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Antibacterial activity of chemical compounds derived from thiosemicarbazones against *Pseudomonas aeruginosa* PN1

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Abstract

Three synthetic chemical compounds derived from thiosemicarbazones (4a, 4c , and 4g) were used to evaluate their antibacterial activities against *Pseudomonas aeruginosa* PN1 strains.

Physiological, biochemical and molecular tests (16S rRNA) were performed to identify the strain PN1.

Three methods for evaluating the bioactive activity of chemical molecules were applied: the diffusion method on MH agar, the microdilution method for determination of MIC and MBC, and the growth kinetics of indicator strains in the presence of chemical molecules.

The results allowed us to conclude that molecules derived from thiosemicarbazones have varying effects against *Pseudomonas aeruginosa* PN1. Molecules 4c and 4g have an inhibitory effect (stationary or slowing bacterial growth) without being bactericidal, while molecule 4a showed a bactericidal effect (decreased bacterial growth).

The MIC of molecule 4a was 62.5µg/ml , and the MBC was 125µg/ml. For molecules 4c , and 4g, the MIC was estimated at 125µg/ml and the MBC was greater than 125µg/ml.

The tested compounds showed interesting and promising antibacterial properties.

Keywords: Thiosemicarbazones, antibacterial activity, CMI, CMB, *Pseudomonas aeruginosa* PN1

I Introduction

Pseudomonas species have a multitude of habitats at their disposal, ranging from soils and waters to plant and animal tissues (Moore *et al.,* 2006). Among these species, *Pseudomonas aeruginosa* is a Gram-negative, strict aerobic opportunistic pathogen widely distributed in various habitats , especially in hospital environments. The survival of *P. aeruginosa* in the environment and hospital settings is due to its intrinsic resistance to natural toxins against this microorganism and its usual resistance to a wide range of antibiotics used in hospitals (Laborda *et al.*, 2022).

The overuse of antibiotics has led to the emergence of antibiotic-resistant pathogens, which is a serious global health problem. Many researchers have focused on developing new molecules that can overcome microbial resistance to antibiotics. Therefore, thiosemicarbazones are a class of chemical compounds derived from thiosemicarbazide, an organic molecule containing sulfur and nitrogen. They have a structure formed by a thioamide and a hydrazone group, giving them unique properties. These molecules are receiving special attention in the field of medicine due to their effective biological effects : Antibacterial, antifungal, antiparasitic, anti-trypanosomal, Antitubercular, Anticancer, Antiinflammtory and analgesic activity Anticonvulsant and antidepressant activity, Antiviral/anti-HIVactivity, Antidiabetic activity…(Jain*et al.*,2012; Alam *et al.,* 2023).

Due to their wide applications as bioactive molecules in the medicinal field, our research group has set the antibacterial activity of three chemical compounds of thiosemicarbazone derivatives towards the strain *Pseudomonas aeruginosa* PN1 as a devalued target.

II Materials and methods

1Chemistry compounds

Three chemical compounds of thiosemicarbazones derivatives were used in this study are:*(E)-2-(4-Nitrobenzylidene)-N-phenylhydrazinecarbothioamide*(**4a**), *(E)-2-((E)-3-(2-Nitrophenylallylidene)-N-phenylhydrazinecarbothioamide*(**4c**) et *(E)-2-(4-(Methylthiobenzylidene)-N-phenylhydrazinecarbothioamide*(**4g**) (figure1).

Thiosemicarbazone derivatives were synthesized from thiosemicarbazide. An article has previously been published describing in detail the method of synthesizing these molecules (Benmohammed *et al*., 2014).

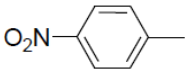
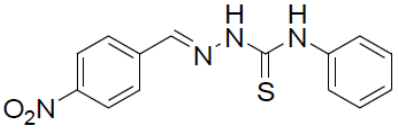
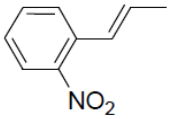
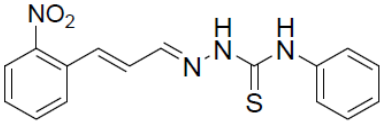
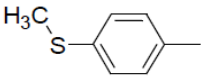
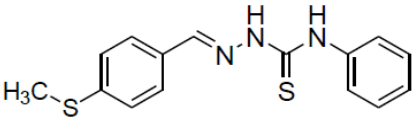
	R	*Structure*
4a		
4c		
4g		

Figure.1: Chemical structure of the three compounds 4-phényl-3-thiosemicarbazones: 4a, 4c and 4g

2 Bacterial Strain

2.1 Isolation, media , and growth conditions

The strain PN1 used in this study has been isolated from agricultural soil in the Mesra province of Mostaganem, Algeria (GPS coordinates; Latitude: 35.8373, Longitude: 0.169785.35° 50’ 14’’ North, 0° 10’ 11’’ East).

The isolation of these bacteria in the soil first involves the enrichment technique in a liquid medium. We carried out the enrichment in vials containing 90 ml of peptone water in 10g of soil. The incubation at 30°C for 24 hours with shaking was used . Cetrimide agar is the culture medium used for isolation (Lowbury and Collins, 1955).

2.2 Phenotypic characterization

After incubation, colonies were picked randomly and purified using the above medium. The purity of the cultures was characterized by observing the appearance of the colonies and the cell composition using Gram staining. For the purified isolates, phenotypic identification was carried out using the following physiological and biochemical tests: Catalase, oxidase, nitrate reductase, arginine dihydrolase, gelatinase, growth in Meat Liver agar (type of bacterial respiration), and growth on King’s media A and B as it appears in the second edition of Bergey’s Manual of Systematic Bacteriology (Garrity *et al*., 2005).

2.3 Genotypic and phylogenetic analysis

Extraction of DNA

Genomic DNA was extracted from bacteria PN1 using the Wizard®, ReliaPrepTM DNA Purification Kit from Promega. The entire manufacturer's recommendations were followed; here are the steps briefly: preparing a 24h bacterial suspension of PN1in nutrient broth.. 2ml of the suspension was centrifuged at 10,000 rpm for 2 minutes. 1ml of bacterial suspension was kept at -20ºC to repeat the experiment if necessary and 1 mL was centrifuged for 2 minutes at 13,000 rpm. The supernatant was thrown away. and the pellet collected and resuspended in 480 µL of TE buffer (10 mMTrisHCl pH 7.6;1 mM EDTA), then with a micropipette 600 µL of nucleic lysis solution and 10 µL of Proteinase K (PK) were carefully added and resuspended (de Brito *et al*., 2022).

PCR amplification

Primers 27F (5'AGTTTGATCCTGGCTCAG-3') and 1492R (5'GTTACCTTGTTACGACTTC-3') were used to amplify the 16S rRNA gene using bacterial DNA.The program respected in the Polymerase Chain Reaction (PCR) is a first hold of temperature at 95°C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30s, annealing at 55 °C for 45s , and extension at 72 °C for 90s. A final elongation is done at 72°C for 10 min (Bekenniche *et al*., 2024).

Phylogenetic analysis

The identical sequence was located in the NCBI nucleotide sequence database (http://www.ncbi.nlm.nih.gov/BLAST) using BLAST.The NCBI 16S microbial database was searched with BLASTn to find the sequence for isolate PN1 with the maximum target_seqs of 20. The results were sorted by value first, then by score, and the highest scoring sequence's taxonomy was published. In Molecular Evolutionary Genetics Analysis (MEGA 11.0), homologous sequences were added to display the phylogenetic positions of strain PN1 and other related species (Kumar et *al.* 2018), available at https://www.megasoftware.net/. Distances from each branch were calculated using: "The Neighbor-Joining Method" by Jukes-Cantor (Saitou and Nei, 1987). The representative sequence of the strain PN1was submitted to the NCBI website (www.submit.ncbi.nlm.nih.gov/subs) to have the GenBank accession number.

3 Sensitivity to antibiotics:

Antibiotic sensitivity was assessed by the agar diffusion method following the general technical conditions recommended by CA-SFM/ EUCAST (2022).The antibiotics used are Erythromycin (E) (15µg, Bio-Rad, France), Chloramphenicol (C) (30µg, Bio-Rad, France), Ampicillin (AM) (10µg, Bio-Rad, France) and Tetracycline(TE) (30µg, Bio-Rad, France).

4 Bio-evaluation of chemical compounds:

To evaluate the effect of chemical compounds on strain PN1, we applied two methods: agar-well diffusion and microdilution.

Both methods are performed according to protocols described by Joffin and Leyral (1997), with minimal modifications.

4.1 Agar well diffusion method:

A solution of each compound at a concentration of 2,5mg/mL was prepared. Solubilization was carried out in dimethyl sulfoxide (DMSO, Acros).

Wells of approximately 6 mm were made on Petri dishes containing Muller Hilton agar (MH) inoculated with bacterial suspension at a final concentration of are~10^6 CFU/ml (microbial suspension of density equivalent to the 0.5 Mac Farland standard diluted 1/100) and filled with a quantity of 50 µl of solutions of chemical compounds. The dishes are left for 1 hour at room temperature and then incubated at 30°C in aerobics for 24 hours. After incubation, the inhibition diameter is measured (Benmohammed, 2015).

4.2 Microdilution method:

96-well U-bottom microplates were used for the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (Kartsev*et al*., 2018). Figure 2 illustrates the operating mode of using the microplate.

The strain PN1 was inoculated in 11 wells containing the compounds at decreasing concentrations.

To obtain decreasing final concentrations, the chemical molecules were diluted using binary dilutions (1/2): 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml, 15.62 µg/ml, 7.81 µg/ml, 3.9 µg/ml, 1.95 µg/ml, 0.97 µg/ml, 0.49 µg/ml, 0.24 µg/ml and 0.12 µg/ml.
Well 12 was used as a negative control: 50 µl of inoculum (10 µl bacterial suspension in MH broth) and 50 µl DMSO without chemical compound.The final cell charge is~10^5 CFU/ml. The microplate was incubatedat 30°C for 24 hours.
After incubation and reading of the results, 10 µl from each well was placed on nutrient agar divided into 12 quadrants.Incubation at 30°C/24h. The results will be read by evaluating the growth in the 12 quadrants.

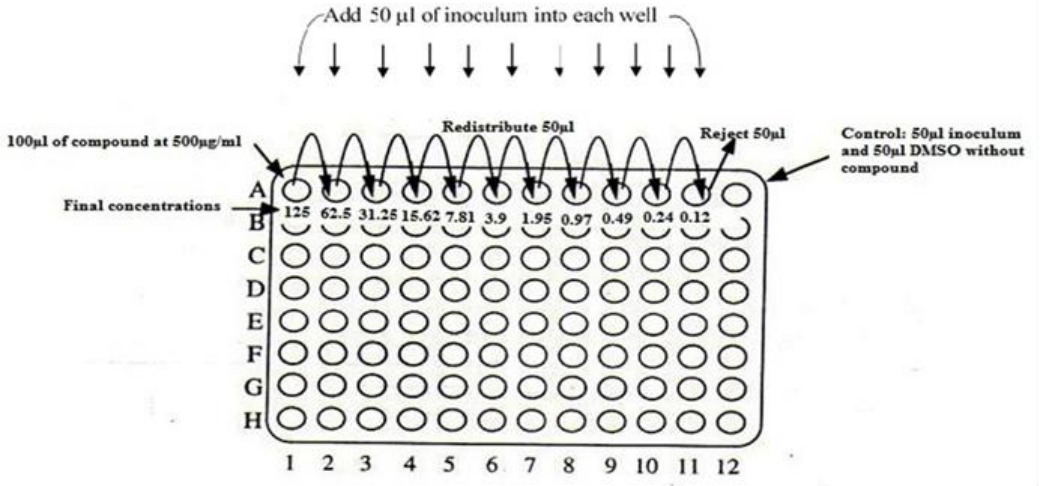


Figure 2 : Schematic of the microdilution method on microplate

4.3 Growth kinetics of strain PN1 in the presence of chemical compounds

Overnight culture of strain PN1 grown in nutrient broth was centrifuged at 8000 rpm for 10 and washed twice in phosphate buffer (pH7.2). The bacterial suspension was adjusted to OD_{600} for a final absorbance of 0.1±0.02 equivalent to a microbial load of ~10^7 CFU/ml (Ghosh and Mukherji, 2021). In 250 ml Erlenmeyer flasks containing 49 ml MH broth, 1 ml bacterial suspension and 50 ml of the chemical compounds.Final concentration of chemical compounds 125µg/ml and the final bacterial load inoculated is ~10^5 CFU/ml. The flasks were incubated at 30°C with shaking. The growth kinetics of strain PN1 in MH medium without chemical compounds and in MH medium with chemical compounds were followed at different times:T_{0h} , T_{1h}, T_{2h} , T_{3h} , T_{4h} and T_{5h} (Tratrat*et al.*, 2022).
Cell viability was determined by counting on nutrient agar. Incubation 30°C/24h to 48h.

III. Results and discussion

1. Bacterial Strain:

1.1 Phenotypic characterization:

The results of macroscopic and microscopic observation of the PN1 strain gave us colonies of green pigmentation on cetrimide agar and Gram-negative rod-shaped cells observed under the optical microscope (figure 3). Cetrimide agar is a differential selective medium that promotes the growth and presumptive identification of *Pseudomonas*.

Cetrimide (cetyltrimethylammonium bromide) is a quaternary ammonium that inhibits the growth of associated microbial flora and minimizes interference with the growth of *P. aeruginosa* (Moore *et al*., 2006). This species stains this medium blue-green by the production of pyocyanin. *P. aeruginosa* is a Gram-negative, rod-shaped bacterium measuring 0.5–1 mm by 1.5–4 mm long and does not form spores (Moore *et al*., 2006).

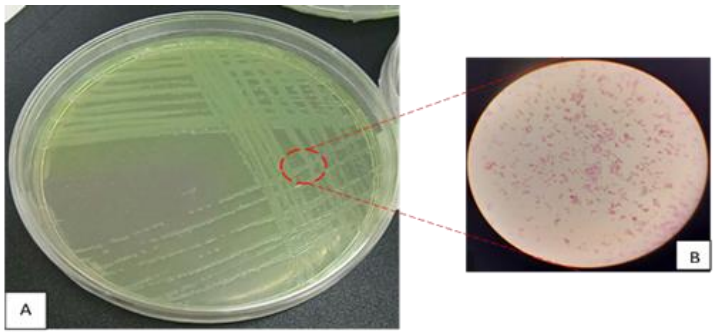


Figure 3 : Macroscopic and Microscopic observation of the strain PN1
A: growth on cetrimide agar medium (incubation at 30°C / 48h in aerobic conditions)
B: observation under optical microscope after Gram staining (X 1000)

The results illustrated in Table 1 confirm the belonging of strain PN1 to the genus *Pseudomonas* according to the phenotypic characters proposed in Bergey's Manual of Systematic Bacteriology (Garrity*et al*., 2005). However, these results did not allow us to confirm the species given that the characters obtained do not allow us to distinguish our strain PN1 between two species *P. fluorescens* and *P. aeruginosa* according to the phenotypic data of the species *P. fluorescens*, *P. aeruginosa* and *P. stutzeri* cited by the authors Ray and Patel (2022).

Table 1:Results of physiological and enzymatic tests of the strain

	Morphological and enzymatic characters										
	colony pigmentation on cetrimide agar	cell shape	Gram	Cat	Ox	Nit	Gel	ADH	TR	King A	King B
Strain PN1	Green	Bacilli	-	+	+	+	+	+	Aero	+	+

Cat: Catalase; **Ox**: Oxydase; **Nit:** Nitrate Reductase; **Gel:** Gelatinase; **ADH**: arginine dihydrolase; **TR**: type of bacterial respiration, **Aero**: Aerobic; **King A (+)**: production of pyocyanin (blue fluoresce); **King B:** production of pyoverdine (pigment fluorescent yellow-green under UV.

1.2 Genotypic characterization:

Genotypic identification by sequencing of the 16S rRNA gene confirmed that our PN1 strain belongs to the species *P. aeruginosa* , with a rate of 99.83% sequence homology . An accession number has been assigned to it (PQ288965).

A dendrogram was performed from the alignment of the 16S rRNA sequences of strain PN1 and the sequences of seven type strains of *Pseudomonas* from the NCBI database: *P. chlororaphis* strain DSM 50083T(Z76673.1),*P. aeruginosa* strain ATCC 10145 (X06684.1),*P. fluorescens* strain ATCC

13525 (D84013.1), *P. pertucinogena* strain ATCC 190 (EF673695.1),*P. putida* ATCC 12633 (D84020.1),*P. stutzeri* strain VKM B-975 (EU883663.1) and *P. syringae* strain ATCC 19310 (AF094749.1) (figure 4).

Phylogenetic distance analysis showed that our strain PN1 was close to 100 nodes from the type species *P. aeruginosa* ATCC 10145 (X06684.1). This confirms the genotypic results obtained by Blast.

*Pseudomonas aeruginosa*is a ubiquitous strain with a genetic repertoire adapted to its habitat. This bacterium is considered one of the most frequent causative agents of nosocomial infections (Green *et al*., 1974). This Gram-negative bacilli bacterium can also be found in various ecological environments such as soil, water, plants , and hospital settings thanks to its great metabolic versatility (Kutshik *et al*., 2022; Ray and Patel, 2022). Several studies on soil flora in different places have shown the species's presence and abundance . *P. aeruginosa* isolates from French agricultural soils, from different soil types and under different land uses (Deredjian *et al*., 2014). *P. aeruginosa* AIN7 isolated from sludge from a sewage treatment pond in an oil field in Algeria (Bekenniche *et al.*, 2021). *P. aeruginosa* AS_23 isolated from oil-contaminated soil in Nigeria **(**Kutshik *et al*., 2022).

P. aeruginosa L3 was isolated from heavy metal-contaminated soil near a smelter in Zhuzhou, Hunan Province, PR China (Xue *et al*., 2024).

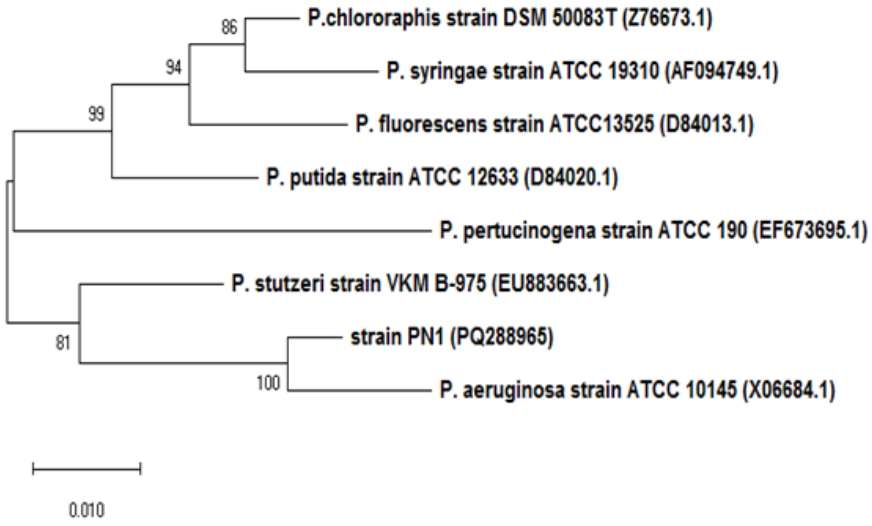


Figure 4 : Dendrogram based on the 16S rRNA gene sequences, showing the relationship swithin the strain PN1 and their positions with representatives of some other related taxa from the NCBI database (*P. chlororaphis* strain DSM 50083T (Z76673.1), *P. aeruginosa* strain ATCC 10145 (X06684.1), *P. fluorescens* strain ATCC 13525 (D84013.1), *P. pertucinogena* strain ATCC 190 (EF673695.1), *P. putida* ATCC 12633 (D84020.1), *P. stutzeri* strain VKM B-975 (EU883663.1) and *P. syringae* strain ATCC 19310 (AF094749.1)). The sequences were aligned using the neighbor-joining method with Jukes-Cantor correction. Bootstrap values (from 1000 replicates) are showed on the nodes. The numbers in brackets mean accession numbers of the strains in the NCBI Genbank database

2 Sensitivity to antibiotics

Strain PN1 shows resistance to all the antibiotics used in this study: Erythromycin (E) (8mm), Chloramphenicol (CL) (8mm), Ampicillin (AML) (8mm) and Tetracycline (TE) (11mm) (figure 5).

These results agree with the data on the sensitivity and resistance of *Pseudomonas aeruginosa* to antibiotics published by CA-SFM/ EUCAST (2022).

One characteristic that sets *P. aeruginosa* apart is its strong resistance to β-lactams, quinolones, and aminoglycosides—three common antibiotics. When dangerous compounds are assaulted , they quickly adapt. The primary causes of antibiotic resistance in *P. aeruginosa* are innate and acquired mechanisms, such as active efflux pumps, reduced membrane permeability, and antibiotic resistance genes (Reem*et al*., 2024). Due to *P.aeruginosa*s capacity to acquire resistance markers through horizontal transfer and develop resistance to almost all antibiotics currently in use, a small number of high-risk, extensively drug-resistant and multidrug-resistant clones have spread throughout the world (Fernandez-Billon *et al*., 2023).As recognized by the World Health Organization, antibiotic resistance has reached a critical global health issue in the twenty-first century, leading to a decline in the efficacy of antibiotics for treating infections.

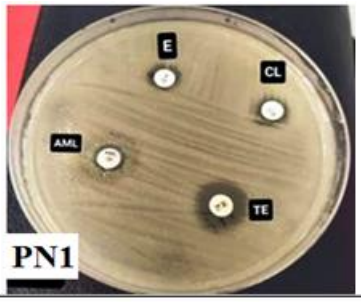


Figure 5 : PN1 strain antibiogram test.
Antibiotics and zones of inhibition: Erythromycin (E) (8mm), Chloramphenicol (CL) (8mm), Ampicillin (AML) (8mm) and Tetracycline (TE) (11mm).

3Bio-evaluation of chemical compounds:

3.1 Agar well diffusion and Microdilution methods

We applied the agar diffusion method to highlight the inhibitory effect of chemical molecules against strain PN1. The results are shown in figure 6. According to the results , the tested products 4a, 4c and 4g seem to have an inhibitory activity against strain PN1. The inhibition diameter size was between 7 and 12 mm.

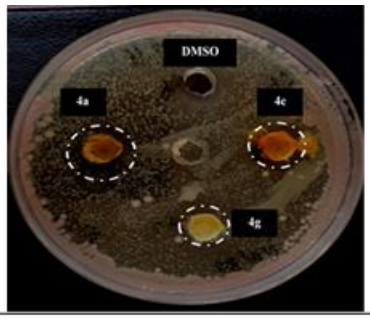


Figure 6 : Evaluation of the inhibitory activity of compounds 4a, 4c and 4g against strain PN1 using the MH agar diffusion method.
The inhibition zone of the compounds: 4a (12 mm), 4c (10 mm) and 4g (7mm).
The DMSO well is a negative control

The MIC and MBC values of the compounds were estimated by observing and comparing the turbidity in the microplate wells and the growth on nutrient agar (Figure 7). According to the results,

the MIC of molecule 4a is 62.5µg/ml and the MBC is 125µg/ml. No significant growth was observed in quadrant 1 of the Petri dish of molecule 4a. For molecules 4c and 4g, the MIC was estimated at 125µg/ml and the MBC greater than 125µg/ml.

This antimicrobial activity has been reported in several scientific works using agar diffusion and microdilution methods. Benmohammed (2015) showed in his study that components of 4-Phenyl-3-Thiosemicarbazones and their derivatives (4b, 4e, 7b and 8b) have inhibitory activities against the strain *Staphylococcus aureus* (ATCC25923). This activity was evaluated using the agar diffusion method. These molecules give inhibition zones on MH agar with of 8 and 15 mm diameters. In another study by Brousse *et al.* (2024), synthetic compounds of 1,3,4-thiadiazolines (TDZ) and their intermediates thiosemicarbazones (TSC) were tested on Gram-positive and Gram-negative bacteria and molds. These authors found antimicrobial activities for several synthesized compound. Screening by the agar diffusion method showed that TSC had similar or stronger activity than TDZ. Out of 22 compounds tested on *Pseudomonas aeruginosa* ATCC9027, 6 (TDZ1, TDZ6, TSC8, TSC10, TSC11 and TSC13) gave inhibition zones of 6 to 8 mm. Aneesrahman et al. synthesized three 5-methoxyisatin thiosemicarbazones and their copper complexes. These compounds were measured as antibacterial and antifungal against many microorganisms by microdilution method. These metal complexes showed higher activity against bacterial strains. Although the complexes were measured to decrease activity against *S. aureus,* although no change in activity could be detected in *P. aeruginosa* at the copper T219 complex, it was found to decrease activity at T220 relative to its ligand. (Aneesrahman *et al*., 2019). In the same line of research, several studies have been conducted on the antimicrobial activity of thiosemicarbazones and their metal complexes (Çakmakçı *et al*., 2023; Fabra*et al*., 2024; Saha *et al*., 2024). These authors found that thiosemicarbazone-based compounds and their metal complexes exhibit antimicrobial activity against various bacterial and fungal strains.

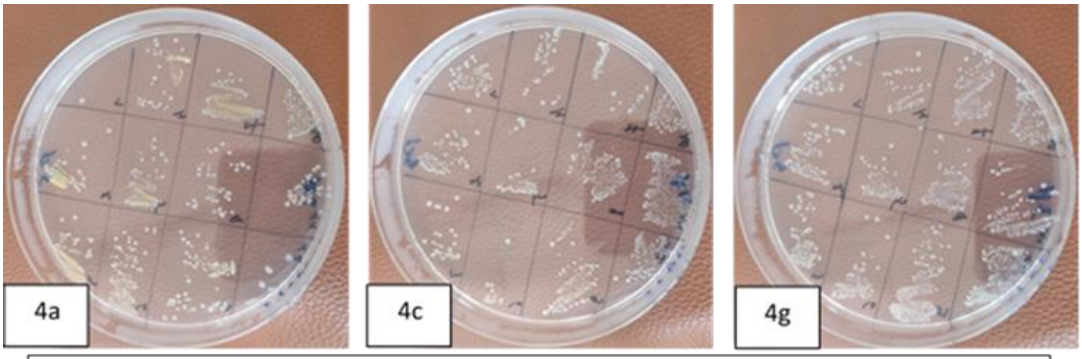


Figure 7: Results of the Microdilution method. Highlight the MIC and the CMB on nutrient agar.Each quadrant on the Petri dish corresponds to the well of the microplate.

3.2 Growth kinetics of strain PN1 in the presence of chemical compounds:

Application of the inhibitory effect of the three thiosemicarbazone compounds on the growth of strain PN1 induced a decrease in the colonies on nutrient agar over time. The results are presented as a curve in Figure 8.

A similar inhibition of the growth of strain PN1 was observed for the three thiosemicarbazone compounds. A significant reduction in the number of CFU of strain PN1 over time was recorded for compound 4a with a rate of 31.08% after 5 hours of incubation. A less significant effect was observed

for compound 4a, where we observed a decrease rate of 6.77%. Compound 4g exerted a slowing effect on the growth of strain PN1. These results provide additional information on the bacteriostatic and bactericidal effect of the three compounds (4a, 4c and 4g). The concertation 125µg/ml of compound (4a) has a bactericidal effect for strain PN1. Both compounds (4c and 4g) with the same concentration have a bacteriostatic effect, except that the growth of strain PN1 is not completely stopped by compound 4g.

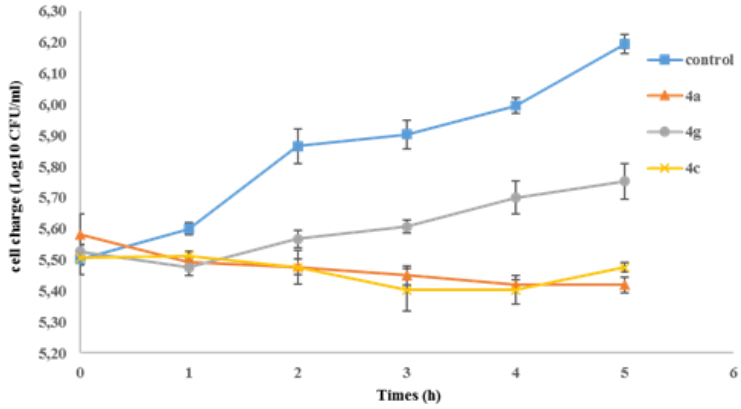


Figure. 8: Effect of compounds on the growth of strain PN1 over time. Five hours of incubation at 30°C. Initial cell charge (T_{0h}) was ~ 3 x 10^5 CFU/ml

IV. Conclusion:

In conclusion, the three chemical compounds of thiosemicarbazone derivatives (4a, 4c , and 4g) have exhibited considerable antibacterial potential against the strain *Pseudomonas aeruginosa* PN1.Compound (4a) at 125µg/ml showed bactericidal activity against the *Pseudomonas aeruginosa* PN1 strain. On the other hand, compounds with the same concentration had shown inhibitory activity against PN1 strain.

These results are promising; however, other complementary tests are necessary to know the mode of action of the molecules on the bacterial cell as well as its non-medical cytotoxicity.

Conflict of interest statement

The authors declare that they have no conflict of interest. All authors have given their final consent for publication.

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