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Investigating the Antimicrobial and Biofilm inhibitory Effects of Gingerol against Multidrug Resistant isolates of *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa is a Gram-negative bacterium and one of the most important opportunistic pathogens causing hospital infections that usually acquires multidrug resistance. This study was conducted to investigate the effect of gingerol in inhibiting the growth and biofilm formation of *Pseudomonas aeruginosa* isolates.

This study was conducted on *P. aeruginosa* isolates with multiple antibiotic resistance. The disk diffusion method was used to determine the antibiotic resistance of the strains. The diffusion and broth microdilution methods were used to investigate the antimicrobial effect of gingerol. The anti-biofilm effect of gingerol was investigated by the microplate method, and its effect on the expression of the biofilm *pslA* gene by real-time PCR.

Gingerol showed an antimicrobial effect against pathogenic *P. aeruginosa* strains, and its minimum inhibitory concentration (MIC) in varied between 512-1024 µg/mL. The use of this substance caused a 44-52% reduction in biofilm formation of isolates ($P<0.05$). Gingerol also significantly reduced (more than 52%) the expression of the *pslA* gene.

The present results indicate the possibility of using gingerol to combat planktonic and biofilm forms of antibiotic-resistant bacteria. The use of this substance with or instead of antibiotics may be a way to reduce the use of existing antibiotic drugs and thus reduce the side effects and treatment costs of infectious diseases.

Key words: Gingerol, Antimicrobial, Anti-Biofilm, *Pseudomonas aeruginosa*

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Introduction

Multidrug resistance (MDR) in Gram-negative bacteria, including *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, is a global health threat and an important cause of infection, especially in intensive care units (ICUs) in hospitals (Leylabadlo HE et al., 2017). *P.aeruginosa* is a non-fermentative Gram-negative *Bacillus* and one of the main microorganisms causing opportunistic and hospital-acquired infections. This bacterium is responsible for a wide range of acute and chronic infections, including respiratory, urinary tract, wound, burn, and septicemia infections. Treatment of infections caused by *P. aeruginosa* is often complex because this organism is inherently resistant to many antibiotics and can easily acquire resistance to most effective antibiotics, as multidrug-resistant strains of this bacterium have been reported in many studies. This is challenging in the treatment of infections caused by this bacterium and often leads to significant complications and mortality (Corehtash et al., 2015). *P. aeruginosa* is one of the most important biofilm-forming bacteria. This bacterium can form biofilms on various surfaces such as living tissues, medical devices, and implants. Biofilm formation by *Pseudomonas aeruginosa* occurs through several steps, including attachment to the surface by flagella and surface proteins, followed by cell division to form microcolonies, and finally biofilm maturation, which is accompanied by the expression of matrix polymers. The production of these biofilms also depends on growth factors, pH, and other biological factors (Bogiel et al., 2021). The spread of antibiotic resistance has caused existing antimicrobial drugs to be less effective or even ineffective. In recent years, various strategies have been proposed to overcome antibiotic resistance. One of the proposed strategies to achieve this goal is the use of plant extracts or compounds found in plants. Another strategy is to combine other molecules with antibiotics to which bacteria have developed resistance. This apparently restores the desired antibacterial activity. These molecules can be non-antibiotic drugs with potential antibacterial properties that can create an opportunity for innovative therapeutic approaches and bring various benefits such as increased efficiency, reduced effective dose, and reduced adverse effects of drugs (Khameneh et al., 2019).

Over the past ten years, the effectiveness of plant compounds in combination with antibiotics has been proven in various studies. Active compounds such as plant extracts have also been recognized as important sources of biofilm destruction. Herbal medicines and their derivatives not only play an effective role in the treatment of biofilm-based diseases but also have fewer side effects compared to chemical drugs. The inhibition of biofilm formation can be explained by the presence of compounds such as the flavonoids quercetin, kaempferol, naringenin, and apigenin, which have the ability to reduce biofilm synthesis by suppressing the activity of the quorum-sensing system and inhibiting cell-cell communication. These compounds suppress the expression of genes involved in bacterial biofilm formation (Mohammadzadeh et al., 2016). Ginger is the most widely consumed and consumed dietary spice in the world. Ginger is rich in flavonoids and polyphenolic compounds such as gingerols, shogaols, zingerone, paradol, terpineol, terpenes, borneol, geraniol, limonene, linalool, and alpha-zingiberene (Urge et al., 2023). Gingerol, a phenolic compound, is one of the most active compounds extracted from ginger and is known for its aromatic and therapeutic effects. Gingerol has attracted attention due to its low toxicity and a wide range of biomedical applications, including antiproliferative, antifungal, antioxidant, anti-inflammatory, antibacterial, and anti-biofilm effects (Pashizeh et al., 2024). In the present study, the effect of gingerol on inhibiting the growth and biofilm formation of *P. aeruginosa* isolates is investigated.

Materials and Methods

Sample collection, isolation and identification of bacteria

Clinical isolates of *P. aeruginosa* were collected from burn samples in Guilan province and transferred to the laboratory. To confirm the diagnosis and purification of bacteria, various biochemical tests were performed, including the ability to produce hemolysin, oxidase, catalase, urease, ability to grow at 42 °C, growth pattern in MacConkey, TSI, SIM and Mueller-Hinton agar media. Also, colony morphology and bacteria and the type of staining in Gram staining were examined.

Antibiotic susceptibility test of *P. aeruginosa* isolates

Determination of the antibiotic resistance pattern of *P. aeruginosa* isolates was performed by antibiogram test (disk diffusion) and according to CLSI standards. The antibiotic discs used included amikacin, gentamicin, ceftazidime, cefotaxime, ceftriaxone, cefoxitin, imipenem, meropenem, piperacillin, erythromycin, azithromycin, cotrimoxazole, ciprofloxacin, and enrofloxacin. Then, isolates that were resistant to more than two antibiotic classes were determined as multidrug-resistant (MDR) strains.

Phenotypic evaluation of biofilm production ability of isolates by microplate method

To examine the biofilm formation ability, first, a suspension equivalent to half McFarland was prepared from the studied isolates in trypticase soy broth (Merck, Germany) containing 1% glucose. Then, 200 µl of this culture was added to each ELISA well and the microplate was placed in an incubator at 37°C for 24 hours. After incubation, the wells were washed three times with PBS to remove non-adherent bacteria. Next, the remaining bacteria attached to the wells were fixed using 250 µL of 96% ethanol for 15 minutes. After that, each well was stained with 200 µL of 0.2% crystal violet and after 5 minutes, the stain was washed off with distilled water. After the plates were dried, quantitative analysis of the biofilm was calculated by adding 200 µL of 33% glacial acetic acid to each well and reading their OD at 492 nm using an ELISA reader. In the evaluation of the biofilm formed based on the optical absorption, the strains were categorized as strong biofilm producers ($OD > 1.500$), moderate biofilm producers ($0.500 < OD \leq 1.500$), and biofilm negative ($OD < 0.500$) (Shafie et al., 2014).

Investigation of the inhibitory effect of gingerol on clinical isolates of *P. aeruginosa* Disk diffusion method

In order to investigate the antimicrobial effect of gingerol against standard and clinical isolates of *P. aeruginosa*, the disk diffusion method was used. For this purpose, first, Mueller Hinton agar medium was prepared. Then, 100 µl of the microbial suspension with a turbidity of 0.5 McFarland was cultured as a lawn on Mueller Hinton agar medium. After cultivation, discs impregnated with 500 µg of gingerol were transferred to culture under sterile conditions using sterile forceps. After placing the discs in a closed plate, they were kept in an incubator at 37°C for 24 hours. After 24 hours, the plate was examined and the diameter of the zone of inhibition was measured using a ruler. This experiment was performed in 2 replicates.

Investigation of the Effect of gingerol on *pslA* gene expression

RNA was extracted from *P. aeruginosa* isolates treated with a concentration of gingerol below the minimum inhibitory concentration (MIC) using a CinnaGen kit. Bacterial cultures without antimicrobial agents were also used as a control. The extracted RNA was immediately used to synthesize cDNA using a CinnaGen RT kit. Specifically, one microgram of extracted RNA was added to 1 µl of random hexamer primer, and the volume was adjusted to 10 µl with DEPC-treated water in a nuclease-free microcentrifuge tube. This mixture was incubated at 65°C for 5 minutes and then placed on ice for 2 minutes. After gentle vortexing, 10 µl of cDNA synthesis mix was added. Following mixing, the microcentrifuge tube was incubated at 25°C for 10 minutes, followed by 60 minutes at 42°C. The reaction was terminated by heating at 85°C for 5 minutes. The integrity of the cDNA synthesis reaction was confirmed by agarose gel electrophoresis. The synthesized cDNA was then used as a template in a real-time PCR reaction. In this study, the rpsL gene was used as a reference gene. The gene-specific primers, with sequences reported by Oliveira et al. (10), were custom synthesized in lyophilized form by Metabion (Germany). The nucleotide sequences of the primers used are shown in Table 1 (or "in the table below," if you prefer).

Polymerase chain reaction was performed in a volume of 20 µl using the Genet Bio kit CAT.NO:Q9210 (South Korea) according to the following protocol:10 µl of 2x master mix containing SYBR Green, 1 µl of forward and reverse primers (10 µM),1 µl of template cDNA (1 µg),7 µl of distilled water. The thermal cycler program consisted of an initial denaturation step at 95°C for 1 minute, followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The change in gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. This experiment was performed in 2 replicates.

Results

Antimicrobial effect of gingerol against *P. aeruginosa*

In a disk diffusion assay, gingerol exhibited a growth-inhibitory effect against all tested bacteria. The diameter of the zone of inhibition produced by 500 µg of gingerol ranged from 14-17 mm in the examined isolates. The minimum inhibitory concentration (MIC) of gingerol against the P. aeruginosa isolates ranged from 512-1024 µg/mL (Table 2).

Inhibitory effect of Gingerol on biofilm formation

Treatment of *P. aeruginosa* isolates with gingerol at a concentration of 1/2 MIC resulted in a reduction in biofilm formation. Gingerol resulted in a 44-52% reduction in biofilm formation in the tested bacteria (Figure 1).

Investigating the effect of Gingerol on the expression of biofilm-associated genes

Examining the expression level of the pslA gene in *P. aeruginosa* isolates treated with a sub-MIC concentration of gingerol resulted in a significant decrease in gene expression. Gingerol decreased *pslA* expression in P. aeruginosa by more than 52% compared to the control ($p < 0.05$) (Figure 2).

Discussion

The emergence of drug resistance in bacteria is a major global health challenge. Within biofilms, bacteria can communicate via quorum sensing, exhibiting greater resistance to antibiotics, disinfectants, and detergents compared to their planktonic counterparts (Foroughi et al., 2022). *P. aeruginosa* is an opportunistic pathogen, and its increasing multidrug resistance poses significant clinical problems. Given the importance of biofilms in the pathogenesis and antibiotic resistance of *P. aeruginosa,* the search for novel antimicrobial agents capable of eradicating biofilm-producing bacteria at lower concentrations is crucial. In this context, medicinal plants offer a promising avenue. Plants with antimicrobial effects, acting through mechanisms distinct from those of conventional antibiotics, can inhibit bacterial growth. This underscores the need for comprehensive research into the potential of medicinal plants (Aminnezhad et al., 2016). Plants produce a wide array of secondary metabolites with antimicrobial potential. Their antimicrobial, antifungal, antiparasitic, and antiviral activities have been investigated and confirmed in various studies. Many medicinal plants not only contribute to the treatment of infectious diseases but may also mitigate some of the adverse effects associated with antibiotic use (Foroughi et al., 2022). In this study, *P. aeruginosa* isolates exhibiting multiple antibiotic resistances and biofilm production were selected for investigation following isolation and laboratory identification. Gingerol inhibited the growth of these drug-resistant isolates, with minimum inhibitory concentrations ranging from 512-1024 µg/mL. Previous studies have reported the inhibitory effect of ginger extract on the growth of bacteria causing urinary tract infections, including *P. aeruginosa* (Momeni and Zamanzad 2009). Furthermore, ginger extract has been shown to inhibit the growth of *Staphylococcus aureus*, *Klebsiella pneumoniae,* and *Escherichia coli* (Abdollahy et al. 2000). However, the anti-biofilm properties of ginger extract and gingerol remain relatively under-explored. In the present study, treatment of P. aeruginosa isolates with gingerol at a concentration of 1/2 MIC resulted in a reduction in biofilm formation. Gingerol resulted in a 44-52% reduction in biofilm formation in the tested bacteria.

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Table 1: Name and nucleotide sequence of primers used in the study

Gene	Primer Nucleotide Sequence (5'-3')	Length (bp)	Reference
pslA	TCCCTACCTCAGCAGCAAGCTGGT (F) CGGATGTCGTGGTTGCGTACCAGGTAT (R)	198	Oliveira et al., 2020
rpsL (reference)	GCAACTATCAACCAGCTGGTG (F) GCTGTGCTCTTGCAGGTTGTG (R)	231	Oliveira et al., 2020

Table 2. Diameter of inhibition zone (IZ) (in mm) and MIC (µg/mL) of Gingerol against *P. aeruginosa.*

Bacterial No.	Gingerol	
	IZ	MIC
P. aeruginosa 19429	14	1024
P1	15	1024
P2	15	1024
P3	17	512

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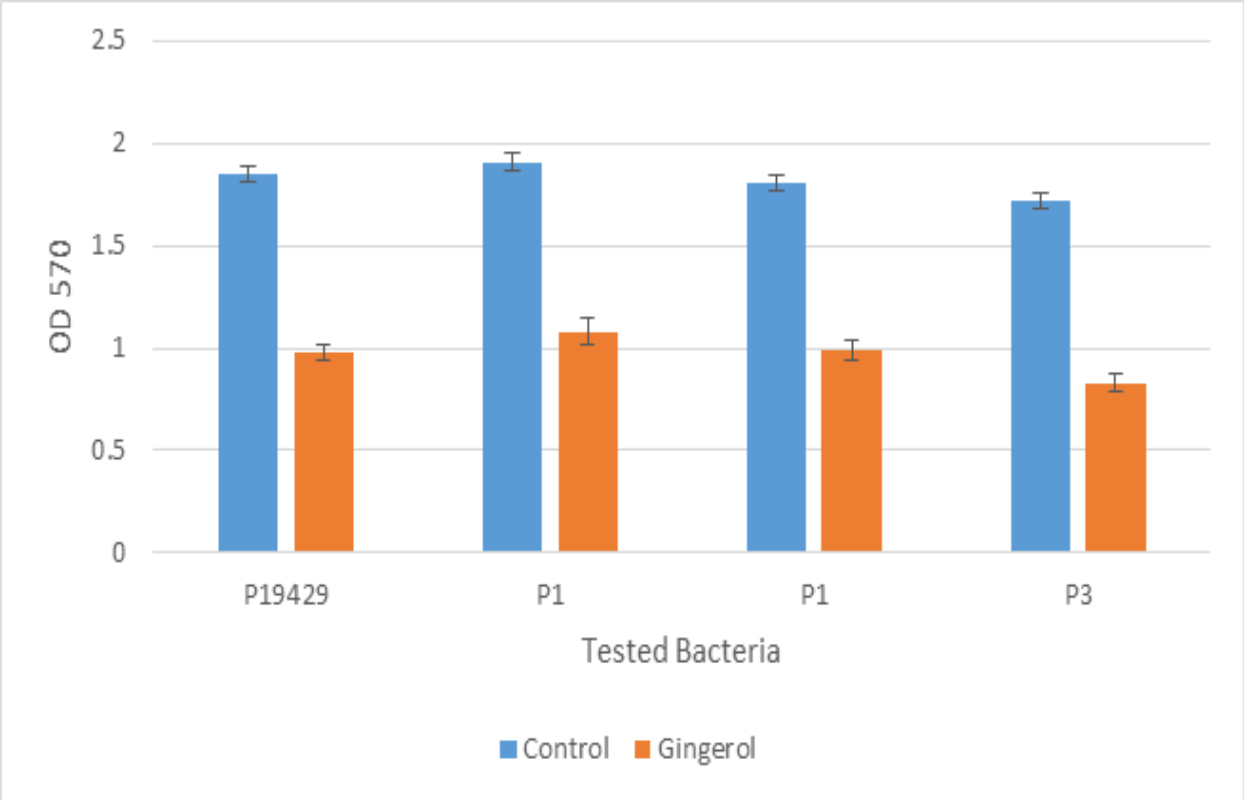

Fig. 1. Anti-biofilm activity of gingerol against *P. aeruginosa* isolates. Mean±SD of OD570 of bacteria treated with a sub-MIC concentration of

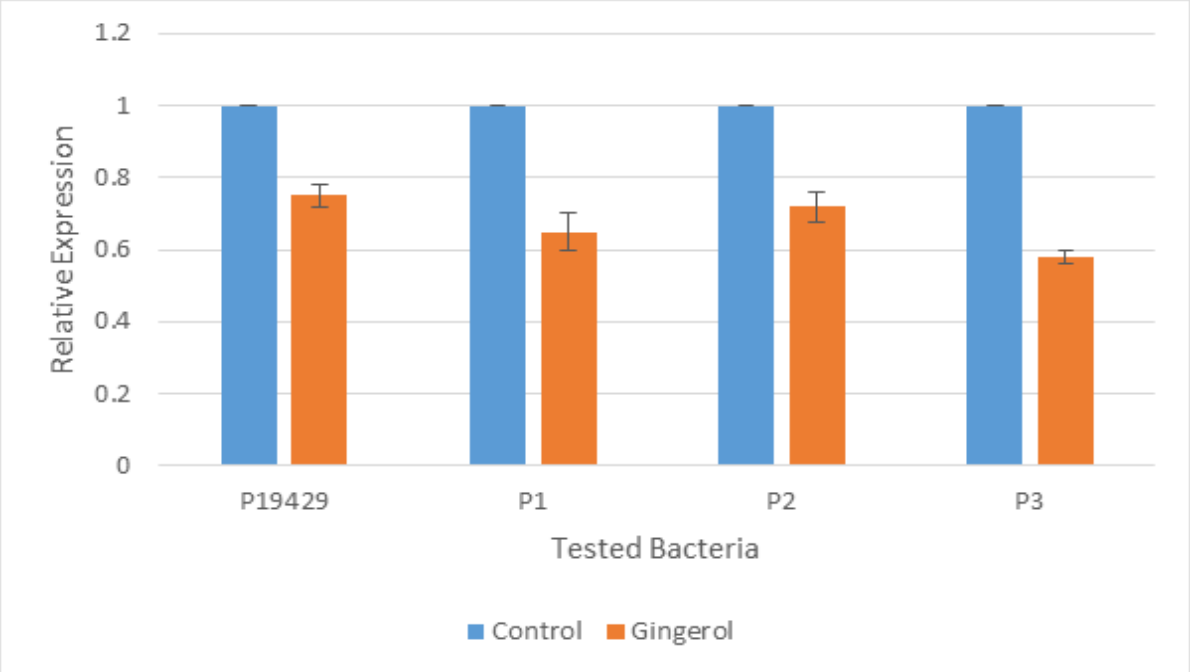

Fig. 2. *pslA* gene expression analysis in *P. aeruginosa* treated with a sub-MIC concentration of gingerol.

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