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Synthesis of N-(2-nitrophenyl) pyrazine-2-carboxamide and N-(4-nitrophenyl) pyrazine-2-carboxamide and evaluation of their antimycobacterial properties using *in vitro* and *in-silico* techniques

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ABSTRACT

N-(2-nitrophenyl) pyrazine-2-carboxamide and N-(4-nitrophenyl) pyrazine-2-carboxamide synthesized and studied as an anti-tuberculosis agents using in-silico and in-vitro analysis. In-silico study reveals the inhibition of Mycobacterium tuberculosis mycolic acid cyclopropane synthase CmaA2 (PDB Code : 3HEM) by both compounds. Whereas in-vitro study, reveals the inhibition of M. tuberculosis H37Rv occurred at concentrations ≥32 µg/ml for n-(2-nitrophenyl) pyrazine-2-carboxamide only.

Keywords: Mycobacterium, Tuberculosis, Inhibition, Drug, Enzyme, N-phenyl pyrazine-2-carboxamide

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INTRODUCTION

Tuberculosis (TB), the deadly disease, account for nearly 1.6 million death and 8.8 million new cases each year [1, 2]. There are certain crucial problems in the treatment of TB. The time involved in the treatment and its complexity results in nonadherence to treatment which further leads to the continuous spread of diseases[3, 4]. Secondly, the multi-drug resistance and resistance particularly to *fluroquinolone* and *aminoglycosides* lead higher rates of mortality [5]. Therefore, there is constant need to identify new anti-mycobacterial molecule.

The classes of Mycobacterium are characterized by lipid biosynthesis. They are synthesizes a large number of lipids with unknown function. The sequencing of the *M. tuberculosis* genome has also revealed that a large number of individual enzymes are dedicated to lipid metabolism. These bacteria are also posses at least two distinct sets of pathways for fatty acid biosynthesis, FAS-I barrier against hydrophilic molecules. This well ordered monolayer barrier is likely to account for the high resistance of mycobacterium to toxic substances. The fatty acids synthesized by FAS-I are then converted into the mycolic acids by FAS-II using Cyclopropane-Fatty-Acyl-Phospholipid Synthes 2 Enzymes[6,7].

The main target for anti-mycobacterium molecules is Quinolinic acid phosphoribosyltransferase (QAPRTase), a key enzyme in the de novo biosynthesis of NAD[8]. It is also reported that mycolic acid cyclopropane synthases (PcaA, CmaA1, and CmaA2) play a vital role in site-specific modification of mycolic acid. Inhibition of one or more of these enzymes will definitely provide novel anti-mycobacterium agent[9].

Numbers of small molecules are found active against Mycobacterium tuberculosis. One among them is substituted N-Phenyl Pyrazine-2-Carboxamide based molecules [10].

The aim of the present study is to synthesize substituted N-Phenyl Pyrazine-2-Carboxamide molecules and evaluate its antimycobacterial properties by inhibition it with Cyclopropane-Fatty-Acyl-Phospholipid Synthes 2 Enzymes (PDB Code: 3HEM) using *in vitro* and *in silico* techniques.

EXPERIMENTAL SECTION

Initially, numbers of compounds were designed *in-silico* having the basic structure of N-phenyl pyrazine-2-carboxamide by substituting various functional groups at R1, R2 and R3 positions. Figure 1 shows general structure of N-phenyl pyrazine-2-carboxamide.

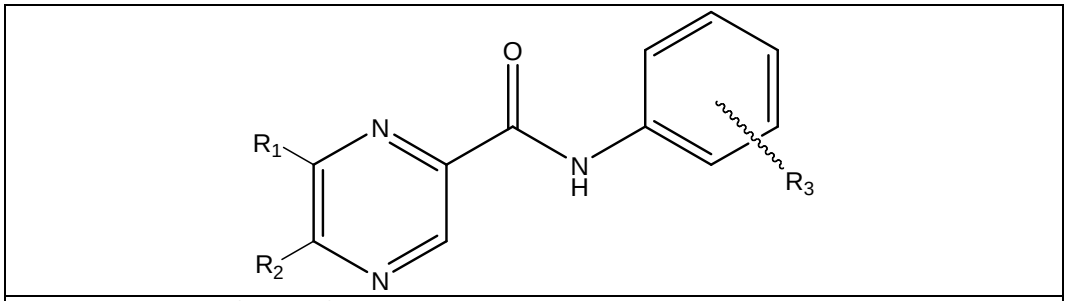


Figure 1. Structural formula of substituted N-Phenyl Pyrazine-2-Carboxamide molecule. (Lead Compound)

Molecular energy minimizations were performed using MM2 techniques [11, 12]. The molecules were designed as per the process provided in *ChemDraw* manual and Ganatra et al.[13-16]. Only those molecules which show very less total energy and achieve global minima are selected for the docking process. Table 1 lists the selected molecules for docking study.

Table 1. List of molecules designed along with groups at R1, R2 and R3

S. No.	Molecules	R1	R_2	R_3
1	$C_{11}H_9N_3O$	-H	-H	-H
2	$C_{11}H_7Cl_2N_3O$	-H	-Cl	*P*-Cl
3	$C_{11}H_7Cl_2N_3O$	-H	-H	*m*-Cl, *p*-Cl
4	$C_{11}H_8Cl_3O$	-H	-H	*P*-Cl
5	$C_{15}h_{16}cln_3o$	-H	*Tert-Butyl*	*P*-Cl
6	$C_{11}H_8N_4O_3$ Synthesized	-H	-H	*O*-NO_2
7	$C_{11}H_8N_4O_3$ Synthesized	-H	-H	*P*-NO_2

Target enzyme Mycobacterium tuberculosis mycolic acid Cyclopropane synthase CmaA2 (PDB Code : 3HEM) downloaded from protein database[17-18]

Using ligand explorer, ligand interaction studies performed to understand the interaction of natural inhibitor within the active site of enzyme[22]. It is reported that this enzyme complex is having two natural inhibitors. N-octyloctan-1-amine (Dioctylamine) and carbonate ion (CO_3^{--}). Dioctylamine is in active pocket of enzyme and showing hydrophobic interaction with atoms of nearby amino acids.

Figure 2 shows the graphical representation of hydrophobic interaction between Dioctylamine and surrounding amino acids and table 2 list the observed possible hydrophobic interactions.

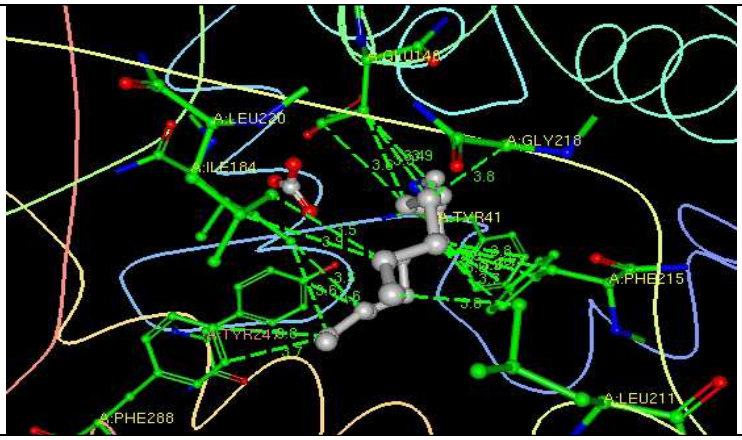


Figure 2. Hydrophobic interaction of Dioctylamine (Natural Inhibitor) with amino acids in active sites of *Mycolic acid cyclopropane synthase CmaA2* (PDB Code : 3HEM).

Table 2. Hydrophobic interaction of Dioctylamine (Natural inhibitor) with amino acids in active sites of Mycolic acid cyclopropane synthase CmaA2 (PDB Code : 3HEM).

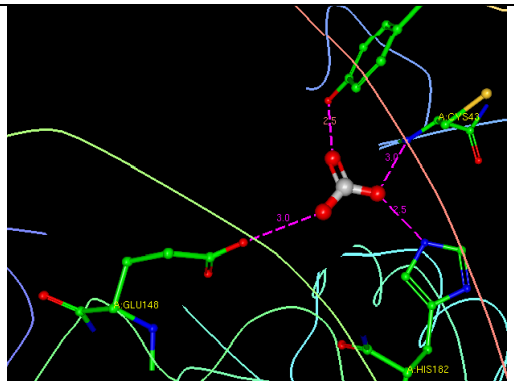
Natural Inhibitor : N-octyloctan-1-amine (Dioctylamine)		
Molecular Formula : : C_{16} $H_{35}N$		
Property : Hydrophobic Interaction		
Atom of Natural Inhibitor	Atom of amino acid of enzyme	Distance
A:303:D22:C2	A:220:LEU:CG	3.9
A:303:D22:C2	A:220:LEU:CD2	3.5
A:303:D22:C3	A:215:PHE:CB	3.3
A:303:D22:C3	A:215:PHE:CG	3.7
A:303:D22:C3	A:215:PHE:CD1	3.7
A:303:D22:C4	A:148:GLU:CB	3.4
A:303:D22:C4	A:218:GLY:CA	3.8
A:303:D22:C5	A:148:GLU:CB	3.3
A:303:D22:C5	A:148:GLU:CG	3.6
A:303:D22:C6	A:148:GLU:CB	3.9
A:303:D22:C16	A:184:ILE:CD1	3.6
A:303:D22:C16	A:288:PHE:CE1	3.7
A:303:D22:C16	A:288:PHE:CZ	3.8
A:303:D22:C15	A:184:ILE:CD1	3.5
A:303:D22:C14	A:247:TYR:CZ	3.6
A:303:D22:C11	A:41:TYR:CD1	3.9
A:303:D22:C11	A:41:TYR:CE1	3.6
A:303:D22:C11	A:41:TYR:CE2	3.8
A:303:D22:C11	A:41:TYR:CZ	3.6
A:303:D22:C11	A:215:PHE:CZ	3.9
A:303:D22:C1	A:211:LEU:CD2	3.8

Selected enzyme also shows the presence of carbonate ion. The ligand explorer study reveals the possibilities of four hydrogen bonds between carbonate ion and surrounding amino acids in active site[22]. Besides hydrogen bonding, there is also the possibility of hydrophobic interactions. Table 3 shows the list of possible hydrogen bonding and figure 3 shows the graphical representation of hydrogen bonding and hydrophobic interactions between carbonate ion and amino acids in active site.

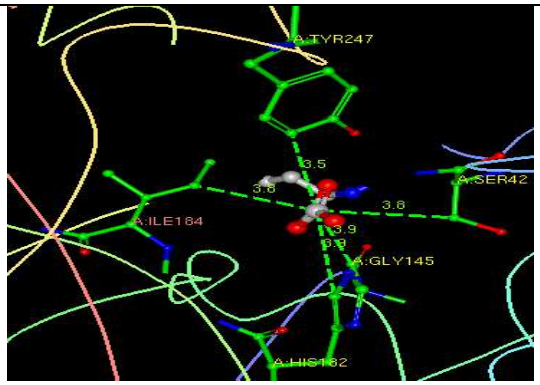
Table 3. Hydrogen bonding between the various oxygen atoms of carbonate ion and atoms of amino acids in active sites of Mycolic acid cyclopropane synthase CmaA2 (PDB Code : 3HEM).

Atom of carbonate ion	Atom of amino acid in active site	Distance A°
CO3:O1	A:247:TYR:OH	2.5
CO3:O2	A:148:GLU:OE2	3.0
CO3:O3	A:43:CYS:N	3.0
CO3:O3	A:182:HIS:NE2	2.5
Note : 'A' stands for Chain A of Enzyme		

This is clearly indicates that carbonate ion play a vital role in enzymatic activities by holding four amino acids in their respective positions.



Hydrogen bonds



Hydrophobic interaction

Figure 3. Hydrogen bonds and hydrophobic interactions between carbonate ions and amino acids in active site of Mycolic Acid Cyclopropane Synthase CmaA2 (PDB Code : 3HEM).

Designed molecules were docked with the 3HEM enzyme using autodock version 4.0. The position of natural inhibitor was selected as the centre of active site and it was removed before docking the ligand.

Table 4 shows the obtained binding energy from docking study. It is reported that molecule number 6 and 7 shows higher binding energy than natural inhibition.

Table 4. The binding energy of docked molecules

S. No.	Molecule	ΔG Kcal/mol.
1	$C_{11}H_9N_3O$	-9.1634
2	$C_{11}H_7Cl_2N_3O$	-9.3775
3	$C_{11}H_7Cl_2N_3O$	-9.0016
4	$C_{11}H_8ClN_3O$	-8.4074
5	$C_{15}H_{16}ClN_3O$	-9.9826
6	$C_{11}H_8N_4O_3$	-12.8654
7	$C_{11}H_8N_4O_3$	-12.1622
8	N-octyloctan-1-amine (Dioctylamine) *Natural inhibitor*	-11.307

The docking study also provides information related to the possible hydrogen bonds between ligand and amino acid of enzyme. The same is reported in table 5.

Table 5. Docking results of selected *N-Phenyl Pyrazine-2-Carboxamide* based molecules with Cyclopropane-Fatty-Acyl-Phospholipid Synthes 2 Enzymes(PDB Code : 3HEM)

Mol. No.	Molecular Formula	Binding Energy in Kcal/mol.	Total Hydrogen bonding	Amino Acids involved in Hydrogen bonding	Hydrogen bonding distance in A^0
1.	$C_{11}H_9N_3O$	-9.1634	--	--	--
2.	$C_{11}H_7Cl_2N_3O$	-9.3775	--	--	--
3.	$C_{11}H_7Cl_2N_3O$	-9.0016	--	--	--
4.	$C_{11}H_8ClN_3O$	-8.4074	--	--	--
5.	$C_{15}H_{16}ClN_3O$	-9.9826	--	--	--
6.	$C_{11}H_8N_4O_3$ *Synthesized*	-12.8654	4	280TYR, 280TYS, 224TYS, 41TYS	2.9942, 2.99, 2.756, 2.88
7.	$C_{11}H_8N_4O_3$ Synthesized	-12.1622	-	--	--
8.	Natural inhibitor	-11.307	--	--	--

The binding energy values for all docked molecules vary from -12. 86 to - 8.40 kcal/mol. This depends upon the steric, electrostatic and hydrophobic interaction properties of the molecules with binding site of enzymes. The binding energy of molecule number 6 and 7 is better than natural inhibitor. Also it is reported that these molecules fits in the binding site and shows number of possible confirmations. Figure 4 shows the complex of natural inhibitor with binding site of 3HEM. Figure 5 and 6 show the molecule 6 and 7 complexes with binding site of 3HEM respectively.

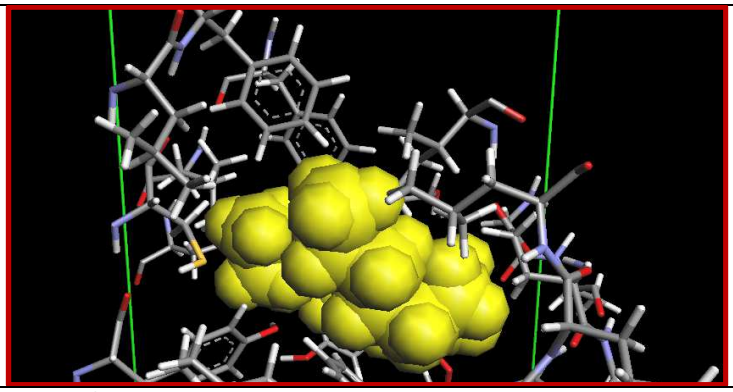


Figure 4. Docking of N-octyloctan-1-amine (natural inhibitor) with 3HEM enzyme within active site.

The docking of compound number 6 reports the presence of four hydrogen bonds. $–NO_2$ groups form the hydrogen bonds with the amino acids 280TYR, 280TYS, 224TYS and 41TYS. Figure 3 shows the docking picture of molecule 7 in the active site of enzyme.

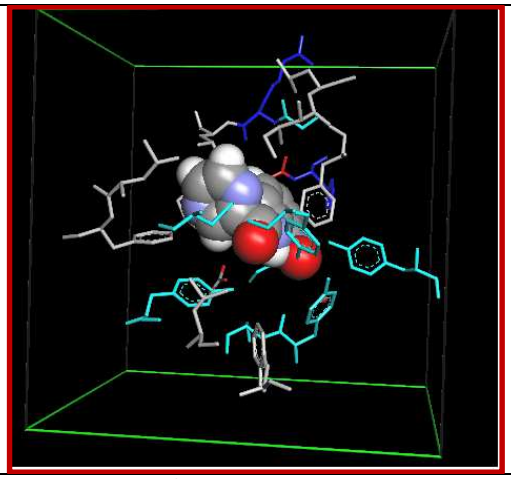


Figure 5. Docking of compound no. 6 with 3HEM enzyme

The figure 6 shows docking picture of molecule number 7 with enzyme.

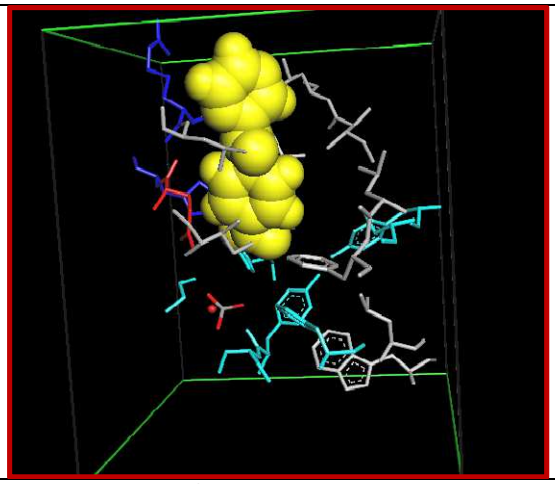


Figure 6. Docking of compound no. 7 with 3HEM enzyme

Synthesis of compounds

N-(2-nitrophenyl) pyrazine – 2-caboxamide (compound no. 6) and N-(4-nitrophenyl) pyrazine – 2-caboxamide (compound no. 7) synthesized and checked for their biological activities against M. tuberculosis.

Synthesis of N-(2-nitrophenyl) pyrazine – 2-caboxamide

A mixture of acid, *i.e.* pyrazinecarboxylic acid, (0.05 mol) and thionyl chloride (5.5 ml, 75.0 mmol) in dry benzene (20 ml) was refluxed for about 1 hour. Excess of thionyl chloride was removed by repeated evaporation with dry benzene *in vacuum*. The crude acyl chloride dissolved in dry acetone (50 ml). It was added drop wise to a stirred solution of o-nitro aniline (50.0 mmol) and dry pyridine (50.0 mmol) in 50 ml keeping the mixture at the room temperature. Stirred the mixture for the next 30 minutes. Poured mixture in 200 ml of cold water and the crude was collected and re-crystallized from aqueous ethanol.

Obtained yield was 85%, molecular formula: $C_{11}H_8N_4O_3$, molecular weight 244.21 m/e , melting point 95°C, and compound is soluble in diethyl ether, acetone and chloroform.

Spectral Analysis: IR (KBR) cm^{-1} ; 3349.3 (NH) ,1619.4 (CO) ,740.7 (Substituted benzene) ^1H NMR (300 MHz, $CDCl_3$) ∂ 7.9 (d, 1H,), 7.8 (d, 1H,), 7.28 (s, 1H, NH), 7.24 (s, 1H,), 7.0 (d, 1H) , 6.9 (d, 1H) , 6.49 (d ,1H,), 6.51(d,1H) ^{13}C NMR (75 MHz, $CDCl_3$) ∂ 145.9, 135.2, 130.4, 125.1, 116.9, 115.2, 39.5, 39.3, 39.1, 38.9, and 38.75.

Compound 7 : N-(4-nitrophenyl) pyrazine – 2-caboxamide

A mixture of acid, *i.e.* pyrazinecarboxylic acid, (0.05 mol) and thionyl chloride (5.5 ml, 75.0 mmol) in dry benzene (20 ml) was refluxed for about 1 hour. Excess of thionyl chloride was removed by repeated evaporation with dry benzene *in vacuum*. The crude acyl chloride dissolved in dry acetone (50 ml).

The crude acyl solution in dry acetone was added dropwise to a stirred solution of p-nitro aniline (50.0 mmol) and dry pyridine (50.0 mmol) in 50 ml keeping the reaction mixture at the room temperature. After the addition was complete, stirring continued for the next 30 min. Reaction mixture was poured into 200 ml of cold water and the crude amide was collected and recrystallized from aqueous ethanol.

The yield was 89 %, molecular formula: $C_{11}H_8N_4O_3$, molecular weight 244.21, melting point 142° C and solubility is in diethyl ether, acetone and chloroform.

IR (KBR) cm^{-1} ; 3362.1 (NH) ,1632.1 (CO) , 842.6(Substituted benzene). ^{1}H NMR (300 MHz, $CDCl_3$) ∂ 7.70, (d, 1H,) , 7.60 (d, 1H,), 6.42 (d, 1H,), 6.40 (d, 1H,), and 7.15 (s, 1H, NH,). ^{13}C NMR (75 MHz, $CDCl_3$) ∂ 145.9, 135.2, 130.4, 125.1, 116.9, 115.2, 39.5, 39.3, 39.1, 38.9, and 38.75.

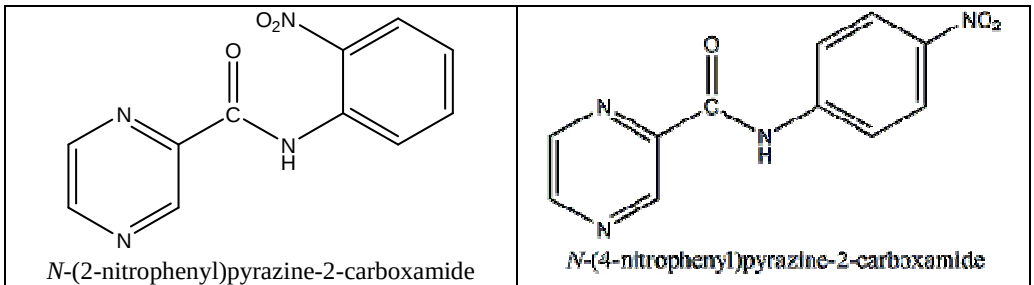
Purification of compound I and compound II

1 gm. of crude product taken in a small beaker, into it 10 ml ethanol was added, content was heated in water bath up to dissolved most of the crude product. Filtrate was obtained while filtering in hot condition, concentrating the filtrate by further heating and by allowing the filtrate to cool, crystals of product start appearing. The crystals was allowed to grow in size, filter the crystal.

Drying- The recrystallised substance was dried by using vacuum desiccators. The recrystallised substance was spread over a watch glass and would be kept in vacuum desiccator.The substance was allowed to stay under these conditions for several hours and then the vacuum was released very slowly.

Storage- Two compounds are to be stored in two vials of glass.

The structure and IUPAC names of synthesized compounds 6 and 7 are shown in figure 7.



N-(2-nitrophenyl)pyrazine-2-carboxamide	*N*-(4-nitrophenyl)pyrazine-2-carboxamide
Compound 6: N-(2-nitrophenyl)pyrazine-2-carboxamide	Compound 7: N-(4-nitrophenyl)pyrazine-2-carboxamide

Figure 7. Structural formula and IUPAC names of synthesized compound 6 and 7.

In-vitro analysis

The synthesized compounds were analyzed for their biological activities using in-vitro analysis. These compounds were tested at lower concentration against M. tuberculosis H37Rv [23,24] to determine the MIC testing by micro dilution assay/ REMA method. N-(2-nitrophenyl) pyrazine-2-carboxamide(compound 6) found active with 32 µg/ml. concentration, whereas activity of N-(4-nitrophenyl) pyrazine-2-carboxamide (compound 7) reported negative.

CONCLUSION

Doležal *et al.* [10] has extensively studied substituted N-phenyl pyrazine-2-carboxamide as an anti-mycobacterial agent. The approach was extend in our work by synthesizing substituted N-phenyl pyrazine-2-carboxamide by replacing R3 by -*o*-NO_2(compound 6) and R3 by -*p*-NO_2 (compound 7). These compounds are newly synthesized and it is reported that substitution at R3 by -*o*-NO_2 makes compound biological active against tuberculosis. It is reported that the substitution at R1 by –H, R2 by –H and R3 by -*o*-NO_2 provides better binding capacity to N-phenyl pyrazine-2-carboxamide. It also shows four hydrogen bonding in docking study, whereas the substitution at R3 by -p-NO_2 reverse the activity and do not show any hydrogen bond.

Hence it is evident from this study that substitution at R1 by –H, R2 by –H and R3 by -*o*-NO_2 positions on N-phenyl pyrazine-2-carboxamide provides antimycobacterial properties to the compound.

The in-silico study also supports the finding from in-vitro study, still it cannot be confirmed from this study that the substituted N-phenyl pyrazine-2-carboxamide inhibits Cyclopropane-Fatty-Acyl-Phospholipid Synthes 2 Enzymes. Study supports the possibilities of inhibition, but it requires cellular level study to confirm the same.

Hence, it can be concluded that the N-phenyl pyrazine-2-carbaxamide based molecules with substitution at R1 by –H, R2 by –H and R3 by *o-*NO_2 and related electron withdrawing groups are potent therapeutic agents for M. Tuberculosis.

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REFERENCES

[1] Global Tuberculosis Control: Surveillance, Planning, Financing, *WHO report 2006*. Geneva: WHO, **2006,** (WHO/HTM/TB/2006.362).
[2] C Dye; S Scheels; P Dolin; V Pathania; MC Raviglion. WHO Global Surveillance And Monitoring Project, *JAMA*. **1999**, 282(7), 677-86.
[3] ED Chan and MD Iseman. *Current Medical Treatment for Tuberculosis*, BMJ, **2002,** 325, 1282-1286.
[4] J Volmink and P Garner. Directly Observed Therapy for Treating Tuberculosis. *Cochrane Database Syst*. **2007**.
[5] Multidrugresistant Tuberculosis (Mdr-Tb) Indicators, *World Health Organization (WHO)*, **2010.**
[6] TS Balganesh; V Balasubramanian and S Anand Kumar, *Current Science*,**2004**,86(1),167-176.
[7] NB Holmgren; I Millman; GP Youmans, *J Bacteriol.* **1954,** 68(4),405-10.
[8] MR Patle; SH Ganatra. *Asian Journal of Research in Chemistry*, **2011**, 4(6), 990-996.
[9] Sharma V, Grubmeyer C, Sacchettini, *Structure*, **1998,** 6(12), 1587-99.
[10] M Doležal et al., *Molecules*, **2010,** 15(12), 8567-81.
[11] DA McQuarrie, Quantum Chemistry. 2nd Edition, University Science Books, Michigan, **2007**. ISBN-10: 1891389505, ISBN-13: 978-1891389504.
[12] C Cohen-Tannoudji ; B Diu; F Laloe, Quantum Mechanics Volumes I, 1st Edition, Wliey, **1991**, ISBN-10: 047116433X, ISBN-13: 978-0471164333.
[13] SH Ganatra; MN Bodhe, *Journal of Computer Science & Systems Biology*, **2013**,
[14] SH Ganatra; AS Suchak, *Journal of Computer Science & Systems Biology*, **2013**, 5(3), 068-078.
[15] HV Sanghani; SH Ganatra; R Pande. *Journal of Computer Science & Systems Biology*, **2012.**
[16] SH Ganatra; MR Patle; GK Bhagat. *Asian Journal of Research in Chemistry*, **2012**,5(10), 1159-1159.
[17] D Barkan, D et al., "Structure of Mycobacterium tuberculosis Mycolic Acid Cyclopropane Synthase CmaA2 in Complex with Dioctylamine" (3HEM), *Protein Data Bank*,**2009**.
[18] HM Berman et al., *Nucleic Acids Research*, **2000**, 28(1), 235-242.
[19] GM Morris et al., *J Computational Chemistry*, **1998**, 1639-1662.
[20] GM Morris et al., *J. Comput. Chem*., **2009**, 30, 2785-279.
[21] GE Terp; BN Johansen; IT Christensen; FS Jorgensen, *J Med Chem.* **2001,** 44(14), 2333-43.
[22] JL Moreland et.al., *BMC Bioinformatics*, **2005**, 6(21),1-7.
[23] "Mycobacterium tuberculosis". Sanger Institute. **2007**-03-29.
[24] JC Camus et al.,*Microbiology*, **2002**,148, 2967-2973.