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.⁴⁵ 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 antimicrobial 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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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. 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.

Screening for Antimicrobial Activity of Pseudomonas spp.

By inoculating bacteria in 100 mL of King’s B broth and incubating them at 30°C 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 37°C for 24 hours, after the incubation period the zone of inhibitory was measured.

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. 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. 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.

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...
roots’ excretion of exudates that contained diverse proteins, sugar molecules, amino acids, organic acids, and secondary metabolite compounds. 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.

**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. pneumoniae) 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. 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.

**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. aeruginosa 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. Mostly from bacteria, antimicrobial substances for clinical application have been identified. Additionally, 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. 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.

**Characterization of Phenazine**

The finding in Figure 3 demonstrated that the isolate P. fluorescens3 produced phenazine that gave a band with a retardation factor (RF) of 0.64 based on the compound’s

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

<table>
<thead>
<tr>
<th>Isolate</th>
<th>P. fluorescens1</th>
<th>P. fluorescens2</th>
<th>P. fluorescens3</th>
<th>P. fluorescens4</th>
<th>P. fluorescens5</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa</td>
<td>11</td>
<td>12</td>
<td>14</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>S. aureus</td>
<td>15</td>
<td>20</td>
<td>23</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>13</td>
<td>15</td>
<td>18</td>
<td>14</td>
<td>17</td>
</tr>
</tbody>
</table>

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

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

CONCLUSION

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

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