**ARAŞTIRMA MAKALESİ /** *RESEARCH ARTICLE*

# The Antibacterial and Antibiofilm Activities of Resveratrol on Gram-positive and Gram-negative Bacteria

## *Resveratrol'ün Gram-pozitif ve Gram-negatif Bakteriler Üzerindeki Antibakteriyel ve Antibiyofilm Aktiviteleri*

**Murat Karamese[1], Yalcin Dicle[2]**

*[1]Department of Medical Microbiology, Kafkas University, Faculty of Medicine, Kars; [2]Department of Nutrition and Dietetics, Mus Alparslan University, Faculty of Health Sciences, Mus, Türkiye*

## ABSTRACT

***Aim:*** *Resveratrol (3,5,4'-trihydroxystilbene) shows antimicrobial activity against many pathogens. It has been detected that subinhibitory concentrations can reduce bacterial motility and interference with quorum sensing, lead to reduced bacterial toxin production, and inhibit biofilm formation. In this study, we aimed to investigate resveratrol's antibacterial and antibiofilm activities on some Gram-positive and Gram-negative bacteria.*

***Material and Method:*** *The bacterial strains, including Staphylococcus aureus (ATCC-29213), Bacillus subtilis (ATCC-6051), Escherichia coli (ATCC-25923), and Pseudomonas aeruginosa (ATCC-27853) were grown overnight in LB broth at 37°C in a humidified chamber. The resveratrol was dissolved in 1.5% of dimethyl sulfoxide (DMSO). Serial two-fold dilutions of the resveratrol, ranging from 16 to 0.5 mg/ml, were prepared in a 96-well plate. The microdilution method determined the minimum inhibitory concentration (MIC) for the resveratrol. Bacterial biofilm formation was assessed using the crystal violet assay. The agar gel diffusion assay was also performed to determine the antimicrobial activity.*

***Results:*** *In these assays, the resveratrol inhibited the growth of both Gram-positive and Gram-negative bacteria strains tested, with inhibition zone diameters ranging from 19.8 to 22 mm and MIC values of 4 mg/ml, confirming its antimicrobial properties. Concerning the effect of resveratrol on biofilm formation, an inhibition ranging from 24% to 99% on the total biofilm mass was achieved for all bacteria strains (Fig. 2 and Fig. 3). 16 mg/ml of resveratrol is the most effective dose for antibiofilm activity.*

***Conclusion:*** *Resveratrol has gained significant scientific and public attention not only for being a possible natural antimicrobial but also for its potential functional and therapeutic applications. Further studies should be planned to understand the molecular mechanism underlying resveratrol's inhibitory effect, investigate the synergistic effects of resveratrol with antibiotics, and apply it in clinical practice.*

**Key words:** *antibacterial activity; antibiofilm activity; minimal inhibitory concentration; resveratrol; gel diffusion*

## ÖZET

***Amaç:*** *Resveratrol (3,5,4'-trihidroksistilben), birçok patojenlere karşı antimikrobiyal aktivite gösterir. İnhibe edici konsantrasyonlarda, bakteri hareketliliğini ve haberleşme ağını azaltabileceği, bakteri toksin üretiminin azalmasına yol açabileceği ve biyofilm oluşumunu engelleyebileceği saptanmıştır. Bu çalışmada, resveratrol'ün bazı Gram-pozitif ve Gram-negatif bakteriler üzerine antibakteriyel ve antibiyofilm aktivitelerini araştırmayı amaçladık.*

***Materyal ve Metot:*** *Staphylococcus aureus (ATCC-29213), Bacillus subtilis (ATCC-6051), Escherichia coli (ATCC-25923) ve Pseudomonas aeruginosa (ATCC-27853) bakteri suşları, 37°C'de LB sıvı besiyerinde bir gece inkübasyon sonrası üretildi. Resveratrol, %1,5 dimetil sülfoksit (DMSO) içinde çözüldü. Doksan altı kuyucuklu bir plakada 16 ila 0,5 mg/ml arasında değişen resveratrol dozları ile seri dilüsyonlar hazırlandı. Resveratrol için minimum inhibitör konsantrasyonu (MİK), mikrodilüsyon yöntemiyle belirlendi. Bakteri suşlarının biyofilm oluşturma yetenekleri, kristal viyole testi ile değerlendirildi. Bunlara ilave olarak, antimikrobiyal aktiviteyi belirlemek için agar jel difüzyon testi yapıldı.*

***Bulgular:*** *Bu testlerde resveratrol, 19,8 ila 22 mm arasında değişen inhibisyon çapları ve 4 mg/ml MİK değerleri ile hem Gram-pozitif hem de Gram-negatif bakteri suşlarının üremesini inhibe ederek antimikrobiyal özelliklerini ortaya koymuştur. Resveratrol'ün biyofilm oluşumu üzerindeki etkisi ile ilgili olarak, tüm bakteri suşları için toplam biyofilm kütlesi üzerinde %24 ila %99 arasında değişen bir inhibisyon elde edilmiştir. On altı mg/ml konsantreli resveratrol uygulaması, antibiyofilm aktivitesi için en etkili dozdur.*

***Sonuç:*** *Resveratrol, yalnızca doğal bir antimikrobiyal olduğu için değil, aynı zamanda fonksiyonel ve terapötik uygulamalar için potansiyel bir ajan olduğu içinde bilimsel alanda dikkat çekmiştir. Resveratrolün inhibitör etkisinin altında yatan moleküler mekanizmayı anlamak, antibiyotiklerle sinerjistik etkilerini araştırmak ve klinik pratikte uygulamak için yeni çalışmalar planlanmalıdır.*

**Anahtar kelimeler:** *antibakteriyel aktivite; antibiyofilm aktivite; jel difüzyon; minimal inhibitör konsantrasyon; resveratrol*

***İletişim/Contact:*** *Murat Karameşe, Kafkas University, Medical Faculty, Department of Microbiology, 36100, Kars, Turkey 36100 Kars - Türkiye* • ***Tel:*** *0553 810 11 12* • ***E-mail:*** *murat_karamese@hotmail.com* • ***Geliş/Received:*** *20.11.2021* • ***Kabul/Accepted:*** *11.04.2022*

***ORCID:*** *Murat Karameşe, 0000-0001-7803-1462* • *Yalçın Dicle, 0000-0002-7658-7763*

## Introduction

In order to prevent and control bacterial infections, broad-spectrum antibiotics and chemical bactericides are used to temporarily counter bacterial colonization and remove their biofilms[1–3]. However, many of the currently available oral antibiotics have side effects such as nausea, diarrhea, and vomiting[4]. At that point, the discovery and use of natural compounds that can be effective for bacteria activities and biofilm formations are inevitable.

Resveratrol is present in numerous plants such as peanuts (*Arachis hypogea*), blueberries and cranberries (*Vaccinium* spp.), Japanese knotweed (*Polygonum cuspidatum*) a traditional Asian herbal medicine, and most importantly as a natural source for human consumption in grape wines (*Vitis vinifera*)[5]. Resveratrol, a natural phytochemical found at high levels in especially red wine and grapes, has been extensively studied for a variety of different kinds of health-beneficial effects, including anti-aging, antioxidant, anti-inflammation, anti-carcinogenesis, anti-proliferative, cardiovascular protection and apoptotic effects. It has also an antimicrobial activity against a wide range of bacterial pathogens. It has been studied that even at subinhibitory concentrations, it can reduce bacterial motility and interference with quorum sensing, lead to reduced bacterial toxin production, and inhibit biofilm formation[6–12].

Current literature supported that the resveratrol has an antibacterial activity by effecting different mechanisms such as inactivating the efflux pump systems[13], binding reversibly to the ATP synthase, partially inhibiting both ATP hydrolysis and ATP synthesis functions of the ATP synthase[14], inhibiting biofilm formation of bacteria[15].

In this study, our aim was to investigate the possible anti-bacterial and anti-biofilm activities of resveratrol on Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*.

## Materials and Methods

### *Preparation of Resveratrol and Bacterial Strains:*

The bacterial strains used in this study were *S. aureus* (ATCC-29213), *B. subtilis* (ATCC-6051), *E. coli* (ATCC-25923), and *P. aeruginosa* (ATCC-27853). All bacteria strains were growth overnight in Luria-Bertani (LB) broth at 37°C and 100 µl of bacterial cells with a concentration of $10^8$ cfu/ml were added into 96-well plates. The resveratrol was commercially purchased (CAS Number: 501–36–0, Sigma Aldrich, Taufkirchen, Germany). The resveratrol was prepared as a stock solution of 16 mg/ml and then was sub-diluted as following concentrations (16, 8, 4, 2, 1 and 0.5 mg/ml).

### *Agar Gel Diffusion Test:*

Mueller-Hinton Agar (MHA) agar plate surface is inoculated by spreading 100 µl of the bacterial inoculum over the entire agar surface and let to dry 10 min at room temperature. Wells were drilled out on seeded MHA agar plates (6 mm in diameter) with a sterile cork borer. Then, 16 mg/ml stock and sub-dilutions of the resveratrol was introduced into the drilled well. Then, MHA agar plates were left to incubate at 37°C for 24 hours in a flat position. At the end of the incubation the bacterial inhibition zone diameters were measured[16].

### *Crystal Violet (CV) Test:*

A hundred µl Mueller-Hinton Broth (MHB) and 100 µl of bacterial cells suspension ($10^8$ cfu/ml) were added to polystyrene 96-well plates and left to static incubation at 37°C for 48 hours. At the end of the period, all the wells were washed 3 times with Phosphate Buffered Saline (PBS). 0.5% CV dye was applied to wells for 20 minutes for staining attached cells. The residual dye was cleaned with tap water. After fixation with 30% acetic acid, measurement was taken at 590 nm by Multiskan™ GO UV/Vis microplate spectrophotometer (Thermo Scientific, Schwerte, Germany)[17].

### *Determination of Minimum Inhibitory Concentration (MIC):*

We performed the MIC assay to determine the lowest concentration of an resveratrol that prevents visible growth of a microorganism. For this reason, "European Antimicrobial Susceptibility Test" (EUCAST)[18] protocols have been applied for determining the MIC values. Briefly, 100 µl of bacterial cells with a concentration of $10^8$ cfu/ml and resveratrol with the specific concentrations (0.5; 1; 2; 4; 8; 16 mg/ml) were added into 96-well plates. After 24 hours of incubation at 37°C, 100 µl of samples were taken from the wells and inoculated on MHA agar plate by the spread

plate method. After 24 hours of incubation of these plates at 37°C, the MIC is the lowest concentration of antimicrobial agent that completely inhibits colony formation.

### *Determination of Minimum Biofilm Inhibitory Concentration (MBIC):*

Inhibitory effects on biofilm formation are commonly assessed by the MBIC, which is the lowest concentration of an antimicrobial substance at which there is no time-dependent increase in the mean number of biofilm viable cells. For this reason, MBIC value was determined by CV test. Briefly, 100 µl of the bacterial cell ($10^8$ cfu/ml) concentration was added to each well of the 96-well plate. Then, resveratrol at specific concentrations (0.5; 1; 2; 4; 8; 16 mg/ml) was added. Total volume was completed to 150 µl with MHB medium. The plate was left to incubate statically at 37°C for 48 hours. At the end of the period, all contents in the wells were discarded and washed 3 times with PBS. 200 µl of 0.5% CV dye was added to wells and incubated in the dark condition for 20 minutes. At the end of the incubation, the wells were washed and fixed with 30% acetic acid. Measurement was taken at 590 nm via a spectrophotometer[19].

## Results

After the Agar Gel Diffusion test, it was seen that the resveratrol inhibited the growth of both Gram-positive and Gram-negative bacteria stains which have been tested in our study with inhibition diameters ranging from 19.8 to 22 mm (Fig. 1).

On the other hand, antibiofilm activity of resveratrol were determined in a dose dependent manner on both Gram-positive and Gram-negative bacteria stains which were tested in our study. As seen in Fig. 2, an inhibition ranging from 61% to 99% on the total biofilm mass was achieved for *B. subtilis* and *S. aureus* strains. 16 mg/ml resveratrol application was the most effective dose for preventing the biofilm formation in tested Gram-positive bacteria.

Additionally, an inhibition ranging from 24% to 99% on the total biofilm mass was achieved for *E. coli* and *P. aeruginosa* strains. 16 mg/ml of the resveratrol was also the most effective dose for preventing the biofilm formation in tested Gram-negative bacteria. The MIC values of all groups were detected as 4 mg/ml dose of resveratrol (Fig. 3).

## Discussion

In this study, we evaluated the possible antibacterial and antibiofilm activities of resveratrol on some Gram-positive and Gram-negative bacteria such as *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa*. For this reason, we used a catalogued resveratrol and standard bacterial strains obtained from ATCC.

After the agar diffusion test and microdilution assay, it was detected that the resveratrol inhibited the growth

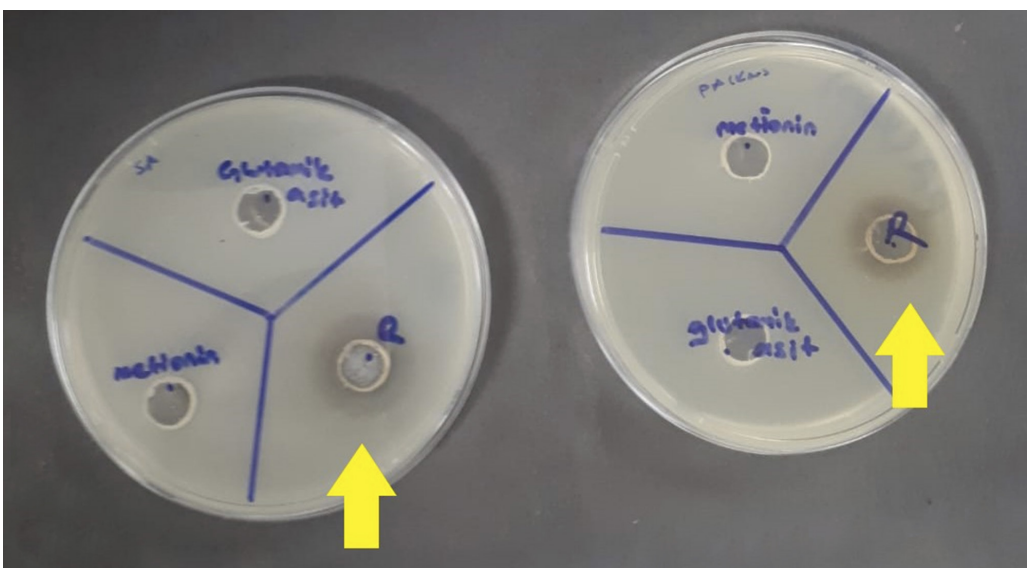


***Figure 1.*** *Agar gel diffusion assay results for resveratrol. The markings (R) show the resveratrol gel diffusion test on agar plate.*

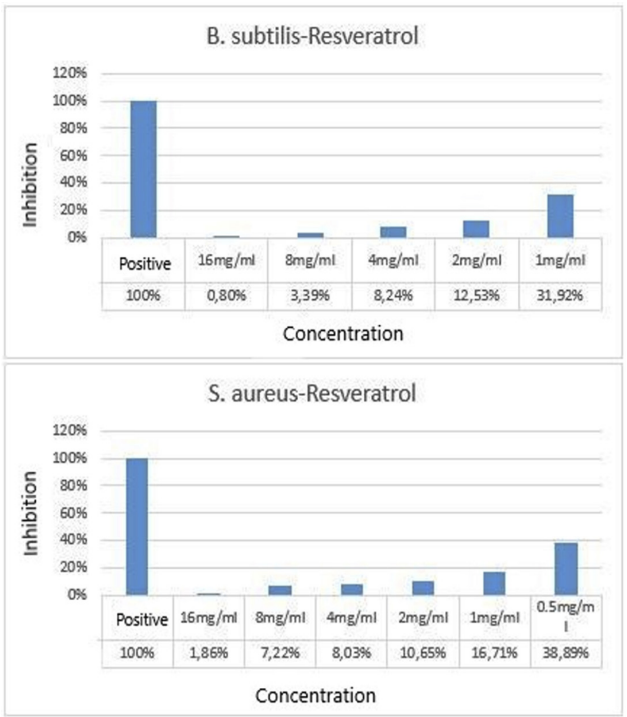


***Figure 2.*** *The antibiofilm effect of resveratrol on gram positive bacteria.*

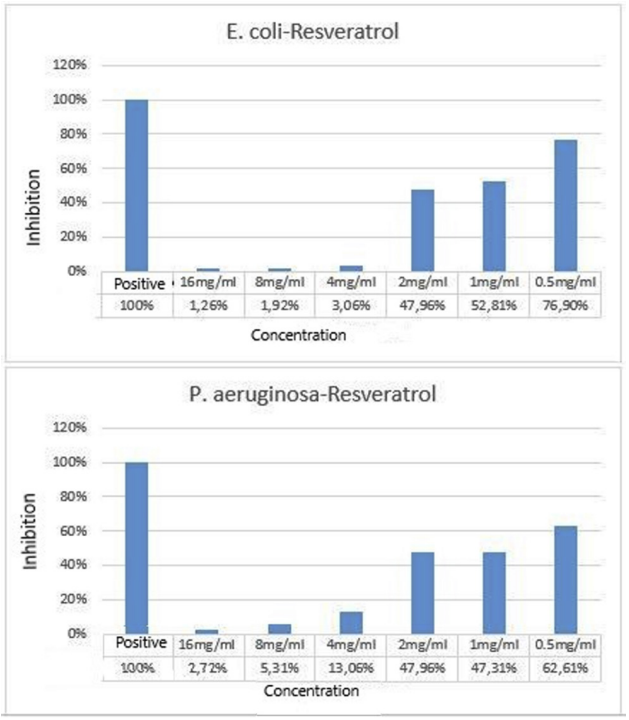


***Figure 3.*** *The antibiofilm effect of resveratrol on gram negative bacteria.*

of both Gram-positive and Gram-negative bacteria strains, with inhibition diameters ranging from 19.8 to 22 mm and MIC values of 16 mg/ml, confirming its antimicrobial properties. Concerning the effect of the resveratrol on biofilm formation, an inhibition ranging from 24% to 99% on the total biofilm mass was achieved for all bacteria strains. The 16 mg/ml application of resveratrol is the most effective dose for antibiofilm activity.

Several studies in current literature[11,20] have demonstrated that the resveratrol has antibacterial, antifungal, antiviral, and antibiofilm effects. These studies have also investigated the therapeutic application of the resveratrol against infectious diseases. The resveratrol inhibits growth of most of pathogens in different concentrations such as *Bacillus cereus* (MIC=50 µg/mL), *Mycobacterium smegmatis* (MIC=64 µg/mL), *Helicobacter pylori* (MIC=25 µg/mL), *Arcobacter cryaerophilus* (MIC=50 µg/mL), *Campylobacter coli* (MIC=64 µg/mL), *Vibrio cholera* (MIC=60 µg/mL), *Neisseria gonorrhoeae* (MIC=75 µg/mL), *Mycobacterium tuberculosis* (MIC=100 µg/mL), *Staphylococcus aureus* (MIC=10 µg/mL), *Enterococcus faecalis* (MIC=20 µg/mL), *Escherichia coli* (MIC >200 µg/mL), *Klebsiella pneumoniae* (MIC >200 µg/mL), and *Pseudomonas aeruginosa* (MIC >200 µg/mL)[21–28]. Our results were closely parallel with the current literature (MIC=16 µg/mL); however, we understand that we should have needed to perform more concentrations of resveratrol on bacterial strains. Several studies have reported lower susceptibilities for several Gram-negative pathogens compared with Gram-positive bacteria. This may be the result of active extrusion of the resveratrol by efflux pump systems[29].

On the other hand, bacteria can live as planktonic cells or in aggregates attached to surfaces, referred to as biofilms. The formation of bacterial biofilms must, necessarily, begin with the adhesion of a small number of bacterial cells to a surface[30]. There are advantages of biofilm formation such as providing phagocytosis and antimicrobial agents and biofilm formation is crucial for bacteria and clinically important in chronic and recurrent infections[31].

The resveratrol has been studied on various bacterial pathogens for its ability to reduce biofilm formation. In our study, 16 µg/mL resveratrol application inhibited the biofilm formation ranging from 24% to 99% on the total biofilm mass was achieved for *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa*. A study reported that resveratrol inhibits biofilm formation at concentrations

of 4–6-fold below the MIC value on some Gram-negative anaerobic bacteria[32]. Furthermore, the resveratrol demonstrated its antibiofilm properties against Gram-negative bacteria including *E. coli* at concentrations of 2–6-fold below the MIC value [24] and Gram-positive bacteria including *Propionibacterium acnes* and *S. aureus* at concentrations of 3–4-fold below the MIC value[33,34]. Same studies claimed that the resveratrol inhibited the biofilm formation in especially *E. coli* by reducing expression of csgA and csgB genes[35].

The resveratrol has gained significant scientific and public attention not only for being a possible natural antimicrobial but also for its potential functional and therapeutic applications. Further studies should be planned to understand the molecular mechanism underlying the inhibitory effect of resveratrol, to investigate the synergistic effects of resveratrol with antibiotics, and to apply in clinical practice.

Our study has some limitations. First, the concentrations of resveratrol should have been in a wider range. Second, more microorganisms should have been tested in this study.

### *Conflict of interest*

The authors have no conflict of interest.

## References


1. Herrera D, Alonso B, Leon R, Roldan S, Sanz M. Antimicrobial therapy in periodontitis: the use of systemic antimicrobials against the subgingival biofilm. J Clin Periodontol. 2008;35(8 Suppl):45–66.
2. Kouidhi B, Al Qurashi YM, Chaieb K. Drug resistance of bacterial dental biofilm and the potential use of natural compounds as alternative for prevention and treatment. Microb Pathog. 2015;80:39–49.
3. Sun M, Zhou Z, Dong J, Zhang J, Xia Y, Shu R. Antibacterial and antibiofilm activities of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) against periodontopathic bacteria. Microb Pathog. 2016;99:196–203.
4. Herrera Herrera A, Franco Ospina L, Fang L, Diaz Caballero A. Susceptibility of Porphyromonas gingivalis and Streptococcus mutans to Antibacterial Effect from Mammea americana. Adv Pharmacol Sci. 2014;2014:384815.
5. Aggarwal BB, Bhardwaj A, Aggarwal RS, Seeram NP, Shishodia S, Takada Y. Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. Anticancer Res. 2004;24(5A):2783–840.
6. Catalgol B, Batirel S, Taga Y, Ozer NK. Resveratrol: French paradox revisited. Front Pharmacol. 2012;3:141.
7. Marques FZ, Markus MA, Morris BJ. Resveratrol: cellular actions of a potent natural chemical that confers a diversity of health benefits. Int J Biochem Cell Biol. 2009;41(11):2125–8.
8. Neves AR, Lucio M, Lima JL, Reis S. Resveratrol in medicinal chemistry: a critical review of its pharmacokinetics, drug-delivery, and membrane interactions. Curr Med Chem. 2012;19(11):1663–81.
9. Renaud S, de Lorgeril M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet. 1992;339(8808):1523–6.
10. Tome-Carneiro J, Larrosa M, Gonzalez-Sarrias A, Tomas-Barberan FA, Garcia-Conesa MT, Espin JC. Resveratrol and clinical trials: the crossroad from in vitro studies to human evidence. Curr Pharm Des. 2013;19(34):6064–93.
11. Vestergaard M, Ingmer H. Antibacterial and antifungal properties of resveratrol. Int J Antimicrob Agents. 2019;53(6):716–23.
12. Vidavalur R, Otani H, Singal PK, Maulik N. Significance of wine and resveratrol in cardiovascular disease: French paradox revisited. Exp Clin Cardiol. 2006;11(3):217–25.
13. Piddock LJ. Multidrug-resistance efflux pumps - not just for resistance. Nat Rev Microbiol. 2006;4(8):629–36.
14. Dadi PK, Ahmad M, Ahmad Z. Inhibition of ATPase activity of Escherichia coli ATP synthase by polyphenols. Int J Biol Macromol. 2009;45(1):72–9.
15. Lee K, Lee JH, Ryu SY, Cho MH, Lee J. Stilbenes reduce Staphylococcus aureus hemolysis, biofilm formation, and virulence. Foodborne Pathog Dis. 2014;11(9):710–7.
16. Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc. 2008;3(2):163–75.
17. Tang M, Wei X, Wan X, Ding Z, Ding Y, Liu J. The role and relationship with efflux pump of biofilm formation in Klebsiella pneumoniae. Microb Pathog. 2020;147:104244.
18. https://www.eucast.org/ast_of_bacteria/mic_determination/?no_cache=1. Accessed 03.03.2022, 2022.
19. Schillaci D, Cusimano M, Russo D, Arizza V. Antimicrobial peptides from echinoderms as antibiofilm agents: a natural strategy to combat bacterial infections. Italian Journal of Zoology 2014;81:312–21.
20. Abedini E, Khodadadi E, Zeinalzadeh E, et al. A Comprehensive Study on the Antimicrobial Properties of Resveratrol as an Alternative Therapy. Evidence-Based Complementary and Alternative Medicine. 2021.
21. Paulo L, Ferreira S, Gallardo E, Queiroz JA, Domingues F. Antimicrobial activity and effects of resveratrol on human pathogenic bacteria. World Journal of Microbiology and Biotechnology. 2010;26:1533–8.
22. Lechner D, Gibbons S, Bucar F. Plant phenolic compounds as ethidium bromide efflux inhibitors in Mycobacterium smegmatis. Joubrla of Antimicrobials and Chemotheraphy. 2008;62:345–8.

23. Sun D, Hurdle JG, Lee R, Lee R, Cushman M, Pezzuto JM. Evaluation of flavonoid and resveratrol chemical libraries reveals abyssinone II as a promising antibacterial lead. ChemMedChem. 2012;7(9):1541–5.
24. Augustine N, Goel AK, Sivakumar KC, Kumar RA, Thomas S. Resveratrol--a potential inhibitor of biofilm formation in Vibrio cholerae. Phytomedicine. 2014;21(3):286–9.
25. Docherty JJ, Fu MM, Tsai M. Resveratrol selectively inhibits Neisseria gonorrhoeae and Neisseria meningitidis. J Antimicrob Chemother. 2001;47(2):243–4.
26. Ferreira S, Silva F, Queiroz JA, Oleastro M, Domingues FC. Resveratrol against Arcobacter butzleri and Arcobacter cryaerophilus: activity and effect on cellular functions. Int J Food Microbiol. 2014;180:62–8.
27. Jung CM, Heinze TM, Schnackenberg LK, et al. Interaction of dietary resveratrol with animal-associated bacteria. FEMS Microbiol Lett. 2009;297(2):266–73.
28. Mahady GB, Pendland SL. Resveratrol inhibits the growth of Helicobacter pylori in vitro. Am J Gastroenterol. 2000;95(7):1849.
29. Singkham-In U, Higgins PG, Wannigama DL, Hongsing P, Chatsuwan T. Rescued chlorhexidine activity by resveratrol against carbapenem-resistant Acinetobacter baumannii via down-regulation of AdeB efflux pump. PLoS One. 2020;15(12):e0243082.
30. Costerton JW. Introduction to biofilm. Int J Antimicrob Agents. 1999;11(3-4):217–21; discussion 237–19.
31. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol. 2004;2(2):95–108.
32. He Z, Huang Z, Zhou W, Tang Z, Ma R, Liang J. Anti-biofilm Activities from Resveratrol against Fusobacterium nucleatum. Front Microbiol. 2016;7:1065.
33. Coenye T, Brackman G, Rigole P, et al. Eradication of Propionibacterium acnes biofilms by plant extracts and putative identification of icariin, resveratrol and salidroside as active compounds. Phytomedicine. 2012;19(5):409–12.
34. Qin N, Tan X, Jiao Y, et al. RNA-Seq-based transcriptome analysis of methicillin-resistant Staphylococcus aureus biofilm inhibition by ursolic acid and resveratrol. Sci Rep. 2014;4:5467.
35. Lee JH, Cho HS, Joo SW, et al. Diverse plant extracts and trans-resveratrol inhibit biofilm formation and swarming of Escherichia coli O157:H7. Biofouling. 2013;29(10):1189–203.