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Synthesis, Characterization and Biological Applications of Benzohydrazide derivatives

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Abstract
Using aroyl hydrazides as a source of amine and substituted aromatic aldehydes as a carbonyl source, we have synthesized aroyl hydrazide derivatives by condensation reaction. The synthesized compounds were characterized by FT-IR, ^{1}H-NMR and ^{13}C-NMR spectral studies. The Synthesized compounds were subjected to antimicrobial and anti-oxidant activities. Finally, molecular docking study had been performed by *in silico* method to analyse their anti-tuberculosis aspect against InhA, the enoyl acyl carrier protein reductase (ENR) from Mycobacterium tuberculosis.

Keywords: Benzohydrazide, Antimicrobial, Tuberculosis, Molecular docking, 2NSD

Introduction
There has been a continual battle between humans and the multitude of microorganisms that cause infection and disease. One third of the world's population is thought to have been infected with *Mycobacterium tuberculosis* and new infections occur at a rate of one per second. World Health Organization estimates that 30 million people will be infected within next 20 years [1]. During recent years, *M. tuberculosis* and microorganisms increased resistance against drugs. When the microorganisms become resistant to anti-mycobacterial drugs they are often referred to as "superbugs". This is a major concern because a resistant infection may kill, can spread to others, and imposes huge costs to individuals and society. Therefore there is a need to develop new, potent, maximum effectiveness and fast acting anti-mycobacterial drugs with low toxicity.

Molecular docking plays an important role in the rational drug design. It is used to determine and predict the interaction of two molecules and to find the best orientation of small molecule, known as ligand which would form a complex with overall minimum energy to target protein. The ligand usually fits within protein's cavity which is predicted by the search algorithm. These protein cavities become active when they contact with any external compounds and thus are called as active sites. The results are analyzed by a statistical scoring function which converts interacting energy into numerical values called as the docking score; and also the interacting energy is calculated. The 3D pose of the bound ligand can be visualized using different visualizing tools like Pymol, Rasmol etc., which could infer the best fit of ligand. Predicting the mode of protein ligand interaction can assume the active site of the protein molecule and further help in protein annotation.

Hydrazone nuclei containing compounds are considered as an important class in the field of medicine and pharmaceutical. They show various biological activities like antimicrobial [2], antidepressant, analgesic [3], anti-inflammatory [4], antitumour [5], anti-HIV [6], anticancer [7], anticonvulsant [8] antitubercular [9], anticancer [10], antimalarial [11] activities etc., Therefore, in the present study, biologically active aroyl hydrazide Schiff base compounds were synthesized and were screened for antibacterial, antioxidant activities and molecular docking studies.

Materials and methods

All anhydrous solvents and reagents of AR grade (Sigma-Aldrich and Merck products) were obtained from commercial suppliers and used without any further purification unless and otherwise noted. All the reactions were carried out at room temperature. Melting points were determined by open capillary and were uncorrected. FT-IR spectral measurements were made for the synthesized compounds using Perkin Elmer Spectrum-1 FT IR spectrometer in 4000-400 cm^{-1}. NMR analysis were recorded with Bruker NMR spectrometers operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}CNMR usingDMSO-d_6 as a solvent and TMS as a internal reference.

General synthesis of benzohydrazide derivatives

To an equimolar mixture of benzohydrazide(1.36g, 0.01mol) and 5-bromo-2-hydroxybenzaldehyde(2.01g, 0.01mol) in 10 mL water, three drops of con. hydrochloric acid was added with stirring for 30 min at room temperature. Insoluble solid was gradually generated, filtered and washed with petroleum ether(40-60%) and dried in vacuum desiccator. The crude solid was recrystallised from absolute ethanol.

Antimicrobial Activity

Antimicrobials were one of most significant weapons against microbial infections. Standard sterilized filter paper disks (5 mm diameter) impregnated with a solution of the test compound in DMSO (1 mg/ml) was placed on agar plate seeded with the appropriate test organisms. All the synthesized compounds were tested for their *in vitro* growth inhibitory activity against *S. aureus* as gram positive and *E. coli* as gram negative bacterial strains and *in vitro* antifungal potential against *A. niger* strain by agar well diffusion method [12] at a concentration level of 100 µg/mL. The plates were incubated at 37 °C for 24 hrs for bacteria and 48 hrs for fungi. Erythromycin and Gentamycin were used as standard drug at a concentration of 100 µg/mL. Nutrient agar was used as a culture media and DMSO was used as a solvent. The results obtained were tabulated.

Estimation of Anti-oxidant effect by DPPH method

One of the quick methods to evaluate antioxidant activity is the scavenging activity on DPPH (2,2-diphenyl-1-picryl hydrazyl). The $DPPH^{\bullet}$ was a stable free radical which surveys the antioxidant potential of pure compounds by an easy colorimetric method. Accepting a hydrogen atom from the antioxidant scavenger compound, $DPPH^{\bullet}$ turns to $DPPH_2$ and the change in color from purple to yellow was screened spectrophotometrically at 516 nm [13].

4.3mg of DPPH was dissolved in 3.3 ml methanol was used as a blank solution and it was protected from light by covering the test tubes with aluminum foil. Required quantity of standard solution of ascorbic acid and synthesized samples were dissolved in methanol separately to give the concentration of 20, 40, 60 and 80 µg/ml. 150µL of DPPH solution was added to 3 ml methanol and absorbance was taken immediately at 516 nm for blank reading. 150 µL DPPH solution was added to each concentration of standard and sample solutions. Their absorbance values were taken at 516nm using UV-Visible perkin Elmer spectrophotometer [14].

The free radical scavenging activity (% antiradical activity) was calculated using the following equation:

$$\text{Free radical scavenging \%} = \frac{A-B}{A} \times 100$$

Where, A = Absorbance of the blank
B = Absorbance of the sample

All the tests and analyses were performed with three triplicates and the results were averaged.

Molecular docking studies

To predict the anti-tuberculosis data on a structural basis, automated docking studies were carried out using Discovery studio software program. 3D crystal structure of the enzyme reported in this work was downloaded from the Protein Data Bank (PDB id: 2NSD) and was processed by the addition of hydrogen, assigning the bond order, identifying overlaps, creating zero order bonds to metals and creating disulphide bonds. The co-factors, unwanted water molecules and chains were deleted. Then energy minimization was done followed by grid generation. The 2D structures of the synthesized ligands were drawn from Chem Draw. After the generation of the grid, the ligands synthesized were docked to identify the interaction with the active site of the protein using LibDock module in the Discovery Studio Accelrys software [15]. The scoring functions and hydrogen bonds formed with the surrounding amino acids were used to predict their binding modes, their binding affinities and orientation of these compounds at the active site of *Mtb* InhA protein.

Results and Discussion

Benzohydrazide derivatives were synthesized via condensation reaction by Schiff base route. The nucleophilic displacement of acid hydrazides with aromatic aldehydes resulted in the products. The reaction was performed employing various acid catalysts (HCl, H_2SO_4, HNO_3, formic acid and acetic acid (Table 1)) and different types of solvents (water, methanol, ethanol, toluene and 1,4-dioxane) (Table 2)) at room temperature. Using con. HCl as a catalyst and water as a solvent gave fascinating results (Table 3). Finally the structures of the synthesized compounds were characterized and confirmed by FT-IR, ^{1}H NMR, ^{13}C NMR spectral studies.

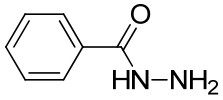
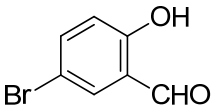
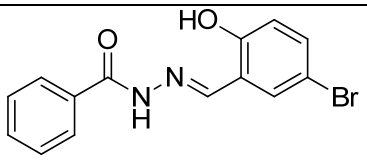
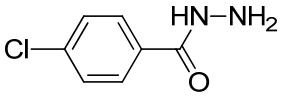
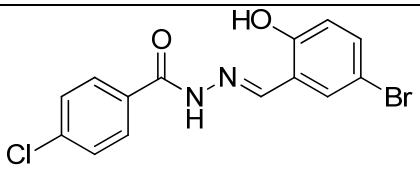
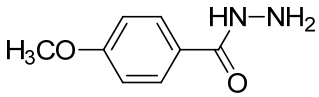
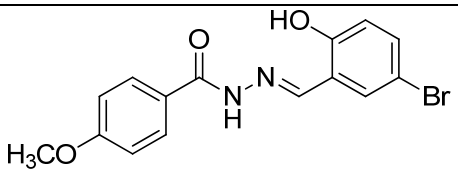
Table 1: Effect of catalyst on reaction of benzohydrazide with 5-bromo-2-hydroxybenzaldehyde

Entry	Catalyst	% Yield of B4
1	HCl	96
2	H_2SO_4	92
3	HNO_3	80
4	HCOOH	40
5	CH_3COOH	45

Table 2: Effect of solvents on reaction of benzohydrazide with 5-bromo-2-hydroxybenzaldehyde

Entry	Solvents	% Yield of B4
1	water	96
2	methanol	85
3	ethanol	90
4	1,4-dioxan	60
5	acetonitrile	65

Table 3: List of products and their corresponding reactants

Sl. No	Benzohydrazide(1a-3a)	5-bromo-2-hydroxybenzaldehyde	Products	Yield (%)
1	 1a	 2b	 B4	96
2	 2a	2b	 P4	94
3	 3a	2b	 J4	95

1. (E)-N'-(5-bromo-2-hydroxybenzylidene) benzohydrazide (B4): was derived from benzohydrazide and 5-bromo-2-hydroxybenzaldehyde (1:1) Yield: 96%.
FT-IR: (ν in cm^{-1}) 3439 (OH) 3219 (NH), 3057 (Ar CH), 1644 (C=O), 1575 (C=N).
^{1}H NMR δ in ppm (300 MHz, DMSO-d_6): 12.1 (s, 1H, Ar-OH), 11.3(s, 1H, enolizable NH proton), 8.6 (s, 1H, CH=N), 7.9 (d, 2H, o-ArH), 7.7 (d, 1H, *o*-ArH bromine ring), 7.6 (t, 1H, *p*-ArH), 7.5 (t, 2H, *m*-ArH), 7.4 (q, 1H, *p*-ArH bromine ring), 6.9 (d,1H, *m*-ArH bromine ring).
^{13}C NMR δ in ppm (100 MHz, DMSO-d_6) 162 (CO), 156 (*o*-ArC bromine ring) 145 (-CH=N azomethine), 131,130,128,127 (6C Phenyl ring), 133, 132, 121, 118, 110 (5C Ar bromine ring).

***(*E)-N'-(5-bromo-2-hydroxybenzylidene)-4-chlorobenzohydrazide (P4):** was derived from 4-chlorobenzohydrazide and 5-bromo-2-hydroxybenzaldehyde (1:1) Yield: 94%.
FT-IR: (ν in cm^{-1}) 3434 (OH) 3218 (NH), 3067 (Ar CH), 1647 (C=O), 1601 (C=N).
^{1}H NMR δ in ppm (300 MHz, DMSO-d_6): 12.2 (s, 1H, Ar-OH), 11.2(s, 1H, enolizable NH proton), 8.9 (s, 1H, -CH=N), 7.9 (d, 2H, o-ArH chlorine ring), 7.7 (d, 1H, *o*-ArH bromine ring), 7.6 (d, 2H, *m*-ArH chlorine ring),7.4 (q, 1H, *p*-ArH bromine ring), 6.9 (d,1H, *m*-ArH bromine ring).
^{13}C NMR δ in ppm (100 MHz, DMSO-d_6) 161 (CO), 156 (*o*-ArC bromine ring) 145 (-CH=N azomethine), 136,130,129,128 (6C chlorine ring), 133, 131, 121, 118, 110 (5C Ar bromine ring).

(E)-N'-(5-bromo-2-hydroxybenzylidene)-4-methoxybenzohydrazide (J4): was derived from 4-methoxybenzohydrazide and 5-bromo-2-hydroxybenzaldehyde (1:1) Yield: 95%.
FT-IR: (ν in cm^{-1}) 3612 (OH) 3307 (NH), 2650 (Ali CH), 1690 (C=O), 1603 (C=N).
^{1}H NMR δ in ppm (300 MHz, DMSO-d_6): 12.0 (s, 1H, Ar-OH), 11.3(s, 1H, enolizable NH proton), 8.5 (s, 1H, -CH=N), 7.9 (d, 2H, o-ArH methoxy ring), 7.7 (d, 1H, *o*-ArH bromine ring), 7.4 t, 1H, *p*-ArH bromine ring),7.0 (q, 2H, *m*-ArH methoxy ring), 6.9 (d,1H, *m*-ArH bromine ring), 3.8 (s, 3H, ArOCH3).
^{13}C NMR δ in ppm (100 MHz, DMSO-d_6) 162.3 (CO), 162.2 (*o*-ArC bromine ring), 156.3(*p*-ArC methoxy ring) 145 (-CH=N azomethine), 133 (*p*-ArC bromine ring),130.5 (*o*′-ArC bromine ring), 129.6 ((*o*-ArC methoxy ring), 124.6 (Ar C-C methoxy ring), 121.2 (Ar C-C bromine ring), 118.6 (*m*-ArC bromine ring), 113.7(*m*-ArC methoxy ring), 110.3 (*m*′- ArC bromine ring), 55.3 (OCH3).

Antimicrobial activity
The synthesized compounds were screened for their in vitro antimicrobial activities against *S. aureus*, *E. coli* and *A. Niger* by minimum inhibitory concentration method. The antimicrobial activities were performed by nutrient agar medium at concentration level of 100µg/ml. Erythromycin and Gentamycin were used as positive control.

Table 4: Antimicrobial activities of benzohydrazide derivatives

Compounds	Zone of inhibition (mm)		
	S. aureus	***E. coli***	***A. niger***
B4	12	11	11
J4	18	17	17
P4	16	14	15
Erythromycin	28	23	25
Gentamycin	10	10	16

Off all the synthesized compounds, compound J4 shows good antimicrobial activity. B4 and J4 show better activity. All the synthesized compounds were revealed a greater antimicrobial activity than gentamycin.

DPPH free radical scavenging activity
DPPH is a stable free radical that accepts hydrogen radical or an electron to become a stable diamagnetic molecule. The absorption maximum of a stable DPPH radical in ethanol was at 516 nm. The decrease in absorbance of DPPH radical caused by antioxidants (synthesized ligands), because of the reaction between antioxidant molecules and radical progresses, which results in the scavenging of the radical by hydrogen donation. The free radical scavenging abilities of the synthesized compounds were shown in Table 5 and in Fig.5 where it was compared with that of ascorbic acid as standard.

Table 5: DPPH radical scavenging activities of synthesized compounds at different concentrations.

Sl. No	Concentrations (µg/mL)	B4	J4	P4	Ascorbic acid
1	20	12.6±0.57	15.78±0.53	11.15±2.70	25.6± 1.79
2	40	37.63±2.63	39.82±2.31	34.88±2.53	61.26± 4.28
3	60	62.7±4.28	67.4±4.23	60.1±4.08	88.98± 6.22
4	80	87.6±6.08	90.6±6.10	86.3±6.23	99.34± 6.95

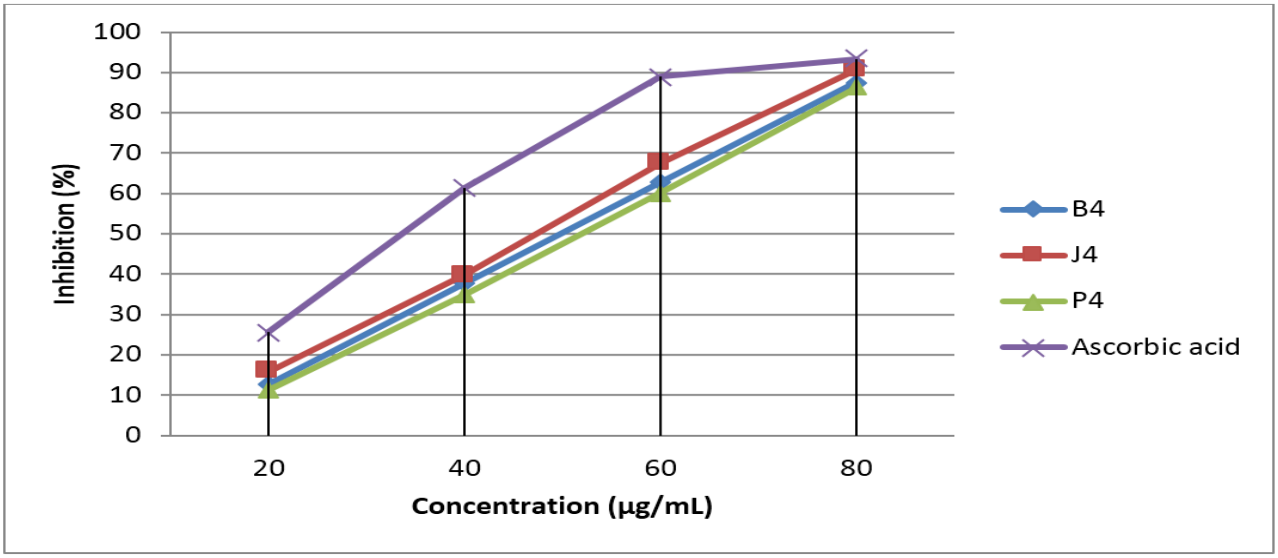


Fig 1: Radical scavenging capacities of new hydrazide derivatives

From the results it was observed that, compound P4 shows good antioxidant activity than J4 and B4 due to the presence of electron releasing methoxy group [give a reference and explain in detailed].

Molecular Docking

Inorder to find suitable inhibitor for *Mycobacterium tuberculosis* docking studies was carried out for InhA protein. InhA catalyzes the reduction of long-chain *trans*-2-enoyl-ACP in the type II fatty acid biosynthesis pathway of *M. tuberculosis*. Inhibition of InhA disrupts the biosynthesis of the mycolic acids that were central constituents of the mycobacterial cell wall. Results of the docking studies revealed that ligand P4 as best ligand which showed high score of Cdocker energy and interaction energy. This compound showed hydrogen bond interaction with Gly A: 192 and electrostatic interaction with Ile A: 194. Therefore the compound P4 may be suitable to overcome the drug resistance of *Mycobacterium tuberculosis* InhA protein

Compound id	Cdockerinteraction energy score	Type and number of interactions	Residues involved
B4	-41.7505	Electrostatic interaction-1	Lys A: 165
J4	-39.8104	pi stacking interaction-1 and water of interaction-1	Phe A: 149 and Hoh W:56
P4	-37.5198	Hydrogen bonding interaction- 1 andelectrostatic interaction-1	Gly A: 192 and Ile A: 194
Isoniazid (Standard)	-22.1896	Hydrogen bonding interaction- 2 and Van der Walls interaction-1	Lys A: 165 and Asp A: 148

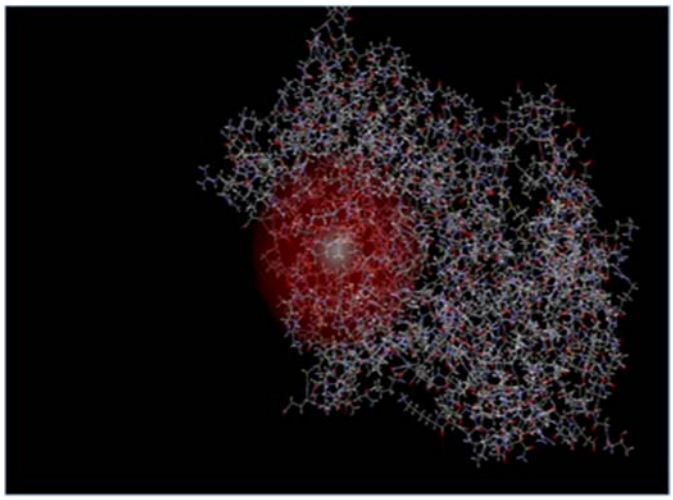


Fig2: Original image of 2NSD in running software

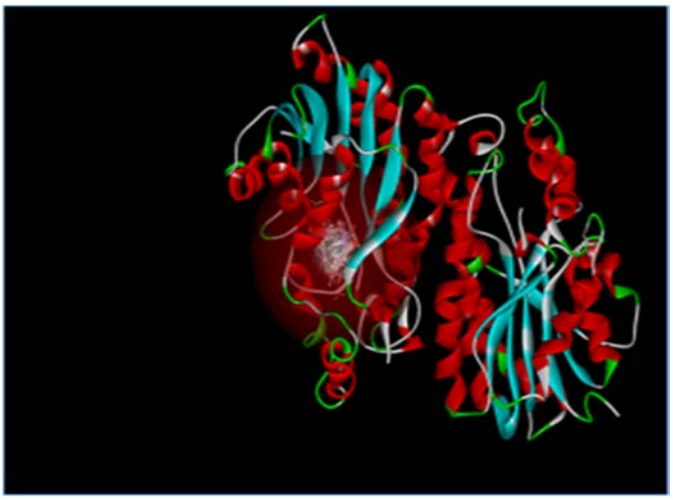


Fig 3: Secondary structure of a protein with binding site in a chain

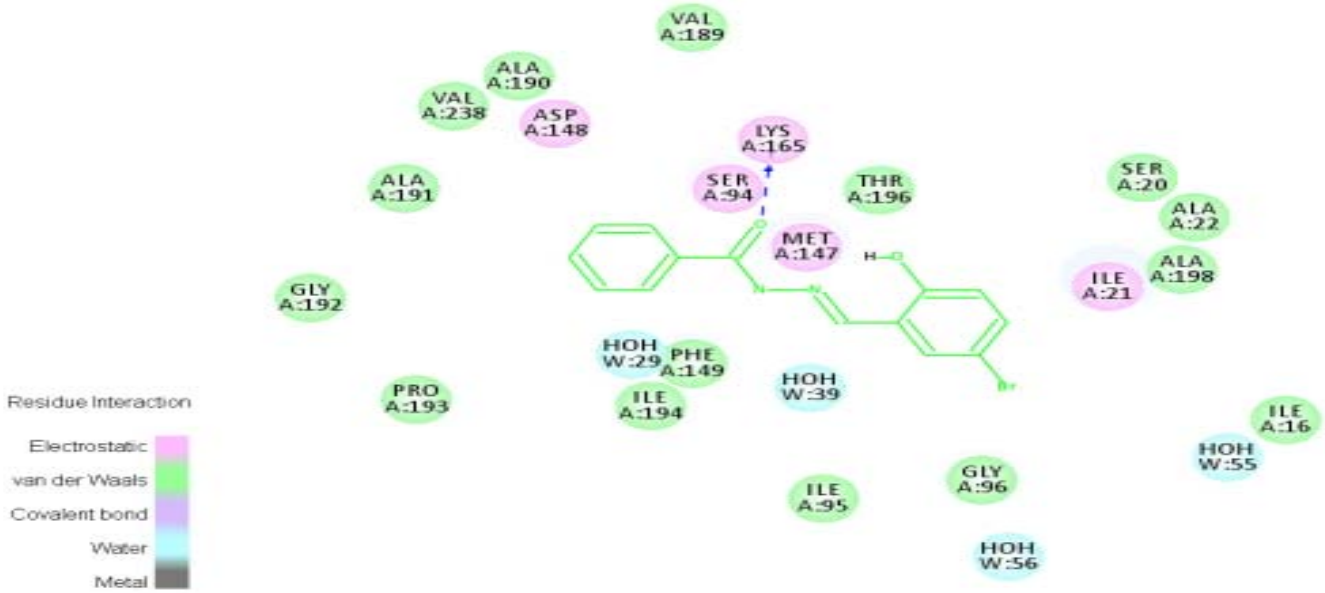


Fig 4: Compound B4 docked in active site of InhA and interaction details.

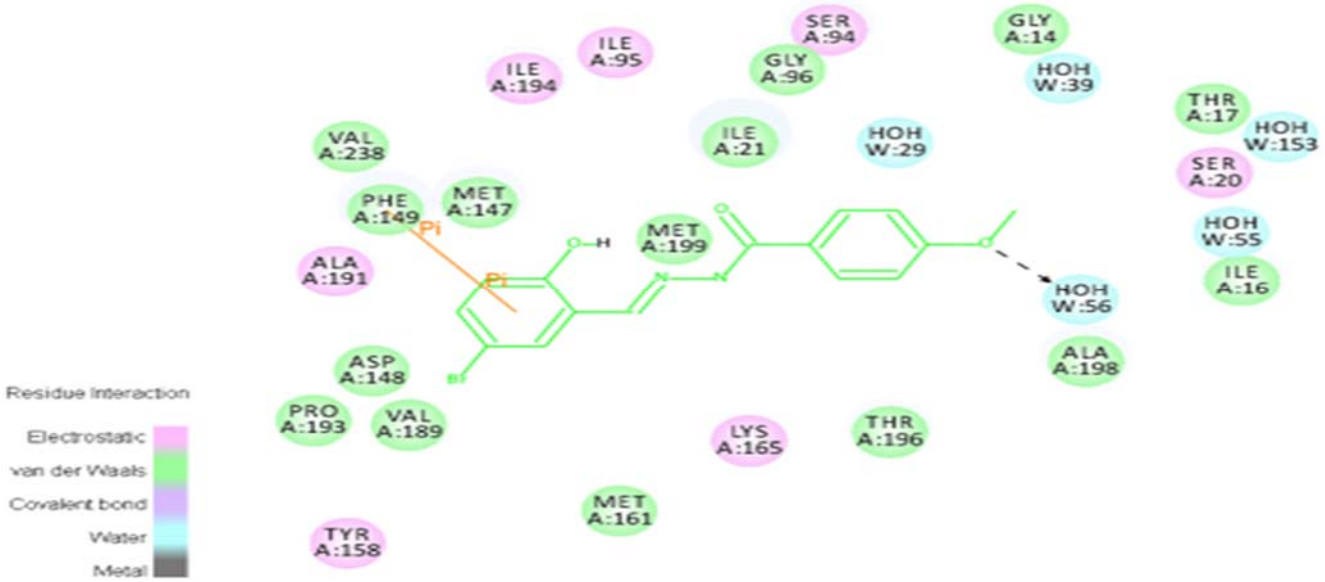


Fig 5: Compound J4 docked in active site of InhA and interaction details.

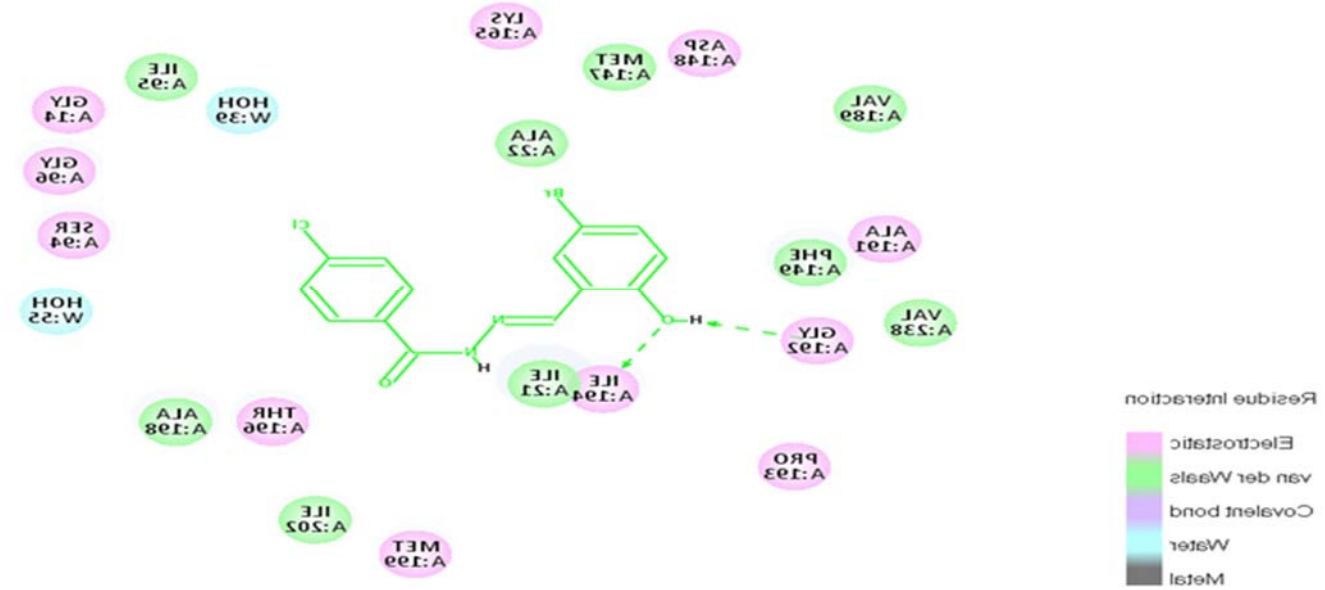


Fig 6: Compound P4 docked in active site of InhA and interaction details.

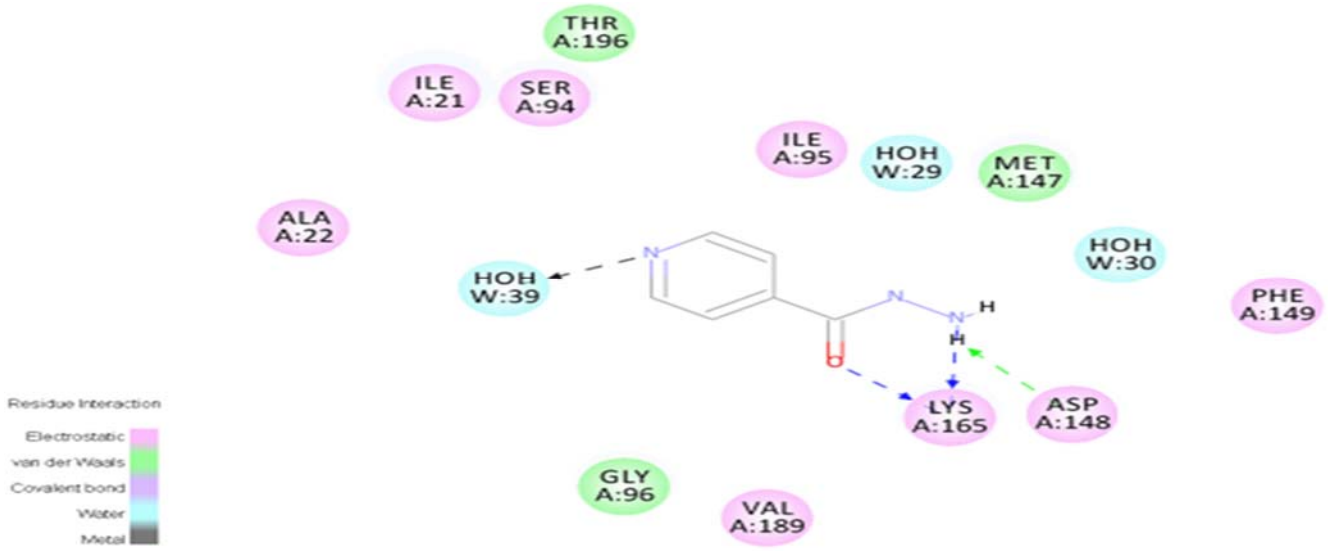


Fig 7: Standard compound Isoniazid docked in active site of InhA and interaction details.

4. Conclusion

To conclude, we have synthesized novel aroyl hydrazide derivatives using simple and convenient method. This study opens up a new area of cost-effective synthesis of biologically active Schiff base compounds. The antimicrobial activities of the synthesized compounds were effectively screened against gram positive *S. aureus* and gram negative *E.coli* bacterial and *A. Niger*fungi strains. Compound J4 shows good antimicrobial and antioxidant activities. To expand the knowledge of anti-tuberculosis activities of hydrazides against *Mycobacterium tuberculosis* InhA protein molecular modeling studies were performed. It revealed that compound P4 which showed high score of Cdocker interaction energy and it may be suitable to overcome the drug resistance of *Mycobacterium tuberculosis* InhA protein.

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