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RESEARCH ARTICLE

IN VITRO ASSESSMENT OF ANTIMICROBIAL PROPERTIES OF THIOSEMICARBAZONE COMPUNDS AGAINST PROVIDENCIA SPECIES

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ABSTRACT

The Thiosemicarbazone derivative of 4-chlorobenzaldehyde represents a promising compound with significant clinical relevance due to its broad-spectrum antimicrobial potential. In this study, two experimental approaches were employed to evaluate its efficacy against both fastidious and non-fastidious microorganisms. Minimum Inhibitory Concentration (MIC) was determined using a colorimetric assay, while antimicrobial susceptibility was assessed via the agar disc diffusion method. Data analysis was carried out using EUCAST and NCCLS guidelines to interpret zone diameters and MIC values. Graph Pad Prism software facilitated the comparison of inhibition zones and MIC values across various antibiotics. Additionally, WHONET 5.6 software was utilized to assess the epidemiological cut-off values (ECOFF) for the test compound, allowing for classification of bacterial isolates as susceptible, intermediate, or resistant. This integrative approach provided a robust assessment of the antimicrobial activity of the test compound in comparison with conventional antibiotics.

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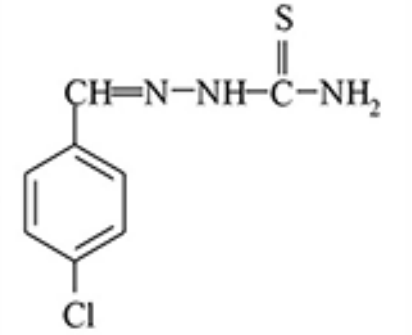
INTRODUCTION

The rise of microbial resistance to conventional antibacterial and anti-fungal agents has become a significant global health concern. This alarming trend has led to an urgent need for the discovery and development of novel therapeutic compounds capable of combating drug-resistant pathogens. Among the various classes of potential antimicrobial agents, Thiosemicarbazone and their derivatives have gained considerable attention due to their broad-spectrum biological activities [1]. In particular, 4-chlorobenzaldehyde-based Thiosemicarbazones have shown promising results in medicinal chemistry, exhibiting antimicrobial, antiviral, antimalarial, and anticancer (including antineoplastic and antimycobacterial) properties. Their structural adaptability, involving both nitrogen and sulfur donor atoms, enables complexation with metal ions, thereby enhancing their pharmacological potential through diverse coordination geometries. In this study, the antimicrobial efficacy of Thiosemicarbazone derivatives synthesized from 4-chlorobenzaldehyde is evaluated, contributing to the ongoing search for effective alternatives to traditional antimicrobial agents.

MATERIALS

An organic crystal of thiosemicarbazone of 4-Chlorobenzaldehyde and Thiosemicarbazone of benzaldehyde was prepared by adopting general procedure [2 - 3].To a hot solution of Thiosemicarbazone in methanol, a solution of benzaldehyde in methanol was added drop wise during thirty minutes. The mixture was stirred and refluxed for 4 hours. It was filtered and the filtrate was concentrated to half the volume. After a slow evaporation of the concentrate at room temperature, Crystals were collected by filtration, washed with cold ethanol and dried in vacuum. The harvested crystals are shown in figure 1. These crystals are suitable for characterization studies.

Structure of Antibiotic-Thiosemicarbazone of 4-Chlorobenzaldehyde



CELL CULTURE

Bacteria had been cultured in liquid medium (DMEM) supplemented 10% Fetal Bovine Serum (FBS), a hundred µg/mL antibiotics, and maintained beneath an environment of five% CO_2 at 37ºC.

MTT ASSAY

Assays that allow for the quantitative assessment of cellular viability and cytotoxicity, particularly those that measure cell death during subculture, are essential in evaluating drug candidates. In preclinical drug discovery, it is crucial to investigate the extent of perturbation a compound may exert on mammalian cell lines, such as CHO or Vero cells. These assessments help verify any cytotoxic effects and provide insight into cellular phenotypes, gene expression, or protein changes. Among these, the MTT assay—first described by Tim Mosmann in 1983—is one of the most commonly used methods for assessing cell viability. This colorimetric assay is based on the reduction of a yellow tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), to purple formazan crystals by mitochondrial NAD(P)H-dependent oxidoreductase enzymes in metabolically active cells. The formazan, an insoluble crystalline product, is solubilized to yield a deep crimson-colored solution. The intensity of the resulting color, measured by absorbance at 560–600 nm using a plate reader, correlates with the number of viable cells. The darker the solution, the greater the number of viable, metabolically active cells.

RESULTS

The antimicrobial efficacy of the synthesized compound TSC4CB was evaluated against *Providencia sp.,* a Gram-negative and fastidious microorganism [4]. The assessment was carried out using several standard methods, including MTT assay, agar disc diffusion, minimum inhibitory concentration (MIC) determination, and data analysis with Graph Pad Prism 8.3, EcoFinder, and WHONET 5.6. The susceptibility results were compared with reference data from EUCAST and NCCLS databases. The TSC4CB compound was synthesized via slow evaporation and recrystallized for purification (Table: 1). The MTT assay was employed to determine the inhibitory effect of TSC4CB on *Providencia sp.,* Optical density (OD) readings were taken at 570 nm to evaluate cell viability, with results analyzed both in the presence and absence of TSC4CB. Colorimetric assessment allowed for the calculation of percentage inhibition using the formula (Table:2).

The compound was tested at varying concentrations ranging from 0.625 µg/mL to 160 µg/mL. The minimum concentration at which maximal inhibition occurred was identified as the MIC, while the MIC break point was determined to be 130 µg/mL(Table:3). TSC4CB exhibited a MIC value of 2.5 µg/mL against *Providencia sp.,* indicating effective susceptibility, as the MIC value was significantly below the break point (Table: 4).For comparison, meropenem was used as a reference antibiotic. According to EUCAST and NCCLS standards, the susceptibility break point for meropenem against Providencia sp. is <2 µg/mL. TSC4CB demonstrated comparable efficacy with an IC_{50} value of 1.03 µg/mL, calculated using the standard formula [5]. Graph Pad Prism 8 software was used for statistical and graphical analysis of the data.

MTT ASSAY TEST

The MTT assay was conducted to evaluate the cytotoxicity of the tested extract concentrations. According to ISO 10993-5 standards, a material is considered cytotoxic if the cell viability falls below 70% compared to the control. The test results, summarized in Table: 4, indicate that all examined extract concentrations maintained cell viability well above this threshold, demonstrating non-cytotoxic behavior. Specifically, cell viability values ranged from 83.2% to 98.6%, confirming the biocompatibility of the material under the experimental conditions.

Table 1. MTT Assay Test

S. No	Tested Sample Concentration (µg/ml)	OD Value at 570 nm (In Triplicates)		
1.	Control	0.49	0.488	0.451
2.	100	0.198	0.201	0.219
3.	90	0.247	0.249	0.229
4.	80	0.253	0.253	0.263
5.	70	0.29	0.273	0.284
6.	60	0.308	0.274	0.292
7.	50	0.32	0.301	0.304
8.	40	0.321	0.31	0.313
9.	30	0.374	0.329	0.321
10.	20	0.375	0.381	0.354
11.	10	0.451	0.453	0.409
12.	5	0.472	0.472	0.418
13.	2.5	0.476	0.475	0.428
14.	1.25	0.464	0.466	0.426
15.	0.625	0.441	0.46	0.42

Table 2. Calorimetrically Study

S. No	Tested Sample Concentration (µg/ml)	Cell Viability (%) (In Triplicates)			Mean Value (%)
1.	Control	100	100	100	100
2.	100	36.56	38.14	42.43	39.05
3.	90	47.62	48.98	44.69	47.10
4.	80	48.98	49.88	52.37	50.41
5.	70	57.33	54.40	57.11	56.28
6.	60	61.39	54.62	58.91	58.31
7.	50	64.10	60.72	61.62	62.15
8.	40	64.33	62.75	63.65	63.58
9.	30	76.29	67.04	65.46	69.60
10.	20	76.52	78.78	72.91	76.07
11.	10	93.67	95.03	85.32	91.34
12	5	98.41	99.32	87.35	95.03
13	2.5	99.32	100	89.61	96.31
14	0.125	96.61	97.96	89.16	94.58
15	0.625	91.42	96.61	87.81	91.94

Table 3. Graph pad Prism 8 Analysis

log(inhibitor) vs. normalized response -- Variable slope	
Best-fit values	
Hill Slope	-0.9369
IC50	0.9026
95% CI (Profile likelihood)	
LogIC50	1.917 to 2.001
Hill Slope	-1.066 to -0.8214
IC50	0.8255 to 0.1002
Goodness of Fit	
Degrees of Freedom	37
R squared	0.9495
Sum of Squares	637.8
Sy.x	4.152
Replicates test for lack of fit	
SD replicates	3.974
SD lack of fit	4.545
Discrepancy (F)	1.308
P value	0.2746
Evidence of inadequate model?	No
Number of points	
# of X values	39
# Y values analyzed	39

ECOFINDER STUDY

The ECOFINDER tool was employed to determine the Epidemiological Cut-Off Value (ECV) for the tested drug against *Providencia sp.,* spp. The Minimum Inhibitory Concentration (MIC) was recorded at 2.5 µg/mL, while the ECV was established at 0.08 µg/mL, representing the 95th percentile of the wild-type distribution for this organism.

Table 4. MIC and MIC Break Point

S. No	Tested sample concentration (µg/ml)	Cell Viability (%) Mean Value
1	160	0
2	150	0
3	140	0
4	130	1.26
5	120	9.48
6	110	30.12
7	100	42.37
8	90	50
9	80	53.13
10	70	58.68
11	60	60.61
12	50	64.24
13	40	65.59
14	30	71.29
15	20	77.42
16	10	91.88
17	5	95.37
18	2.5	96.58
19	1.25	94.94
20	0.625	92.45

Since the observed MIC exceeds the ECV, it indicates that the *Providencia sp.,* strain under investigation likely harbors an acquired resistance mechanism to the tested drug. Consequently, the strain cannot be classified as part of the wild-type population for this antimicrobial agent. Based on this result, the TSCHCB material exhibits resistance when tested against *Providencia sp.,* and potential treatment failure should be considered. Notably, the ECV value of 0.08 µg/mL remains well below the clinical breakpoints set by CLSI and EUCAST, as further illustrated in Figures 2 and 3.

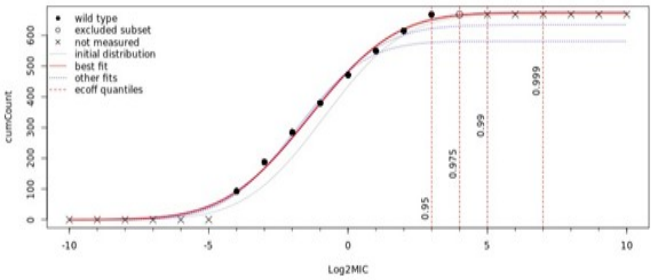


Figure 2. Density Curve

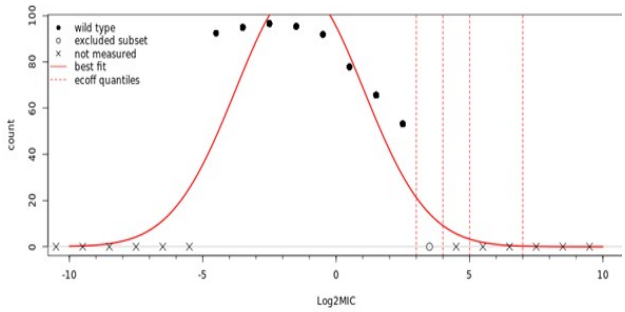


Figure 3. Cumulative curves

WHONET 5.6 SOFTWARE ANALYSIS

The Whonet 5.6 software was employed to assess the susceptibility profile of Providencia sp., isolated from a blood sample. Analysis was conducted using data on multiple antibiotics, including their class, code, method, break point, susceptibility range, isolate number, and resistance pattern [6]. According to the data summarized in Table 5, all antibiotics tested showed resistance, as the MIC values for the isolate were higher than the established breakpoints (Figures 2 and 3). This indicates that the isolate is resistant to the tested antibiotics and that the drugs are ineffective in this context.

Providencia sp.,
Number of isolates = 1
Use expert interpretation rules
Specimen type-Blood

Interestingly, TSCHCB demonstrated MIC values below the respective breakpoints, indicating potential antimicrobial activity against the *Providencia sp.,* isolate. This suggests that TSCHCB may be a viable alternative for treating infections involving resistant strains.From a broader perspective, empirical antibiotic selection should be guided by species-specific resistance trends. *Providencia*species are often intrinsically resistant to several antibiotics, including older generations of penicillins and cephalosporins, with variable susceptibility to agents like aztreonam, imipenem, meropenem, fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole (TMP-SMX). Hence, careful selection and validation through susceptibility testing remain critical in managing such infections.

AGAR DISK DIFFUSION STUDY

The agar disk diffusion method was employed to evaluate the antibacterial activity of TSCA4CB against *Providencia sp.,* Petri dishes containing Mueller-Hinton agar were inoculated with the test organism, and disks impregnated with the TSCA4CB compound were placed on the surface. At an optimal concentration of 2.5 µg/mL[6], TSCA4CB exhibited a maximum inhibition zone diameter of 30 mm against *Providencia sp.,* indicating significant antimicrobial potential. The corresponding zone of inhibition is illustrated in Figure 4.As per EUCAST and NCCLS standards, inhibition zones less than 16 mm are typically considered indicative of resistance [7]. In the present study, TSCA4CB demonstrated an inhibition zone of 30 mm at a concentration of 2.5 µg/mL, signifying strong antibacterial activity against *Providencia sp.,* Compared to conventional antibiotics, TSCA4CB exhibited superior efficacy in vitro. This suggests that TSCA4CB is a promising antimicrobial candidate for treating infections caused by *Providencia*species. These include conditions involving the skin, wound cultures, blood, stool, and potentially serious complications such as purple urine bag syndrome and fever-related infections.

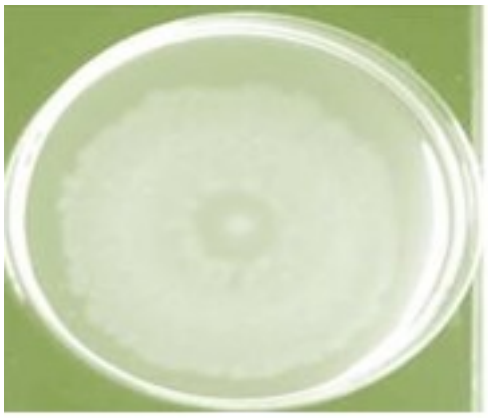


Figure: 5 Agar Disk Diffusion Methods (Inhibition Zone With) *Providencia sp.,* concentration of antibiotic 2.5 (µg/ml) TSC4CB (30 mm)

SUMMARY

Carbonyl-based compounds are known for their broad clinical potential in combating various diseases. In this study, two classes of organic compounds were synthesized and modified via solution growth techniques and evaluated for their antimicrobial activity against ***Providenciasp***including both Gram-positive and Gram-

negative strains. The minimum inhibitory concentration (MIC) for ***Providenciasp***was observed as low as 0.625 µg/mL for the most active derivative, TSCA4CB. Although MIC breakpoints typically range between 1.0 and 13.0 µg/mL,[9] the compounds demonstrated substantial activity below these thresholds.Graph Pad Prism software was used to determine the IC_{50} values, which were consistently below 1 µg/mL (ranging from 0.7 to 0.9 µg/mL), confirming the strong efficacy of TSCA4CB derivatives. The steep dose-response curve and negative log (C_{50}) further support their potent antibacterial effect. Compared to existing antibiotics, TSCA4CB derivatives exhibited superior performance in both inhibition and binding efficiency, likely due to their smaller molecular size and greater ability to penetrate bacterial cells[10,11].WHONET analysis also confirmed that TSCA4CB derivatives outperformed several conventional antibiotics, showing lower MICs and better activity profiles. Furthermore, ECOFINDER analysis revealed that MIC values for TSCA4CB were higher than the ECV (Epidemiological Cut-off Value)[12], indicating that the tested organisms do not belong to the wild-type distribution, and resistance is unlikely. These findings suggest that TSCA4CB compounds hold significant promise as alternative antimicrobial agents with minimal risk of treatment failure.

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