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Characterization and Anti-Tubercular Evaluation of Mannich base Substituted Imidazole Derivatives

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Abstract: ***The present research has focused to characterize and evaluate anti-tubercular activity of Mannich base substituted imidazole derivatives. A new series of substituted imidazole derivatives were synthesized by condensation of O-Phenylenediamine with glacial acetic acid and also with different amino acids (valine, glycine) which on further treated with secondary amines in the presence of formaldehyde. All these compounds were evaluated for in-silico anti-tubercular activity (docking on Cytochrome P450 14 α-sterol demethylase) and also screened in-vitro anti-tubercular activity at various concentrations against the Mycobacterium tuberculosis H37RV strain by Proportional Sensitivity test method. All compounds were characterized by FT-IR, ^{13}CNMR, ^{1}HNMR and elemental analysis (CHNO). The titled compound exhibited good binding property with molecular target. The compounds (1a&2a) showed Sensitivity against H37RV strain.***

Keywords: Imidazole, Docking, H37RV, Proportional Sensitivity test

1. Introduction

Tuberculosis (TB) is a threat to worldwide public health, caused mainly by mycobacterium tuberculosis (M.tb) bacteria species [1]. It is the only disease which does not require any vector for the transportation from one person to another. The primary site of infection is the lungs, followed by dissemination via the circulatory and lymphatic system to secondary sites including the bones, joints, liver, and spleen [2-3]. Being air born disease with no vaccine, it is the single largest disease encountered by both developing and developed countries [4-5]. Recent survey showed that in 2010, there were 8.8 million (range: 8.5–9.2 million) incident cases of TB, 1.1 million (range: 0.9–1.2 million) deaths from TB among HIV-negative people, and an additional 0.35 million (range: 0.32–0.39 million) deaths from HIV associated TB [6]. Para-aminosalicylic acid, Streptomycin, and isoniazid used as the first-line drugs for treatment of some 50 years ago has witnessed a remarkable decline in TB cases all over the world [7-8]. Other one is the development of resistance due to non completion of treatment regime by patients and hence gene mutation by organisms made its management more difficult [9]. However, the disease has been undergoing resurgence in the last two decades driven by variety of changes in social, medical, and economic factors as well as M. tuberculosis resistance to the aforementioned drugs itself. The resurgence of TB is now one of the most serious public health concerns worldwide. Despite its global impact on world health, TB is considered a neglected disease, and no new anti-TB therapeutics have been introduced into the market over the last half-century[10]. Such circumstances forced the scientists across the globe to search newer molecules that can be used as lead compounds for the development of newer anti-tubercular drugs with better and safer therapeutic effects. These facts allowed us to search novel anti- diabetic compounds with greater efficacy and fewer side effects [11-18].

The imidazole ring is an important pharmaco-phor in modern drug discovery. The synthesis of novel substituted imidazole derivatives remains a main focus of medicinal research. Recent observations suggest that substituted imidazoles and heterocyclic, show easy interactions with the biopolymers, possess potential activity with lower toxicities in the chemotherapeutic approach in man [19]. Mannich base substituted imidazole derivative are not intermediates, it is very effective organic compounds in their own right. It has become an important construction motif for the development of new drugs. Compounds containing imidazole cores have a broad biological activity spectrum such as anti-microbial [20], anti-convulsant [21-22], anti-inflammatory [23-24], anti-proliferative, anthelmintic [25-27], anti-tubercular [28], anti-HIV, anti-hypertensive [29], anti-tumor [30], anti-ulcer [31] and anti-trichinellosis.

The aim of the present study was to synthesize, characterize novel mannich base substituted imidazole derivative and to evaluate their anti-tubercular activity.

2. Experimental Protocol

2.1 Chemistry

All the chemicals and solvents were purchased form Merck (India), S. D. Fine, Spectrochem (India) and used without further purification. The purity of synthesize compounds were ascertained by thin layer chromatography on silica gel G (coated to a thickness of about 0.3 mm on previously cleaned TLC plates of 20x5 cm using conventional spreader) in Chloroform /Methanol (9:1) solvent system. Elemental Melting points were determined by using open capillary melting point apparatus and are uncorrected. FT-IR spectra (KBr) were recorded on a Perkin-Elmer 1800 FT-IR spectrophotometer. Proton 1H-NMR spectra and carbon (13C

NMR) nuclear magnetic resonance spectra were recorded on Bruker 300 MHz High Resolution NMR spectrometer. Chemical shifts were reported in ppm (d) and signals were described as singlet (s), doublet (d), triplet (t), and multiplet (m).The mass spectra were recorded on a Thermo Finnigan LCQ Advantage MAX 6000 ESI Mass spectrometer. Elemental analysis was carried out on Thermo Finnigan EA1112 Elemental analyser and the values were found within 0.4% of the theoretical values.

2.2 Synthesis

Synthesis of 1(1*H*-Benzimidazol-2yl)-2 methylpropan-1-amine (1)

O- phenylenediamine 1.296 g (12mmol) and Valine 1.89 g (36mmol) are stirred together in 4N HCl (40ml). Reflux for 4hours, cool at room temperature and monitor the completion of reaction by TLC. The pH is adjusted to 7.2 by adding NaOH pellets. The resulting brown solid is filtered, washed with water, dry in a vacuum and recrystallized from acetone.

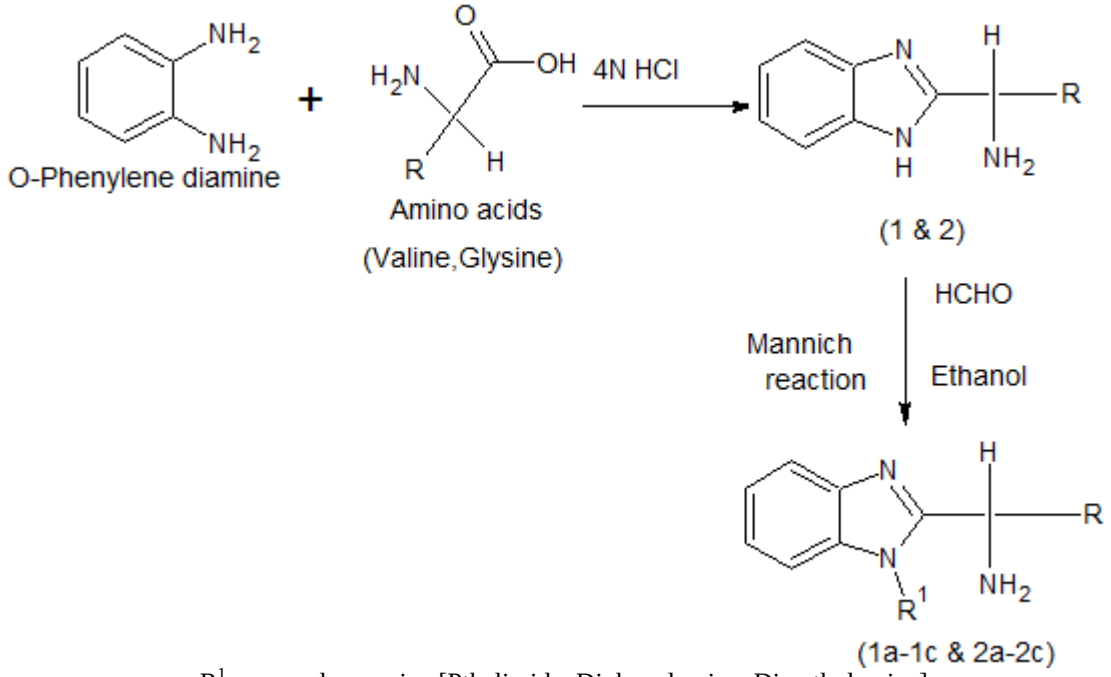
Synthesis of amino (1*H*-Benzimidazol-2yl) acetic acid (2)

O- phenylenediamine 1.296 g (12mmol) and Glycine 1.47 g (36mmol) are stirred together in 4N HCl (40ml). Reflux for 4hours, cool at room temperature and monitor the completion of reaction by TLC. The pH is adjusted to 7.2 by adding NaOH pellets. The resulting brown solid is filtered, washed with water, dry in a vacuum and recrystallized from acetone.

General Procedure for the synthesis of Mannich bases of substituted imidazole derivatives.

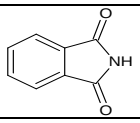
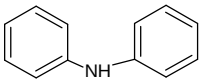
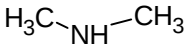
Various substituted benzimidazole in formaldehyde (15mmol) 37%w/v and Pthalimide/ diphenylamine/ dimethyl amine (10mmol) were reflux for 5-6 hrs at 70-72° C in presence of ethanol to obtain the title products (1a-1c) & (2a-2c). The reaction mixture is allowed to keep in refrigeration for overnight. The solvent is removed and product is recrystallized by using ethanol. The synthetic pathways were given in scheme 1 and its details were shown in table 1.

Scheme 1



R^1 = secondary amine [Pthalimide, Diphenylamine, Dimethylamine]

Table 1: Physico-chemical properties of the synthesized compounds

Compound code	R	R^1	Molecular formula	Molecular weight	% yield	Melting point (°C)	Rf value	Log p
1a	$CH(CH_3)_2$		$C_{20}H_{20}N_4O_2$	348.398	62.75	140	0.98	4.12
1b	$CH(CH_3)_2$		$C_{24}H_{26}N_4$	370.490	68.52	250	0.72	5.96
1c	$CH(CH_3)_2$		$C_{14}H_{22}N_4$	246.351	70.15	245	0.63	2.63

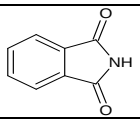
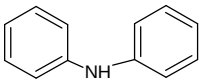
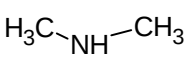
2a	O=C ϴH		$C_{18}H_{14}N_4O_4$	350.328	69.56	175	0.81	3.76
2b	O=C ϴH		$C_{22}H_{20}N_4O_2$	372.419	66.10	285	0.66	5.03
2c	O=C ϴH		$C_{12}H_{16}N_4O_2$	248.281	59.80	276	0.53	4.61

Table 2: Elemental analysis of the synthesized compounds

Compound code	Molecular formula	Molecular weight	Melting point (°C)	Elemental analysis % calculated/ found			
				C Carbon	H Hydrogen	N Nitrogen	O Oxygen
1a	$C_{20}H_{20}N_4O_2$	348.398	140	68.95	5.79	16.08	9.18
1b	$C_{24}H_{26}N_4$	370.490	250	77.80	7.07	15.12	-
1c	$C_{14}H_{22}N_4$	246.351	245	68.26	9.00	22.79	-
2a	$C_{18}H_{14}N_4O_4$	350.328	175	61.71	4.03	15.99	18.27
2b	$C_{22}H_{20}N_4O_2$	372.419	285	70.95	5.41	15.04	8.59
2c	$C_{12}H_{16}N_4O_2$	248.281	276	58.05	6.50	22.57	12.89

2.3 Spectral characterization of synthesized compounds

Compound 1a: IR (KBr cm^{-1}): N-H [Stretch] - 3487.63, C=C [Aromatic stretch] - 1590.99, C-N Stretch - 1056.8, CH_3 Bend - 1395.25, C=O Stretch - 1660.41; ^{13}CNMR δ: 47.114, 47.28, 47.45, 47.62, 47.79, 47.96, 48.13, 60.06, 123.0, 130.65, 131.01, 131.96, 133.95, 134.27, 167.69; ^{1}HNMR (ppm)δ: 7.5-8.0 (m, 8H, Ar- H) , 4.905 (s, 2H, NH_2) , 3.330 (s, 2H, $-CH_2$) , 0.8-1.3 (m, 6H, $-CH_3$); MS: 348.12 m/z

Compound 1b: IR (KBr cm^{-1}): N-H Stretch - 3425.92, Aromatic C=C Stretch - 1589.06, Aromatic C-H Stretch - 2973.7, C-N Stretch - 1139.72, CH_3 Bend – 1395; MS: 369. 95m/z

Compound 1c: IR (KBr cm^{-1}): N-H Stretch - 3423.03, C-H [Aromatic stretch] - 2921.63, C=C [Aromatic stretch] - 1655.59, C-N Stretch - 1105.98, CH_2 bend - 1396.21; MS: 245.92 m/z

Compound 2a: IR (KBr cm^{-1}): C=O Stretch - 1658.48, N-H Stretch - 3486.67, Aromatic C-H stretch - 2952.48, Aromatic C=C Stretch - 1465.63, C-N Stretch - 1145.51, CH_2 bend - 1465.63; MS: 350.23 m/z

Compound 2b: IR (KBr cm^{-1}): C=O Stretch - 2158.12, N-H Stretch - 4361.09, Aromatic C-H stretch - 2412.24, Aromatic C=C Stretch - 1576.74, C-N Stretch - 1255.16, CH_2 bend - 1546.13; MS: 372.52 m/z

Compound 2c: IR (KBr cm-1): C=O Stretch – 1764.52, N-H Stretch - 3569.27, Aromatic C-H stretch - 3140.48, Aromatic C=C Stretch - 1605.36, C-N Stretch - 1369.01, CH2 bend - 1615.73,;13CNMR : δ 47.11, 47.28, 47.45, 47.62, 47.79, 47.97, 48.13, 60.06, 123.01, 131.95, 134.27, 167.66; ^{1}HNMR (ppm) δ: 7.8 -7.9 (m, 8H, Ar-H) ,5.149 (s, 2H, NH2) ,3.3 (s, 2H, $-CH_2$) , 1.0 (s, 1H, -CH) , 4.882 (s, 1H, -OH); MS: 248.28m/z.

2.4 Molecular docking

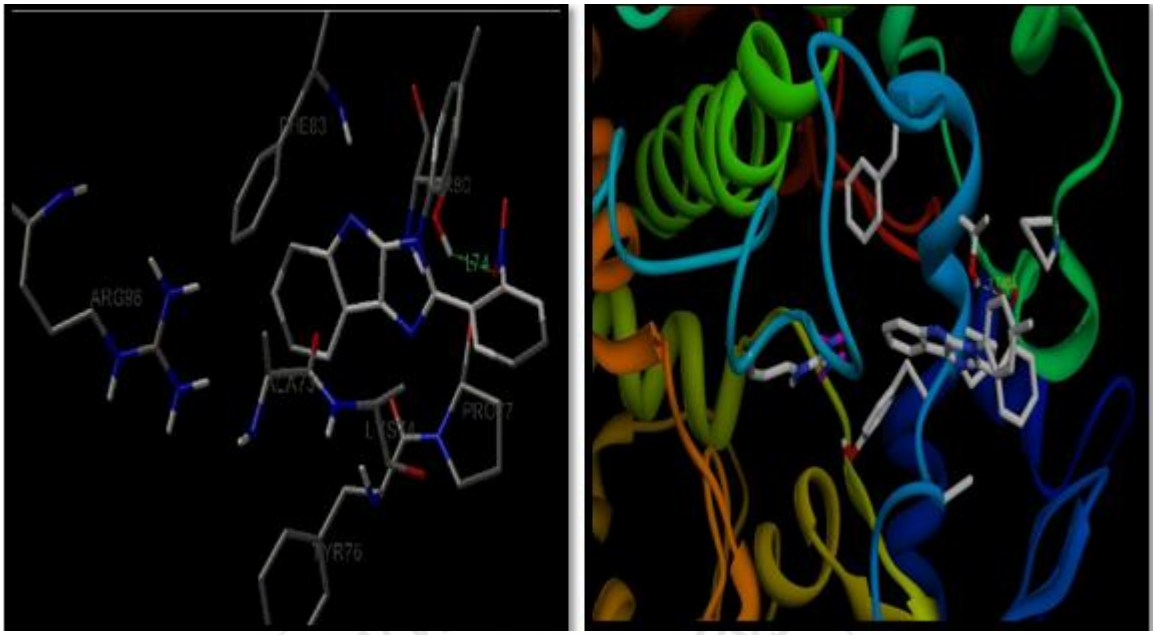
Docking is concerned with predictions of interactions of drugs or small biological substrates (less than about 600-700 Da) to pockets of larger, more rigid, receptors (typically, protein molecules, DNA or RNA). For accurate ligand docking, the goal is to have an adequate three-dimensional model of the receptor pocket you are planning to dock ligands. If this is the case then ICM docking has been shown to be very accurate in a number of independent assessments.

Auto dock

Auto Dock is a suite of automated docking tools. It is designed to predict how small molecules, such as substrates or drug candidates, bind to a receptor of known 3D structure. Current distributions of Auto Dock consist of two generations of software: Auto Dock 4 and Auto Dock Vina. Auto Dock 4 actually consists of two main programs: auto dock performs the docking of the ligand to a set of grids describing the target protein; auto grid pre-calculates these grids. In addition to using them for docking, the atomic affinity grids can be visualized. This can help, for example, to guide organic synthetic chemists design better binders. In the present study the synthesized compounds are reported for the docking study of anti-tubercular activity.

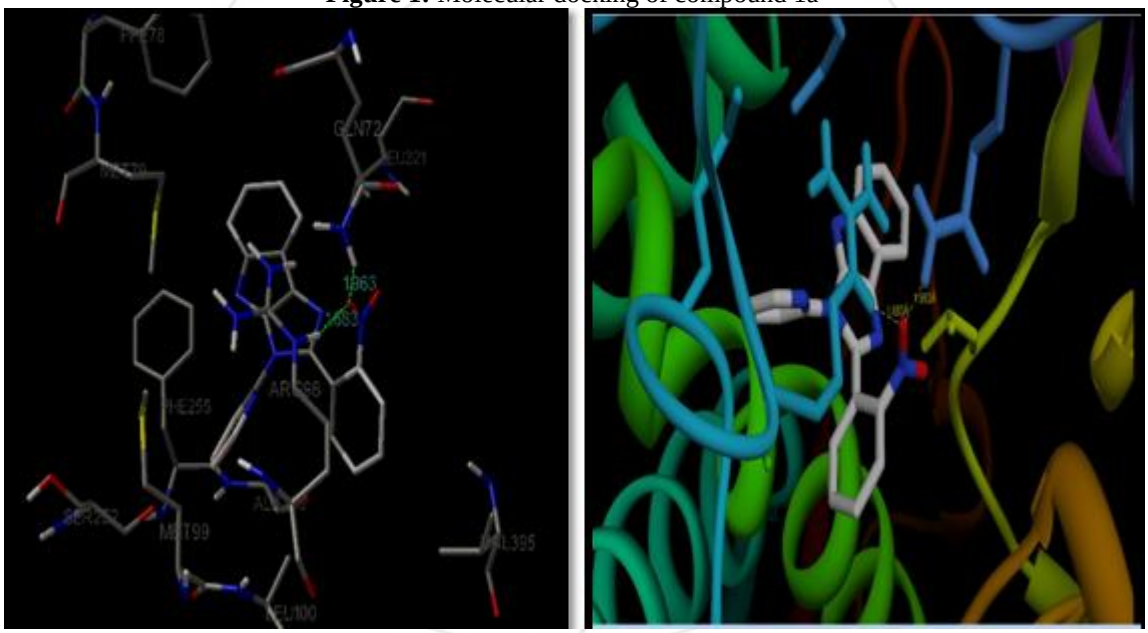
CYTOCHROME P450 14 α –STEROL DEMETHYLASE (14DM) [32] PDB Id: 1EA1

In Mycobacterium tuberculosis, Cytochrome P450 14 α-sterol demethylase enzyme is a key enzyme that constitutes an important target for the development of new anti-mycobacterial agents. 14α-Sterol demethylase catalyzes the removal of a methyl group at position C14 in the sterol molecule. The molecular docking of compound 1a & 2a and the docking score were mentioned in fig 1& fig 2 and table 3 respectively.



H-bond interaction: THR 80:HG1 with score -7.42

Figure 1: Molecular docking of compound 1a



H-bond interaction: GLN 72:HE22, ARG 96: HE with score -8.44

Figure 2: Molecular docking of compound 2a

Table 3: Energy minimization/ Auto docking results

Compound code	H-Bond interaction	H-Bond distance	Vdw interacting residue	Auto dock score
1a	THR 80:HG1	1.740	Phe255,Thr80, Phe83, Ala73, Lys74, Pro77, Tyr76	**-7.42**
1b	LYS 48:HZ3	1.931	Ls48, Gly47, Lys74, Ala75, Tyr181, Val182	-5.96
1c	ASP 90:HN	2.072	Arg96, Pro93, Glu94, Met99, Phe83, Glu85, Thr80	-6.39
2a	GLN 72:HE22 ARG 96:HE	1.963 1.683	Ala256, Gly257, Phe255, Arg96, Gln72, Met99, Leu100	**-8.44**
2b	CYS 394:HG	2.19	Ala256, Gly257, Gys394, Val395, Gly396, Phe399,Ala400	-4.52
2c	TRY 181:O	2.029	Leu179, Ala180, Gly47, Lys48, Val182, Asp183, Lys74, Pro77	-4.98

3. Anti-tubercular activity

3.1 Lowenstein Jensen Medium slant Preparation

About 37.24gm of Lowenstein Jensen media was suspended in 600ml of distilled water containing 12ml of glycerol. The medium was sterilized by autoclaving at 15lbs pressure (121°C) for 15 minutes. Sterile fresh hen's eggs were cleaned with soap water, wiped with 70% ethanol and dried under aseptic condition. The egg contents were collected in a pre-sterilized beaker and were homogenized under aseptic conditions. The egg fluid was filtered through a presteriled gauze cloth and 1litre of egg emulsion was added to 600ml of pre-sterilized L.J medium base. The medium was mixed well and was distributed approximately 7-10ml into each pre-sterilized McCartney bottles. The medium was incubated at 90ºC for 50 minutes and stored at 37°C to check the sterility of the medium. The solid contamination free L.J slants were then used for inoculation.

3.2 Preparation of synthesized compounds:

100mg of synthesised compounds 1a and 2a is dissolved in 1000ml of DMSO. Various concentrations have been prepared 200µg, 400µg, 800µg, 1600µg, 3200µg. The compound 1a and 2a were evaluated for anti-tubercular activity and the results were listed in table 4

Proportional sensitivity test method:

3.3 Preparation of McFarland Nephelometer Barium chloride Standards :

1) Prepare 1% aqueous barium chloride (0.1g of Barium chloride (anhydrous) in 10 ml of SDW).
2) Prepare 10ml of 1% sulphuric acid solution (9.0ml of distilled water and 1ml of concentrated sulphuric acid).
3) Add 0.1ml of 1% Barium Chloride solution to 9.9 ml of 1% Sulphuric acid solution to obtain the McFarland standard, which matches with 1mg/ ml of M.tuberculosis.
4) Seal the tubes with parafilm and label as No.1 McFarland standard tube with date of preparation.
5) Once prepared, standard can be stored & used for up to 4 months. 6. Wire meshes to hold McCartney bottles and bijou bottles.

3.4 Inoculum preparation

With a 3mm wire loop, a representative sample of approximately 4-5mg (loop full) is taken from the primary culture and placed on the side wall of a McCartney bottle containing 1ml SDW and 6 glass beads of diameter 3mm. Emulsify the bacterial inoculum, (with a loop of water, if required), on to the side wall of McCartney bottle in round rotatory movements with inoculation loop, till the bacterial mass is emulsified, (this is visible by reduction in the clumpy hydrophobic to aqueous hydrophilic nature of suspension). Let the emulsified suspension be fully dissolved in the 1ml of SDW. Vortex the bottle up to 20-30 seconds and 4ml of distilled water is added slowly. Allow the coarse particles to settle down (leave it on stand for app. 5mins). Decant the suspension carefully into another clean, sterile McCartney bottle. Match the opacity/turbidity of inoculum with McFarland standard no.1, against a black background. This is the neat bacterial suspension, standardized at 1mg/ml, equaling to 107 to 108 CFU/ml. Make sure that no clumps are taken. If required, the opacity of the bacterial suspension is then adjusted by the addition of distilled water to obtain a concentration of 1mg/ml of tubercle bacilli by matching with McFarland's standard No.1. Make further two log dilutions to achieve 10-2 and 10-4 dilutions as given below: i. The dilution 10-2 is produced by discharging two loopfuls of the neat bacterial suspension, into a small tube containing 2ml of distilled water, and shaking. ii. Similarly, the dilution 10-4 is produced by discharging two loopfuls of the dilution 10-2 into a small tube containing 2ml of distilled water, and shaking.

3.5 Inoculation Procedure

Label with L.J slopes with lab number of culture, and serially arrange in the stand. Heat the loop to red hot (incandescence) in flame for each dilution separately; ensure the loop is cooled by touching the insides of medium slope, before using the loop. Inoculate a loop-full (3mm) of each dilution on to media slopes. Should be inoculate uniform suspension in to all slopes.

3.6 Incubation and Reading

Incubate the inoculated slopes at 37^{o}C. Read the growth at 28 days and again at 42 days. Record growth as Confluent growth = 3+; More than 100 colonies = 2+; Record actual number of colonies = 1-100 colonies. When the number of colonies on a given dilution is less than 5, count the number of colonies with the next larger inoculum, or estimate if more than 100. Make no attempt to estimate the number of colonies if the growth is 3+.

3.7 Interpretation of tests

First reading is taken at 28th day after inoculation. Count the colonies only on the slopes seeded with the inoculum that has produced exact readable counts or actual counts (up to 100 colonies on the slope). This inoculum may be the same for the control slopes and the drug-containing slopes, or it may be the low inoculums (10–4 dilution) for the control slopes and the high inoculum (10–2 dilution) for the drug-containing slopes. The average number of colonies obtained for the drug-containing slopes indicates the number of resistant bacilli contained in the inoculum. Dividing the number of colonies in drug containing slopes by that in drug free slopes gives the proportion of resistant bacilli existing in the inoculum. Below a standard value-the critical proportion-the strain is classified as sensitive; above the value, it is classified as resistant. The proportions are reported as percentages. In case growth on the control media is poor even after six weeks (i.e., few or no colonies on the 10-4 bacterial dilution), the test should be repeated.

3.8 Criteria of resistance

Any strain with above 1% (the critical proportion) of value to any of the four drugs – Rifampicin, Isoniazid, Ethambutol, and Streptomycin – is classified as resistant against respective drug. For calculating the proportion of resistant bacilli, the highest count obtained on the drug free and on the drug-containing medium should be taken (regardless of whether both counts are obtained on the 28th day, both on the 42nd day, or one on the 28th day and the other on the 42nd day.)

Table 4: Proportionality sensitivity test method

Compound code	Concentration of synthesized compound																			
	200µg				400 µg				800 µg				1600 µg				3200 µg			
	weeks				weeks				weeks				weeks				weeks			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1a	-	1+	2+	3+	Sc	1+	2+	3+	Sc	1+	2+	2+	Sc	1+	2+	2+	-	Sc	1+	1+
2a	Sc	1+	2+	3+	-	1+	3+	3+	1+	2+	3+	3+	Sc	1+	2+	2+	-	-	-	-

Confluent growth = 3+ More than 100 colonies = 2+ Sc= scanty (≤10)

4. Result

The structures of the synthesized derivatives of Mannich base imidazoles were established by IR, ^{13}C-NMR, ^{1}H-NMR, Mass spectra and elemental analysis. The purity of synthesized compounds had been checked on TLC plates using Chloroform: Methanol (9:1) as a mobile phase. The IR, ^{13}C-NMR, ^{1}H-NMR and Mass data reported in manuscript under section of spectral data. All the synthesized compounds shows hydrogen bonding interactions with active sites of the receptor **Cytochrome P450 14α-sterol demethylase** with binding energy ranges between -4.42 to -8.52 k cal/mol. All the synthesized compounds were evaluated for their Anti-tubercular activity at 200, 400, 800, 1600, 3200 µg concentration against the Mycobacterium tuberculosis H37Rv strain by Proportional Sensitivity test method (Solid culture method). Compounds (1a, 2a) showed Sensitivity at higher concentration against the Mycobacterium tuberculosis H37Rv strain.

5. Conclusion

From the present research, it is conclude that the compound substituted with pthalimide at 1st position to nitrogen exhibited maximum anti-tubercular activity. Hence the study fit for the further *in-vivo* studies.

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