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Synthesis of 6-(4-(4-propoxyphenyl) piperazin-1-yl)-9*H*-purine derivatives as antimycobacterial and antifungal agents: *In-vitro* evaluation and *in-silico* study

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Abstract

A series of novel alkyl substituted purines were synthesized. 6-(4-(4-propoxyphenyl) piperazin-1-yl)-9*H*-purine was used as the key starting material, which was synthesized via a multistep protocol and finally subjected for N-alkylation with various alkyl halides with an aim to get prospective antimicrobial agents. The structures of the novel compounds were established by substantiating them through spectral techniques like ^{1}H NMR, ^{13}C NMR, FT-IR and EI-MS. They were explored for antitubercular activity against *Mycobacterium tuberculosis* H37RV. Furthermore, they were checked for their antimicrobial activity concerning bacterial and fungal strains. The title compounds exhibited considerable antimicrobial activity without any significant toxicity. *In-silico* studies depicted their good binding profile against *Mycobacterium tuberculosis* enoyl reductase (InhA) (PDB ID: 4TZK) and *Candida albicans* dihydrofolate reductase (PDB ID: 1AI9). The title compounds obeyed Lipinski's parameters and have exhibited good drug-like properties.

Key words: Propoxyphenyl piperazin-1-yl; Antitubercular activity; Antimicrobial; *Mycobacterium tuberculosis* H37RV; Docking Study.

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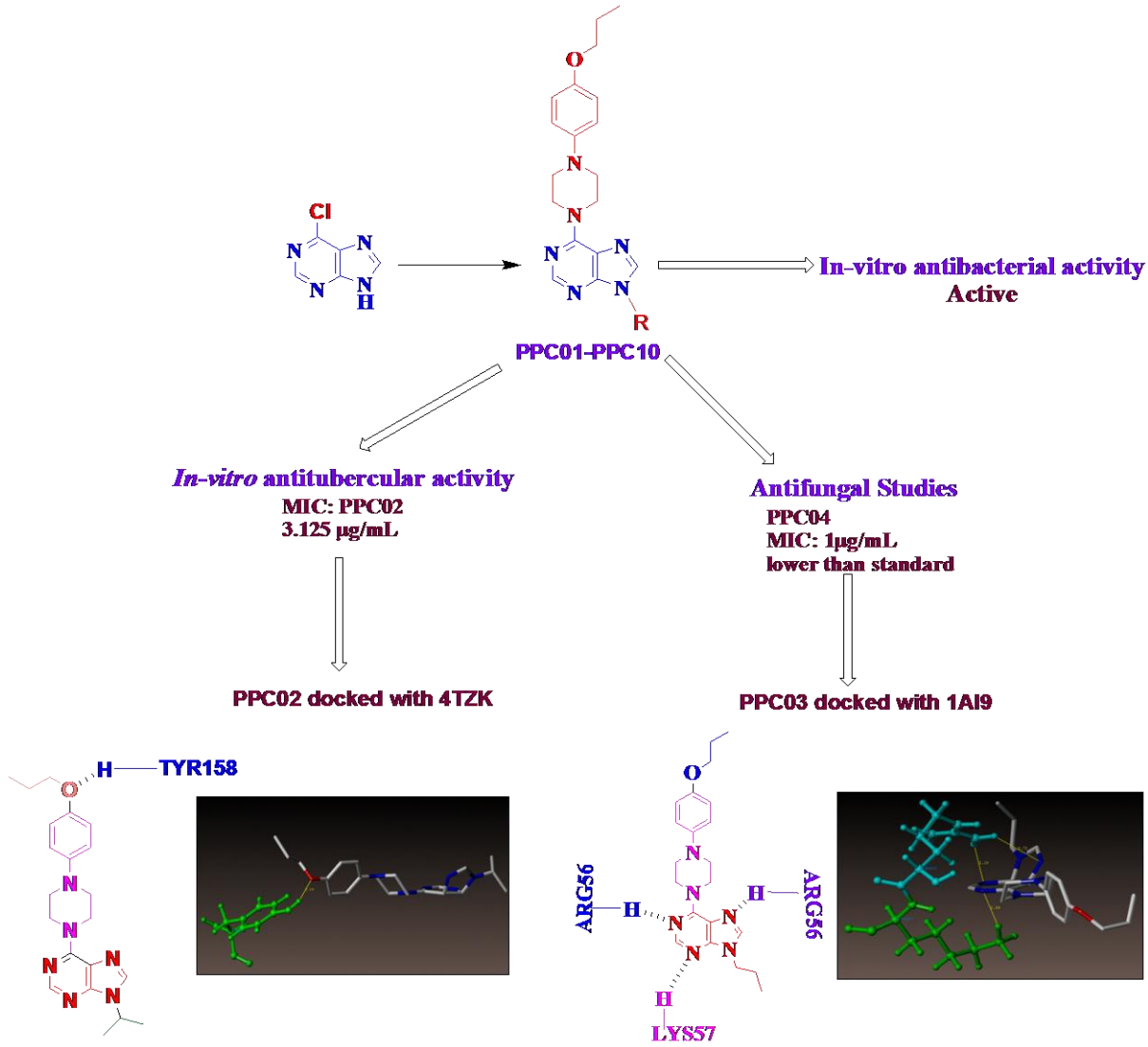
Graphical Abstract



1. Introduction

There is a formidable urge for the synthesis of novel leads in order to develop potent drugs for the treatment of life-threatening as well as infectious diseases. Tuberculosis is one of the most pervasive communicable disease accounting for millions of death globally. The appearance of unfamiliar virulent strains of TB like the multi drug resistance TB (MDR-TB), extremely drug resistance TB (XDR-TB) and their association with human immuno deficiency virus (HIV) aggravates the situation.[1-3] Also, the advent of multi-drug resistance strains of bacteria and fungi have constituted a challenge for the treatment of infectious diseases.[4-6] An indispensable component of drug designing programs is the search of new leads by synthesis of low molecular weight compounds, which are novel but still resemble well known pharmacologically active fragments having imperative structural components.[7-8]

It has been entrenched that heterocyclic scaffolds play a vital role in designing new structural motifs with medicinal applications. Amongst them, purine nuclei have contributed immensely for augmentation of the antimicrobial activity comparatively to the other hetero moieties. Being one of the most ubiquitous nitrogen-containing heterocycles, purine occurs abundantly in nature and also forms a core moiety of nucleic acids, which are of prime importance for genetic and proper functioning of most of the metabolic processes in living organisms.[9] Purines are involved with multiple number of enzymes and receptors which exhibit crucial roles in different phases of the cell cycles, in cell signaling and other fundamental reactions in the biological system. [10-11] Concisely, purines and their substituted analogues have grabbed considerable attention of research in medicinal chemistry.

Literature reports have shown that naturally occurring purine as well as their synthetic counterparts exhibit a wide range of biological activity.[12-16] Di, tri and tetra substituted purines have been accounted for possessing antiviral and antitubercular properties. They also modulate CRH-R1 and act as inhibitors of Hsp90, sulfotransferase, leukotriene A4 hydrolase, and kinases phosphodiesterases.[17] Furthermore, there are reports available regarding their role in enzyme inhibition,[18-19] as antihyperglycemic,[20] antifungal and antibacterial agents.[21-24] According to a report; WHO stresses for research on purines as potential anti-mycobacterials.[25] As a result, a broad range of bioactive moieties with purine skeletal have been designed and synthesized which have added to the launch of several powerful drugs with varied applications.[26] The rationale for the display of biological activities is conferred to the diversity of substituents that can be combined

on the C-2, C-6, C-8 and N-9 centers of purine nucleus whereas C-6 and N-9 positions are the most anchored ones.[27] The explanation of the selectivity and efficacy of derivatives of purine is attained principally by the cell surface transporters which mediate access to the cell and later the enzymes of the metabolic pathways modify the prodrug into an active metabolite.[28]

Piperazine and its derivatives are reported for inhibiting tumor metastasis in most of the cancer cell lines. Mechanistic studies of the analogues containing piperazine have showed that this property is accomplished by impeding synthesis of microtubules, progression of the cell cycle, angiogenesis and obliteration of the tumor cells through the initiation of apoptosis.[29-30] Further, literature proves that incorporation of piperazine into the first generation quinolines had significantly improved the bioavailability of the second generation fluoroquinolones. This indicates that piperazine skeleton might aid to regulate the physiochemical properties and enhance their pharmacokinetic and pharmacodynamic behavior. SQ786 and LL-3858 from Lupin Ltd., with a piperazine moiety in their framework showed promising antitubercular activity.[31] In addition, biologically active compounds with varying alkyl/acyl groups have realized to regulate their lipophilicity and permeability[32-33] whereas the alkenyl and alkynl purines are often cytotoxic to the human cells.[34]

With this outlook and in extension of our work with purines; we emphasized on the design and development of potent molecules with purine as the core moiety, hybridized with phenyl piperazine at its C-6 position and exploring its N-9 position with various alkyl groups. Hence we attempted the synthesis of alkyl-substituted purines by simple and efficient multi-step protocol. The title compounds were explored for their *in-vitro* and *in-silico* antimicrobial and antitubercular properties. Also, their physiochemical properties have been investigated.

2. Results and Discussion

2.1. Chemistry

N-9 substituted alkyl derivatives of purine (**PPC01-PPC10**) are synthesized as shown in **Scheme 1** as per the reported procedures.[35] *N*-protected 4-hydroxyphenyl piperazine (PP1) and propyl bromide which are commercially available were refluxed using triethyl amine to get the O-alkylated product (PP2). This (PP2) was now subjected to de-acetylation with HCl (4 N, 20 mL) to obtain the first precursor 4-propoxyphenyl piperazine (PP3). The key intermediate (PP4) was obtained by nucleophilic substitution of PP3 with 6-chloro purine. Finally, the reaction of this

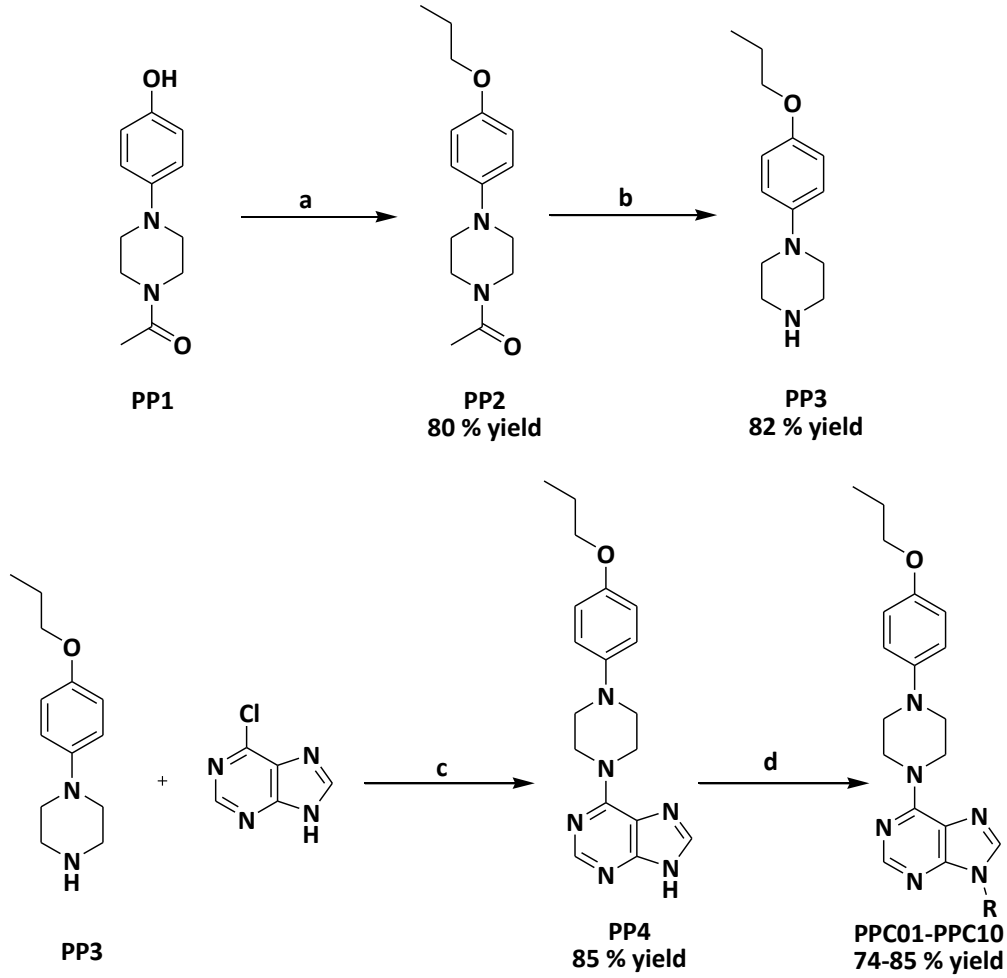
key intermediate (PP4) with different alkyl halides accorded the target compounds i.e., *N*-9 alkyl substituted purines with yields about 74-85 %. The chemical structures of the final compounds were confirmed by spectroscopic techniques like ^{1}H and ^{13}C NMR, FT-IR and EI-MS. The characterization data of the final molecules **PPC01-PPC10** are in accordance with the synthesized compounds and are specified in the experimental section.

In **PPC01**, the ^{1}H NMR spectrum showed two singlet's at δ 8.38 and δ 7.78 ppm, accounting for the C-H proton of pyrimidine and imidazole of the purine scaffold respectively. The characteristic quartet-triplet pattern of the ethyl chain appeared at δ 4.25 and δ 1.52 ppm whereas the propyl chain of propoxy phenyl piperazine moiety resonated as triplet multiplet triplet pattern at δ 3.88, δ 1.78 and δ 1.02 ppm respectively. It was further established by recording ^{13}C NMR spectrum. The signals present in the spectrum interpreted for all the C atoms present in **PPC01**. The EI-MS of **PPC01** revealed a molecular ion peak at 366[M^{+}]. This coincides with its respective molecular formula $C_{20}H_{26}N_6O$.

The ^{1}H NMR spectrum of **PPC04** showed a signal at δ 8.36 ppm as singlet relating to the C-H of pyrimidine moiety whereas the C-H proton of imidazole in the purine scaffold appeared as singlet at δ 7.73 ppm. The butyl substituent appeared as a triplet corresponding to 2H linked to nitrogen at δ 3.86 ppm, multiplet corresponding to 4H of 2 CH_2 at δ 1.79 ppm and triplet corresponding to 3H of CH_3 at δ 1.00 ppm. The EI-MS spectrum showed a molecular ion peak at 394[M^{+}] which further supported the formation of compound **PPC04**. The characterization details of the remaining molecules are in accordance with their expected chemical structures.

2.2. Biological Evaluation

All the synthesized title compounds were evaluated for their antimycobacterial activity against *Mycobacterium tuberculosis* by making use of microplate Alamar Blue assay (MABA). The title compounds were also tested for antibacterial activity against Gram-positive *Staphylococcus aureus* and *Bacillus subtilis*, Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*; antifungal activity against *Aspergillus flavus, Trichoderma harzianum, Penicillium chrysogenum and* yeast *Candida albicans* by macrodilution broth method.



R= **Eth-1-yl, Isoprop-1-yl, Prop-1-yl, But-1-yl, Pent-1-yl, Hex-1-yl, Hept-1-yl, Oct-1-yl, Non-1-yl, Dec-1-yl**

Scheme 1:
Synthesis of PPC01-PPC10
Reagents and conditions:
(a) Propyl bromide (1.2 eq); anhyd. K_2CO_3(2 eq), DMF, 120 ^{0}C, 12 h; (b) 4 N HCl (20 mL), 80 ^{0}C, 3 h.
(c) triethyl amine (2.7 eq), n-butanol, 110 ^{0}C, 12 h; (d) R–Br (1 eq), K_2CO_3(1.2 eq), DMF, RT, 10 h.

Scheme 1: Synthetic route for **PPC01-PPC10**

2.3. Antimycobacterial Activity

PPC01-PPC10 were assessed for antimycobacterial activity against *M. tuberculosis* H37Rv by employing microplate Alamar Blue assay (MABA).[36] Pyrazinamide, Ciprofloxacin and Streptomycin were used as positive controls. Among the tested compounds PPC01- PPC04 having ethyl, isopropyl, propyl and butyl groups have shown good results whereas rest of the

compounds have appeared moderate. **PPCO2** and **PPC03** have shown good activity with MIC 3.125 μg/mL similar to the two positive standards; pyrazinamide and ciprofloxacin, whereas **PPC01** and **PPC04** exhibited MIC 6.25 μg/mL which is the same as that of streptomycin standard. The Screening results are briefed in **Table 1.**

Table 1: Anti-mycobacterial activity of **PPC01-PPC10**

COMPOUNDS	MIC μg/mL	STANDARDS	MIC μg/mL
PPC01	**6.25**	**Ciprofloxacin**	**3.125**
PPC02	**3.125**	**Pyrazinamide**	**3.125**
PPC03	**3.125**	**Streptomycin**	**6.25**
PPC04	**6.25**		
PPC05	12.5		
PPC06	12.5		
PPC07	12.5		
PPC08	12.5		
PPC09	12.5		
PPC10	12.5		

2.4. Antibacterial studies

The antibacterial screening of **PPC01-PPC10** against human pathogenic bacterial strains by macrodilution broth method [37] have shown moderate to good activity. Tetracycline was the positive control. Screening results are encapsulated in **Table 2. PPCO1-PPC06** have shown good activity in comparison with the standard whereas rest of them have exhibited moderate activity against all the strains.

2.5. Antifungal Studies

PPC01-PPC10 were explored for their antifungal activity against four fungal strains[37] using nystatin and fluconazole as standards. Among the tested compounds **PPCO1-PPC06** have emerged active against all the tested fungal strains, whereas rest of them have shown good activity. The results are tabulated in **Table 3**.

Table 2: Antibacterial Activity of **PPC01-PPC10** (minimum inhibitory concentration, μg/mL)

	Test organism			
Compounds	***Staphylococcus aureus***	***Bacillus subtilis***	***Escherichia coli***	***Pseudomonas aeruginosa***
PPC01	**4**	**2**	**2**	**4**
PPC02	**4**	**2**	**2**	**4**
PPC03	**4**	**2**	**2**	**2**
PPC04	**4**	**2**	**4**	**4**
PPC05	**4**	**2**	**4**	**2**
PPC06	**2**	**2**	**4**	**2**
PPC07	16	32	32	32
PPC08	16	8	16	16
PPC09	64	32	32	64
PPC10	32	64	64	32
Tetracycline	4	2	2	4

2.6.Cytotoxicity of the synthesized compounds

The synthesized compounds were screened for their toxicity against normal Human Embryonic kidney cell line (HEK293). As depicted in **Table 4**, the compounds have exhibited moderate to low levels of cytotoxicity with IC_{50} values in the range of 580.8-753.6 μg/mL. None of the compounds have exhibited any considerable cytotoxic effects, recommending good potential for their further use.

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Table 3: Antifungal Activity of **PPC01-PPC10** (minimum inhibitory concentration, μg/mL)

	Test organism			
Compounds	***Aspergillus flavus***	***Trichoderma harzianum***	***Penicillium chrysogenum***	***Candida albicans***
PPC01	**4**	**4**	**2**	**2**
PPC02	**2**	**2**	**4**	**2**
PPC03	**2**	**2**	**4**	**2**
PPC04	**1**	**2**	**4**	**4**
PPC05	**8**	**16**	**8**	**16**
PPC06	**8**	**16**	**16**	**8**
PPC07	16	32	16	32
PPC08	32	32	64	16
PPC09	64	32	64	64
PPC10	64	64	64	64
Nystatin	2	2	2	4
Fluconazole	2	4	2	2

Table 4: Cytotoxicity of **PPC01-PPC10** against normal human embryonic kidney cells using MTT assay

Compound Name	**IC_{50} (μg/mL)**	**Compound Name**	**IC_{50} (μg/mL)**
PPC01	750.4	PPC06	695.4
PPC02	659.8	PPC07	710.1
PPC03	646.1	PPC08	686.6
PPC04	753.6	PPC09	702.4
PPC05	624.4	PPC10	580.8

2.7. Prediction of Physio-chemical properties of PPC01-PPC10

The correlation between physio-chemical properties of the chemical moieties and the drug pharmacokinetics in the human body is calculated by the Lipinski rule of five.[38-39] As stated by this rule a chemical moiety is more likely to exhibit poor absorption or permeation if

i. The molecular weight of the moiety is greater than 500
ii. The measure of lipophilicity i.e., Log P (Octanol/Water partition coefficient) is more than 5
iii. The number of H-bond donors exceeds 5,
iv. The number of H-bond acceptors i.e., the sum of Nitrogen and Oxygen atoms is over 10.

Despite of some exceptions, it is widely accepted method to screen all patterns of drugs. The results deduced using molinspiration server are summarized in **Table 5.** The data disclosed that the molecular weights of compounds are below 500 and range between 366.47- 478.69. The Log P values lie in the limits of 2.97- 6.83. All the compounds have shown 4 hydrogen bond acceptors. Six compounds have shown no violations of Lipinski.

2.8. Structure-activity relationship study

The SAR studies have interpreted the role of the length of alkyl chains in deciding the biological activities of the synthesized pharmacophores. In case of anti-mycobacterial activity, it has been seen that PPCO1-PPCO4 have shown good activity with MIC of 3.125 to 6.25 µg/mL equivalent to the positive control whereas the remaining compounds have shown moderate activity with MIC of 12.5 µg/mL. PPC01- PPC04 bears smaller length alkyl substituents namely ethyl, isopropyl, propyl and butyl. The molecular docking studies against InhA was carried out and all of the molecules exhibited higher C-score values than the standard which suggested that the all the compounds should have good activity but a look into their Lipinski's parameters showed that the compounds of chain length 7 to 10 violated the rules in the parameter of lipophilicity. This may be the reason for the decrease in the *in-vitro* activity.

The antimicrobial activity results showed that **PPC01** and **PPC03** have appeared active against *E. coli* with MIC 2 µg/mL, whereas **PPC02** is active against *B. subtilis* with MIC 2 µg/mL which is as same as the standard tetracycline. **PPC04** is active towards *S. aureus* having MIC 4 µg/mL. The remaining compounds have exhibited good antibacterial activity towards all the four strains. Molecules with smaller chain alkyl groups are more active than their longer counterparts.

Table 5: Predicted properties of PPC01-PPC10

Lipinski's Parameters						Bioactivity score								
Compounds	***Log P***	***MW***	***HDA***	***HBD***	**Violations**	**TPSA**	**Molar volume (A^3)**	**%ABS**	**Drug Likeliness**	**GPCR ligand**	**Ion channel modulator**	**Kinase inhibitor**	**Nuclear receptor ligand**	**Protease inhibitor**
PPC01	2.97	366.47	4	0	0	59.32	346.79	88.53	0.16	0.32	0.14	0.41	-0.58	-0.22
PPC02	3.32	380.50	4	0	0	59.32	363.37	88.53	0.40	0.35	0.14	0.48	-0.51	-0.28
PPC03	3.45	380.50	4	0	0	59.32	363.59	88.53	0.16	0.35	0.17	0.39	-0.55	-0.18
PPC04	3.93	394.52	4	0	0	59.32	380.39	88.53	-0.03	0.35	0.17	0.27	-0.53	-0.15
PPC05	4.42	408.55	4	0	0	59.32	397.19	88.53	-0.07	0.35	0.17	0.36	-0.51	-0.14
PPC06	4.90	422.58	4	0	0	59.32	413.99	88.53	-0.07	0.34	0.16	0.35	-0.49	-0.13
PPC07	5.38	436.60	4	0	1	59.32	430.80	88.53	-0.07	0.33	0.16	0.34	-0.48	-0.13
PPC08	5.86	450.63	4	0	1	59.32	447.60	88.53	-0.07	0.32	0.15	0.33	-0.46	-0.12
PPC09	6.34	466.46	4	0	1	59.32	460.40	88.53	-0.07	0.31	0.15	0.32	-0.45	-0.12
PPC10	6.83	478.69	4	0	1	59.32	481.20	88.53	-0.07	0.30	0.13	0.31	-0.43	-0.12

Calculated from online server [38] http://www.molinspiration.com/cgi-bin/properties
[39] http://www.molsoft.com/mprop/

HDA-Number of hydrogen bond acceptors (n-ON)

HBD-Number of hydrogen bond donors (n-OHNH)

MW-Molecular weight

LogP-Logarithm of partition coefficient between n-octanol and water (miLogP)

TPSA-Topological polar surface area

%ABS-Percent of absorption; GPCR-G-Protein-Coupled Receptor

A molecule having bioactivity score more than 0.00 is likely to exhibit considerable biological activity, while value -0.50 to 0.00 are expected to be moderately active and if the score is less than -0.50 it is presumed to be inactive

In case of antifungal activity PPC01 to PPC04 have appeared active against all the fungal strains with MIC ranging between 1-4 µg/mL. PPC05 and PPC06 also possess good activity with MIC of 8-16 µg/mL whereas the rest of the compounds are moderate with MIC ranging between 16-64 µg/mL. **PPC02** and **PPC03** have appeared active against *C. albicans, A. flavus and T. harzianum* whereas **PPC01** exhibited good activity towards *P. chrysogenum.* However, **PPC04** appeared active towards *A. flavu*s. All of the compounds discussed above have shown MIC comparable to the standards.

The C-score values also show that the molecules with alkyl chain between 1-4 chain length have higher values than the standard and hence possess good binding interactions. The substituents with 5 and 6 chain length are having lower C-score values; hence there is a decrease in the in-vitro activity. On the other hand, the compounds with the 7 to 10 chain length also exhibit high C-score values but due to violations of lipophilicity parameter, the *in-vitro* activity decreases in comparison to the smaller alkyl chains. This explanation also holds accounts for the antibacterial studies.

It has been analyzed that the compounds containing smaller alkyl chains (i.e., chain length 1-4) have exhibited better activities which is also supported by their *in-silico* as well as physicochemical studies. It has also been noticed that with increase in the length of an alkyl chain, the activity of the compounds decreases.

2.9. *In-Silico* Evaluation

Among the enzymes involved in FAS-II, the NADH-dependent enoyl-ACP reductase encoded by *Mycobacterium* gene *InhA* is the key catalyst in mycolic acid biosynthesis. Studies in the past have established that InhA is the primary molecular target for INH which has been the frontline drug for over 40 years used in the treatment of TB. The INH-NADH adduct functions as a potent inhibitor of InhA and the well-known non-selective InhA inhibitors diazoborines [40-41] and triclosan, [42] InhA direct inhibitors, pyrrole[43] derivatives and piperazines[44] have all been reported to exhibit *in vivo* as well as *in vitro* antitubercular activities.

On the other hand, Dihydro folate reductase (DHFR) is an important enzyme in both mammals and microorganism including *Mycobacterium tuberculosis* (Mtb). It catalyzes folic acid conversion to dihydro and tetrahydro folic acid which is a crucial step in the folate pathway. Inhibition of the folate pathway leads to the interruption of thymidine supply resulting in cell

death. Hence molecular docking was performed on these two targets in order to find active molecules.

Molecular modelling was employed to comprehend the effectiveness of the title molecules by examining their interactions which also reinforced the *in-vitro* results. Molecular modelling was done on *Mycobacterium tuberculosis* enoyl reductase (InhA) coordinated with 1-cyclohexyl-N-(3,5-dichlorophenyl)-5-oxopyrrolidine-3-carboxamide (PDB ID: 4TZK) from *M. tuberculosis* and *Candida albicans* DHFR (PDB ID: 1AI9) from *C. albicans.* This was also utilized to explicate the binding approach of the title molecules. The docking study was carried out using Surflex-Dock programme of Sybyl-X software extracting proteins from the Protein Data Bank (PDB).[45-46]

2.9.1 Docking studies of PPC01-PPC10 with *Mycobacterium tuberculosis* enoyl reductase (InhA) coordinated with 1-cyclohexyl-N-(3,5-dichlorophenyl)-5-oxopyrrolidine-3-carboxamide (PDB ID: 4TZK) from M. tuberculosis

The docking investigation unfolded that all the title molecules have shown reasonably good docking score for InhA (**Table 6)**. **Fig.1** represents the docked view of **PPC01-PPC10** in the active pocket of the enzyme PDB ID: 4TZK.

Fig.2 illustrates that compound **PPC02** makes one H-bonding interaction at the active pocket of the enzyme (PDB ID: 4TZK). Oxygen of the propoxy group makes an interaction with hydrogen atom of TYR158 (O------H-TYR158, 2.14 Å). The pyrrolidine carboxamide (4TZK ligand) exhibited two H-bonding interactions through its carbonyl oxygen with Tyr158 and co-factor NAD^+ as portrayed in **Fig.3**

PPC10 makes three hydrogen bonding connections on active pocket of the enzyme (PDB ID: 4TZK), amongst those two interactions are from nitrogen atom of the first position of purine with hydrogen of NAD500 and TYR158 (N------H-NAD500, 2.26 Å; N------H-TYR158, 2.41 Å) and the last one raised from the nitrogen of the third position of purine with hydrogen atom of TYR158 (N------H-TYR158, 2.58 Å). **(Supplementary Fig.1 & 2)**

Fig.4 (A and B) illustrate the hydrophobic and hydrophilic amino acids encircling **PPC10 & PPC02.** Docking studies interpreted that most of the title compounds occupied analogous binding sites as that of pyrrolidine carboxamide. The compounds have exhibited C score between the range 10.42-6.21, signifying the outline of all the forces of interaction between **PPC01-PPC10** and InhA and indicate that molecules competitively bind to InhA than 4TZK reference ligand.

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Table 6: Surflex Docking score (kcal/mol) of **PPC01-PPC10** for *M. tuberculosis* enoyl reductase (InhA) (PDB ID: 4TZK)

Compounds	C Score[a]	Crash Score[b]	Polar Score[c]	D Score[d]	PMF Score[e]	G Score[f]	Chem Score[g]
4TZK	6.13	-1.39	1.18	-168.11	-49.19	-285.29	-37.47
PPC01	6.87	-2.82	0.00	-144.964	-43.371	-298.556	-30.210
PPC02	6.95	-2.03	1.04	-131.628	-47.238	-248.902	-28.037
PPC03	6.59	-1.57	0.00	-135.489	-53.734	-249.958	-22.968
PPC04	6.21	-2.30	0.00	-149.141	-48.685	-279.305	-31.068
PPC05	6.67	-3.46	0.00	-165.361	-30.275	-335.324	-32.075
PPC06	8.80	-1.83	0.00	-161.880	-70.888	-309.389	-29.905
PPC07	8.85	-2.49	0.00	-173.274	-38.750	-335.916	-35.675
PPC08	8.74	-2.21	0.00	-180.275	-73.876	-331.317	-32.648
PPC09	10.42	-2.40	0.00	-195.518	-76.801	-396.089	-35.680
PPC10	9.08	-6.41	0.78	-219.898	-45.133	-460.212	-39.608

[a] C Score (Consensus Score) integrates a number of popular scoring functions for ranking the affinity of PPC01-PPC10 bound to an active pocket of a receptor and reports output of total score.

[b] Crash-score discloses the inappropriate penetration into the binding pocket. The scores close to 0 are favorable whereas Negative numbers indicate penetration.

[c] Polar score indicates the contribution of polar interactions to the total score. It may be useful for excluding docking results that make no H-bonds.

[d] D-score depicts charge and Vander Waals interactions between protein and ligand.

[e] PMF-score specifies the Helmholtz free energies of interactions for protein-ligand atom pairs (Potential of Mean Force, PMF).

[f] G-score shows hydrogen bonding, complex (ligand-protein) and internal (ligand-ligand) energies.

[g] Chem-score points for H-bonding, lipophilic contact and rotational entropy including an intercept term.

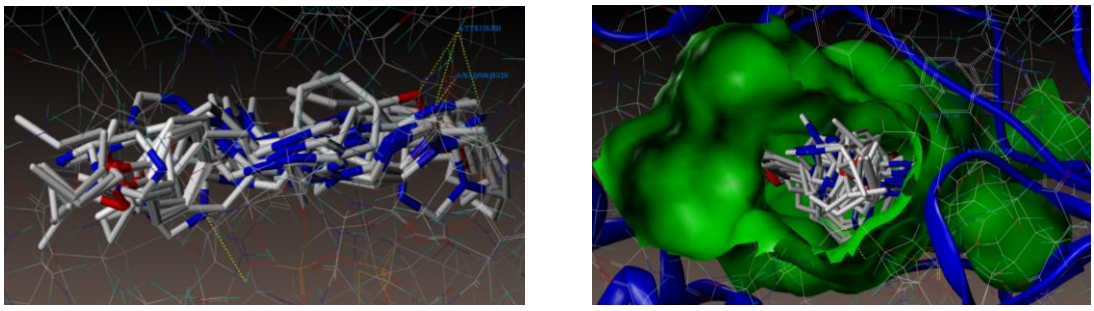

Fig.1 Docked view of **PPC01-PPC10** in the active pocket of the enzyme (PDB ID: 4TZK)

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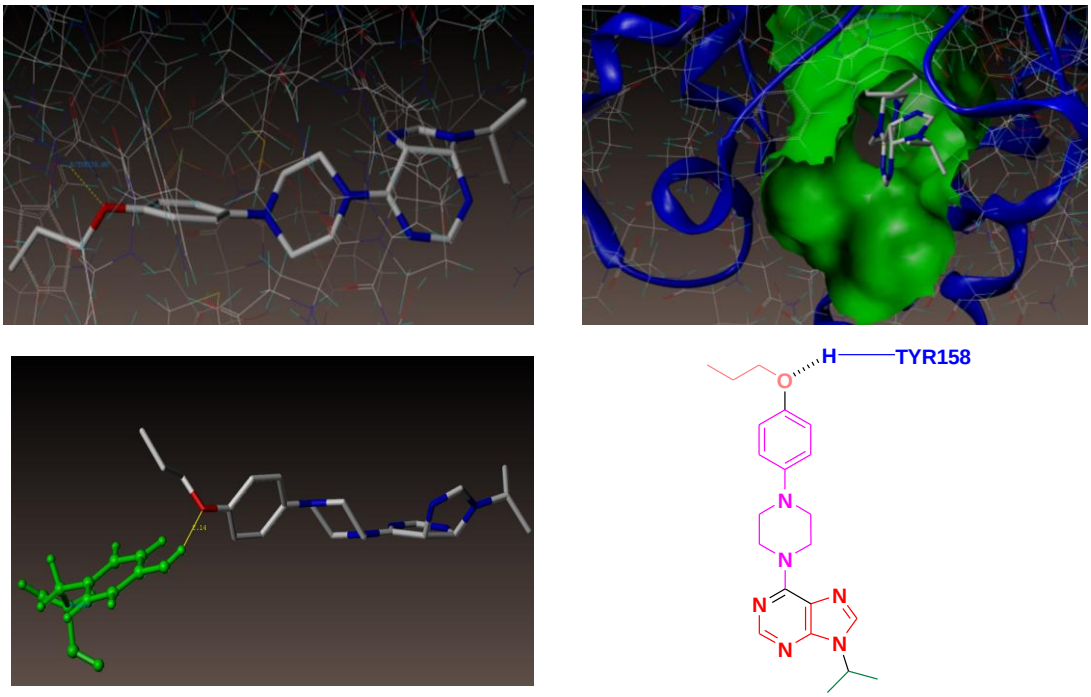

Fig.2 Docked view of **PPC02** in the active pocket of the enzyme (PDB: 4TZK)

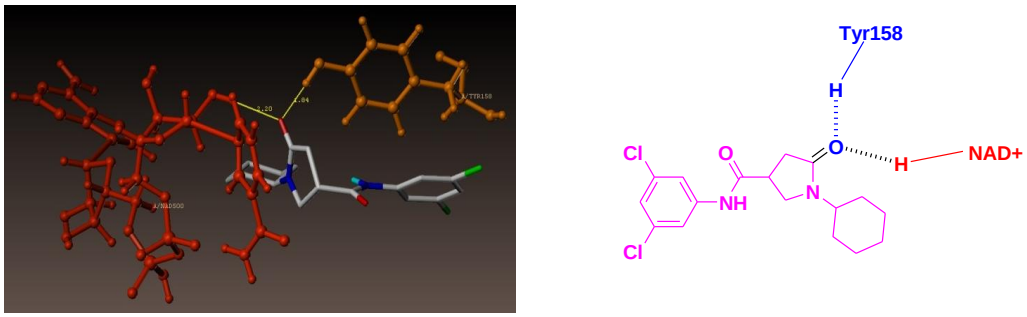

Fig.3 Interaction of (InhA) at the binding site of the enzyme (PDB ID: 4TZK)

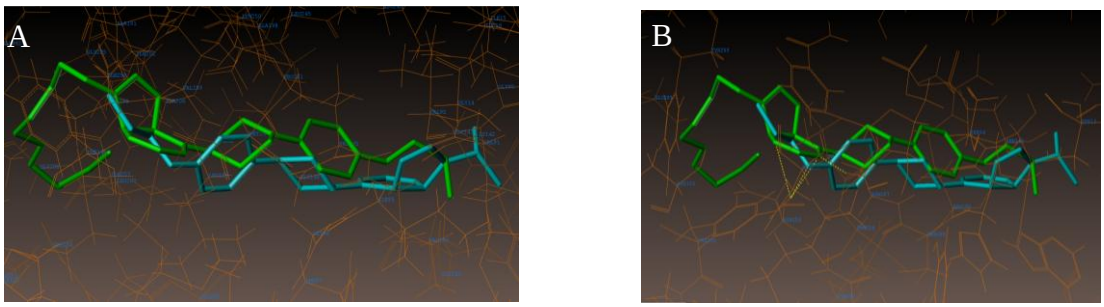

Fig.4 A) Hydrophobic amino acids encircling PPC02 (cyan colour) and PPC10 (green colour)

B) Hydrophilic amino acids encircling PPC02 (cyan colour) and PPC10 (green colour)

2.9.2. Docking studies of PPC01-PPC10 with *Candida albicans* DHFR (PDB ID: 1AI9) from *C. albicans*

The studied compounds have shown good docking scores against the enzyme dihydrofolate reductase of *Candida albicans* as revealed by the docking study. **(Table 7)** summarizes the results. **Fig.5** represents the docked view of **PPC01-PPC10** in the active pocket of the enzyme PDB ID: 1AI9.

PPC03 has three H-bonding interactions in the active pocket of the enzyme (PDB ID: 1AI9). The first interaction is through the nitrogen of 7th position of purine with hydrogen of ARG56 (N------H-ARG56; 2.72 Å), the second one through the nitrogen of 1st position on purine ring with hydrogen atom of LYS57 (N------H-LYS57; 2.08 Å). The third interaction is raised through the nitrogen of 3rd position of purine with hydrogen atom of ARG56 (N------H-ARG56; 2.72 Å) as depicted in **Fig.6**.

PPC08 has three H-bonding interactions in the active pocket of the enzyme (PDB ID: 1AI9). Amongst that an interaction is from the nitrogen atom present on the 7th position of purine with hydrogen atom of LYS57 (N------H-LYS57; 1.96 Å), nitrogen of 1st position of purine binds with hydrogen atom of LYS57 (N------H-LYS57; 2.09 Å). The last one is from nitrogen of 3rd position of purine with hydrogen atom of GLY114 (N------H-GLY114; 2.86 Å). **(Supplementary Fig.3** and **4)**

As depicted in **fig.7** fluconazole, shows three H-bonding interaction at the active pocket of *Candida albicans* DHFR (PDB ID: 1AI9) and all of these are from only one nitrogen of triazole with hydrogen of SER78 (-N----H-SER78), LYS57 (-N----H-LYS57) and ARG56 (-N----H-ARG56).

Fig.8 illustrates the hydrophobic and hydrophilic amino acids encircling **PPC03 & PPC08**. The synthesized molecules have shown binding interactions with LYS57 and ARG56 as similar to that of fluconazole. Thus, from these studies, the experimental findings can be substantiated, wherein the purine derivatives inhibit DHFR.

As DFHR is a very universal enzyme across all living organisms from bacteria to mammals including humans, docking studies with human dihydrofolate reductase complexed with NADPH and 2,4-diamino-5-((7,8-dicarbaundecaboran-7-yl)methyl)-6-methylpyrimidine, a novel boron-containing, non classical antifolate (PDB ID: 2C2T) was explored as shown in **Fig. 9 (A and B)**. This was done to check whether the synthesized compounds are selective towards DHFR of *C.*

albicans or also interact with other DHFR's. The study showed that compounds did not show any interactions with the human DHFR and are selective towards dihydrofolate reductase (DFHR) enzyme of C. albicans which also supports the toxicity study on the normal cell line.

Table 7: Surflex Docking score (kcal/mol) of **PPC01-PPC10** for *Candida albicans* dihydrofolate reductase (PDB ID: 1AI9)

Compounds	C Score[a]	Crash Score[b]	Polar Score[c]	D Score[d]	PMF Score[e]	G Score[f]	Chem Score[g]
Flucanazole	6.12	-2.43	4.76	-25.709	-38.035	-201.77	-11.08
PPC01	6.35	-0.91	1.19	-101.680	-6.412	-187.612	-17.642
PPC02	7.03	-3.39	0.03	-151.299	27.538	-330.283	-22.817
PPC03	6.23	-1.76	0.01	-106.269	-43.415	-257.610	-19.191
PPC04	4.78	-7.45	0.21	-175.798	45.507	-345.851	-21.530
PPC05	5.13	-3.51	0.01	-137.091	-12.721	-273.537	-16.253
PPC06	5.79	-4.66	0.00	-158.458	26.196	-306.378	-22.519
PPC07	6.05	-5.46	0.02	-169.192	30.734	-339.918	-26.555
PPC08	6.22	-6.18	1.77	-171.586	4.711	-312.986	-22.591
PPC09	7.81	-1.44	0.00	-133.758	2.210	-274.778	-17.349
PPC10	6.78	-2.35	0.00	-147.730	11.838	-314.429	-21.645

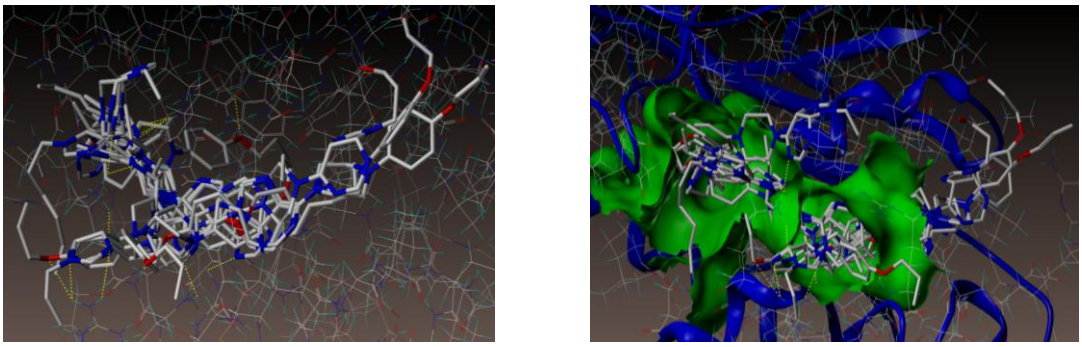

Fig.5 Docked view of PPC01-PPC10 in the active site of the enzyme (PDB ID: 1AI9)

10.1002/cbdv.202000053

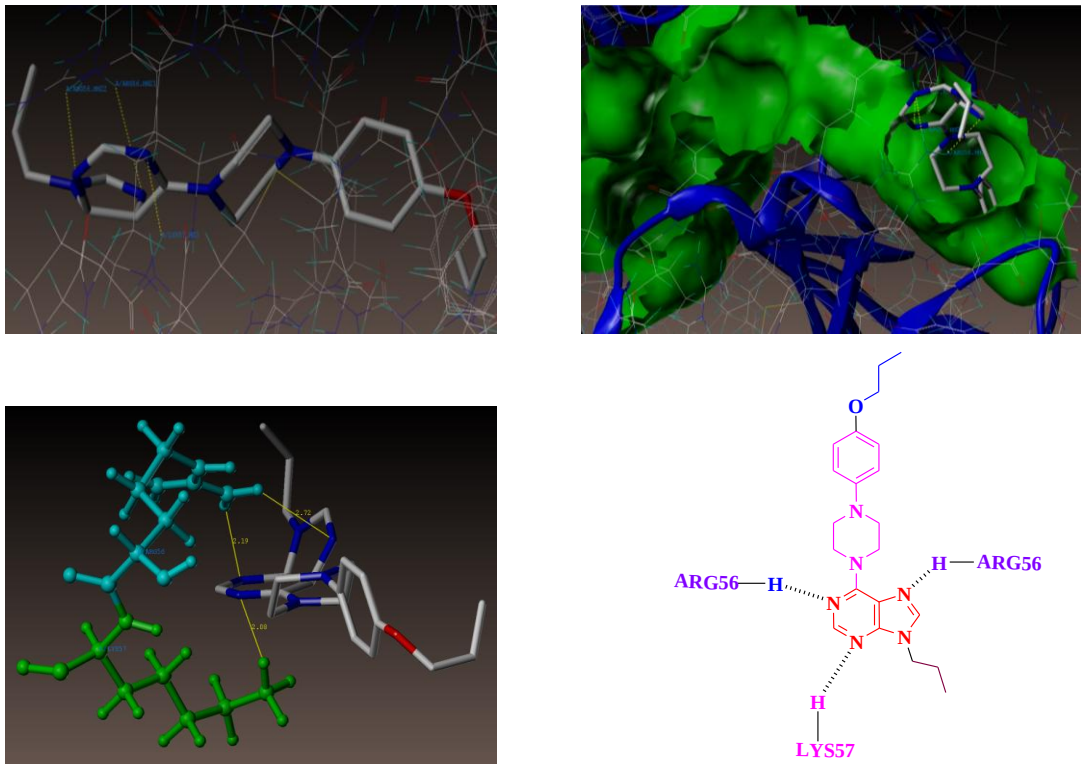

Fig.6 Docked view of **PPC03** in the active pocket of the enzyme (PDB: 1AI9)

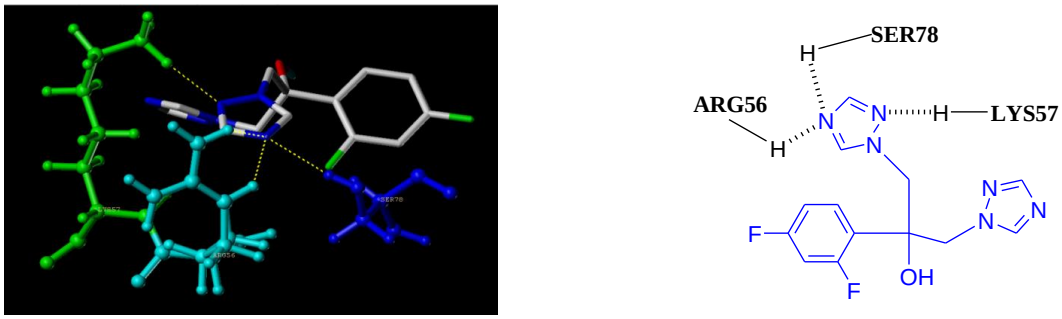

Fig.7 Interaction of Fluconazole at the binding pocket of the enzyme (PDB ID: 1AI9)

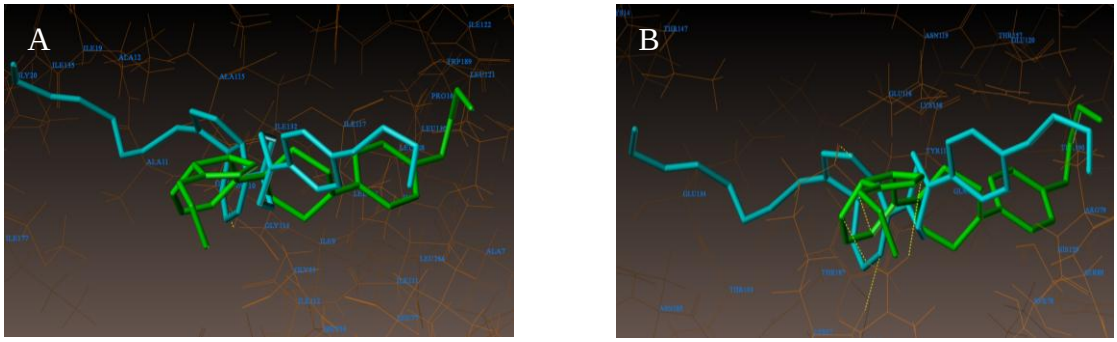

Fig.8 A) Hydrophobic amino acids encircling PPC03 (green colour) and PPC08 (cyan colour)
B) Hydrophilic amino acids encircling PPC03 (green colour) and PPC08 (cyan colour)

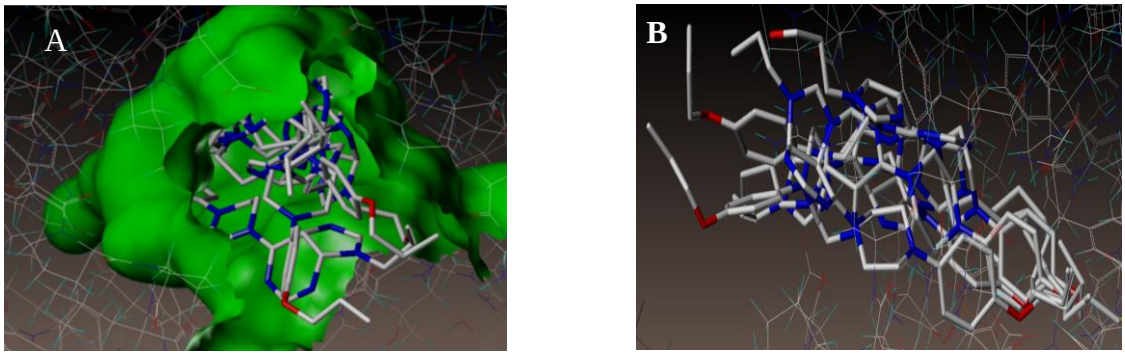

Fig.9 A) Docked view of PPC01-PPC10 in the active site of the enzyme (PDB ID: 2C2T)
B) Interaction of PPC01- PPC10 at the binding pocket of the enzyme (PDB ID: 2C2T)

3. Conclusion

An efficient and simple synthetic protocol was chosen to synthesize novel N-9 alkyl-substituted purines employing mild reaction conditions. The title compounds obtained were characterized by ^{1}H NMR, ^{13}C NMR, FT-IR and EI-MS. A dominant display of antimicrobial activity including antitubercular activity has been shown depending on the length of the alkyl chain. In general, expression of activity is manifested for the compounds with alkyl substituent of 2 to 7 chain length. The title compounds exhibited strong antitubercular activity with MIC similar to the standards used. PPC01-PPC04 appeared better with MIC (3.125-6.25µg/mL) whereas the rest compounds were moderate (MIC 12.5 µg/mL).

The studied compounds showed pronounced antimicrobial activity expressed against bacterial and fungal strains with MIC values lower than the standards used. The molecules with smaller alkyl chain length showed better activity with MIC's lower than the standards while their longer chain counterparts exhibited moderate activity.

The docking study with *M. tuberculosis* enoyl reductase (InhA) depicted that all of the compounds possessed better docking scores, indicating that the molecules occupy similar binding sites as that of pyrrolidine carboxamide and preferentially bind to InhA.

The docking study of the molecules with *Candida albicans* dihydrofolate reductase shows that the synthesized molecules have shown binding interactions with LYS57 and ARG56 as similar to that of fluconazole. Thus, from these studies, the experimental findings can be substantiated, wherein the purine derivatives inhibit DHFR of *Candida albicans* selectively.

The promising activities shown by the title molecules established them as crucial pharmacophores that can be further explored and used as leads to design further novel derivatives with enhanced activity.

4. Experimental Section

4.1. Chemistry

Chemicals and solvents of reagent grade from Sigma Aldrich were used without further purification. The open capillary method was used to record melting points and they are uncorrected. Perkin Elmer FT-IR spectrometer was used for recording FT-IR spectra were recorded on. ^{1}H and ^{13}C NMR data were collected by using JEOL 400-MHz FT NMR spectrometer. $CDCl_3$ was the solvent used. Chemical shifts (δ) are represented in parts per million (ppm) with respect to Tetramethylsilane (TMS) as an internal standard. Mass spectra were obtained by using QP2010S SHIMADZU instrument.

4.2. General procedure for the synthesis of PP2

N-protected 4-hydroxyphenyl piperazine (PP1) (4.0 g, 18.1 mmol) and propyl bromide (2.66 g, 21.72 mmol) were reacted with anhyd. K_2CO_3 (5.0 g, 36.2 mmol). Dry DMF was used as solvent. It was refluxed at 120 ^{0}C at a time period of 12 h. TLC was used to monitor the progress of the reaction. After the completion, the reaction mixture was cooled to room temperature, the unreacted K_2CO_3 was filtered off and the residue was washed with a small volume of solvent. The filtrate was concentrated under reduced pressure to obtain crude solid which was washed with cold water to yield the pure product as white colored solid (PP2) with 80 % yield.

4.3. General procedure for the synthesis of PP3

PP2 obtained in the earlier step (3.5 g, 13.3 mmol) was taken in a round bottom flask containing distilled water (15 mL) and stirred for 5-10 min. To this, 20 mL of HCl (4 N) was added slowly. This reaction mixture was refluxed at 80 ^{0}C for 3 h and the progress of reaction was examined by thin-layer chromatography. After the completion of reaction, the reaction mixture was cooled to 5 ^{0}C and neutralized using aq. NaOH (25%) to get crude solid. Ethyl acetate was used to recrystallize the crude compound to afford pure light brown colored solid as (4-propoxyphenyl) piperazine (PP3) with 82 % yield.

4.4. General procedure for the synthesis of PP4

(4-propoxyphenyl)piperazine (PP3) (4.0 g, 18.17 mmol) and 6-chloro purine (2.8 g, 18.17 mmol) were taken in n-butanol. The reaction mixture was stirred for 5 minutes followed by drop wise addition of triethylamine (5.83 g, 57.64 mmol). On completion of addition, the reaction mixture was refluxed at 120 ^{0}C for 8 h. After the completion of reaction, solvent was evaporated using rota evaporator to obtain the crude solid. This upon recrystallization using ethyl alcohol to get pure C-6 substituted purine (PP4) with 85 % yields.

4.5. General procedure for the synthesis of PPC01-PPC10

C-6 substituted purine (PP4) (500 mg, 1.47 mmol) was taken in dry DMF and anhyd. K_2CO_3 (250 mg, 1.77 mmol) was added. This reaction mixture was stirred at room temperature for 10-15 min and substituted alkyl bromides (160 mg, 1.47 mmol) were added and maintained at room temperature for about 12 h. The completion of reaction was monitored by TLC and the reaction mixture was quenched with ice to obtain crude solid. This was filtered and washed with water to obtain the crude products which were purified by eluting through hexane and ethyl acetate in 80-90% yields.

4.5.1. 9-ethyl-6-(4-(4-propoxyphenyl)piperazin-1-yl)-9*H*-purine (PPC01)

Obtained as colorless solid; Yield: 80%; m.p: 98-100 ^{o}C; FT-IR (cm^{-1}): 3110 (C-H), 2927 (C-C-H),1580 (C=N), 1250 (C-O), 1154 (C-N); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.38 (s, 1 H), 7.78 (s, 1 H), 6.94 (d, *J* 6.8 Hz, 2 H), 6.86 (d, *J* 9.1 Hz, 2 H), 4.47 (s, 4 H), 4.25 (q, *J* 7.3 Hz, 2 H), 3.88 (t, *J* 6.6 Hz, 2 H), 3.19 (s, 4 H), 1.74-1.83 (m, 2 H), 1.52 (t, *J* 7.3 Hz, 3 H), 1.02 (t, *J* 7.4 Hz, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 153.96, 153.82, 152.40, 150.98, 145.51, 138.65, 120.17, 118.81, 115.26, 69.83, 51.26, 45.44, 22.87, 14.35, 10.69; EI-MS: m/z calcd for $C_{20}H_{26}N_6O$ 366.46, found 366 (M^+).

4.5.2. 9-isopropyl-6-(4-(4-propoxyphenyl)piperazin-1-yl)-9*H*-purine(PPC02)

Obtained as colorless solid; Yield: 85%; m.p: 96-98 ^{o}C; FT-IR (cm^{-1}): 3104 (C-H), 2924 (C-C-H),1592 (C=N), 1255 (C-O), 1198 (C-N); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.37 (s, 1 H), 7.74 (s, 1 H), 6.93 (d, *J* 9.0 Hz, 2 H), 6.84 (d, *J* 9.0 Hz, 2 H), 4.46 (s, 4 H), 4.15 (t, *J* 7.2 Hz, 2 H), 3.19 (s, 4 H), 2.86-2.97 (m, 1H) 1.85-1.94 (m, 2 H), 1.10 (d, *J* 6.5 Hz, 6H), 0.95 (t, *J* 7.4 Hz, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 153.85, 153.72, 152.02, 150.65, 145.22, 137.95, 119.32, 117.61, 114.34, 69.86, 51.02, 22.68, 21.47, 10.62; EI-MS: m/z calcd for $C_{21}H_{28}N_6O$ 380.49, found 380 (M^+).

4.5.3. 6-(4-(4-propoxyphenyl)piperazin-1-yl)-9-propyl-9*H*-purine (PPC03)

Obtained as colorless solid; Yield: 82%; m.p: 92-94 ^{o}C; FT-IR (cm^{-1}): 3104 (C-H), 2935 (C-C-H),1615 (C=N), 1265 (C-O), 1158 (C-N); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.37 (s, 1 H), 7.74 (s, 1 H), 6.93 (d, *J* 9.0 Hz, 2 H), 6.84 (d, *J* 9.0 Hz, 2 H), 4.46 (s, 4 H), 4.15 (t, *J* 7.2 Hz, 2 H), 3.87 (t, *J* 6.6 Hz, 2 H), 3.19 (s, 4 H), 1.81-1.95 (m 2 H), 1.73-1.80 (m, 2 H), 1.01 (t, *J* 7.4 Hz, 3 H), 0.95 (t, *J* 7.4 Hz, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 153.93, 153.84, 152.17, 150.99, 145.40, 138.28, 120.10, 118.81, 115.30, 69.97, 51.15, 43.71, 26.76, 22.68, 14.06, 10.77; EI-MS: m/z calcd for $C_{21}H_{28}N_6O$ 380.49, found 380 (M^+).

4.5.4. 9-butyl-6-(4-(4-propoxyphenyl)piperazin-1-yl)-9*H*-purine (PPC04)

Obtained as colorless solid; Yield: 78%; m.p: 90-92 ^{o}C; FT-IR (cm^{-1}): 3100 (C-H), 2926 (C-C-H),1585 (C=N), 1270 (C-O), 1152 (C-N); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.36 (s, 1 H), 7.73 (s, 1 H), 6.93 (d, *J* 9.0 Hz, 2 H), 6.84 (d, *J* 9.1 Hz, 2 H), 4.45 (s, 4 H), 4.16 (t, *J* 7.2 Hz, 2 H), 3.86 (t, *J* 6.6 Hz, 2 H), 3.18 (s, 4 H), 1.74-1.87 (m, 6 H), 1.00 (t, *J* 7.4 Hz, 3 H), 0.86 (t, *J* 7.6 Hz, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 153.93, 153.84, 152.19, 150.81, 145.47, 139.13, 120.33, 118.55, 115.00, 70.07, 50.77, 44.04, 31.98, 26.13, 22.57, 14.06, 10.01; EI-MS: m/z calcd for $C_{22}H_{30}N_6O$ 394.51, found 394 (M^+).

4.5.5. 9-pentyl-6-(4-(4-propoxyphenyl)piperazin-1-yl)-9*H*-purine (PPC05)

Obtained as colorless solid; Yield: 80%; m.p: 90-92^{o}C; FT-IR (cm^{-1}): 3105 (C-H), 2929 (C-C-H), 1592 (C=N), 1260 (C-O), 1154 (C-N); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.38 (s, 1 H), 7.74 (s, 1 H), 6.94 (d, *J* 9.1, 2 H), 6.86 (d, *J* 9.1 Hz, 2 H), 4.47 (s, 4 H), 4.19 (t, *J* 7.2 Hz, 2 H), 3.88 (t, *J* 6.6 Hz, 2H), 3.20 (s, 4 H), 1.81-1.89 (m, 2 H), 1.76-1.79 (m, 2 H), 1.29-1.42 (m, 4 H), 1.02 (t, *J* 7.4 Hz, 3 H), 0.95 (t, *J* 7.4 Hz, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 153.94, 153.84, 152.40, 150.86, 145.52, 138.06, 120.21, 118.83, 115.26, 69.87, 51.37, 38.99, 31.35, 29.26, 22.48, 20.27,15.45, 10.38; EI-MS: m/z calcd for $C_{23}H_{32}N_6O$ 408.54, found 408 (M^+).

4.5.6. 9-hexyl-6-(4-(4-propoxyphenyl)piperazin-1-yl)-9*H*-purine (PPC06)

Obtained as colorless solid; Yield: 85%; m.p: 88-90^{o}C; FT-IR (cm^{-1}): 3070 (C-H), 2925 (C-C-H), 1582 (C=N), 1249 (C-O), 1154 (C-N); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.38 (s, 1 H), 7.74 (s, 1 H), 6.94 (d, *J* 9.1 Hz, 2 H), 6.86 (d, *J* 9.1 Hz, 2 H), 4.47 (s, 4 H), 4.19 (t, *J* 7.2 Hz, 2 H), 3.88 (t, *J* 6.6 Hz, 2 H), 3.20 (s, 4 H), 1.84-1.88 (m, 2 H), 1.74-1.79 (m, 2 H), 1.29-1.40 (m, 4 H), 1.20-1.23 (m, 2 H), 1.02 (t, *J* 7.4 Hz, 3 H), 0.95 (t, *J* 7.4 Hz, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 153.87, 153.77,

152.32, 151.03, 145.37, 138.30, 120.08, 118.50, 115.10, 69.93, 50.84, 44.91, 43.36, 32.02, 29.66, 22.52, 22.02, 13.50, 10.38; EI-MS: m/z calcd for $C_{23}H_{32}N_6O$ 422.57, found 422(M^+).

4.5.7. 9-heptyl-6-(4-(4-propoxyphenyl)piperazin-1-yl)-9*H*-purine (PPC07)

Obtained as colorless solid; Yield: 76%; m.p: 84-86°C; FT-IR (cm^{-1}): 3115 (C-H), 2923 (C-C-H), 1592 (C=N), 1262 (C-O), 1157 (C-N); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.36 (s, 1 H), 7.73 (s, 1 H), 6.92 (d, *J* 9.1 Hz, 2 H), 6.84 (d, *J* 9.1 Hz, 2 H), 4.45 (s, 4 H), 4.16 (t, *J* 7.2 Hz, 2 H), 3.86 (t, *J* 6.6 Hz, 2 H), 3.18 (s, 4 H), 1.86-1.90 (m, 2 H), 1.74-1.80 (m, 2 H), 1.28-1.34 (m, 8H), 1.00 (t, *J* 7.4 Hz, 3 H), 0.87 (t, *J* 7.0 Hz, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 153.99, 153.87, 152.24, 151.24, 145.38, 138.57, 120.05, 118.76, 115.24, 70.12, 51.12, 44.01, 31.98, 31.62, 29.82, 29.78, 28.81, 22.70, 22.22, 13.99, 10.63; EI-MS: m/z calcd for $C_{25}H_{36}N_6O$ 436.59, found 436 (M^+).

4.5.8. 9-octyl-6-(4-(4-propoxyphenyl)piperazin-1-yl)-9*H*-purine (PPC08)

Obtained as colorless solid; Yield: 80%; m.p: 80-82°C; FT-IR (cm^{-1}): 3100 (C-H), 2916 (C-C-H),1588 (C=N), 1242 (C-O), 1182 (C-N); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.36 (s, 1 H), 7.73 (s, 1 H), 6.92 (d, *J* 9.1 Hz, 2 H), 6.84 (d, *J* 9.1 Hz, 2 H), 4.45 (s, 4 H), 4.16 (t, *J* 7.2 Hz, 2 H), 3.86 (t, *J* 6.6 Hz, 2 H), 3.18 (s, 4 H), 1.86-1.92 (m, 2 H), 1.74-1.80 (m, 2 H), 1.42-1.48 (m, 2 H), 1.30-1.36 (m, 8 H), 1.00 (t, *J* 7.4 Hz, 3 H), 0.87 (t, *J* 7.0 Hz, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 153.99, 153.84, 152.24, 151.24, 145.38, 138.57, 120.05, 118.76, 115.24, 70.12, 51.12, 44.01, 31.98, 31.62, 29.82, 29.78, 28.81, 22.70, 22.22, 13.99, 10.63; EI-MS: m/z calcd for $C_{26}H_{38}N_6O$ 450.62, found 450 (M^+).

4.5.9. 9-nonyl-6-(4-(4-propoxyphenyl)piperazin-1-yl)-9*H*-purine (PPC09)

Obtained as colorless solid; Yield: 75%; m.p: 82-84°C; FT-IR (cm^{-1}): 3115 (C-H), 2920 (C-C-H), 1580 (C=N), 1252 (C-O), 1152 (C-N); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.36 (s, 1 H), 7.73 (s, 1 H), 6.92 (d, *J* 9.1 Hz, 2 H), 6.84 (d, *J* 9.1 Hz, 2 H), 4.45 (s, 4 H), 4.16 (t, *J* 7.2 Hz, 2 H), 3.86 (t, *J* 6.6 Hz, 2 H), 3.18 (s, 4 H), 1.88-1.94 (m, 2 H), 1.74-1.80 (m, 2 H), 1.34-1.42 (m, 10 H), 1.25-1.31(m, 2 H), 1.00 (t, *J* 7.4 Hz, 3 H), 0.87 (t, *J* 7.0 Hz, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 153.89, 153.75, 152.24, 151.24, 145.38, 138.57, 120.05, 118.76, 115.24, 70.12, 51.12, 44.01, 31.98, 31.62, 29.82, 29.78, 28.81, 22.70, 22.22, 21.34, 13.99, 10.63; EI-MS: m/z calcd for $C_{27}H_{40}N_6O$ 464.65, found 464 (M^+).

4.5.10. 9-decyl-6-(4-(4-propoxyphenyl)piperazin-1-yl)-9*H*-purine (PPC10)

Obtained as colorless solid; Yield: 74%; m.p: 80-82^{o}C; FT-IR (cm^{-1}): 3125 (C-H), 2910 (C-C-H), 1588 (C=N), 1256 (C-O), 1162 (C-N); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.36 (s, 1 H), 7.73 (s, 1 H), 6.92 (d, *J* 9.1 Hz, 2 H), 6.84 (d, *J* 9.1 Hz, 2 H), 4.45 (s, 4 H), 4.16 (t, *J* 7.2 Hz, 2 H), 3.86 (t, *J* 6.6 Hz, 2 H), 3.18 (s, 4 H), 1.82-1.90 (m, 2 H), 1.74-1.82 (m, 2 H), 1.43-1.49 (m, 2 H),1.31-1.37 (m, 12 H), 1.00 (t, *J* 7.4 Hz, 3 H), 0.87 (t, *J* 7.0, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 153.84, 153.79, 152.24, 151.24, 145.38, 138.57, 120.05, 118.76, 115.24, 70.12, 51.12, 44.01, 31.98, 31.62, 29.82, 29.78, 28.81, 22.70, 22.22, 13.99, 10.63; EI-MS: m/z calcd for $C_{28}H_{42}N_6O$ 478.67, found 478 (M^+).

4.6. Biological Activity

4.6.1. Anti-mycobacterial activity

The synthesized compounds were assessed for their anti-mycobacterial activity against *Mycobacterium tuberculosis* employing microplate Alamar Blue assay (MABA). This methodology is a parallel method to BACTEC radiometric method and is non-toxic. To all outer perimeter wells of sterile 96 wells plate, 200μL of sterile de-ionized water was added to reduce evaporation of media during incubation. 100 μL of the middlebrook 7H9 broth was added to the 96 wells plate and serial dilution of synthesized compounds PPC01-PPC10 was done on the plate. The final concentrations of the title molecules tested were 100 to 0.2 μg/mL. Parafilm was used to cover the plates and the plates were incubated for five days at 37 ºC. After 5 days, 1:1 mixture of Almar Blue reagent and 10% tween 80 were poured onto the plates (25μL, freshly prepared) and incubated for 24 h. The colour of the well was used to interpret the bacterial growth. A blue colour in the well was interpreted as no bacterial growth, and pink colour was scored as growth. The lowest drug concentration at which the color change from blue to pink is prevented is termed as MIC. The Standard Strain used: *Mycobacterium tuberculosis* (Vaccine strain, $H_{37}R_V$ strain): ATCC No- 27294.

4.6.2. Antibacterial activity

The title compounds PPC01-PPC10 were screened for antibacterial activity against four bacterial strains by macro dilution broth method. The synthesized compounds in concentrations of 0.125 - 256 μg/mL respectively were poured into the labeled sterile Mueller Hinton Broth (MHB) medium tubes. 1 mL lag phase cultures of the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus*

subtilis; Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* were inoculated in the MHB tubes. These were incubated for 24 h at 37 ºC followed by which the inhibition of growth of the bacteria was observed in the tubes. The lowest concentration of the synthesized compounds containing tubes which did not show any visible growth of the test bacteria is termed as MIC.

4.6.3. Antifungal activity

PPC01-PPC10 were screened for antifungal activity by macro dilution broth method. The synthesized compounds in concentrations of 0.125 - 256 μg/mL respectively were added to labeled sterile saboraud dextrose broth (SDB) tubes. The lag phase cultures of the fungi *Aspergillus flavus, Trichoderma harzianum, Penicillium chrysogenum* and yeast *Candida albicans* were inoculated in the SDB tubes and incubated for 48 h at 27 ºC. The inhibition of the growth of test fungi in the tubes was observed after 48 h. The lowest concentration of the synthesized compounds containing tubes which did not show any visible growth of the test fungi is termed as MIC.

4.6.4. Cytotoxicity

To evaluate the effects of **PPC01-PPC10** on cell viability, Human Embryonic kidney Cells (HEK293) were employed to perform the cytotoxic assay by MTT method. The measurement in the reduction of yellow colour of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase is measured through this colorimetric assay. 96-well flat-bottom micro plate were seeded with HEK293 cells at a density of about 5×10^3 cells/well and maintained in 95% humidity and 5% CO_2 overnight at 37 °C. The synthesized compounds in six concentrations (600, 300, 150, 75, 37.5, 18.75 μg/mL) were seeded in the cells and incubated for 48 h. After this time, the cells in the well were washed with phosphate buffer solution twice and MTT staining solution (20 μL) was added to each well. The plate was incubated at 37 °C. 100 μL of Dimethyl sulfoxide (DMSO) was poured in each well after 4 h to dissolve the formazan crystals formed and using micro plate reader the absorbance at 570 nm was recorded.

4.6.5. Molecular docking studies

Information regarding 3D arrangement of the target protein was extracted from Protein Data Bank (PDB). Surflex Dock program was employed to examine the complete intermolecular interactions between the ligand and the target protein. A Gasteiger-Huckel charge was used to add polar hydrogen atoms in the proteins for docking and the water molecules were removed. 3D structure

was generated using SKETCH module present in the SYBYL program and Tripos force field was used to obtain its energy-minimized conformation using Gasteiger-Huckel charges. Surflex-Dock program interfaced with Sybyl- X 2.0. was utilized for performing the molecular docking. The default values of the miscellaneous parameters were assigned by the software.

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Author Contribution Statement

AfraQuasar A. Nadaf performed the experiments, analyzed the data and wrote the first draft of the manuscript. Mahesh S. Najare, Manjunatha Garbhagudi and Shivaraj Mantur helped with the interpretation of spectral data of the synthesized compounds. Manjunath G. Sunagar Supreet Gaonkar helped in designing the experiment. Shrinivas Joshi carried out molecular docking work and Imtiyaz Ahmed M. Khazi supervised the whole work along with drafting it. All the authors studied and approved the final draft of the manuscript.

Conflict of interest

Authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Representative spectral data for the synthesized compounds are provided in supporting information.

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