Synthesis, *In Vitro* Antimicrobial, and *In Silico* Analysis of Camphor-Based Schiff Base Derivative

By: Tseganesh Abate (B. Pharm.)

Advisors: Dr. Daniel Bisrat (PhD.)

Prof. Kaleab Asres (PhD.)

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This is to certify that the thesis prepared by Tseganesh Abate, entitled**: "Synthesis, *In Vitro* Antimicrobial, and *In Silico* Analysis of Camphor-Based Schiff Base Derivative"** and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Medicinal Chemistry complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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Advisor: Dr. Daniel Bisrat	_____	_____
Prof. Kaleab Asres (PhD.)	_____	_____

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Abstract

Synthesis, *In Vitro* Antimicrobial, and *In Silico* Analysis of Camphor-Based Schiff Base Derivative

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Microbial diseases are among the leading causes of mortality and morbidity worldwide. The rise of antimicrobial resistance has become a significant challenge, as microorganisms have developed resistance to many existing drugs. Therefore, the search for new antimicrobials is critically important. Schiff bases (SB) and their derivatives exhibit a wide range of biological activities, including antimicrobial, anti-inflammatory, antimalarial, anticancer, and antioxidant effects, among others. Camphor (**1**) is a terpenoid (1, 7, 7-trimethylbicyclo [2.2.1]-2-heptanone), exists in two enantiomeric forms (1*S*)-m (-) is a synthetic camphor and (1*R*)-(+) is a natural camphor. This study aims to enhance antimicrobial activity of camphor by synthesizing its Schiff base derivative through condensation reaction of *R*-camphor with 2, 4-dinitrophenylhydrazine in a methanolic solution using an acid catalyst, and also evaluate its antimicrobial activity against twenty-six bacterial strains and four fungal strains. Furthermore, molecular docking studies were performed to explore the potential mechanism of action of the synthesized compound, while ADMET analysis was carried out to evaluate its drug-likeness properties. The synthesized compound (**19)** was purified using column chromatography, and its chemical structure was determined based on ^{1}H-NMR and ^{13}C-NMR spectral data, as (*E*)-1-(2,4-dinitrophenyl)-2-(1,7,7-trimethylbicyclo [2.2.1] heptan-2-ylidene) hydrazine. The synthesized compound (**19**) exhibited broad-spectrum antibacterial activity, including effectiveness against multidrug-resistant (MDR) strains. Notably, it exhibited the strongest activity against *Escherichia coli* and *Staphylococcus*

aureus, with a minimum inhibitory concentration (MIC) of 25 μg/mL for both, which is twice as potent as camphor (**1**), whose MIC is 50 μg/mL. To predict the potential mode of action, we conducted a molecular docking study using the Gyr24B of *E. coli* (PDB ID: 7P2X) as the target enzyme. The synthesized compound (**19**) exhibited a docking score of –5.368kcal/mol, suggesting a moderate binding affinity. Additionally, ADMET predictions indicated that the synthesized compound (**19)** adhered to Lipinski's rule of five. In the end, Schiff base derivative of camphor may hold significant promise in boosting the antimicrobial properties of camphor and addressing drug-resistant microbes.

Key words**:** *R*-camphor, Schiff bases, synthesis, antimicrobial activity, molecular docking, ADMET

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Acronyms and Abbreviations

^{13}C-NMR	Carbon thirteen nuclear magnetic resonance
^{1}H-NMR	Proton nuclear magnetic resonance
ADMET	Absorption distribution metabolism excretion and toxicity
AMR	Antimicrobial resistance
DMSO	Dimethyl sulfoxide
MDR	Multidrug resistance
MRSA	Methicillin-resistant *Staphylococcus aureus*
MIC	Minimum inhibitory concentration
PDB	Protein Data Bank
SB	Schiff bases
TLC	Thin layer chromatography
TMS	Tetra methyl saline
VAP	Ventilator-Associated Pneumonia
WHO	World Health Organization

1. Introduction

1.1. Overview of microbial disease and drug resistance microbial

Microbial diseases, caused by pathogenic microorganisms such as bacteria, viruses, parasites, fungi, and their toxic products, are among the leading causes of global mortality and morbidity (Cole & Kramer, 2016). Despite the remarkable advancements in science and technology, the prevalence of infectious diseases has also significantly increased, posing serious and widespread public health threats (Debta *et al*., 2020). Since 2000, the rapid growth of urbanization and global travel has led to more frequent and complex infectious disease pandemics, notable examples of which are Ebola, severe acute respiratory syndrome (SARS), Middle East respiratory syndrome corona virus (MERS-CoV), Nipah, influenza A subtype H5N1, and Zika (Gong *et al*., 2021). According to the World Health Organization (WHO), 32% of fatalities globally, 68% of deaths in Africa, and 37% of deaths in Southeast Asia were due to infectious diseases. These diseases are responsible for 90% of the health problems worldwide and kill about 14 million people each year, 90% of whom are from the developing world (Ismahene, 2022). Poverty and poor health care exacerbate the health problem in developing countries (Fenollar & Mediannikov, 2018).

Antimicrobial resistance (AMR) is one of the biggest global public health challenges. AMR impacts financial sustainability, global health, food sustainability and security, environmental wellbeing, and socio-economic development(Ahmed *et al*., 2024). The six main pathogens responsible for deaths associated with resistance are *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii,* and *Pseudomonas aeruginosa*. In 2019, these pathogens were accountable for approximately 929,000 deaths attributed to AMR and around 3.57 million deaths associated with AMR (Collaborators, 2022).

In addition, the occurrence of drug resistance to certain antifungal drugs has increased over the last two decades (Peraman *et al*., 2018). The World Bank estimated that up to 3.8% of the global gross domestic product could be lost due to AMR by 2050. The World Health Organization (WHO) predicts that by 2050, antimicrobial resistance (AMR) will cause 10 million deaths annually (Shinu *et al*., 2022).

Many new antibiotic molecules were developed, however they were prevented from further development due to various reasons. According to available reports, more than 90% of the developed hit molecules were deemed unsuitable for treating drug-resistant infections, while the remaining few were either low in potency against super bugs or highly cytotoxic to humans. As a result , the WHO declared this era as the antibiotic crisis era, highlighting the urgent need for novel treatments and approaches to prevent antimicrobial resistance from taking millions of lives (Peraman *et al*., 2021).

1.2. Statement of problem

Antimicrobial resistance (AMR) occurs when bacteria, viruses, fungi, and parasites evolve to resist the effects of antimicrobial drugs, making infections harder to treat (Tang *et al*., 2023). This resistance leads to ineffective treatments, delays in appropriate therapy, and increased reliance on more toxic or expensive drugs (French, 2005). The 2022 GLASS report highlights alarming resistance rates, such as 42% resistance of *E. coli* to third-generation cephalosporins and 35% resistance of Methicillin-resistant *Staphylococcus aureus* (MRSA) across 76 countries. *E. coli* and *Klebsiella pneumoniae* are showing declining susceptibility to commonly used antibiotics, increasing dependence on last-resort drugs like carbapenems, which themselves are facing rising resistance (WHO, 2022). Since few new antibiotics are in development and the threat of untreatable infections is growing, there is an urgent need to search for new safe and

effective antibiotics. Recent studies suggest that camphor possesses promising antimicrobial properties by enhancing the effectiveness of various antibiotics through synergistic effects (Abdollahi *et al*., 2024). However, when administered alone, camphor exhibits a moderate antimicrobial activity against various microorganisms. While it possesses some inherent ability to inhibit the growth of certain microorganisms, its effectiveness as a standalone antimicrobial agent is limited. This suggests that camphor alone may not be sufficient to combat more resistant or aggressive pathogens, highlighting the need to explore ways to enhance its antimicrobial potential, such as through chemical modification or combination with other agents. Therefore, synthesizing new camphor derivatives with diverse mechanisms could be a valuable strategy to improve antimicrobial efficacy and combat AMR.

1.3. Objectives

1.3.1. General objectives

To enhance the antimicrobial efficacy of camphor by synthesizing its Schiff base derivative and predicting its mode of action through *in silico* analysis.

1.3.2. Specific objectives

- To synthesize a camphor-based Schiff derivative via the condensation reaction of camphor and 2, 4- dinitrophenylhydrazine.
- To purify the synthesized compound using column chromatography.
- To characterize the synthesized compound using spectroscopy (^{1}H-NMR and ^{13}C-NMR).
- To assess the *in vitro* antimicrobial activity of the camphor-based Schiff derivative against 26 bacterial and 4 fungal strains using disk diffusion and Broth dilution methods.

- To conduct an *in silico* study of camphor-based Schiff derivatives using Schrödinger suite.

1.4. Significance of the study

As multidrug-resistant microbial pathogens continue to spread worldwide, the need for new and effective antimicrobial agents to treat life-threatening infections is becoming increasingly urgent. Developing new drugs that can serve as alternatives to existing therapies and expand treatment options is a critical global priority. Recent studies have shown that camphor has a relevant antimicrobial properties. When used in combination, it significantly enhances the efficacy of antibiotics, demonstrating a synergistic effect. It has been proven that this compound not only contributes to lowering the MIC values of antimicrobial drugs, but also helps strains that had previously demonstrated antibiotic resistance to regain their sensitivity (Duda-Madej *et al*., 2024).

In this study, a camphor-based Schiff base derivative was synthesized using a Schiff base reaction and evaluated for its antimicrobial activity against 26 bacterial and 4 fungal strains. The results revealed that the new compound significantly improved the antimicrobial potency of camphor. Furthermore, molecular docking studies suggest that the synthesized compound may target the DNA Gyrase subunit B of *E. coli* (PDB ID: 7P2X), providing insight into its possible mechanism of action. These findings highlight the potential of the camphor-derived Schiff base as a promising lead compound for the development of new antimicrobial agents. This study lays the groundwork for further structural optimization and drug design aimed at combating resistant microbial infections.

2. Literature review

2.1. Camphor and its biological activities

Camphor (**1**) is a natural product extracted from *Cinnamomum camphora* tree, commonly found in Asian countries including Japan, Taiwan and China, but it has also been naturalized in other parts of the world. Camphor(**1**) is a terpenoid (1, 7, 7-trimethylbicyclo [2.2.1]-2-heptanone), that exists in two enantiomeric forms (1*S*)-(-), which is synthetic camphor and (1*R*)-(+),which is a natural camphor (Ali *et al*., 2019). It has a wide range of applications, ranging from being used to treat medical conditions in humans to being used as a natural insecticide and fumigation-based disinfectant, which may seem contradictory. Recent biomedical research studies have shown that camphor has antibacterial, anthelmintic, antiinflammatory, analgesic, and anticancer properties (N. Sharma, 2021). Nowadays, camphor is commonly used as a topical medication to treat respiratory depression, as well as poisoning from sedative and narcotics, acute and chronic heart failure (Sokolova *et al*., 2015). In chemistry, camphor has been utilized as a chiral auxiliary, a shift reagent in nuclear magnetic resonance (NMR), a selector in chiral separations, a component of catalytic systems, and a versatile chemical precursor (da Silva *et al*., 2016).

Camphor's diverse chemical structure and biological activity make it a promising building block for the preparation of biologically active derivatives (Duan *et al*., 2022). The ketone functional group of the camphor molecule is the primary means to produce various potent derivatives. Natural (+)-camphor-based imines have shown promise as a source of antiviral and antibacterial compounds (Zielińska-Błajet & Feder-Kubis, 2020). The ketone in camphor has been found to have strong anticonvulsant and nicotinic receptor-blocking properties. The camphor moiety is particularly significant because it gives the produced compounds their lipophilic nature, which is necessary for them to pass across the blood-brain barrier (Agrawal *et al*., 2014).

2.2. Schiff Bases and their biological activities

Imine derivatives, also known as Schiff bases (compounds with a C=N bond), are formed when aldehydes and ketones react with ammonia or primary amines, as indicated in figure 1. Schiff base compounds have a wide range of applications in the pharmaceutical industries and chemical industries. Schiff bases and their derivatives have exhibited antimicrobial, antiinflammatory, antimalarial, anticancer, antioxidant and other biological effects (Kajal *et al*., 2013). Because of its electrophilic carbon and nucleophilic nitrogen, the imine group offers a high binding potential with numerous nucleophiles and electrophiles, which inhibits targeted diseases, enzymes, or DNA replication. Hydrazone derivatives and anti pyrine-derived Schiff bases are an interesting class of compounds, especially in antimicrobial drug research (Senthil Kumar Raju *et al*., 2022). In addition, Schiff base compounds play a significant role as ligands in co-ordination chemistry. They are polydentate ligands that can be macro-cyclic or macro-acyclic, containing both nitrogen and oxygen donor atoms (Das, 2021).

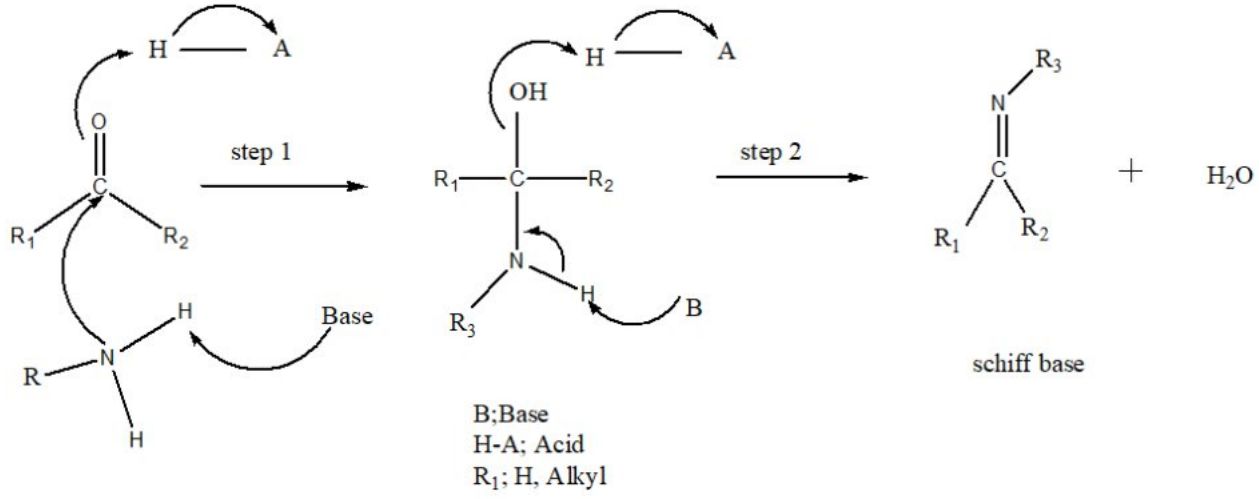


Figure 1: General reaction mechanism of Schiff base synthesis

2.3. Antimicrobial activity of camphor derivatives

Many studies have shown that camphor derivatives have antimicrobial activities. For example, the antibacterial and antibiofilm properties of camphor's sulfur derivatives have been tested against both Gram-positive and Gram-negative bacteria. These compounds exhibited significant impacts on the biofilms of Gram-positive strains, indicating that they may find application as antiseptics, disinfectants, and therapies for skin infections (Peraman *et al*., 2021). Another investigation on camphor hybrids showed enhanced antibacterial action against drug-resistant pathogens such as Methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant *Klebsiella pneumoniae.* The increased lipophilicity of these compounds contributed to their improved antibacterial activity (Peraman *et al*., 2018).

2.3.1. Camphor derivatives as antiviral agents

Yarovaya *et al.*, (2019) described the synthesis and structure-activity relationships of novel camphecene (**2)** analogues as anti-influenza agents. The modifications included replacing the camphecene's terminal hydroxyl group with mono and bicyclic heterocyclic moieties. Camphecene was treated with phosphorus tribromide(PBr_3) to produce key intermediate **3**, which was then used immediately in further reactions. Fourteen compounds were prepared by nucleophilic substitution of secondary amines or thiols with bromide (**3)** in the presence of potassium carbonate and 1, 8- Diazabicyclo[5.4.0]Undec-7-ene(DBU). Among the 15 tested compounds two compounds (compound **4** and **5**) demonstrated the highest virus-inhibiting activity with Selectivity Index (SI) of 106 and 183, bearing pyrrolidine and piperidine moieties, respectively. Compound (**6)** was shown to interfere with viral reproduction at early stages of the viral life cycle.

Sokolova *et al.*, (2021) developed a derivatives of (+)-camphor and (-)-borneol as potential antiorthopox virus agents. In this research the 1, 7, 7-trimethylbicyclo [2.2.1] heptane core structure, N-acylhydrazone motif, and thiosemicarbazone group were found to be advantageous for antiorthopox virus activity by the SAR analysis. Among the tested compounds, the acyl hydrazones (**7** and **8)** were identified with broad-spectrum activity (SI values of 76, 117, and 157; 206, 460, and 854, respectively) against various OPVs, including VARV, CPV and ECTV.

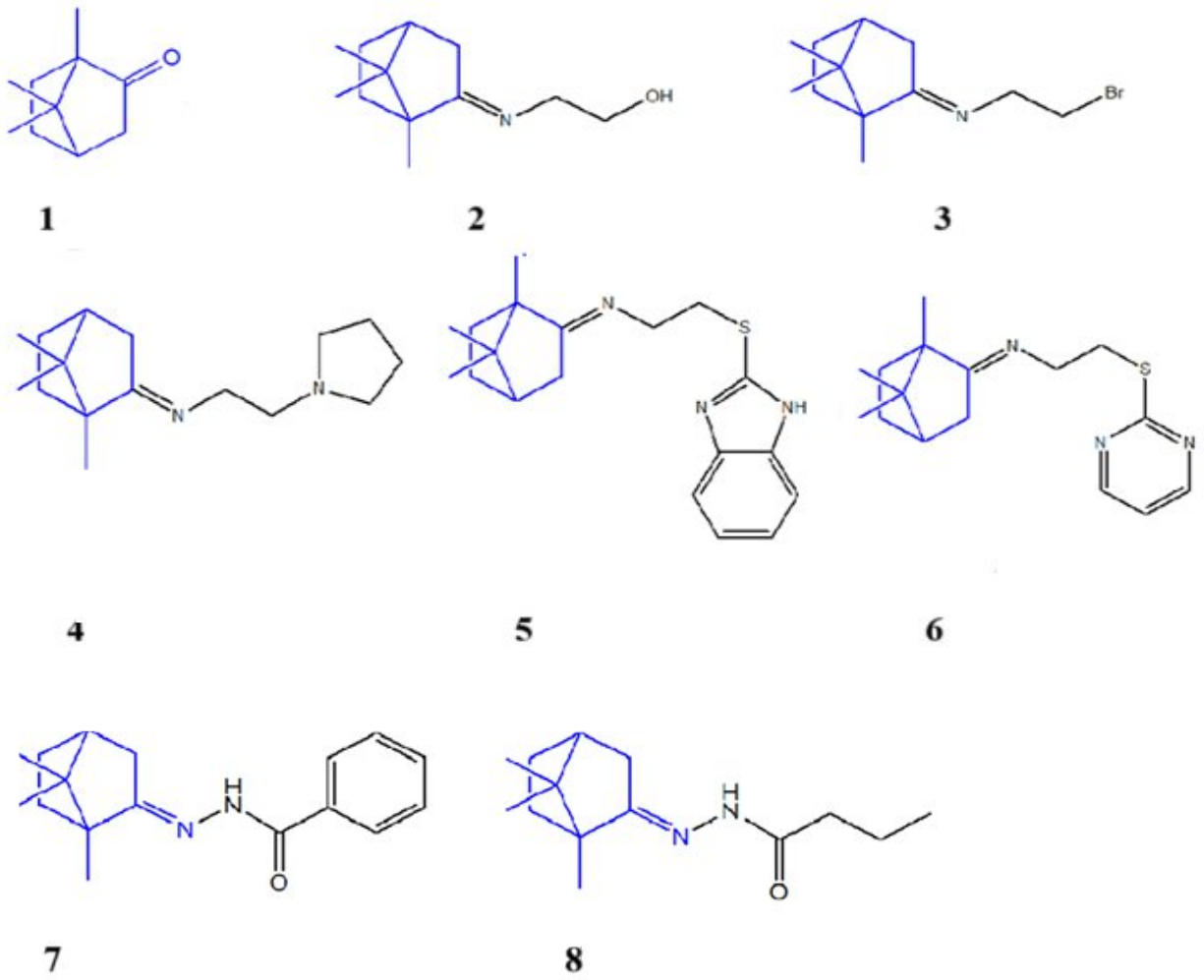
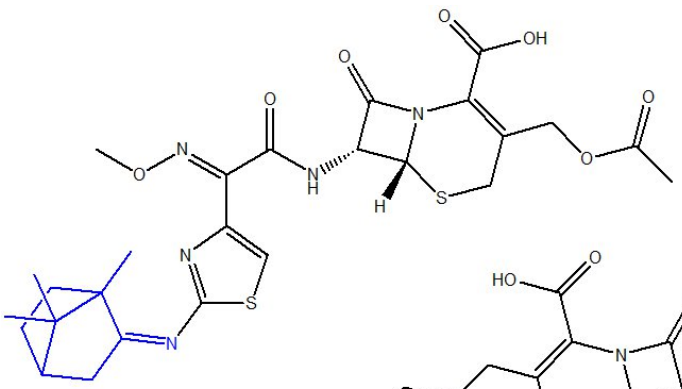


Figure 2: SB derivatives of camphor that have antiviral activities

2.3.2. Camphor derivatives as antibacterial agents

Five new Schiff bases were synthesized through the condensation reaction of *R*-camphor and five third-generation cephalosporins (cefotaxime, ceftazidime, ceftriaxone, cefdinir, and cefixime) (Ali *et al.*, 2019). The *in vitro* antibacterial activity was assessed against selected bacteria, including gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and one-gram positive bacteria (*Staphylococcus aureus*) using disk diffusion method. All compounds exhibited maximum antibacterial activity against all tested bacteria compared to the reference drug. Compounds (**9** and **10)** showed a greater effect on *S. aureus* than on *E. coli* and *P. aregions,* while Compounds (**11** and **12)** yielded similar results with the highest antibacterial activity against all tested bacteria compared to the reference drug.

The silver camphor imine complexes were synthesized as novel antibacterial compound (Leitão *et al*., 2018). Silver nitrate (AgNO3) binds one or two camphor ligands per metal atom to create the complex. Their antibacterial activity towards Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli, P. aeruginosa, Burkholderia contaminans*) has been screened. according to the MIC values for complexes (**13**), (**14**) and (**15**), the presence of one aromatic group between the two camphor moieties (**14**) enhanced the antimicrobial activity towards the Gram-negative strains, whereas two consecutive aromatic groups between the camphor moieties (**15**) led to a general loss of antibacterial activity. A marked decrease in activity was observed upon replacement of the Para by Meta substituted aromatic spacer.



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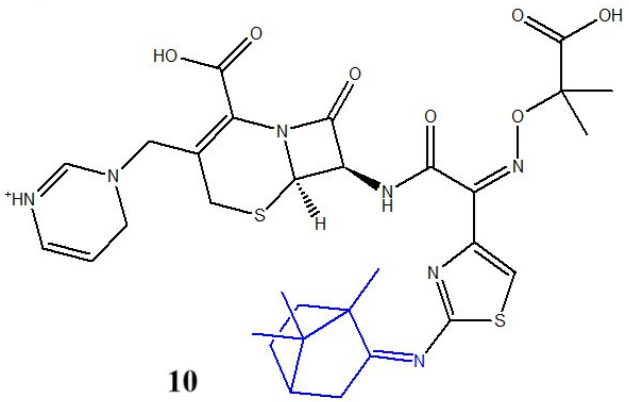

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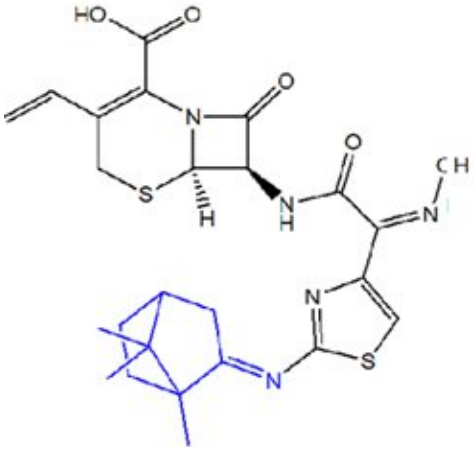

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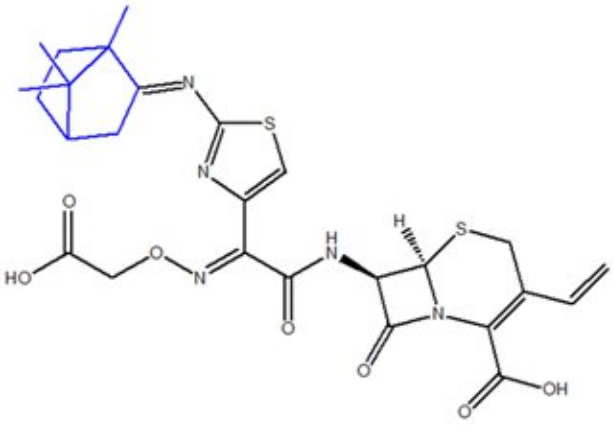

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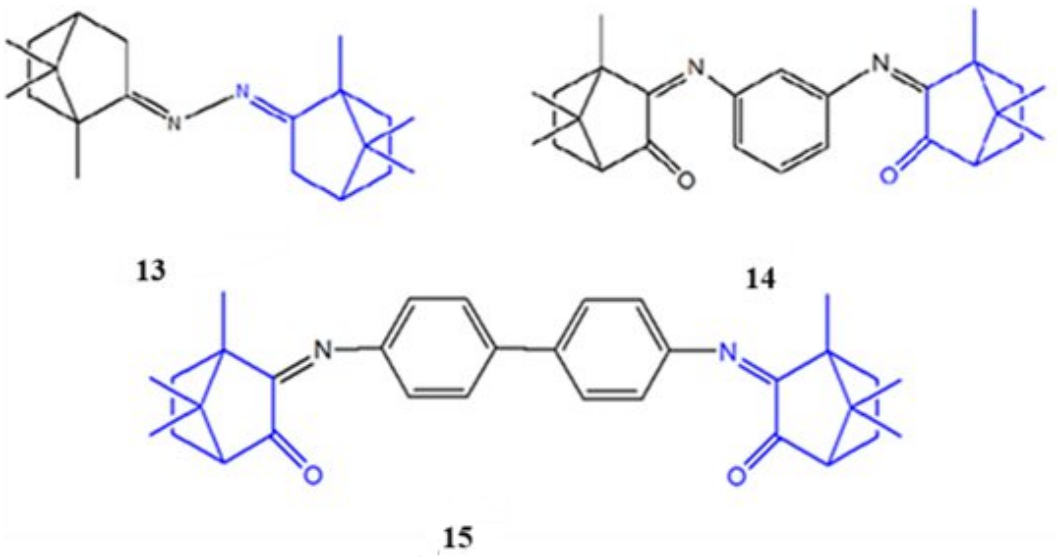


Figure 3: SB derivatives of camphor that have antibacterial activities

2.3.3. Camphor derivatives as antifungal agents

The synthesis, antifungal activity, cytotoxicity and quantitative structure–activity relationship (QSAR) study of camphor derivatives were conducted in 2022 (Duan *et al*., 2022). Out of 37 compounds, (**16**), (**17**) and (**18**) demonstrated strong antifungal activity against *Trametes versicolor*, with EC_{50} values of 0.43, 6.80 and 4.86mg/L, respectively, these value were better than that of tricyclazole (EC_{50} 118.20 mg/L) and comparable to or better than that of carbendazim (EC_{50} 1.20 mg/L). The most effective compound (**18**), displayed broad-spectrum antifungal activity against six fungi with EC_{50} values ranging from 0.43 to 40.18 mg/L. Derivatives with a substituent group at the para-position generally exhibited superior antifungal activity against *T. versicolor*, based on the effect of the substituent position in the benzene ring. Additionally, fluorine mono substituted compounds had a stronger antifungal effect than fluorine-poly substituted derivatives.

Costa *et al.*, (2019) synthesized camphor imine complexes using Ag (OAc) or AgCl as metal sources. The reaction media's basic characteristics led to the formation of hydroxide or oxide complexes rather than acetate or chloride complexes. By selecting of the camphor imine ligands (L) that included mono and bicamphors, the design of complexes with distinct electronic and steric properties and thus different biological activities was made possible. In conclusion, the study's most significant achievement was that the novel oxo and hydroxo silver camphor imine complexes overcome *C. albicans'* resistance. Furthermore, the complexes achieved MIC_{50} values for *C. glabrata* even lower than those previously reported for the camphor imine nitrate complexes [Ag (NO3) L]. The oxo [Ag (OH) L] and hydroxo [{AgL}2}(μ-O)] silver camphor imine complexes exhibited even greater antifungal activity against *Candida albicans* than against *Candida glabrata*.

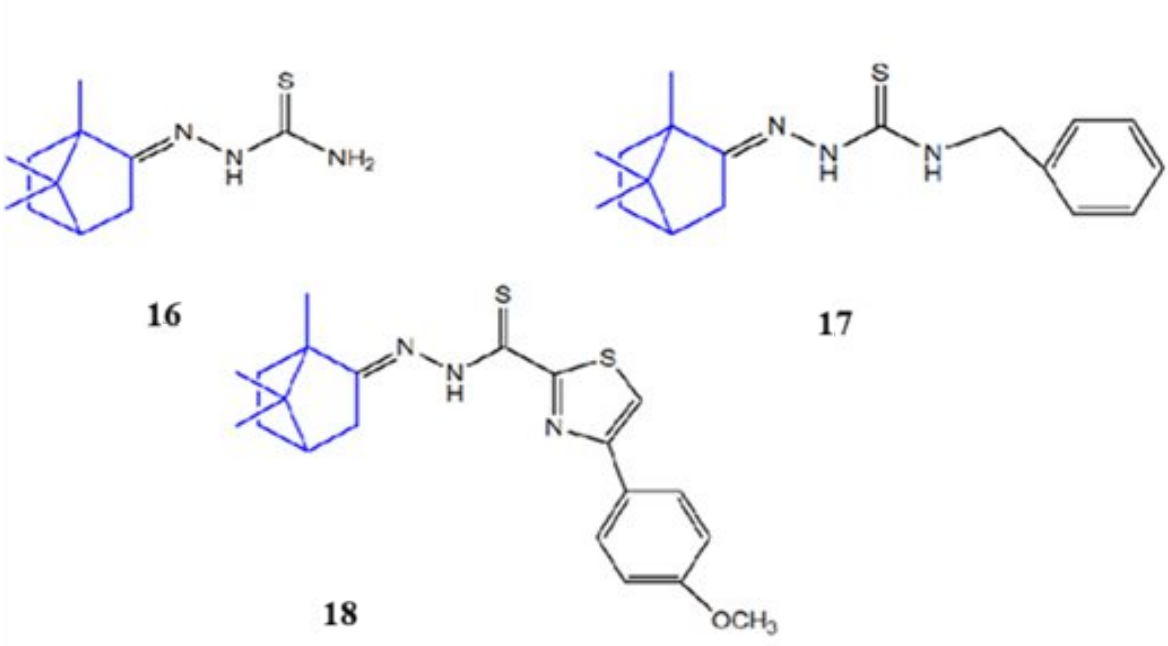


Figure 4: SB derivatives of camphor that have antifungal activities

2.4. Mechanism of action of camphor

Although the antibacterial mode of action of terpenes is not completely understood, the majority of terpenoids have the ability to inhibit two crucial processes that are necessary for microbial survival: oxygen absorption and oxidative phosphorylation (Costa *et al*., 2019). In addition, monoterpenes are likely to disrupt the permeability and fluidity of the bacterial membrane, changing topology of the membrane proteins and halting the respiratory. Studies have investigated the antibacterial mechanism of the bicyclic monoterpene 1, 8-cineol (CN) against carbapenemase producing *Klebsiella pneumoniae* (KPC-KP). Oxidative stress induced by CN led to the rupture of the bacterial membrane through lipid peroxidation and leakage of intracellular material, ultimately resulting in cell death (Moo *et al*., 2021). *Cupressus funebris* (EO) also induced significant cell morphological changes including bacterial cell wall damage in MRSA bacterial strains. *In-silico* molecular docking analysis showed that the two major components of the essential oil (citronellal and terpinene-4ol) exhibited strong binding with the DNA-gyrase B target protein. This suggests their ability to inhibit this bacterial protein, leading to antibacterial action of the essential oil (Yuan & Hao, 2023). While the complete mechanism of action of camphor as antibacterial agent remains unknown, many experts and specialists believe that its antibiotic properties include destabilizing the structure of the phospholipid bilayer, interacting with membrane enzymes and proteins, functioning as a proton exchange, and reducing the $_{P}$H gradient across the membrane (Ali *et al*., 2019). Studies have shown that the mechanism of action of camphor as antifungals is cytomembrane destruction, enhancing the permeability of the cytomembrane, and releasing intracellular macromolecules, such as nucleic acids and proteins (Kong *et al*., 2022).

3. Materials and Methods

3.1. Materials

3.1.1. Chemicals and reagents

Chemicals and reagents used in this study were: L-camphor (BDH, England), 2,4-dinitrophenylhydrazine, hexane, glacial acetic acid, dimethyl sulfoxide, methanol, chloroform and ethyl acetate (all from Sigma-Aldrich Co., MO, USA) were used for the synthesis, purification and chromatogram of the compounds. All the solvents were of analytical grade.

3.1.2. Instruments

Silica gel analytical TLC plates (60 F254, 0.2 mm thick, Merck KGaA, Darmstadt, Germany) and column silica gel (60 F254, 70 - 240 mesh, Merck KGaA, Darmstadt, Germany) were used for the analysis and purification for the compounds during synthesis. Rotary evaporator (BUCHI Rotavapor™ R-300, Switzerland) was used for the removal of solvents. Incubator (Modell 100-800 memmert), autoclave, biosafety cabinet (biostarmodell50/60), water bath, inoculating loops, electrical weighing balance, magnetic stirrer, test tube, pipette tips, pipette filler, micro-pipette (10-200µl), micro-titter plates, flask, capillary tube and measuring cylinder were used for synthesis and antimicrobial test.

A UV viewing cabinet (CAMAG UV cabinet) was used to visualize the TLC chromatogram plates, which used for monitoring reaction progress. NMR spectra were recorded using 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR on a Bruker Avance DMX400 FT-NMR spectrometer (Bruker, Billerica, 18 Massachusetts, USA), tetramethyl silane (TMS) as internal standard and $CDCl_3$ was used as solvent.

3.1.3. Media, microbial strains and standard drugs

Mueller-Hinton Broth (MHB), Mueller Hinton Agar (MHA), Sabouraud Dextrose Agar (SDA) (HiMedia Laboratories Pvt. Ltd. India), Nutrient Broth (NB) and Sabouraud Dextrose Broth (SDB) (Sisco Research Laboratories Pvt. Ltd. India) were used for the media preparation.

The gram-positive bacteria strains were used; *Bacillus pumilus 82, B.subtilis ATCC 6633, Staphylococcus aureus* MDR10*, S.aureus* MDR 1 *and S.aureus* MDR 2*. The gram-negative bacterial strains include; Escherichia coli* 3:37C*, E.coli HB101, E.coli13 C600, E. coli 7360, E. coli 872, E. coli CD/99/1, E. coli K 88, E. coli T 37, E. coli ROW 7/12 E. coli 5933, Salmonella enterica TD 01, S. typhi Ty2, Shigella boydii D13629, S. dysentery 8, S. flexneri Type 6, S. soneii 1, Vibrio cholerae NCTC 5596, V. cholerae NCTC 10732, V. cholerae NCTC 11501, V. cholerae NCTC 4693 and Pseudomonas aeruginosa MDR.*

Moreover, *in vitro* antifungal activity testing was done on four fungal strain; *Candida albicans ATCC 10231, Aspergillus niger ATCC 6275, Penicillium notatum ATCC 11625 and Penicillium funiculosum NCTC 287*. All microbial strains were obtained from the Department of Technology, Jadavpur University; Central Drugs Laboratory, Kolkata and Institute of Microbial Technology, Chandigarh, India. Ciprofloxacin and Griseofulvin were used as a positive control for antibacterial and antifungal tests**,** respectively.

3.2.Methods

3.2.1. Synthesis of **(*E*)-1-(2, 4-dinitrophenyl)-2-(1, 7, 7-trimethyl bicyclo [2.2.1] heptan-2-ylidene) hydrazine (19)**

Compound **19** was synthesized by using the Schiff base reaction as described by Da Silva et al, (2015). Camphor methanolic solution was prepared by dissolving 3.045g of camphor in 20 ml of

methanol. Similarly, 3.963 g of 2, 4- dinitrophenylhydrazine was dissolved in 20ml of methanol in separate flask. 3ml acetic acid was added in camphor solution. Then after, The 2, 4-dinitrophenylhydrazine solution was gradually added to camphor-acetic acid solution. The reaction was refluxed for 6 hours at 70℃, and the reaction progress was monitored by using analytical TLC with the hexane: ethyl acetate (4:1) ratio solvent system. The completion of the reaction was confirmed by the consumption of starting material. After the reaction completion, the mixture was allowed to dry at room temperature; an orange solid product was obtained. The final product was gained through purification with column chromatography. The structure of the compound was determined by using their ^{1}H and ^{13}C- NMR spectra data.

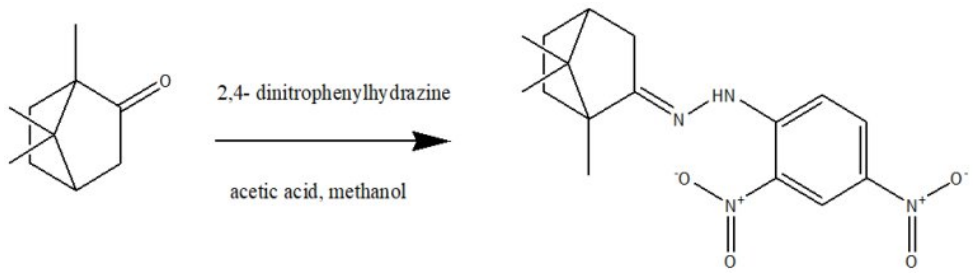


Figure 5: Synthesis of camphor Schiff base derivative

3.2.2. Antimicrobial activity assay

3.2.2.1. Preparation of media, inoculum and standardization

The media used to conduct the experiments was prepared in accordance with the manufacturer's instructions. Mueller Hinton Agar (MHA) and Mueller Hinton Broth (MHB) for bacteria, Sabouraud Dextrose Broth (SDB) and Sabouraud Dextrose Agar (SDA) for fungi were used and NB (Nutrient Broth) for general bacterial growth comparison as a control. To use these media, 38g of MHA, 65g of SDA, 30g SDB and 8g of NB were weighed and each dissolved in 1 L of distilled water then boiled by shaking frequently in a separate media bottle according to

manufacturer's instructions. After that, the media was sterilized by autoclaving at 121℃ for 15 minutes by using Autoclave (JSR JSAT-105 Autoclave Korea). A media was removed from autoclave and put in a 50℃ hot water bath to cool down. Then the media was dispensed on the sterilized 100mm Petri dishes in a Bio-safety Cabinet (Bioair instruments, Eurolone Company, Italy) and allowed some time to solidify. All the selected microorganisms were sub-cultured on Petri dishes containing respective culture media. Each microorganism was incubated for18-24 h at 37 °C and up to 7 days at 25°C, respectively. Four–Five colonies for each tested microorganism were inoculated in to Petri dishes containing media by using inoculating loop for antimicrobial testing. For standardization, 3-5 inoculations of a fresh, pure culture of the specimen bacterium suspension were prepared in Mueller Hinton broth. The absorbance of the prepared suspension was measured by using an ultraviolet-visible (UV) spectrophotometer (Thermo Scientific Evolution 60s CAT 840210100) at 625 nm having a path length of 1cm until the absorbance reading was 0.08 to 0.1. The antimicrobial activity testing was conducted based on Eloff and the Clinical and Laboratory Standards Institute (CLSI) guideline.

3.2.2.2. Disc diffusion method

The *in vitro* antibacterial activity of produced compounds was screened using the disc diffusion method (Hegstad, 2014). To generate nutritional agar plates, molten medium was placed into sterile Petri dishes. The plates were then incubated for 24 hours at 37°C to conduct a sterility test. A standardized inoculum was equally swabbed onto the agar plates and then allowed to dry. The 6 mm filter paper discs (Whatman no. 1) were then coated with the 200 µg/ml test samples and placed on the top medium. The Petri plates were then incubated for 24 hours at 37 °C. The antifungal experiment was conducted using the Saborauds dextrose medium, except that the test samples were employed at a concentration of 2000 µg/ml and the petri dishes were cultivated for

three days at room temperature. Lastly, the inhibitory zone's diameter was determined in millimeters. For antibacterial and antifungal assays, ciprofloxacin and glimeofulvin served as standard references, and 1% DMSO served as a negative control

3.2.2.3. Broth dilution method

Broth dilution method was performed to determine Minimum Inhibitory Concentration of synthesized compound. Stock solution of synthesized compound was prepared by dissolving in 1% DMSO and distilled water. For each tested microorganism three 96-well micro plates were prepared, and Muller Hinton broth and sabouraud dextrose broth were added into each of the 96-well microplates for bacteria and fungi, respectively. Serial dilutions of the stock solution were made ranging from 800µg/ml to 5µg/mL for the antibacterial activity test, and from 2000µg/mL to 50µg/mL for the antifungal activity test in each of the 96-well microplates. The bacterial suspension containing approximately 5×10^5 colony-forming units/ml was prepared from a refreshed culture. Similarly, the fungal suspension containing approximately 5×10^4 colony-forming units/ml was prepared. From these suspensions, 100µl was inoculated into each well. Additionally, growth control (broth and tested microorganism) and sterility control (only broth) were run parallel to the experimental tests on the remaining columns. After that, all tests were incubated for 18-24 hours at 37°C. To measure the MIC value, 40µl of a 0.4mg/ml solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added and incubate for 30 min at 37℃.The MIC values were visually determined by observing the presence or absence of purple color on each plate. The lowest concentration of every well that produced no apparent purple color considered as MIC value. A negative control trial with only 5% DMSO was also carried out. The experiment was done in triplicate.

3.3. *In Silico* Studies

3.3.1. Molecular docking study

Molecular docking of the synthesized compounds was performed on the three-dimensional (3D) crystal structure of the *E. coli* DNA gyrase subunit B (GyrB24) with inhibitor KOB20 downloaded from the PDB (7P2Xpdb) (www.rcsb.org). Molecular docking study was performed using the Schrödinger suite 2023 version 1 (Schrödinger Inc., New York, USA). The molecular docking process comprised four steps: protein preparation, ligand preparation, grid receptor generation, and molecular docking, utilizing default values.

3.3.1.1. Protein Preparation

The crystal structure of *E. coli* GyrB24 (PDB ID: 7P2X) was retrieved from protein data bank. The downloaded protein was run through the Schrödinger suite's protein preparation wizard module in order to establish the right ionization states, assign suitable bond ordering, add missing hydrogen atoms and remove water molecules in order to carry out the best possible docking investigation.

3.3.1.2. Ligand preparation

Ligand preparation was carried out by using the Ligprep module of the Schrödinger suite. The Compound drawn in ChemDraw SD format were imported and prepared.

3.3.1.3. Receptor grid generation

A receptor grid generation panel was carried out in order to create a grid box around the co-crystallized ligand in the receptor protein that permits docking into the active site. The protein structure was selected on the workspace to define the ligand-binding site, which has a radius of 6.0 Å from the advanced setting option of the site. The van der Waals radii of the receptor atoms

was set with partial atomic charge scaling factor of 0.8 and partial cut-off of 0.15 to soften the receptor's non polar parts.

3.3.1.4. Ligand docking

Extra precision (XP) docking was applied to the synthesized compound. The docking protocol was validated by re-docking the inhibitor (KOB20) into the protein's active site.

3.3.2. Prediction of ADMET property

The absorption, distribution, metabolism, and excretion (ADME), toxicity and physicochemical properties prediction of synthesized compound was made using online tools ADMETlab 2.0 software.

3.4. Statistical analysis

The Statistical Package for Social Science (SPSS) version 20 was used to enter the data into an Excel spreadsheet for statistical analysis. The results were presented as mean standard deviation of mean (M ±SD). To compare antimicrobial activity assay values between treatment and control groups, statistical significance was established using one-way ANOVA followed by the Tukey post hoc test. Statistically significant differences were declared at a p-value of less than 0.05.

4. Results and Discussion

In this study, three camphor-based Schiff base derivatives were targeted for synthesis, but only one —(*E*)-1-(2,4-dinitrophenyl)-2-(1,7,7-trimethylbicyclo[2.2.1]heptan-2-ylidene)hydrazine (**19**)— was successfully synthesized and characterized; the others likely failed due to steric or reaction limitations.

4.1. Synthesis of (*E*)-1-(2, 4-dinitrophenyl)-2-(1,7, 7-trimethylbicyclo [2.2.1] heptan-2-ylidene) hydrazine (19)

(*E*)-1-(2, 4-Dinitrophenyl)-2-(1, 7, 7-trimethylbicyclo [2.2.1] heptan-2-ylidene) hydrazine (**19**) was synthesized via a condensation reaction between camphor (**1**) and 2, 4-dinitrophenylhydrazine in the presence of acetic acid. Compound **19** (62%, yield) was obtained as an orange solid with an R_f value of 0.6 using hexane: ethyl acetate (4:1) as solvent system, as indicated in Table 1.

Table 1: Physical properties of the synthesized compound (**19**)

Code	M.F.	M. Wt. g/mol	R_f value (hexane: ethyl acetate(4:1))	Yield (w/w)	Physical appearance
Compound **19**	$C_{16}H_{20}N_4O_4$	332.35	0.6	62%	Orange colure

The purity of compound **19** was monitored using TLC. It quenched fluorescence at 254 nm, appearing as a dark spot under 254 nm UV light, and displayed a brown color under 366 nm UV light (Figure 7)

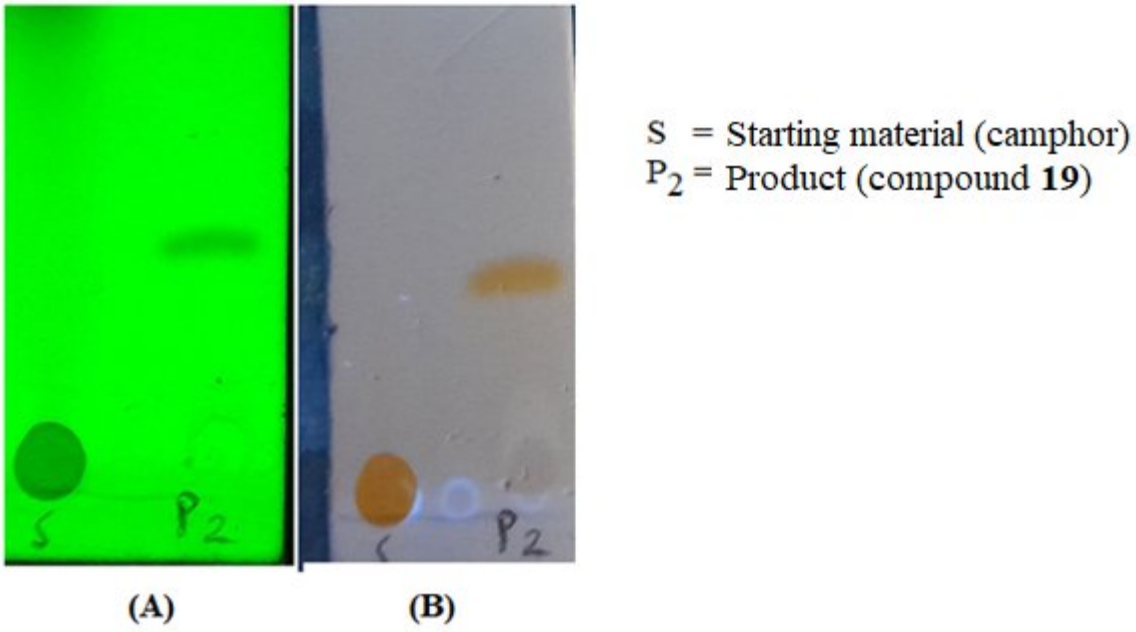


Figure 6 : TLC Chromatograms of the synthesized compound (**19)** (A: viewed under 254 nm; B: viewed under 366 nm)

The reaction mechanism for the formation of compound **19** proceeds through five distinct steps, as illustrated in Figure 7. The synthesis begins with the activation of the carbonyl group in camphor, where the carbonyl oxygen is protonated by acetic acid. This protonation increases the electrophilicity of the carbonyl carbon, making it more susceptible to nucleophilic attack. Next, the lone pair of electrons on the nitrogen atom of the primary amine, 2,4-dinitrophenylhydrazine, attacks the activated carbonyl carbon, resulting in the formation of a tetrahedral intermediate. Following this, a proton transfer occurs within the intermediate, typically moving from the nitrogen to the oxygen atom, which leads to the creation of an unstable carbinolamine structure characterized by both hydroxyl (OH) and amine (NH) groups attached to the same carbon. Under acidic conditions, the hydroxyl group is then protonated and eliminated as water in a dehydration step, forming an iminium ion intermediate. Finally, this iminium ion undergoes deprotonation to

yield compound 19, the Schiff base, which features a characteristic carbon-nitrogen double bond (C=N).

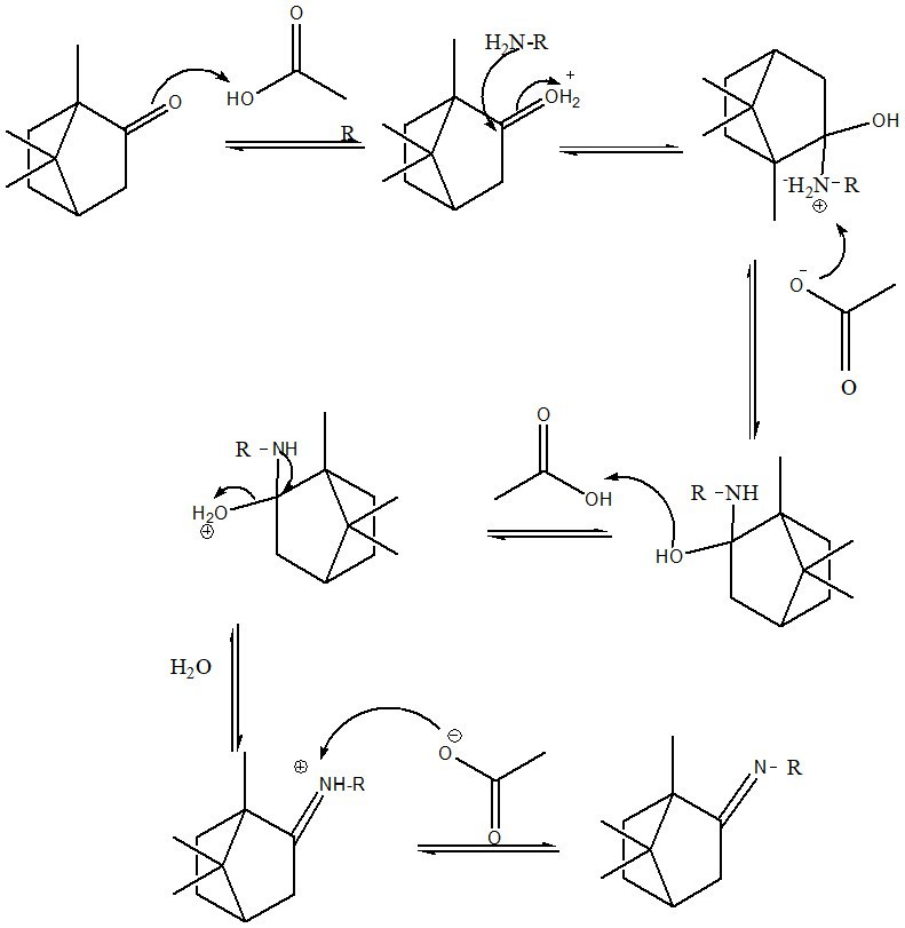


Figure 7: Proposed mechanism of reaction for the synthesized compound (**19)**

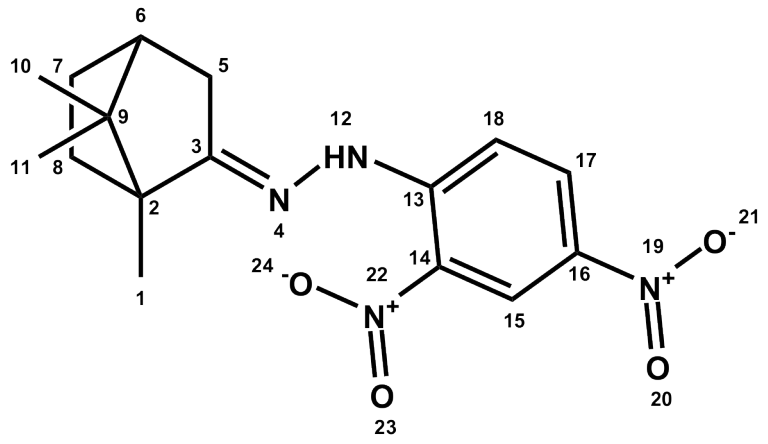
4.2.Structural elucidation of the synthesized compound (19)

The structure of compound **19** was confirmed by the ^{1}H and ^{13}C NMR spectral data. The ^{1}H-NMR spectrum of compound **19** showed signals at d 9.15 (1H, *s,*), d 8.32 (1H, *d*, *J*=12H) and d 8.09 (1H, *d*, *J*=12H), corresponding to the phenyl protons H-15, H-17, and H-18. The presence of secondary amine (NH) moiety in compound **19** was evident from a signal resonate at δ10.8 (1H, *brs*, H-12). Additionally, there are three important methylene protons, that appeared as *exo*

and *endo* configuration at δ 2.6 (*d*, 1H, H-5, *exo*), 2.1(*s*, 1H,H-5,*endo*), 1.98(*m*,1H,H-7,*exo*), 1.32(*s*,1H,H-7,*endo*), 1,86(*m*,1H,H-8,*exo)* and 1.27(*s*,1H,H-8,*endo*). The signal at δ1.49 (1H, *m*, H-6) indicated that the presence of methine proton. All other proton signals are indicated in Table 2.

The ^{13}C-NMR spectrum of compound **19** revealed a total of sixteen carbon atoms. A distinct signal at δ 171.55 in the ^{13}C-NMR spectrum strongly indicates the presence of an azomethine carbon (C=N). Additional key carbon signals are summarized in Table 2.

Table 2: ^{1}H-NMR and ^{13}C-NMR spectral data of compound **19** in $CDCl_3$



Position	^{1}H-NMR (δH, ppm)	^{13}C-NMR (δC, ppm)
1	0.83(1H,*s*)	11.12
2	-	53.43
3	-	171.55
5	2.62(1H,*d*,J=16H) &2.12(1H,*s*)	34
6	1.49 (1H,*m,J*=16H)	44.07
7	1.98(1H,*m,J=16H*)&1.32(*s*)	27.2
8	1.86(1H,*m,J*=12H)&1.27(*s*	32.65

	)	
9	-	48.46
10	1.15(*s*)	18.64
11	1.02(*s*)	19.59
12	10.8(*s*)	
13	-	145
14	-	128.7
15	9.15 (*s*)	123.64
16	-	137
17	8.32 (1H, *d*, *J*=12H)	129.89
18	8.09 (1H, *d*, *J*=12H)	116.42

4.3.Antimicrobial activity of the synthesized compounds

4.3.1. Antibacterial Activity

The synthesized compound (**19**) demonstrated broad-spectrum antibacterial activity against 26 bacterial strains, as evaluated by disk diffusion and broth dilution methods, as shown in Table 3. Overall, the compound exhibited antibacterial activity with MIC values ranging from 25 µg/mL to 200 µg/mL. Interestingly, compound **19** demonstrated significantly enhanced activity against all *E. coli* strains and *Staphylococcus aureus* MDR 10, with an MIC value of 25 µg/mL—twice as effective as camphor (1), which had an MIC of 50 µg/mL. These findings are particularly important, as *E. coli* is a major pathogen responsible for diarrheal infections worldwide, as well as neonatal meningitis, septicemia, and urinary tract infections (Makvana & Krilov, 2015) .

S. aureus is a common pathogen responsible for a wide spectrum of infections, ranging from mild skin conditions to severe, life-threatening illnesses(Chen *et al*., 2022). According to the World Health Organization, nearly 80% of *S. aureus* infections in Africa are resistant to

methicillin, rendering many conventional antibiotics ineffective for treatment. Indeed, *S. aureus* remarkable ability to develop resistance to multiple antibiotics further complicates treatment strategies (Moglad & Altayb, 2022). Interestingly, Compound **19** also demonstrated promising antibacterial activity against AMR strains. For instance, compound **19** was effective against *S. aureus* MDR1 and MDR2, with MICs of 50 μg/mL—significantly more potent than camphor (**1**), which required concentrations of 200 μg/mL to achieve similar effects. Furthermore, compound **19** exhibited strong activity against *P. aeruginosa* MDR1, showing comparable effectiveness to ciprofloxacin, with a ZOI of 11.5 mm and an MIC of 100 μg/mL. This suggests that incorporating hydrazone moieties into the camphor structure can enhance its biological activities. Hydrazones are known for their structural stability and strong interactions with microbial targets, making them promising candidates for antimicrobial drug development (P. C. Sharma *et al*., 2020).

In addition to *E. coli* and *S. aureus*, Compound **19** showed activity against *Vibrio cholerae* strains, showing ZOIs of 15.0–15.5 mm and MICs of 50 μg/mL. The level of activity was slightly lower than that of ciprofloxacin but superior to camphor (**1**), providing further confirmation of its enhanced antibacterial potential. The activity against *Salmonella typhi* and *Salmonella enterica*, was moderate, with ZOIs of 13.5 mm and MICs of 100 μg/mL for both compounds (**1** and **19**). Similar patterns were observed for various Shigella species, where Compound **19** exhibited relatively weaker activity, with ZOIs between 12.0–13.0 mm and MIC values ranging from 100 to 200 μg/mL—considerably lower than the standard, which achieved ZOIs up to 20.5 mm. On the other hand, compound **19** displayed poor activity against Gram-positive *Bacillus* species, namely *B. pumilus* and *B. subtilis*, with low ZOIs (6.0 mm) and high

MICs (800 µg/mL), indicating limited effectiveness against these strains. The remaining antibacterial activity is compiled in Table 3.

Table 3: Zone of inhibition (ZOI) and minimum inhibition concentration (MIC) of the synthesized compound (**19)** against the tested bacterial strains

Bacterial strains	ZOI in mm (200 µg/ml)			MIC (µg/ml)	
	Cpd**19**	Cpd**1**	Std	Cpd**19**	Cpd**1**
E.coli NCTC 5933	14.5	13.0	16.0	25	50
E.coli K88	15.5	13.5	17.0	25	50
E.coli NCTC 7360	15.0	14.0	17.0	25	50
E.coli LT37	15.0	13.5	16.0	25	50
E.coli 872	15.5	14.0	16.0	25	50
E.coli ROW 7/12	15.5	14.0	16.5	25	50
E.coli 3:37C	15.5	14.5	16.5	25	50
E.coli CD/99/1	16.0	14.0	17.0	25	50
Salmonella typhi Ty2	13.5	13.5	16.0	100	100
Salmonella enterica TD 01	13.5	13.5	19.0	100	100
Shigella dysentery 8	12.0	11.5	20.0	100	200
Shigella soneii 1	12.5	13.0	19.5	100	200
Shigella boydii D13629	12.0	12.0	20.0	100	200
Shigella flexneri Type 6	13.0	12.0	20.5	100	200
Staphylococcus aureus MDR10	14.0	13.5	18.0	25	50
Bacillus pumilus 82	6.0	6.0	19.0	800	800
Bacillus subtilis ATCC 6633	6.0	6.0	18.0	800	800
Vibrio cholerae NCTC 4693	15.5	14.0	17.5	50	50
Vibrio cholerae NCTC5596	15.0	14.5	18.5	50	50
Vibrio cholerae NCTC 10732	15.0	14.5	19.0	50	50
Vibrio cholerae NCTC 11501	15.5	14.5	18.5	50	50
*E.coli HB101**	12.0	12.0	14.0	100	200
*E.coli C600**	11.5	11.5	13.5	100	200
*S.aureus MDR 1**	11.0	10.0	11.5	50	200
*S.aureus MDR 2**	11.0	11.5	12.0	50	200
*Pseudomonas aeruginosa MDR 1**	11.5	10.5	12.5	100	100

*Cpd = Compound, Std= Ciprofloxacin

4.3.2. Antifungal Activity

Compound **19** exhibited antifungal activity against all four tested fungal strains, with ZOI ranging from 10.5 to 11.5 mm at 1500 μg/mL (Table 4). The MIC values of compound **19** were 800 μg/mL for all strains. While its activity was generally comparable to that of camphor (**1**), both were less potent than the standard antifungal agent, griseofulvin. Camphor (**1**) showed slightly better MIC activity against *Candida albicans* (400 μg/mL) compared to compound **19**.The antifungal activity of compounds **1** and **19** is presented in Table 4.

Table 4: Zone of inhibition (ZOI) and minimum inhibition concentration (MIC) of the synthesized compound (**19)** against the tested fungal strains

Fungal strain	ZOI mm (1500 μg/ml)			MIC (μg/ml)	
	Cpd **19**	Cpd 1	Std	Cpd **19**	Cpd **1**
Candida albicans ATCC 10231	11.5	12.0	16.0	800	400
Aspergillus nigerATCC 6275	11.5	11.0	15.0	800	800
Penicillium notatum ATCC 11625	10.5	11.0	13.5	800	800
Penicillium funiculosum NCTC 287	10.5	10.5	14.0	800	800
*Cpd = Compound, Std= Griseofulvin					

4.4. *In silico* analysis

4.4.1. Molecular docking

The potential mechanism of action of the synthesized compound **19** was explored through molecular docking studies, using *Escherichia coli* DNA gyrase subunit B (GyrB24) as the target enzyme. The crystal structure of GyrB24 was retrieved from the Protein Data Bank (PDB ID: 7ZPX) via the RCSB database (www.rcsb.org). Docking simulations were performed using the Schrödinger molecular modeling suite to assess the binding affinity and interaction profile of compound **19** within the enzyme's active site.

Compound **19** demonstrated a moderate binding affinity toward the GyrB24 enzyme, with a docking score of –5.368 kcal/mol, indicating a reasonably stable interaction with the active site.

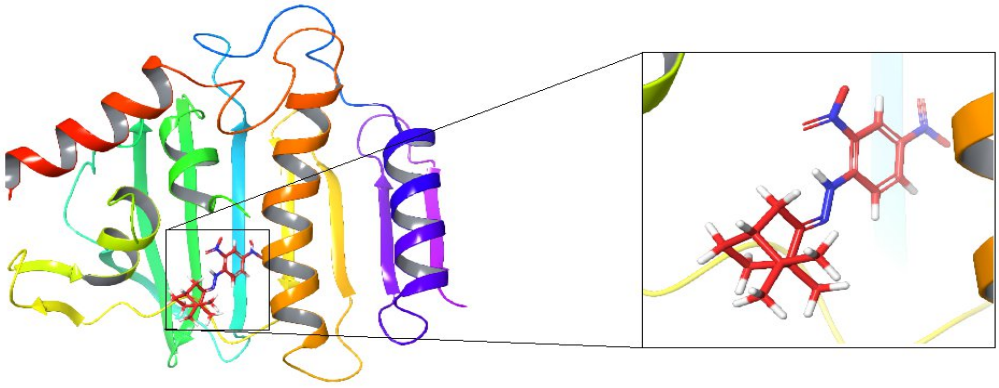
Detailed analysis of the docking results, as depicted in Figure 8, revealed that compound **19** formed a key hydrogen bond with the amino residue ASP73 (Table 5). Furthermore, additional polar interactions were observed with GLN72 and ASN46, which likely contribute to the compound's specificity and orientation within the binding pocket. Hydrophobic interactions involving VAL71 and ALA47 further stabilized compound **19** within the active site, enhancing the overall binding profile.

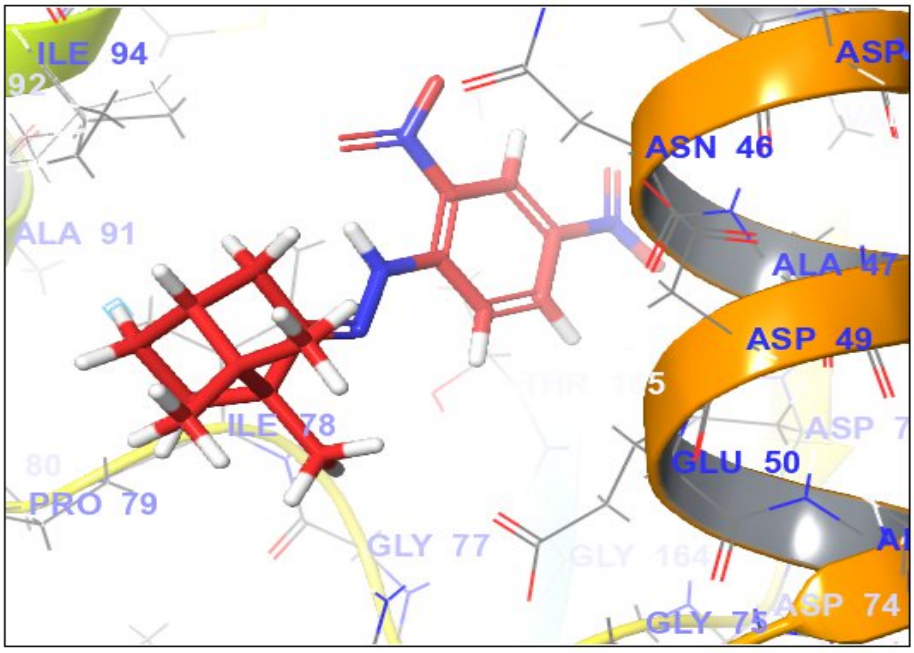
To ensure the reliability and accuracy of the docking protocol, a validation step was conducted using the native co-crystallized inhibitor KOB20. The redocking of KOB20 into the active site of GyrB24 reproduced its experimentally observed binding pose and yielded a docking score of –8.773 kcal/mol. This close alignment with the known structural data confirms the robustness and predictive validity of the docking methodology employed in this study.

Thus, in this study, Compound **19** is proposed to inhibit the DNA gyrase enzyme, a critical target in bacterial DNA replication and cell survival. DNA gyrase, particularly its GyrB subunit, plays an essential role in introducing negative supercoils into DNA, which is necessary for processes such as transcription and replication. Inhibiting this enzyme can effectively halt bacterial growth, making it a valuable target in the development of antibacterial agents. Although its binding affinity is lower than that of some known antibiotics, the compound fits well into the active pocket and forms stabilizing interactions, suggesting potential inhibitory activity. This finding supports the hypothesis that Compound **19** may exert its antibacterial effect, at least in part, by targeting DNA gyrase. Further validation through *in vitro* enzyme inhibition assays and structure–activity relationship (SAR) studies would help confirm its mechanism of action and optimize its potential as an antimicrobial agent.

Table 5: Docking scores of the synthesized compound with 72PX according to Schrodinger 2023 suite docking software.

Ligand	Docking score (kcal/mol)	Interaction with amino acid residues	
		H-bonds	Non-H-bonds
Compound **19**	-5.368	ASP73	GLN72,ASN46, VAL71,VAL71 ALA47 and ALA47





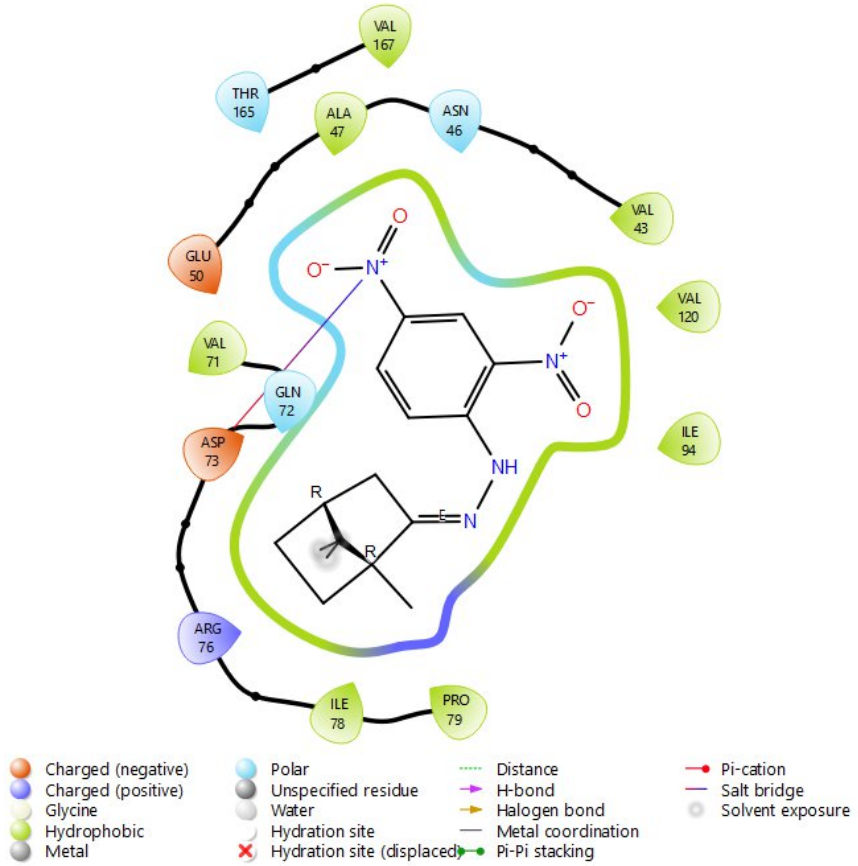


Figure 8: Binding interaction of the synthesized compound (**19**) in *E. coli*

4.4.2. *In Silico* Prediction of ADMET property

ADMETlab2.0 online software tools were utilized to determine the ADMET profiles of the synthesized compound (**19**). The objectives of the analysis were to assess a key pharmacokinetic property of the synthesized compound that determines the drug likeness. The findings are outlined in Table 6. The absorption properties of synthesized compound were predicted with cell permeability (CACO-2), Pgp Inhibitor, Pgp Substrate, Human Intestinal Absorption (HIA) and 20% &30% Bioavailability. The synthesized compound (**19**) has Caco-2Permeability value of -4.801 is within an optimal range, indicating good oral bioavailability. Strong plasma protein binding was found in synthesized compound, which reduces the free plasma fraction and causes

a longer half-life and a smaller volume of distribution. The compound is predicted to be inhibitors of CYP3A4, CYP2C19 and CYP2C9, it leads to drug- drug interaction causing to toxicity. Toxicity profile of the compound was predicted in terms of drug-induced liver injury (DILI), mutagenicity and cardiotoxicity. The result shows that the compound has a probability of being mutagenic and hepatotoxic.

Table 6: ADMET prediction the synthesized compound (**19**)

ADMET Property	**Value**	**Remark**
Absorption		
Caco-2	-4.801	Optimal: higher than -5.15 Log unit
Pgp-Inhibitor	0.071	Category 1: Inhibitor; Category 0: Non-inhibitor. The output value is the probability of being Pgp-inhibitor
Pgp-Substrate	0.0	Category 1: substrate; Category 0: Non-substrate; The output value is the probability of being Pgp-substrate
HIA	0.0	Category 1: HIA+(HIA < 30%); Category 0: HIA-(HIA >= 30%); The output value is the probability of being HIA+
F (20%)	0.006	Category 1: F 20% + (bioavailability < 20%); Category 0: F 20% - (bioavailability ≥ 20%); The output value is the probability of being F 20%
F (30%)	0.0	Category 1: F 30% + (bioavailability < 30%); Category 0: F 30% - (bioavailability ≥ 30%); The output value is the probability of being F 30%
Distribution		
PPB	99.431	plasma Protein Binding; Optimal: < 90%
VD	0.137	Volume Distribution; Optimal: 0.04-20L/kg
BBB	0.0	Category 1: BBB+; Category 0: BBB-; The output value is the probability of being BBB+
Metabolism		

CYP1A2	0.009	Category 1: Inhibitor; Category 0: Non-inhibitor
CYP3A4	0.467	Category 1: Inhibitor; Category 0: Non-inhibitor
CYP2C19	0.593	Category 1: Inhibitor; Category 0: Non-inhibitor
CYP2C9	1.0	Category 1: Inhibitor; Category 0: Non-inhibitor
CYP2D6	0.043	Category 1: Inhibitor; Category 0: Non-inhibitor
Excretion		
Clearance	5.911	>15 ml/min/kg: high clearance; 5-15ml/min/kg: moderate clearance; < 5 ml/min/kg: low clearance.
T1/2	0.835	Ultra-short half-life drugs: 1/2 < 1 hour; short T1/2 between 1-4 hours; intermediate short; T1/2 between 4-8 hours; long T1/2 > 8 hours.
Toxicity		
hERG	0.092	Molecules with IC50 ≤10μM or ≥50% inhibition at 10 μM were classified as hERG+ (Category 1), while molecules with IC50 >10μM or < 50% inhibition at 10μM were classified as hERG -(Category 0)
Ames	0.984	Category 1: Ames positive (+); Category 0: Ames negative (-)
DILI	0.994	Category 1: drugs with a high risk of DILI; Category 0: drugs with no risk of DILI.

The synthesized compound does fulfill the Lipinski rule: molecular weight, logP (which predicts octanol/water partition coefficient, log), the number of H-bond donors, and hydrogen bond acceptors. The findings are outlined in Table 7.

Table 7: Prediction of physicochemical and medicinal chemistry friendliness of compound **19**

Compound	nHA	nHB	nRot	M.wt	LogS	LogP	LogD	Lipinski
Compound **19**	8	1	4	332.15	-5.614	3.842	3.601	accepted

5. Conclusions

In this study, we successfully synthesized a camphor-based Schiff base derivative, (E)-1-(2, 4-dinitrophenyl)-2-(1, 7, 7-trimethylbicyclo [2.2.1] heptan-2-ylidene)hydrazine (**19**), via a Schiff base condensation reaction, achieving a high yield of 62%. The compound exhibited strong antibacterial activity, particularly against *E. coli and S. aureus*, with an MIC value of 25 μg/mL. Particularly, it also showed potent activity against AMR strains, including *S. aureus* MDR1 and MDR2, with an MIC of 50 μg/mL. Molecular analysis suggests that the compound may act by inhibiting DNA gyrase, a critical bacterial enzyme. Additionally, ADMET predictions confirmed the compound's compliance with Lipinski's rule of five, indicating good drug-likeness. Overall, the synthesized camphor Schiff base derivative demonstrates significant potential as a lead compound for the development of new antibacterial agents to combat rising antimicrobial resistance and infectious diseases.

6. Recommendation

Based on the major findings and conclusions, the following possible recommendations are suggested for future studies.

- ✓ To further conduct comprehensive toxicity studies to evaluate the safety profile of the synthesized compound and ascertain any potential adverse effects.
- ✓ Considering the potential therapeutic applications of the synthesized compounds, future research should focus on further structural modification of these lead compounds to enhance its activity, pharmacokinetics and safety profiles.
- ✓ To investigate the synergistic effects of the synthesized compound (**19**) with other antimicrobial agents.

7. Reference

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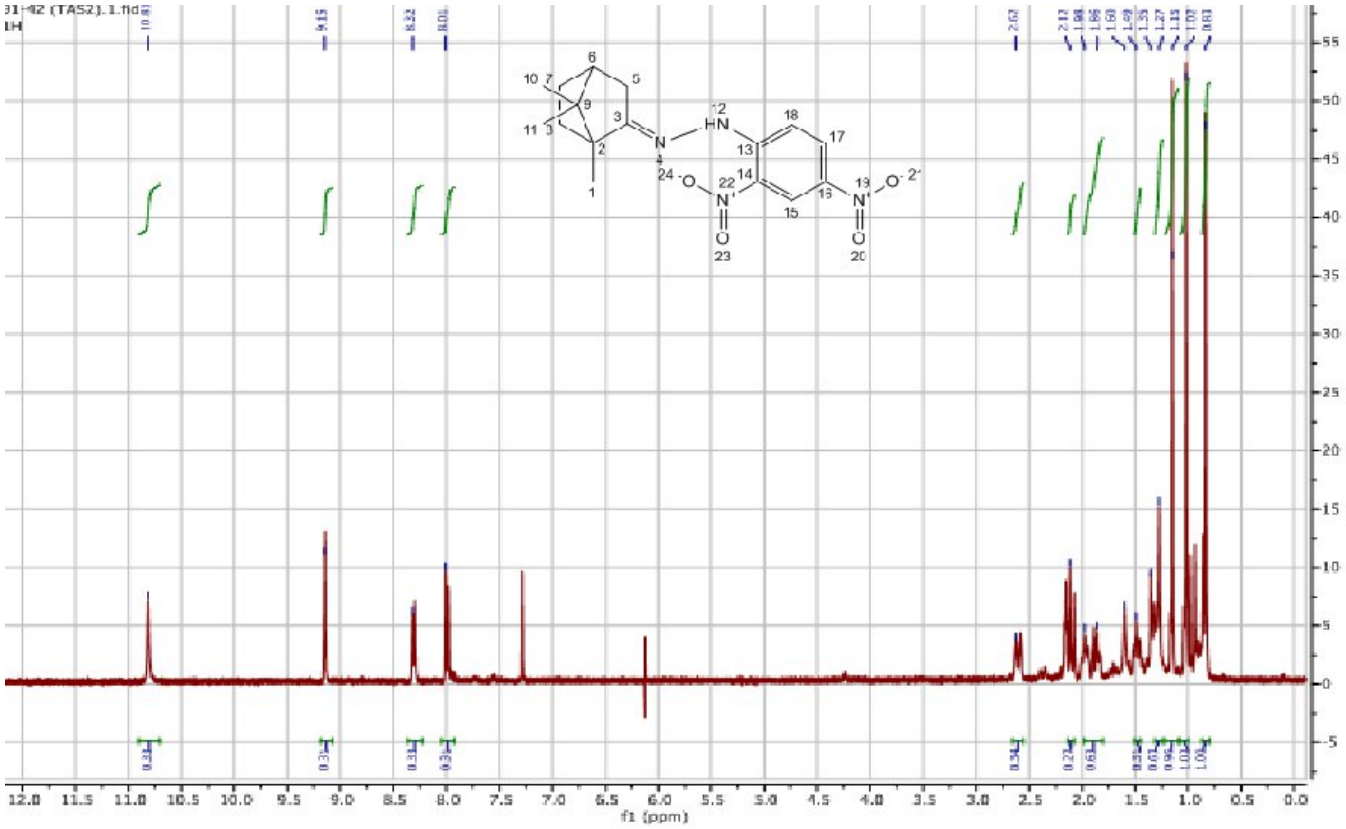
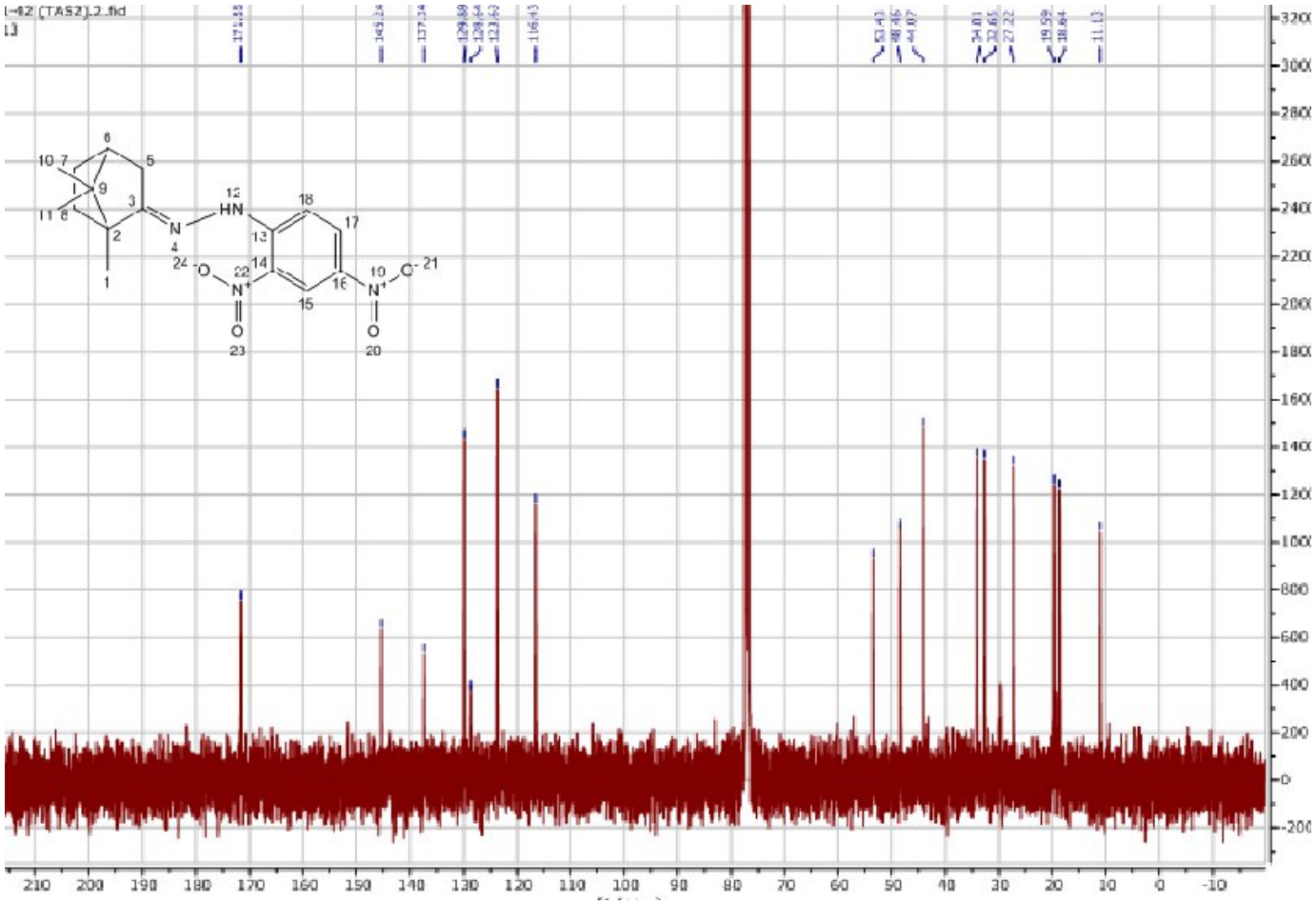
8. Appendex

8.1.[1]H-NMR spectra of the synthesized Compound (**19**)



8.2.[13]C-NMR spectra of the synthesized Compound (19)



Schiff Base Derivative of camphor: Synthesis, *In vitro* Antimicrobial and *InSilico* Studies

Tseganesh Abate[1], Avijit Mazumder[2], Kaleab Asres[1] and Daniel Bisrat[1]

[1]Department of Pharmaceutical Chemistry and Pharmacognosy, School of pharmacy, College of Health Sciences,Addis Ababa University, Addis Ababa, Ethiopia;

[2]Department of Pharmaceutical Technology, Noida Institute of Engineering and Technology, 19 Knowledge ParkII, Institutional Area, Greater Noida, 201306, India

Abstract

Microbial diseases are among the leading causes of mortality and morbidity worldwide. The rise of antimicrobial resistance has become a significant challenge, as microorganisms have developed resistance to many existing drugs. Schiff bases (SB) and their derivatives exhibit a wide range of biological activities, including antimicrobial, anti-inflammatory, antimalarial, anticancer, and antioxidant effects, among others. Therefore, the objectives of this study is to enhance antimicrobial activity of camphor by synthesizing its Schiff base derivative through condensation reaction of *R*-camphor (**1**) with 2, 4-dinitrophenylhydrazine, and also evaluate its antimicrobial activity against twenty six bacterial strains and four fungal strains. The synthesized compound (**19)** chemical structure was determined based on ^{1}H-NMR and ^{13}C-NMR spectral data, as (*E*)-1-(2, 4-dinitrophenyl)-2-(1, 7, 7-trimethylbicyclo [2.2.1] heptan-2-ylidene) hydrazine. Compound **19** showed the highest antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, with an MIC value of 25 µg/ml each, as compared to the starting material (camphor, MIC=50 µg/ml). Molecular docking analysis targeting Gyr24B of *E. coli* predicted moderate ligand-protein interactions of synthesized compound. ADMET predictions indicated that the synthesized compound (**19)** adhered to Lipinski's rule of five. In the end, Schiff base derivative of camphor may hold significant promise in boosting the antimicrobial properties of camphor and addressing drug-resistant microbes.

Key words**:** *R*-camphor, Schiff bases, synthesis, antimicrobial activity, molecular docking, ADMET

1. Introduction

Microbial diseases, caused by pathogenic microorganisms such as bacteria, viruses, parasites, fungi, and their toxic products, are among the leading causes of global mortality and morbidity (Cole & Kramer, 2016). Despite the remarkable advancements in science and technology, the prevalence of infectious diseases has also significantly increased, posing serious and widespread public health threats (Debta *et al*., 2020). Antimicrobial resistance (AMR) is one of the biggest global public health challenges. AMR impacts financial sustainability, global health, food sustainability and security, environmental wellbeing, and socio-economic development(Ahmed *et al*., 2024).

Schiff bases (compounds with a C=N bond), are formed when aldehydes and ketones react with ammonia or primary amines. Schiff bases and their derivatives had exhibit antimicrobial, antiinflammatory, antimalarial, anticancer, antioxidant and other biological effects. Nowadays, camphor is commonly used as a topical medication to treat respiratory depression, as well as poisoning from sedative and narcotics, acute and chronic heart failure (Sokolova *et al*., 2015). In chemistry, camphor has been utilized as a chiral auxiliary, a shift reagent in nuclear magnetic resonance (NMR), a selector in chiral separations, a component of catalytic systems, and a versatile chemical precursor (da Silva *et al*., 2016). Recent studies suggest camphor possesses promising antimicrobial properties by enhancing the antibiotic effectiveness of various antibiotics through synergistic effects (Abdollahi *et al*., 2024). Therefore, synthesizing new camphor derivatives with diverse mechanisms could be a valuable strategy to improve antimicrobial efficacy and combat AMR.

2. Materials and Methods

2.1.Materials

2.1.1. Chemicals and reagents

Chemicals and reagents used in this study were: L-camphor (BDH, England), 2,4-dinitrophenylhydrazine, hexane, glacial acetic acid, dimethyl sulfoxide, methanol, ethanol, Chloroform and ethyl acetate (all from Sigma-Aldrich Co., MO, USA) were used for the synthesis, purification and chromatogram of the compounds. All the solvents were of analytical grade.

2.1.2. Instruments

8.2.1. Nuclear Magnetic Resonance (NMR) spectra were recorded using 400 MHz for proton NMR

8.2.2. (1H-NMR) and 100 MHz for carbon-13 NMR (13C-NMR) on a Bruker Avance DMX400 FT-

NMR spectrometer(Bruker, Billerica, Massachusetts, USA), tetramethyl silane(TMS) as internal standard and deuterated dimethylsulfoxide (DMSO-d) was used as solvent.

2.1.3. Media, microbial strains and standard drugs

Mueller-Hinton Broth (MHB), Mueller Hinton Agar (MHA), Sabouraud Dextrose Agar (SDA) (HiMedia Laboratories Pvt. Ltd. India), Nutrient Broth (NB) and Sabouraud Dextrose Broth (SDB) (Sisco Research Laboratories Pvt. Ltd. India) were used for the media preparation.

The gram-positive bacteria strains were used; *Bacillus pumilus 82, B.subtilis ATCC 6633, Staphylococcus aureus* MDR10*, S.aureus* MDR 1 *and S.aureus* MDR 2*. The gram-negative bacterial strains include; Escherichia coli* 3:37C*, E.coli HB101, E.coli13 C600, E. coli 7360, E. coli 872, E. coli CD/99/1, E. coli K 88, E. coli T 37, E. coli ROW 7/12 E. coli 5933, Salmonella enterica TD 01, S. typhi Ty2, Shigella boydii D13629, S. dysentery 8, S. flexneri Type 6, S. soneii 1, Vibrio cholerae NCTC 5596, V. cholerae NCTC 10732, V. cholerae NCTC 11501, V. cholerae NCTC 4693 and Pseudomonas aeruginosa MDR.*

Moreover, *in vitro* antifungal activity testing was done on four fungal strain; *Candida albicans ATCC 10231, Aspergillus niger ATCC 6275, Penicillium notatum ATCC 11625 and Penicillium funiculosum NCTC 287*. All microbial strains were obtained from the Department of Technology, Jadavpur University; Central Drugs Laboratory, Kolkata and Institute of Microbial Technology, Chandigarh, India. Ciprofloxacin and Griseofulvin were used as a positive control for antibacterial and antifungal tests**,** respectively.

2.2.Methods

2.2.1. Synthesis of **(*E*)-1-(2, 4-dinitrophenyl)-2-(1,7,7-trimethyl bicyclo [2.2.1] heptan-2-ylidene) hydrazine (19)**

Compound **19** was synthesized by using the Schiff base reaction as described by Da Silva et al, (2015). Camphor methanolic solution was prepared by dissolving 3.045g of camphor in 20 ml of methanol. Similarly, 3.9628 g of 2, 4- dinitrophenylhydrazine was dissolved in 20ml of methanol in separate flask. 3ml acetic acid was added in camphor solution. Then after, The 2, 4-dinitrophenyl hydrazine solution was gradually added to camphor-acetic acid solution. The reaction was refluxed for 6 hours at 70℃, and the reaction progress was monitored by using analytical TLC with the hexane: ethyl acetate (4:1) ratio solvent system. After the reaction completion, the mixture was allowed to dry at room temperature; an orange solid product was obtained.

2.2.2. Antimicrobial Activity Assay

2.2.2.1. Disc diffusion method

The *in vitro* antibacterial activity of produced compounds was screened using the disc diffusion method (Hegstad, 2014), by determining zones of inhibition produced by the test samples and comparing them with that of ciprofloxacin. The antifungal potential of the test samples were screened by disc diffusion method against the fungal pathogens on Saborauds dextrose media. The Petri dishes were incubated at room temperature for 3 days and the diameter of zone of inhibition was measured in mm. Griseofulvin was used as a reference standard.

2.2.2.2. Broth dilution method

Broth dilution method was performed to determine Minimum Inhibitory Concentration of synthesized compound. The MIC determination of synthesized compound was performed according to Eloff (1998) and the Clinical and Laboratory Standards Institute (CLSI) guideline. The MIC values were visually determined by observing the presence or absence of purple color on each plate. The lowest concentration of every well that produced no apparent purple color considered as MIC value.

2.3.*In Silico* Studies

2.3.1. Molecular docking study

Molecular docking of the synthesized compounds was performed on the three-dimensional (3D) crystal structure of the *E. coli* GyrB24 with inhibitor KOB20 downloaded from the PDB

(7P2Xpdb) (www.rcsb.org). Molecular docking study was performed using the Schrödinger suite 2023 version 1 (Schrödinger Inc., New York, USA).

2.3.2. Prediction of ADMET property

The absorption, distribution, metabolism, and excretion (ADME), toxicity and physicochemical properties prediction of synthesized compound was made using Online tools ADMETlab 2.0 software.

2.4.Statistical analysis

The Statistical Package for Social Science (SPSS) version 20 was used to enter the data into an Excel spreadsheet for statistical analysis.

3. Results and Discussion

3.1. Synthesis of (*E*)-1-(2, 4-dinitrophenyl)-2-(1,7, 7-trimethylbicyclo [2.2.1] heptan-2-ylidene) hydrazine (19)

(*E*)-1-(2, 4-Dinitrophenyl)-2-(1, 7, 7-trimethylbicyclo [2.2.1]heptan-2-ylidene)hydrazine (**19**) was synthesized via a condensation reaction between camphor (**1**) and 2,4-dinitrophenylhydrazine in the presence of acetic acid. Compound **19** (62%, yield) was obtained as an orange solid with an R_f value of 0.6 using hexane: ethyl acetate (4:1) as solvent system, as indicated in Table 1.

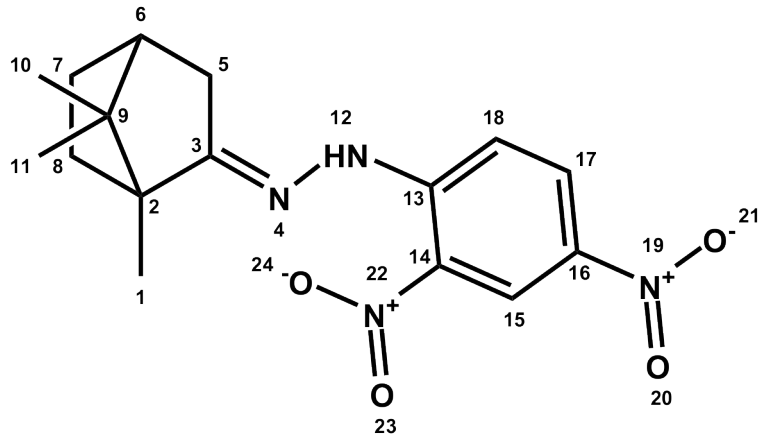
Table 1: Physical properties of the synthesized compound (**19**)

Code	M.F.	M. Wt. g/mol	R_f value (hexane: ethyl acetate(4:1))	Yield (w/w)	Physical appearance
Compound **19**	$C_{16}H_{20}N_4O_4$	332.35	0.6	62%	Orange colure

3.2.Structural elucidation of the synthesized compound (19)

The structure of compound **19** was confirmed by the ^{1}H and ^{13}C NMR spectral data. The ^{1}H-NMR spectrum of compound **19** showed signals at d 9.15 (1H, *s*), d 8.32 (1H, *d*) and d 8.09 (1H, *d*) corresponding to the phenyl protons H-15, H-17, and H-18. The presence of secondary amine (NH) moiety in compound **19** was evident from a signal resonate at δ10.8 (1H, *brs*, H-12). The ^{13}C-NMR spectrum of compound **19** revealed a total of sixteen carbon atoms. A distinct signal at δ 171.55 in the ^{13}C-NMR spectrum strongly indicates the presence of an azomethine carbon (C=N). Additional key carbon signals are summarized in Table 2.

Table 2: ^{1}H-NMR and ^{13}C-NMR spectral data of compound **19** in $CHCl_3$



Position	^{1}H-NMR (δH, ppm)	^{13}C-NMR (δC, ppm)
1	0.83(s)	11.12
2	-	53.43
3	-	171.55
5	2.60(d) &2.1(s)	34
6	1.49(m)	44.07
7	1.98(m)&1.32(d)	27.2
8	1.86(m)&1.27(d)	32.65
9	-	48.46
10	1.15(s)	18.64
11	1.02(s)	19.59
12	10.8(s)	
13	-	145

14	-	128.7
15	9.15 (s)	123.64
16	-	137
17	8.32 (d)	129.89
18	8.09 (d)	116.42

3.3.Antimicrobial activity of the synthesized compound

3.3.1. Antibacterial Activity

Broth Dilution method and Disk Diffusion method were used to evaluate the antibacterial activity of the synthesized compounds, and the results are presented in Table 3. The compound showed antibacterial activity with MIC values ranging from 25 µg/ml to 200 µg/ml. Interestingly, Compound **19** has demonstrated superior performance against all *E. coli* strains and *Staphylococcus aureus* MDR 10 compared to the starting compound (**1**); with an MIC value of 25 µg/ml. Additionally, the synthesized compound displayed better performance against *S.aureus MDR 1 &MDR 2* and *Vibrio cholerae* with MIC value of 50µg/ml. Compound **19** showed a comparable ZOI for *S.aureus 872* and *S.aureus MDR 1* to ciprofloxacin. Furthermore, compound **19** showed closer ZOI for *E.coli LT37*, *S. aureus MDR 2*, and *Pseudomonas aeruginosa MDR 1* to the standard drugs. The lowest activity of synthesized compounds was for *Bacillus pumilus* and *Bacillus subtilis ATCC 6633* (800 µg/ml).

Table 3: Zone of inhibition (ZOI) and minimum inhibition concentration (MIC) of the synthesized compound (**19)** against the tested bacterial strains

Bacterial strains	ZOI in mm (200 µg/ml)			MIC (µg/ml)	
	Cpd**19**	Cpd**1**	Std	Cpd**19**	Cpd**1**
E.coli NCTC 5933	14.5	13.0	16.0	25	50
E.coli K88	15.5	13.5	17.0	25	50
E.coli NCTC 7360	15.0	14.0	17.0	25	50
E.coli LT37	15.0	13.5	16.0	25	50
E.coli 872	15.5	14.0	16.0	25	50
E.coli ROW 7/12	15.5	14.0	16.5	25	50
E.coli 3:37C	15.5	14.5	16.5	25	50
E.coli CD/99/1	16.0	14.0	17.0	25	50
Salmonella typhi Ty2	13.5	13.5	16.0	100	100

Salmonella enterica TD 01	13.5	13.5	19.0	100	100
Shigella dysentery 8	12.0	11.5	20.0	100	200
Shigella soneii 1	12.5	13.0	19.5	100	200
Shigella boydii D13629	12.0	12.0	20.0	100	200
Shigella flexneri Type 6	13.0	12.0	20.5	100	200
Staphylococcus aureus MDR10	14.0	13.5	18.0	25	50
Bacillus pumilus 82	6.0	6.0	19.0	800	800
Bacillus subtilis ATCC 6633	6.0	6.0	18.0	800	800
Vibrio cholerae NCTC 4693	15.5	14.0	17.5	50	50
Vibrio cholerae NCTC5596	15.0	14.5	18.5	50	50
Vibrio cholerae NCTC 10732	15.0	14.5	19.0	50	50
Vibrio cholerae NCTC 11501	15.5	14.5	18.5	50	50
*E.coli HB101**	12.0	12.0	14.0	100	200
*E.coli C600**	11.5	11.5	13.5	100	200
*S.aureus MDR 1**	11.0	10.0	11.5	50	200
*S.aureus MDR 2**	11.0	11.5	12.0	50	200
*Pseudomonas aeruginosa MDR 1**	11.5	10.5	12.5	100	100

*Cpd = Compound, Std= Ciprofloxacin

3.3.2. Antifungal Activity

The antifungal activity of compound **19** was evaluated by using Broth Dilution & Disc diffusion method. Compound **19** displayed moderate antifungal activities against four fungal strains; with MIC value of 800µg/ml. the ZOI of synthesized compound on all tested fungal strain was closer to starting compound(**1**).

Table 4: Zone of inhibition (ZOI) and minimum inhibition concentration (MIC) of the synthesized compound (**19)** against the tested fungal strains

Fungal strain	ZOI mm (1500 µg/ml)			MIC (µg/ml)	
	Cpd **19**	Cpd 1	Std	Cpd **19**	Cpd **1**
Candida albicans ATCC 10231	11.5	12.0	16.0	800	400
Aspergillus nigerATCC 6275	11.5	11.0	15.0	800	800

Penicillium notatum ATCC 11625	10.5	11.0	13.5	800	800
Penicillium funiculosum NCTC 287	10.5	10.5	14.0	800	800

*Cpd = Compound, Std= Griseofulvin

3.3. *In silico* analysis

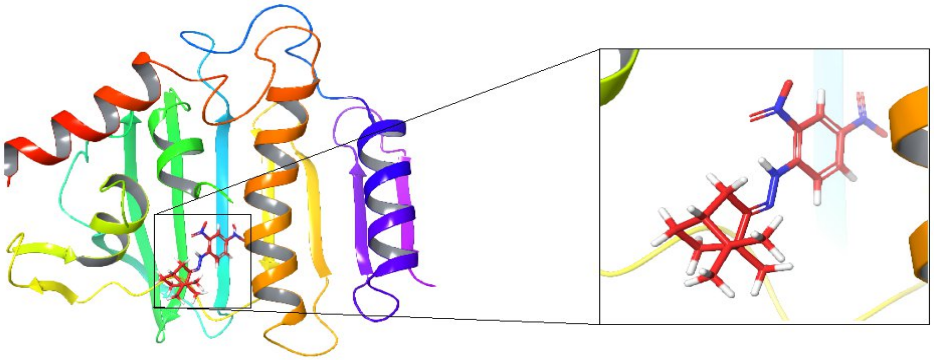
3.3.2. Molecular docking

The potential mechanism of action of the synthesized compound **19** was explored through molecular docking studies, using *Escherichia coli* DNA gyrase subunit B (GyrB24) as the target enzyme. The crystal structure of GyrB24 was retrieved from the Protein Data Bank (PDB ID: 7ZPX) via the RCSB database (www.rcsb.org). Docking simulations were performed using the Schrödinger molecular modeling suite to assess the binding affinity and interaction profile of compound **19** within the enzyme's active site.

Compound **19** demonstrated a moderate binding affinity toward the GyrB24 enzyme, with a docking score of –5.368 kcal/mol, indicating a reasonably stable interaction with the active site. Detailed analysis of the docking results, as depicted in Figure 8, revealed that compound **19** formed a key hydrogen bond with the amino residue ASP73 (Table 5). Furthermore, additional polar interactions were observed with GLN72 and ASN46, which likely contribute to the compound's specificity and orientation within the binding pocket. Hydrophobic interactions involving VAL71 and ALA47 further stabilized compound **19** within the active site, enhancing the overall binding profile.

Table 5: Docking scores of the synthesized compound with 72PX according to Schrodinger 2023 suite docking software.

Ligand	**Docking score (kcal/mol)**	**Interaction with amino acid residues**	
		H-bonds	**Non-H-bonds**
Compound **19**	-5.368	ASP72	GLN72,ASN46, VAL71 and VAL71 and ALA47 ALA47



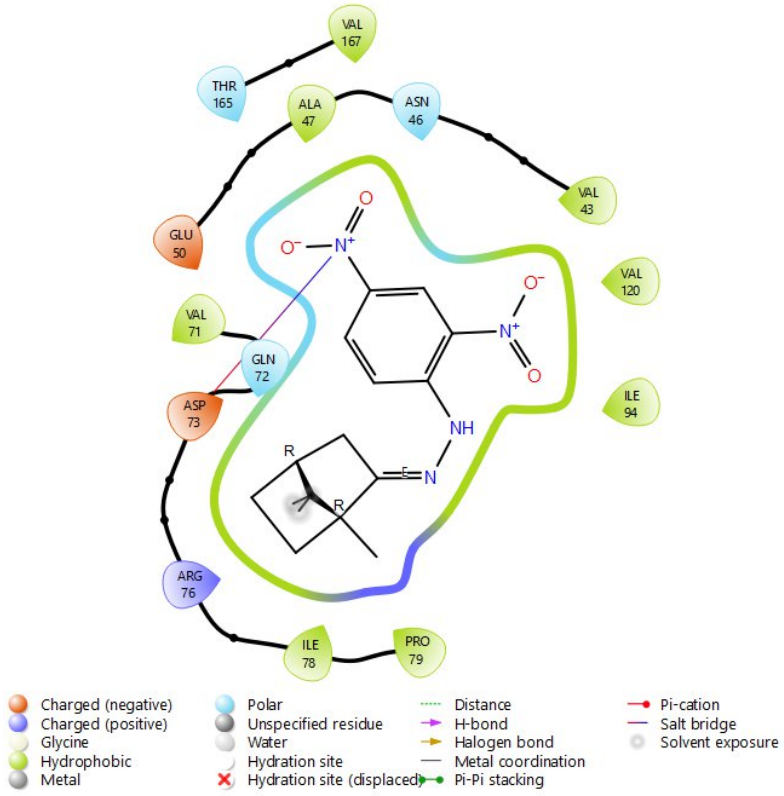


Figure 8: Binding interaction of the synthesized compound (**19**) in *E. coli*

3.3.3. *In Silico* Prediction of ADMET property

ADMETlab2.0 online software tools were utilized to determine the ADMET profiles of the synthesized compound (**19**). The objectives of the analysis were to assess a key pharmacokinetic property of the synthesized compound that determines the drug likeness. The findings are outlined in Table 6. The absorption properties of synthesized compound were predicted with cell permeability (CACO-2), Pgp Inhibitor, Pgp Substrate, Human Intestinal Absorption (HIA) and 20% &30% Bioavailability. The synthesized compound (**19**) has Caco-2Permeability value of -4.801 is within an optimal range, indicating good oral bioavailability. Strong plasma protein binding was found in synthesized compound, which reduces the free plasma fraction and causes a longer half-life and a smaller volume of distribution. The compound is predicted to be inhibitors of CYP3A4, CYP2C19 and CYP2C9, it leads to drug- drug interaction causing to toxicity. Toxicity profile of the compound was predicted in terms of drug-induced liver injury (DILI), mutagenicity and cardiotoxicity. The result shows that the compound has a probability of being mutagenic and hepatotoxic.

Table 6: ADMET prediction the synthesized compound (**19**)

ADMET Property	**Value**	**Remark**
Absorption		
Caco-2	-4.801	Optimal: higher than -5.15 Log unit
Pgp-Inhibitor	0.071	Category 1: Inhibitor; Category 0: Non-inhibitor. The output value is the probability of being Pgp-inhibitor
Pgp-Substrate	0.0	Category 1: substrate; Category 0: Non-substrate; The output value is the probability of being Pgp-substrate
HIA	0.0	Category 1: HIA+(HIA < 30%); Category 0: HIA-(HIA >= 30%); The output value is the probability of being HIA+
F (20%)	0.006	Category 1: F 20% + (bioavailability < 20%); Category 0: F 20% - (bioavailability ≥ 20%); The output value is the probability of

		being F 20%
F (30%)	0.0	Category 1: F 30% + (bioavailability < 30%); Category 0: F 30% - (bioavailability ≥ 30%); The output value is the probability of being F 30%
Distribution		
PPB	99.431	plasma Protein Binding; Optimal: < 90%
VD	0.137	Volume Distribution; Optimal: 0.04-20L/kg
BBB	0.0	Category 1: BBB+; Category 0: BBB-; The output value is the probability of being BBB+
Metabolism		
CYP1A2	0.009	Category 1: Inhibitor; Category 0: Non-inhibitor
CYP3A4	0.467	Category 1: Inhibitor; Category 0: Non-inhibitor
CYP2C19	0.593	Category 1: Inhibitor; Category 0: Non-inhibitor
CYP2C9	1.0	Category 1: Inhibitor; Category 0: Non-inhibitor
CYP2D6	0.043	Category 1: Inhibitor; Category 0: Non-inhibitor
Excretion		
Clearance	5.911	>15 ml/min/kg: high clearance; 5-15ml/min/kg: moderate clearance; < 5 ml/min/kg: low clearance.
T1/2	0.835	Ultra-short half-life drugs: 1/2 < 1 hour; short T1/2 between 1-4 hours; intermediate short; T1/2 between 4-8 hours; long T1/2 > 8 hours.
Toxicity		
hERG	0.092	Molecules with IC50 ≤10μM or ≥50% inhibition at 10 μM were classified as hERG+ (Category 1), while molecules with

		IC50 >10μM or < 50% inhibition at 10μM were classified as hERG -(Category 0)
Ames	0.984	Category 1: Ames positive (+); Category 0: Ames negative (-)
DILI	0.994	Category 1: drugs with a high risk of DILI; Category 0: drugs with no risk of DILI.

The synthesized compound does fulfill the Lipinski rule: molecular weight, logP (which predicts octanol/water partition coefficient, log), the number of H-bond donors, and hydrogen bond acceptors. The findings are outlined in Table 7.

Table 7: Prediction of physicochemical and medicinal chemistry friendliness of compound **19**

Compound	nHA	nHB	nRot	M.wt	LogS	LogP	LogD	Lipinski
Compound **19**	8	1	4	332.15	-5.614	3.842	3.601	accepted

4. Conclusions

In this study, we successfully synthesized a camphor Schiff base derivative, (*E*)-1-(2,4-dinitrophenyl)-2-(1,7,7-trimethylbicyclo[2.2.1]heptan-2-ylidene)hydrazine (17), via a Schiff base condensation reaction. The compound showed highest antibacterial activity against *E.coli and S.aureus* according with an MIC value of 25μg/ml and also demonstrated better activity against *S.aureus* MDR1&MDR2 with an MIC value of 50μg/ml. The synthesized compound were subjected to molecular docking with the *E. coli* DNA gyrase sub unit B(Gyr24B) structure, obtained from PDB (7P2X.pdb), it had -5.368K/cal docking score. ADMET predictions for synthesized compound confirmed its adherence to the Lipinski rule of five. In summary, the camphor SB derivatives showed considerable potential as lead compounds in developing novel

antibacterial agents to address the escalating threats of antibacterial resistance and infectious diseases.

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