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SYNTHESIS AND EVALUATION OF NOVEL HETEROCYCLIC DERIVATIVE AS ANTI-TUBERCULAR AGENT.

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1. INTRODUCTION:

Tuberculosis remains one of the major global health threats leading to morbidity and mortality. One in three people worldwide, representing 2–3 billion individuals, are known to be infected with Mycobacterium Tuberculosis, of whom 5–15% are likely to develop active TB disease during their lifetime. [1] There has not been much development in the first-line/classes of TB drugs, which has led to the emergence of drug-resistant strains (TDRs) that have reached a dead end to the current line of treatment.[2] Recently, the USFDA granted accelerated approval to the Johnson and Johnson drug "Bedaquiline" for the treatment of drug-resistant TB. [3] There is therefore an urgent call for the development of faster-acting and effective new antituberculosis agents, ideally belonging to new structural classes, to better fight TB, including MDR-TB and XDR-TB, and to shorten the duration. current treatments, improve patient compliance, and provide effective treatment for dormant tuberculosis infection.[2]

Current antitubercular drugs primarily target cellular processes involved in bacterial growth and are either bacteriostatic or bactericidal. These include cell wall synthesis inhibitors, nucleic acid synthesis inhibitors, protein synthesis inhibitors, and energy inhibitors. Newer drugs with new targets are being used to address the problems of multidrug tolerance and dormant TB populations.[5]

Recent developments in mycobacterial molecular genetics tools have aided in the identification and validation of new drug targets essential for tubercle bacilli not only in vitro but also for their survival and persistence in vivo. [6]

2. Importance of heterocyclic compounds in medicinal chemistry

Heterocyclic compounds have a broad spectrum of pharmacological activities and for this reason continue to provide new therapeutic agents. The biological activity of heterocycles is due to their potential to bind to various enzymes either at active sites or enzyme pocket structures through a wide range of intramolecular interactions such as van der Waals and hydrophobic forces, hydrogen bonds, and metal coordination bonds, making them an important scaffold in medicinal chemistry.

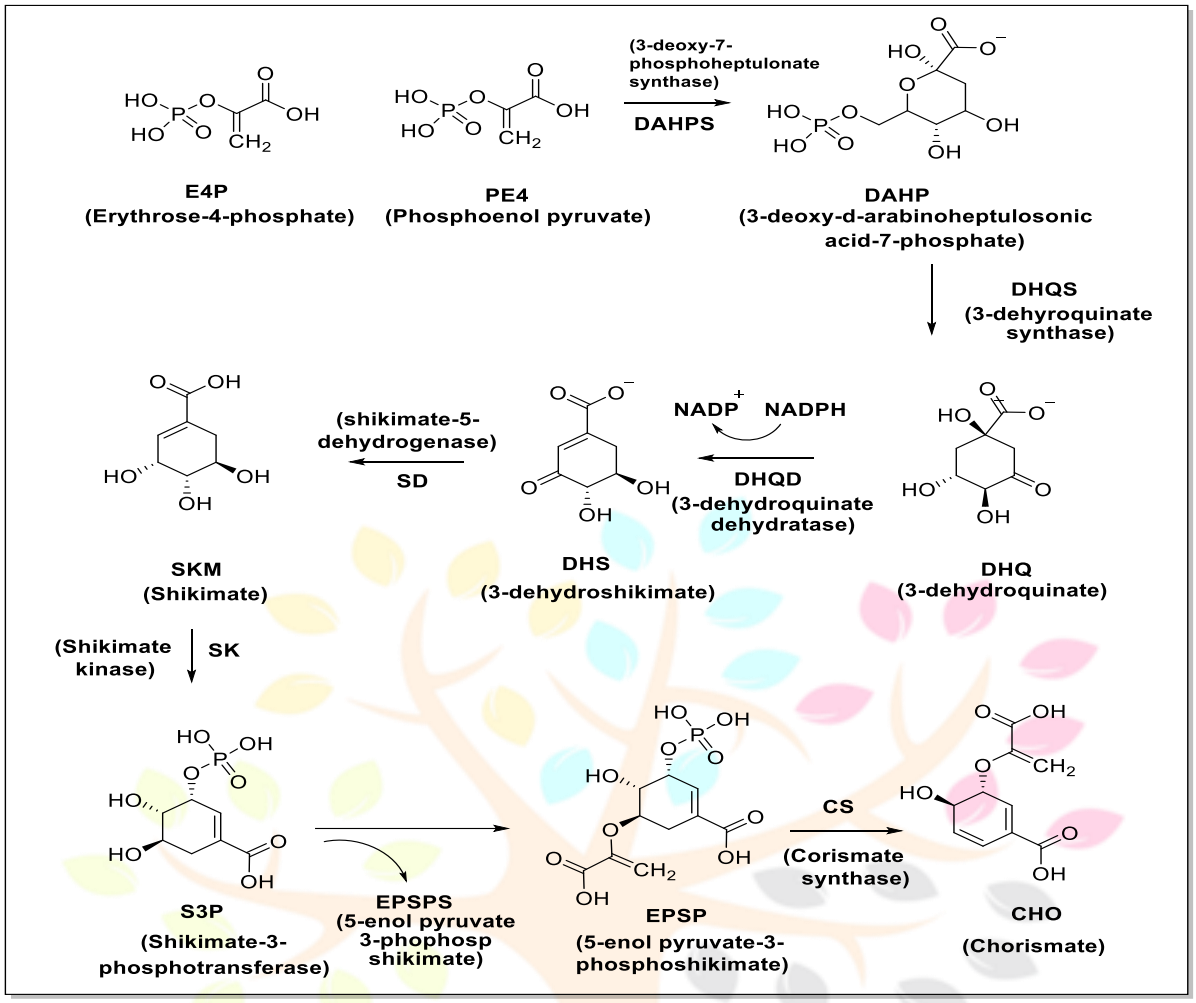
3. Therapeutic targeting of the hallmarks of tuberculosis (TB)

Tuberculosis, a communicable disease caused by the bacterium Mycobacterium tuberculosis, is one of the 10 leading causes of death. At the UN's first-ever high-level meeting in September 2018 at its headquarters in New York, heads of state came together and made firm commitments to end TB. The title of the meeting was "United to End TB: An Urgent Global Response to a Global Epidemic" (WHO, Global Tuberculosis Report 2023). In the field of TB drug discovery, although many drugs are currently in use, the development of multidrug-resistant Mycobacterium tuberculosis (MDRTB) and extremely drug-resistant TB (XDRTB) represent two major challenges. Currently, the development of an effective anti-TB drug that has a short duration of treatment, is simpler, less toxic, drug-resistant, and has minimal drug-drug interactions is an urgent requirement in the field. For TB, the main targets chosen by researchers are mycobacterial transcriptional repressor (EthR), enoyl ACP-reductase (InhA), decaprenyl phosphoryl-βD-ribose 2′epimerase (DprE1), shikimate kinase, alanine racemase, etc. current anti-TB -the drug contains a new chemical scaffold and different targets, including thiadiazole, oxadiazole, and triazole, because both cores can coordinate well with these targets. [7]

4. SHIKIMIC ACID PATHWAY:

The shikimic acid pathway, also known as the chorismate biosynthetic pathway, is a seven-micron enzymatic reaction for the conversion of two metabolites, phosphoenolpyruvate (PEP) of the glycolysis pathway and erythrose-4-phosphate of the non-oxidative pentose branch. phosphate way, to chorismate.[8] It is a potential and favorable target for drug design. It is vital for the survival of microorganisms and is exclusive to microorganisms. This pathway is not present in mammals, allowing the detection of targets that can potentially reduce the toxicity of drug candidates. The fact that the shikimate pathway is essential for M. tuberculosis even in the presence of exogenous supplements such as p-aminobenzoate, p-hydroxybenzoate, and aromatic amino acids highlights its importance as a drug target. Thus, inhibition of critical enzymes involved in this pathway appears to be an attractive target for the development of new anti-infective agents.[2]

One of the most potential enzymes of the Shikimic acid pathway is Shikimate Kinase (SK), which participates in the fifth step of the pathway. It is responsible for catalyzing the ATP-dependent phosphorylation of shikimate to form shikimate-3-phosphate. In M. tuberculosis, the aroK gene encodes the M. tuberculosis shikimate kinase (MtSk), which catalyzes the SK reaction. Disruption of the arok gene is essential for the viability of M. tuberculosis, making it an attractive target for designing new molecules. [9]



The crystal structure of MtSK-MgADP-shikimate provides essential information for the design of SK inhibitors that target both the shikimate and ATP binding pockets or, exceptionally, the shikimate binding site.[10] The shikimate binding site is characterized by a hydrophobic surface along with several hydrophilic charged residues that protrude into the cavity. The binding of shikimate to its cavity represents essential residues that create possible interactions of the ligand with its protein. [2]

A literature survey shows that structure-based virtual screening protocols have been used to predict MtSk inhibitors. Docking simulations found potential inhibitors to be structurally related to a triazole or tetrazole heteroaromatic system, which may offer a candidate for MtSk inhibitor discovery.[11] The presence of a sulfur moiety as an electron-rich center can improve lipophilicity and modulate the electron density of the triazole ring, as well as its interaction with hydrogen bond donors of the organism.[12] The top-ranked compounds were reported to contain a mercapto group and a triazole or tetrazole ring in the scaffold.[9]

Triazoles are an important group of heterocyclic compounds that are biologically active and of great importance in medicinal chemistry. It is known to exhibit antitubercular activity along with other biological activities such as antimicrobial, CNS depressant, anti-HIV, cytotoxic, anti-inflammatory, analgesic, anticonvulsant, and many other

activities and can cross the blood-brain barrier. 13] Therefore, it was decided to synthesize heterocyclic derivatives without mercapto group or sulfur atom to clarify their importance in antitubercular activity using aminoguanidine bicarbonate.

5. EXPERIMENTAL WORK:

5.1 SYNTHESIS OF THE HETEROCYCLIC MOLECULES:

The purity of the starting materials used in the reaction was confirmed by thin-layer chromatography (TLC). The purity and structures of the synthesized compounds were confirmed by melting point, thin-layer chromatography, infrared spectroscopy, nuclear magnetic resonance spectroscopy, and, where applicable, mass spectroscopy. The melting points of the synthesized compounds were uncorrected and recorded by the open glass capillary method on "DBK Prog. Melting Point Apparatus' and complied with the reported melting points whenever possible. Analytical thin-layer chromatography (TLC) was performed on pre-coated TLC plates (Silica gel GF254). 1H NMR spectra were recorded on "FT-NMR 400 MHz Analyzer" at SAIF NMR RESEARCH CENTRE, INDIAN INSTITUTE OF SCIENCE, BANGALORE. IR spectra were recorded using "Bruker Optik GmbH, ALPHA-T" at Dr. L.H. Hiranandai College of Pharmacy, Ulhasnagar. Mass spectra of several compounds were recorded on a "Shimadzu LC-MS 8040 System" at Powai, Mumbai.

All chemicals, reagents and solvents used in this study were obtained from Molychem, Sigma Aldrich.

5.1.1. Synthesis of new molecule:

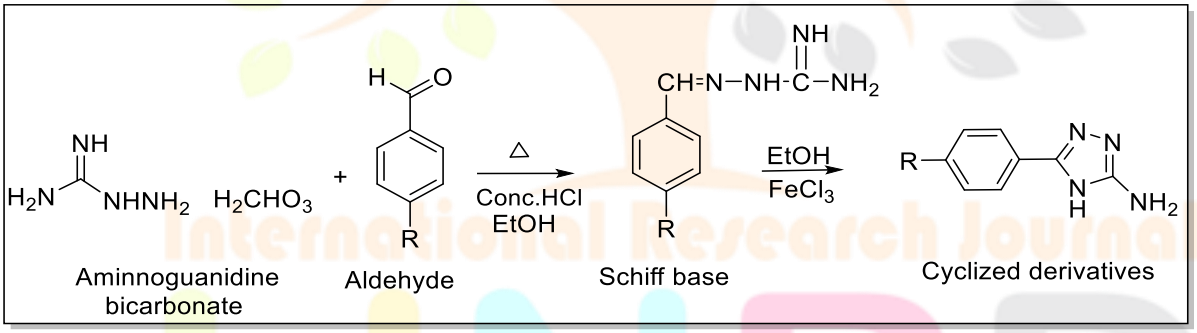


Figure No. 5.1.1. Scheme for synthesis cyclized derivatives.

Where R is,

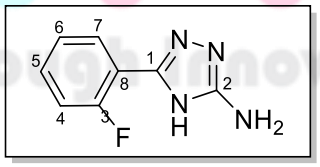
Compound	R
TVD7	

Table No.5.1.1. aldehyde substitution for the synthesis of substituted aminoguanidine derivatives.

5.1.2. Step 1- Synthesis of Schiff base:

General Reaction:

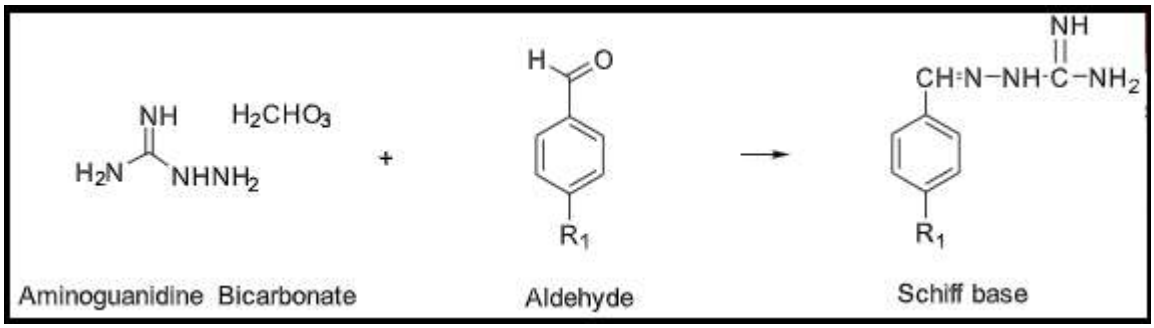
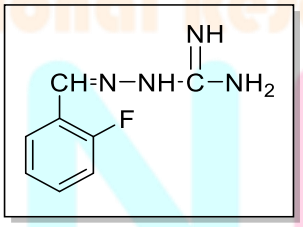


Figure No. 5.1.2.1. Scheme for synthesis Schiff bases.

General Procedure:

A mixture of aminoguanidine bicarbonate (1 mol) and substituted aldehyde (1 mol) in ethanol was heated in a microwave oven at power 7 (420 W) for 10 min and 5 cycles, and the progress of the reaction was monitored by TLC. After the reaction was complete, the solution was cooled and added to crushed ice with constant stirring. Dilute HCl was then added until the color changed. Sodium bicarbonate solution was then added for neutralization and complete precipitation. The product thus obtained was filtered under vacuum, washed with cold water, dried and recrystallized from ethanol. All derivatives (TVD2) were synthesized similarly and characterized by spectral analysis.

TVD7: Synthesis of 5-(2-fluorophenyl)-4H-1, 2, 4-triazol-3-amine



Molecular formula: $C_8H_9FN_4$

Molecular weight: 180.1 g/m

TLC:

Stationary Phase: Silica Gel GF254

Mobile Phase: Ethyl Acetate: Benzene: Methanol (7:3:1)

Chromatogram: Single spot with Rf value = 0.41

Detection: Under short UV lamp in UV chamber.

Yield (%):76.37 %

Melting Point (M.P.): 178-182°C

5.1.3. Step 2- Cyclization of Schiff Base:

General Reaction:

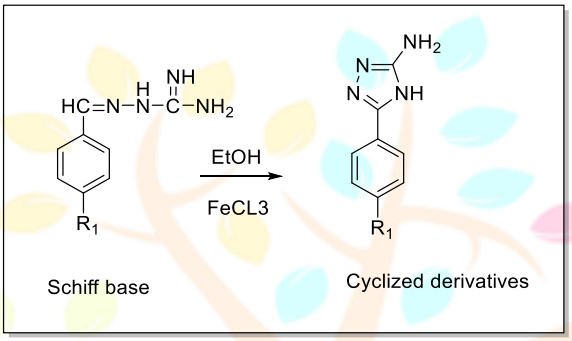
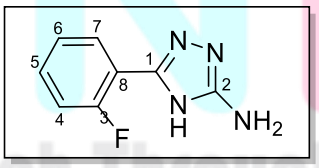


Figure No. 5.1.3.1. Scheme for synthesis Cyclized derivative from Schiff base.

General Procedure:

A catalytic amount of FeCl3 was added to a solution of Schiff's base in 10-20 ml of ethanol. The contents were stirred at 60°C on a magnetic stirrer until the reaction was complete. The completeness of the reaction mixture was checked by thin-layer chromatography. The product formed was filtered, washed with cold water, dried, and recrystallized from ethanol. All derivatives were synthesized similarly and characterized by spectral analysis.**TVD2:** 5-(4-methylphenyl)-4H-1, 2, 4-triazole-3-amine



Molecular formula: $C_8H_7FN_4$

Molecular weight: 179.2 g/m

TLC:

Stationary Phase: Silica Gel GF254

Mobile Phase: Ethyl Acetate: Benzene: Methanol (1:1:2)

Chromatogram: Single spot with Rf value = 0.41

Detection: Under short UV lamp in UV chamber.

Yield (%):74.11 %

Melting Point (M.P.): 178-182°C

FT-IR Spectra of compound TVD7:

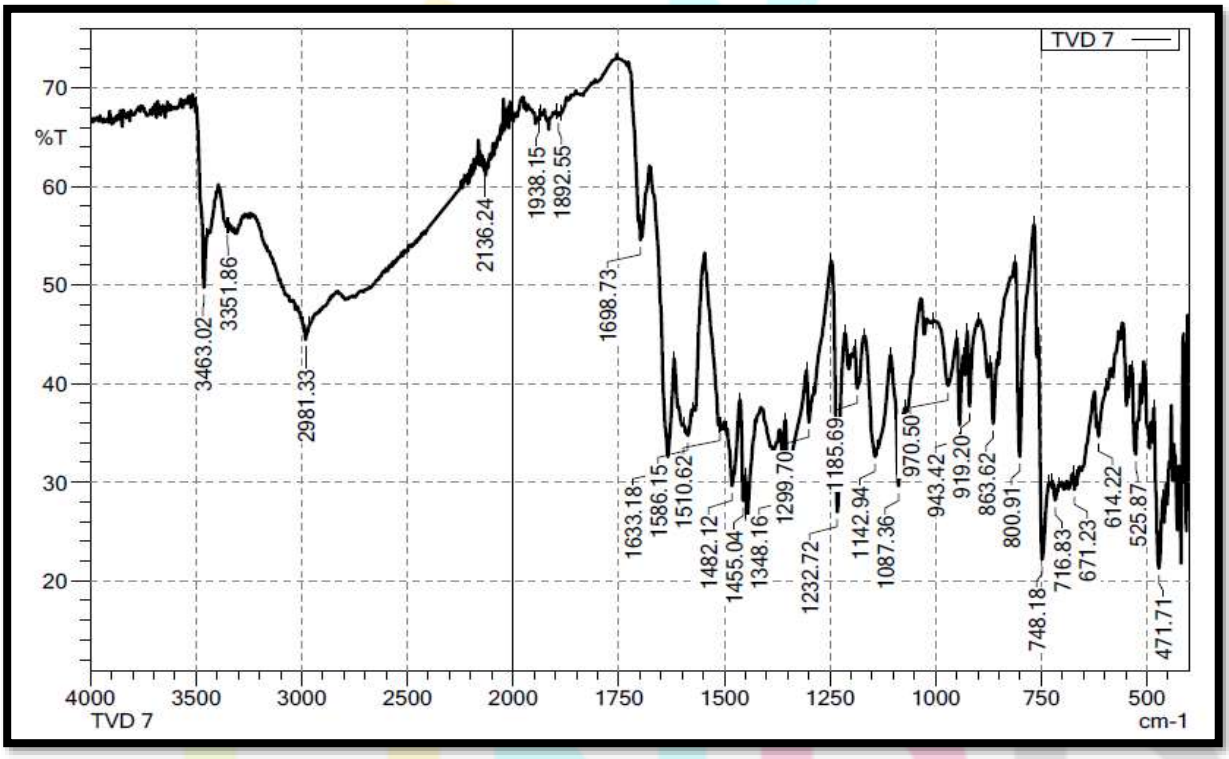


Figure No. 5.1.3.1. IR Spectra of compound TVD7

Peak value (cm-1)	Groups
3425.97	-NH_2 stretching vibration
3320.51	-NH stretching vibration
2981.33	Aromatic CH stretching vibration
1640.30	-C=N stretching vibration
1586.15	-CH stretching vibration
1232.72	C–F stretching vibration

Table No. 5.1.3.1. IR values of compound TVD2.

13C NMR of compound TVD2

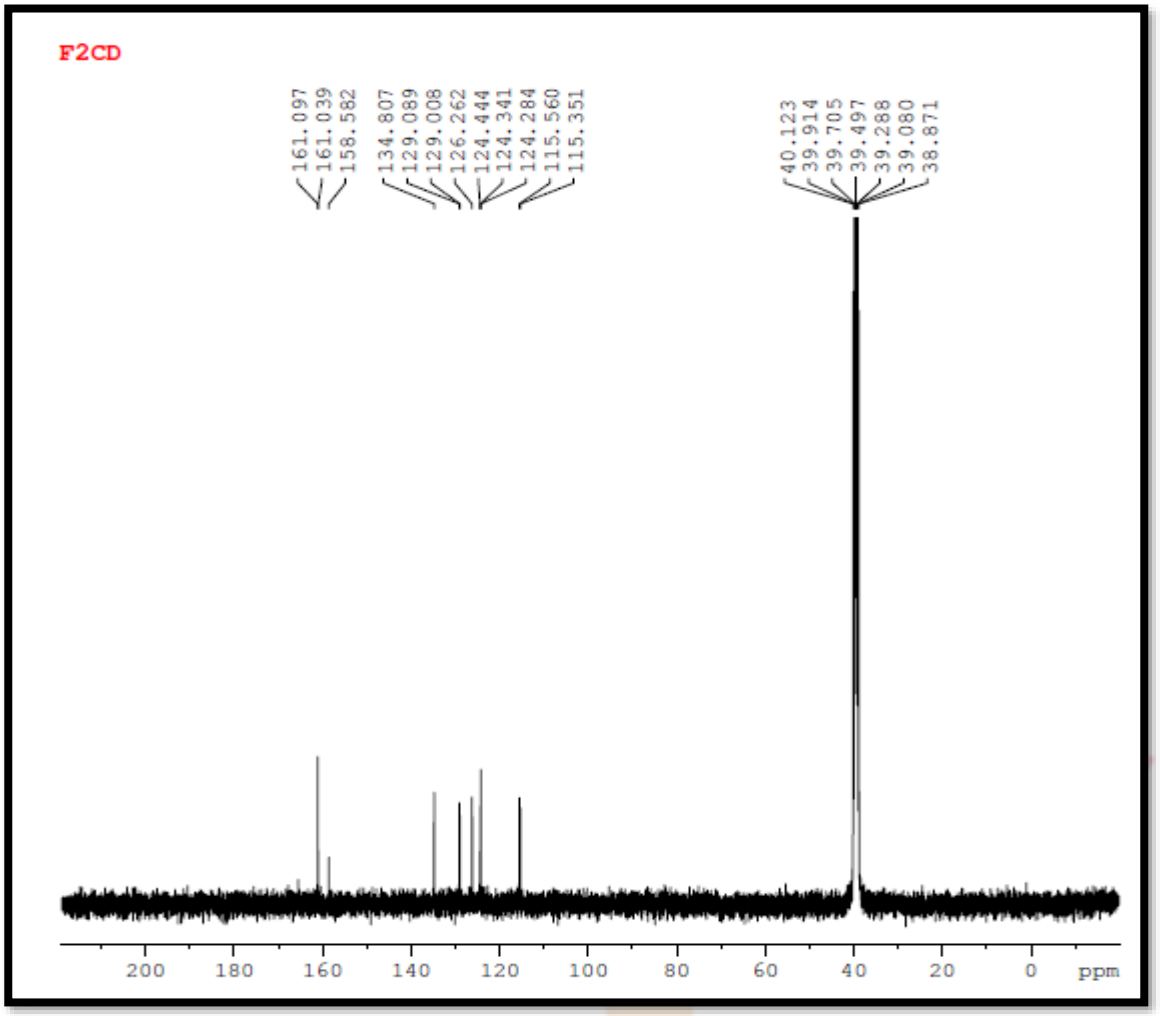


Figure No. 5.1.3.2. ^{13}C NMR of compound TVD7

Assignment	Carbon Type	Delta Value (ppm)
C1	>C-C	161.09
C2	>C-N	161.03
C3	F-C_6H_4 Aromatic Carbon	158.58
C4	C_6H_4 Aromatic Carbon	134.80
C5	C_6H_4 Aromatic Carbon	129.08
C6	C_6H_4 Aromatic Carbon	129.00
C7	C_6H_4 Aromatic Carbon	126.16
C8	C_6H_4 Aromatic Carbon	124.44

Table No. 5.1.2.1. ^{13}C NMR values of compound TVD7

Table No. 5.1.3.3. ^{13}C NMR values of compound TVD2

1H NMR of compound TVD7

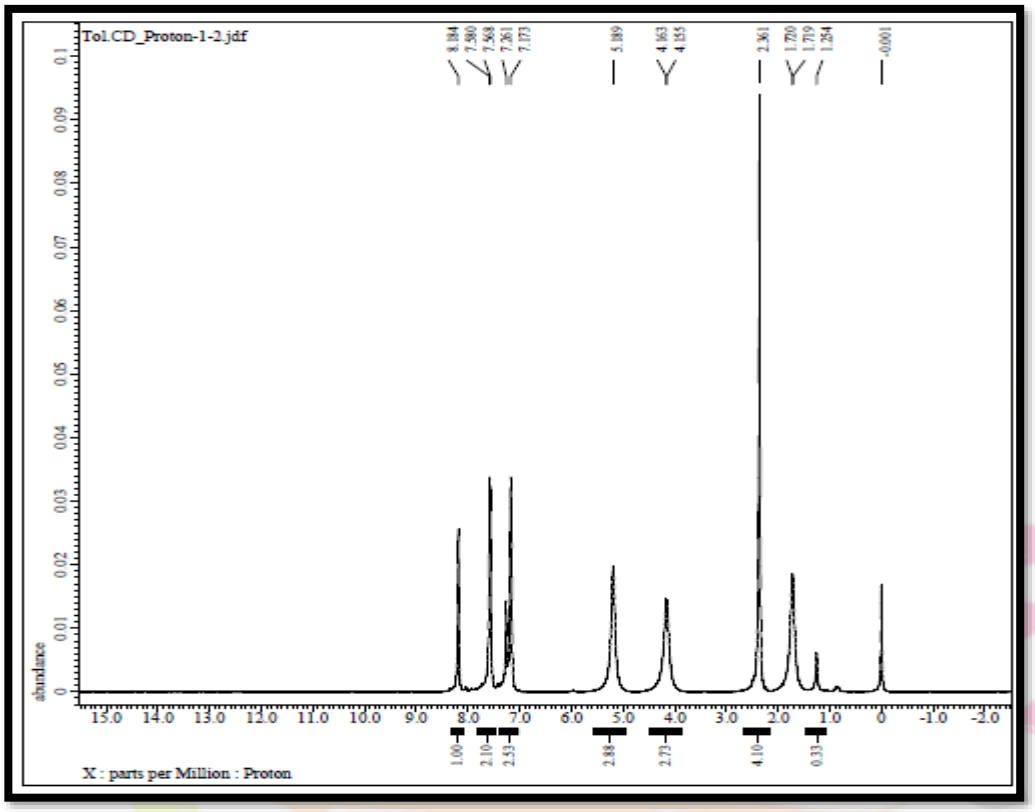


Figure No. 5.1.3.3. 1H NMR of compound TVD7

Group	Delta Value (ppm)	Number of protons	Multiplicity
-NH	2.461	1H	Singlet
-NH2	3.300	2H	Singlet
-Ar-H	8.078	1H	Doublet
-Ar-H	7.240	1H	Doublet
-Ar-H	5.955	2H	Multiplet

Table No. 5.1.3.4. 1H NMR values of compound TVD2

Mass Spectroscopy of compound TVD2:

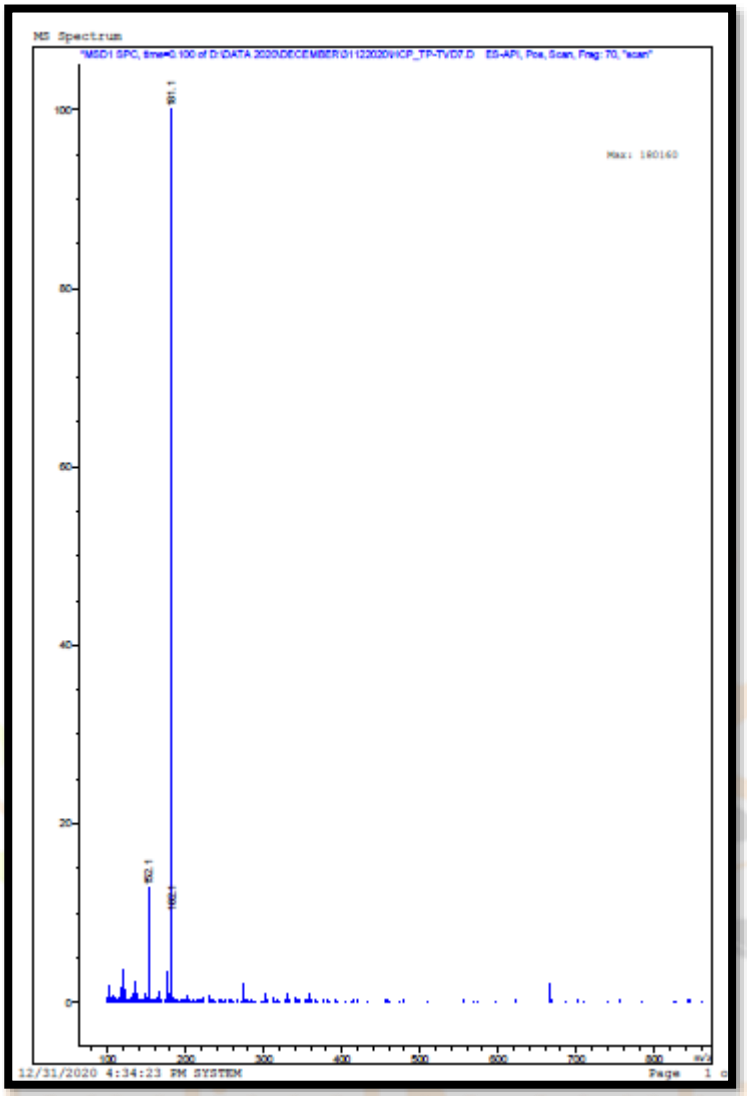


Figure No. 5.1.3.4. Mass of compound TVD7

Compound	m/z Value
TVD2	177.7

Table No. 5.1.3.5. Mass values of compound TVD2.

5.2 BIOLOGICAL TESTING OF THE SERIES OF COMPOUNDS:

The series of compounds synthesized were tested for their antitubercular activity against *MTSK*H37Rv using the MABA (MABA) method.

PROCEDURE:

1) The antimycobacterial activity of compounds was assessed against MTB using MABA.

2) This methodology is non-toxic, uses a thermally stable reagent, and shows good correlation with proportional and BACTEC radiometric methods.

3) Briefly, 200µl of sterile deionized water was added to all outer perimeter wells of sterile 96-well plate to minimize evaporation of medium in the test wells during incubation.

4) The 96-well plate received 100 µl of the Middlebrook 7H9 broth and serial dilution of compounds was made directly on the plate.

5) The final drug concentrations tested were 100 to 0.2 µg/ml.

6) Plates were covered and sealed with parafilm and incubated at 37ºC for five days.

7) 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.

8) A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth.

9) The MIC was defined as the lowest drug concentration which prevented the color change from blue to pink.

6. SYNTHESIS OF MOLECULES:

A series of molecules were synthesized by initially reacting substituted aromatic aldehydes with aminoguanidine bicarbonate to form a Schiff base as an intermediate. In step 2, the Schiff base was further cyclized to form 1,2,4-triazol-3-amine derivatives in the presence of FeCl3. **Figure 6.1** summarizes the general way of synthesizing molecules and outlines the work done.

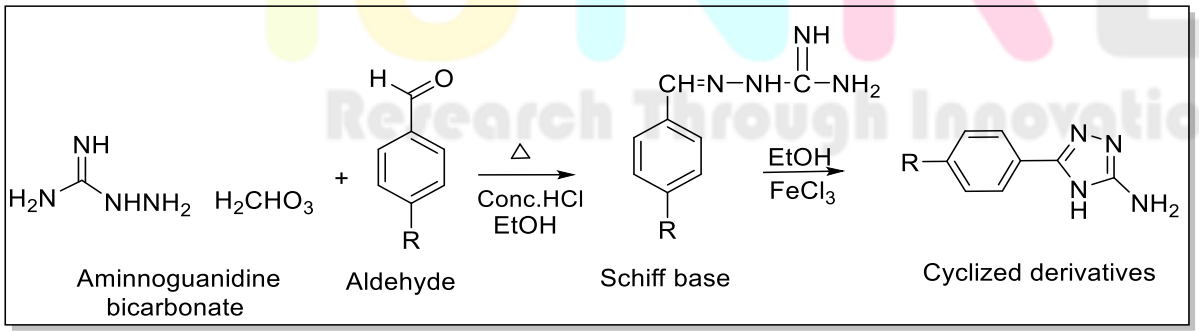


Figure No. 6.1. Scheme for synthesis cyclized derivatives.

6.1 Characterisation of the synthesized compounds:

The synthesized compounds were purified by recrystallization and characterized spectroscopically using IR, ^{1}H NMR, ^{13}C NMR, and mass spectroscopy.

IR spectra of the compounds show NH stretching vibration of primary amine in the range of 3400- 3350 cm-1. NH stretching vibration of secondary amine near 3200-3100 cm-1. C=N stretching vibration is observed in the 1570-1450 cm-1 region which disappeared in the Schiff base. C-N stretching vibration of 1, 2, 4-triazol-3-amine is in the range of 1650-1550 cm-1 which was not seen in Schiff bases. Aromatic C=C stretching vibration is observed in the 1150-1100 cm-1 region. Aromatic C-H stretching vibration is observed in 3300-3200 cm-1.

1H NMR spectra of Schiff bases show a singlet of -NH group of Aminoguanidine bicarbonate in the range of δ 11.5-11.1 and a singlet for secondary amine is observed in the range of δ 9.5-9.0, aromatic protons show multiplet in the range of singlet for CH group of Schiff base is observed in the range of Final 1,2,4-triazol-3-amine derivatives show singlet of -NH group is in the range of δ 5-6, NH2 group is observed in the range of δ 3-5. Ar-CH group peaks are observed in the range of δ 6.5-8.0. Ar-CH_3 group is observed in the range of δ 2.4-2.7 of 1, 2, 4-triazol-3-amine derivatives from Schiff bases.

13C NMR spectra of synthesized compounds are as follows. Chemical shift values of all the other aromatic carbons were observed at the expected regions. The numbering of the carbons and their corresponding values are shown in the following section.

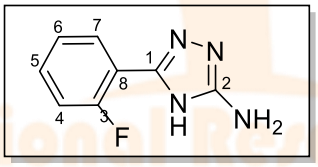


Figure No. 6.1.1. Structure of compound TVD2

Assignment	Carbon Type	Delta Value (ppm)
C1	>C-C	161.09
C2	>C-N	161.03
C3	F-C_6H_4 Aromatic Carbon	158.58
C4, C6	C_6H_4 Aromatic Carbon	134.80, 129.00
C5	C_6H_4 Aromatic Carbon	129.08
C7	C_6H_4 Aromatic Carbon	126.16
C8	C_6H_4 Aromatic Carbon	124.44

Table No. 6.1.1 13C NMR values of compounds TVD 2

Mass spectrometry of the synthesized compound was recorded and was found (M+1) as molecular ion peak.

Thus, from the available spectral data of compounds (See Experimental Section), it can be concluded that the compounds synthesized are substituted 1, 2, 4-triazol-3-amine derivatives according to the predicted structure.

Characterization of molecules confirms the structure of molecules and hence the mechanism of reaction of substituted 1, 2, 4-triazol-3-amine derivatives as shown in **Figure No.6.2.1.**

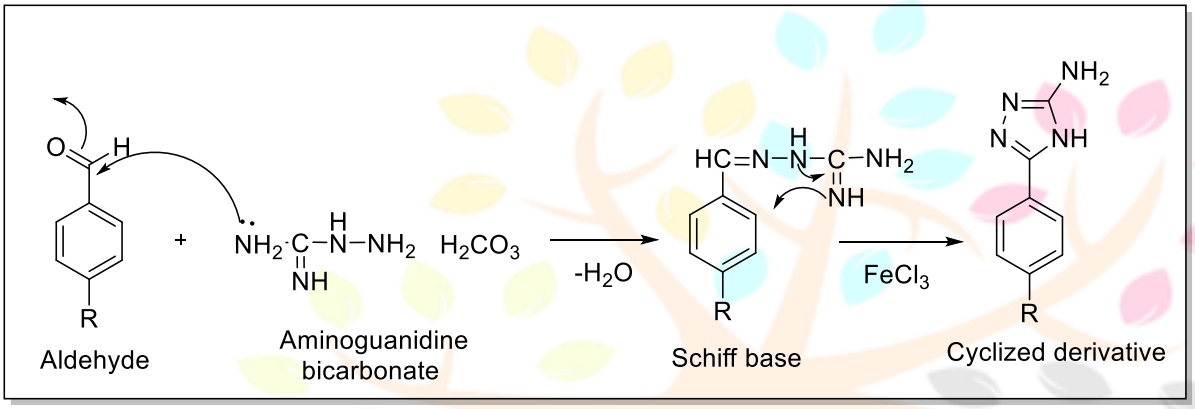


Figure No.6.2.1. Mechanism for the synthesis of 1, 2, 4-triazole-3-amine derivatives

In the first step, free aminoguanidine bicarbonate acts as a nucleophile and attacks the carbonyl group of aldehydes. The loss of water molecules results in the formation of a Schiff base.

In the next step, the free amino acid Schiff base acts as a nucleophile and attacks the carbonyl group of aldehydes. The loss of water molecules results in the formation of Schiff base cyclization in the presence of FeCl3, which results in the formation of 1,2,4-triazol-3-amine derivatives.

6.2 ***IN-SILICO* STUDIES:**

6.2.1 DRUG-LIKENESS PROPERTIES:

The medicinal properties of the synthesized compounds are compared with standard drugs based on their molecular weight, number of atoms, hydrogen bond acceptors, hydrogen bond donors, total polar surface and number of rotating bonds, log P and N violation parameters in Table No. 4.3.1.

Molecules (Compound Code)	Mol. wt. (Dal)	Total no. of Atom	nON	TPSA	nOHNH	No. of rotating bonds	Log P	N Violation	Volume
ISONIAZID	137.14	10	4	68.01	3	1	-0.97	0	122.56
RIFAMPICIN	822.95	59	16	220.16	6	5	2.62	3	755.91
TVD7	178.17	13	4	67.60	3	1	1.43	0	148.34

Table No. 4.3.1. Drug likeness score of the synthesized derivatives.

Based on the results obtained, all the compounds displayed drug-like characteristics based on Lipinski's rule of 5 that states if the compound, has certain pharmacological or biological activity, to make it an orally active drug in humans.

- The molecular weights of all Schiff bases and 1, 2, 4-triazol-3-amine derivatives were found to be less than 500 Daltons, and thus these molecules are predicted to be easily transported, diffused, and absorbed as compared to large molecules.
- The number of hydrogen bond acceptors (nON) (Oxygen and Nitrogen atoms) is not observed more than 10.
- The number of hydrogen bond donors (nOHNH) (nitrogen or oxygen atoms with one or more H atoms) was found to be not more than 5.
- The calculated log P values of all derivatives were below 5 which is an indication of good water solubility.
- The topological polar surface area (TPSA) is calculated from the number of oxygen and nitrogen atoms and by hydrogen atoms attached to them. Thus, the TPSA mimics the hydrogen bonding characteristic of a compound. TPSA was found to be not more than 140 Å for all derivatives indicating good intestinal absorption.
- A less than 10 No. of rotating bonds were observed.
- Zero N violations were observed for all derivatives.

Hence all molecules obey Lipinski's rule of five and therefore these molecules are anticipated to be easily transported, diffused, and absorbed and are likely to be orally bioavailable.

6.2.2 **PREDICTION OF ADMET PROPERTIES:**

The biological permeability of synthesized compounds is compared with standard drugs based on blood-brain barrier (BBB), human intestinal absorption (HIA), P-glycoprotein substrate, acute oral toxicity, carcinogenicity, rat acute toxicity, and AMES toxicity in **Table No. 4.3.2.**

Molecules	**HIA**	**BBB**	**P-glycoprotein Substrate /inhibition**	**AMES Toxicity**	**Carcinogenicity**	**Acute Oral Toxicity**	**LD50 in Rats**
ISONIAZID	**1.000**	**0.9961**	**Non-substrate/ Non-inhibitor**	**Toxic**	**Carcinogenic**	**0.8032**	**1.167**
RIFAMPICIN	**0.8597**	**0.9738**	**Substrate/ inhibitor**	**Toxic**	**Non-Carcinogenic**	**0.7763**	**3.21**
TVD7	1.0000	0.9492	Non-substrate/ Non- inhibitor	Non-Toxic	Non-Carcinogenic	0.5574	2.8492

Table No. 6.2.2.1. Predicted biological permeability of synthesized derivatives

- ADMET properties as derived from the admet SAR server show that the molecules had a better human intestinal absorption (HIA) score than the control molecules, Greater HIA indicates that the compound could be better absorbed from the intestinal tract after oral administration.
- Penetration of molecules through the blood-brain barrier (BBB) showed good results.
- Efflux prediction of the P-glycoprotein (P-gp) molecule was shown to be a non-substrate and non-inhibitory P-gp similar to the control molecule.
- The carcinogenic profile also revealed that all ligands were non-carcinogenic similar to the control molecule RIF. However, the control molecule INH was found to be carcinogenic.
- The molecule showed lower acute oral toxicity than the control.
- The important information obtained was the calculated dose LD50 in the rat model. When comparing LD50 doses, the compound with the lower dose is more lethal than the compound with the higher LD50. From our study, we found that the molecule had a higher LD50 compared to the control.

6.3.PREDICTION OF BIOACTIVITY SCORE:

The bioactivity scores of the synthesized derivatives are compared with standard drugs based on GPCR ligand, ion channel modulator, nuclear receptor ligand, kinase inhibitor, protease inhibitor, and enzyme inhibitor in **Table No. 6.3.1.**

Molecules	GPCR Ligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor	Ligand protease inhibitor	Enzyme inhibitor
ISONIAZID	-1.39	-1.45	-1.05	-2.33	-1.23	-0.66
RIFAMPICIN	-2.10	-3.27	-3.04	-2.89	-1.61	-2.42
TVD7	-0.72	-0.22	-0.48	-1.95	-1.19	-0.44

Table No. 6.3.1. Bioactivity score of the synthesized compounds

- A molecule with a biological activity score greater than 0.00 is most likely to exhibit significant biological activities, while values between −0.50 and 0.00 are expected to be moderately active, and if the score is less than −0.50, it is predicted that it is inactive.
- The results show that the physiological action of Schiff bases and 1,2,4-triazol-3-amine derivatives involves a mechanism of inhibiting some enzymes, which confirms our design principle of Shikimate kinase inhibition.
- Compounds' bioactivity scores suggest moderate interaction with the target drug.
- Compounds showed promising biological activity scores. The compounds showed better biological activity scores compared to the standards used for the study.

6.4.BIOLOGICAL TESTING OF THE SERIES OF COMPOUNDS:

The synthesized compounds in the present study were tested for their antitubercular activity against *M. tuberculosis* H37Rv by microplate alamar blue assay (MABA) method.

Compound	MIC values (µg/ml)
INH	6.25
RIF	1.6
TVD2	50

Table No. 6.4.1. MIC values of synthesized and standard compounds

- The results of *in vitro* antitubercular activity show that the synthesized 1,2,4-triazol-3-amine series is effective against *M. tuberculosis* at 12.5 µg/ml Isoniazid.

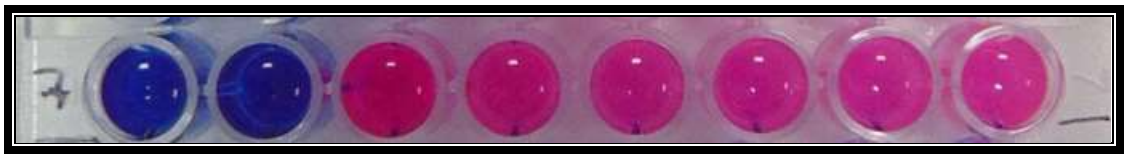


Figure No. 6.4.1. (a) Anti-TB activity of synthesized compounds using Alamar Blue Dye

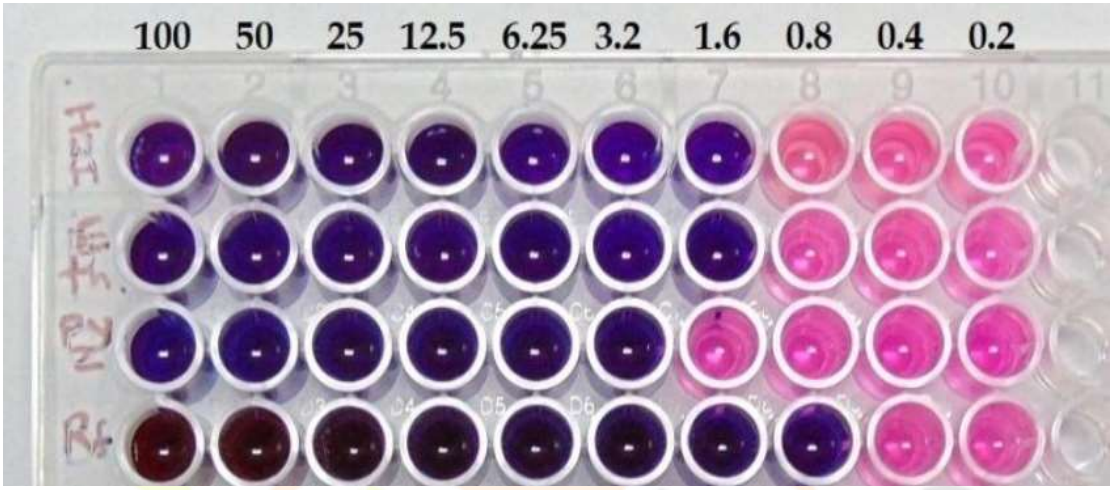


Figure No. 6.4.1. (b) Anti-TB activity of standard compounds using Alamar Blue Dye

(Isoniazid, Ethambutol, Pyrazinamide, Rifampicin)

Result and discussion:

Bacterial resistance to current antimicrobial agents and treatment failure have necessitated the discovery of new molecules and a more targeted, target-specific approach to the development of new therapeutics. A literature review shows that structure-based virtual screening protocols have been used to predict Mycobacterium tuberculosis shikimate kinase (MtSK) inhibitors. To synthesize and evaluate new compounds active against TB, this project planned to synthesize and evaluate heterocyclic derivatives as potential antitubercular agents.

This chapter contains a detailed overview of the results of studies of heterocyclic derivatives, a discussion of synthesis and characterization of molecules, in-silico studies that include ADMET properties, drug-like properties, and bioactivity score prediction, results of in vitro testing against M tuberculosis H37RV.

CONCLUSION

In vitro antitubercular activity results show that the synthesized series of 1,2,4-triazol-3-amines is effective against M. tuberculosis at 12.5 µg/ml of isoniazid. Thus, it can be concluded that the synthesized series of 1,2,4-triazol-3-amines is not very active compared to standard drugs and previously synthesized molecules in the laboratory with an S in the ring or an SH group on the ring. This means that it is important for Shikimate kinase inhibition activity. Therefore, future work in the laboratory will focus on modifying these molecules to improve their antitubercular activity.

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