Synthesis, molecular docking studies and anti-mycobacterial evaluation of N-(Malon-substituted-anilic)-4-phenyl thiosemicarbazide

Mohd. Shahnawaaz[*1], Arshi Naqvi[#2] and Asif Naqvi[3]

[1]Department of Chemistry J.K. Lakshmipat University, Jaipur- 302026, India.
*Email: shaan_organic@yahoo.co.in

[2] Department of Chemistry, School of Chemical Sciences, St. John's College, Agra (U.P)-282002 (India),
[#]Present Affiliation: Pharmaceutics Division, Central Drug Research Institute, Chattar Manzil Palace, M. G. Road, Lucknow (U.P)-226001 (India),
Email: arshi_84@yahoo.com

[3] BioDiscovery-Solutions for future Plot No 29, FF2, 2nd street, Pearl Astragal Apartment, Perumbakkam, off Medavakkam, Solinganallur Main Road, Chennai (Tamil Nadu)-600100 (India)
Email: ceo@biodiscoverygroup.com

* Author to whom correspondence should be addressed.

Abstract: Tuberculosis remains the most important communicable disease in the world. Tuberculosis (TB) is an infection, primarily in the lungs (a pneumonia), caused by bacteria called *Mycobacterium tuberculosis*. Along with the recent increase in cases of tuberculosis, there is a progressive increase in multidrug resistant (MDR) tuberculosis. Therefore, the increasing clinical importance of drug-resistant mycobacterial pathogens has lent additional urgency to microbiological research and new anti-mycobacterial compound development. For this purpose, new malonamic acid thiosemicarbazide derivatives were synthesized and evaluated for anti-tubercular activity. N-(Malon-substituted-anilic)-4-phenyl thiosemicarbazides were prepared by the reaction of malonamic acid hydrazides and substituted phenyl isothiocynates. The structures were confirmed by their ^{1}H-NMR, IR and mass spectral data. Computational studies were undertaken to test the inhibitory effect of the synthesized molecule on protein kinase PKnB from *Mycobacterium tuberculosis.* The docking result of Thiosemicarbazides demonstrated that the binding energies were in the range of -7.32 kcal/mol to -8.95 kcal/mol, with the minimum binding energy of –8.95 kcal/mol. The synthesized thiosemicarbazides were screened for anti-tubercular activity. Nine compounds were tested for their in vitro activity against *Mycobacterium Tuberculosis* (M.TB) H_{37}Rv employing REMA (Resazurin microtitre assay) method and three compounds (3c, 3g &3h) were found to be active against M.TB.

Keywords: thiosemicarbazide, synthesis, molecular docking, anti-mycobacterial activity

Introduction:

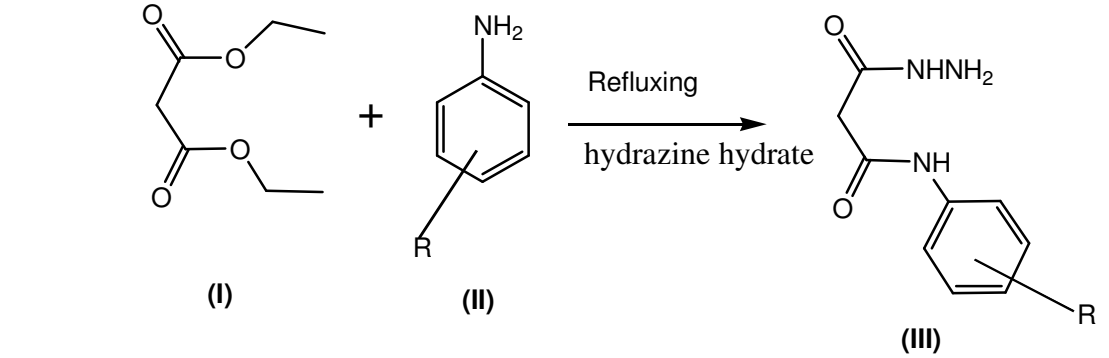
Tuberculosis is presently regarded as the most dangerous infective diseases worldwide. And it is one of the leading causes of death due to a single infectious pathogen. Approximately one-third of the world's population has been infected with the causative pathogens *Mycobacterium tuberculosis* (M.TB). Eight billion people fall sick with TB every year and globally it accounts for almost three deaths annually [1]. The resurgence of TB over the last 15 years [2], one-fifth of all deaths of adults in developing countries are due to TB, even in industrialized countries where it was almost eradicated has been favored by the pathogenic synergy with Human Immunodeficiency Virus (HIV) infection [3]. It was reported that around 19-43% of the world population might get infected with M.TB between 2000 and 2020, if control measure are not effective [2,4]. Moreover the emergence of TB has also been accompanied by the appearance of Single-Drug-Resistant (SDR) and Multi-Drug-Resistant (MDR) is particularly alarming [5]. MDR-TB has already caused several fatal out brakes [6] and poses a significant threat to the treatment and control of the disease in some parts of the world, where the incidence of MDR-TB can be as high as 14% [7]. Therefore all of these serious concerns require particular attention and stimulate the continuing search for new anti-mycobacterial agents and therapeutic regimens. However in the last 40 year, only few drugs have been approved by the Food and Administration (FDA) to treat TB because of the lack of pharmaceutical industry research in the areas [6,8]. A variety of compounds are under investigation as potential anti-mycobaterial drugs. Among them, thiosemicarbazide derivatives have been reported as anti-mycobaterial agents [9-11]. They also posses interesting biological activity like antifungal [12] hypoglycemic [13] anti-viral[14] and bacteriostatic [15]. Therefore it was thought worthwhile to synthesize some thiosemicarbazides. These synthesized thiosemicarbazides were subjected to molecular docking and anti-mycobacterial screenings against *Mycobacterium tuberculosis*.

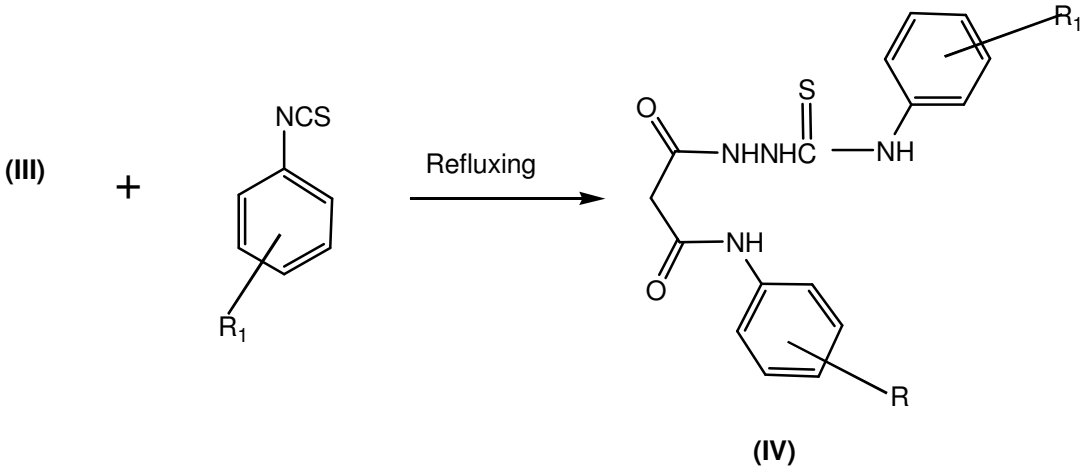
Experimental Section:

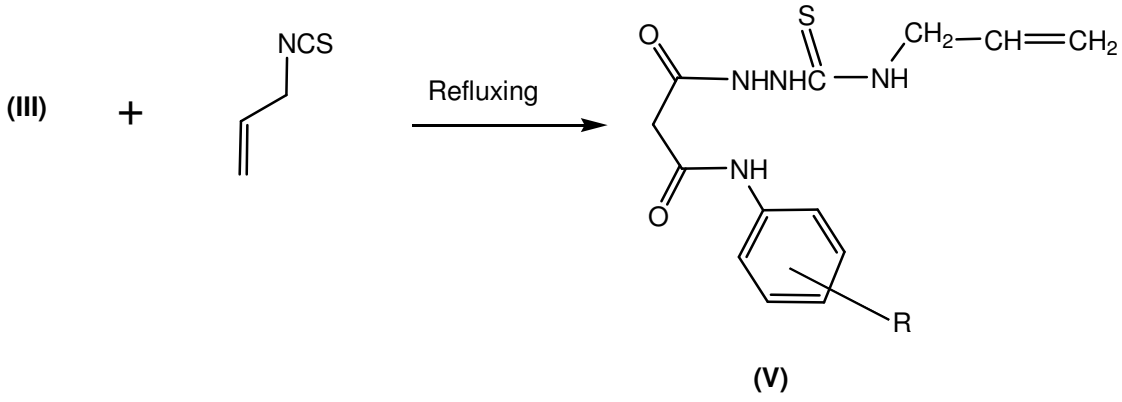
Melting points were determined in open capillary tubes and are uncorrected. The purity of the compound was checked on silica-gel-coated Al plates (Merck). The structures of the compounds are confirmed on the basis of their Infra red spectra (IR) using KBr discs, on a Perkin Elmer Spectrum RX1 infra red spectrophotometer. ^{1}H NMR spectra were recorded in DMSO on Bruker DRX-300 (300 MHz) and Jeol AL300 FT-NMR (300 MHz) systems; chemical shift (δ) are reported in ppm using TMS as an internal reference. Elemental analysis was performed on Elementor Vario EL III. All the compounds gave satisfactory microanalysis. Synthesized molecules were docked into the nucleotide-binding pocket of the M. tuberculosis PKnB structure (PDB ID 2FUM)[16] using the program AutoDock4 [17]. Anti-tubercular screening of compounds was carried out using REMA method.

General procedure of synthesis: N-(substituted)phenyl malonamic acid hydrazides have been synthesized according to our reported method [18]. A mixture of N-(substituted)phenyl

malonamic acid hydrazide (0.001mol) and (4-substituted)phenyl isothiocynate (0.001mol) dissolved in 7 ml and 3 ml of ethanol respectively, were refluxed for two hours. The white product obtained on cooling was recrystallized with hot absolute ethanol and was found to be N-(Malon-substituted-anilic)-4-phenylthiosemicarbazide. As shown in scheme 1







Scheme 1

*N-(Malon-4-bromo-2-fluoroanilic)-4-(4'-bromophenyl)thiosemicarbazide (**3c**):*
White crystal; Yield: 0.13g, 28.88%; m.p: 178 ^{o}C; IR (KBr) (cm^{-1}): 3435 (N-H, stret.), 2994 CH_2(C-H stret.), 1533 (N-C=O, stret., amide-II), 1646 (>C=C< / Ar-C-C, stret.), 1335 (C=S), 1262 primary aromatic amine (C-N, stret.), 1220 (-N-N-, stret.),1012 (ArC-Br, stret.), 771 (mono substituted ring); Anal. Calc. for $C_{16}H_{13}O_2N_4Br_2FS$: C 38.09, H 2.57, N 11.11, S 6.34;
Found: C 38.18, H 2.47, N 11.17,S 6.40

*N-(Malon-4-bromo-2-fluoroanilic)-4-(4'-fluorophenyl)thiosemicarbazide (**3g**):*
White crystal; Yield: 0.17g, 38.46%; m.p: 176 ^{o}C ; IR (KBr) (cm^{-1}): 3433 (N-H, stret.), 2997 CH_2(C-H stret.), 1529 (N-C=O, stret., amide-II), 1646 (>C=C< / Ar-C-C, stret.), 1339 (C=S), 1262 primary aromatic amine (C-N, stret.), 1219 (-N-N-, stret.), 1110 (ArC-F, stret.), 1012 (ArC-Br, stret.), 771 (mono substituted ring); ^{1}H-NMR (300 MHz, DMSO-d6) (δ ppm): 2.25 (s, H, NH), 2.25-2.30 (s, 1H, HN-C=S), 3.42 (s, 2H, CH_2), 4.02-4.05 (s, 1H, NH), 6.32-6.85(m, 4H, Ar-H), 7.16(s,1H, Ar-H), 7.61(s,1H, Ar-H), 7.22(s,1H, Ar-H), 9.43 (s, 1H, CONH), 10.06(s, 1H, CONH); Anal. Calc. for $C_{16}H_{13}O_2N_4BrF_2S$: C 43.34, H 2.93, N 12.64, S 7.22; Found: C 43.42, H 2.89, N 12.70, S 7.15.

*N-(Malon-4-bromo-2-fluoroanilic)-4-allyl thiosemicarbazide(**3i**):*
White; Yield: 0.13g, 33.51%; m.p: 182 ^{o}C; IR (KBr) (cm^{-1}): 3433 (N-H, stret.), 2997 CH2(C-H stret.), 1529 (N-C=O, stret., amide-II), 1646 (>C=C< / Ar-C-C, stret.), 1339 (C=S), 1262 primary aromatic amine (C-N, stret.), 1219 (-N-N-, stret.), 1110 (ArC-F, stret.), 1012 (ArC-Br, stret.), 771(mono substituted ring);^{1}H-NMR (300 MHz,DMSO-d6) (δ-ppm): 2.25-2.30 (s, 1H, HN-C=S), 3.42 (s, 2H, CH_2), 4.02-4.05 (s, 1H, NH), 4.11 (s, 2H, N-CH_2), 5.06 (s, 1H, >C=CH_a), 5.10 (s, 1H, >C=CH_b), 5.16 (s, 1H, CH=CH_2), 9.43 (s, 1H, CONH), 10.06(s, 1H, CONH); Anal. Calc. for $C_{13}H_{15}O_2N_4BrFS$: C 40.10, H 3.85, N 14.39, S 8.25; Found: C 40.23, H 3.80, N 14.45, S 8.24.

Molecular Docking: Molecular Docking of Thiosemicarbazides was carried out using Lamarckian Genetic Algorithm [19] on the basis of calculated ligand-protein pair wise interaction energies. Study was carried out on 9 molecules and the grid maps representing the protein were calculated using auto grid and grid size was set to 60*60*60 points with grid spacing of 0.375 Å. Docking was carried out with standard docking protocol on the basis of a population size of 150 randomly placed individuals; a maximum number of 2.5 *107 energy evaluations, a mutation rate of 0.02, a crossover rate of 0.80 and an elitism value of 1. Fifteen independent docking runs were carried out for each ligand and results were clustered according to the 1.0 Å rmsd criteria.

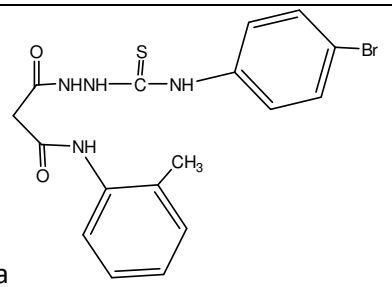
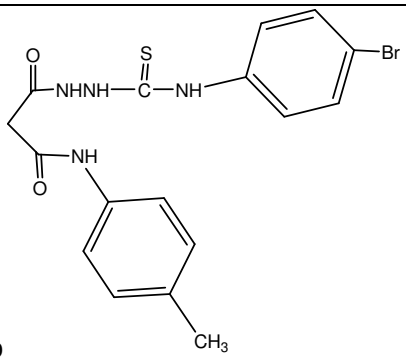
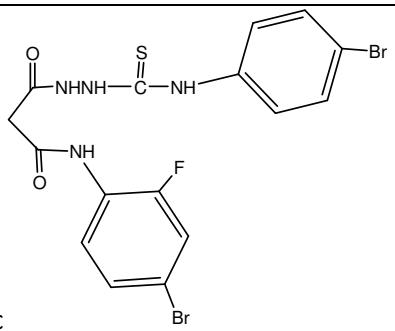
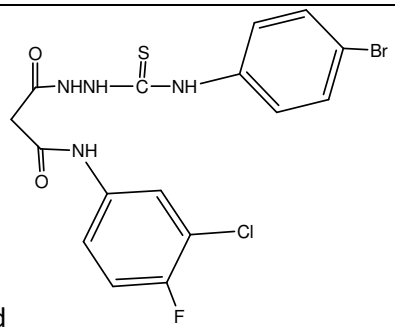
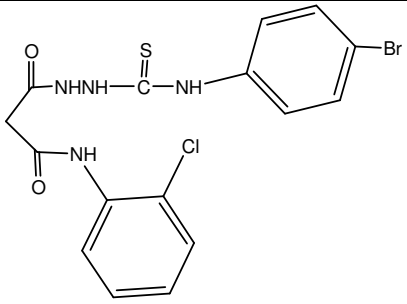
Antimycobacterial Activity: The synthesized compounds were tested for their anti-tubercular activity in vitro against M.TB(H37Rv) by REMA method [3], in middle brook 7H9 supplemented with OADC(Hi-Media) using double dilution technique. A 100µL volume of middle brook (difco USA) was dispensed in each well of a 96 well cell culture plate (nune, Denmark), the compounds were tested against M.TB at different drug concentration (i.e. 3.25, 6.26, 12.5, 25, 50, 100, 200, 400, 500, 1600µg/ml) for the determination of minimum inhibitory concentration (MIC). The MIC was defined as the minimum concentration of the compounds required to inhibit the complete bacterial growth. Rifampicin & Ethambutol were used as standard drug.

Result and Discussion:

The aim of this study was to determine the conditions of synthesis to study the chemical compositions and structure, and to characterize the physicochemical properties of new antimicrobial agents. A series of new compounds were synthesized according to the scheme 1.

The IR spectra contained signature peak of NH_2 at 3435cm^{-1}, sec.amide(N-C=O) 1531cm^{-1}, and carbonyl peak(C=O) at 1798-1742 cm^{-1}. The title compounds i.e. Thiosemicarbazide characteristic peak (C=S) occurs at 1396-1339 cm^{-1}, sec.amide (N-C=O) at 1541-1529 cm^{-1} and the methylene group(CH_2) peak in malonamic acid hydrazide and thiosemicarbazide occur at 2930-2897 cm^{-1}. The proton spectra revealed the featured characteristic methylene proton (CH_2) signal at δ 3.46-3.51 and proton signal of sec.amide (CONH) at δ 7.91-8.30 δ(ppm), while in acid hydrazide methylene proton (CH_2) group signal occur at δ 3.34-3.46 , NH_2 proton of compound occurs at δ 2.52-2.90.

The docking result of Thiosemicarbazides demonstrated that the binding energies were in the range of -7.32 kcal/mol to -8.95 kcal/mol, with the minimum binding energy of –8.95 kcal/mol (Table 1). The molecules were then tested for structure analysis by the visualization tool. The coordinate of the docked protein along with the ligand was visualized using UCSF chimera [20] within 6.5 Å region. Out of 9 molecules 7 protein-ligand complex showed H - bond with the active site residue VAL 95 (Table 1). The molecule, 3c, showed Drug Likeness score of -0.25 with Mol PSA as 56.11 A^2 and MolVol as 352.70 A^3. The MolLogS was -6.03 (in Log(moles/L)) 0.46 (in mg/L)(Fig 1). The molecule showed H-Bond interaction with VAL 95 with bind length of 3.275 Å and TYR 94 with bond length of 3.301 Å (Fig 2a & 2b).

STRUCTURE	Min Binding Energy	H-Bond Info
 3a	-8.16	ASP 96 2.868 Å
 3b	-8.40	VAL 95 2.740 Å TYR 94 3.381 Å GLY 97 3.166 Å
 3c	-8.95	VAL 95 3.275 Å TYR 94 3.301 Å
 3d	-8.27	GLU 93 3.295 Å
 3e	-8.35	VAL 95 2.646 Å TYR 94 3.126

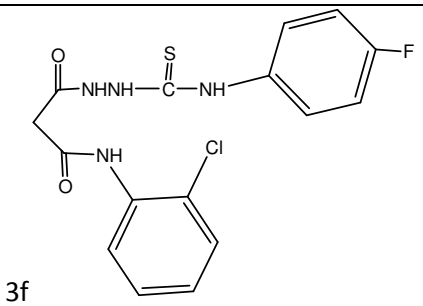
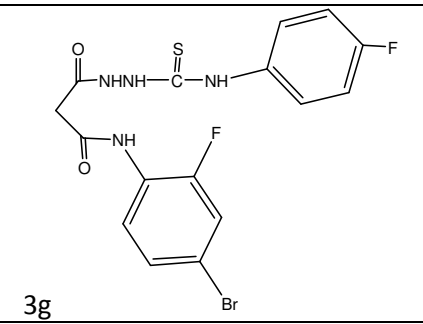
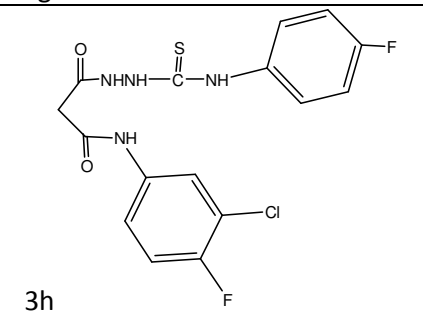
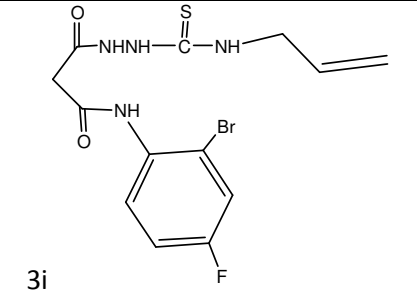
 3f	-7.77	VAL 95 2.753 Å
 3g	-8.21	VAL 95 2.855 Å
 3h	-8.22	VAL 95 2.475 Å TYR 94 3.254 Å
 3i	-7.32	VAL 95 3.501 Å

Table 1: Binding energies (kcal/mol) of synthesized thiosemicarbazides.

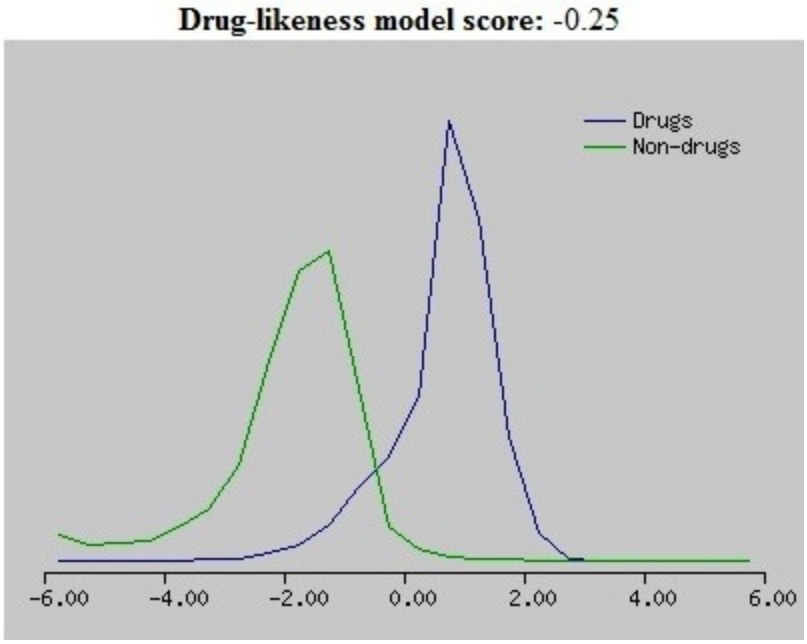


Fig. 1: Drug Likeness Model Score

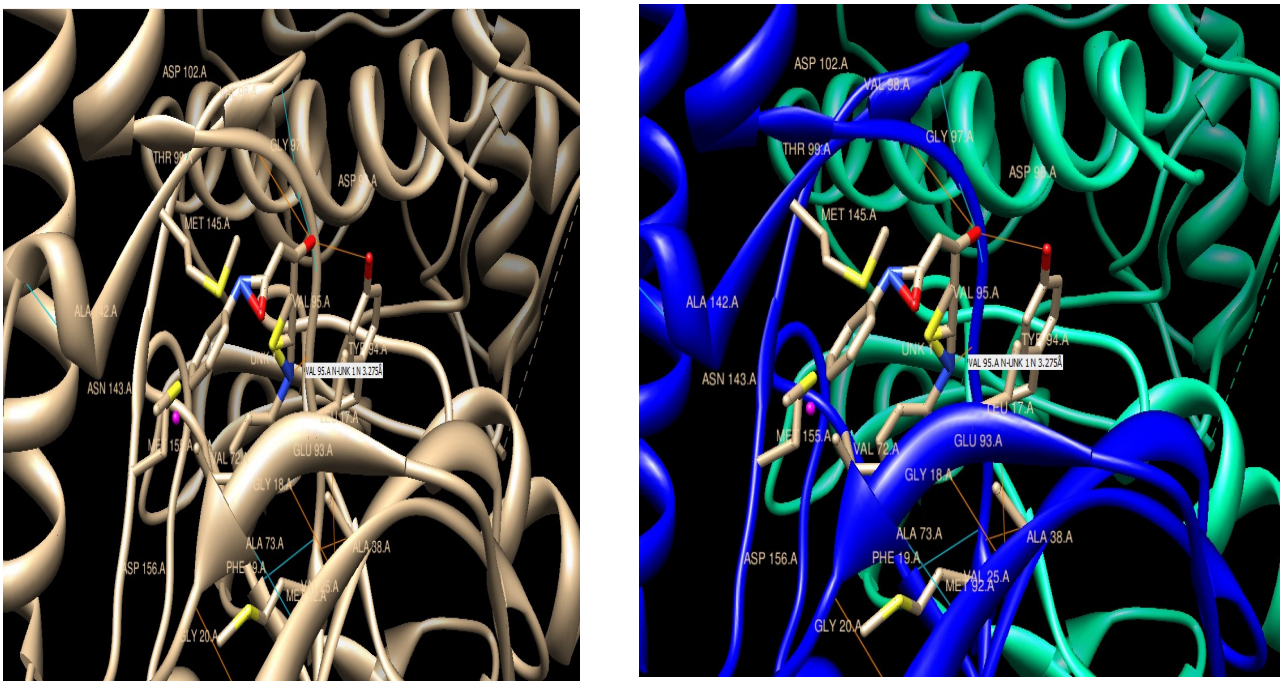


Fig.2a and 2b: H- Bond interaction with active site residue

MICs of the compounds is reported in Table-2. The obtained result revealed that the compounds (3a,3b,3d,3e,3f,3i) exhibited nil activity at these concentration against (H37Rv), indicated by

change in color from blue to pink which shows bacterial growth in culture plate, while compounds (3c, 3g,3h) were found to be active at 1600µg/ml concentration with no color change in culture plate.

Compound	R	R1	Mp (^{0}C)	Yield (%)	MIC(µg/ml) (1600µg/ml)
3a	2-CH_3	4-Br	208	30.71	Inactive
3b	4-CH_3	4-Br	205	50.43	Inactive
3c	4-Br, 2-F	4-Br	178	28.88	Active
3d	3-Cl, 4-F	4-Br	174	31.12	Inactive
3e	2-Cl	4-Br	178	25.03	Inactive
3f	2-Cl	4-F	168	50.06	Inactive
3g	4-Br, 2-F	4-F	176	38.46	Active
3h	3-Cl, 4-F	4-F	98	25.20	Active
3i	4-Br, 2-F	Allyl	182	33.51	Inactive

Table 2: Melting point, % yield and anti-tubercular activity screenings

Conclusion:

In conclusion the docking results demonstrated that the binding energies were in the range of -7.32 kcal/mol to -8.95 kcal/mol, with the minimum binding energy of –8.95 kcal/mol. Three compounds (3c, 3g and 3h) showed activity against M.TB (H37Rv) at 1600 µg/ml concentration and here no color change of resazurin solution in culture plate has taken place. Substituent with strong electron withdrawing group like fluoro, in the phenyl isothiocynate ring showed good antimicrobial activity. While bromo group in the phenyl isothiocynate ring did not showed remarkable antimicrobial activity.

REMA method is better than other anti-tubercular screening methods as it give result for all the isolates after two days of addition of resazurin solution. However REMA can be performed with minimum labors inputs compared to other methods for testing. The cost of REMA can be reduced further by decreasing the number of tested drug concentrations. One disadvantage of REMA is associated with bio-safety since the plates use liquid medium and can generate aerosols. However, these concerns can be taken care of by adapting this assay to screw-up tube format.

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