RESEARCH ARTICLE

Efficiency of Phenazine Compound Produced by Rhizospheric *Pseudomonas fluorescens* against Few Pathogenic Bacteria Isolated from Ear Infections

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ABSTRACT

The most typical bacterial infection in children of pediatric age is acute otitis media. Because resistance to these microorganisms grows, management expenses for each pathogen must be specifically adapted to that pathogen. *Pseudomonas fluorescens* isolated from the rhizosphere rice soil revealed a higher level of antibacterial activity against ear infection pathogenic bacteria than other species of *Pseudomonas.* Phenazine was extracted *P. fluorescens* by using organic solvents such as benzene with 3.76 µg/ml then characterized with thin layer chromatography and gave a band with a retardation factor (RF) of 0.64 based on the compound's mobility on the silica plate. In comparison to the antibacterial activity of crude phenazine, the purified phenazine revealed the highest activity against *Staphylococcus aureus* at 32 mm, and the lowest activity was recorded on bacteria *P. aeruginosa* with an 18 mm diameter of inhibition. This leads to the encouragement of the use of phenazine compound for the treatment of the increased ear infections due to multi-resistance by causing pathogens.

Keywords: Ear infections, Phenazine, Rhizosphere rice soil.

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INTRODUCTION

There are three clinical stages to acute otitis media: The most severe symptoms and indications arise during the first phase, which is the exudative inflammatory phase.[1] A sense of fullness in the ear is brought on by eustachian tube obstruction. The causes of that include tubal kinematic abnormalities and nasopharyngeal obstructive processes. Pus and middle ear exudate are spontaneously ejected during the second phase, which is characterized by resistance and demarcation. Antibiotic medication during this stage is ineffective and unable to stop the perforation of the tympanic membrane.[2] The discharge dries up and the hearing returns in the final stage, which marks the beginning of healing. The most prevalent method of infection is by the tubal route, followed by the hematogenous method, which also includes measles, scarlet fever, and septicemia.[3] Recurrent ear infections are caused by mixed aerobic and anaerobic microorganisms, primarily *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Certain bacteria will not be killed by antibiotics when utilized *in-vivo* or *in-vitro*.[4,5] A biofilm is a collection of microorganisms that are bonded to a surface or one another within an exopolysaccharide matrix. The inability of conventional antibiotics to treat recurrent infections may be explained by the creation of biofilms.[6]

One class of antibiotic is phenazine, a heterocyclic redox agent that contains nitrogen.[7] Phenazines had been investigated as secondary metabolites produced by bacteria with broad-spectrum antibacterial activities that contributed to bacterial pathogenicity.[8] Many phenazines generated by fluorescent *Pseudomonas* researched extensively for their biological function. However, some bacteria, including gram-positive and gram-negative species, such as *Nocardia, Erwinia, Burkholderia, Pantoea,* and *Vibrio*, were also capable

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of producing phenazine.[9] Phenazine and its derivatives are employed in medical applications as antifungal agents against some microbes, such as *Candida albicans* and *Aspergillus fumigatus*, which are risk factors for pulmonary candidiasis in people with *P. aeruginosa* colonization.[10] The objectives of this study were to identify the *Pseudomonas* species that produce phenazine, extract phenazine, and assess phenazine's effectiveness as an antibacterial agent against ear pathogenic bacteria.

MATERIALS AND METHODS

Isolation and Identification of *Pseudomonas* spp.

A total of 22 soil samples from the rhizosphere of rice were collected from various locations in the farmland. Each sample was suspended in 10 mL sterile distilled water and aggressively agitated for 10 minutes. The surface of the sample was then treated with 0.1 mL of the suspending liquid. On MacConkey agar and *Pseudomonas* agar plates, each sample was infected, and the plates were subsequently incubated for 18 to 24 hours at 30°C. Testing of the expanding colonies' physiological and biochemical processes was conducted.[11] The VITEK 2 system verified these isolates.

Isolation and Identification of Pathogenic Bacteria

A total of 19 samples were taken from patients with various forms of ear infections. All samples were taken using sterile, clean swabs under the supervision of trained medical professionals. All of the isolates were first cultured on nutrient agar, and then they were recultured using the *S. aureus*-specific mannitol salt agar, which is then incubated for 24 hours at 37°C. Other isolates were recultured using the gram-negative bacteria-only MacConkey agar. The VITEK 2 system is used to confirm bacterial isolates after the diagnosis is mostly based on the morphological, microscopic, and biochemical characteristics of the colonies.[11]

Screening for Antimicrobial Activity of *Pseudomonas* spp.

By inoculating bacteria in 100 mL of King's B broth and incubating them at 30ºC for 72 hours in a shaker incubator spinning at 150 rpm, the primary screening for *P. fluorescens* isolate with antibacterial activity was carried out. After the incubation, the culture was centrifuged at 6000 rpm for 15 minutes, and filtered the supernatant with millipore filter paper (0.22 µm) by utilizing the agar well diffusion method to identify the antibacterial activity against ear infection bacterial isolates. With a sterile cork borer, 5 mm diameter wells were punched into the agar. Using distilled water alone as a negative control, 100 µL of the produced filtrate was added to the wells of agar plates containing the test microorganisms and incubated at 37ºC for 24 hours, after the incubation period the zone of inhibitory was measured.[12]

Extraction of Phenazine

The selected isolate was cultured at 37°C in King's B broth agar (KBA) medium in a rotary shaker for 3 days. The culture liquid was used to extract phenazine as follows: After centrifuging five mL of culture at 5000 rpm for 30 minutes, the supernatant was adjusted to pH 2 with 0.1 N HCl. Samples were mixed with 5 mL of benzene for 1-hour, then centrifuged at 5000 rpm for 30 minutes. The benzene layer was decanted into 4 mL and allowed to air dry. After resuspending the samples in 1-mL of 0.1 N NaOH, the absorbance at 248 nm was measured.[13] In comparison to the phenazine antibacterial standard curve, the concentration of phenazine was estimated.

Characterization of Phenazine

Samples were examined using the sheet (silica gel 20 x 20 cm). One centimeter from the plate's bottom edge, a slotting line was marked. In 20 µm of the sample were applied to a thin layer chromatography plate coated with a 250 mm layer of silica gel used to analyze the sample and created with phenazine as the solvent system in a 9:1 v/v ratio of chloroform to methanol. The plate was taken out and inspected under UV light at 254 nm after drying. The spot was then scrubbed with a spatula, dispersed in benzene solvent, and allowed to dry in a desiccated vacuum at 40°C before being dispersed in methanol.[14] Each dye's RF value was calculated using the following formula: RF is the product of component and solvent travel distance.

Antibacterial Activity of Phenazine

The agar well diffusion method was used to differentiate between the effects of phenazine by spreading 0.1 mL of each ear pathogenic isolate, such as *K. pneumoniae, P. aeruginosa*, and *S. aureus*, on Muller Hinton agar. Wells of 5 mm diameter was then punched in the agar loaded with 100 µL of purified phenazine, and a control well was made in the center of the plate filled with methanol and used The resulting inhibitory zone was measured on a millimeter scale.[15]

RESULTS AND DISCUSSION

Isolation and Identification of *Pseudomonas* spp.

Different species of *Pseudomonas* were detected following the culturing of rhizosphere rice soil samples on *Pseudomonas* agar medium. Of them, 6 (55%) isolates of *P. fluorescens*, 2 (18%) isolates of *P. aeruginosa*, and 3 (27%) isolates of *P. putida* were found (Figure 1).

Numerous investigations indicated that the presence of numerous bacteria in the rhizospheric zone was caused by the

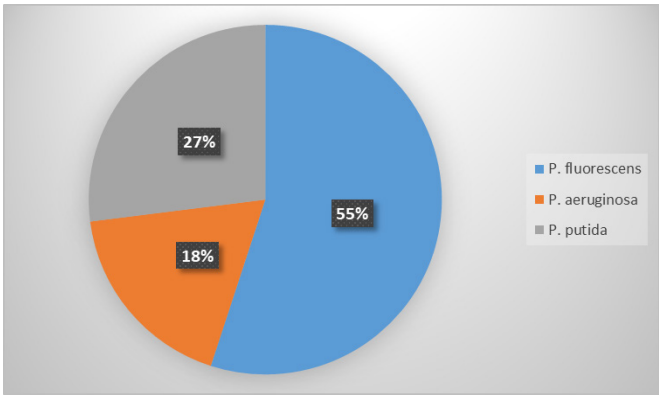

Figure 1: Distribution of *Pseudomonas* in rhizosphere rice soil

roots' excretion of exudates that contained diverse proteins, sugar molecules, amino acids, organic acids, and secondary metabolite compounds.[16] Through a variety of physical, chemical, or biological interactions, these exudates are known to establish a network of contacts between plant roots and the rhizospheric microorganisms that surround them.[17]

Isolation and Identification of Pathogenic Bacteria

On various selective media, 19 samples of various ear infection pathogens have been collected and isolated. The findings showed that there were 11 isolates total, with 3 isolates of each genus (*S. aureus* and *K. pneumonia*e) and 5 isolates of *P. aeruginosa*, as shown in Figure 2.

Acute or chronic otitis externa are the two categories. A chronic infection is one in which the symptoms or signs persist for longer than three months. The chronic form is primarily fungal or allergic in origin or a symptom of dermatitis.[3] The acute form is typically caused by excess moisture or local trauma, is 90% bacterial in origination, and the remaining 10% is caused by a fungal infection. The majority of instances of otitis externa are caused by *P. aeruginosa*, while *S. aureus* is the second most prevalent infection.[18]

Screening for Antimicrobial Activity of *Pseudomonas* spp.

Filtrates from 11 isolates of *Pseudomonas* spp. were used in agar well diffusion procedures to investigate the antibacterial activity of these isolates against the tested pathogens. According to the data, the majority of *P. aeruginos*a and *P. putida* isolates lack any antimicrobial action against the pathogenic bacteria under test, and the remaining isolates only exhibit modest levels of such activity. In contrast, all isolates of *P. fluorescens* are antimicrobial efficient against the tested bacterium, with two isolates having greater activity as shown in Table 1. The highest activity for *P. fluorescens* 3 was measured against *S. aureus* with a diameter of inhibition of 23 mm, and the lowest activity was measured at 14 mm against *P. aeruginosa.*

P. aeruginosa isolated from soil showed good activity against several pathogenic bacteria.[19] Mostly from bacteria, antimicrobial substances for clinical application have been identified. Additionally,[20] noted that *S. aureus* was significantly inhibited from growing by the putative antibiotic compounds isolated from *P. aeruginosa*.

Extraction of Phenazine

Phenazine was extracted from *P. fluorescens* by using organic solvents such as benzene and the concentration of extracted phenazine was quantified from the respective standard curve of phenazine and recorded as 3.76 µg/mL. The color of the culture media changed over the incubation time, indicating the production of organic compounds by *P. fluorescens*. The presence of several compounds, including phenazine (PHZ), phenazine -1-carboxylic acid (PCA) and pyocyanin (PYO), as well as their extraction with an equal volume of benzene and chloroform, was indicated by the development of blue, orange, and lemon yellow hue in liquid KBA medium throughout a range of incubation times.[21] The blue-green solution of *P. aeruginosa* in KA liquid medium and the crude extraction eluted with dichloromethane and methanol revealed yellow or yellow-green and blue hue indicating phenazine and pyocyanin synthesis.[22-24]

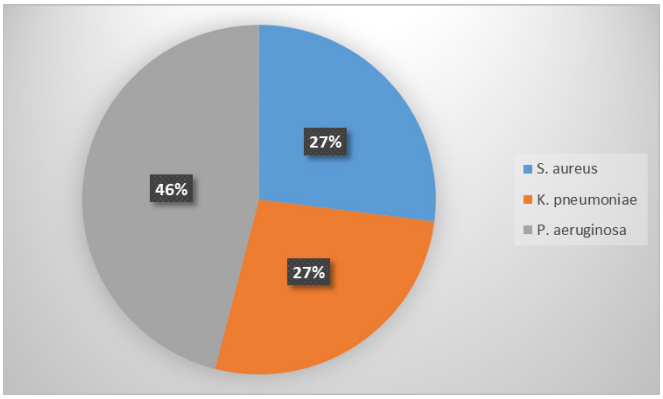

Figure 2: Percentages for the presence of ear infection pathogenic bacteria

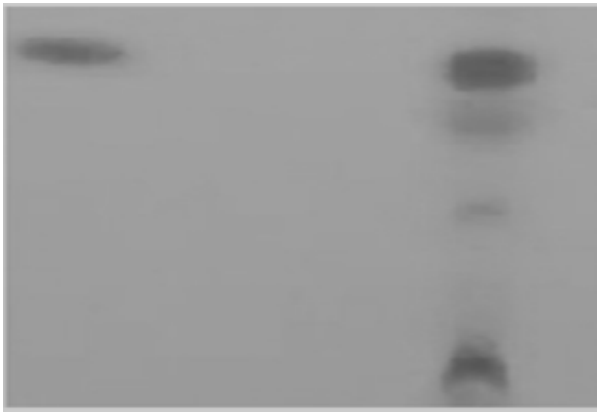

Figure 3: Thin layer chromatography for purified phenazine and the standard phenazine antibiotic

Characterization of Phenazine

The finding in Figure 3 demonstrated that the isolate *P. fluorescens*3 produced phenazine that gave a band with a retardation factor (RF) of 0.64 based on the compound's

Table 1: Antibacterial activity of *P. fluorescens* isolates against pathogenic bacteria causing ear infections

Isolate	*Diameter of inhibition zone(mm)*				
	P. fluorescens1	*P. fluorescens2*	*P. fluorescens3*	*P. fluorescens4*	*P. fluorescens5*
P. aeruginosa	11	12	14	11	10
S. aureus	15	20	23	19	16
K. pneumoniae	13	15	18	14	17

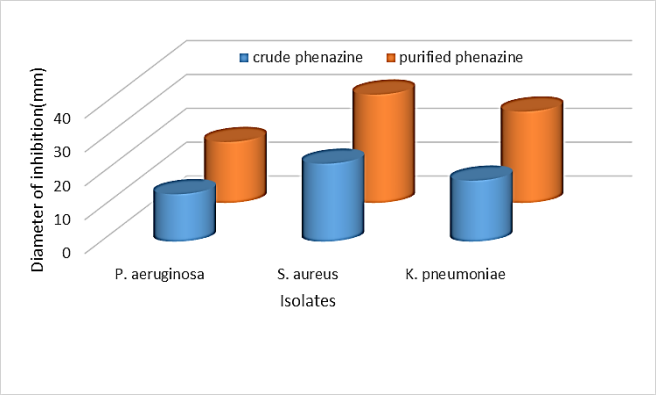

Figure 4: Antimicrobial activity of crude and purified phenazine against ear infection pathogenic bacteria

mobility on the silica plate. According to Viviana *et al*., (2015). The RF value of 0.7 corresponds to the phenazine antibiotic in a 9:1 v/v solvent solution containing chloroform and methanol. The outcomes were discovered to be comparable to those discovered by (22) using TLC analysis to purify phenazine with RF 0.70.

Antibacterial Activity of Phenazine

Agar well diffusion was used to test the pure phenazine's antibacterial activity against pathogenic bacteria that cause ear infections. The results are summarized in Figure 4. In comparison to the antibacterial activity of crude phenazine, the results showed that the highest activity of phenazine emerged on bacteria *S. aureus* was 32 mm, and the lowest activity was recorded on bacteria *P. aeruginosa* was 18 mm.

Numerous investigations indicated that *P. aeruginosa* might develop a variety of secondary metabolic products that could play a significant role in controlling infections, one of which was the broad-spectrum bacterial and fungicidal chemical phenazine.[25] The mechanism of action of phenazine was considered to be that they diffuse across or insert into the membrane and function as a reducing agent, causing the uncoupling of oxidative phosphorylation and producing poisonous intracellular superoxide radicals and hydrogen peroxide that are bad for the organisms.[26-28]

CONCLUSION

Encouragement of the use of phenazine compound for the treatment of the increased ear infections due to multi-drug resistant pathogens.

REFERENCES

1. Behrbohm H; Kaschke O; Nawka T and Swift A : Ear, Nose, and Throat Diseases with Head and Neck Surgery 2019, 3rd Ed., Thieme, Berlin.
2. Al-Shawwa BA and Wegner D Trimethoprim-Sulfamethoxazole Plus Topical Antibiotics as Therapy for Acute Otitis Media with Otorrhea caused by community-acquired Methicillin-Resistant *Staphylococcus aureus* in children. Arch Otolaryngol Head Neck Surg. 2015;131:782-784.
3. Bansal M, Diseases of Ear, Nose and Throat/Head and Neck Surgery, 1st Ed. Jaypee Brothers. New Delhi. 2013
4. Bronner S, Monteil H and Prevost G , Regulation of virulence determinants in *Staphylococcus aureus*: complexity and applications. FEMS Microbiology Reviews, (2004), 28, 183-200.
5. Bazire A,Diab F, Jebbar M, and Haras D, Influence of high salinity on biofilm formation and benzoate assimilation by *Pseudomonas aeruginosa*. J Ind Microbiol Biotechnol , 2007, 34: 5-8.
6. Korres AMN; Aquije GMF, Buss, DS; Ventura, JA; Fernandes P.MB and Fernandes AAR, (Comparison of Biofilm and Attachment Mechanisms of a Phytopathological and Clinical Isolate of *Klebsiella pneumoniae* Subsp. Pneumoniae. Scientific World Journal, 2020, Article ID 925375.
7. Chen, JJ; Chen, W; He H; Li DB; Li WW; Xiong L and Yu HQ, Manipulation of microbial extracellular electron transfer by changing molecular structure of phenazine- type redox mediators. Environmental Science Technology. 2013, 47: 1033–1039.
8. Pierson S and Pierson E, Metabolism and function of phenazines in bacteria:impacts on the behavior of bacteria in the environment and biotechnological processes. Applied Microbiology Biotechnology. 2020, 86:1659–1670.
9. Pan X; Wu J; Xu S; Duan Y and Zhou M, The ankyrin-like protein AnkB interacts with CatB, affects catalase activity, and enhances resistance of Xanthomonas oryzae pv. oryzae and Xanthomonas oryzae pv. oryzicola to phenazine-1-carboxylic acid. Applied Environmental. Microbiology. 2019, 84(4): e02145–e02117
10. Mahamda, HA and Al Alwany AA, Influence of Syphilis Infection on Abortions in Iraq. Journal of Communicable Diseases, 2022. 54(4): 41-45
11. Al Alwany AA, Effect and benefit of percutaneous coronary intervention in chronic total occlusion on ventricular repolarization: QT correction and dispersion, Journal of Medicine and Life, 2022, 15 (8),1025 - 1030, DOI: 10.25122/jml-2022-0207
12. Brooks FG; Carroll KC; Butel S and Morse AS Medical Microbiology, Edetors, J.E.; and Adelberg, E.A. 24th ed. Megraw hill. NewYork. 2007.
13. Thavasi R; Jayalakshmi S and Banat IM, Application of biosurfactant produced from peanut oil cake by Lactobacillus del brueckii in biodegradation of crude oil. Bioresource Technology. 2011,102(3): 3366– 3372.
14. Kavitha K; Mathiyazhagan, S; Sedhilvel V; Nakkeeran S; Chandraseker G and Fernando W, Broad spectrum action of phenazine against active and dormant structures of fungal pathogens and root knot nematode. Archives of Phytopathol. Plant production. 2015, 38(1): 69- 76.
15. Genevieve G; Sharief B; Sebastien R; Tjeerd V; Ben J; Lugtenberg J and Guido V. Pip, a novel activator of phenazine biosynthesis in P. chororaphis PCL 1391. Journal of Bacteriology. 2006, 188(23): 8283-8293.
16. Collee, JG; Duguid, JP; Fraser, AG and Marmion, BP, Laboratory strategies in the diagnosis of infectious syndromes in Practical Medical Microbiology, eds Collee JG, Barrie, MP, Fraser, AG and Simons A, 14th ed. Churchill Livingstone, New York, 1996, 53-93.
17. Rashid MH.; Krehenbrink M and Akhtar MS, Nitrogen-fixing plant-microbe symbioses. In: Lichtfouse E (ed) Sustainable agriculture reviews,vol 15.Springer International Publishing, Switzerland, 2015, 193-234.
18. Nielsen TH; Sorensen D ;Tobiasen C; Andersen JB; Christophersen C; Givskov M. and Sorensen J, Antibiotic and biosurfactant

properties of cyclic lipopeptides produced by fluorescent Pseudomonas spp. from the sugar beet rhizosphere. Applied Environmental Microbiology, 2002, 68: 3416–3423.

19. Al Alwany AA, Echocardiographic Assessment of the Aortic Stenosis Valve Area: Parameters and Outcome. Journal of Medicinal and Chemical Sciences, 2022. 5(7): 1281-1288.
20. Samanta S, Thavasi R and Jayalakshmi, S, Phenazine pigments from P. aeruginosa and their application as antibacterial agent and food colourants. Research Journal of Microbiology. 2008,3(3): 122-128.
21. Zhang L, Tian, X, Kuang S, Liu G, Zhang C, and Sun, C, Antagonistic Activity and Mode of Action of Phenazine-1-Carboxylic Acid, Produced by Marine Bacterium Pseudomonas aeruginosa PA31x, Against Vibrio anguillarum In vitro and in a Zebrafish In vivo Model. Frontiers in Microbiology. 2017, 8: 289.
22. Parvin,W, Othman, R, Jaafar H and Wong, M,. Detection of Phenazines from UPMP3 Strain of *Pseudomonas aeruginosa* and its Antagonistic Effects against *Ganoderma boninense*. Int. J. Agric. Biol., 2016, 18(3): 483–488.
23. Saosoong, K, Wongphathanakul, W, Poasiri C and Ruangviriyachai, C. Isolation and analysis of antibacterial substance produced from P. aeruginosa. TISTR 781. Kkv. Journal of Science. 2009, 37 (20): 163-172.
24. Mahmoud ZH, Hammoudi OG, Abd AN, Ahmed YM, Altimari US, Dawood AH, Shaker R, Functionalize cobalt ferrite and ferric oxide by nitrogen organic compound with high supercapacitor performance, Results in Chemistry,2023, 5, art. no. 100936. DOI: 10.1016/j.rechem.2023.100936
25. Abbas HH, Al-Alwany AA, and FS Dleikh FS, Impact of smoking on cardiac electrophysiological parameters of symptomatic sinus node patients in Iraq. Pakistan Journal of Medical and Health Sciences, 2020, 14(4): 1643-1650.
26. Vinay JU, Naik MK; Rangeshwaran R; Chennappa G; Shaikh S and Sayyed RZ, Detection of antimicrobial traits in fluorescent pseudomonads and molecular characterization of an antibiotic pyoluteorin. 3 Biotechology, 2016 6(2): 227.
27. Jaaffar AKM,Parejko, JA, Paulitz, TC, Weller DM and Thomashow LS,. Sensitivity of Rhizoctonia isolates to phenazine-1-carboxylic acid and biological control by phenazine-producing Pseudomonas spp. Phytopathology, 2017, 107:692–70
28. Jaloud RE, Hassan FF, Al-Aamery RA, Hashim RT, Histopathological and Immunohistochemical Study of Aflatoxin B1 in Freshly Slaughtered Iraqi Sheep Meat, using CD Marker of TNF-α, International Journal of Drug Delivery Technology, 2022, 12 (2), 798 - 804, DOI: 10.25258/ijddt.12.2.58