Synthesis, Molecular Docking Studies of Novel 4-(Substituted Phenyl Amino)-6-(Substituted Aniline)-N'-Aryl-1,3,5-Triazine-2-Carbahydrazide Derivatives As Potent Antitubercular Agents

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Abstract

A series of 4-(substituted phenyl amino)-6-(substituted aniline)-N'-aryl-1,3,5-triazine-2-carbahydrazide derivatives were synthesized under microwave irradiation and evaluated for their antimycobacterial activity and in-silico (molecular docking studies) to recognize the hypothetical binding motif of the title compounds using VLifeMDS software. The binding mode of the title compounds has been proposed based on the docking studies. They have interesting pharmacophore that display a broad spectrum of biological activity. Triazine derivatives have shown diverse pharmacological actions. Different derivatives have been prepared by synthesizing the hybrid structure of triazine and existing drugs. While taking into consideration we have tried to make hybrid with antitubercular drug Isoniazid. Simultaneously triazine ring is substituted with different various aromatic hydrazides and aromatic amines. Among all heterocycles, the triazine scaffold occupies a prominent position, possessing a broad range of biological activities and their structure activity relationship has generated interest among medicinal chemists and this has culminated in the discovery of several lead molecules. The antimycobacterial 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.

Keywords: *Triazine, Molecular docking studies, M. tuberculosis, Antimycobacterial activity, Microplate Alamar Blue assay*

1. INTRODUCTION

The outstanding development of triazine derivatives in diverse diseases within very short span of time proves its magnitude for medicinal chemistry research. Therefore, these compounds have been synthesized as target structure by many researchers, and were further evaluated for their biological activities[1]. Tuberculosis is a chronic disease which caused by causative agent mycobacterium tuberculosis responsible for for death about 1 billion people during last 2 centuries. In addition, the evolution of its new virulent forms like multidrug resistant and extensively drug resistant has become major threat to human life[2]. The present chemotherapy DOTS has a cure rate upto 95% if patient gives compliance. In developing countries, the prevalence of MDR-TB was reported due to inadequate supply of drugs as a consequences of poor financial resources. DHFR catalyzes the dihydrofolate to tetrahydrofolate, the folate cofactor involved the DNA replication as well as synthesis or catabolism of several amino acids[3]. Thus above mentioned factors reveal the necessary to develop effective and affordable anti-tubercular agents. Dihydrofolate reductase is a key enzyme of folate biocycle and also a prime target for infectious disease[4]. About 90% of those infected with Mycobacterium tuberculosis have asymptomatic, latent TB infection (sometimes called LTBI), with only a 10% lifetime chance that a latent infection will progress to TB disease[5]. However, if untreated, the death rate for these active TB cases is more than 50%. TB infection begins when the mycobacteria reach the pulmonary alveoli, where they invade and replicate within the endosomes of alveolar macrophages. The primary site of infection in the lungs is called the Ghon focus and is generally located in either the upper part of the lower lobe or the lower part of the upper lobe. Bacteria are picked up by dendrite cells which do not allow replication, although these cells can transport the bacilli to local (mediastinal) lymph nodes[6]. Further spread is through the bloodstream to other tissues and organs where secondary TB lesions can develop in other parts of the lung (particularly the apex of the upper lobes), peripheral lymph nodes, kidneys, brain, and bone. All parts of the body can be affected by the disease, though it rarely affects the heart, skeletal muscles, pancreas and thyroid[7]. Tuberculosis is classified as one of the granulomatous inflammatory conditions. Macrophages, T lymphocytes, B lymphocytes and fibroblasts are among the cells that aggregate to form a granuloma, with lymphocytes surrounding the infected macrophages. The granuloma functions not only to prevent dissemination of the mycobacteria, but also provides a local environment for communication of cells of the immune system[8]. Within the granuloma, T lymphocytes secrete cytokines such as interferon gamma, which activates macrophages to destroy the bacteria with which they are infected. Cytotoxic T cells can also directly kill infected cells by secreting perforin and granulysin[9]. Importantly, bacteria are not always eliminated within the

granuloma, but can become dormant resulting in a latent infection. Another feature of the granulomas of human tuberculosis is the development of cell death also called necrosis, in the center of tubercles. To the naked eye this has the texture of soft white cheese and was termed caseousnecrosis[10]. If TB bacteria gain entry to the bloodstream from an area of damaged tissue they spread through the body and set up many foci of infection, all appearing as tiny white tubercles in the tissues[11-16]. This severe form of TB disease is most common in infants and elderly and is called miliary tuberculosis. The present study was aimed at synthesizing some novel compounds and to screen the synthesized compounds for antitubercular activity[17-24]. Conventional methods for different chemical synthesis is very well documented and practiced. The methods for synthesis of organic compounds had continuously modified from the last 10 years. The application of green chemistry principles and practices renders regulation, control, clean-up, and remediation of the environment. In year 1855 Robert Bunsen invented the burner acts as energy source for heating reaction vessel this was latter superseded by isomental, oil bath but the drawback of the heating though method remains the same[25-29]. Microwave Assisted Organic Synthesis had developed in now years which has been considered superior to traditional heating. Microwave assisted organic synthesis has as a new “lead” in the organic synthesis. The technique offers clean, simple, efficient, fast and economic for the synthesis of a number of organic molecules such reaction has new tool in the organic synthesis. Important advantage of this technology includes highly accelerated rate of the reaction time with an improvement in yield and quality of product[30-41]. This technique is considered as important approach toward green chemistry because this technique is more environments friendly and this technology is used in the laboratory and has the potential to have a large impact on the fields of combinatorial chemistry, screening, medicinal chemistry and drug development. Conventional method of organic synthesis usually requires longer heating time, tedious apparatus setup which result in higher cost of process and the excessive use of solvents or reagents lead to environmental pollution. Computational studies are the crucial steps in the drug designing. Docking study is the computational routine to determine probable binding manners of a ligand to the dynamic site of a receptor. It makes an image of the dynamic site with interaction points known as grid. Then it fits the ligand in the binding site either by grid search or energy search[42-45]. Due to failure of ADME so it necessary to perform docking studies before pharmacological activity. An outbreak of coronavirus disease (COVID-19) caused by the novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) raises an unparalleled challenge in the discovery of appropriate drugs for prevention and treatment. Given the rapid pace of scientific research and clinical data produced by the large number of people quickly infected with SARS-CoV-2, clinicians need reliable proof of successful medical care for this infection as in intial stage with help of

molecular docking software it is easy to do in-silico study[46-55]. The chemical modification of drug delivery system for protein and peptide drugs is important in improving both enzymatic stability and membrane permeations can help to have good biological activity from any heterocyclic compound modification. Someday soon, you might be making your own medicines at home. That's because researchers have tailored a 3D printer to synthesize pharmaceuticals and other chemicals from simple, widely available starting compounds fed into a series[56-66].

2. MATERIALS AND METHODS

Chemistry:

All chemicals and solvents were procured from commercial sources, purified and dried using standard procedures from literature whenever required the regents were purchased from Sigma Aldrich and solvents and anilines were purchased from Research Lab. Melting points were determined by open capillary tube method and are uncorrected. Thin layer chromatography was used to assess the course of reaction and the purity of the intermediate and the final compounds were confirmed by applying a single spot on TLC plate (silica gel G) using various solvents such as toluene, acetone system.

General procedure for Synthesis of 4, 6-dichloro-N-(substituted phenyl)-1, 3, 5-triazin-2-amine

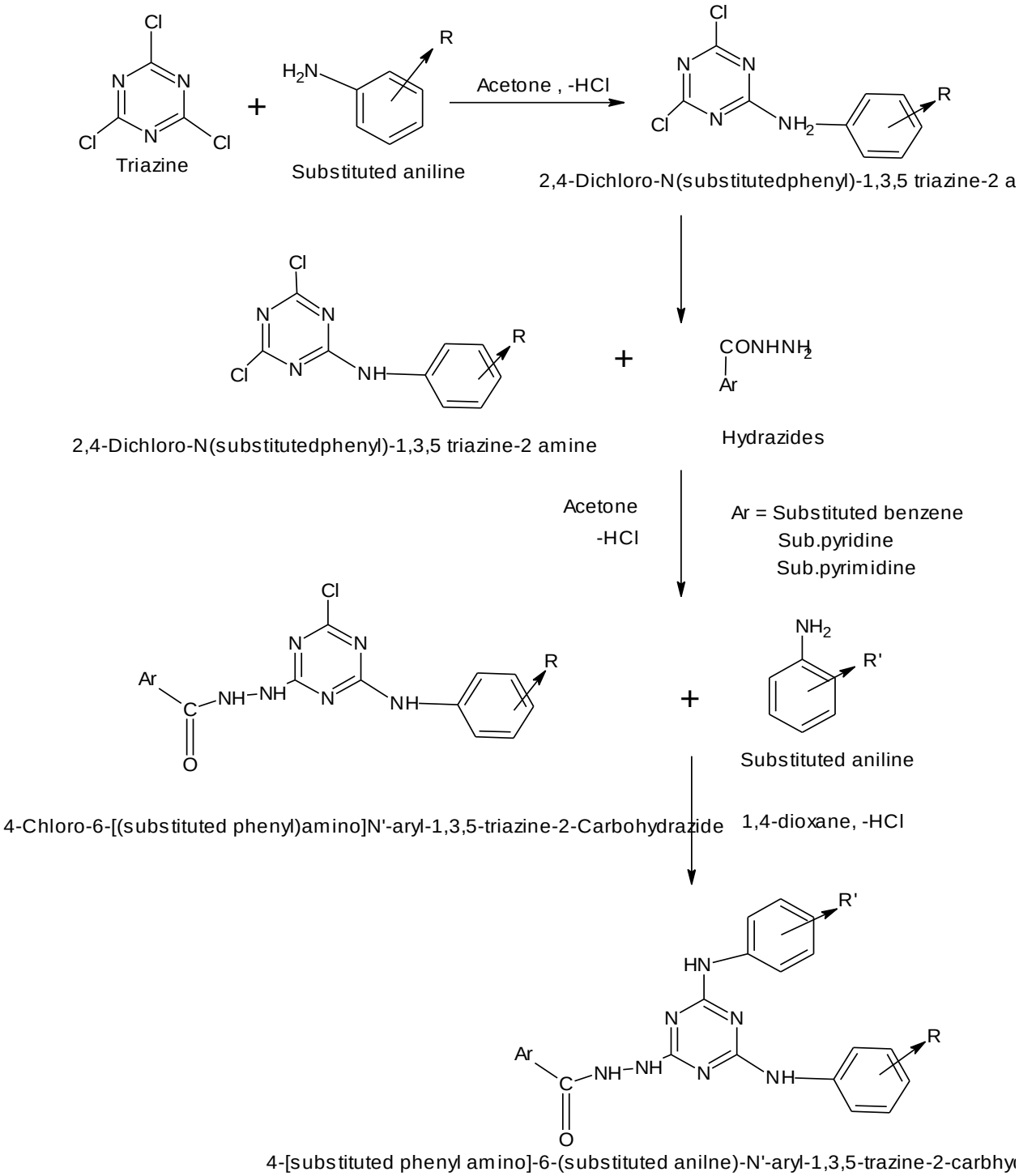
In a conical flask, 2,4,6-trichloro-3,5-triazine (0.01 mol) will be taken in acetone (20 mL) and substituted aryl amine (0.01 mol) will be added to it. To this mixture, 4% NaOH will be added drop wise at 0-5ºC temperature. The solution will be stirred and irradiated in microwave for 25min. The reaction mixture will be poured onto crushed ice with constant stirring. The solution will be neutralized with dil. HCl. The solid will be filtered and washed with water. The product will be dried and recrystallized from acetone.

General procedure for Synthesis 4-chloro-6-[(substituted phenyl)amino]-N'-aryl-1,3,5-triazine-2-carbohydrazide

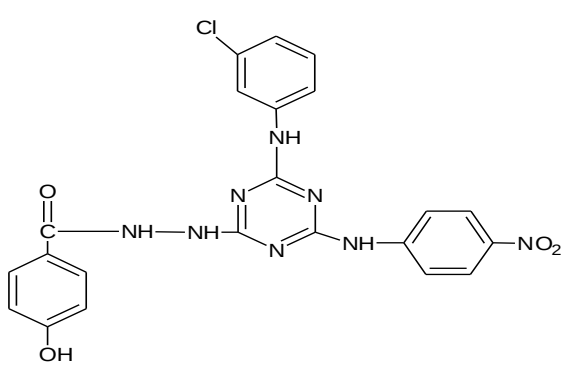
In a conical flask, 4, 6-dichloro-*N*-(substituted phenyl)-1, 3, 5-triazin-2-amine (0.01mol) will be taken in acetone (20 mL) and aryl hydrazide (0.01 mol) will be added to it. To this mixture, 4% NaOH will be added drop wise at room temperature. The solution will be stirred for irradiated for 15min. The reaction mixture will be poured onto crushed ice with constant stirring. The solution will be neutralized with dil. HCl. The solid will be filtered and washed with water. The product will be dried and recrystallized from acetone.

General procedure for Synthesis 4-[(substitutedphenyl)amino]-6-(substituted aniline)-N'-aryl-1,3,5-triazine-2-carbohydrazide

In a round bottom flask, 4-chloro-6-[(substituted aryl) amino]-*N*'-pyridin-4-yl-1, 3, 5-triazine-2-carbohydrazide (0.01 mol) and 1,4-dioxane (20 mL) will be taken. To this mixture, substituted amine (0.01 mol) will be added. The pH will be adjusted to neutral by adding 8% NaOH. The reaction mixture was irradiated in microwave for 30 min and poured onto crushed ice with constant stirring. This will be neutralized with dil. HCl. The product will be filtered and washed with cold water. The product will be dried and recrystallized from methanol.



Scheme 1. *Synthetic route for the preparation of the title compound (3a-c)*



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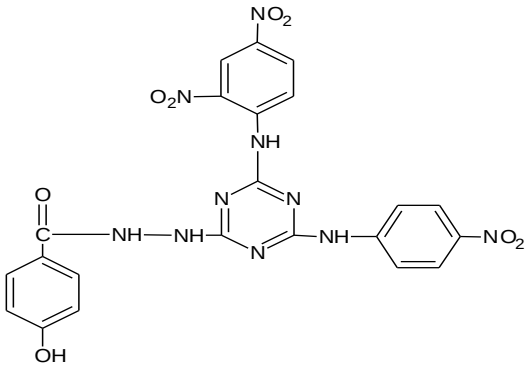

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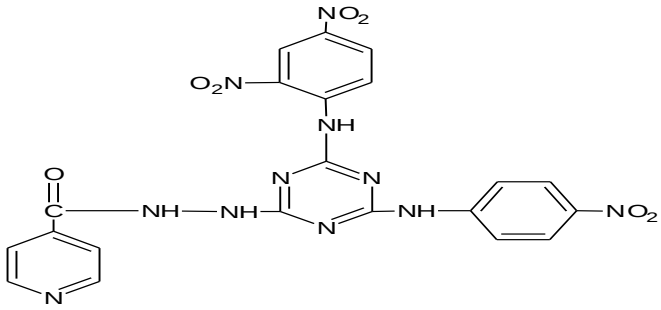

III

Molecular Docking Studies:

1LQU receptor (mycobacterium tuberculosis FprA in complex with NADPH) was obtained from protein data bank and water molecules in the crystal structure were deleted. optimized receptor was then saved as mole and used for docking simulation.

Ligand Preparation

2D structures of the compounds were built and then converted into the 3D. 3D structures were then energetically minimized up to the rms gradient of 0.01 using MMFF[67].

Identification of cavities

By using cavity determination option of software, cavities of enzyme were determined. The cavities in the receptor were mapped to assign an appropriate active site. The basic feature used to map the cavities were the surface mapping of the receptor and identifying the geometric voids as well as scaling the void for its hydrophobic characteristics. Hence, all the

cavities that are present in receptor are identified and ranked based on their size and hydrophobic surface area[68].

Scoring Function

Distinction of good or bad docked conformation is based on scoring function. MDS uses functions on only electrostatic and both steric and electrostatic interactions between receptor ligand as well as dock score scoring function. The dock score compute binding affinity of a given protein-ligand complex with known 3D structure[69-71].

Biological Activity:

In-vitro antitubercular activity

Materials and Methods

Standard Strain used: Mycobacteria tuberculosis (Vaccine strain, H37 RV strain): ATCC No-27294.

Standard values for the Anti-Tb test which was performed.

Pyrazinamide- 3.125µg/ml

Ciprofloxacin-3.125µg/ml

Streptomycin- 6.25µg/ml.

NOTE:

S – Sensitive

R- Resistant

The antimycobacterial activity of compounds were assessed against M. tuberculosis using microplateAlamar Blue assay (MABA). This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with propotional and BACTEC radiometric method. Briefly, 200µl of sterile deionzed 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[72-74].

3. RESULT AND DISCUSSION

Chemistry

4,6-Dichloro-N-(substituted phenyl)-1, 3, 5-triazin-2-amine were prepared in conical flask by adding 2,4,6-trichloro-3, 5-triazine in acetone and substituted aryl amine. To this mixture, 4% NaOH will be added drop wise at 0-5ºC temperature. The solution will be stirred and irradiated in microwave. The reaction mixture will be poured onto crushed ice with constant stirring. The solution will be neutralized with dil. HCl. The solid will be filtered and washed with water. The product will be dried and recrystallized from acetone. In next step 4-Chloro-6-[(substitutedphenyl)amino]-N'-aryl-1,3,5-triazine-2-Carbohydrazide were prepared by, In a conical flask, 4,6-dichloro-N-(substituted phenyl)-1,3,5-triazin-2-amine (0.01mol) will be taken in acetone and aryl hydrazide will be added to it. To this mixture, 4% NaOH will be added drop wise at room temperature. The solution will be stirred stirred and irradiated in microwave. The reaction mixture will be poured onto crushed ice with constant stirring. The solution will be neutralized with dil. HCl. The solid will be filtered and washed with water. The product will be dried and recrystallized from acetone. In further step for Synthesis 4-[(substituted phenyl)amino]-6-(substituted aniline)-N'-aryl-1,3,5-triazine-2-Carbohydrazide in a round bottom flask, 4-chloro-6-[(substituted aryl) amino]-N'-pyridin-4-yl-1,3,5-triazine-2-carbohydrazide and 1,4-dioxane will be taken. To this mixture, substituted amine will be added. The pH will be adjusted to neutral by adding 8% NaOH. The reaction mixture was irradiated in microwave and poured onto crushed ice with constant stirring. This will be neutralized with dil. HCl. The product will be filtered and washed with cold water. The product will be dried and recrystallized from methanol.

Anti-tb Activity Molecular Docking Results

Table 1. : Inflammatory activity result of molecular docking studies by using GRIP Batch docking.

Sr. no	Compound	Final energy	Final GRMS	Dock score
1	3a	55.4134	0.2970	-65.53
2	3b	61.5375	0.8997	-69.47
3	3c	46.1430	0.5164	-68.00

The dock score of compound 3a-c are shown in table and in that compound 3b dock score is found to -69.47 shown minimum dock score than other compounds. As we compared result of compound 3b to the literature this docking score indicated that designed compounds have good binding affinity for binding to 1LQU receptor (mycobacterium tuberculosis FprA in complex with NADPH). The best pose obtained by docking results is reported where main interaction between ligand and receptor can be observed. All designed compound adopt a very similar conformation binding pocket, showing Hydrogen bonding Vander Waals binding with amino acid of GLN 185B,ALA196B, GLN185 A,GLY 298B, GLY298A, ASP296B, LYS295B, ARG140B, GLY294A,PHE297B.Which shown by 2D representation diagram.

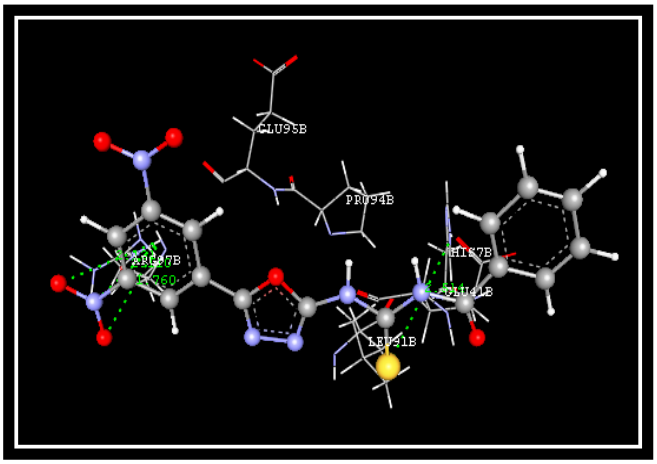

Fig 1. Docking Poses of compound 3b

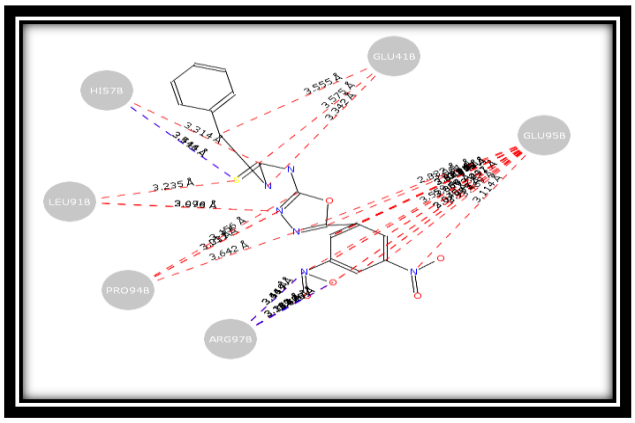

Fig 2. 2D representation of docking poses of compound 3b and standard

Table 2. Data for interaction of compound 3b with Amino Acid

Amino acid	Atom of ligand	Type of interaction
HIS 7 B	2S	H-Bond Interaction
ARG 97 B	27N,28O,29O	H-Bond Interaction
GLU 95 B	3C,8C,28O,31H	VDW Interaction
LEU 91 B	12N, 2S ,12N	VDW Interaction
PRO 94 B	10N,12N,12N,13C	VDW Interaction
GLU 41 B	15C,16N,17C	VDW Interaction

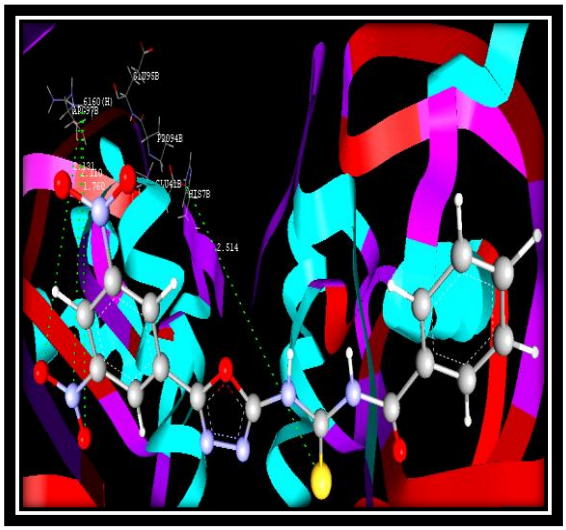

Fig 3. Superimpose image of docking poses of compound 3b

Antitubercular Activity

Antitubercular activity was performed by Alamar dye blue method using microtitre plate. Pyrazinamide, Ciprofloxacin and Streptomycin were used as standards. Standard compound 60% activity whereas samples were in between 20-50%.

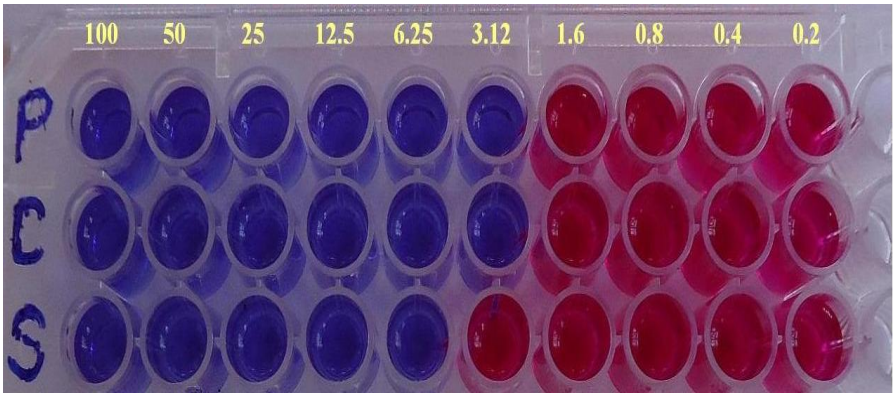

Fig 4. Standard Drug Sensitivity

P = 60% activity ……. (P = Pyrazinamide)

C = 60% activity…….(C = Ciprofloxacin)

S = 50% activity …….(S = Steptomycin)

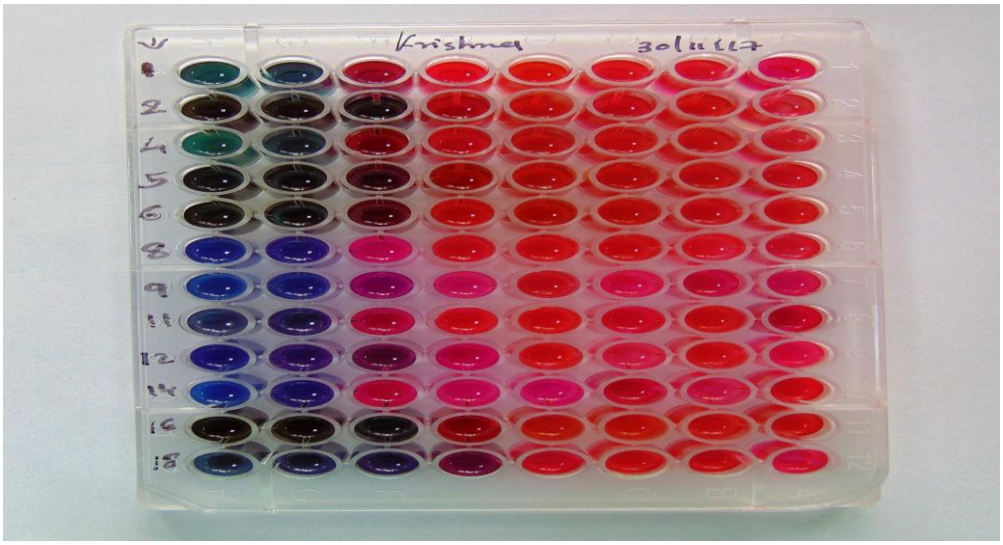

Fig 5. Synthesized compound Sensitivity

Table 4. In-vitro anti-tb activity of targeted compound.

Compound	Activity Percentage
3a	25
3b	37.5
3c	28

Table 5. *In-Vitro* Anti-TB Activity evaluation by using Alamar Blue Dye

Sr. No.	Samples	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	3.12 µg/ml	1.6 µg/ml	0.8 µg/ml
1	3a	S	S	R	R	R	R	R	R
2	3b	S	S	S	R	R	R	R	R
3	3c	S	S	S	R	R	R	R	R

NOTE:

S – Sensitive

R- Resistant

4. CONCLUSION

Development of antimycobacterial agents based on identified target and their mechanisms of action is a productive approach for lead generation. In this perception, we have chosen to explore 1,3,5-triazine moiety known to be targeting DHFR enzyme, which was also identified as a prime target for antitubercular as a prime target for anti-tubercular drug isoniazid. With this aim, we designed and synthesized easily accessible. Activity results suggest that the incorporated of anti-tubercular drug INH increased the potency of 1,3,5-triazines. The docking study were carried out for all the synthesized compounds and compared the docking score with reference compounds for antitubercular activity. The compounds code 3b showed higher binding score, which are further

attributed to the antitubercular activity of these compound. We have described an efficient and benign synthesis of triazine systems gives more yields and requires less time by microwave method. 1,3,5-triazines is the key intermediate in the formation of these heterocyclic compounds.Thus our studies reveal that these 1,3,5-triazines are compound 3b is novel anti-tubercular agents can be proved as potential drug candidates.

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