Antibacterial Property of Schiff-based Piperazine against MRSA: Design, Synthesis, Molecular Docking, and DFT Computational Studies

Honnegowdanaahalli Shivabasappa Nagendra Prasad 1,* , Navyatha Prashanth Gaonkar 1, Agasanapura Puttaswamy Ananda 2,3 , Amogh Mukarambi 1, Guddappa Charan Kumar 1, Tumakuru Nagarajappa Lohith 4, Hampapura Sunderraj Jayanth 3, Nidaghatta Beeregowda Krishnamurthy 6, Mandayam Anandalwar Sridhar 4, Puttaswamappa. Mallu 1

1 Department of Chemistry, Sri Jayachamarajendra College of Engineering, JSS Science and Technology University, Mysuru-570 006, Karnataka, India; nprasad@jssstuniv.in (H.S.N.P.);
2 Research and Development Centre, Bharathiar University, Coimbatore, 641046, Tamil Nadu, India
3 Ganesh Consultancy and Analytical Services, Hebbal Industrial Area, Mysuru, 570016, Karnataka, India
4 Department of Studies in Physics, University of Mysuru, Manasagangothri, Mysuru-570006, Karnataka, India
5 Department of Microbiology, Yuvaraja’s, University of Mysore, Mysuru-570005, Karnataka, India
6 Department of Biotechnology, Shridevi Institute of Engineering & Technology, Tumkur, 572106, Karnataka, India

* Correspondence: nprasad@jssstuniv.in (H.S.N.P.);

Received: 2022; Accepted: 2022; Published: 2022

Abstract: In this investigation, Schiff-based Piperazine was synthesized characterized with different spectral studies. Synthesized compound was subjected to Density functional theory (DFT), Adsorption, Distribution, Metabolism and Excretion (ADME), Blood-Brain Barrier (BBB), and their prediction of activity spectra of computational screening (PASS) for their application in the biological science was predicted, drug-likeness and total surface area was calculated. Membrane disassembly was studied based on the fatty acid profile test, compound exhibit potent in vitro biocidal activity against Methicillin-resistant Staphylococcus aureus (MRSA) at 30±0.45µg/mL. The membrane damage property was validated by SEM analysis; then, the fatty acid profile test addressed membrane disassembly. The synthesized compound shows significant activity in the fatty acid profile test. It confirms involvement in the membrane disassembly of MRSA. Molecular docking approach for the validation for understanding membrane damage and miss loading of FMM. This work suggests that Schiff-based piperezine is a potent antibacterial candidate against MRSA.

Keywords: piperazine; antibacterial activity; molecular docking; DFT; fatty acid profile; membrane damage; MRSA.

© 2022 by the authors. This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Schiff-base structures are among the most critical stereochemical aspects in collecting and changing metal coordination science because of their essential variety [1]. Piperazine is utilized as a structure block in macrocycles compounds once in a while. Piperazine is a fair hydrogen bond acceptor, which alongside its metal complexing capacities, makes it a fascinating moiety for complex supramolecular science [2]. We have been captivated for a long time in the arrangement and blend of new macrocyclic Schiff base structures, particularly in the compounds of CR-type ones [3]. Piperazine is a notable structure because of two weakly
held terminal amino protons. Macrocyclic Schiff-based structures have been extensively focused on their particular chelation to certain metal particles dependent upon the number, type, and position of the particles, the ionic range of the metal molecule, and the arranging properties of any counter particles. The chair is thermodynamically stable for two promptly interconvertible piperazine structures than the boat structure [4]. Macrocyclic Schiff base compounds containing the piperazine moiety are significant in coordination chemistry and natural chemistry. Sometimes, the presence of piperazine compounds brings about upgrading their organic property [5]. Schiff base compounds have been utilized as medications, and they have a wide collection of antimicrobial action against microscopic organisms, growths, and particular atoms. A few medications have expanded action when directed as metal chelates and hinder the development of tumors [6]. Schiff bases assumed a significant part in improving coordination chemistry, and a few metals chelates have been appeared to resist tumor development. Theoretical calculations like density functional theory (DFT) are used to explore molecular properties like structural, physicochemical properties, etc. Reactivity is one of the physicochemical properties because it is closely associated with reaction mechanisms, thus allowing the understanding of chemical reactions. A set of global and local descriptors to measure the reactivity of molecular systems has emerged using DFT calculations. Due to the diverse biological and industrial importance of the piperazine derivatives. The present paper furnishes a complete description of the molecular geometry, global and local reactivity descriptors, MEP features of the compound, and RDG analysis of the compound.

Schiff base ligands have a collection of utilizations, including clinical, logical, modern, and natural; in any case, their significant work in catalysis and natural complexity. Molecular italic docking is a convincing and competent instrument for in silico screening. It is expected to be a huge and genuinely growing part of a reasonable medicine plan. With everything taken into account, it is a determination of the rate of the two molecules, for instance, ligand and protein. It is routinely understood that nuclear definitive of one molecule (the ligand) to the pocket of another molecule (the receptor), which is generally a protein. Multidrug-resistant bacteria (MDRB) are microorganisms resistant to one or more antimicrobial agents. They are usually resistant to all but one or two commercially available antimicrobial agents. The MDRB of clinical interest includes Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus* with resistance to vancomycin (these are Vancomycin-intermediate *Staphylococcus aureus* (VISA) and Vancomycin-resistant *Staphylococcus aureus* (VRSA)), Vancomycin-resistant *Enterococci* (VRE), Extended-spectrum beta-lactamase’s (ESBLs) producing gram-negative *Bacilli*, Multidrug-resistant *Streptococcus pneumoniae* (MDRSP), Carbapenem-resistant *Enterobacteriaceae* (CRE) and Multidrug-resistant *Acinetobacter baumannii* [7-9]. They have for centuries been among the leading causes of death, disability, growing challenges to health security, and human progress, especially in developing countries [10]. Although many new antibacterial drugs have been produced, bacteria exhibiting resistance to them have increased and become a global concern as we are fast running out of therapeutic options [11]. The challenges of antimicrobial resistance are faced in health care and community settings, necessitating a broad approach with multiple partners across the continuum of care [12].

*S. aureus* is a Gram-positive coccoid bacterium. It is ubiquitous, and 30-40% of adults are asymptomatic carriers. It is also a major pathogen of humans and can cause various infections, from mild skin infections and food poisoning to life-threatening infections [13-18]. Resistance to methicillin by *S. aureus* was initially observed in 1961, shortly after the
antibacterial agent was introduced clinically. Since then, there has been a global epidemic of Methicillin-resistant *Staphylococcus aureus* (MRSA) in healthcare and community settings [19-21]. *S. aureus* is widely spread in the human population, with many asymptomatic carriers. It can also cause life-threatening infections, and its strains have evolved into MRSA and strains with reduced vancomycin susceptibility. This compound may be employed as an alternative candidate for drug development to halt or/and control the infections of multidrug-resistant *S. aureus*. In the previous studies conducted by Nagendra Prasad et al. [22]. Piperazine-based metal complexes and Piparizine sulphonyl analogs were designed, synthesized, characterized, and elaborately tested their medicinal properties and antibacterial activity against superbugs-MRSA. In the metal complexes, metals boost the antibacterial activity against MRSA. In the same way, the sulphonyl group showed enhanced antibacterial activity. The incremental study was conducted to check the Schiff-based piperazine antibacterial activity against superbugs-MRSA.

2. Materials and Methods

All the chemicals and reagents used in the present study were of analytical grade. Thin-layer chromatography was performed using silica gel sheets(silica GF254) in the presence of UV light Cary 630 FTIR spectrometer. All the spectra were run at 400-4000 cm-1 at room temperature. The NMR spectra were recorded using DRX 400 spectrometer at 400 MHz for 1H NMR with tetramethylsilane as the internal standard. All chemical shift is reported in ppm relative to TMS; Mass spectroscopic analysis was performed in a water micro TQF QII mass spectrometer.

2.1. General procedure for the synthesis of piperazine Schiff base.

Commercially available 1,4-perazine carboxaldehyde (0.15g) and 2-(piperazine-1-yl) ethamine (0.30g) were refluxed for 4-5 h in methanol (25 ml), and then 2-3 drops of acetic acid were added to the mixture. The reaction completion was confirmed by thin-layer chromatography (TLC). The solvent was concentrated, and the solid was dried and recrystallized from methanol. The orange-yellowish product was obtained with a good yield of (1E,1’E)-1,1’-(piperazine-1,4-diyl) bis(N-(2-(piperazin-1-yl) ethyl) methanimine) (Figure 1).

![Figure 1](https://nanobioletters.com/)

**Figure 1.** Scheme of the synthesis of piperazine Schiff base compound 3.

2.1.1. Synthesis of (1E,1’E)-1,1’-(piperazine-1,4-diyl) bis(N-(2-(piperazin-1-) ethyl) methanimine).

Petroleum ether/ethyl acetate (2:3) v/v solvent system has used in column chromatography by 85% yield. $^1$H NMR:(400MHz,CDCl$_3$) 2.34(T,Pip-H,2H) 2.65(Q,Pip-H,2H) 2.34(T,Pip-H,2H) 2.4(D,CH$_2$,2H) 1.61(T,CH$_2$,2H) 7.15(S,CH,1H) 3.73(T,Pip-H,2H) 3.73(T,Pip-H,2H) 3.73(T,Pip-H,2H) 3.73(T,Pip-H,2H) 3.73(T,Pip-H,2H) 7.15(S,CH,1H) 1.61(T,CH$_2$,2H) 2.4(D,CH$_2$,2H,J=Hz) 2.34(T,Pip-H,2H) 2.65(Q,Pip-H,2H) 1.07(M,Pip-H,1H) 2.65(Q,Pip-H,2H) 2.34(T,Pip-H,2H) 2.4(D,CH$_2$,2H) 1.61(T,CH$_2$,2H) 7.15(S,CH,1H) 3.73(T,Pip-H,2H) 3.73(T,Pip-H,2H) 3.73(T,Pip-H,2H) 3.73(T,Pip-H,2H) 3.73(T,Pip-H,2H) 7.15(S,CH,1H) 1.61(T,CH$_2$,2H) 2.4(D,CH$_2$,2H,J=Hz) 2.34(T,Pip-H,2H) 2.65(Q,Pip-H,2H) 1.07(M,Pip-H,1H) 2.65(Q,Pip-H,2H) 2.34(T,Pip-H,2H). $^{13}$C NMR:(400Hz, CDCl$_3$) 156.2, 61.8, 57.3, 52.5, 51.2, 46.2. LCMS m/z Calculated for C$_{18}$H$_{36}$N$_8$: (M-H) 364.54.
Found IR $\nu_{max}$ (cm$^{-1}$) 1660(N=C), 1120(C-N), 3100(C-H), 3360(N-H), Elemental Analysis: Calculated: C, 59.31; H, 9.95; N, 30.74; Experimental: C, 59.30; H, 9.92; N, 30.7

2.2. Absorption, Distribution, Metabolism, and Excretion analysis (ADME).

The drug is considered complicated in clinical trials of novel pharma due to improper ADME analysis. Pharmacokinetics assessment for new drug selection is a vital step in drug development that can correspond to optimizing efforts into recovered analogs. In addition to the worth of development of a new drug, this study was carried out using the swiss ADME free web tool. The present ADME property is carried out using a web tool to evaluate the ADMET properties, like aqueous solubility (log S), skin permeability (log Kp), synthetic accessibility score (SA), percentage absorption, pharmacokinetics, drug-likeness, and medicinal chemistry, friendliness properties of small molecules [23].

2.3. Prediction of Activity Spectra for Substances (PASS).

The prediction of the biological activity spectra of newly synthesized compounds was carried out using http://www.pharmaexpert.ru/PASSonline/predict.php. This software designed a tool for evaluating the general biological potential of an organic drug-like candidate. This software predicts above 6400 types of biological activity. Thus, PASS can estimate the biological activity of newly designed molecules. Based on the decomposition of chemical structures using 2D or 3D descriptors, this tool can provide information about the quantitative structure-activity relationship followed by the generation of models obtained from bioactive ligands [24]. The activity was estimated in Pa (probable activity) and Pi (probable inactivity). Structures with Pa greater than Pi were considered for a particular pharmacological activity.

2.4. Density Functional Theory (DFT).

All computational calculations were performed on a computer using the Gaussian 09 software. Becke’s three-parameter hybrid functional using the LYP correlation function (B3LYP), one of the most robust functionalities of the hybrid family, was used for all the DFT calculations with a 6-31G (d,p) basis set. Output files of the Gaussian software were visualized by using Gauss view 5. MEP and RDG calculations were done using Multiwfn 3.7 and visualized using Visual Molecular Dynamics (VMD) software [25].

2.5. Antibacterial activity against MRSA.

2.5.1. Preparation of bacterial culture and maintenance.

From the frozen glass beads of glycerol vial stock, the single purified colony was used (previously culture coated on glass beads and stored at -18 to -22°C). The glass bead was incubated at 37°C for 24 h and inoculated to Brain heart fusion (BHI) broth. The cell density was adjusted to 1x10$^6$ CFU/mL using UV-vis spectroscopy at 600 nm [26].

2.5.2. Minimum inhibitory concentration by resazurin assay.

The synthesized piperazine derivative underwent biological screening, and the MIC value of sterile BHI broth was dispensed in each of 96 wells. The piperazine derivative of different concentrations was dissolved in dimethyl sulfoxide (DMSO) and mixed with media. 10 μL of resazurin indicator (270 mg in 40 mL sterilized distilled water) solution were
dispensed to all the wells. Finally, 10 μL of bacterial suspension (5×10^6 CFU/mL) was added to give 5×10^5 CFU/mL. Streptomycin served as standard bactericidal agents from broad-spectrum antibiotics. A combination with all except the test compound served as a positive control. The ones with all solutions without bacterial culture were negative control. The plates were incubated at 37°C for 24 h after being wrapped with thin plastic films [27].

2.5.3. Disc diffusion method.

Antibacterial activity was assessed by the disc fusion method with different synthesized compound concentrations. The bacterial cultures were prepared from the overnight culture and 1×10^7 CFU/mL cells and inoculated onto nutrient agar. Then sterile disc (6 mm) was loaded with different concentrations of piperazine derivatives. Streptomycin (10μg/disc) was used as a positive control, and the sterile saline water was used as a negative control. To examine the zone of inhibition (ZOI), the plates were inverted and incubated at 37°C for 24 h [28].

2.6. Bacterial cell microscopy.

2.6.1. Membrane damage stud

To understand the effect of the sample on MRSA membrane, scanning electron microscopy (SEM) was carried out by treating MIC of Compound 3 for 2 h. Pelleted cells were centrifuged (10,000 rpm for 5 min) at 4°C. Further, cells were fixed by using 2.5% glutaraldehyde in PBS, pelleted, and smeared on a glass slide, dried, followed by stepwise treatment of 30% to 100% ethanol. After drying at room temperature, the sample was analyzed for SEM as per Manu Kumar et al. [29-32].

2.6.2. Total lipid extraction.

MRSA control and treated membrane fatty acid were analyzed by centrifuging the broth for 10 min at 5000 rpm; harvested pellets were immediately used for Boron-Trifluoride (BF₃) extraction described by Manu Kumar et al. [33]. Briefly, the cell pellet was washed in sterile 2 mL distilled water in a 50 ml tube, added (3.75 × 2) mL of methanol/chloroform (2:1, v/v), then mixed for 2 h. After incubation, supernatant (S1) was collected by centrifuge at 2500 rpm for 15 min. The remaining pellet was suspended in (4.75 × 2) mL of methanol/chloroform/water (2:1:0.8, v/v) vortextes, then left for 2 h to collect supernatant (S2) by centrifugation. Pool an S1 and S2 and add ((S1 + S2)/3.8) mL of chloroform followed by an equal volume of water. Finally, after vortex and settling of 2 phase system, the lower lipid-containing chloroform fraction was collected, warmed, and evaporated under nitrogen gas. The final small volume of chloroform was added, sealed, and stored at 4°C for the short term, -20°C for long-term storage.

2.7. Analysis of fatty acid.

2.7.1. FAME.

The transmethylation was carried out for extracted samples to analyze their acyl groups as fatty acids methyl esters (FAME) by gas-liquid chromatography (GLC). Add 2 mL of H2SO4(2.5%, v/v) to the extracted total lipid fraction in dry methanol (stored over anhydrous sodium sulfate). Heated for 2 h at 70°C, then the reaction was stopped by the addition of 3 mL
of NaCl (9%, v/v) in water. The FAME was extracted with 3×2 mL of light petroleum spirit, then extracts were evaporated under a stream of nitrogen gas, and the residue was re-suspended in a small volume of light petroleum for GLC analysis [34].

2.7.2. Gas-liquid chromatography.

The prepared FAME sample was analyzed using gas chromatography with column (1 m×4 mm) packed with 10% SP2330 on 100–120 supersport. The initial temperature of the column was 50°C, held for 2 min, and then a 10°C/min ramp to 200°C. Carrier nitrogen gas was adjusted with a pressure of 2 kPa. Separated FAME was calculated using the following formula:

$$\text{FAME} = \frac{\text{Retention time on column}}{\text{Peak height}}$$

Peaks were identified by their retention time relative to those of authentic standards.

2.8. Molecular docking validation.

The molecular docking simulation was performed to analyze drug-target interactions of the synthesized compound using the Auto dock 4.2 software [35]. The chemical structures of the compounds were drawn, and their 3D structures were optimized in the chem draw 16.0 software. A docking study of antimicrobial activity was carried out against two different protein targets, i.e., *S. aureus* (3VMF and 6FTB) was selected for *in silico* docking simulations for antibacterial activity of the synthesized compound. The X-ray crystallographic structure of the target proteins was retrieved from the protein data bank [36]. Before the simulations, all bound ligands, cofactors, and water molecules were removed from the proteins. Kollman charges were computed, and the Auto Dock atom types were defined using Auto Dock version 4.5. The three-dimensional grid boxes were created, and the grid maps representing the intact ligand in the actual docking target site were calculated with the Auto Grid algorithm. Finally, Auto Dock was used to calculating the binding free energy of a given inhibitor conformation in the macromolecular structure. Lamarckian Genetic Algorithm (LGA) parameters were set to default settings, which includes 300 runs, 200 conformational possibilities, 100 populations, and 3,50,0000 energy evaluations. A maximum of 20 conformers were considered during the docking process for each compound. The results were evaluated based on the binding compatibility, i.e., binding energy in kcal/mol and inhibition constant. The resultant protein-ligand complex structure was determined using Biovia discovery studio [37].

2.9. Toxicity.

2.9.1. Preparation of L6 cell lines and MTT assay.

The monolayer cell culture was trypsinzed, and the cell count was adjusted to 5.0 x 10⁵ cells/mL using recommended media containing 10% FBS (Fetal Bovine Serum). 100 µL of the diluted cell suspension (50,000 cells/well) was dispensed to 96 well microtiter plate. After 24h, when a partial monolayer was formed, the supernatant was flicked off, the monolayer was washed once with medium, and 100 µL of different concentrations of test drugs were mixed with cell line culture. The plate was incubated at 37°C for 24 h in a 5% CO2 atmosphere. After incubation, the test solutions in the wells were discarded, and 100 µl of MTT (5 mg/10 mL of MTT in PBS) was added to each well. The plates were incubated for 4 h at 37°C in a 5% CO2 atmosphere.
atmosphere. The supernatant was removed, 100 µL of DMSO was added, and the plate was gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 590 nm. The percentage growth inhibition was calculated based on the concentration of test drug needed to inhibit cell growth by 50% (IC₅₀) values cell line [38].

3. Results and Discussion

3.1. Pharmacophore model, Absorption, Distribution, Metabolism and Excretion (ADME) and Blood-Brain Barrier (BBB) analysis of synthesized compounds.

The synthesized analog was designed and categorized into two parts (Figure 2) shows the hydrophilic character of the piperazine ring as part B and the electron donor imine group as part A. The physicochemical, pharmacokinetic, and pharmacodynamic properties of piperazine analog with high biological activity were analyzed.

![Pharmacophore model of synthesized compound 3.](image)

Figure 2. Pharmacophore model of synthesized compound 3.

For a molecule to become a good drug candidate, its pharmacological and pharmacokinetics play important roles. While developing a new drug, it is important to examine the adsorption, distribution, metabolism, and excretion (ADME) and to evaluate compliance with Lipinski's rules [39]. According to Lipinski’s rule, oral bioavailability drug has no more than one violation of the following rules: No more than 5 hydrogen bond donors. No more than 10 hydrogen bond acceptors. Molecular weight (MW) less than 500 D.

An octanol-water partition coefficient (milogP) is not greater than 5. Synthesized piperazine moieties fully agreed with the rules and no violation of rules from any synthesized compound, as shown in table 1. Molecule 3 have the potential to be a drug; on the other hand, compound show less oral bioavailability if their topological polar surface area (TPSA) values are higher than 140 Å². The calculated value of TPSA for the synthesized molecule is tabulated in table 1. Figure 3A and 3B shows the Gastrointestinal absorption (GI) and blood-brain barrier (BBB) permeation properties of the synthesized molecules; in this figure, the white region, the physicochemical field of the molecule can be absorbed by the gastrointestinal system, on the other hand, yellow region indicates the physicochemical field of the synthesized compound that can penetrate the brain (Figure 3). To calculate the percentage of absorption of the compounds using the formula % Abs = 109 -0.345xTPSA. The predicted Bioactivity score of synthesized analogs was tabulated in table 2, which shows that synthesized is a potent enzyme inhibitor and Kinase inhibitor.
Table 1. *In silico* some physicochemical and pharmacokinetic parameters of the compound 3.

<table>
<thead>
<tr>
<th>Comp</th>
<th>Mi LogP&lt;5</th>
<th>TPSA (oA)</th>
<th>MW &lt;500</th>
<th>p&lt;ON-10</th>
<th>p&lt;OH</th>
<th>NICS</th>
<th>p&lt;ethyl</th>
<th>MV</th>
<th>%ABS</th>
<th>Vis&lt;1</th>
<th>BBB</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>-1.41</td>
<td>61.73</td>
<td>364.54</td>
<td>8</td>
<td>2</td>
<td>8</td>
<td>8</td>
<td>371.40</td>
<td>85</td>
<td>0</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Table 2. Bioactivity of synthesized analog.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>GPCR ligand</th>
<th>Ion channel modulator</th>
<th>Kinase inhibitor</th>
<th>Nuclear receptor ligand</th>
<th>Protease inhibitor</th>
<th>Enzyme inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.25</td>
<td>0.03</td>
<td>0.09</td>
<td>-0.20</td>
<td>0.09</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Figure 3. The BOILED-Egg representation of GI and BBB properties of the synthesized and bioavailability radar graph of 3 (pink area reflects the allowed values of drug-likeness properties of the molecule.

Table 3. Predicted biological activities of compounds 3, Pa (probability "to be active"), Pi (probability "to be inactive").

<table>
<thead>
<tr>
<th>Activity</th>
<th>Pa</th>
<th>Pi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antineoplastic (melanoma)</td>
<td>0.213</td>
<td>0.087</td>
</tr>
<tr>
<td>Antihelmintic (Fasciola)</td>
<td>0.15</td>
<td>0.098</td>
</tr>
<tr>
<td>Antiprotozoal (Coccidial)</td>
<td>0.261</td>
<td>0.458</td>
</tr>
<tr>
<td>Antiprotozoal (Trichomonas)</td>
<td>0.128</td>
<td>0.248</td>
</tr>
<tr>
<td>Antiprotozoal (Amoeba)</td>
<td>0.258</td>
<td>0.178</td>
</tr>
<tr>
<td>Antimyopathies</td>
<td>0.147</td>
<td>0.358</td>
</tr>
<tr>
<td>Antineoplastic (non-small cell lung cancer)</td>
<td>0.369</td>
<td>0.267</td>
</tr>
<tr>
<td>Antiseborrheic</td>
<td>0.153</td>
<td>0.346</td>
</tr>
<tr>
<td>Antineoplastic (lung cancer)</td>
<td>0.351</td>
<td>0.221</td>
</tr>
<tr>
<td>Antiseborrheic</td>
<td>0.562</td>
<td>0.165</td>
</tr>
<tr>
<td>Antineoplastic (lung cancer)</td>
<td>0.456</td>
<td>0.221</td>
</tr>
<tr>
<td>Anti-bacterial</td>
<td>0.235</td>
<td>0.011</td>
</tr>
</tbody>
</table>

Pa (Probability 'to be active') and Pi (Probability 'to be inactive') values, which predicted the biological activities of synthesized analog, are tabulated in Table 3.

3.1.1. synthesis and characterization of piperazine Schiff base.

A piperazine moiety of (1E,1’E)-1,1’-(piperazine-1,4-diyl) bis(N-(2-((piperazin-1-yl) ethyl) methanimine) was synthesized as shown in the Figure 1. The structure of the synthesized compound was established based on spectral studies shown in Supplementary figure S1(A,B,C&D). The elemental analyses data indicated admirable results between the
experimentally determined values and the theoretically calculated values within ±0.4%. Synthesis of (1E,1'E)-1,1'-((piperazine-1,4-diyl) bis(N-(2-(piperazin-1-yl) ethyl) methanimine) was single-step reaction between commercially available 1,4-piperazine dicarboxaldehyde and 2-(piperazine-1-yl) ethamine(1equi). The spectral analysis of synthesized compounds shows in the FT-IR spectra the band at 1120 cm⁻¹ due to the stretching vibration of C=N. The absorption bands at 1660 cm⁻¹ are assigned to the C=N stretch and the band's appearance at 3100 cm⁻¹ due to the stretching of vibration C-H. The absorption band at 3360 cm⁻¹ due to N-H stretching. The predicted strain of synthesized is in good agreement with the ¹H -NMR spectral analysis values. Spectra analysis of the synthesized analog shows multiple peaks at δ1.07; it attributes NH proton in piperazine molecule. Triplet peak at δ2.34 indicates the presence of CH₂ in piperazine. The singlet peak at δ7.15 shows the presence of CH proton (HC=N). The total number of protons in synthesized compound 3 is the same as that obtained in ¹H NMR spectral analysis. According to LCMS, the molecular ion peak of the synthesized compound 3 confirmed its formula weight, which is the same as the calculated molecular ion value (C₁₈H₃₆N₈). From the ¹³C NMR spectra, the signals of C=N can be seen at 156.2. The signals at 61.8 and 51.2 represent nonaromatic carbon. Signal values at 57.3, 52.5, and 46.2 show the presence of nonaromatic carbon of piperazine ring.

3.2. DFT.

3.2.1 Molecular geometry and Frontier Molecular Orbitals (FMOs).

The molecular geometry of the title compound was optimized using DFT calculations with a 6-31G (d,p) basis set. The optimized structure of the title compound is shown in figure 4.

![Figure 4. Optimized structure of the title compound.](image)

The frontier molecular orbital determines how the molecule interacts with the other molecules. The highest occupied molecular orbital (HOMO), the outermost orbital containing electrons, tends to give these electrons such as an electron donor. On the other side, the lowest unoccupied molecular orbital (LUMO) can be thought the innermost molecular orbital has vacant places to accept electrons. Therefore, the energy of the HOMO is directly related to the ionization potential, and LUMO is related to the electron affinity. The energy difference between these two is called an energy gap, which is important for the stability of the structure. HOMO-LUMO helps to depict the kinetic stability and chemical reactivity of the molecule. A molecule with a small gap is more polarized and is a soft molecule; the larger the gap, the harder the molecule is. The FMOs of these derivatives with the B3LYP/6-31G (d,p) method are plotted in Figure 5.

The chemical reactivity of the molecular systems has been determined by the conceptual density functional theory. Electronegativity (χ), chemical potential (μ), global
hardness (\(\eta\)), global softness (\(S\)) and electrophilicity index (\(\omega\)) are called global reactivity parameters. These global reactivity parameters are calculated using the energies of frontier molecular orbitals \(E_{\text{HOMO}}\), \(E_{\text{LUMO}}\) as 
\[ \chi = -\frac{1}{2}(E_{\text{HOMO}}+E_{\text{LUMO}}), \ \mu = -\chi = \frac{1}{2}(E_{\text{HOMO}}+E_{\text{LUMO}}), \ \eta = \frac{1}{2}(E_{\text{HOMO}}-E_{\text{LUMO}}), \ S = \frac{1}{2}\eta \] and \(\omega = \mu^2/2\eta\). The energies of FMOs and global parameters for all the compounds are listed in Table 4.

\[
\begin{align*}
\text{Table 4. Various kinds of global reactive parameters.} \\
\text{Parameters} & \quad \text{Values} \\
E_{\text{HOMO}} \ (\text{eV}) & \quad -3.808 \\
E_{\text{LUMO}} \ (\text{eV}) & \quad -0.247 \\
\text{Energy gap (\(\Delta\)) (eV)} & \quad 3.561 \\
\text{Ionization energy (I) (eV)} & \quad 3.808 \\
\text{Electron affinity (A) (eV)} & \quad 0.247 \\
\text{Electronegativity (\(\chi\)) (eV)} & \quad 2.027 \\
\text{Chemical potential (\(\mu\)) (eV)} & \quad -2.027 \\
\text{Global hardness (\(\eta\)) (eV)} & \quad 1.780 \\
\text{Global softness (\(S\)) (eV}\ ^{-1}) & \quad 0.562 \\
\text{Electrophilicity index (\(\omega\)) (eV)} & \quad 1.154
\end{align*}
\]

The FMO gap helps to characterize molecular electrical transport properties, chemical reactivity, and the kinetic stability of the molecule. A molecule with a small frontier orbital gap is generally associated with a high chemical reactivity and low kinetic stability. The FMO energy gap for the title compounds was found to be 3.561 eV. Larger the HOMO-LUMO energy gap, the harder the molecule. The region of HOMO spread over the entire molecule, and the region of LUMO spread over the piperazine ring. When two molecules react, the electrophilic character will depend upon the value of the electrophilicity index. The higher the value of the electrophilicity index better is the electrophilic character. Thus, the electrophilicity
index for the title compound was found to be 1.154 eV. To analyze the chemical behavior of some piperazine derivatives, we evaluated their global and local reactivity parameters. The values of \( \mu, \chi, S, \) and \( \omega \) were calculated, and the results are listed in Table 4.

### 3.2.2. Molecular Electrostatic Potential Map (MEP).

Electrostatic surface potential (ESP) maps, also known as electrostatic potential energy maps, or molecular electrical potential surfaces, illustrate the three-dimensional charge distributions over molecules. MEP provides a visual method to understand the reactive polarity of the molecule. The negative electrostatic potential corresponds to an attraction of the proton by the concentrated electron density in the molecules. The positive electrostatic potential corresponds to the repulsion of the proton by the atomic nuclei in regions where low electron density exists. The electrostatic potential surface was calculated by DFT/B3LYP at 6-31G(d,p) basis set and plotted in figure 6. Different colors represent the different values of the electrostatic potential; the most negative electrostatic potential (red), the regions of the most positive electrostatic potential (blue), and the region of zero potential (green). From Figure 6, one can observe that the more negative electrostatic potential is observed near the nitrogen atoms, and the more positive electrostatic potential is observed around the hydrogen atom, which is attached to the nitrogen atom in the piperazine ring of the compound.

![Figure 6. The molecular electrostatic potential surface of the compound.](https://nanobioletters.com/)

### 3.2.3. Reduced density gradient analysis.

Reduced density gradient (RDG) is used to explore weak interactions in real space based on the electron density and its derivatives. The RDG is a dimensionless quantity obtained from electron density (\( \rho \)),

\[
\text{RDG}(r) = \frac{\Delta \rho(r)}{2(3\pi^2)^{1/3} \rho(r)^{4/3}}
\]

The weak interactions are shown in the region with low electron density and low RDG. The interaction type can be found with the electron density \( \rho \) multiplied by the sign of \( \lambda_2 \) with RDG. The RDG calculations were performed by Multiwfn 3.7. The scattered reduced density gradient for compound C1 (see supplementary file for another molecule) is shown in Figure 7.

The RDG versus sign of \( \lambda_2 \) \( \rho \) peaks (electron density value) provides information about the nature of the interaction. The RDG=0.2 lines are evaluated in these molecules and crossed the repulsive spikes. Large negative values of sign \( \lambda_2 \) \( \rho \) are indicative of stronger attractive interactions, while positive ones indicate strong repulsion interactions. Values near zero indicate very weakly Van der Waals interactions. The weak interaction region can be located by generating RDG isosurface enclosing the corresponding regions in the real molecular space using the VMD program, as shown in Figure 7. The color from blue to red
means stronger attraction to repulsion, respectively. The green color circles can be identified as the Van der Waals interaction region, which means that the density of electrons in these regions is low. Figure 7 displayed that the molecule has strong repulsion or steric effect in the ring, and the vdw interactions are observed between the nitrogen atom and the hydrogen atoms. Still, there is no strong attraction present in the molecule.

![Figure 7](https://doi.org/10.33263/LIANBS122.054)

**Figure 7.** Plots of the RDG versus the electron density multiplied by the sign of the second Hessian Eigenvalue ($\lambda_2$) and the colored surfaces of the compound according to values of sign $\lambda_2$.

### 3.3. Antibacterial activity of Piperazine Schiff base against MRSA.

The minimum inhibitory concentration of the synthesized analog was determined by resazurin assay. The parental structural piperazine (3) was tested against MRSA. The analog 3 showed a good MIC value of $30 \pm 0.45 \mu g/mL$ was tabulated in table 5. Piperazine analogs showed moderate inhibition of zone in radius (ZIO) $3.21 \pm 0.02$, $4.12 \pm 0.01$ and $5.35 \pm 0.02$, $6.10 \pm 0.03$, $6.80 \pm 0.02$, and $7.22 \pm 0.03$mm (figure 8). The data confirms that the synthetic piperazine derivative produced significant results as a result of its structure and chemical moieties in the compound. The synthesized analog showed promising biological activity to govern physicochemical, pharmacokinetic, and pharmacodynamics properties.

**Table 5.** Antibacterial activity of the synthesized piperazine derivative against methicillin-resistant *Staphylococcus aureus* (MRSA).

<table>
<thead>
<tr>
<th>Synthetic compound</th>
<th>MIC µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schiff based Piperazine</td>
<td>30±0.45</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10±0.04</td>
</tr>
</tbody>
</table>
Figure 8. (A) MRSA tested with standard drug streptomycin 10 µg/disc (12.12±0.02) ZOI in mm; (B) Antimicrobial activity 3 against MRSA.

3.4. Membrane damaging effect on MRSA.

The membrane damage effect of Schiff-based Piperazine has been analyzed using SEM and showed there are alterations on the membrane (B), such as pores or breaks, indicating the potential inhibition of MRSA growth compared to the control (A) after 12 h of incubation.

Figure 9. The membrane-damaging property of Schiff-based Piperazine against MRSA.

In this study, the active Schiff-based Piperazine was evaluated for MRSA membrane damage effect and revealed significant membrane alterations such as pores, cracks, and breaks compared to control untreated by SEM (Figure 9). The MIC of Schiff-based Piperazine in 12h showed a significant effect on MRSA membrane, comparable to previous studies conducted better to understand the mechanism of synthetics against drug-resistant MRSA. This result further confirms that the role of Schiff-based Piperazine at the membrane or membrane-bound factors are responsible for the biocidal cause excreted, which leads to cellular content leakage, cell death, and adverse inflammatory conditions can be induced during MRSA infection in the host.

3.5. Effect on MRSA membrane fatty acid content.

To better understand the role of Schiff-based Piperazine on MRSA, the study examined the disappearance of cellular fatty acid content during bacterial growth using GC-MS and compared the MIC value of Schiff-based piperazine to the control which reported significant changes in the fatty acid profile of MRSA. This implies that the effect of synthetic on the
biosynthetic pathway was confirmed by the decreased or increased contents of fatty acid in MRSA. The saturated (Lauric acid, Myristic acid, and Palmitic acid) and unsaturated (Oleic acid, Linoleic acid, and Linolenic acid) contents of treated showed significant changes after treatment when compared to control, implying that analog 3 involved in lipid biosynthesis was deduced (Table 6). The loss of MRSA cell wall components leads to a loss of membrane integrity, which becomes susceptible to any antibiotic treatments during pathogenic conditions [40].

### Table 6. The fatty acid profile of MRSA. The treatment of Schiff-based Piperazine to MRSA showing an alteration in the fatty acid content confirms involvement in cell wall lipid biosynthetic pathway.

<table>
<thead>
<tr>
<th>Methyl ester of</th>
<th>Control (%)</th>
<th>Analogue5 (%)</th>
<th>MUFA/PUFA/SF</th>
<th>Lipid number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caproic acid</td>
<td>12.20</td>
<td>19.87</td>
<td>SF</td>
<td>C6:0</td>
</tr>
<tr>
<td>Caprylic acid</td>
<td>2.65</td>
<td>Nil</td>
<td>SF</td>
<td>C6:0</td>
</tr>
<tr>
<td>Capric acid</td>
<td>2.20</td>
<td>1.07</td>
<td>SF</td>
<td>C10:0</td>
</tr>
<tr>
<td>Lauric acid</td>
<td>16.15</td>
<td>0.98</td>
<td>SF</td>
<td>C12:0</td>
</tr>
<tr>
<td>Myristic acid</td>
<td>20.10</td>
<td>14.23</td>
<td>SF</td>
<td>C14:0</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>20.96</td>
<td>11.70</td>
<td>SF</td>
<td>C16:0</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>6.50</td>
<td>4.02</td>
<td>SF</td>
<td>C18:0</td>
</tr>
<tr>
<td>Behenic acid</td>
<td>0.63</td>
<td>Nil</td>
<td>SF</td>
<td>C22:0</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>19.03</td>
<td>3.15</td>
<td>MUFA</td>
<td>C18:1 cis-9</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>15.15</td>
<td>2.18</td>
<td>PUFA</td>
<td>C18:2 cis, cis9,12</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>3.80</td>
<td>Nil</td>
<td>PUFA</td>
<td>C18:0</td>
</tr>
</tbody>
</table>

### 3.6. Molecular docking validation.

In the present investigation, the Schiff-based Piperazine was effectively involved in the prevention of MRSA development which is validated using invitro antibacterial study and involved in significant changes of fatty acid profile. In silico molecular docking, the study was used to confirm the interaction of the synthesized molecules with various proteins of MRSA [41]. The molecular docking was performed to investigate the dynamics of biological components by targeting MRSA proteins such as 3VMT and 6FTB from the RCSB protein data bank, which is membrane-bound proteins. The molecular docking study shows that compound 3 has the active binding site with respect to the above proteins of MRSA, and the study provides a high docking score and good interactions such as hydrogen, Vander Waals, and hydrophobic with all possible amino acid residues (Table 7).
Compound 3 contains a piperazine ring, eight The active binding site of compound 3 with respect to protein 3VMT and 6FTB shows in Figure 10 and 11(3D), the Schiff based piperazine demonstrated Vander Waals interactions with GLN136, THR115, PHE158, PHE110, and hydrogen bond interaction with LEU157 in 3VMT protein. The protein 6FTB interaction with compound 3 shows both Vander Waals and hydrogen bond interactions, Vander Waals interactions with SER149, ASP147, ASP145, and hydrogen bond interaction with ARG148, MOE301; due to these interactions, compound 3 exhibits a high docking score with good binding energy (Supplementary figures S2 and S3). In this study, the 3 has a good docking score compared to other analogs, and also this theoretical data strongly correlated to our experimental results.

![Figure 11](image_url)

**Figure 11.** (A) Molecular docking of compound 3 against 3VMT; (B) 6FTB and antibiotic streptomycin; (C) of binding deep inside the active site, depicting the best docking pose showing 3D, respectively.

<table>
<thead>
<tr>
<th>PDB id</th>
<th>Compounds</th>
<th>3VMT Docking Score</th>
<th>Glide Energy (kcal/mol)</th>
<th>6FTB Docking Score</th>
<th>Glide Energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Schiff based Piperazine</td>
<td>-9.21</td>
<td>-45.215</td>
<td>-7.558</td>
<td>-49.97</td>
</tr>
<tr>
<td></td>
<td>Streptomycin</td>
<td>-10.65</td>
<td>-44.065</td>
<td>-9.293</td>
<td>-48.084</td>
</tr>
</tbody>
</table>

3.7. Toxic effect of Schiff-based Piperazine.

In this present work, Schiff-based piperazine undergoes cytotoxicity assay with different doses to predict the anti-proliferative effect of Schiff-based piperazine against skeletal muscle cells (L6 myotubes). The obtained data of cytotoxicity study of Schiff-based piperazine showed IC$_{50}$ value is 150.45 µg/mL. Figure 12A and 12B shows the microscopic images of the control and treatment of cytotoxicity study, and Figure 13 indicates the % of inhibition with different concentration from 10-320 µg/mL.

![Figure 12](image_url)

**Figure 12.** Toxicity of Schiff-based Piperazine. (A) L6 cell lines; (B) Toxic effect of compound Schiff-based Piperazine.
4. Conclusions

A new Schiff-based Piperazine was synthesized, and the compound was characterized by elemental analysis, H\textsuperscript{1} NMR, IR, and mass spectroscopy. The ADME, PASS, and DFT theoretical analysis of the structure was also carried out, showing the potent result. In this investigation, the synthesized piperazine analog derived from piperazine amine and piperazine aldehyde, Schiff-based piperazine showed excellent antibacterial activity against MRSA. Also, the potential bactericidal activity of Schiff-based piperazine was validated for its potency within-silico molecular docking studies on 3VMT and 6FTB protein of MRSA. Additionally, the Schiff-based piperazine insignificant level of toxicity on L6 cell lines. This potent compound can be utilized for future drug design and biomedical applications against superbug MRSA.

Funding

This research received no external funding

Acknowledgments

The authors are greatly thankful to Sri Jayachamarajendra College of Engineering (SJCE), JSS Science and Technology University, Mysuru and Ganesh Consultancy & Analytical Services, Mysuru, for providing instrumentation facility.

Conflicts of Interest

The authors declare no conflicts of interest.

References


Supplementary materials

Figure S1. NMR and LC-MS data of new compounds (compound 3); A. $^1$H- NMR spectra, B. $^{13}$C- NMR spectra, C. FTIR and D. LC-MS
**Figure S2.** Docking view of compound 3 with protein 6FTB.

**Figure S3.** Docking view of compound 3 with protein 3VMT.