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DESIGN, SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF NOVEL ANTI-TUBERCULAR AGENTS TARGETING GLUTAMINE SYNTHETASE-1 AND CYCLOPROPANEMYCOLIC ACID SYNTHASE-2

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Keywords:

Tuberculosis, Glutamine synthetase-1, Thiophene, Gewald reaction, Aminothiophenes, MABA

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ABSTRACT: The World Health Organization 2015 (WHO) reports indicate that there were 9.0 million new tuberculosis (TB) cases and 1.5 million tuberculosis (TB) deaths during 2015. Co-infection with the HIV fuels the global TB crisis, and successful TB treatment is further complicated and hampered by the existence of multidrug- resistant (MDR) TB and extensively drug resistant (XDR) TB. Hence there is an urgent need for newer anti tubercular agents. Heterocyclic moieties possess profound biological activities including anti inflammatory, antitumour, antitubercular activity. The Benzothiazole nucleous and the thiophene nucleous exhibit profound pharmacological activity and so series of 2-amino thiophene derivatives were designed and docked against the enzyme glutamine synthetase-1 (PDB-ID- 4ACF) which plays an important role in the metabolism of nitrogen. Similarly, a series of Schiff bases with benzothiazole scaffolds were designed and docked against cyclopropanemycolic acid synthase-2 (PDB-ID-1KPI), the enzyme specific for synthesis of cell wall of Mycobacterium tuberculosis. Based on the docking score, interactions and docking pose molecules were screened and the selected compounds were synthesized. The synthesized compounds were purified by re-crystallization and characterized by spectral analysis (FT-IR, NMR, and Mass Spectroscopy). Anti-tubercular activity was evaluated by MABA. The present study concludes that the synthesized compounds (MMF, TM, CM and MC) show promising activity at 1.6 mcg/ml, 3.12 mcg/ml and 6.25mcg/ml against Mycobacterium tuberculosis when compared to the standard drugs like Pyrazinamide and Streptomycin.

INTRODUCTION: Tuberculosis is a major health concern [1]. There is a surge in the number of patients being infected with TB, is due to infection with human immunodeficiency virus, bacterial resistance to medications, increased international travel and immigration from countries with high incidence, Vulnerability of hospitalized patients and drug abusers are some of the reasons for the high prevalence of tuberculosis [2].

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The long duration of treatment is a reason for noncompliance which leads to the emergence of Multi Drug Resistant (MDR) and Extremely Drug Resistant (XDR) strains of TB. Therefore we have an emergency situation to introduce not only new TB drugs but also new regimens that will be effective and which will also reduce the duration of therapy.

Glutamine synthetase (GS) is an enzyme that plays an essential role in the metabolism of nitrogen by catalyzing the condensation of glutamate and ammonia to form glutamine Glutamine synthetase (GS; EC 6.3.1.2, also known as γ-glutamyl: ammonia Ligase) catalyzes the adenosine 5´-triphosphate (ATP) dependent condensation

between glutamate and ammonia, to give glutamine. There are three types of GS, each with a different structure. These enzymes are present in all organisms; eukaryotes express GS type II while prokaryotes mainly express GS types I and III. All GS enzymes have an amino-acid binding site as well as a nucleotide-binding site.

They also have three metal ions in the active site between the two pockets, which are necessary for stability and catalytic activity. The amino-acid-binding site that is fairly well preserved amongst species, but the nucleotide-binding site differs between mammalian and bacterial enzymes [3]. Cyclopropanemycolic acid synthase enzyme is a part of FAS II pathway for the biosynthesis of mycolic acid in mycobacteria and this enzyme acts on a long acyl chain, which is linked to the acyl carrier protein (AcpM).

Recent studies show that the cyclopropane synthases of *M. tuberculosis* is consider as a novel class of persistent genes and the need for new inhibitors for the persistent phase of tuberculosis infection. Thus cycloprpane synthase is an attractive target for new anti-tb drug development.

Thus the main focus of this research work is to design and synthesize new molecules which inhibit target enzymes Glutamine synthetase and Cyclopropanemycolic acid synthase of mtb.

A series of thiophene derivatives were designed and docked against glutamine synthase-1. Thiophene nucleus comprises of a five membered ring containing a sulphur atom. Gewald reaction involves the reaction between α-methyl ketones with elemental sulphur in the presence of malononitrile and diethylamine which yields 2-amino thiophenes [4 - 9]. By using various reagents such as glacial acetic acid, formic acid or carbondisulphide, the pyrimidine derivatives are formed by cyclization such as Thiophene derivatives [10 - 19].

Schiff bases are reported for a variety of biological activities including anticonvulsant, insecticidal and herbicidal activities, antibacterial, anti inflammatory, antitumor, antitubercular, antifungal, antiHIV, analgesics. A series of Schiff bases containing benzothiazole moiety were designed, and docked against specific anti tubercular enzyme. Cyclopropanemycolic acid synthase - 2.

MATERIALS AND METHODS:

Drug Design: Drug design predicts a binding interaction between a small molecule ligand and an enzyme protein that results in activation or inhibition of the enzyme. About 200 molecules were designed and docked against specific mtb enzyme. Docking of ligands was carried out by Argus lab® docking software 4.0 and Molegro® molecular viewer is an application which helps in analyzing the energies and interaction of the binding sites.

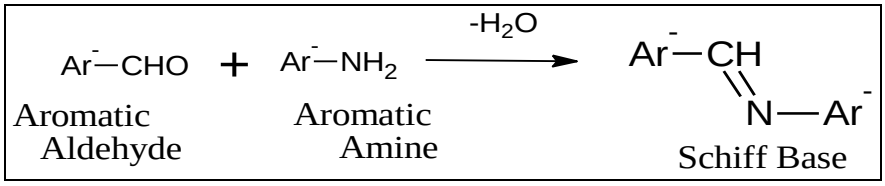
Toxicity Prediction: All the molecules were subjected to the toxicity risk assessment by using OSIRIS® property Explorer. Prediction results are color coded. Red color indicates presence of toxicity and green color indicates absence of toxicity for that particular compound.

Experimental Procedure:

Scheme 1: Equimolar quantity of aromatic amine and substituted aromatic aldehydes was refluxed for 10 - 15 hours in 20 ml of ethanol. Completion of the reaction was monitored by TLC. After the completion of the reaction, the mixture was poured into a beaker containing ice cold water. The precipitates formed was filtered out and dried. Finally purified by recrystallization by using ethanol.

Aromatic Aldehyde used: 2, 4- Dichloro Benzaldehyde and Benzyloxy Benzaldehyde.

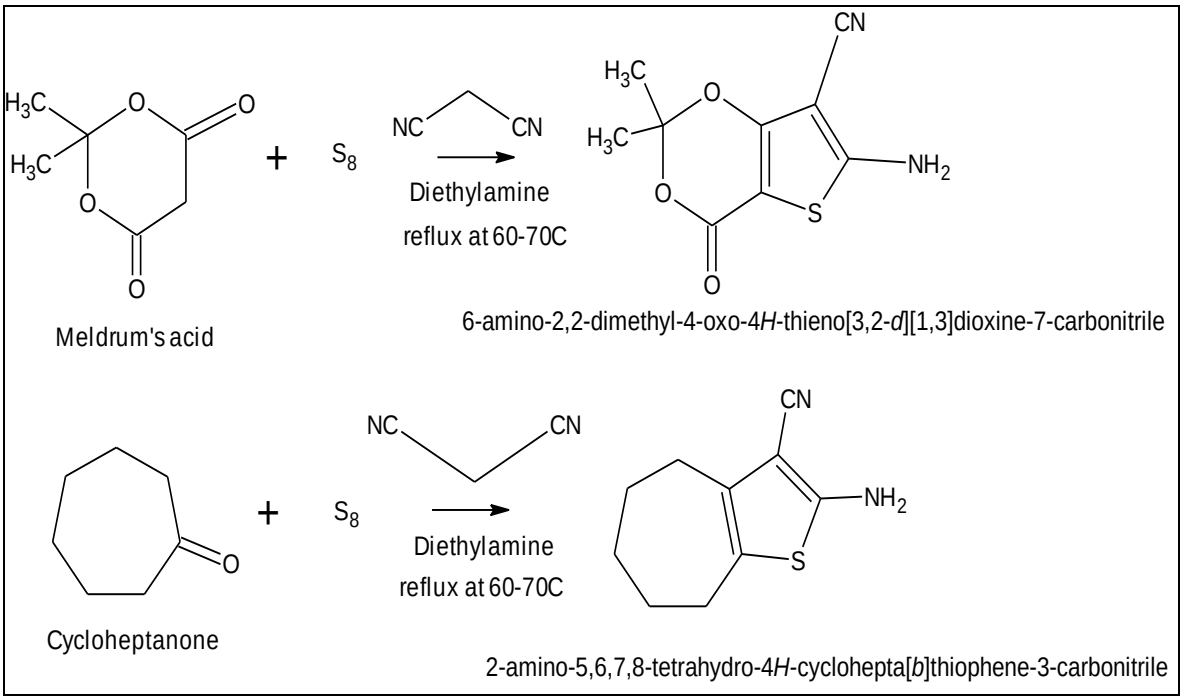
Aromatic Amines used: 2-Amino Benzothiazole and 2-Amino 4-Methyl Pyridine.



Scheme 2: In the synthetic methodology, novel compounds were synthesized as per Gewald reaction. Alpha methyl ketone (like Meldrum's acid or Cycloheptanone (Cyclic ketone), and malononitrile is dissolved in ethanol which is used as a solvent. Diethylamine was used as the catalyst in this reaction. The reaction mixture was stirred at room temperature for 18 hrs. Completion of the reaction was confirmed by TLC and detected by UV chamber.

To the reaction mixture the remaining quantity of diethylamine and crushed elemental sulphur was added and refluxed with stirring until sulphur was completely dissolved and the temperature was maintained at 60 - 80 ºC (over 5 - 6 hrs). The mixture was again stirred at room temperature for 24 hrs. The completion of the reaction was again confirmed by TLC and the reaction mixture poured into crushed ice. Finally the product was obtained which was recrystallized by using various organic solvents like ethanol, ethyl acetate, *etc*.

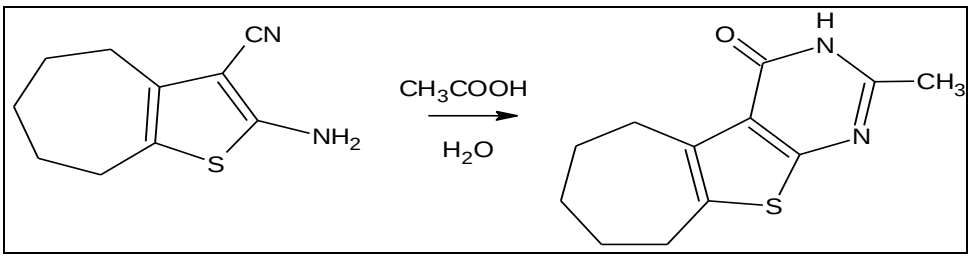
Compounds CM and MM:



Synthetic Derivatives of CM and MM:
Sample Code: CMG

2-methyl-4a,5,6,7,8,9- hexahydro- 4H-cyclohepta [4,5]thieno[2,3-d]pyrimidine: To compound MM, 30 ml of glacial acetic acid (used as a solvent) was added, and the temperature was maintained at 80 ºC for 12 hrs when 2 methyl pyrimidine analogue is formed.

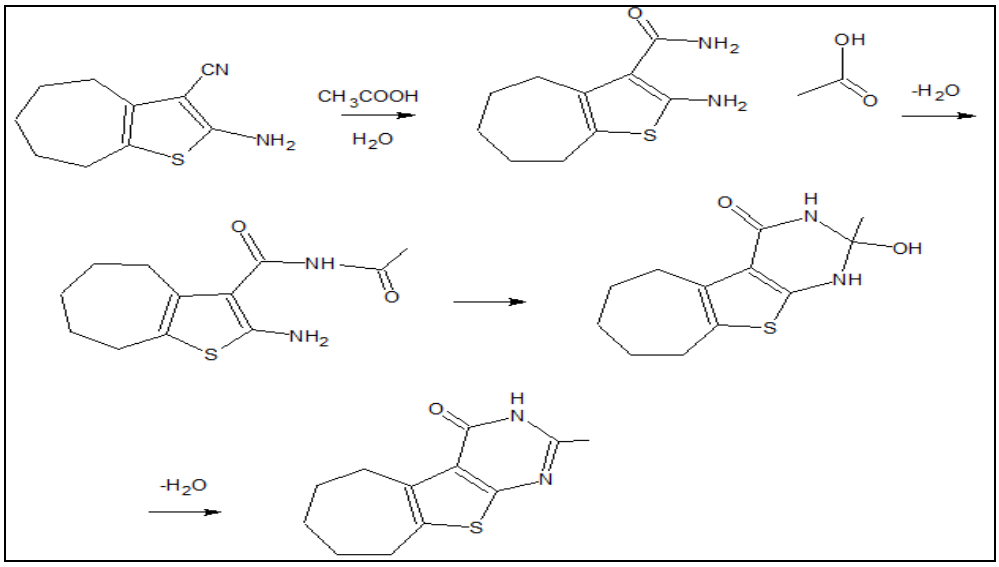
Reaction:



MM

2-methyl-4a, 5, 6, 7, 8, 9-hexahydro-4H-cyclohepta [4, 5]thieno[2,3d]pyrimidine

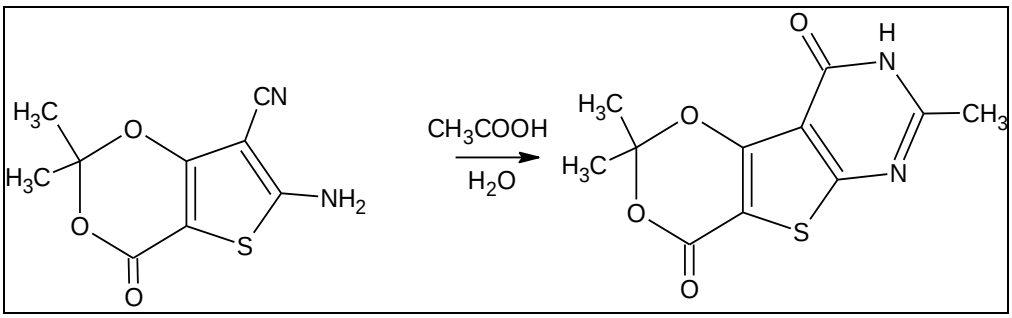
Mechanism:



Sample Code: MMG

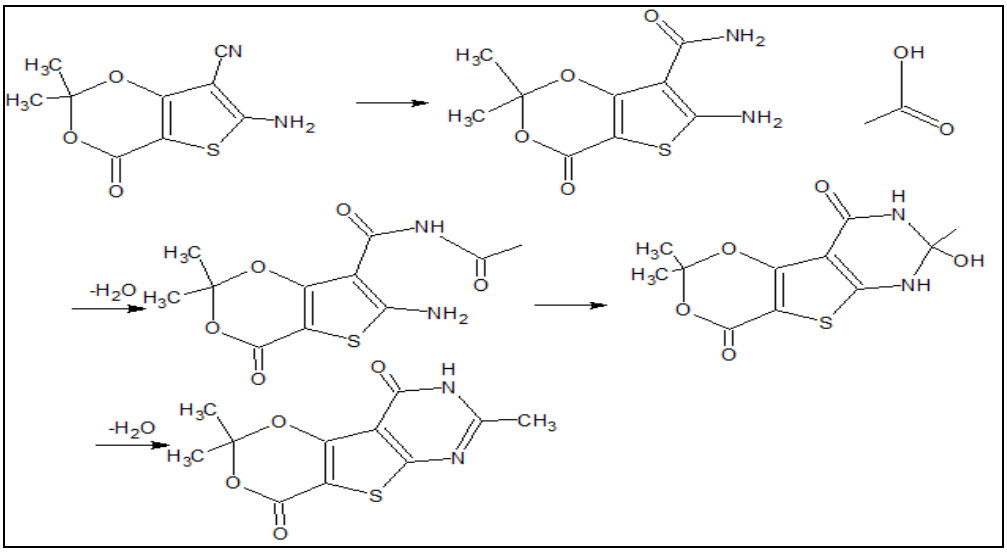
2,2,7- trimethyl- 9,9a-dihydro- 4H- [1,3] dioxino [4'5':4,5]thieno[2,3-d]pyrimidin-4-one: Thirty ml of glacial acetic acid was added to compound CM and the temperature maintained at 80 ºC for 12 hrs. The 2 methyl pyrimidine analogue was formed.

Reaction:



MM **2,2,7-trimethyl-9,9a-dihydro-4H-[1,3] dioxino[4',5':4,5]thieno[2,3-d]pyrimidin-4-one**

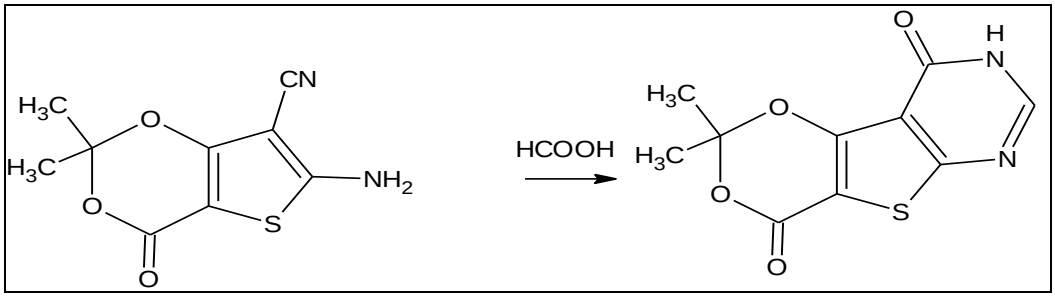
Mechanism:



Sample Code: MMF

2,2,7- trimethyl-9, 9a-dihydro-4H- [1,3] dioxino [4',5':4,5]thieno[2,3-d]pyrimidin-4-one: Twenty ml of formic acid and 3 drops of concentrated sulphuric acid was added to compound CM and the temperature maintained at 80 ºC for 12 hrs. The pyrimidine analogue was formed.

Reaction:

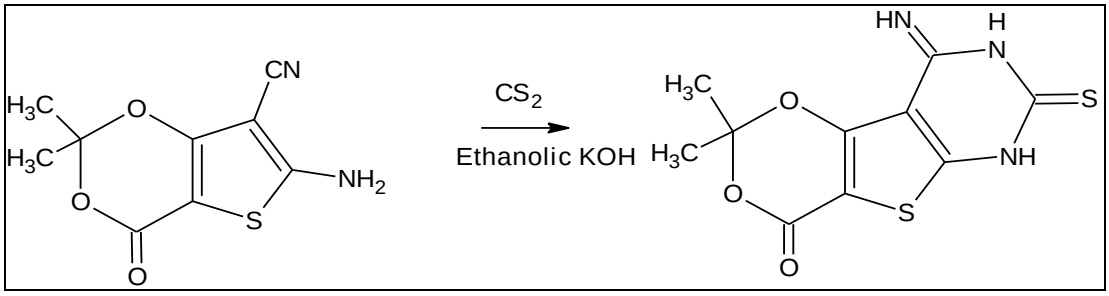


CM **2,2-dimethyl-9,9a-dihydro-4H-[1,3] Dioxino[4',5':4,5]thieno[2,3-d]pyrimidin-4-one**

Sample Code: MC/MMC

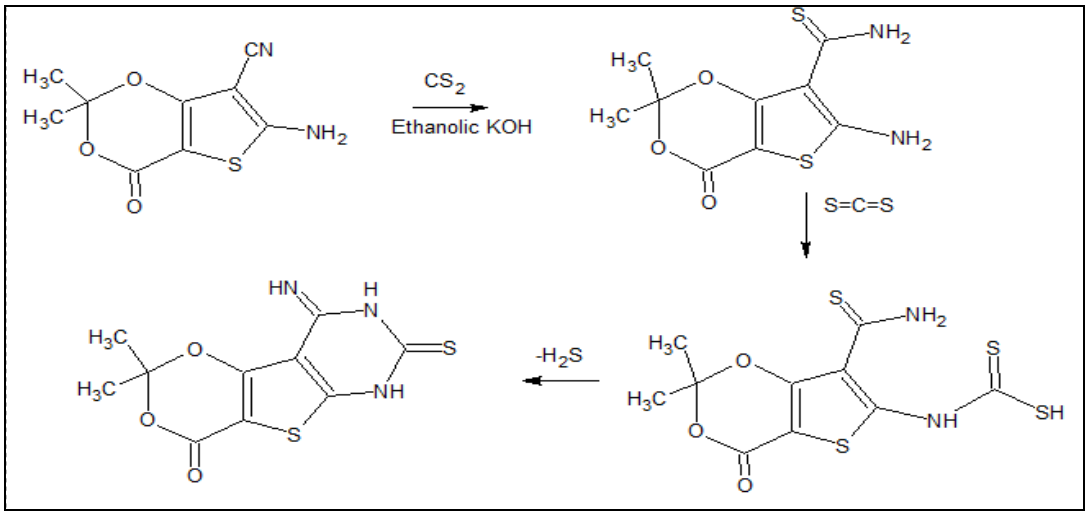
9-imino-2,2-dimethyl-7-thioxo-6,9-4H,7H-[1,3]dioxino[4'5':4,5]thieno[2,3-d][1,3]thiazin-4-one: To compound CM, 10 ml of carbon-disulphide and ethanolic potassium hydroxide as a solvent was added and refluxed for 12 hrs and temperature maintained at 80 ºC.

Reaction:



CM **9-imino-2,2-dimethyl-7-thioxo-6,9dihydro-4H,7H-[1,3]dioxino[4',5':4,5]thieno[2,3-d][1,3]thiazin-4-one**

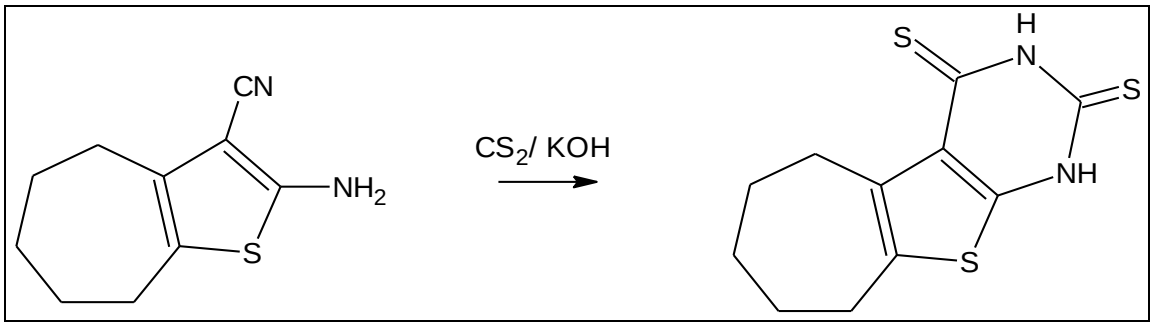
Mechanism:



Sample Code: CC/TM

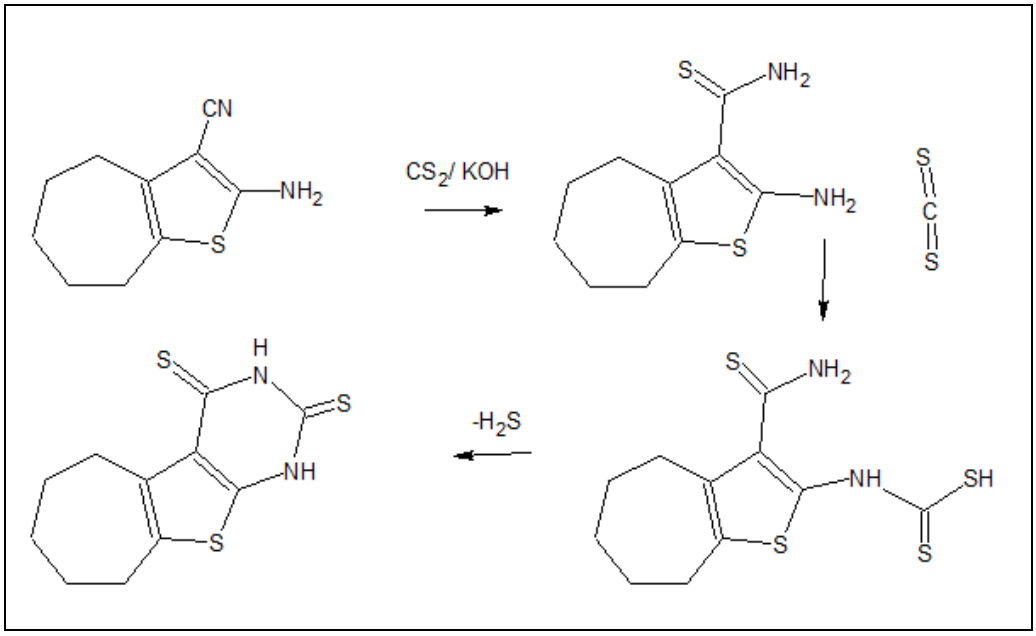
4-imino-1,5,6,7,8,9-hexahydro-2H,4H-cyclohepta [4, 5]thieno[2, 3-d] [1, 3] thiazine- 2- thione: To compound MM, 10 ml of carbon-disulphide and ethanolic potassium hydroxide as a solvent was added and refluxed for 12 hrs, at 80 ºC.

Reaction:



MM **4-imino-1,5,6,7,8,9-hexahydro-2H,4H-cyclohepta[4,5]thieno[2,3-d][1,3]thiazine-2-thione**

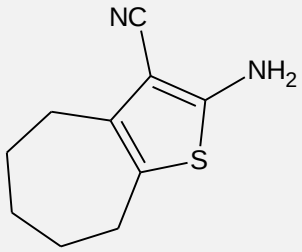
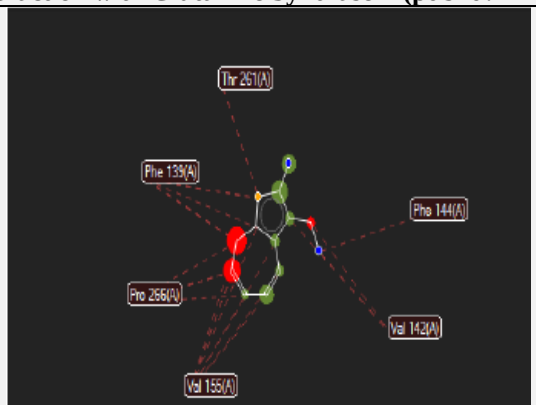
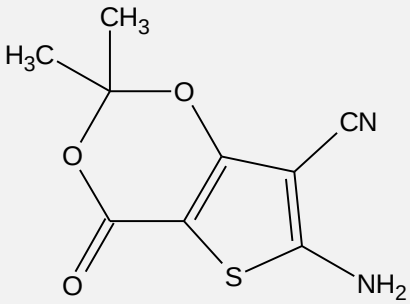
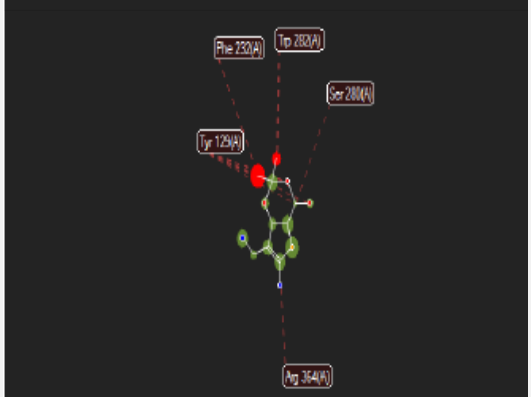
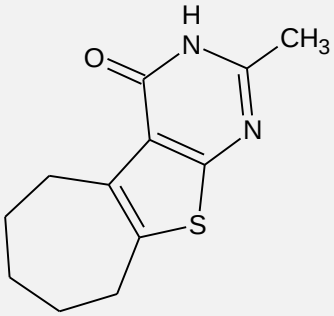
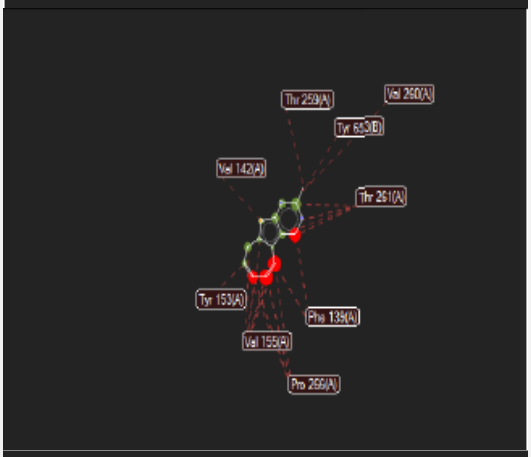
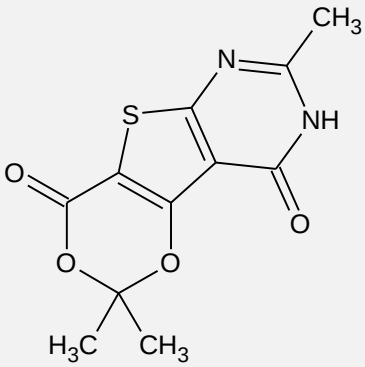
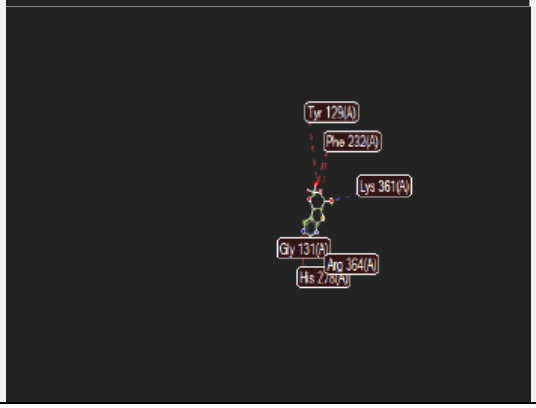
Mechanism:



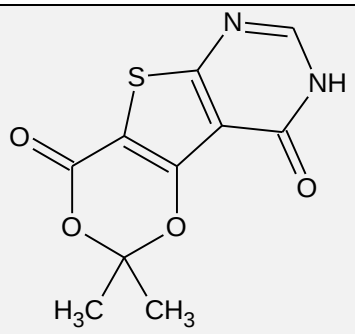
Assay Procedure for Estimating Anti-Tb Activity Using Alamar Blue Dye: The anti-mycobacterial activity of the synthesised compounds was assessed against M. tuberculosis using Alamar Blue micro plate assay (MABA). This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with proportional and BACTEC radiometric method. Briefly, 200 micro litter of sterile de-ionized water is added to all outer perimeter wells of sterile 96 wells plate to minimize evaporation of medium in the test wells during incubation. The 96 wells plate receives 100 micro litter of the middle brook 7H9 broth and serial dilutions of compounds are made directly on plate. The final drug concentrations tested are 100 to 0.2 micro gram/ml. Plates are covered and sealed with paraffin and incubated at 37 ºC for five days. Further, 25 micro litters of freshly prepared 1:1 mixture of Alamar blue reagent and 10% tween 80 is added to the plate and incubated for 24 hrs. A blue colour in the well is interpreted as no bacterial growth, and pink colour was scored as growth. The MIC is defined as lowest drug concentration which prevents the colour change from blue to pink [20].

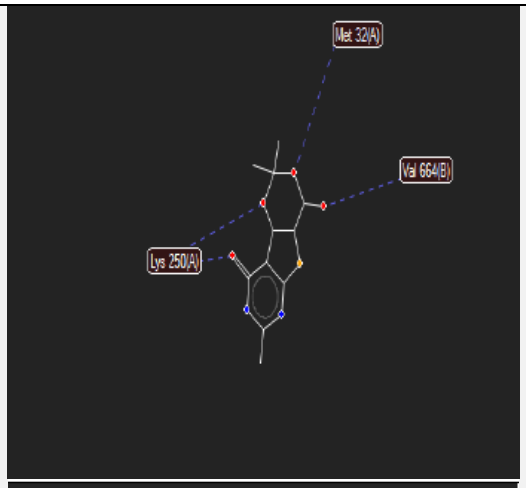
RESULTS AND DISCUSSIONS:

TABLE 1: LIGAND INTERACTION WITH GLUTAMINE SYNTHETASE-1(pdb id: 4ACF)

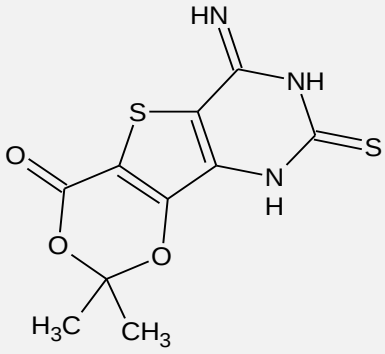
Sample Code	Docking View	Interaction with Glutamne Synthase -1(pdb id: 4ACF)
MM		
CM		
CMG		
MMG		

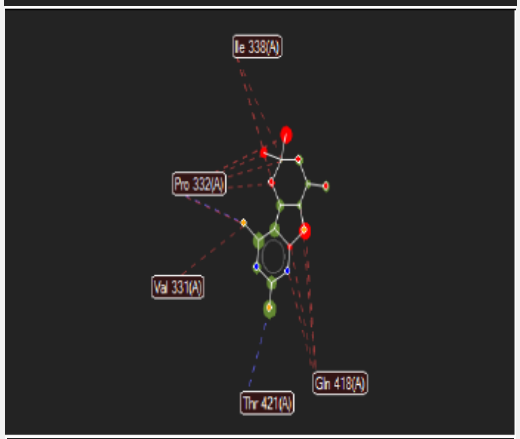
MMF



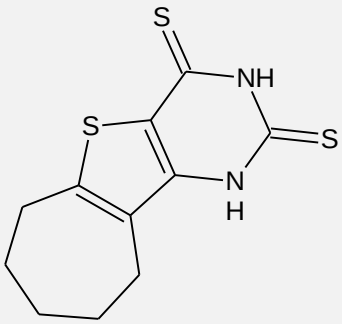


MMC/MC





CC/TM



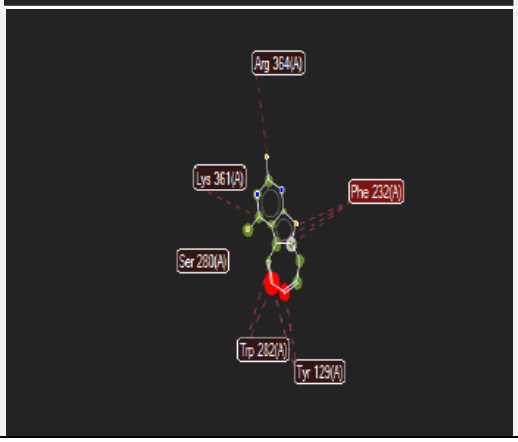
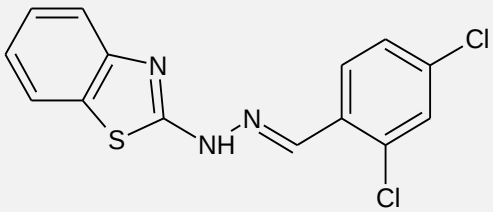
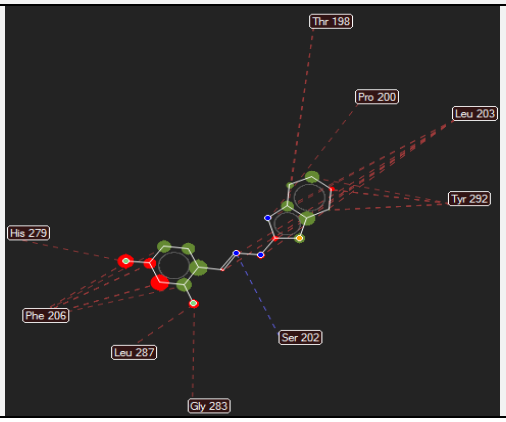
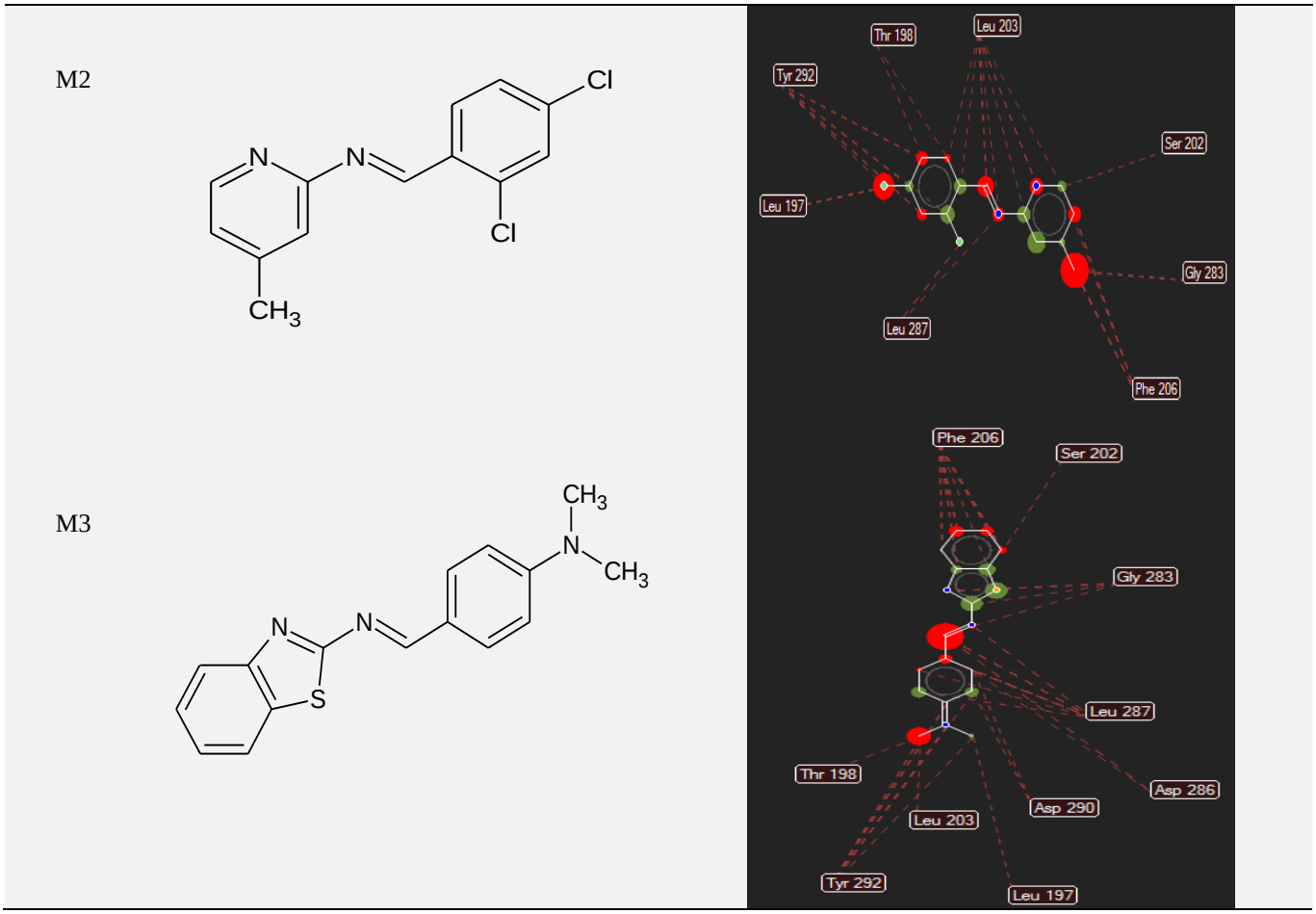


TABLE 2: LIGANDS INTERACTION WITH 1KPI

S. no.	Interaction with amino acids
M1 	



Spectral Studies:

1. Compound: CM

6-amino-2, 2-dimethyl-4-oxo-4H-thieno[3,2-d][1, 3]dioxine-7-carbonitrile: IR- 1645.13 cm^{-1} (C=O str), 1627.80 cm^{-1} (C=C Str), NMR: δ (3.01 ppm, singlet, 6H), 6.78 ppm, doublet, 2H), (7.25 ppm, multiplet, 1H), (7.45 ppm, singlet, 1H), (7.62 ppm, doublet, 2H), (7.75 ppm, singlet, 1H), (7.85 ppm, multiplet, 1H), (8.05 ppm, multiplet, 1H), MASS: 224.23(M^+).

1)

2. Compound: MM

2-amino-5, 6, 7, 8-tetrahydro-4H-cyclohepta [b] thiophene-3-carbonitrile: IR- 1644.53 cm^{-1} (C=C Str), 1188.60 cm^{-1} (-C-C- Str), NMR: δ (7.65 ppm, singlet, 1H), (7.30 ppm, multiplet, 1H), (7.71-8.07 ppm, multiplet, 5H), (8.11 ppm, singlet, 1H), (8.15 ppm, triplet, 1H), MASS: 192.13(M^+).

2)

3. Compound: CMG

2-methyl-4a,5,6,7,8,9-hexahydro-4H-cyclohepta [4,5]thieno[2,3-d]pyrimidine: IR: 1600.73 cm^{-1} (C=C Str), 2348.43 cm^{-1} (C=N Str), 1082.11 cm^{-1} (-C-C- Str), NMR: δ (1.05 ppm, singlet, 2H), (1.25 ppm, doublet, 2H), (1.38 ppm, multiplet, 5H), (1.41 ppm, singlet, 1H), (2.01 ppm, singlet, 1H), (2.23 ppm, doublet, 1H), MASS: 220.33 M+

4. Compound: MMG

2,2,7-trimethyl- 9, 9a- dihydro-4H- [1,3]dioxino [4',5':4,5] thieno [2,3-d] pyrimidin-4-one: IR: 2347.47 cm^{-1} (C=N Str), 1639.54 cm^{-1} (C=O Str), 1600.37 cm^{-1} (C=C Str), NMR: δ (3.01 ppm, singlet, 6H), 6.78ppm, doublet, 2H), (7.25 ppm, multiplet, 1H), (7.45 ppm, singlet, 1H), (7.62 ppm, doublet, 2H), (7.75 ppm, singlet, 1H), (7.85 ppm, multiplet, 1H), (8.05 ppm, multiplet, 1H), MASS: 266.27(M^+)

5. Compound: MMF

2,2-dimethyl-9,9a-dihydro-4H-[1,3]dioxino[4',5': 4,5] thieno [2, 3-d]pyrimidin-4-one: IR: 2347.47 cm^{-1} (C=N Str), 1639.54 cm^{-1} (C=O Str), 1600.37 cm^{-1} (C=C Str), NMR: δ (0.92 ppm, triplet, 1H), (1.43ppm, multiplet, 4H), (1.61 ppm, doublet, 1H),

(2.34ppm, singlet, 1H), (2.66 ppm, singlet, 1H), (2.72ppm, multiplet, 1H), (8.09 ppm, multiplet, 1H), MASS: 238.26(M^+).

6. Compound: MC/MMC

9-imino-2, 2-dimethyl-7-thioxo-6, 9-4H,7H-[1, 3] dioxino[4 5 :4,5]thieno [2,3d][1,3] thiazin-4-one: IR: 1633.43 cm^{-1} (C=C Str), 1688.47 cm^{-1} (C=O Str), NMR: δ (3.01 ppm, singlet, 6H), 6.78 ppm, doublet, 2H), (7.25 ppm, multiplet, 1H), (7.45 ppm, singlet, 1H), (7.62 ppm, doublet, 2H), (7.75 ppm, singlet, 1H), (7.85 ppm, multiplet, 1H), (8.05 ppm, multiplet, 1H), MASS: 300.38(M^+).

7. Compound: CC/TM

4-imino-1,5,6,7,8,9-hexahydro-2H,4H-cyclohepta [4,5] thieno [2,3-d][1,3] thiazine-2-thione: IR: 1633.43 cm^{-1} (C=C Str), 1688.47 cm^{-1} (C=O Str), NMR: δ (3.01 ppm, singlet, 6H), 6.78 ppm, doublet, 2H), (7.25 ppm, multiplet, 1H), (7.45 ppm, singlet, 1H), (7.62 ppm, doublet, 2H), (7.75 ppm, singlet, 1H), (7.85 ppm, multiplet, 1H), (8.05 ppm, multiplet, 1H), MASS: 270.44 (M^+)

8. Compound: M1

2- {(2E)- 2- [4- (benzyloxy) benzylidene] hydrazinyl}-1, 3-benzothiazole: FT-IR (cm^{-1}): 3046.50 (Ar-CH), 1584.59 (C=N), 2934.82 (Aliphatic CH), 3152.78 (NH) ^{1}H NMR (δ ppm): 7.0-8.0 (13H, Ar-H), 3.5 (1H, NH), 5.0 (2H, CH_2), 8.1(1H, N=CH), LC-MS (g/mol): Calculated mass = 359.4441, Actual mass = 360.1167.

9. Compound: M2

2- [(2E)- 2- (2, 4- dichloro-benzylidene) hydrazinyl]-1, 3-benzothiazole: FT-IR (cm^{-1}): 3064.06 (Ar-CH), 1600.99 (C=N), 2927.10 (Aliphatic CH), 3170.15 (NH), ^{1}H NMR (δ ppm): 7.0-8.0 (13H, Ar-H), 3.5(1H, NH), 5.0 (2H, CH_2), 8.1 (1H, N=CH), LC-MS: Calculated mass = 322.2123, Actual mass = 321.9984.

10. Compound: M3

(E)-1-(2, 4-dichlorophenyl)-N-(4-methylpyridin-2- yl) methanimine: FT-IR (cm^{-1}): 2928.07 (Aliphatic CH), 3057.30 (Ar-CH), 1693.57 (C=N), 261.50 (C-C) ^{1}H NMR (δ ppm): 7.0-8.0 (13H, Ar-H), 3.5 (1H, NH), 5.0 (2H, CH_2), 8.1 (1H, N=CH), LC-MS: Calculated mass = 265.1329, Actual mass = 265.0306.

TABLE 3: DESCRIPTION OF THE COMPOUNDS

Sample Code	Mol. Wt	Solubility	Melting Point	% Yield
CM	224.23	Soluble in Ethanol	200-204 ºC	80%
MM	192.13	Soluble in Ethanol	168-170 ºC	75%
CMG	220.33	Soluble in ethyl acetate, Methanol, Ethanol	198-200 ºC	80%
MMG	252.29	Soluble in Ethyl acetate, Methanol, Ethanol	224-226 ºC	85%
MMF	238.26	Soluble in Ethyl acetate	Above 200 ºC	70%
MC	300.38	Soluble in Ethanol	Above 200 ºC	60%
TM/CC	268.42	Soluble in Ethanol, Methanol	173-175 ºC	65%
M1	360.11	Soluble in Ethanol, Methanol	270-272 ºC	87%
M2	321.99	Soluble in Ethanol, Methanol	228-230 ºC	90%
M3	265.03	Soluble in Ethanol, Methanol	98-90 ºC	79%

RESULTS: The anti-tubercular activities of the synthesized compounds were determined by Microplate Alamar Blue Assay method (MABA against *Mycobacterium tuberculosis* strain H37Rv.

TABLE 4: ANTI TB RESULTS

S. no.	Samples	100µg/ml	50µg/ml	25µg/ml	12.5µg/ml	6.25µg/ml	3.12µg/ml	1.6µg/ml	0.8µg/ml
1	CM	S	S	S	S	**S**	R	R	R
2	MM	S	S	S	**S**	R	R	R	R
3	MMG	S	S	S	**S**	R	R	R	R
4	CMG	S	S	**S**	R	R	R	R	R
5	MMF	S	S	S	S	S	S	**S**	R
6	MC	S	S	S	S	**S**	R	R	R
7	TM/CC	S	S	S	S	S	**S**	R	R
8	M1	**S**	R	R	R	R	R	R	R
9	M2	S	**S**	R	R	R	R	R	R
10	M3	S	**S**	R	R	R	R	R	R

S – Sensitive; R – Resistant

Photograph of Standard Drugs:

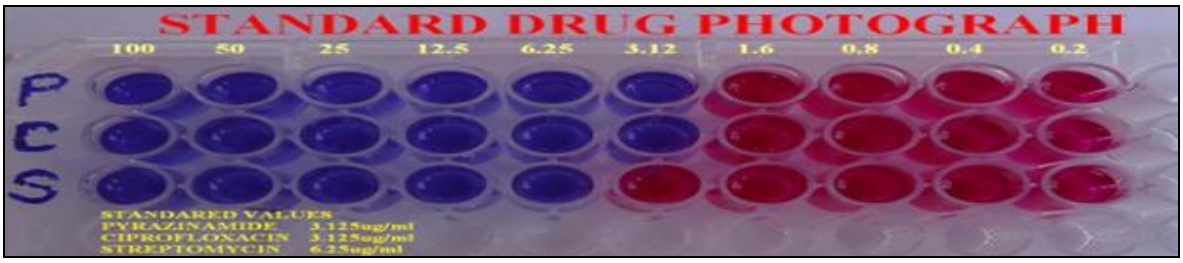
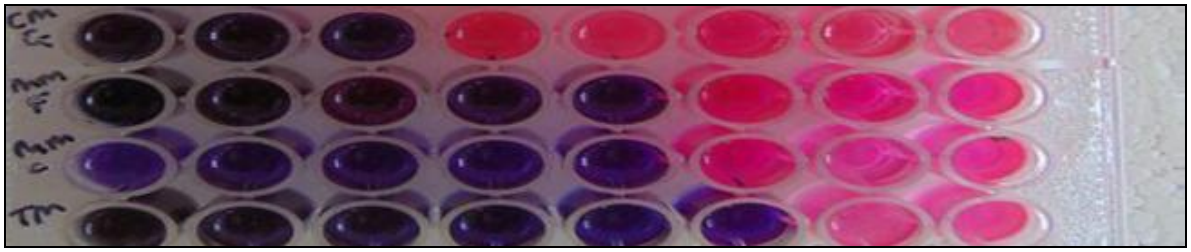


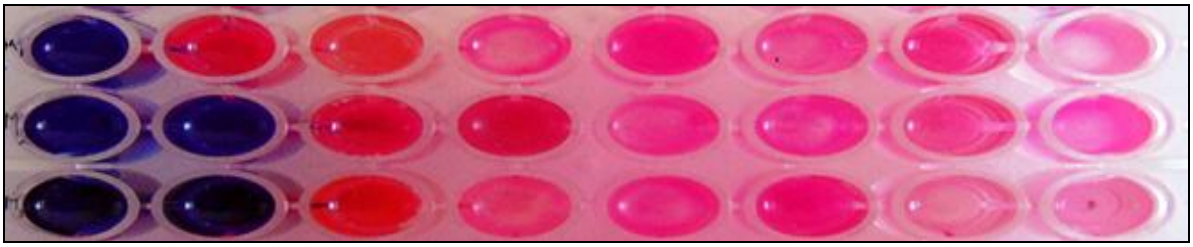
FIG. 1: PHOTOGRAPH OF STANDARD DRUGS

Photograph of Synthesized Compounds:

SAMPLE CODE: 3. CM, 4. MMG, 5.MM



SAMPLE CODES: CMG, MMF, MMC, TM



SAMPLE CODES: M1, M2, M3

FIG. 2: PHOTOGRAPH OF SYNTHESIZED COMPOUNDS

CONCLUSION:

- All the synthesized compounds were screened against Mycobacterium tuberculosis H37RV to determine the actual MIC, using the Micro plate Alamar Blue Assay (MABA) method.
- Compounds MMF, TM, CM and MC exhibited MIC values of 1.6 µg/ml, 3.12 µg/ml and 6.25 µg/ml (CM, MC) respectively which is comparable to the activity of the standard drugs like Pyrazinamide and Streptomycin. Whereas rest of the compounds were moderately effective with a MIC of 12.5 µg/ml (MM, MMG), 25 µg/ml (CMG), 50 µg/ml (M2, M3) 100 µg/ml (M1) respectively.
- Further structural refinement to the structure of the synthesized compounds are expected to yield promising molecules against the tuberculosis causing pathogen *Mycobacterium tuberculosis.*

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CONFLICT OF INTEREST: The authors declare that there is no conflict of interest

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