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In Silico Design, Synthesis and Evaluation of *In-Vitro* Anti-Tuberculosis Activity of New 2-Amino 6-Methyl Pyridine Derivatives

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ABSTRACT

The discovery of new anti-Tubercular agent is become necessary for effective TB treatment. Nowadays the strategy for new drug development is directed towards Computer Aided Drug Design (CADD) techniques such as Molecular Docking. In the present investigation docking study of different 2', 5-dimethyl-4-phenyl [2,3'-bipyridin]-6'-amine derivative's on different targets was carried out with the reference of as standard pyrazinamide, streptomycin and ciprofloxacin drug. The docking experiments were performed by using Mcule docking software. With the aim of developing new biologically active compound, a series of derivatives were 2',5-dimethyl-4-phenyl[2,3'-bipyridin]-6'-amine synthesized. The chemical structures of compounds were characterized by IR and NMR. The In-vitro anti-TB activity of synthesized compound was performed by using *microplate Alamar Blue assay* (MABA) and compound 1C, 2C & 4C showed moderate to good anti-tubercular activity.

1. INTRODUCTION

Tuberculosis (TB) is a chronic infectious disease caused by mycobacterium of the ‘‘tuberculosis complex’’, including primarily *Mycobacterium tuberculosis*, but also *Mycobacterium bovis* and *Mycobacterium africanum.* Currently, near about one third of the world population is latently infected with Mycobacterium TB. Despite the availability of the BCG vaccine and chemotherapy, TB still remains a leading infectious disease globally. Although its incidence has diminished significantly in the industrially more developed countries, it remains a major public health problem in most of the developing nations. Recent efforts have been directed toward exploring new, potent anti- TB agents with low toxicity profiles when compared with currently used anti-TB drugs [13, 14].

In this work, study was focused to synthesize new anti-TB agent. The study includes docking study, synthesis and In-Vitro anti-TB evaluation of synthesized compound.

2. MATERIAL AND METHODS

Tools and Materials Used: - For our present study, we used biological database like PDB (protein data bank) and software like ACD chem. Sketch sand molecule docking. ACD/chem. sketch is the powerful all-purpose chemical drawing and graphics package from ACD/labs developed to help chemist to draw schematic diagrams, molecules, reactions and calculate chemical properties quickly and easily. ACD chem. sketch can convert “SMILES” notations to structure and vice versa [16].

PDB contains structural information of the various macromolecules determined by X-ray crystallographic study, NMR methods etc. Docking allows the scientist to virtually screen a database of compounds and based on various scoring functions they predict the strongest binders. The collection of drug analogs and receptor complexes was identified via docking and their relative's stabilities were evaluated using molecular dynamics and their binding affinities, using free energy simulations. The software Mcule docking is used for docking of pyridine derivatives on various targets such as *1tpy: Cyclopropanemycolic acid synthase MmaA2, 1mru: Serine/threonine-protein kinase pknB, 2vhx: Alanine dehydrogenase, 3i59: Transcriptional Regulatory Protein, 2b37: Enoyl-[acyl-carrier-protein] reductase [NADH].*

Melting points of compounds were determined routinely in open capillary tubes. ^{1}H-NMR spectra were obtained on Bruker (500 MHz), chemical shifts were given on the delta scale as

parts per million (ppm) with tetramethylsilane (TMS) as the internal standard. Infrared spectra were measured with KBr pellet on a FTIR-Shimadzu IRAffinity-1S in the range 4000-400 cm^{-1}. Thin layer chromatography (TLC) was performed on silica coated aluminum plate.

3. EXPERIMENT

The procedure used for Preparation of 2', 5-dimethyl-4-phenyl [2,3'-bipyridin]-6'-amine derivative's is similar to that reported by Krishna N and Mohite M [10].

3.1. STEP 1: PREPARATION OF CHALCONE

Equimolar mixture 2-amino-6-methyl pyridine and different substituted aldehydes dissolved in 15ml of ethanol and added 40% KOH and stirred the entire reaction mixture for 6 hrs. Then the mixture is kept for overnight at room temperature. Then pour the above mixture in crushed ice and acidified with HCl. The obtained chalcone was recrystallized from ethanol [10].

3.2 STEP 2: PREPARATION OF MICHAEL ADDUCTS

NaOH-1.0M was add to the stirred solution of chalcones (2.08g,10mmol) at room temperature in DMF (10ml) and nitromethane (0.61g,10mmol) the resulting mixture was stirred until the reaction was complete (TLC) [10].

3.3 STEP 3: REDUCTION AND RING CYCLIZATION

The granular zinc (3.27g: 50mmol) was added to the mixture and was stirred at 80°C and conc. HCl (20ml) was added very slowly. The mixture was stirred at 80°C under the reducing conditions for about 90min and then allowed to come room temperature. Neutralized with Saturated aqueous $NaHCO_3$ (30ml) and extracted with diethyl ether (3×20ml), filtered and concentrated. The crude product was purified by silica gel chromatography [10].

Table No. 1 Substitutions of Michael adduct pyridine derivatives and Smiles File.

Sr. No	SUBSTITUTIONS	SMILE FILES
1	Biphenyl-4-carboxaldehyde	Nc1ccc(c2ncc(C)c(c2)c2ccc(cc2)c2ccccc2)c(C)n1
2	2-chlorobenzaldehyde	Nc1ccc(c2ncc(C)c(c2)c2ccccc2Cl)c(C)n1
3	3-chlorobenzaldehyde	Nc1ccc(c2ncc(C)c(c2)c2cccc(Cl)c2)c(C)n1
4	4-Chlorobenzaldehyde	Nc1ccc(c2ncc(C)c(c2)c2ccc(Cl)cc2)c(C)n1
5	4-dimethylaminobenzaldehyde	Nc1ccc(c2ncc(C)c(c2)c2ccc(cc2)N(C)C)c(C)n1

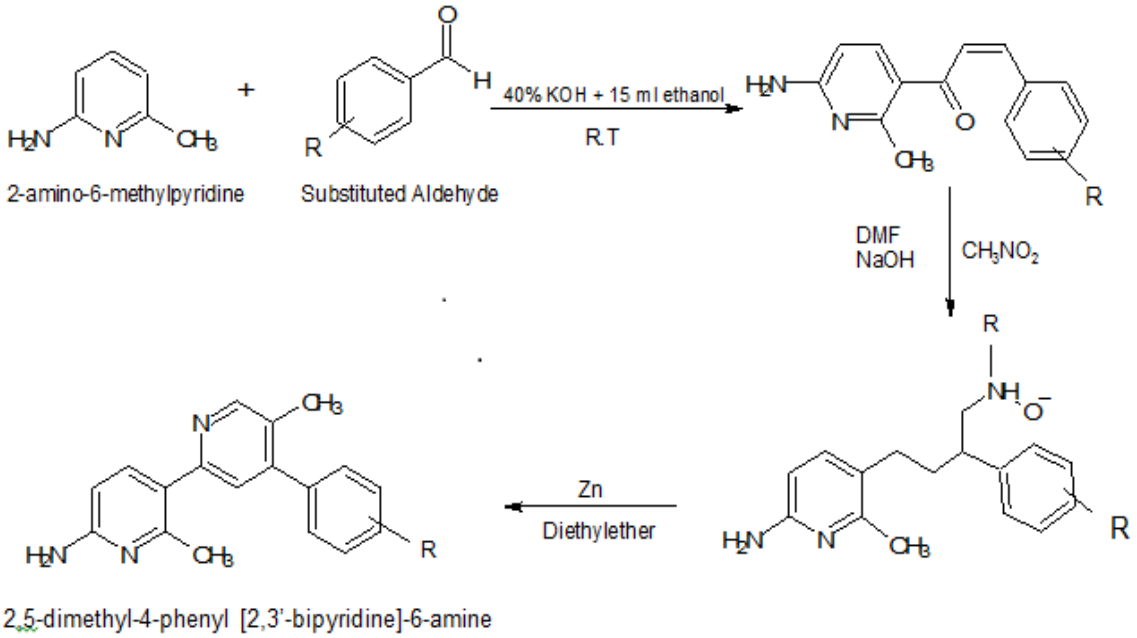
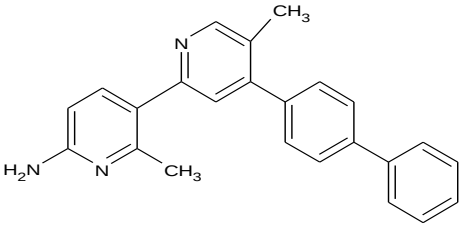
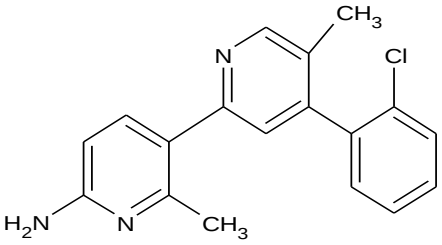
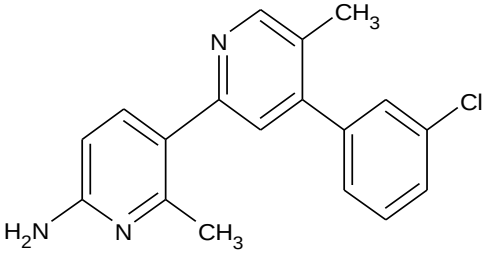
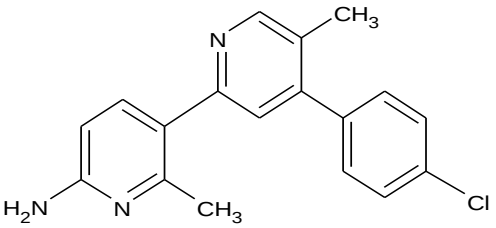
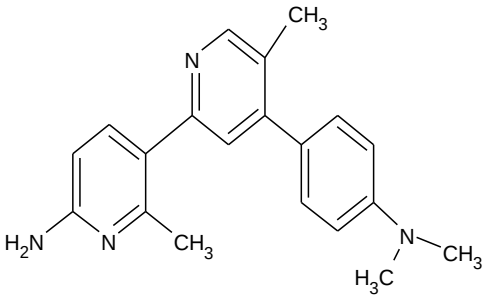


Figure No. 1 Reaction scheme for preparation of compounds 1C-5C.

Table No. 2 List of synthesized compounds

Sr. No	CODE	MOL. FORMULA	STRUCTURE	IUPAC NAME
1	1C	$C_{24}H_{21}N_3$		4-([1,1'-biphenyl]-4-yl)-2',5-dimethyl[2,3'-bipyridin]-6'-amine
2	2C	$C_{18}H_{16}ClN_3$		4-(2-chlorophenyl)-2',5-dimethyl[2,3'-bipyridin]-6'-amine
3	3C	$C_{18}H_{16}ClN_3$		4-(3-chlorophenyl)-2',5-dimethyl[2,3'-bipyridin]-6'-amine
4	4C	$C_{18}H_{16}ClN_3$		4-(4-chlorophenyl)-2',5-dimethyl[2,3'-bipyridin]-6'-amine
5	5C	$C_{20}H_{22}N_4$		4-[4-(dimethylamino)phenyl]-2',5-dimethyl[2,3'-bipyridin]-6'-amine

4. RESULTS AND DISCUSSION

4.1. DOCKING STUDY:

The docking score was obtained by using software module docking with reference to standard drugs pyrazinamide, streptomycin and ciprofloxacin as mentioned in **Table No. 3** The compound 1C, 3C and 4C given good binding score but compound 3C does not show in-vitro anti-TB activity. The Docking poses of compound 1C and 4C with Transcriptional Regulatory Protein and Alanine dehydrogenase as shown in **Figure No. 2.**

Table No. 3 The docking score, of synthesized Michael adducts pyridine derivatives.

COMPOUND CODE	DOCKING SCORE OF DIFFERENT TARGETS				
	1tpy: Cyclopropanemycolic acid synthase MmaA2.	1mru: Serine/threonine-protein kinase pknB	2b37: Enoyl-[acyl-carrier-protein] reductase [NADH].	3i59: Transcriptional Regulatory Protein (Probably CRP/FNR-Family)	2vhx: Alanine dehydrogenase.
1C	-9.6	-9.2	-9.4	-10.4	-9.9
2C	-6.6	-8.2	-8.8	-9.2	-8.5
3C	-7.9	-9.0	-8.9	-8.8	-8.5
4C	-7.2	-8.5	-8.3	-9.3	-8.5
5C	-7.3	-8.5	-8.1	-8.9	-8.2
Pyrazinamide	-5.8	-4.4	-4.8	-5.3	-5.3
Streptomycin	-5.4	-7.2	-7.5	-8-1	-7.7
Ciprofloxacin	-6.4	-8.7	-7.3	-8.2	-8.0

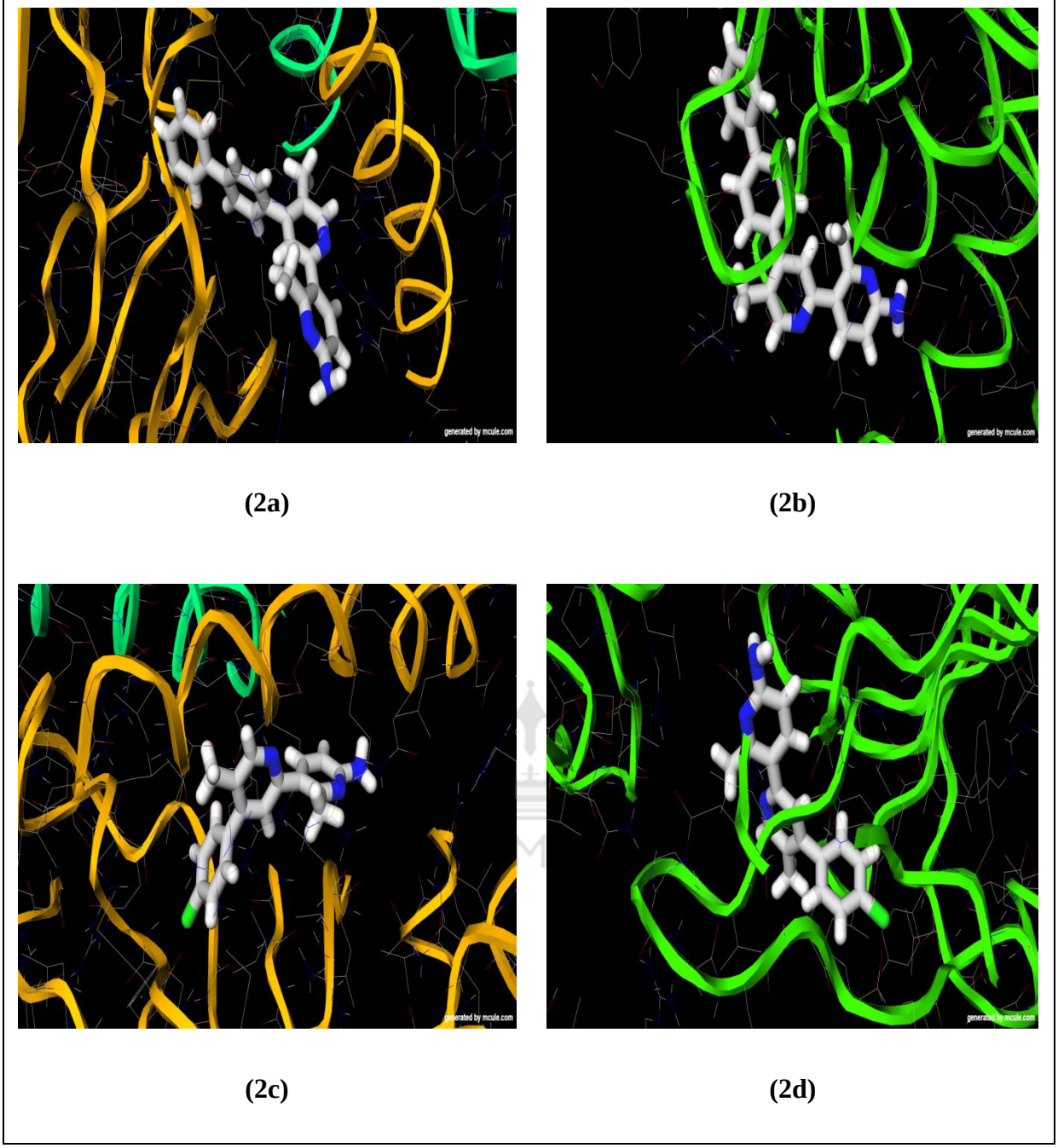


Figure No.2 The Docking poses of compound 1C and 4C with different Targets; (2a) C1 with Transcriptional Regulatory Protein, (2b) C1 with Alanine dehydrogenase, (2c) C4 with Transcriptional Regulatory Protein and (2d) C4 with Alanine dehydrogenase.

4.2. MOLECULAR PROPERTIES AND SPECTRAL CHARACTERIZATION:

We had synthesized five 2', 5-dimethyl-4-phenyl [2,3'-bipyridin]-6'-amine derivative's by using standard procedures with slight modification in the substituted aldehyde usage. In the current course of study we had observed several variations like practical yield, melting point,

molecular weight and R_f values as shown in **Table No.4.** Thin layer chromatography (TLC) was performed on silica coated aluminum plate using mobile phase Benzene: Ethyl acetoacetate: Methanol (0.5:1.5:1.5).

Table No. 4 Physico-chemical characterization data synthesized compounds.

Sr. No.	Code	Mol. Formula	Mol. Weight	% Yield	Melting point (°C)	Rf value	Colour
1	1C	$C_{24}H_{21}N_3$	351.17	68.2	98-103	0.60	Pale Yellow
2	2C	$C_{18}H_{16}ClN_3$	309.10	50.5	171-174	0.57	Yellow
3	3C	$C_{18}H_1ClN_3$	309.10	63.1	188-192	0.61	Creamish White
4	4C	$C_{18}H_{16}C N_3$	309.10	56.0	162-165	0.51	Pale Brown
5	5C	$C_{20}H_{22}N_4$	318.18	47.2	246-249	0.46	Brown

The ^{1}H NMR Analysis was done in Bruker 500 MHz instrument. Infrared spectra were measured with KBr pellet on a FTIR-Shimadzu IRAffinity-1S in the range 4000 - 400 cm^{-1}. The results obtained are shown in in **Table No. 5.** The spectral data shown the appropriate spectra's for all the reported synthesized compound except compound 5C. The NMR spectrum of the compound was not obtained because the compound is not completely soluble in any NMR solvent.

Table No. 5 Spectral characterization of synthesized compound.

Sr. No.	Code	IR Frequencies (cm^{-1})	1H-NMR Values (δ)
1	1C	1410, 1500 (Ar C=C), 3020 (Ar C-H), 1180 (Amine C-N), 1280, 1340 (Ar amine C-N), 1755 (Ar amine C-H), 2900 (C-H).	1.4 ppm Aliphatic C-H, 2 ppm Aliphatic C-H, 2.7 ppm C=C-C-H, 7.5 ppm Benzene C-H, 8.1 ppm Benzene C-H.
2	2C	840(C-Cl), 740 (O-or P- sub benzene), 1400 ($-CH_3$), 1520 (Ar C=C), 1690 (C=N).	1.2 ppm Aliphatic C-H, 2 ppm Aliphatic C-H, 7-8 PPM Aromatic C-H, 3.1 ppm Cl-C-H.
3	3C	840 (C-Cl), 710 (m-sub Benzene), 1380 ($-CH_3$), 1490, 1600 (Ar C=C), 1180 (C-N).	1.3 ppm Aliphatic C-H, 2.7 ppm C=C-C-H, 3.2 ppm Cl-C-H, 3.7 ppm Cl-C-H, 5.4 ppm Alkene =C-H, 7-8 ppm Aromatic C-H. :
4	4C	760, 690 (C-Cl), 1375 ($-CH_3$), 1490, 1600 (Ar C=C), 1020, 1190 (Amine C-N), 1280 (Ar C-N), 1750 (Ar C-H).	2.5 ppm –C=C-C-H, 3.2 ppm Cl-C-H, 3.5 ppm Cl-C-H, 5.5 ppm Alkene =C-H, 7.8 ppm Aromatic C-H, 3-5 ppm Aromatic NH_2.
5	5C	1400 ($-CH_3$), 1500 (Ar C=C), 1030 (Amine C-N), 1320 (Ar amine C-N), 1680 (C=N).	The NMR spectrum of the compound was not obtained because the compound is not completely soluble in any NMR solvent.

4.3. *IN-VITRO* ANT-TUBERCULAR ACTIVITY:

The anti-tubercular activities of the synthesized compounds (1C-4C) against *Mycobacterium tuberculosis (H37 RV strain)* were assessed at the Department of Microbiology, Maratha Mandal's NGH Institute of Dental Sciences and Research Centre, Belgaum-590010, India. The method applied is similar to that reported by Maria and Lourenco. [28]

4.3.1. Procedure:

The anti-mycobacterial activity of compounds were assessed against *M. tuberculosis* using *Microplate Alamar Blue Assay* (MABA). This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with proportional and BACTEC radiometric method. Briefly, 200µl of sterile deionized water was added to all outer perimeter wells of sterile 96 wells plate to minimized evaporation of medium in the test wells during incubation. The 96 wells plate received 100 µl of the Middlebrook 7H9 broth and serial dilution of compounds were made directly on plate. The final drug concentrations tested were 100 to 0.2 µg/ml. Plates were covered and sealed with parafilm and incubated at 37ºC for five days. After this time, 25µl of freshly prepared 1:1 mixture of Almar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs. A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth. The MIC was defined as lowest drug concentration, which prevented the color change from blue to pink. For standard tests MIC value of Pyrazinamide-3.125 µg/mL, Streptomycin-6.25 µg/mL and Ciprofloxacin-3.125 µg/mL, were determined each time.

However, compounds 1C, 2C & 4C showed moderate to good anti-tubercular activity as represented in **Figure No.3 and Table No.6** This is due to the presence of active structural moieties like 2', 5-dimethyl-4-phenyl [2,3'-bipyridin]-6'-amine group, which might interfere in the mechanism of cell wall synthesis and hence stop further growth of *Mycobacterium tuberculosis.*

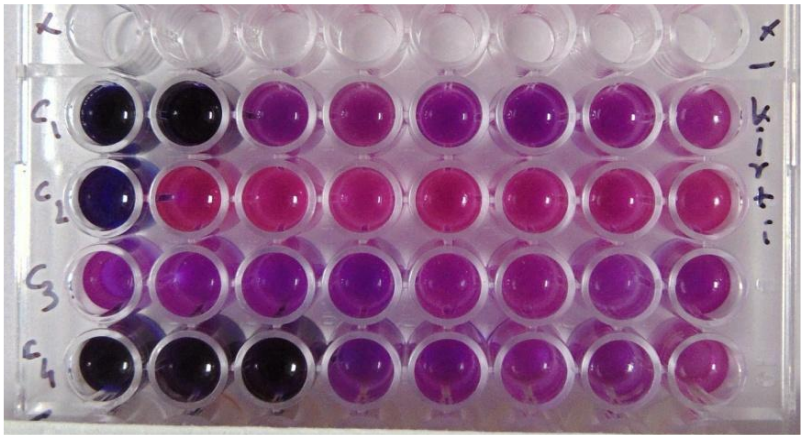


Figure No.3 Image showing Anti-tubercular results for synthesized compounds.

Table No.6 Results of anti-tubercular activity of the synthesized compounds

Sr. No	Sample code	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	312 µg/ml	1.6 µg/ml	0.8 µg/ml
1	1C	S	S	R	R	R	R	R	R
2	2C	S	R	R	R	R	R	R	R
3	3C	R	R	R	R	R	R	R	R
4	4C	S	S	S	R	R	R	R	R
Std.	Pyrazinamide-	S	S	S	S	S	S	R	R
Std.	Ciprofloxacin	S	S	S	S	S	S	R	R
Std.	Streptomycin	S	S	S	S	S	R	R	R

Where,

S = Sensitive

R = Resistant

From the above data, the compounds 1C, 2C and 4C shown significant anti-TB activity against Mycobacteria *tuberculosis (Vaccine strain, H37 RV strain).* All the studied compounds are showing different potency due to the effective barrier of cell wall membrane of Mycobacterium tuberculosis for entrance of external substances like test compounds under this study. Among this compound 4C shown best activity at 25 µg/ml concentration, compound 1C shows inhibitory activity at concentration 50 µg/ml and 2C at 100 µg/ml.

5. CONCLUSION

On the basis of docking score obtained from docking study the various 2', 5-dimethyl-4-phenyl [2,3'-bipyridin]-6'-amine derivatives were synthesized. 2-amino-6-methyl pyridine reacts with different aldehydes to get derivatives. Anti-tubercular activity was performed for the synthesized compounds by MABA (*microplate Alamar Blue assay*) Method. Among all the synthesized compounds, compound 4C has shown highest activity at **25 µg/ml** and the compound C1 shown activity at **50 µg/ml** against *mycobacterium tuberculosis pathogen H37 RV strain.* This study would pave the way for future development of more effective 2', 5-dimethyl-4-phenyl [2,3'-bipyridin]-6'-amine derivative's for applications TB control.

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