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Synthesis, *in silico* molecular docking studies and antimicrobial activity of levofloxacin schiff bases

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ABSTRACT

The molecular docking and antimicrobial activity studies of synthesized levofloxacin Schiff bases were performed, in order to provide insights into the mechanism of action of potential antimicrobial drugs for resistant micro-organisms. antimicrobial activity of compounds was investigated in vitro under aseptic conditions, using the disk diffusion method, against various gram positive and gram negative pathogenic microorganisms such as Pseudomonas aeruginosa (P.A.), Staphylococcus aureus (S. aureus), Helicobacter pylori (H. pylori), Escherichia coli (E. coli), Methicillin-resistant Staphylococcus aureus (MRSA) and some fungal strains such as, Aspergillus fumigatus, Pneumocystis carinii and Aspergillus niger. A series of these compounds were prepared and have been shown to inhibit pathogenic growth, judging from the area of the zone of inhibition. The area of zone of inhibition of compounds found from 6 mm^2 to 48 mm^2. Among the synthesized compounds; Compound SV-15 showed good activity against P.A.(zone of inhibition 6 mm^2 at 40 µg/ml); Compound SV-16 showed good activity against S. aureus(zone of inhibition 15 mm^2 at 100 µg/ml). Compounds SV-15, SV-16, SV-17, SV-18, SV-19, SV-20 and SV-21 exhibited promising antibacterial activity. The target compounds showed in vitro antibacterial & antifungal activity less than reference antibiotic levofloxacin.

Keywords: Antimicrobial, Schiff base, Molecular Docking, Zone of Inhibition, fluoroquinolone

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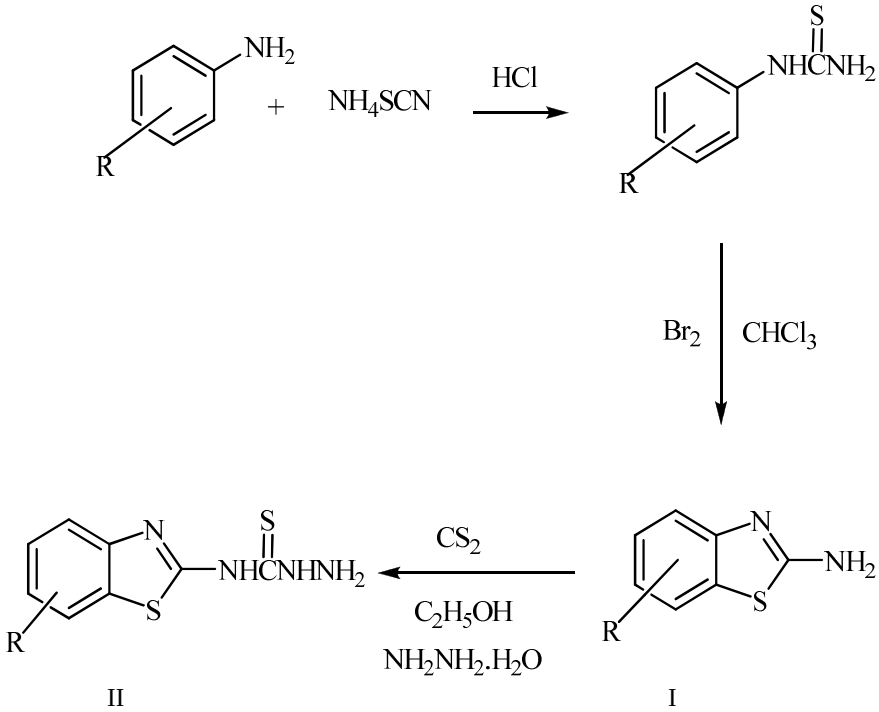
INTRODUCTION

There is an urgent need to synthesize novel, potent selective antimicrobial (Antifungal and Antibacterial) drugs, in view of the fact that pathogenic microorganisms develop resistance to pathogens [1]. This is as a result of pathogenic organisms undergoing genetic mutations which change the proteins and other components of cells. Pathogens also produce enzymes that destroy or inactivate antimicrobials. Added to this, pathogens can alter the permeability of their cell membrane, making it difficult for antimicrobials to enter [2, 3].

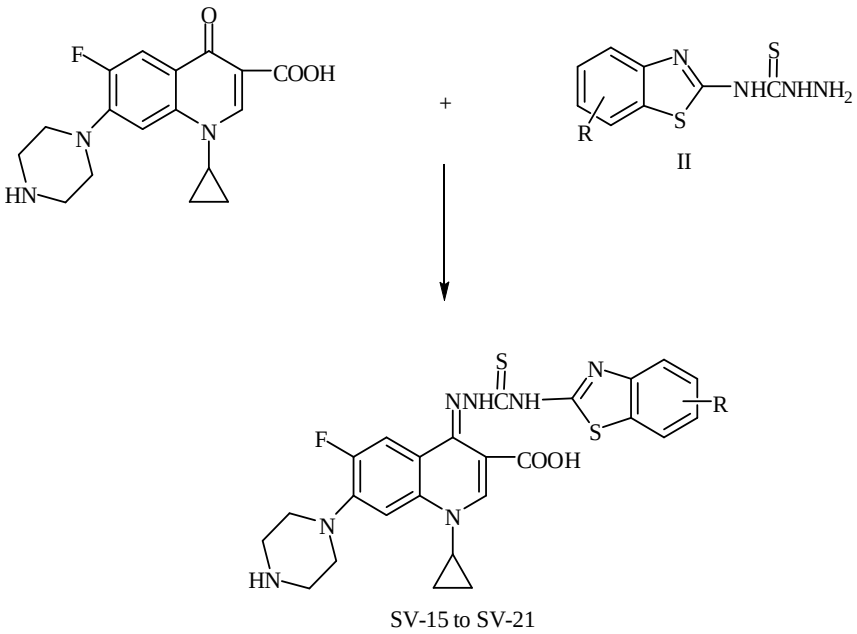
Recently a relatively new approach to the rational design of antimicrobial agents has been introduced based on some new quinolone molecules [4]. Levofloxacin, [-9-fluoro-2,3-dihydro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-

pyrido-1,4-benzoxazine-6-carboxylic acid], a typical third generation fluoroquinolone, has been in clinical use for more than a decade [5, 6].

Step 1:-



Step 2:-



Scheme 1: Synthesis of Schiff base of levofloxacin

Different structural modifications in the quinoline nucleus have been made to increase antimicrobial activity and improve its performance. During 1980's, it was discovered that a fluorine atom at position 6 and piperazine ring at position 7 greatly enhance the spectrum of activity of these antibiotics [7, 8]. In a structure activity relationship 4-oxo group is considered essential for antibacterial activity and therefore, modifications of this moiety has been not much explored. In the present study the modification of 4-oxo group has been explored to confirm whether this group is really essential or not. On the other hand 2- amino benzthiazole derivatives have shown promising antibacterial activities [9]. Therefore, schiff bases of 2-amino benzthiazole with 4-oxo group of fluoroquinolones are expected to enhance antibacterial activity of fluoroquinolones. These compounds were prepared as per Scheme 1.

MATERIALS AND METHODS

Melting points of the synthesized compounds were determined by open capillary method. The purity of the compounds was checked using TLC Plates, using chloroform**:** methanol (8:2) solvent system. The developed chromatographic plates were visualized in saturated Iodine chamber. IR Spectra were recorded using KBr on BRUKER Spectrophotometer, NMR spectra in $CDCl_3$on FT-NMR instrument using TMS as internal standard.

General Procedure for Synthesis of Schiff bases

Compound I: Benzothiazole-2-yl amine

Compound [I] was synthesized by heating aniline (0.3moles) and concentrated hydrochloric acid (25ml). 0.4mole of saturated solution of ammonium thiocynate in water (30gm in 60ml water) was added slowly in above solution. The reaction mixture was boiled until the solution got turbid. The solution was poured in ice water. The precipitate was filtered and recrystallized from ethanol to give phenylthiourea. Phenylthiourea (0.1mole) in glacial acetic acid (75ml) was brominated by using bromine solution in glacial acetic acid (5%) till the orange yellow color appeared. The slurry was poured in cold water and make alkaline with 50% aq. Ammonia solution. The precipitate was filtered and washed with water, dried and recrystallize by using ethanol. The melting point was found to be 156°C [9].

Compound II: N-(benzothiazol-2-yl)hydrazinecarbothioamide

0.01mole of product I was dissolved in ethanol using potassium hydroxide as base. An equi-molar amount of CS_2 and hydrazine hydrate were then separately added drop wise to the solution of product I with stirring at 0-5°C temperature. A light yellow solid was precipitated at the end of the reaction. The product obtained was filtered and recrystallized with ethanol.

Schiff base of levofloxacin with N-(benzothiazol-2-yl) hydrazine carbothioamide

Equimolar quantities (approx. 0.01mole) of Compound II and levofloxacin were separately dissolved in a minimum amount of ethanol and then they were mixed together followed by addition of 5ml glacial acetic acid. The solution was refluxed for 10hrs. Then cooled to room temperature and poured into ice cold water .The solid product was collected through filtration and then were air dried. The product was re-dissolved in ethanol for re-crystallization and filtered to give a product. The physicochemical properties of the Schiff bases are described in Table 1.

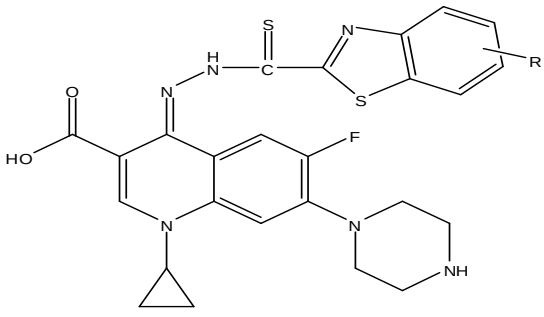


Figure No-1: Test Compound

Preparation of ligand structures-

Ligand file of Test compound (Figure 1) was edited using chemdraw developed by the cheminformatics compony ChambridgeSoft in .mol format. These files could not directly used by Autodock 4.0 tools [10] thus they were converted it into .pdb files using Discovery Studio Visualizer version 2.5.5. Discovery Studio is a software package

of biological molecular design solutions for computational chemists and computational biologists. Discovery Studio makes it easier to examine the properties of large and small molecules. Further the ligand was submitted for minimization using Chimera version 1.5.3 using with Genetic Algorithm Steps 2000 and 0.5 grid units Optimized [11].

Preparation of protein structures

The structures of Proteins involved in this study Mycobacterium Tuberculosis DNA Gyrase Type-A, PDB ID-4G3N was obtained from RCSB Protein Data Bank. Published structures were edited to remove HETATM using Discovery Studio Visualizer (Version 3.1). Chimera was used for energy minimization, removal of steric collision with the steepest descent steps 1000, steepest descent size 0.02 Å, Conjugated gradient steps 1000 and the conjugate gradient step size 0.02 Å for the conjugate gradient minimization [12, 13].

Protein-Ligand Docking Studies

Docking studies were performed by Autodock version 1.5.4 suit [14, 15] and Cygwin interface was used in the Microsoft Windows 7 professional Version 2008, Service pack 3 operating System on Intel (R) i5 (TM), CPU @ 3.30 GHz and 8.0 GB of RAM of Intex Machine. We implemented molecular docking methods followed by the searching the best conformation of enzymes and carcinogens complex on the basis of binding energy. Water molecules were removed from the protein structures before docking and hydrogen atoms were added to all target proteins. Kollman united charges and salvation parameters were added to the proteins. Gasteiger charge was added to the ligands. Grid box was set to cover the maximum part of proteins and ligand. The values were set to 60×60×60 Å in X, Y and Z axis of grid point. The default grid points spacing was 0.375 Å. Lamarckian Genetic Algorithm (LGA) [16] was used for proteins ligands flexible docking calculations. The LGA parameters like population size (ga_pop_size), energy evaluations (ga_num_generation), mutation rate, crossover rate and step size were set to 150, 2500000, 27000, 0.02, 0.8 and 0.2 Å, respectively. The LGA runs were set to 20 runs. All obtained 40 conformations of proteins and ligand complex were analysed for the interactions and binding energy of the docked structure using Discovery Studio Visualizer version 3.1.

Antimicrobial Activity:

Preparation of Suspension of Bacteria:

2 ml normal saline (0.85% w/v) was taken in test tubes and then plugged with cotton, raped with news paper with help of cello tape. Test tubes were put in autoclave for sterilization for 15lbs for 20min. After autoclaving take 1-2 colonies of bacteria from sub cultured bacterial plate with the help of loop. Colonies were dissolved in normal saline with rub on side of test tube with stirring. Turbidity of tubes were marked and add more colonies if needed.

Procedure for Sensitivity Test:

Preparation of Muller Hinton Agar Plates:

3.0mg Muller Hinton agar media was dissolved properly in 80mL Distilled water in 250mL conical flask with stirring (For preparation of four plates).The mouth of conical flask was plugged with cotton, rapped with news paper with help of cello tape. Conical flask was put in autoclave for sterilization for 15lbs for 20min. After autoclaving, warm 20-25mL media was poured on petri dish per plate in front of laminar flow. The media was left until solidified in petri disk. After that plate was put in incubators for drying the water vapour in plate. Now agar plate was ready for use [17-20].

Preparation of Compounds Solution:

5mg compound was dissolved in 1ml DMSO: PEG=1:10 in test tube with vertex stirring, heated if required (conc. of DMSO was not increased above 1%). Each tube was given a code number. The prepared plate was divided into four quadrants with the help of marker. The same code was given to each quadrant as given to code to test tube containing solution of compound. One plate was swab from one bacterial suspension with the help of cotton swab, coded the name of bacteria on each plate.

With the help of micro pipette, 10-20µl solution of compound was dropped on same code of quadrant as given on the test tube containing solution of compound. All plates were put in incubator for incubation for 18-24hrs. After18-24hrs, the plates were viewed. If the specific compound was sensitive for specific bacteria, then growth was found in whole plate except where solution of compound was dropped. If the specific compound was not sensitive for specific bacteria, then growth was found in whole plate including where solution of compound was dropped.

Source of microorganisms:
For the bacterial organisms, different Gram negative (-) and Gram positive (+) bacteria used were *P. aeruginosa*, *E. coli*, *H. pylori*, *S. aureus* and *MRSA* were used. For the fungi, yeast of the *P. carinii*, *A. fumigates* and *A. niger* species was investigated. These microorganisms were obtained from the Dept. of Biotechnology, SITM, Lucknow, and stored in a refrigerator at the microbiology laboratory at Dept. of Pharmacy.

Reference and Control:
The references were antibiotic in nature. Levofloxacin was chosen as the reference for all bacterial species. Nystatin was used as the reference for the fungus. The control experiment consists of a plate of solidifying agar onto which was inoculated pure solvent with microorganism mixed in a 1:1 portion as reported [21].

Aseptic conditions:
The aseptic chamber consists of a wooden box (1m x 1m x 0.5m) with a door which was cleaned with 70% ethanol and irradiated with short wave UV light for 1 hour.

RESULTS AND DISCUSSION

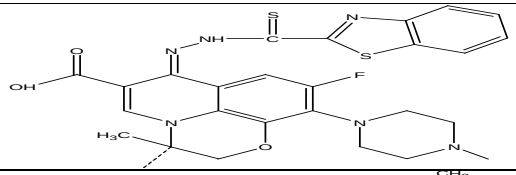
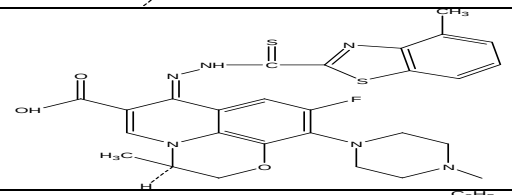
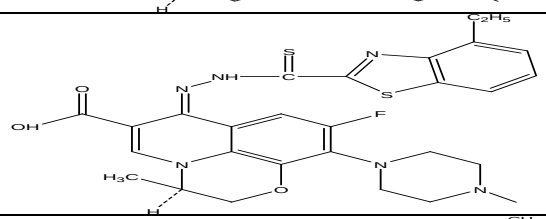
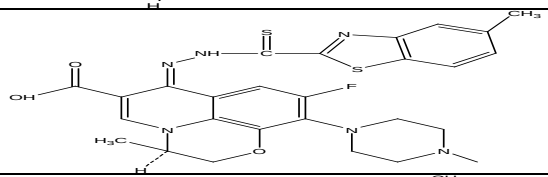
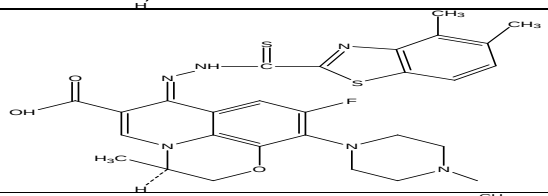
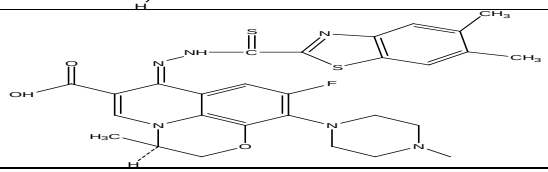
***In silico* Molecular Docking Study:** We performed the molecular docking simulation study of *Mycobacterium tuberculosis* DNA Gyrase Type-A, PDB ID- 4G3N as a macromolecule with test compounds as a ligands by the use of AutoDock 1.5.4, in this study we found that all the test compounds are binding with the *Mycobacterium tuberculosis* DNA Gyrase Type-A, very efficiently as compare to exciting drugs. The binding energy Results are summarized in Table: 2

Antibacterial Activity: The *in vitro* antibacterial activity of Schiff bases of levofloxacin was investigated against gram positive organisms (*Staphylococcus aureus, Methicillin-resistant Staphylococcus aureus)* and gram negative organisms *(Helicobacter pylori, Escherichia coli* and *Pseudomonas aeruginosa).* Results are summarized in Table: 3 along with standard drug.

All analogues, showed comparable antibacterial activity at the dose 300µg/ml against all the tested strains. Results indicate that compounds SV-15 showed maximum activity against P.A (zone of inhibition=40mm^2 and MIC=30µg/ml at the dose of 300µg/ml) in comparison to other strains used by us. Compounds **SV-16** Showed maximum activity *H. Pylori (*zone of inhibition-45 mm^2 and MIC-30µg/ml at the dose of 300µg/ml) **SV-17** showed maximum activity against *pseudomonas aeruginosa* (zone of inhibition=22mm^2, and, MIC=100µg/ml at the dose of 300µg/ml) **SV-18** (zone of inhibition=28mm^2 and MIC=86µg/ml at the dose of 300µg/ml). **SV-19**-showed maximum activity against *pseudomonas aeruginosa* (zone of inhibition-34mm^2, MIC=40µg/ml at the dose of 300µg/ml).

Antifungal Activity: Levofloxacin is an antibacterial drug and inactive against fungi, in order to evaluate the result of addition of different functional groups to its basic structure, the antifungal activity of its derivatives was carried out against; *A. fumigatus***,** *P. carinii and A. niger*, and results are summarized in Table: 4. It was found from the result that compound SV- 21 has got enhanced activity against all the antifungal strains used. The compounds SV-17 and SV- 18 also showed moderate activity against *A. fumigatus.* The compound SV-16 showed moderate activity against *A. niger*. The compounds SV-15 and SV- 19 showed moderate activity against *P. carinii* and *A. niger* respectively.

Table 1: Physicochemical properties of the Schiff bases

S. No.	Code	Compound Structure	Mol. Formula	Mol. Weight	Melting Point	% Yield	Solubility	Elemental Analysis (%) Calculated/Found		
								C	H	N
1.	SV-15		$C_{25}H_{23}FN_6O_3S_2$	538.13	262°C	50%	Ethanol	55.75 55.73	4.30 4.31	15.60 15.59
2.	SV-16		$C_{26}H_{25}FN_6O_3S_2$	552.14	278°C	34%	Ethanol	56.51 56.53	4.56 4.58	15.21 15.24
3.	SV-17		$C_{27}H_{27}FN_6O_3S_2$	566.16	235°C	45%	Ethanol	57.23 57.25	4.80 4.82	14.83 14.86
4.	SV-18		$C_{26}H_{25}FN_6O_3S_2$	552.14	210°C	40%	Ethanol	56.51 56.53	4.56 4.58	15.21 15.24
5.	SV-19		$C_{27}H_{27}FN_6O_3S_2$	566.16	215°C	30%	Ethanol	57.23 57.25	4.80 4.82	14.83 14.86
6.	SV-20		$C_{27}H_{27}FN_6O_3S_2$	566.16	222°C	45%	Ethanol	57.23 57.26	4.80 4.82	14.83 14.86

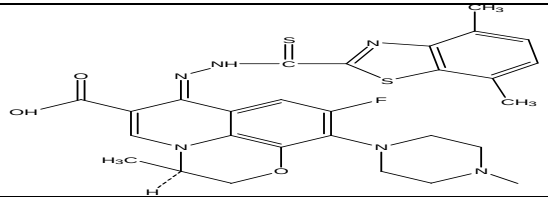
7.	SV-21		$C_{27}H_{27}FN_6O_3S_2$	566.16	285°C	40%	Ethanol	57.23 57.26	4.80 4.82	14.83 14.86

Table 2: in silico docking studies of the Schiff bases

S. No.	PROTEIN NAME	LIGAND NAME	BINDING ENERGY	Ki CONSTANT	RESIDUE ACTIVE SITES	HYDROGEN BOND	DISTANCE
1	4G3N	SV15	-8.81 kcal/mol	347.59 Nm	Thr521,Glu522,Gly524,Ala556,His557,Thr572 Arg607,Ile608,Ala609,Gln610,Leu659Val660 Gly661,Arg710,Leu712Ser713,Arg817,Ala819	A:HIS557:HD1 - :UNK0:O :UNK0:H - A:VAL660:O :UNK0:H - A:ALA556:O	2.38729 2.21184 1.95366
2	4G3N	SV16	-9.04 kcal/mol	235.34 Nm	Thr521,Glu522,Gly524,Ala556,His557,Thr572 Arg607,Ile608,Ala609,Gln610,Leu659,Val660 Gly661Ser713,Arg817,Ala819	:UNK0:H - A:ILE608:O :UNK0:H - A:ALA556:O	2.23695 1.82395
3	4G3N	SV17	-9.23 kcal/mol	170.73 Nm	Thr521,Glu522,Gly,5,24Ala556,His557,Thr572 Arg607,Ile608,Ala609,Gln610,Leu659,Val660 Gly661,Arg710,Leu712,Ser713Arg817,Ala819	A:HIS557:HD1 - :UNK0:O :UNK0:H - A:VAL660:O :UNK0:H - A:ALA556:O	2.30877 2.2215 1.93081
4	4G3N	SV18	-9.04 kcal/mol	238.18 nM	Thr521,Glu522,Gly524,Ala556,His557,Thr572 Arg607,Ile608,Ala609,Gln610,Leu659,Val660 Gly661,Ser713,Arg817,Ala819	A:ARG817:HN - :UNK0:O :UNK0:H - A:ILE608:O :UNK0:H - A:ALA556:O	2.3897 2.22279 1.82627
5	4G3N	SV19	-9.29 kcal/mol	155.23 Nm	Thr521,Glu522,Gly524,Ala556,His557,Thr572 Arg607Ile608,Ala609,Gln610,Leu659,Val660 Gly661,Leu712,Ser713,Arg817,Ala819	:UNK0:H - A:VAL660:O :UNK0:H - A:ALA556:O	2.23544 1.90484
6	4G3N	SV20	-9.60 kcal/mol	92.33 Nm	Thr521,Glu522,Gly524,Ala556,His557,Thr572 His595,Val596,Arg607,Ile608,Ala609,Gln610 Leu659,Val660,Gly661,Ser713,Arg817,Ala819	:UNK0:H - A:ILE608:O :UNK0:H - A:ALA556:O	2.25907 1.76259
7	4G3N	SV21	-8.38 kcal/mol	720.44 Nm	Ile520,Thr521,Glu522,Gly524,Ala556,His557 Thr572,Ile608,Arg628,Glu658,Val660,Arg710 Leu712,Ser713,Gly764,Leu766,Ala814,Ile815 Ala816,Arg817	A:ARG710:HH22 - :UNK0:O A:ARG817:HN - :UNK0:N :UNK0:H - A:GLU522:OE1 :UNK0:H – A:GLU522:OE2 :UNK0:H - A:GLU658:OE1	2.10055 2.32866 2.42268 1.7908 1.87669

Table 3: Toxicology analysis of the Schiff bases

Ligand Name	ADMET_ BBB	ADMET_ BBB_ Level	ADMET_ Absorption_ Level	ADMET_ Solubility	ADMET_ Solubility_ Level	ADMET_ hepatotoxicity	ADMET_ hepatotoxicity_ Probability	ADMET_ CYP2D6	ADMET_ CYP2D6_ Probability	ADMET_ PPB_ Level	TOPKAT_ Ames_ Prediction	TOPKAT_ Ames_ Probability	TOPKAT_ Ames_ Score
SV15	-1.338	3	0	-5.853	2	0	0.41	0	0.346	0	Non-Mutagen	0.239722	-13.18
SV16	-1.187	3	0	-6.283	1	0	0.496	0	0.415	0	Non-Mutagen	0.326337	-11.26
SV17	-1.046	3	0	-6.527	1	0	0.49	0	0.415	0	Non-Mutagen	0.217692	-13.71
SV18	-1.187	3	0	-6.263	1	0	0.463	0	0.415	0	Non-Mutagen	0.34163	-10.93
SV19	-1.037	3	0	-6.691	1	1	0.602	0	0.435	0	Non-Mutagen	0.365568	-10.43
SV20	-1.037	3	0	-6.671	1	1	0.549	0	0.435	0	Non-Mutagen	0.380827	-10.11
SV21	-1.037	3	0	-6.71	1	0	0.47	0	0.366	0	Non-Mutagen	0.385046	-10.02

Table 3: Antibacterial activity of the Schiff bases

S. N.	Compound Code	Conc. (µg/ml)	*P. aeruginosa*		*H. pylori*		*E. coli*		*S. aureus*		*M.R.S.aureus*	
			ZOI	MIC (µg/ml)	ZOI	MIC (µg/ml)	ZOI	MIC (µg/ml)	ZOI	MIC (µg/ml)	ZOI	MIC (µg/ml)
1.	SV-15	300	40	30	27	80	37	40	18	133	8	300
		100	15		10		14		6		0	
		30	6		0		6		0		0	
2.	SV-16	300	38	40	45	30	32	40	12	133	10	240
		100	15		18		12		6		0	
		30	6		8		6		0		0	
3.	SV-17	300	22	100	22	133	18	133	15	133	18	133
		100	8		6		6		6		6	
		30	0		0		0		0		0	
4.	SV-18	300	28	80	30	66	20	114	22	100	12	200
		100	10		12		7		8		0	
		30	0		0		0		0		0	
5.	SV-19	300	34	40	30	40	22	100	16	133	15	133
		100	15		13		8		6		6	
		30	6		6		0		0		0	
6.	SV-20	300	24	100	28	80	24	100	25	100	23	100
		100	8		10		8		8		8	
		30	0		0		0		0		0	
7.	SV-21	300	18	133	28	88	28	80	23	100	6	400
		100	6		9		10		8		0	
		30	0		0		0		0		0	
8.	Levofloxacin (Control)			0.12		0.09		0.21		0.09		2.08

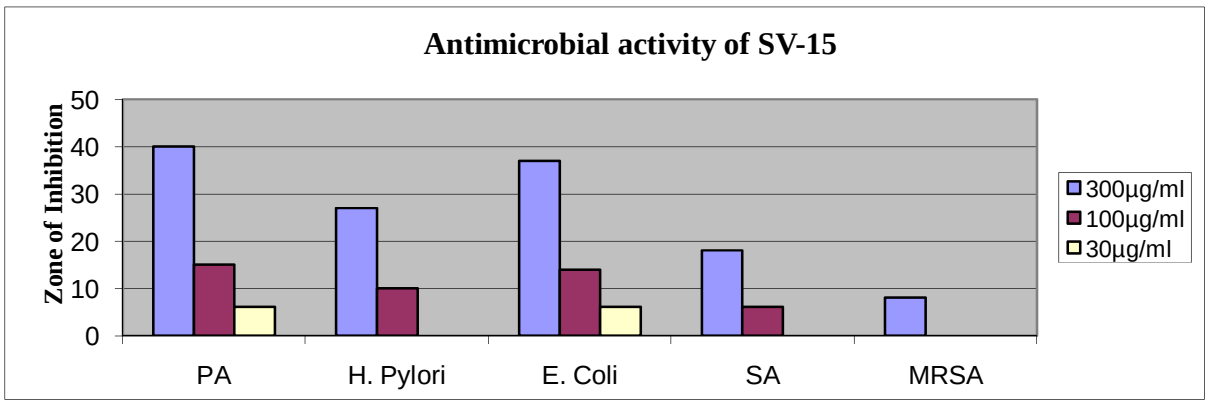
PA= Pseudomonas aeruginosa, SA= Staphylococcus aureus, H. Pylori= Helicobacter pylori, E.Coli= Escherichia coli, MRSA=Methicillin-resistant Staphylococcus aureus, ZOI= Zone of Inhibition

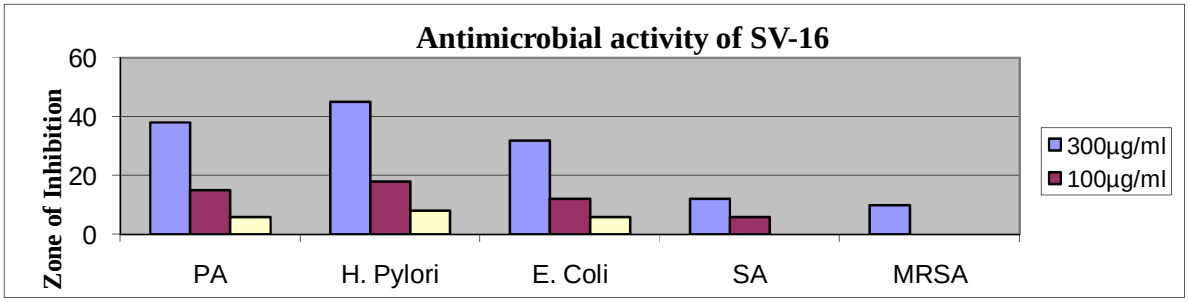
Table 4: Antifungal activity the Schiff bases

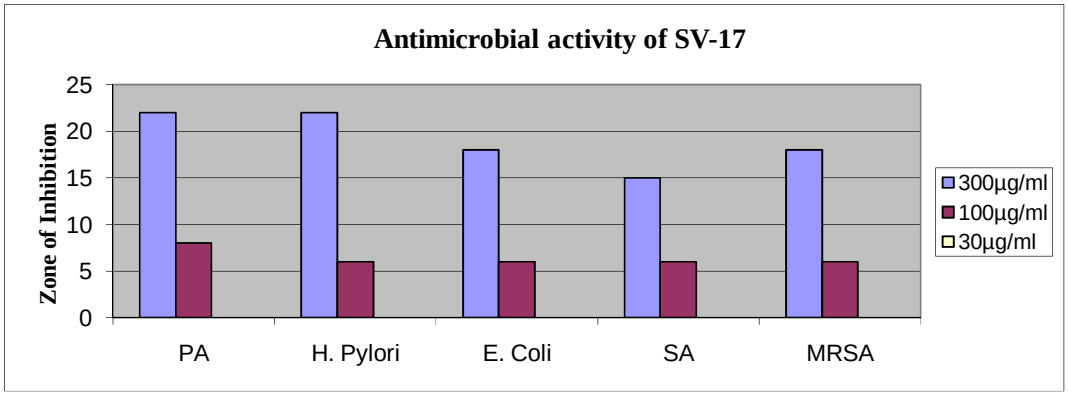
Compound Code	*A. niger*	*P. carinii*	*A. fumigatus*
SV-15	0	10	0
SV-16	16	0	0
SV-17	0	0	0
SV-18	14	12	8
SV-19	8	0	0
SV-20	0	0	8
SV-21	8	0	0

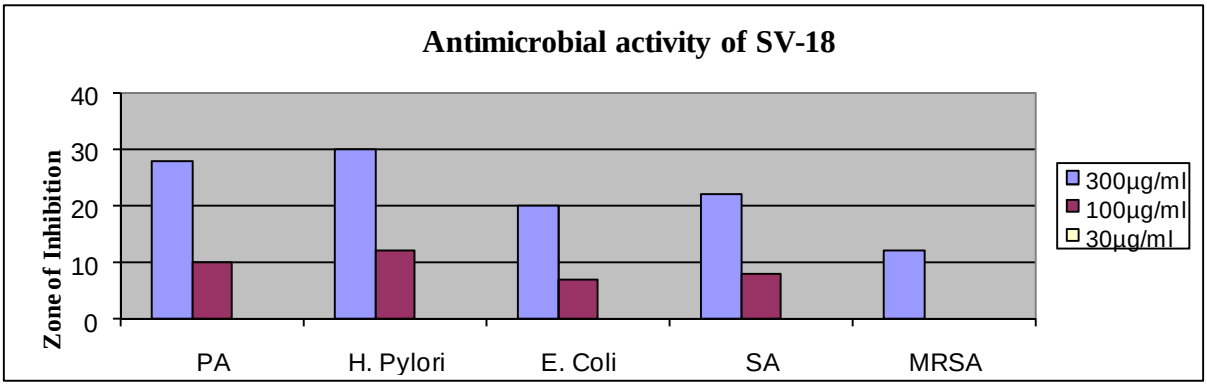
A. niger= Aspergillusniger, P. carinii= Pneumocystis carinii,A. fumigates=Aspergillusfumigatus

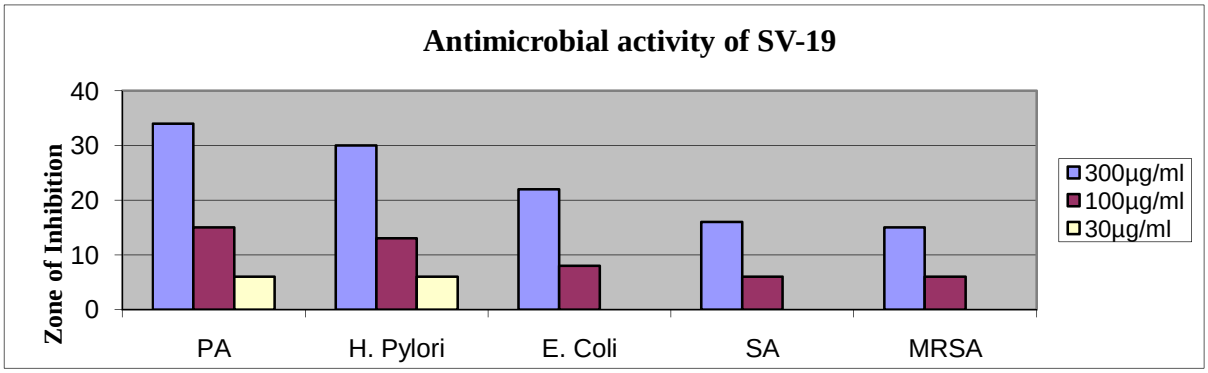
Figure 1(a-g): Zone of Inhibition of the Schiff bases (SV-15 to SV-21) at Different Concentrations




Antimicrobial activity of SV-16
Zone of Inhibition
60
40
20
0
PA
H. Pylori
E. Coli
SA
MRSA
300µg/ml
100µg/ml


Antimicrobial activity of SV-17
Zone of Inhibition
25
20
15
10
5
0
PA
H. Pylori
E. Coli
SA
MRSA
300µg/ml
100µg/ml
30µg/ml


Antimicrobial activity of SV-18
Zone of Inhibition
40
30
20
10
0
PA
H. Pylori
E. Coli
SA
MRSA
300µg/ml
100µg/ml
30µg/ml


Antimicrobial activity of SV-19
Zone of Inhibition
40
30
20
10
0
PA
H. Pylori
E. Coli
SA
MRSA
300µg/ml
100µg/ml
30µg/ml

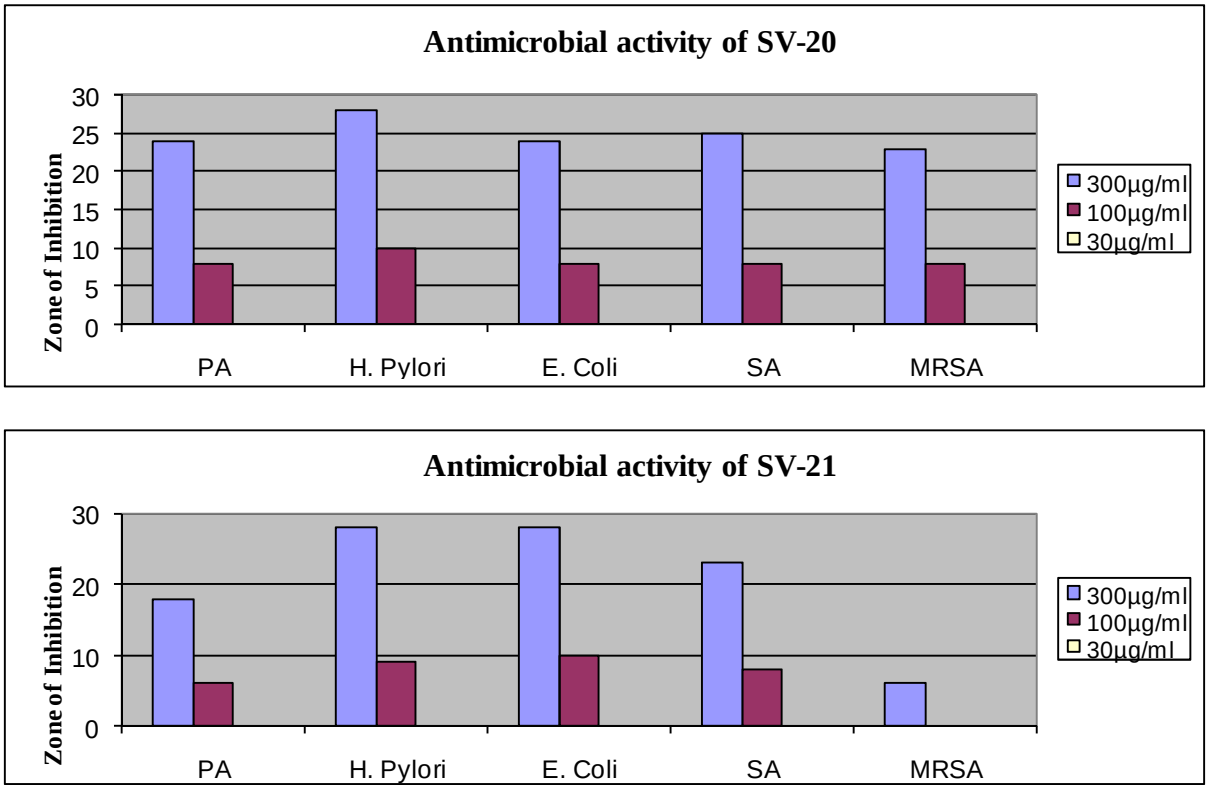
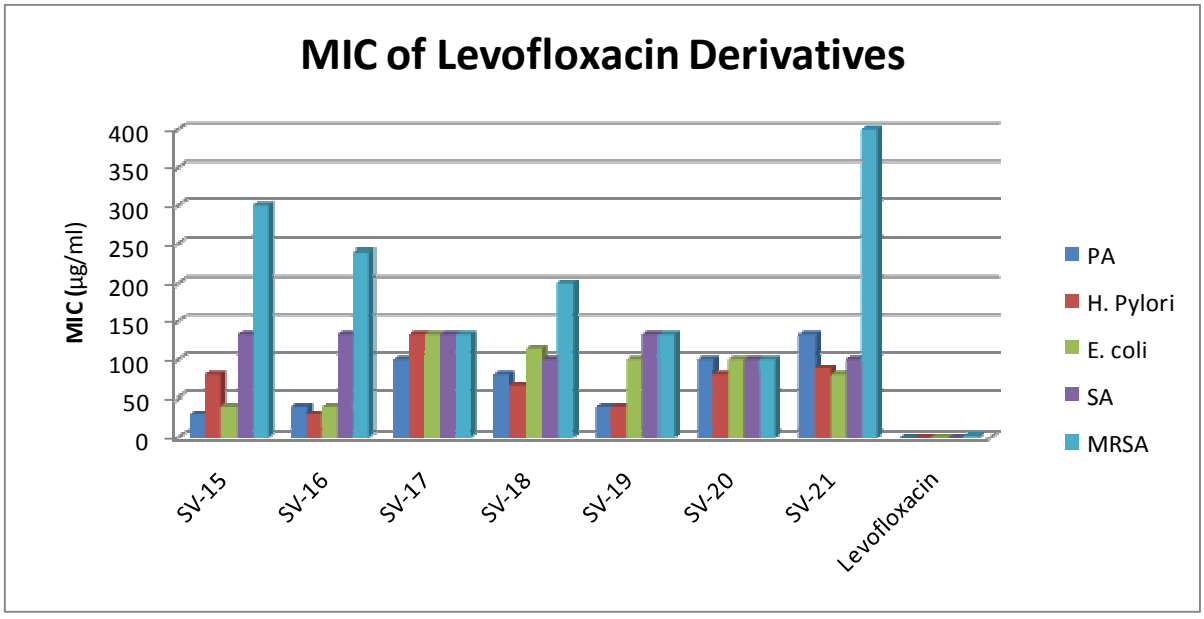


Figure 2: Minimum Inhibitory Concentration (MIC) the Schiff bases



CONCLUSION

In silico molecular Docking simulation Study was performed and it was found that all the test compounds are binding with the *Mycobacterium tuberculosis* DNA Gyrase Type-A, very efficiently as compare to exciting drugs.

Antimicribial activity was performed on all synthesized compounds. From all the synthesized compounds, compound Compounds SV-15 and SV-16 showed good activity against *E. coli*; Compound SV-15, SV-16 and SV-19 showed good activity against P.A. Compounds SV-15, SV-16, SV-17, SV-18, SV-19 SV-20 and SV-20 exhibited promising antibacterial activity against all the selected bacterial strains at 300µg/ml dose.

Compound SV-21 shows good activity against different fungal strains like *A. fumigatus*, *P. carinii* and *A. niger*. Compounds SV-17 and SV-18 were found active against *A. fumigates* and compound SV-15 was active against *P. carinii.*

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