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ORIGINAL ARTICLE / ÖZGÜN MAKALE

DETERMINATION OF ANTIBACTERIAL AND ANTIBIOFILM ACTIVITY OF TERPINEN 4-OL AGAINST SOME STANDARD BACTERIAL STRAINS

TERPINEN 4-OL'ÜN ÇEŞİTLİ STANDART BAKTERİ SUŞLARINA KARŞI ANTİBAKTERİYEL VE ANTİBİYOFİLM AKTİVİTESİNİN BELİRLENMESİ

Merve SAVLUK[1]*, Merve Eylul KIYMACI[1], Nilgün ÜNAL[1]

[1]University of Health Sciences, Gülhane Faculty of Pharmacy, Department of Pharmaceutical Microbiology, 06018, Ankara, Türkiye

ABSTRACT

Objective: *Antimicrobial resistance in bacteria is becoming increasingly common and poses a serious challenge, particularly in treatment of infections where biofilm formation is involved. Biofilms enable bacteria to survive under harsh conditions and increase their resistance to antibiotics, thereby reducing the success of standard treatments. Terpinen-4-ol, the main compound of Melaleuca alternifolia (tea tree) essential oil, has shown notable antimicrobial effects. This study focuses on evaluating the antimicrobial effectiveness of terpinen-4-ol against selected standard bacterial strains and also determinating the antibiofilm activity at subMIC concentrations.*

Material and Method: *In this study, Enterococcus faecalis WHO3 (vanA), Pseudomonas aeruginosa PAO1, Staphylococcus aureus ATCC 43300 (MRSA), Staphylococcus epidermidis ATCC 35984, and Streptococcus mutans ATCC 12575 were used to be tested. Minimum inhibitory concentrations (MICs) were determined by broth microdilution method according to EUCAST standards. Biofilm formation and inhibition assays were tested by the modified crystal violet method. The antibiofilm activity of terpinen-4-ol was evaluated at a sub-inhibitory concentration corresponding to MIC/2, and the extent of inhibition was quantified as the percentage reduction relative to untreated control biofilm formation.*

Result and Discussion: *The MIC values of terpinen-4-ol against the tested bacteria ranged from 0.0625% to 0.5% (v/v). Even at subMIC levels, terpinen-4-ol significantly reduced biofilm formation. Inhibition rates were 79.95% for P. aeruginosa PAO1, 77.79% for S. epidermidis ATCC 35984, and 73.23% for S. mutans ATCC 12575. These results suggest that terpinen-4-ol is effective not only in inhibiting bacterial growth but also in reducing biofilm formation, highlighting its potential as a promising candidate for alternative or complementary treatment strategies, especially against biofilm-associated and resistant infections.*

Keywords: *Antibacterial activity, antibiofilm activity, terpinen-4-ol*

ÖZ

Amaç: *Bakteriler tarafından antimikrobiyal direnç giderek daha yaygın hale gelmekte ve özellikle biyofilm oluşumunun etkili olduğu enfeksiyonlarda ciddi bir sorun oluşturmaktadır. Biyofilmler,*

* **Corresponding Author / Sorumlu Yazar:** Merve Şavluk
e-mail / e-posta: merve.savluk@sbu.edu.tr, **Phone / Tel.:** +905392001962

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bakterilerin zorlu koşullarda hayatta kalmasına yardımcı olarak antibiyotiklere karşı direnç geliştirmelerine neden olur ve standart tedavilerin etkinliğini azaltır. Melaleuca alternifolia (çay ağacı) uçucu yağının ana etken maddelerinden biri olan terpinen-4-ol, belirgin antimikrobiyal etkiler göstermektedir. Bu çalışma, seçilen standart bakteri suşlarına karşı terpinen-4-ol'ün antimikrobiyal etkinliğini ve aynı zamanda subMİK konsantrasyonda antibiyofilm aktivitesini araştırmayı amaçlamaktadır.

Gereç ve Yöntem: *Bu çalışmada, Enterococcus faecalis WHO3 (vanA), Pseudomonas aeruginosa PAO1, Staphylococcus aureus ATCC 43300 (MRSA), Staphylococcus epidermidis ATCC 35984 ve Streptococcus mutans ATCC 12575 test bakterileri olarak kullanılmıştır. Minimum inhibitör konsantrasyon (MİK) değerleri, EUCAST standartlarına göre broth mikrodilüsyon yöntemi ile belirlenmiştir. Biyofilm oluşumu ve inhibisyonu modifiye kristal viyole yöntemi ile incelenmiştir. Terpinen 4 ol'ün antibiyofilm aktivitesi MIK/2 konsantrasyonda belirlenip, ana biyofilm oluşumuna göre farkı yüzde azalma olarak belirtilmiştir.*

Sonuç ve Tartışma: *Terpinen-4-ol'ün test edilen bakterilere karşı MİK değerleri %0.0625 ile %0.5 (v/v) arasında değişmiştir. Sub MİK düzeylerinde bile biyofilm oluşumunu önemli ölçüde azaltmıştır. Biyofilm inhibisyon oranları; P. aeruginosa PAO1 için %79.95, S. epidermidis ATCC 35984 için %77.79 ve S. mutans ATCC 12575 için %73.23 olarak ölçülmüştür. Bu bulgular, terpinen-4-ol'ün yalnızca bakteri çoğalmasını değil, aynı zamanda biyofilm oluşumunu da azaltmada etkili olduğunu göstermekte ve biyofilmle ilişkili dirençli enfeksiyonlara karşı alternatif veya destekleyici tedavi stratejileri için umut vadeden bir aday olduğunu göstermektedir.*

Anahtar Kelimeler: *Antibakteriyel aktivite, antibiyofilm aktivite, terpinen-4-ol*

INTRODUCTION

Antimicrobial resistance (AMR) in bacteria has emerged as a major global public health threat, significantly complicating the treatment of infectious diseases. A key contributor to this problem is the formation of biofilms by bacteria, which act as a crucial virulence factor. Biofilms enable bacterial communities to survive under hostile environmental conditions, protect themselves from the host immune system, and exhibit increased tolerance to antimicrobial agents [1,2]. As part of the bacterial defense mechanism, biofilm formation often renders traditional antibiotic therapies ineffective, contributing to persistent and recurrent infections.

The inappropriate and excessive use of antibiotics has accelerated the development of resistant bacterial strains and has led to inadequate treatment outcomes, especially in chronic and device-associated infections [1,2]. This has created an urgent need for novel antimicrobial strategies and alternative therapeutic agents capable of overcoming biofilm-mediated resistance mechanisms.

Among these alternatives, natural products, particularly essential oils (EOs), have attracted attention due to their broad-spectrum antimicrobial activity. *Melaleuca alternifolia* (tea tree) essential oil, which contains terpinen-4-ol as its major bioactive component, has demonstrated potent antimicrobial activities against a wide range of Gram-positive and Gram-negative bacteria [3,4]. Terpinen-4-ol has been shown to inhibit clinically important human and animal pathogens, such as *Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus* —organisms frequently associated with resistant and hard-to-treat infections [3]. Recent studies have further confirmed the antimicrobial efficacy of *M. alternifolia* and its derivatives against mastitis-associated pathogens and other clinically relevant microorganisms [2-4]. These findings highlight the potential of terpinen-4-ol as a natural antimicrobial and antibiofilm agent with promising veterinary and clinical applications. Despite its broad-spectrum activity, the clinical application of terpinen-4-ol and other essential oil components can be limited by factors such as volatility, oxidative degradation, and low aqueous solubility, which affect their stability and delivery [5]. However, terpinen-4-ol's dual antimicrobial and antibiofilm action makes it a particularly attractive candidate for future therapeutic development.

Therefore, the present study aimed to investigate the antibacterial activity of terpinen-4-ol against selected standard bacterial strains known for their involvement in resistant and recurrent infections, and to evaluate its antibiofilm activity against biofilm-producing bacteria at sub-inhibitory concentrations.

MATERIAL AND METHOD

Bacterial Strains

Enterococcus faecalis WHO3 (vanA), *Pseudomonas aeruginosa* PAO1 (biofilm producer strain), *Staphylococcus aureus* ATCC 43300 (MRSA), *Staphylococcus epidermidis* ATCC 35984 (biofilm producer strain), *Streptococcus mutans* ATCC 12575 (biofilm producer strain), and were used as test bacteria.

Terpinen-4-ol Solution

The intermediate stock solution was prepared according to the % µl/ml method of Cordeiro et al. [6], containing 20% terpinen-4-ol (Sigma-2438-10-0, Germany), 2% Tween-80 and 5% DMSO.

Antibacterial Activity

The antibacterial activity of terpinen-4-ol was determined by the broth microdilution method as the minimum inhibitory concentration (MIC). Two-fold serial dilutions of terpinen-4-ol were prepared in cation-adjusted Mueller-Hinton Broth. Bacterial suspensions adjusted to a 0.5 McFarland standard were diluted 1:100 and 100 µl were added to each well to reach a final concentration of 5×10^5 CFU/ml. Plates were incubated at 35 ± 1°C for 18–20 hours. All experiements were performed three times. MIC was defined as the lowest concentration at which no visible bacterial growth occurred. Ciprofloxacin (Sigma-85721-33-1, Germany) was used as a positive control.

Biofilm Production and Antibiofilm Activity

Biofilm formation and inhibition assays were determined using a modified crystal violet method [7]. Bacterial suspensions at a turbidity of 0.5 McFarland were diluted in TSB supplemented with 1% glucose and incubated in 96-well plates at 35 ± 1°C for 24 h. After incubation, the planktonic cells were carefully removed, all wells were washed three times with sterile phosphate-buffered saline (PBS) and then stained with 0.1% crystal violet for 15 min. The stained wells were washed three times with sterile PBS and air-dried. Dried wells were treated with 33% glacial acetic acid. OD values were measured at 620 nm. The antibiofilm activity of terpinen-4-ol was evaluated at a sub-inhibitory concentration corresponding to MIC/2, and the extent of inhibition was quantified as the percentage reduction relative to untreated control biofilm formation. The percentage of biofilm inhibition was calculated using the following equation:

$$\text{Biofilm inhibition (\%)} = \left[1 - \frac{\text{OD}_{\text{treated}}}{\text{OD}_{\text{control}}}\right] \times 100$$

All experiments were performed in triplicate and repeated independently three times. The results were expressed as mean ± standard deviation (SD), and differences were considered statistically significant at $p < 0.05$.

RESULT AND DISCUSSION

The minimum inhibitory concentration (MIC) values of terpinen-4-ol ranged from 0.0625% to 0.5% (v/v) (Table 1), with *P. aeruginosa* PAO1 exhibiting the highest MIC at 0.5%. This is in line with previous findings by Johansen et al. [8], who reported MIC values up to 2.5% for *P. aeruginosa*, although the present study indicates a notably lower MIC, suggesting enhanced potency of terpinen-4-ol under the experimental conditions used.

Our results showed that clinically important bacteria such as *P.aeruginosa*, *S.epidermidis*, and *S.mutans* had relatively low MIC values between 0.125% and 0.5%, with biofilm inhibition exceeding 70% (Figure 1) even when terpinen-4-ol was applied at half the MIC concentration (Table 2). The MIC for *E. faecalis* was found to be 0.25%, which is in agreement with the findings of Kamiya et al. [9] who reported similar values for bacteria involved in apical periodontitis and demonstrated that terpinen-4-ol can reduce biofilm viability.

Table 1. Antibacterial activity of terpinen-4-ol against strains

Isolate name	MIC (v/v)
***Pseudomonas aeruginosa* PAO1**	0.5
***Staphylococcus epidermidis* ATCC 35984**	0.25
***Streptococcus mutans* ATCC 12575**	0.125
Enterococcus faecalis* WHO3 (vanA)	0.25
Staphylococcus aureus* ATCC 43300 (MRSA)	0.25

* These strains are not biofilm producers

Table 2. Antibiofilm activity of terpinen-4-ol against strains

Isolate name	Biofilm production	Biofilm inhibition %
***Pseudomonas aeruginosa* PAO1**	1.4362 ±0.047	79.95%
***Staphylococcus epidermidis* ATCC 35984**	2.106 ±0.065	77.79%
***Streptococcus mutans* ATCC 12575**	3.054 ±0.414	73.23%

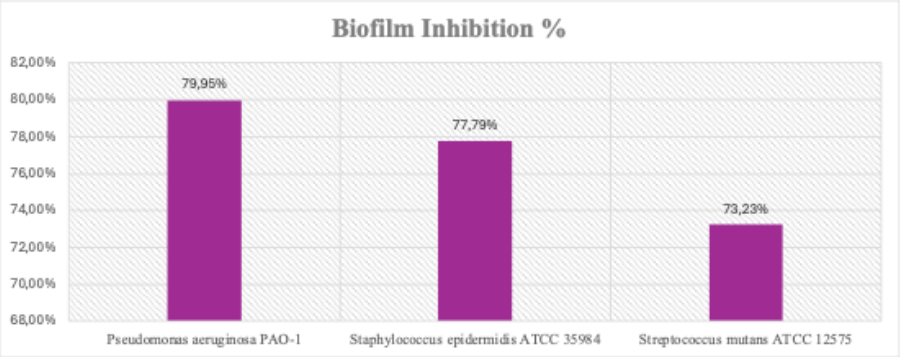

Figure 1. Biofilm inhibitions of biofilm-producer strains

Biofilm formation was quantified through optical density (OD) measurements, revealing that *S. mutans* ATCC 12575 was the strongest biofilm producer among the tested strains (3.054 ± 0.414), followed by *S. epidermidis* ATCC 35984 (2.106 ± 0.065), and *P. aeruginosa* PAO1 (1.4362 ± 0.047) (Figure 2). When treated with terpinen-4-ol at sub-MIC concentration (MIC/2), all three strains showed significant inhibition of biofilm formation: 79.95% inhibition for *P. aeruginosa*, 77.79% for *S. epidermidis*, and 73.23% for *S. mutans*. These substantial reductions highlight the strong antibiofilm capacity of terpinen-4-ol even at concentrations below those required for complete microbial growth inhibition (Figure 3).

In a separate study, Alves et al. [10] demonstrated that a 2% *Melaleuca alternifolia* essential oil formulation containing terpinen-4-ol significantly lowered bacterial counts and improved healing in wounds infected with *S. aureus*, effects attributed in part to the anti-inflammatory and antimicrobial actions of terpinen-4-ol. Furthermore, Chadha et al. [11] found that terpinen-4-ol reduced quorum sensing activity and virulence factor production in *P. aeruginosa* strains PAO1 and PA14, indicating that its benefits extend beyond antimicrobial effects to antivirulence properties.

Pontanayodsakorn et al. [12] also confirmed terpinen-4-ol's antibiofilm activity by showing it effectively inhibits the formation of *S. aureus* biofilms, although it did not eradicate mature biofilms. These findings suggest that terpinen-4-ol primarily prevents biofilm development rather than eliminating established biofilms, and that its antibiofilm effect may occur independently from direct antibacterial activity.

Supporting these findings, this study found terpinen-4-ol MIC values ranging from 0.0625% to 0.5% (v/v), with *P. aeruginosa* PAO1 showing the highest MIC at 0.5%, notably lower than earlier reports by Johansen et al. [8], who observed MICs as high as 2.5%, suggesting increased potency under

the current experimental conditions. Biofilm quantification by optical density showed that *S. mutans* ATCC 12575 was the strongest biofilm former (3.054 ± 0.414), followed by *S. epidermidis* ATCC 35984 (2.106 ± 0.065), and *P. aeruginosa* PAO1 (1.4362 ± 0.047). Treatment at sub-MIC levels resulted in significant biofilm inhibition: 79.95% for *P. aeruginosa*, 77.79% for *S. epidermidis*, and 73.23% for *S. mutans*, highlighting terpinen-4-ol's effectiveness even at doses below full growth inhibition.

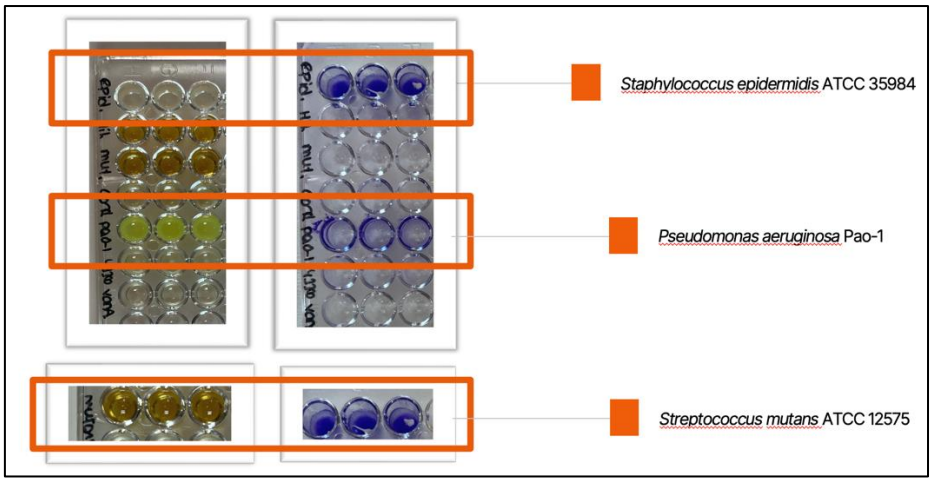

Figure 2. Biofilm productions of biofilm-producer strains

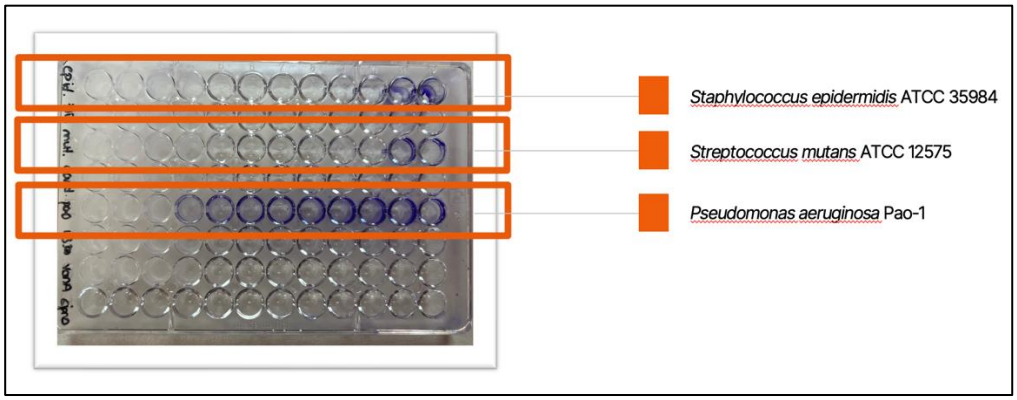

Figure 3. Antibiofilm activity of terpinen-4-ol against biofilm-producer strains

In contrast, *Enterococcus faecalis* WHO3 (vanA), and *Staphylococcus aureus* ATCC 43300 (MRSA) exhibited MIC values of 0.25%, and 0.25%, respectively, but produced barely detectable biofilm as indicated by very low OD values (0.0032 ± 0.054, and 0.0893 ± 0.007). Consequently, biofilm inhibition was not assessed for these isolates. This finding suggests that the antibiofilm effect of terpinen-4-ol is particularly relevant for strains with substantial biofilm-forming capacity.

These results are consistent with previous studies indicating the antibiofilm efficacy of terpinene-4-ol. For instance, Bordini et al. [13] observed MIC and MBIC values around 0.24% against *S. mutans*, closely matching the MIC of 0.125% found here. Cordeiro et al. [6] demonstrated over 80% inhibition of *S. aureus* biofilm formation at 0.5× MIC concentrations, paralleling the strong biofilm inhibition detected in this study. Additionally, Merghni et al. [14] showed that terpinen-4-ol disrupts established *S. aureus* biofilms and possesses notable antibacterial activity against *P. aeruginosa* PAO1, aligning well with the potent antibiofilm and antimicrobial effects observed herein.

The MIC values reported by Johansen et al. [8] (ranging from 1.25% to 2.5% for Gram-positive and up to 2.5% for Gram-negative bacteria) contrast with the generally lower MICs recorded in this

study, possibly reflecting differences in bacterial strains, experimental protocols, or terpinen-4-ol formulations. Nevertheless, the consistently strong antibiofilm activity at sub-MIC levels suggests that terpinen-4-ol may effectively inhibit biofilm formation without exerting excessive selective pressure, which is crucial for minimizing the development of antimicrobial resistance.

Similar observations have been reported by other researchers using *M.alternifolia* essential oil and its main active compound, terpinen-4-ol. Cordeiro et al. [6] demonstrated that terpinen-4-ol effectively inhibited *S. aureus* biofilm formation, while Merghni et al. [14] found comparable antibiofilm and antivirulence effects against *P. aeruginosa* and *S. aureus*. Likewise, Bordini et al. [13] reported potent antimicrobial activity of terpinen-4-ol against oral pathogens, which is consistent with the low MIC values observed in this study. In addition, Zhan et al. [3] and Corona-Gómez et al. [4] highlighted the protective and antimicrobial properties of tea tree oil in mammary epithelial infections, further supporting its broad biological potential. Together, these findings reinforce the current results, indicating that terpinen-4-ol exhibits strong and wide-ranging antibacterial and antibiofilm activity.

In conclusion, the findings of this study confirm that terpinen-4-ol possesses strong antimicrobial and antibiofilm activities against both Gram-positive and Gram-negative pathogens, particularly effective against clinically relevant biofilm producers such as *P. aeruginosa, S. epidermidis*, and *S. mutans*. Its capacity to prevent biofilm formation at sub-inhibitory concentrations makes it a promising natural compound for treating biofilm-associated infections in medicine. Additionally, its antivirulence effects and ability to support wound healing suggest terpinen-4-ol may offer multiple therapeutic benefits while helping to limit antimicrobial resistance.

AUTHOR CONTRIBUTIONS

Concept: M.Ş., M.E.K., N.Ü.; Design: M.Ş., M.E.K., N.Ü.; Control: M.E.K., N.Ü.; Sources: M.Ş., M.E.K., N.Ü.; Materials: M.Ş., M.E.K., N.Ü.; Data Collection and/or Processing: M.Ş.; Analysis and/or Interpretation: M.Ş., M.E.K., N.Ü.; Literature Review: M.Ş., M.E.K., N.Ü.; Manuscript Writing: M.Ş.; Critical Review: M.Ş., M.E.K., N.Ü.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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