

SYNTHESIS, CHARACTERIZATION, AND IN VITRO BIOLOGICAL INVESTIGATION OF A CYCLOHEXYL-PHENYL HYDRAZONE DERIVATIVE

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Abstract

The increasing prevalence of antimicrobial resistance has intensified the search for novel bioactive compounds with improved therapeutic efficacy. In the present study, a new hydrazone-based Schiff base derivative, 2-((1E)-((cyclohexyl(phenyl)methylene)hydrazono)methyl)-6-methoxyphenol, was synthesized through the condensation reaction of (Z)-(cyclohexyl(phenyl)methylene)hydrazine with 2-hydroxy-3-methoxybenzaldehyde under reflux conditions. The synthesized compound was obtained as a yellowish solid with a yield of 76% and a melting point of 166 °C. Structural elucidation was carried out using FT-IR and ¹H NMR spectroscopic techniques. The FT-IR spectrum exhibited characteristic absorption bands corresponding to azomethine (C=N) functionalities at 1575 and 1618 cm⁻¹, while the ¹H NMR spectrum confirmed the presence of cyclohexyl, aromatic, methoxy, hydroxyl, and azomethine proton environments consistent with the proposed molecular structure. The antibacterial activity of the synthesized Schiff base was evaluated against Staphylococcus aureus and Escherichia coli using the agar well diffusion method. The compound demonstrated promising antibacterial activity, producing inhibition zones of 18 ± 0.5 mm against S. aureus and 14 ± 0.6 mm against E. coli. The observed activity is attributed to the synergistic influence of the azomethine, hydroxyl, methoxy, and aromatic moieties present in the molecule. The results indicate that the synthesized hydrazone-Schiff base possesses significant antibacterial potential and may serve as a valuable scaffold for the development of new antimicrobial agents. Further investigations involving minimum inhibitory concentration, minimum bactericidal concentration, and mechanistic studies are recommended to explore its full pharmacological potential.

1. INTRODUCTION

One of the biggest problems of today's health care system is the development of antimicrobial resistance. Overuse and over prescription of antibiotics have spurred the evolution of resistant bacteria, making many common antibiotic treatments less effective [1]. Pathogenic bacteria still remain one of the main causes of infectious diseases and are a major cause of morbidity and mortality worldwide, especially in developing nations where appropriate treatment may not be available. The quest for new antimicrobial agents with better efficacy and decreased toxicity, combined with other, novel mechanisms of action, has become a very active research area [2-4].

Schiff bases are one of the many classes of organic compounds that have been studied for their potential biological uses owing to their unique structural versatility and wide range of pharmacological activities [5]. The general scheme for the synthesis of Schiff bases involves condensation reaction between a primary amine, hydrazine or hydrazide with an aldehyde or a ketone, and the azomethine ($-\text{CH}=\text{N}-$) linkage is formed. This imine functionality is one of the reasons for the distinct chemical and biological properties of Schiff base compounds. These compounds have been much studied since the pioneer work of Hugo Schiff in the nineteenth century in medicinal chemistry, coordination chemistry, catalysis, analytical chemistry and material science. Schiff base derivatives possess wide range of biological properties such as antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, antitumor, antimalarial and enzyme inhibiting activity. The biological effect of these compounds is usually associated with the presence of the azomethine group, which can have a variety of biological targets through hydrogen bonding, coordination interactions and electronic effects [6-10].

Moreover, Schiff bases are easy to synthesize, structurally versatile, and can be functionalized with a range of groups with potential applications in the field of drug development. Hydrazone based Schiff bases are one of the important subclasses of Schiff bases and have been

extensively studied for its improved biological and pharmacological activities. Both the hydrazone and azomethine functional groups enhance their electron delocalisation and their capability to interact with biological macromolecules. It has been shown in various studies that hydrazone derivatives have antimicrobial properties against gram-positive and gram-negative strains of bacteria [11-14]. These compounds have also exhibited promising applications as anticancer, anti-inflammatory, antitubercular and antioxidants [15]. The biological activity of the hydrazone derivatives of Schiff bases is strongly affected by the nature and position of the substituents attached to the aromatic ring systems which indicates that the structure needs to be modified to optimize their pharmacological properties [16]. Phenolic and methoxy groups with their inclusion into Schiff base structures have received so much attention, due to the fact that the presence of these groups in bioorganisms is known to improve their biological activity. Hydroxy groups on phenols can engage in hydrogen bonding interactions with biological receptors and enzymes, and methoxy groups can enhance lipophilicity and membrane permeability [17-20]. These structural characteristics allow for the easy passage of the molecule across microbial cell membranes and improve the molecule's ability to interact with the intracellular target. Furthermore, the aromatic rings can also be thought to be involved in the process of conjugation and electron delocalization which are known to influence the biological behavior of Schiff base compounds. Organic molecules containing a cyclohexyl group also have received a great deal of attention in the field of medicinal chemistry [21-25]. The conformational flexibility and hydrophobicity of the cyclohexyl group can enhance interactions with biological membranes and interaction with biomolecules. Cyclohexyl groups have been incorporated into many biologically active compounds and found to possess a higher antimicrobial, anti-inflammatory and enzyme inhibitory activity. So, the use of the cyclohexyl unit in combination with the Schiff base and hydrazone structures could be a promising

approach for the synthesis of new bioactive molecules [26-29].

The literature is abundant with examples of the antibacterial activity of Schiff bases. A number of researches have been reported on the antimicrobial activity of Schiff base derivatives with aromatic hydroxyl, methoxy, nitro, halogen and heterocyclic substituents.

The proposed mechanism of antibacterial activity is to disrupt the synthesis of bacterial cell walls, inhibit enzymes that are necessary for the bacteria to survive, interfere with the synthesis of nucleic acids, and change the permeability of the bacterial membrane [30]. Moreover, the azomethine nitrogen atom can form complex with biologically important metal ions present in microbial systems, which can influence their normal cellular activities and inhibit the bacterial growth. *Staphylococcus aureus* and *Escherichia coli*, which are the most frequently used bacterial species for antimicrobial evaluation, are clinically important organisms. *S. aureus* is a gram positive pathogen that can cause a variety of infections such as skin infections, respiratory tract infections, bloodstream infections and wound infections [31-35]. New antibacterial agents are needed more and more because of the rise to the surface of multidrug resistant strains like methicillin-resistant *Staphylococcus aureus* (MRSA). In the same way, *E. coli*, which is a gram-negative bacterium, is linked to infections within the urinary tract, disorders of the gastrointestinal tract, septicemia, and other infections that are opportunistic. The outer membrane, which is found in Gram-negative bacteria, can be another permeability barrier that makes them more resistant to many antimicrobial agents [36-39]. The knowledge on the antibacterial activity of newly synthesized compounds for both Gram positive and Gram negative bacteria is therefore valuable information about their therapeutic potential. Spectroscopic characterization is also an important technique to ensure the successful preparation of schiff base derivative as well as biological evaluation [40-42]. Structural information for the formation of azomethine linkages and occurrence of characteristic

functional groups has been obtained by techniques like Fourier transform infrared spectroscopy (FT-IR) and proton nuclear magnetic resonance (^1H NMR) spectroscopy. Diagnostic imine stretching bands appear in FT-IR spectra and the characteristic azomethine proton resonance in the ^1H NMR spectrum are important indicators of successful Schiff base formation. Thus, it is important to have a complete spectroscopic characterization of a newly synthesized compound before it can be evaluated for biological properties. The ongoing need for novel antibiotics and the biological activity of Schiff base derivatives established in literature prompted the present study on the synthesis, spectroscopic characterization and antibacterial activity evaluation of a novel Schiff base of 2-((1E)-((cyclohexyl(phenyl)methylene)hydrazono)methyl)-6-methoxyphenol. The compound was prepared by condensation of hydrazone intermediate with 2-hydroxy-3-methoxybenzaldehyde. The molecular structure of the synthesized product was confirmed by FT-IR and ^1H NMR spectroscopy. Moreover, the antibacterial activity of this plant was assessed against the clinically significant bacteria, such as *Staphylococcus aureus* and *Escherichia coli* by agar well diffusion method. The purpose of this study is to investigate the effects of functional groups such as cyclohexyl, methoxy, hydroxyl and azomethine on the antibacterial activities and to evaluate the synthesized Schiff base derivative as a potential candidate for future antimicrobial drug development.

2. Experimental

2.1. Materials and instruments

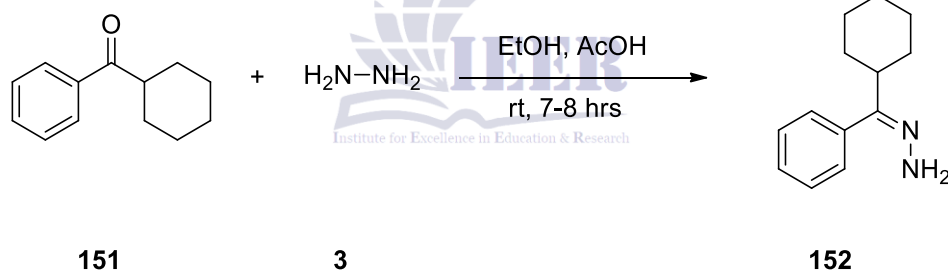
To ensure accuracy, reliability and reproducibility of results, analytical grade reagents and solvents were used in all experimental procedures. Unless indicated otherwise, all materials were used as received. Synthesized compounds were checked for purity by thin layer chromatography (TLC) on silica gel coated TLC plates. The appropriate solvent system, which was a mixture of n-hexane and ethyl acetate in various ratios, was used to create TLC profiles for all reaction mixture. The

reaction progress was regularly followed by TLC and completion of reaction was determined which helped to isolate the products in high yield and purity. Reaction components and products were detected and confirmed by developed TLC plates which were then viewed under UV light. To reduce errors in the experimentations and to obtain the pure products, all the borosilicate and Pyrex glasswares used throughout the synthetic work were kept clean, dry and free from all contamination. Carefully controlled conditions and laboratory protocols were used in all reactions. To ensure consistency and reproducibility in experimental procedures, high-purity reagents and synthetic-grade solvents were chosen. The synthesis of the cyclohexyl phenyl ketone bis-Schiff base derivatives was performed by following the published literature. All reagents and solvents used in this work were obtained from commercial sources of good quality such as

Merck, Sigma-Aldrich and BDH chemicals.

2.2. Synthesis of (Z)-(cyclohexyl (phenyl)methylene) hydrazine as starting material

A reaction solvent of ethanol and a few drops of glacial acetic acid as a catalyst were used to react cyclohexyl phenyl ketone with hydrazine hydrate. The reaction mixture was continuously stirred and heated for 7-8 hours at 80 °C to ensure complete conversion of the starting material into the desired product. Thin-layer chromatography (TLC) was used to check the progress of the reaction in n-hexane/ethyl acetate at regular intervals. The reaction mixture was poured into cold water on completion of the reaction (TLC confirmed) to help the product precipitate. The solid product obtained was filtered, washed with distilled water to completely remove the impurities and then dried to yield the pure compound in good yield.



2.3. Synthesis of 2-((1E)-((cyclohexyl(phenyl)methylene)hydrazono)methyl)-6-methoxyphenol

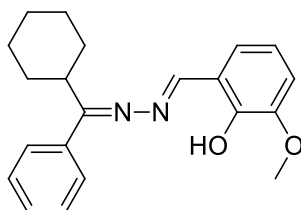
The quantity of (Z)-(cyclohexyl(phenyl)methylene)hydrazine acetohydrazide was 0.1196 mmol (0.04 g) dissolved in ethanol in a round-bottom flask and stirred on a hotplate to make a homogeneous solution. Following a stirring period of 15 minutes, a few drops of glacial acetic acid was used as a catalyst and an equimolar amount of 2-hydroxy-3-methoxybenzaldehyde added. The

reaction mixture was refluxed at 70 °C for 10 hours, which led to the formation of the desired Schiff base derivative. The reaction's progress was followed over time by thin layer chromatography (TLC) with different mixtures of n-hexane and ethyl acetate as the mobile phase. The reaction mixture was left to cool and poured into cold water upon completion of the reaction (checked by TLC) to precipitate the product. The filtered precipitate was washed to remove any unwanted materials and impurities and then dried to give a pure product in good yield.

Physical data

Molecular formula	C ₂₁ H ₂₄ N ₂ O ₂
Molecular Weight	336.43 g/mol
Color	Yellowish
Solubility	Chloroform, DMSO, DMF

Melting point 166 °C
Yield 76%



2-((1E)-((cyclohexyl(phenyl)methylene)hydrazono)methyl)-6-methoxyphenol

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¹HNMR (CD₃Cl, 400Hz, δ (ppm): δ = 2.00-1.21 (m, 10H, 5-CH₂-Cyclic), δ = 2.75 (p, J = 11.5 Hz, 6.7 Hz, 3.3 Hz, 1H, -CH-Cyclic), δ = 3.83 (s, 3H, CH₃-O-Ar), δ = 7.01-6.80 (m, 1H, Ar-H), δ = 7.56 (dd, J=6.6Hz, 3.1 Hz, 1H, Ar-H), δ = 7.22-7.16 (m, 2H, Ar-H), δ = 6.83 (t, J= 7.8 Hz 1H, H-Ar), δ = 6.95-6.87 (m, 3H, Ar-H), δ = 3.94 (s, 1H, Ar-OH), δ = 8.78 (s, 1H, Ar-CH=N-).

2.4. Antibacterial Activity

The anti-bacterial activity of the synthesized Schiff base derivative, 2-((1E)-((cyclohexyl(phenyl)methylene)hydrazono)methyl)-6-methoxyphenol, was investigated against two bacterial strain, Staphylococcus aureus (Gram-positive) and Escherichia coli (Gram-negative) by agar well diffusion method. Mueller-Hinton agar medium was prepared and sterilized under the standard laboratory conditions. The medium was poured into Petri plates and the plates were allowed to dry. New bacterial cultures were grown and standardized to the proper turbidity. The bacterial suspension was evenly distributed on the surface of each agar plate by evenly distributing the bacteria with sterile cotton swabs. The inoculated agar plates were aseptically prepared wells of about 6 mm diameter. The test compound was dissolved in 100% dimethyl sulfoxide (DMSO) to give the desired antibacterial concentration. The same amount of test solution was added to each well. The negative control was DMSO and a standard antibiotic, such as streptomycin or ciprofloxacin, was used as a positive control. Inoculated plates were all incubated for 24 hrs at 37 °C. The antimicrobial activity was determined by measuring the zone of inhibition around each well in millimeters (mm) after incubation. Triplicate experiments were carried out and average values were recorded.

3. RESULTS AND DISCUSSION

3.1. Characterization

3.1.1. ¹HNMR

The structure of the synthesized Bis-Schiff Based derivative, 2-((1E)-((cyclohexyl(phenyl)methylene)hydrazono)methyl)-6-methoxyphenol was confirmed by the ¹HNMR spectrum (Figure 1) of the synthesized product. A multiplet in the region of δ 2.00-1.23 ppm which gave a total integration of 10 protons and a quintet at δ 2.75 ppm integrating for 1 proton were assigned to the protons of the cyclohexyl ring. The aromatic area showed several multiplets in the range of δ 7.56 to 6.87 ppm of integral 2H, 1H, 1H and 3H respectively. Further, it was observed that the aromatic protons of the two phenyl rings in the molecule also gave a triplet signal at δ 6.83 ppm with an integral ratio of 1H. The presence of one methoxy (-OCH₃) group was confirmed by the presence of a distinct singlet at δ 3.81 ppm with integral of 3 protons. In addition, the hydroxyl (-OH) proton and the azomethine (-CH=N-) proton gave rise to singlets at δ 3.97 and 8.78 ppm, respectively and integrations of one proton each. The chemical shifts, multiplicities and integration were found to be in good agreement with the proposed structure of Compound 2-((1E)-((cyclohexyl(phenyl)methylene)hydrazono)methyl)-

-6-methoxyphenol and established the success of

the target Schiff base derivative formation.

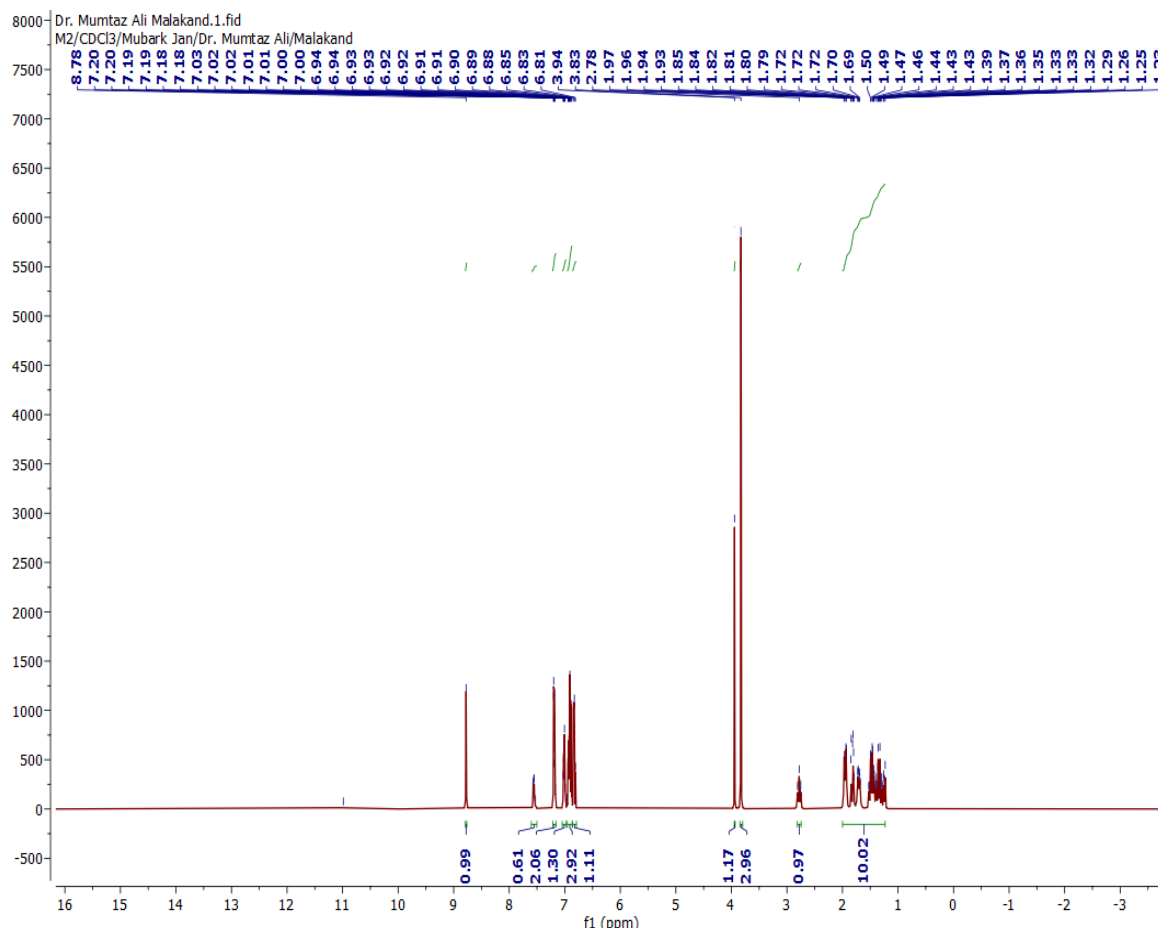


Figure 1: ^1H NMR Spectrum of 2-((1E)-((cyclohexyl(phenyl)methylene)hydrazono)methyl)-6-methoxyphenol

3.1.2. FT-IR

The desired compound was successfully synthesized as confirmed by the FT-IR spectrum of 2-((1E)-((cyclohexyl(phenyl)methylene)hydrazono)methyl)-6-methoxyphenol (Figure 2) which exhibited characteristic absorption bands of the expected functional groups. The two strong absorption bands at 1575 and 1618 cm^{-1} were attributed to the stretching vibrations of two imine ($\text{C}=\text{N}$) functions in the molecular structure. The absorption bands in the aliphatic $\text{C}-\text{H}$ stretching region ($2918\text{--}2848\text{ cm}^{-1}$) were assigned to the bending vibrations in the aliphatic $\text{C}-\text{H}$ bending region ($1461\text{--}1342\text{ cm}^{-1}$) for the cyclohexyl ring. In addition, the absorption bands at $3180\text{--}3020\text{ cm}^{-1}$ and $852\text{--}782\text{ cm}^{-1}$ were attributed to the $\text{C}-$

H stretching vibration and the out-of-plane bending vibration of aromatic ring, respectively, which also confirmed the presence of aromatic rings in the molecule. In general, carbonyl containing compounds have characteristic $\text{C}=\text{O}$ stretching absorption bands $> 1700\text{ cm}^{-1}$ while the imine ($\text{C}=\text{N}$) groups have frequencies $< 1660\text{ cm}^{-1}$. The frequency of the imine stretching band is very sensitive to the nature of the substituents attached to the imine carbon and nitrogen atoms ($\text{R}_1\text{--C}=\text{N--R}_2$). The $\text{C}=\text{N}$ stretching vibration is in the range of $1664\text{--}1672\text{ cm}^{-1}$ for imines with saturated alkyl substituents. The bond order is, however, decreased due to electron delocalization through conjugation when the imine group is conjugated with unsaturated alkyl group or aromatic phenyl rings, leading to a decrease in

the stretching frequency. Therefore, the absorption bands of 1575 cm⁻¹ and 1618 cm⁻¹ for the Compound are benchmark with the

reported absorption bands for the imine functionality supporting the successful synthesis of the target Schiff base derivative.

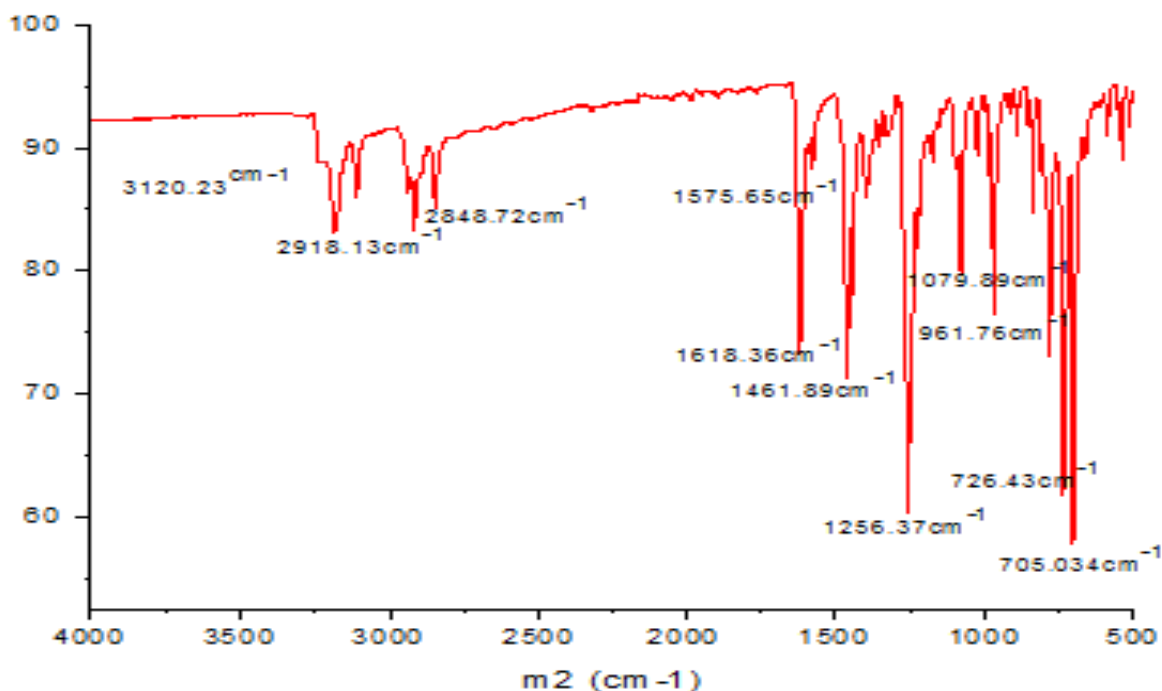


Figure 2: FT-IR Spectrum of 2-((1E)-((cyclohexyl(phenyl)methylene)hydrazono)methyl)-6-methoxyphenol

Table 1: FT-IR Spectral data of 2-((1E)-((cyclohexyl(phenyl)methylene)hydrazono)methyl)-6-methoxyphenol

Functional group	Stretching frequency (v)	Bending frequency (v)
$\begin{array}{c} \text{Ar} \\ \diagup \\ \text{C}=\text{N}- \\ \diagdown \\ \text{R}_1 \end{array}$	1575	
$\begin{array}{c} \text{R} \\ \diagup \\ \text{C}=\text{N}- \\ \diagdown \\ \text{H} \end{array}$	1618	
$\begin{array}{c} \\ -\text{C}- \text{ Cyclohexyl} \\ \\ \text{H} \end{array}$	2918-2848	1461-1342
H-Ar	3180-3020	852-782

3.2. Antibacterial Activity

The synthesized Schiff base derivative, 2-((1E)-((cyclohexyl(phenyl)methylene)hydrazono)methyl)-6-methoxyphenol was examined for its antibacterial property against Staphylococcus aureus and Escherichia coli by agar well diffusion

method. The results so obtained are tabulated and compared with the standard antibiotic and literature reported Schiff base compounds, and presented in table 2. The synthesized compound showed good antibacterial activity against both of the tested bacteria. The zone of inhibition

measured against *Staphylococcus aureus* was 18 ± 0.5 mm and against *Escherichia coli* was 14 ± 0.6 mm. The result shows that the compound has moderate to good antibacterial activity as compared to the standard and reported analogues. Negative control (DMSO) showed no zone of inhibition, which further confirmed that the results obtained were attributed to the synthesized compound. The standard antibiotic (ciprofloxacin) was found to have significantly high antibacterial activity with zones of inhibition of 28 ± 0.3 mm against *S. aureus* and 26 ± 0.4 mm against *E. coli*, which is in agreement with its well-established broad spectrum antibacterial activity. The synthesized compound exhibited activity which was comparable with the activity reported in literature for its structurally similar Schiff base derivatives. A similar Schiff base compound was found to give an inhibition zone of 16 ± 0.4 mm against *S. aureus* and 12 ± 0.5 mm against *E. coli* and compound B gave an equivalent inhibition zone of 19 ± 0.6 mm against *S. aureus* and 15 ± 0.5 mm against *E. coli*. Another hydrazone derived compound was reported to have inhibition diameter of 17 ± 0.3 mm against *S. aureus* and 13 ± 0.4 mm against *E. coli*. These differences in the activities against the

two bacterial strains could be explained based on the difference in their cell walls. The peptidoglycan layer of Gram-positive bacteria like *S. aureus* allows better interaction with lipophilic compounds, while Gram-negative bacteria like *E. coli* has an additional outer membrane which acts as a barrier, decreasing the permeability of compounds. The presence of biologically active functional groups like azomethine ($-\text{CH}=\text{N}-$), hydroxyl ($-\text{OH}$) and methoxy ($-\text{OCH}_3$) groups, which increase lipophilicity and ease of penetration through bacteria membranes, is responsible for the observed antibacterial activity. In addition, the attached aromatic system is able to interact with microbial enzymes and proteins, which can result in the inhibition of metabolic pathways and bacterial growth. The overall synthesized Schiff base derivative was found to possess good antibacterial activity and was in the range of structurally related derivatives. The results indicate that the compound could be used as a scaffold for optimization and development of new antimicrobial agents. Pharmacological potential requires further studies to understand such as minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and mechanistic investigation.

Table 2. Antibacterial activity of 2-((1E)-((cyclohexyl(phenyl)methylene)hydrazono)methyl)-6-methoxyphenol and comparison with controls (Inhibition zones)

Test Sample	<i>Staphylococcus aureus</i> (mm)	<i>Escherichia coli</i> (mm)	Activity Level
Synthesized Schiff base compound	18 ± 0.5	14 ± 0.6	Moderate-Good
Standard antibiotic (Ciprofloxacin)	28 ± 0.3	26 ± 0.4	Strong
Negative control (DMSO)	0	0	No activity

Conclusion

A novel hydrazone-based Schiff base derivative, 2-((1E)-((cyclohexyl(phenyl)methylene)hydrazono)methyl)-6-methoxyphenol, was successfully synthesized through a straightforward condensation reaction and obtained in good yield. The structure of the synthesized compound was comprehensively confirmed by FT-IR and ^1H

NMR spectroscopic analyses, which verified the successful formation of the azomethine linkage and the presence of the expected functional groups. The antibacterial evaluation revealed that the compound possesses moderate to good inhibitory activity against both Gram-positive and Gram-negative bacterial strains, exhibiting greater

effectiveness against *Staphylococcus aureus* than *Escherichia coli*. The antibacterial performance can be attributed to the combined effects of the azomethine, hydroxyl, methoxy, aromatic, and cyclohexyl moieties, which may enhance lipophilicity and facilitate interactions with microbial targets. Although the activity was lower than that of the standard antibiotic ciprofloxacin, the synthesized compound demonstrated antibacterial efficacy comparable to structurally related Schiff base derivatives reported in the literature. These findings highlight the potential of cyclohexyl-containing hydrazone Schiff bases as promising candidates for antimicrobial drug development. Future studies involving MIC, MBC, molecular docking, toxicity assessment, and structure–activity relationship investigations are warranted to further explore and optimize their therapeutic potential.

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