, vancomycin antibiotics were used in the treatment, but this time vancomycin-resistant *S. aureus* (VRSA) strains came to the fore. The formation of the resistance observed in *S. aureus* against existing antibiotics has made the treatment difficult, and this has caused the search for new methods and antimicrobial agents in the treatment (Culos et al., 2013). However, interest in essential oils has increased in the last years due to the antimicrobial and antibiofilm effects of bioactive components in essential oils (Sharifi-Rad et al., 2017; Maniki et al., 2023). It is thought that they do not have toxic effects due to their natural structure, and the development of bacterial resistance to chemical substances found in essential oils becomes difficult (Al-Jabri and Hossain, 2014).

Essential oils obtained from medicinal plants have been known to have biological activity since ancient times (Baratta et al., 1998; Bounatirou et al., 2007). Essential oils contain terpenoids, phenolic compounds, aliphatic hydrocarbons, aldehydes, acids, acyclic esters, alcohol, and lactones (Rota et al., 2004). The antimicrobial effects of these oils are primarily attributed to their chemical composition and the specific functional groups present (Celiktas et al., 2007; Omidbeygi et al., 2007). Essential oils work by sensitizing the bacterial cell membrane, increasing the permeability of intracellular components, and disrupting bacterial enzyme systems (Singh et al., 2002). In this regard, *Laurus nobilis* are considered to have significant antibacterial potential (Chouhan et al., 2017). *Laurus nobilis* (*L. nobilis*) grows in many warm regions of the world and its leaves are used in a variety of food applications (Jirovetz et al., 1997; Barla et al., 2007). Studies have determined that the main component of *Laurus nobilis* essential oil (LnEO) is 1,8-cineole (Marzouki et al., 2009; Aydın, 2019). This compound is also known as eucalyptol, is found in other plants such as *Rosmarinus officinalis* and *Eucalyptus globulus*. Additionally, 1,8-cineole is an agent used in the treatment of gastrointestinal and respiratory diseases (Saraiva et al., 2021).

The increase in antibiotic resistance in recent years has led to the need for the discovery of new plant-derived drugs to combat infections, against which bacteria cannot develop resistance. Essential oils and their components have shown potential to be used as adjuvants in antimicrobial drugs to prevent the development of antibiotic resistance in pathogenic strains (Chouhan et al., 2017). The synergistic interaction between essential oils or their components and antimicrobial agents appears to be a promising alternative strategy for combating antimicrobial resistance. Some studies have shown that the synergy between essential oils and antibiotics increases their effectiveness against antibiotic-resistant bacteria (Yang et al., 2017; Owen and Laird, 2018). For instance, aminoglycosides such as gentamicin are often used in combination with beta-lactams to treat severe infections caused by both Gram-negative and Gram-positive bacteria (Paul et al., 2014). However, there is limited literature on the interaction between 1,8-cineole and gentamicin. The aim of this study is to determine the antibacterial activity of *Laurus nobilis* essential oil (LnEO) on MRSA and to explore the interaction between its main component, 1,8-cineole, and gentamicin to develop new and more effective treatment options.

2. Material and Methods

2.1. Essential Oil

LnEO was commercially purchased from the pharmacy. The oil was obtained from the *L. nobilis* leaves by steam distillation method. LnEO was diluted with 10% dimethyl sulfoxide (DMSO), passed through a 0.45 μm millipore filter, transferred to dark-colored, sterile, and aluminum foil-coated dark-colored bottles, and stored at +4 °C until use.

2.2. Test Microorganism

Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300 reference strain was used in the study. MRSA was cultured by passage from stock culture to freshly prepared Nutrient Agar (NA) medium under sterile conditions using a loop. The petri dishes with bacterial inoculation were incubated in a 37 °C incubator for 18-24 h.

2.3. Chemicals

Gentamicin (10µg; Bioanalyse) antibiotic disc was used as reference material in the disk diffusion method. In the combination study, 1,8-cineole (purity ≥98.0%; Sigma-Aldrich; CAS 470-82-6) was used.

2.4. Gas Chromatography-Mass Spectrometry (GC-MS) *Analysis*

LnEO was analyzed with the Shimadzu QP 5050 (Kyoto, Japan) brand GC-MS system. Varian CP WAX 52 CB capillary column as separation column (50 m x 0.32 mm ID, d_f: 1.2 µm) was used. Helium (99.999%) was used as carrier gas with a constant column inlet pressure of 10 psi (1 psi = 6894.76 Pa). The sample volume injected was 1 µL. The GC furnace was programmed as follows: The initial column temperature was set to 60 °C, after this temperature was kept constant for 1 min, it was increased to 220 °C with a temperature increase of 2 °C/min and kept constant for 20 min at the final temperature of 220 °C. Compound identification was performed by comparing the mass spectra and linear retention indices (LRI) of the compounds with published data from reputable sources, including those from the Wiley and NIST mass spectral libraries. Reference compounds for each identified peak were confirmed using commercial standards, and retention indices (RI) were compared to those available in the literature for further confirmation. The ion source temperature and injection block temperatures were set at 250 and 280 °C, respectively. The emission current of the ionization filament was set to 70 eV.

2.5. Disk Diffusion Method

The *in vitro* antibacterial activity of LnEO was determined by the disk diffusion method, according to the Clinical Laboratory Standards Institute (CLSI, 2012a) standards. Concentrations of the essential oil were prepared as 1/1, 1/5, 1/10, 1/20, and 1/40 (v/v) in 10% DMSO. MRSA was incubated in Mueller Hinton broth (MHB) medium at 37 °C for 18-24 h and the turbidity of the bacterial suspension was adjusted to 0.5 McFarland ($1.0x10^8$ CFU/mL). According to the spread plate method, 100 µL of the bacterial suspension was taken and planted on Mueller Hinton agar (MHA) medium and left to dry for 5-10 min. Sterile commercial blank discs (6 mm in diameter) were impregnated with 20 µL of prepared essential oil samples. The DMSO-impregnated disc was served as a negative control, and a gentamicin antibiotic disc (10 µg) was used as a positive control. The discs were transferred on a solidified agar medium using sterile forceps. The media were incubated for 24 h at 35 °C. After incubation, the diameter of the zone of inhibition around the disc was measured in millimeters. The test was repeated three times

and the mean values were determined as the result value. Based on the width of the inhibition zone diameter, expressed in mm, the results were evaluated as follows: insensitive for diameters less than 8 mm (-), sensitive (+) for diameters 9 to 14 mm, very sensitive for diameters 15 to 19 mm (++) and has been interpreted as being extremely sensitive (+++) for diameter greater than or equal to 20 mm (Moreira et al., 2005).

2.6. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Various approaches exist for assessing antimicrobial activity. One of the most widely used methods is the microbroth dilution technique, which is standardized by the CLSI. This method yields important parameters, including the MIC and MBC of the antimicrobial agent being tested (Buldain et al., 2021). In this study, the broth macro-dilution method was chosen for MIC determination due to its suitability for larger test volumes, ease of application in manual settings, and its inclusion among the validated methods described by CLSI (CLSI, 2012b). The bacterial suspension was incubated for 18-24 h at 37 °C in Mueller Hinton broth (MHB) medium and its turbidity was adjusted to 0.5 McFarland ($1.0x10^8$ CFU/mL). The final two-fold serial dilutions of LnEO were prepared in the concentration range of 1:1-1:128 (v/v). 100 µL of the inoculum was inoculated into the test medium to achieve the final test concentration of $5.0x10^5$ CFU/mL. Positive control (bacteria+medium) and negative controls (medium alone and essential oil only) were also established. Following the overnight incubation at 37 °C, the lowest concentration of essential oil that inhibited the visible growth of bacteria was recorded as the MIC value. On the other hand, MBC was determined as the lowest concentration of LnEO needed to kill MRSA. To determine the MBC value, 100 µL of samples were taken from wells with no bacterial growth and spread onto MHA media and incubated at 37 °C for 24 h. At the end of the incubation, the concentration of essential oil in the Petri dish without bacterial growth was recorded as the MBC value (Yilmaz, 2012). Each experiment was repeated three times.

2.7. Time-kill Assay

MIC and MBC parameters provide static information. The time-kill test defines the antimicrobial activity of a compound dynamically and allows for the analysis of bacterial behavior during the exposure period in the presence of the antimicrobial agent (Buldain et al., 2021). A concentration equal to the 1xMIC and MBC=2xMIC of LnEO were chosen as the oil concentration to be used in the study. Essential oil and 0.5 McFarland suspension were transferred to 2 mL of Mueller-Hinton medium to get a final inoculum of $5.0x10^5$ CFU/mL. In addition, a tube containing the medium and bacteria ($5.0x10^5$ CFU/mL) without essential oil, was also prepared to be used as a growth control. The tubes were incubated at 35 °C at agitation speed of 250 rpm. 100 µL were taken from the tubes at certain interaction times (0., 10., 30., 60., 90., 120., 180., 240. and 360. min) and 1/10, 1/100, 1/1000 dilutions were made in physiological saline. 10 µL and 100 µL of each of these dilutions were taken and they were spread plated on the commercially purchased 5% sheep blood agar. The plates were incubated at 35 °C for 18-

24 h. At the end of the incubation period, the number of viable bacteria (CFU/mL) for each time point was determined by counting the colonies grown on the plates. The experiment was conducted three times. The arithmetic mean values of viable bacterial counts at each time point were calculated and converted into log10 values. The same calculations were applied to the control group. The time-kill curve of LnEO against MRSA was plotted using the logarithmic values of the arithmetic means over time. Antibacterial activity of LnEO against the bacteria was found at each interaction time from the relation as given below (Li et al., 2009).

$$\frac{(N_0 - N_1)}{N_0} \times 100 \quad (1)$$

In this relation, N_0; refers to the number of viable bacteria in the essential oil-free control tube, and N_1; refers to the number of viable bacteria in the test series containing essential oil.

2.8. Determination of Cell Membrane Permeability Using Crystal Violet Assay

The permeability of the cell membrane was illustrated through crystal violet (CV) staining assay (Topçu and Şeker, 2022). Fresh bacterial cultures were adjusted to 0.5 McFarland turbidity and inoculated to 2% (v/v) nutrient broth, followed by incubation at 30 °C overnight. After incubation, the cultures were centrifuged at 4000 rpm for 20 min to remove the supernatant. The cells were then treated with 1xMIC, 1/2xMIC, and 1/4xMIC concentrations of LnEO and incubated overnight at 30 °C. Cell suspension that was not treated with LnEO in physiological saline served as the negative control. Subsequently, the cultures were centrifuged for 5 min at 5000 rpm. Pellets obtained were resuspended in phosphate-buffered saline containing 10 µg/mL CV and incubated at 30 °C for 20 min. After incubation, the samples were centrifuged again at 5000 rpm for 15 min, and the optical density (OD) of the supernatant was recorded at a wavelength of 590 nm. The experiment was repeated three times. The percentage of CV uptake results were calculated using the following equation:

$$\text{CV uptake} = 100 - [(\text{OD sample} / \text{OD control}) \times 100] \quad (2)$$

2.9. Combination study: Checkerboard Method

The efficacy of the combination of 1,8-cineole and gentamicin for MRSA strain was tested by checkerboard method using a 96-well and U-bottom sterile microplate. Firstly, the MIC values of gentamicin and 1,8-cineole were determined. According to the recommendations of CLSI, a stock solution of gentamicin (32 µg/mL) was prepared using sterile distilled water and serial dilutions were prepared two-fold at a concentration range of 16 - 0.03125 µg/mL. Likewise, a stock solution of 1,8-cineole was prepared in 10% dimethyl sulfoxide (DMSO) as 128 mg/mL and two-fold serial dilutions were prepared in the concentration range of 64 - 0.125 mg/mL (CLSI, 2012b). 1,8-cineole was diluted from 2-3 dilutions above the MIC value to 4-5 dilutions below the MIC value in the vertical plane

(column 1) from bottom to top, and gentamicin was diluted from 2-3 dilutions above the MIC value to 4-5 dilutions below the MIC value in the horizontal plane (row A) from right to left. The MIC values were checked using the first horizontal row (row A) for gentamicin and the first vertical row (column 1) for 1,8-cineole. The double dilutions of gentamicin were distributed starting from column 9 to column 2 of the microdilution plate. The double dilutions of 1,8-cineole were transferred to rows B and H of the microdilution plate, with each dilution being placed in an entire row. Thus, different combinations of both antibacterial agents were obtained in each well. A positive control well without antibiotics and a negative control well without bacteria were prepared on the plate. 10 µL of bacterial suspension was pipetted into the wells at a final bacterial inoculum concentration of $5.0x10^5$ CFU/mL and the plates were visually evaluated after 18 h of incubation at 35 ± 2 °C (Table 1). The checkerboard test was repeated twice.

Table 1. Final combination dilutions of gentamicin and 1,8-cineole in the panel to be tested for combination effect*

	1	2	3	4	5	6	7	8	9	10
A	PC	G 0.125	G 0.25	G 0.5	G 1	G 2	G 4	G 8	G 16	
B	C$2.5x10^2$	G 0.125 C$2.5x10^2$	G 0.25 C $2.5x10^2$	G 0.5 C $2.5x10^2$	G 1 C $2.5x10^2$	G 2 C $2.5x10^2$	G 4 C $2.5x10^2$	G 8 C$2.5x10^2$	G 16 C$2.5x10^2$	
C	C $5x10^2$	G 0.125 C $5x10^2$	G 0.25 C $5x10^2$	G 0.5 C $5x10^2$	G 1 C $5x10^2$	G 2 C $5x10^2$	G 4 C $5x10^2$	G 8 C $5x10^2$	G 16 C $5x10^2$	
D	C $1x10^3$	G 0.125 C $1x10^3$	G 0.25 C $1x10^3$	G 0.5 C $1x10^3$	G 1 C $1x10^3$	G 2 C $1x10^3$	G 4 C $1x10^3$	G 8 C $1x10^3$	G 16 C $1x10^3$	
E	C $2x10^3$	G 0.125 C $2x10^3$	G 0.25 C $2x10^3$	G 0.5 C $2x10^3$	G 1 C $2x10^3$	G 2 C $2x10^3$	G 4 C $2x10^3$	G 8 C $2x10^3$	G 16 C $2x10^3$	
F	C $4x10^3$	G 0.125 C $4x10^3$	G 0.25 C $4x10^3$	G 0.5 C $4x10^3$	G 1 C $4x10^3$	G 2 C $4x10^3$	G 4 C $4x10^3$	G 8 C $4x10^3$	G 16 C $4x10^3$	
G	C $8x10^3$	G 0.125 C $8x10^3$	G 0.25 C $8x10^3$	G 0.5 C $8x10^3$	G 1 C $8x10^3$	G 2 C $8x10^3$	G 4 C $8x10^3$	G 8 C $8x10^3$	G 16 C $8x10^3$	
H	C $16x10^3$	G 0.125 C $16x10^3$	G 0.25 C $16x10^3$	G 0.5 C $16x10^3$	G 1 C $16x10^3$	G 2 C $16x10^3$	G 4 C $16x10^3$	G 8 C $16x10^3$	G 16 C $16x10^3$	NC

* PC: Positive control, NC: Negative control, G: Gentamicin, C: 1,8-cineole. The numbers represent gentamicin and 1,8-cineole concentrations in µg/mL. White colored wells have no bacterial growth, black wells have bacterial growth. ■: wells with bacterial growth, □: wells without bacterial growth.

Discordant results were retested twice, and samples from wells showing no growth were incubated on 5% sheep blood agar. The results were confirmed after verifying the absence of bacterial growth. The fractional inhibitory concentration (FIC) was obtained by proportioning the MIC values obtained from the combination of gentamicin and 1,8-cineole to the MIC values obtained individually. Then, the FIC values of the substances in the combination were summed and the FIC index (FICI) was calculated. The interpretation of the calculated FICI values was made according to the limit values: FICI ≤ 0.5 synergistic, 0.5 < FICI < 1 partially synergistic, FICI = 1 additive, 1 < FICI ≤ 4 indifferent and FICI > 4 antagonistic (Bonapace et al., 2002).

Statistics

Data were presented as the arithmetic mean ± standard deviation (SD) and analyzed using SPSS 16.0 software with one-way ANOVA. $p < 0.05$ was considered statistically significant.

3. Results

3.1. GC-MS Analysis

GC-MS identification, mass percent ratios, retention times and peak area contributions (%) of the compounds present in LnEO were given in Table 2. The peak numbers were also shown in the chromatogram in Figure 1. A total of 45 components have been determined, constituting 100% of LnEO. It was shown that the major components detected in LnEO were 1,8-cineole (51.43%), beta-terpinyl acetate (10.75%), sabinene (9.54%), alpha-pinene (6.34%), beta-pinene (4.93%), 4-terpineol (3.38%), p-cymene (2.83%), beta-fenchyl alcohol (2.03%), linalool (1.43%), respectively.

Table 2. Chemical profile of *L. nobilis* essential oil.

Peak number	Retention time	Component	% Ratio	Area
1	6.345	alpha-thujene	0.46	1186930
2	6.611	alpha-pinene	6.34	16372935
3	6.934	2,4(10)-thujadien	0.06	145512
4	7.171	campus	0.29	758612
5	8.028	Sabinene	9.54	24649970
6	8.222	beta-pinene	4.93	12732020
7	8.646	beta-myrcene	0.71	1825037
8	8.710	2,3-dehydro-1,8 cineole	0.27	704514
9	9.386	Alpha-phellandrene	0.14	367809
10	9.510	delta 3-carene	0.07	181839
11	9.880	alpha-terpinene	0.33	860992
12	10.252	p-cymene	2.83	7323006
13	10.779	1,8-cineole	51.43	132922302
14	11.303	Ocimene <(E)-, beta >	0.07	179767
15	11.890	gamma-terpinene	0.50	1293456
16	12.545	trans-sabinene hydrate	0.25	645934
17	13.336	alpha-terpinolene	0.16	419301
18	14.197	linalool	1.43	3696053
19	15.423	carveol	0.04	115747
20	15.566	p-menth-2-en-1-ol	0.13	344850
21	16.495	trans -pinocarveolus	0.05	127243
22	16.632	alloocimene	0.13	339955
23	17.751	pinocarvone	0.07	177067
24	18.321	alpha-terpineol	0.39	1013291
25	18.978	4-terpineol	3.38	8741208
26	19.916	beta -fenchyl alcohol	2.03	5237416
27	25.647	bornyl acetate	0.16	422613
28	26.001	myrtenyl acetate	0.05	130637
29	26.375	(-)-trans-pinocarvyl acetate	0.08	213262
30	27.566	Pseudolimonene	0.73	1892092
31	29.224	Limonene oxide	0.17	443955

Peak number	Retention time	Component	% Ratio	Area
32	29.858	beta -terpinyl acetate	10.75	27788103
33	30.073	Eugenol	0.26	675867
34	30.662	neryl acetate	0.10	246004
35	31.959	alpha-bourbonene	0.02	56009
36	32.443	beta-element	0.20	528007
37	33.292	Methyleugenol	0.73	1883736
38	34.216	Trans-caryophyllene	0.21	547444
39	38.561	Selinene <beta->	0.06	149114
40	40.145	Cadinene <gamma>	0.05	131927
41	40.534	delta-cadinene	0.02	61076
42	41.974	Cis-alpha-bisabolene	0.04	103895
43	43.954	Spathulenol	0.04	102345
44	44.188	(-)-caryophyllene oxide	0.21	555482
45	48.500	Elemol <alpha->	0.05	136164
			=100.00	258430498

According to the chromatogram, compounds with higher peaks indicated high values in the essential oil (Figure 1). The highest peak value seen in the chromatogram belongs to the 1,8-Cineole at the peak of 13.

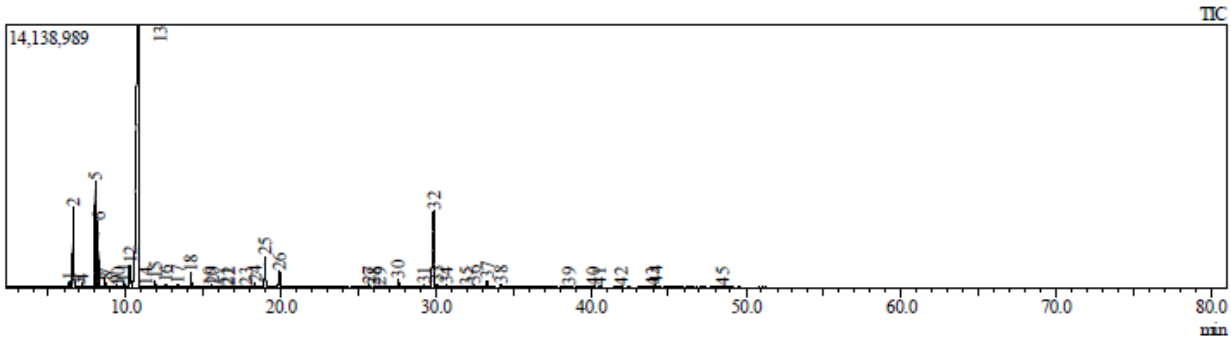

Figure 1. Chromatogram of the *L. nobilis* essential oil by GC-MS. In the chromatogram, the X-axis represents the time taken for the components to pass through the column and reach the mass spectrometer detector. The peaks correspond to the time at which each component reaches the detector, while the Y-axis, or peak area, reflects the amount of each respective component.

3.2. Evaluation of the Antibacterial Activity

In this study, *in vitro* antibacterial efficacy of LnEO was tested against MRSA strain by disk diffusion method. The results are given in Figure 2. The inhibition zone diameter of the gentamicin antibiotic disc was measured as 24.6±0.5 mm. 10% DMSO which was used to dilute LnEO did not show an inhibitory effect on MRSA. To better determine the antibacterial effect, LnEO was diluted at the ratios of 1/1, 1/5, 1/10, 1/20, and 1/40 (v/v). LnEO showed no inhibitory effect on the growth of MRSA at dilution ratios of 1:40 and 1:20. LnEO formed 14.2±0.5 mm, 11.8±0.6 mm, and 8.7±0.7 mm inhibition zones at dilution ratios of 1/1, 1/5, and 1/10, respectively. It was determined that there was a statistically significant difference ($p < 0.05$) between the results obtained at the 1/1, 1/5, and 1/10 dilutions of LnEO and DMSO, but no significant difference was observed between the results obtained at the 1/20 and 1/40 dilutions ($p > 0,5$).

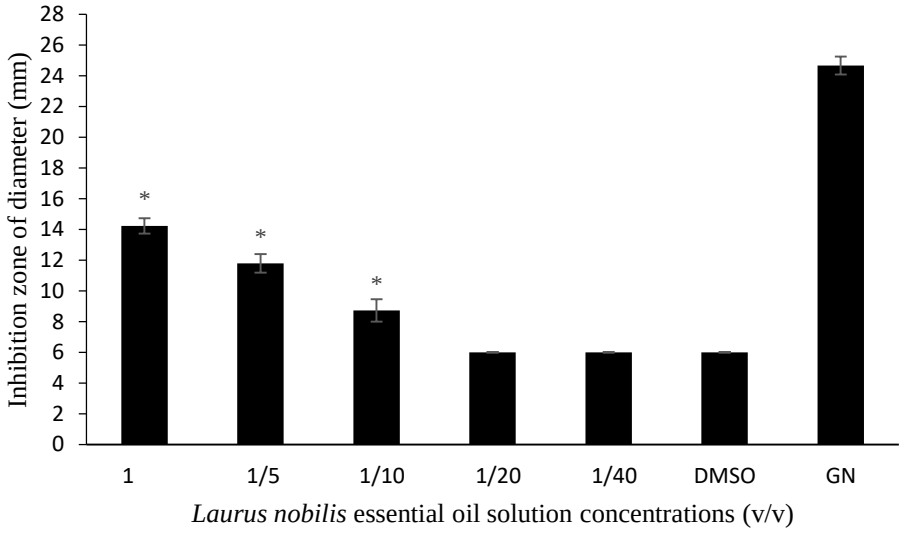

Figure 2. Antibacterial activity of the *L. nobilis* essential oil at different concentrations on MRSA. GN, Gentamicin (10 µg) antibiotic disc was used as the positive control and DMSO was used as the negative control. Zones of inhibition also include disc diameter (6 mm). The data are expressed as means ± SD (n=3). *Indicates a statistically significant difference between the results obtained with LnEO dilutions and those obtained with DMSO ($p < 0.05$).

3.3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC test results of LnEO against MRSA are shown in Table 3. The MIC value was determined as 1:4 (v/v).

Table 3. MIC test results of the *L. nobilis* essential oil against MRSA*

	Dilution rates (v/v)										
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	PC	NC1	NC2
MRSA	-	-	-	+	+	+	+	+	+	-	-

*+, there is growth; -, no growth; PC, positive control (bacteria+medium); NC1, negative control1 (medium alone); NC2, negative control2 (essential oil only).

MBC test results of LnEO against MRSA are presented in Table 4.

Table 4. MBC test results of the *L. nobilis* essential oil against MRSA*

	Dilution rates (v/v)					
	1:1	1:2	1:4	PC	NC1	NC2
MRSA	-	-	+	+++	-	-

*-, no growth (bactericidal); +, low growth; ++, moderate growth (bacteriostatic); +++, high growth (no antibacterial potential); PC, positive control (bacteria+medium); NC1, negative control1 (medium alone), NC2: negative control2 (essential oil only).

One hundred microliters (100 µL) were taken from the dilution tubes (1:1, 1:2, and 1:4) that showed no visible growth in MIC test and were spread onto MHA plates. After overnight incubation at 37 °C, the MBC value, defined as the essential oil concentration in Petri dishes with no bacterial growth, was determined to be 1:2 (v/v).

3.4. Time-kill Kinetics of LnEO

The time-kill curves of LnEO are presented in Figure 3. In the control series, bacterial counts increased as the incubation time progressed. The initial bacterial population was $3.3x10^5$ CFU/mL, which gradually increased throughout the incubation period, reaching $9.2x10^6$ CFU/mL after 6 h.

In the presence of LnEO at a concentration of 2xMIC, bacterial viability progressively decreased at the 10th, 30th, and 60th min. However, no viable bacteria were detected after the 90th min. It was observed that MRSA count decreased to 4.2 log10 by the 10th min, dropped to below 1.7 log10 by the 30th min, and no viable bacteria were detected at the 90th min. When MRSA was exposed to LnEO at a 1xMIC concentration, the initial population ($3.0x10^5$ CFU/mL) decreased gradually during incubation, reaching a reduction of $1.3x10^3$ CFU/mL (2.4 log10 reduction) after 6 h.

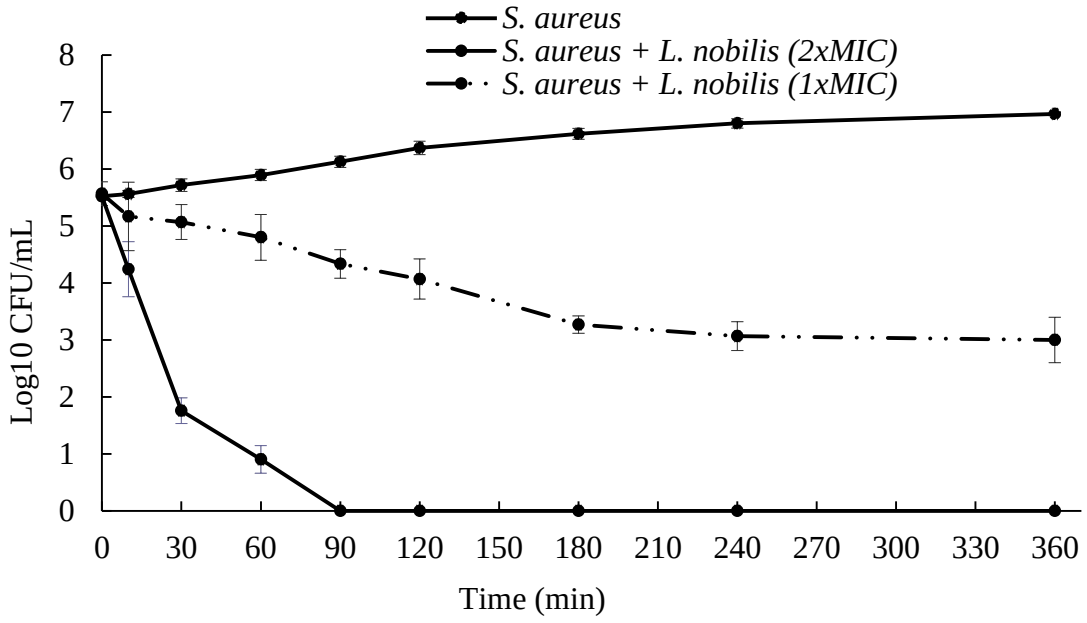

Figure 3. Time-kill curve of *L. nobilis* essential oil (1xMIC, 2xMIC) against MRSA at 6 h incubation time. Each point represents log of the mean ± SD CFU per milliliter (n=3).

The antibacterial activity values of LnEO were calculated using the formula specified in the methods section. These calculations were based on the arithmetic mean values of viable bacterial counts at each incubation period in which MRSA treated with LnEO, as well as the arithmetic mean values obtained from the control experiments. The values found are plotted against time and shown in Figure 4. It was determined that the antibacterial activity of LnEO (2xMIC) against MRSA reached 68.8% at the 30th min, 84.4% at the 60th min (≥ 4 log10 drop in CFU/mL), and 100% at the end of the 90th min. On the other hand, after a 6 h exposure, the antibacterial activity of LnEO at 1xMIC concentration reached 45.1%.

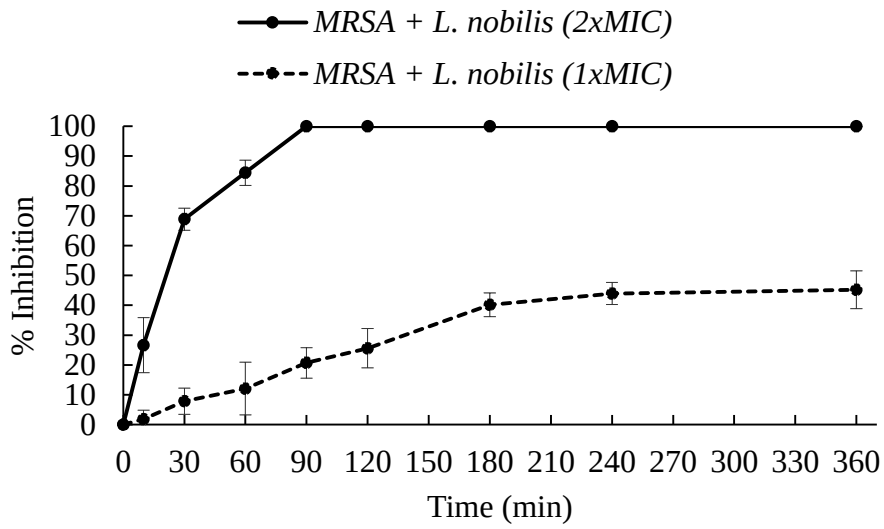

Figure 4. Antibacterial activity of the *L. nobilis* essential oil against MRSA. The data are expressed as means ± SD (n = 3).

3.5. Determination of Cell Membrane Permeability Using Crystal Violet Assay

Membrane permeability was determined using CV uptake assay. CV uptake was 21.8% in the absence of LnEO, while it increased to 65.3% and 38.7% with treatment at 1xMIC and 1/2xMIC concentrations of LnEO, repectively ($p < 0.05$) (Figure 5).

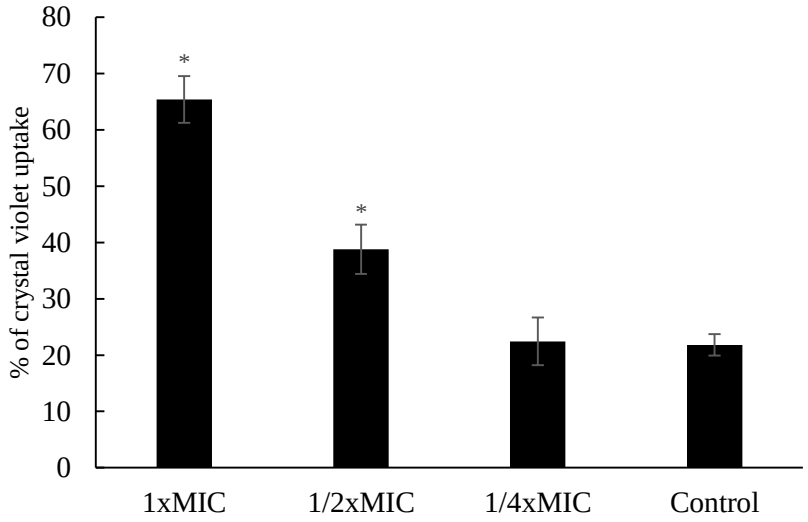

Figure 5. Crystal violet uptake ratio (%). The ability of MRSA to take up crystal violet was evaluated after exposure to LnEO at 1×MIC, 1/2xMIC and 1/4×MIC concentrations. The data are expressed as means ± SD (n = 3). *P < 0.05 compared with the control.

Although CV uptake with 1/4xMIC LnEO treatments was much weaker than with 1xMIC LnEO treatment, a slight increase was observed compared to the control group ($p > 0.05$).

3.6. Interaction of 1,8-cineole with Gentamicin

Firstly, MIC values of 1,8-cineole and gentamicin alone on MRSA were determined. The concentration range of 16 – 0.03125 μg/mL for gentamicin and 64 - 0.125 mg/mL for 1,8-cineole were used to

determine the MIC values. In column 1 for 1,8-cineole and row A for gentamicin, the concentration in the first well without growth was read as the MIC value. The MIC values of gentamicin and 1,8-cineole on MRSA were 4 μg/mL and 4 mg/mL, respectively (Table 5).

Table 5. MIC values of gentamicin and 1,8-cineole against MRSA*

	Dilution rates												
Gentamicin	16	8	4	2	1	0.5	0.25	0.125	0.0625	0.03125	PC	NC1	NC2
(μg/mL)	-	-	-	+	+	+	+	+	+	+	+	-	-
1,8-cineole	64	32	16	8	4	2	1	0.5	0.25	0.125	PC	NC1	NC2
(mg/mL)	-	-	-	-	-	+	+	+	+	+	+	-	-

*+, there is growth; -, no growth; PC, positive control (bacteria+medium); NC1, negative control1 (medium alone); NC2, negative control2 (essential oil only).

The wells containing 1,8-cineole and gentamicin combinations were then evaluated for growth. Wells with the lowest drug concentration without growth in all rows and columns (E2, D3, C4, B5, and B6) were used to calculate the FICI value (Table 1). A synergistic effect was determined between 1,8-cineole and gentamicin against MRSA (FICI=0.25).

4. Discussion

In previous studies on *Laurus nobilis*, the monoterpene 1,8-cineole has been widely identified as the dominant compound in its essential oil (Marzouki et al., 2009). Similarly, in this study, 1,8-cineole (51.43%) was identified as the major active component in the composition of LnEO. Other notable compounds included beta-terpinyl acetate (10.75%), sabinene (9.54%), alpha-pinene (6.34%), beta-pinene (4.93%), 4-terpineol (3.38%), p-cymene (2.83%), beta-fenchyl alcohol (2.03%), and linalool (1.43%). Earlier study indicates that 1,8-cineole is generally expected to be present in LnEO at concentrations ranging from 45% to 60% (Aydın, 2019), and the amount of 1,8-cineole detected in this study falls within this expected range.

Upon reviewing studies conducted by other researchers on the major components of LnEO, the following findings were obtained. Previous study conducted in France, the major components of LnEO were found to be 1,8-cineole (39.1%), sabinene (4.4%), alpha-pinene (2.2%), and beta-pinene (1.7%) (Fiorini et al., 1997). Another study reported that the primary component of LnEO was 1,8-cineole (51.73%-68.48%), with significant amounts of alpha-terpinyl acetate (4.04-9.87%), sabinene (4.44-7.75%), alpha-pinene (2.93%-4.89%), beta-pinene (2.58-3.91%), terpinene-4-ol (1.33-3.24%) (Özcan and Chalchat, 2005). A study conducted in Northern Cyprus also identified 1,8-cineole (58.59%), terpinene-4-ol (4.25%), alpha-pinene (3.39%-3.2%), and sabinene (3.32%) as the major components (Yalçın et al., 2007). In research conducted in Iran, the dominant componentswere 1,8-cineole (55.8%), alpha-terpinyl acetate (15.14%), sabinene (3.42%), terpinene-4-ol (5.27%), and alpha-pinene (5.26%) (Verdinan-rizi, 2009). Pala et al. (2011) reported that 1,8-cineole (27.14-46.37%), linalool (4.27-8.60%), alpha-pinene (0.79-2.99%), beta-pinene (0.90-1.86%), and eugenol (0.66-4.90%) were the major

components of LnEO. These findings are largely consistent with the results of our study, with the main variation observed in the concentration of these compounds. This variability is likely influenced by factors such as geographical conditions, harvesting period, and distillation technique, all of which can affect the chemical composition and yield of the oil (Panizzi et al., 1993; Figueiredo et al., 2008). When comparing our results with studies conducted both in our country and internationally, it was observed that 1,8-cineole is the primary component of LnEO.

In this study, LnEO exhibited no antibacterial activity against MRSA at 1/40 and 1/20 dilutions, while inhibition zones of 14.2 mm, 11.8 mm, and 8.7 mm were observed at 1/1, 1/5, and 1/10 dilutions, respectively. It was noted that the inhibition zone diameters decreased proportionally with the reduction in LnEO concentration. Based on the classification of antimicrobial activity of essential oils according to their inhibition diameters (Moreira et al., 2005), the study concluded that *S. aureus* ATCC 25923 was susceptible to LnEO at dilution ratios of 1:1, 1:5, and 1:10. The inhibition zone of pure LnEO against *S. aureus* ATCC 25923 was reported to be 15 mm. However, zones of inhibition were reported to be 9.1 mm, 8.3 mm, and 7.4 mm at 1/2, 1/4, and 1/8 dilutions, respectively (Ouibrahim et al., 2013). Similarly, pure LnEO formed an inhibition zone of 18.75 mm on *S. aureus*, and when diluted with DMSO at a 1/2 and 1/4 ratios, the inhibition zone diameters were 23.35 mm and 9.65 mm, respectively. Therefore, they reported that *S. aureus* is very sensitive to LnEO at these concentrations (Bennadja et al., 2013). These findings align with the results of Kulaksız et al. (2018), who reported an inhibition zone diameter of 13 mm for *S. aureus* ATCC 25923. As *S. aureus* is a Gram-positive bacterium lacking an outer membrane, its cell wall's permeability promotes the passage of hydrophobic compounds like essential oils (Burt, 2004). Phenolic compounds in essential oils increase the permeability of bacteria by causing sensitization of the phospholipid layer in the cell membrane. Leakage of intracellular components out of the cell disrupts bacterial enzyme systems, resulting in the inhibition of the microorganism (Seow et al., 2014).

In this study, LnEO demonstrated an inhibitory effect against MRSA. The MIC value, which is the lowest concentration of essential oil that visually inhibits the growth of MRSA, was determined as 1:4 (v/v). The MBC value, which is the concentration of essential oil in the Petri dishes without bacterial growth, was determined as 1:2 (v/v). Plant extracts are generally considered bactericidal when the MBC/MIC ratio is ≤4 and bacteriostatic when the ratio exceeds 4 (Mujawah et al., 2022). In this study, the MBC/MIC value was found to be 2, indicating that LnEO is bactericidal against MRSA. Similar results were reported by Kulaksız et al. (2018), who found the MIC and MBC values of LnEO against *S. aureus* ATCC 25923 to be above 50%. Parallel to these results, it was determined that LnEO showed antibacterial activity against *S. aureus* ATCC 27706 at 15% concentration (Özcan and Erkmen, 2001).

The time-kill assay is a valuable method for understanding the interactions between microbial strains and antimicrobial agents. This assay demonstrates the concentration- or time-dependent effects of antimicrobial agents on microbial species. It also determines antibacterial agents as bacteriostatic or bactericidal (Balouiri et al., 2016). To this end, the time-kill curve was evaluated against MRSA at

1xMIC and 2xMIC concentrations of LnEO. The results demonstrated a concentration- and time-dependent response. Upon evaluating the time-kill assay results, it was observed that MRSA treated with 2xMIC LnEO exhibited a reduction in bacterial viability over time, with no viable bacteria remaining after 90 min. The bactericidal effect of LnEO against MRSA was confirmed through the time-kill assay results.

CV dye, due to its hydrophobic nature, exhibits limited penetration across the bacterial cell wall. However, it is able to penetrate cells with compromised membranes and enhanced permeability (Nogueira et al., 2021). This characteristic makes the CV assay a reliable tool for detecting cell membrane damage (Tsuchido et al., 1985; Devi et al., 2010). In the present study, the impact of LnEO on the outer membrane permeability of MRSA was assessed by measuring the uptake of CV dye. The uptake of CV was evaluated after exposing MRSA to LnEO at concentrations of 1xMIC, 1/2xMIC, and 1/4xMIC. The control group exhibited a retention of 21.8% CV, while the retention levels in the 1xMIC, 1/2xMIC, and 1/4xMIC treatment groups were recorded as 65.3%, 38.7%, and 22.4%, respectively. These results suggest that exposure to 1xMIC and 1/2xMIC concentrations of LnEO compromised the integrity of the cell membrane.

Essential oils, owing to their lipophilic and hydrophobic properties, tend to preferentially partition from aqueous environments into the lipid bilayers of bacterial membranes. This interaction causes various alterations, including membrane expansion, increased fluidity, and enhanced permeability. Moreover, essential oils disrupt membrane-embedded proteins, inhibit respiratory processes, and interfere with bacterial ion transport mechanisms, all of which contribute to the overall antimicrobial effect (Trombetta et al., 2005). The findings from this study align with these established mechanisms, suggesting that LnEO may facilitate the entry of large molecules, such as CV, by disrupting the bacterial cell membrane structure.

Currently, the prevalence of multi-drug resistant organisms is increasing, prompting the search for new antibiotics. Given the prolonged timeline required for the discovery of new antibiotics, antibiotic combinations are frequently employed in the treatment of infections (Marques et al., 1997). However, due to side effects such as drug-drug interactions and the possibility of one antibiotic in a combination counteracting the lethal effect of the other, there has been increasing interest in incorporating natural compounds derived from plants. These compounds are believed to help reduce antibiotic resistance and potentially mitigate adverse effects (Ayaz, 2001). Combining antibiotics with essential oils or their active ingredients against resistant bacteria may broaden the antibacterial spectrum to reduce the emergence of resistant variants and minimize the dose of a single antibiotic. Previous studies have demonstrated the antibacterial activity of 1,8-cineole to reduce the emergence of resistant variants and minimize the dose of a single antibiotic (Schürmann et al., 2019; Farhanghi et al., 2022). In this study, 1,8-cineole in LnEO was thought to be related to the antibacterial activity of the oil and the effect of its combination with gentamicin on MRSA was investigated. The MIC values of gentamicin and 1,8-cineole were determined as 4 μg/mL and 4 mg/mL, respectively. However, in a study using the

microdilution method, the MIC values of gentamicin and 1,8-cineole obtained for the MRSA strain were 2 μg/mL and 32 mg/mL, respectively (Hriouech et al., 2020). On the other hand, the MIC value of 1,8-cineole against *S. aureus* ATCC 29213 was reported as 12.4 μg/mL (Yáñez Rueda and Cuadro Mogollón, 2012). This may be due to the use of different techniques or the differentiation of the bacterial growth phase. Because it is known that the bactericidal activity of antibiotics decreases when the inoculum increases (Diaz-Tang et al., 2022). As a result of the checkerboard analysis performed in this study, a synergistic effect between 1,8-cineole and gentamicin was observed. 1,8-cineole has been shown to increase the antibacterial effect capacity of the antibiotic gentamicin. Similarly, Hriouech et al. (2020) reported that the combination of gentamicin and 1,8-cineole showed a complete synergistic effect against MRSA strain. The incorporation of essential oils in the prevention of bacterial resistance represents a highly promising strategy, as many conventional antibiotics are singular compounds with a limited target site, whereas essential oils, comprising multiple active constituents, exert their effects at various levels (Yap et al., 2014). Currently, essential oils or their components are utilized in wound dressings, either independently or in conjunction with antibiotics, based on their therapeutic and antibacterial properties (Altaf et al., 2021). Furthermore, certain studies have demonstrated the effectiveness of essential oils in enhancing skin permeability through the use of foams, hydrogels, dermal patches, films, and electrospun polymer dressings, thus promoting the wound healing process (Krysiak et al., 2020; Sroczyk e al. 2022). In combination with antibiotics, 1,8-cineole may be useful in the clinical management of some infectious diseases caused by *S. aureus*, especially nosocomial infections. However, further research should be conducted in the future to have a complete view on the toxicity of 1,8-cineole and to determine its optimal concentration for clinical applications.

5. Conclusion

The results indicate that LnEO and its primary constituent, 1,8-cineole, exhibit antibacterial activity by enhancing the permeability of the cell membrane in MRSA. Furthermore, 1,8-cineole potentiates the antibacterial efficacy of gentamicin through a synergistic interaction between the two agents. These findings suggest that 1,8-cineole may prove clinically beneficial when combined with gentamicin in the treatment of infections, particularly those caused by nosocomial *S. aureus*. The combination of 1,8-cineole with antibiotics could potentially reduce the required doses of standard antibiotics such as gentamicin, thereby mitigating their associated toxic effects while also preventing the emergence of resistance. By potentiating the activity of antibiotics, 1,8-cineole not only enhances the effectiveness of current treatment regimens but also provides alternative therapeutic options for conditions such as polymicrobial infections, which cannot be adequately addressed with a single standard drug.

Future research is required to further assess the clinical efficacy and safety of these compounds in more detail. Specifically, large-scale clinical trials should be conducted to evaluate the synergistic effects of 1,8-cineole and other essential oil constituents in combination with various antibiotics. Such studies may facilitate the development of plant-based therapeutics with low toxicity profiles that are resistant to

bacterial resistance, offering more affordable alternatives to the currently available antibiotics. Furthermore, additional investigations into the potential toxic effects of the 1,8-cineole and gentamicin combination are essential. Examining these agents in combination with other antibiotics and utilizing clinically relevant microbial strains will provide a clearer understanding of their therapeutic potential and limitations. Future studies will offer valuable insights into how these compounds can be more effectively utilized, in conjunction with antibiotics, to address the growing issue of antibacterial resistance.

Acknowledgments

The author would like to thank 'Innovative Technologies Application and Research Center (YETEM)' in Süleyman Demirel University, Isparta/Turkey, for expert cooperation in performing the GC-MS analysis.

Conflict of Interest

The author declares that there is no conflict of interest.

Contribution Rate Statement Summary of Researchers

The author declare that she contributed 100% to the article.

Ethical Approval

The author reports that formal ethics committee approval is not required for this type of study.

Funding

No funding or support was used in this study.

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