International Journal of Biological & Pharmaceutical Research

e- ISSN 0976 - 3651
Print ISSN 2229 - 7480

www.ijbpr.com

Research Article

SYNTHESIS OF CERTAIN AZETIDIN-2-ONE DERIVATIVES FOR ANTITUBERCULAR ACTIVITIES BY ALAMAR BLUE ASSAY METHOD

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ABSTRACT

Isoniazid condensed with different derivatives of acetophenone to form hydrazones, using Vilsmeier-Haack reagent to form free aldehyde which on reacts with different free amide (R-NH_2) group to form imines(C=N) which on react with Chloro acetyl chloride and Triethylamine to gives Azetidin-2-one derivatives. The structures of the all newly synthesized compounds have been established on the basis of their spectral data and elemental analysis. The selected compound was evaluated for anti-tuberculosis activity by using Alamar Blue Assay method.

Key Words: Azetidin-2-ones, Vilsmeier-Haack reagent, Anti-tubercular activity and Alamar Blue Assay method.

Access this article online		
Home page: http://ijbpr.com/ DOI: http://dx.doi.org/10.21276/ijbpr.2018.9.3.6		Quick Response code
Received:05.07.18	**Revised**:12.07.18	**Accepted**:22.07.18

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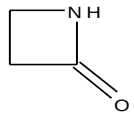
INTRODUCTION



azetidin-2-one

Azetidine-2-one is carbonyl derivatives of azetidines containing carbonyl group at the position-2, which are generally known as azetidine-2-ones or commonly known as β-lactams, are well-known heterocyclic compounds among the organic and medicinal chemists. The β-lactam Heterocycles are still the most prescribed antibiotics used in medicine A large number of β-lactams possess powerful antimicrobial (MaGharaby MA *et al*., 1989; Abdulla FB and Fuhr Knneth H, 1975; Barot VM, 1996; Mohammad EA *et al*., 1995; Riaz F A and Furh H K, 1975; Shastry CS *et al*., 2003; Sharma SD and Khurana J P S, 1989; Srivastav S K and Srivastav S D, 2000) antihistaminic (Reddy CV *et al.*, 1989) antihelmentic (Purohit M and Srivastav SK, 1991), antiamoebic (Ahuwalia VK *et al.*, 1989), antiparasitic (Pathak RB and Bahel SC, 1980), antiprotozoal (Eid AI *et al.*, 1978), anticancer (Freddy HH and Abhay R, 2008), anti-tubercular (Matti G and Farmaco P, 1985; Shafei K and Hassan KM, 1983; Trivedi PB *et al.*, 1993; Gangiee A and Adar G, 1999; Feigelson GB *et al.*, 1995), anti-inflammatory (Menozzi G and Filippelli W, 1994; Balakrishnan K and David B, 1999), CNS, anti-HIV (Pathak RB and Bahel SC, 1980), anti-diabetic (Khalafallah AK *et al.*, 1995), anti-convulsant (Hrib NJ and Jurcak JG 1991; Sharma MC *et al.*, 2009) and analgesic (Fred H *et al.*, 1992) activities.

MATERIALS AND METHODS

Melting points were determined in open capillaries and are uncorrected. Purity of the all newly synthesized compounds was routinely checked by TLC using plates coated with silica gel-G. UV spectra were recorded on ELICO SL 169spectrophotometer. IR spectra were recorded using SHIMADZU IRspectrophotometer. NMR spectra were recorded using BRUKER 300MHZ NMR spectrophotometer (Sayyed MA *et al.*, 2009).

EXPERIMENTAL PROCEDURE

Preparation of N-(1-(4-Substituted Phenyl) Ethylidene) Isonicotinicacidhydrazide

An equal molar (0.036 mol) solution of isonicotinicacidhydrazide and substituted acetophenone in ethanol were taken in a round bottom flask. Add 2 drops of glacial acetic acid and refluxed for 4 hours. The solid separated on cooling filtered and washed with cold ethanol. Finally solids recrystallized from chloroform.

Preparation of 1-Isonicotinoyl-3-(4-Substituted Phenyl) 1h-Pyrazole-4- Carbaldehyde

The N-(1-(4-substituted phenyl) ethylidene) isonicotinicacidhydrazide (0.009mole) was added to a vilsmeier Haack reagent[31](20ml) { prepared by drop wise addition of 1.2ml phosphorousoxychloride to ice cooled dimethyl formamide (10 ml)} was added portion wise and thereaction mixture was heated to 60°C for about 4 hr and poured into crushed ice. The mixture was then neutralized with sodium hydrogen carbonate, heated to 50-60°C, cooled and acidified to pH-6 with 10 M HCl. The solid thus separated was filtered and recrystallized from chloroform (Jiaxi X, 2009; Arunakumar DB *et al.*, 2007; Jigisha AP *et al.*, 2008; Gerona N *et al.*, 2008).

Preparation of N'-((1-Isonicotinoyl-3-(4-Substituted Phenyl) 1h-Pyrazol-4-Yl) Methylene) Free Amines

An equal molar (0.007mole) solution of free amines and 1-isonicotinoyl-3-(4-methylphenyl) 1h-pyrazole-4- carbaldehyde in ethanol were taken in a round bottom flask. Add 2 drops of glacial acetic acid and refluxed for 4 hours. The solid separated on cooling filtered and washed with cold ethanol. Finally solids recrystallized from chloroform.

Preparation of Different Azetidin-2-One Derivatives

All freeamines Compounds (0.01mol) was dissolved in N,N-dimethyl formamide(40ml) and triethylamine(2.80ml,0.02ml) was add to it. Chloroacetylchloride (1.60ml, 0.02mol) was added drop wise over a period of 15min.The reaction mixture was refluxed for 8 hours .The reaction mixture was concentrated, cooled and poured into crushed ice. The solid was obtained, washed with water and recrystallized with chloroform (Ajay KR *et al.*, 2001; Franzblau SG *et al.*, 2002).

Spectral Studies of Prototype Compounds

Structure of Azetidin-2-ones synthesized were established on the basis of **IR, NMR, MASS** spectral data.

3-Chloro-1-Isonicotinohydrazide-4-(1-Isonicotinyl-3-Ethylphenyl-1h-Pyrazole-4-Yl) Azetidin-2-One **(AM)**

IR - NH_{str}(3350.1), Methyl C-H_{str}(2937.4), C-H_{str}Aromatic(3000- 3300),C=O_{str}(1720),C=N_{str}(1590) N=O_{str}(1527.5), C=C_{str}in pyridine moiety(1610), C=C_{str}in aromatic ring(1450) C-N_{str}(1280),C-O_{str}(1101.3), C-N_{str} for Ar NO_2 (856.3), Out of plane bend C-H_{str}(750.3).**^{1}HNMR -** 7.416 – 7.440 (4H of Benzene ring), 4.816 (1H of Azetidinone), 3.30 (1H of CH-Cl Azetidinone), 2.441 (3H of Methyl). **^{13}CNMR –** 166.96 (C=O of Azetidinone),139.12 (C of benzene), 49.64 (CH of Azetidinone).

3-Chloro-1-Isonicotinyl-4-(1-Isonicotinyl-3-Methylphenyl-1h-Pyrazol-4yl) Azetidin-2-One **(BR)**

IR - 3031.89-3448.49(Ar C-H stretching and N-H stretching), 1660.60(C=O stretching), 1290.29(C=C stretching in aromatic), 869.84(C-H stretching), 58.90(C-Cl stretching), **^{1}HNMR -**7.41 – 7.57 (4H of Benzene ring), 4.818 (1H of Azetidinone), 3.308 (1H of CH-Cl Azetidinone), 2.438 (3H of Methyl), **^{13}CNMR -** 169.81, (C=O of Azetidinone),137.28(C of benzene),49.64 (CH of Azetidinone).

3-Chloro-1-Benzoicacid-4-(1-Isonicotinyl-3-Methylphenyl-1h-Pyrazol-4-Yl) Azetidin-2-One **(AL)**

IR - 2864.09-3016.46(Ar C-H stretching and N-H stretching), 1666.38(C=O stretching), 1334.65(C=C stretching in aromatic), 844.76(C-H stretching), 675.04(C-Cl stretching), **^{1}HNMR -** 7.318 – 7.426 (4H of Benzene ring), 4.43 (1H of Azetidinone), 3.308 (1H of CH-Cl Azetidinone), 2.437 (3H of Methyl), **^{13}CNMR -** 170.70(C=O of Azetidinone),139.11(C of benzene), 49.64 (CH of Azetidinone).

3-Chloro-4-[1- Isonicotinoyl-3-(4 -Chlorophenyl)-1h-Pyrazol-4-Yl]-1-Isonicotinamido-Azetidin-2-One **(A - AZT)**

IR- 'H'bonded, coupled,Ar C-H_{str},Lactum C-H and N-H_{str}(2400-3400),C=O_{str}(1710),C=N_{str}(1494) N=O_{str}(1535), C=C_{str}in pyridine moiety(1590), C=C_{str}in aromatic ring(1400)C-N_{str}(1249),C-O_{str}(1101), C-N_{str} for Ar NO_2 (846), C-Cl(866), Out of plane bend C-H_{str}(750). **^{1}HNMR** 2.5 (-CH-CH- LACTUM), 3.4 (–NH),4.6 (-CH Cl),7.7(Ar.benzene proton),8.7(Pyridine proton), 10.2 (Tautomeric enolic OH).

3-Chloro-4-[1 - Isonicotinoyl-3- (4 -Chlorophenyl)-1h-Pyrazol-4-Yl]-1-(2 -Hydroxy Benzoicacid) Azetidin-2-One **(B- AZT)**

IR - 'H' bonded, coupled, ArC-H_{str},Lactum C-H and N-H_{str}(2400-3400),C=O_{str}(1740),C=N_{str}(1477), C=C_{str}in pyridine moiety(1570), C=C_{str}in aromatic ring(1398), C-N_{str}(1290), C-O_{str}(1090), C-Cl(850), Out of plane bend C-H_{str}(759). **^{1}HNMR** 2.4 (-CH-CH- Lactum), 3.4 (–NH), 4.7 (-CH Cl),7.8(Ar.benzene proton),8.7(Pyridine proton), 10.2 (Tautomeric enolic OH).

***3-Chloro-4-[1 - Isonicotinoyl-3- (4 -Chlorophenyl)-1h-Pyrazol-4-Yl]-1- Benzoicacid) Azetidin-2-One*(C-AZT)**
IR - 'H' bonded, coupled, ArC-H_{str}, Lactum C-H and N-H_{str}(2400-3400),C=O_{str}(1700),C=N_{str}(1494) N=O_{str}(1535), C=C_{str}in pyridine moiety(1624), C=C_{str}in aromatic ring(1570)C-N_{str}(1319), C-O_{str}(1014), C-Br (1249), C-Cl(866), Out of plane bend C-H_{str}(759). **^{1}HNMR** 2.5 (-CH-CH- Lactum), 3.4 (–NH), 4.7 (-CH Cl), 7.7(Ar.benzene proton), 8.8(Pyridine proton). **EI –MS:** 283(M^+).

***3-Chloro-4-[1 - Isonicotinoyl-3- (4 -Nitrophenyl)-1h-Pyrazol-4-Yl]-1-(2 -Hydroxy Benzoicacid) Azetidin-2-One* (BM)**
IR - 'H' bonded, coupled,Ar C-H_{str} and N-H_{str}(3000-3400), Methyl C-H_{str}(2933.5), C=O_{str}(1690),C=N_{str}(1580) C=C_{str}in pyridine moiety(1560.3), C=C_{str}in aromatic ring(1411) C-N_{str}(1284),C-O_{str}(1101), C-Cl_{str}(993), Out of plane bend C-H_{str}(752). **^{1}HNMR** 2.6 (-CH-CH- Lactum), 3 (-CH Cl), 3.75 (COOH),4.7 (Ar.OH) 8.4 and 8.6(Ar.benzene proton), 7.9(Pyridine proton).

***3-Chloro-4-[1- Isonicotinoyl-3-(4 -Bromophenyl)-1h-Pyrazol-4-Yl]-1-Isonicotinamido-Azetidin-2-One* (AP)**
IR - 'H' bonded, coupled,Ar C-H_{str} and N-H_{str}(3000-3400), Methyl C-H_{str}(2933.5), C=O_{str}(1690),C=N_{str}(1580) C=C_{str}in pyridine moiety(1560.3), C=C_{str}in aromatic ring(1411) C-N_{str}(1284),C-O_{str}(1101), C-Cl_{str}(993), Out of plane bend C-H_{str}(752).**^{1}HNMR** 2.3 (-CH-CH- Lactum), 3.4 (-CH Cl),4 (COOH),4.9 (Ar.OH), 5.9 (Pyrazole CH) 8.3(Ar.benzene proton), 8.9(Pyridine proton).

***3-Chloro-4-[1 - Isonicotinoyl-3- (4 -Bromophenyl)-1h-Pyrazol-4-Yl]-1-(2 -Hydroxy Benzoicacid) Azetidin-2-One* (BP)**
IR - 'H' bonded, coupled,Ar C-H_{str} and N-H_{str}(3000-3400), Methyl C-H_{str}(2935.5), C=O_{str}(1745),C=N_{str}(1519) C=C_{str}in pyridine moiety(1590), C=C_{str}in aromatic ring(1420) C-N_{str}(1280),C-O_{str}(1105), C-Br_{str0}(1220), Out of plane bend C-H_{str}(752).**^{1}HNMR** 2.5 (-CH-CH- Lactum), 3.6 (-CH Cl),11 (COOH), 7.3 (Pyrazole CH) 9(Ar.benzene proton),9.5(Pyridine proton).

***3-Chloro-4-[1 - Isonicotinoyl-3- (4 -Bromophenyl)-1h-Pyrazol-4-Yl]-1- Benzoicacid) Azetidin-2-One* (CP)**
IR - 'H' bonded, coupled, Ar C-H_{str} and N-H_{str}(3000-3400), C=O_{str}(1745),C=N_{str}(1510) C=C_{str}in pyridine moiety(1678), C=C_{str}in aromatic ring(1350), C-N_{str}(1299), C-Br_{str} C-O_{str}(1105), Out of plane bend C-H_{str}(750).**^{1}HNMR** 2.4 (-CH-CH- Lactum), 3.3 (-CH Cl),11 (COOH), 8.9(Ar.benzene proton), 9.4(Pyridine proton).**EI –MS:** 299(M^+).

ANTIMYCOBACTERIAL STUDIES

Conventional Agar diffusion technique for susceptibility tests, which rely on the size of a zone of inhibition surrounding a drug-containing disc are not suitable for the slowly growing Mycobacterium species because the drug diffuses throughout the medium before the organism has the chance to grow. So, the following principles are recognized for the Anti Mycobacterial screening.

- The composition of medium should have a minimal effect on drug inactivation. So, Middle Brook 7H9 Broth Base is used.
- Drug containing medium should be stored in a refrigerator shielded from light and kept in plastic tubes tightly closed inorder to protect them from evaporation.
- Homogenization of inoculum is essential to eliminate large clumps of cells.

SUSCEPTIBILITY TESTING

DIRECT METHOD IN DIRECT METHOD

Direct Method

This was done if acid-fast bacilli are seen on the smear of the concentrated clinical specimen. Dilutions are made and inoculated.

Indirect Method

Bacterial mass is suspended in Middle brook 7H9 broth containing three or four small sterile glass beads. Mixture is placed on a vortex mixer is placed on a vortex mixer and precautions are taken to prevent aerosol production. Tube is allowed to stand for 15 mts. The stock suspensions is diluted and 0.1 ml is inoculated into control and drug containing media.

Alamar Blue Assay Method

Alamar Blue**,** is an oxidation - reduction dye used for screening of antitubercular activity. Alamar blue (oxidisied form) turns into pink colour upon reduction. Since Mycobacterium is an aerobic organism, its presence of growth turns alamar blue pink. This principle has been used to predict the presence or absence of growth of *Mycobacterium tuberculosis* for testing antimycobacterial agents. Pink colour indicates the presence of growth (no anti-tubercular activity) and blue colour indicates the

absence of growth. (Inhibitory or cidal activity of agents tested).

Materials Used In Antitubercular Screening Studies

Middle Brook 7H9 Broth Base: Himedia, Mumbai
Alamar Blue Solution :Serotec
Media used :Middle Brook 7H9 Broth base

Preparation Of Medium

2.35 gm of Middle Brook 7H9TB broth base was suspendedin 450ml distilled water which contains 5 ml glycerol sterilized by autoclaving at15 psi pressure at 121ºC for 15 minutes. Cooked to 45º C or below and enriched with dextrose to a final concentration of 0.5% of either bovine albumin fraction V or serum.

Procedure For Antitubercular Screening Using Alamar Blue Assay

M. tuberculosis $H_{37}Rv$ maintained on L-J medium (Lowenstein –Jensen medium) procured from Center for Biotechnology, Trivandrum and stored at Sri Vijay Vidyalaya College of Pharmacy, Dharmapuri, Tamilnadu was used as a test organism for anti tubercular screening studies (Table:01) (Luzcaviede T *et al.,* 2001)

Stock solutions of newly synthesized compounds were prepared in Water, chloroform : water (80 : 20), filtered, sterilized and were added to 450 µl of Middle Brook 7H9 TB broth in 1.5 ml sterile micro centrifuge tubes to achieve final concentrations of 100, 10, 1, 0.1, 0.05, 0.01, µg/ml, INH (at 100,10,1, 0.1,0.01 µg/ml) and Rifampicin (at 0.01µg/ml) was set up simultaneously as a positive control and three tubes that did not have any test compounds were used as negative control.

Colonies from four week old sub cultures were transferred to the tubes containing 0.85% saline, thoroughly vortex mixed and the suspension was allowed to stand for five minutes. 50 µl of the supernatant culture were inoculated into all the tubes containing different concentrations of newly synthesized compounds and standard drugs. The tubes were mixed well and incubated at 37º C without shaking.

On the seventh day, 25 µl of Alamar blue solution was added to the first control tube. The colour changed from blue to pink and therefore the dye was added to all tubes and observed for six hours. Blue colour in the tube indicated sensitivity of *M.tuberculosis* to the newly synthesized compounds and pink colour indicated resistance of *M.tuberculosis* to them.

Note :

If colour change was not observed on seventh day of incubation, Alamar blue solution was added to second control tube (C_2) on ninth day and third control tube (C_3) on the eleventh day of incubation. If not, methodology has to be repeated.

RESULT AND DISCUSSION

ANTITUBERCULAR ACTIVITY

In this present work, all the compounds were tested for antitubercular activity by Alamar Blue assay method (Scott G *et al.,* 1998). $H_{37}Rv$ strain of Mycobacteriumtuberculosiswas employed as the test organism. Middlebrook 7H9 TB broth was employed as the medium for inoculation.

The results of the experiment were noted by observing the presence or absence of Mycobacterium tuberculosis $H_{37}Rv$ manifested as a colour change. The absence of any growth indicates inhibition. At the end of 7th day, results were observed and presented in the Table No: 01

It was found that all the compounds inhibited the growth of tubercle bacilli at different concentrations like 100, 10, 1, 0.1, 0.05 and 0.01 µg/ml respectively which was compared with that of standard isoniazid (100, 10, 1, 0.1, 0.05, 0.01 µg/ml.)

The compounds like **AP, BP, CP** and **C-AZT** were found to be effective at the concentration of 0.05µg/ml and all the compounds were found to be ineffective at the lower concentration of 0.01 µg/ml which was compared with standard drugs like isoniazid (100 µg, 10 µg, 1µg, 0.1 µg, 0.05 µg/ml , 0.01 µg/ml) and rifampicin (0.01 µg/ml).

Most compounds were effective even at 0.05 µg/ml concentration making them promising moiety for further anti tubercular studies which may include comparison of our results with sophiscated methods like Tetrazolium microplate assay (Mackie M, 1996), Bactech 460 method and other method like resistance ratio method and MIC screening by absolute concentration method.

Screening For Antimycobacterial Activity By Alamar Blue Assay Method

Organism used :*M. tuberculosis* H_{37} R v
Vehicle used :Water, Water:Ethanol (80 : 20)
Standards : Isoniazid, Rifampicin at various concentration

Table 1. Compound Code and Concentration of the Compounds

S. No	Compound Code	Concentration of the Compounds (µg/ml)					
		100	**10**	**1**	**0.1**	**0.05**	**0.01**
1.	AM	B	B	P	P	P	P
2.	BM	B	B	B	P	P	P
3.	CM	B	B	B	B	P	P

4.	AP	B	B	B	B	B	P
5.	BP	B	B	B	B	B	P
6.	CP	B	B	B	B	B	P
7.	A-AZT	B	B	B	P	P	P
8.	B-AZT	B	B	B	P	P	P
9.	C-AZT	B	B	B	B	B	P
10.	Isoniazid	B	B	B	B	B	P
11.	Rifampicin	-	-	-	-	-	B
12.	Blank	P	P	P	P	P	P
13.	Control	P	P	P	P	P	P

B : Blue (Sensitive) P : Pink (Resistant)

SCHEME

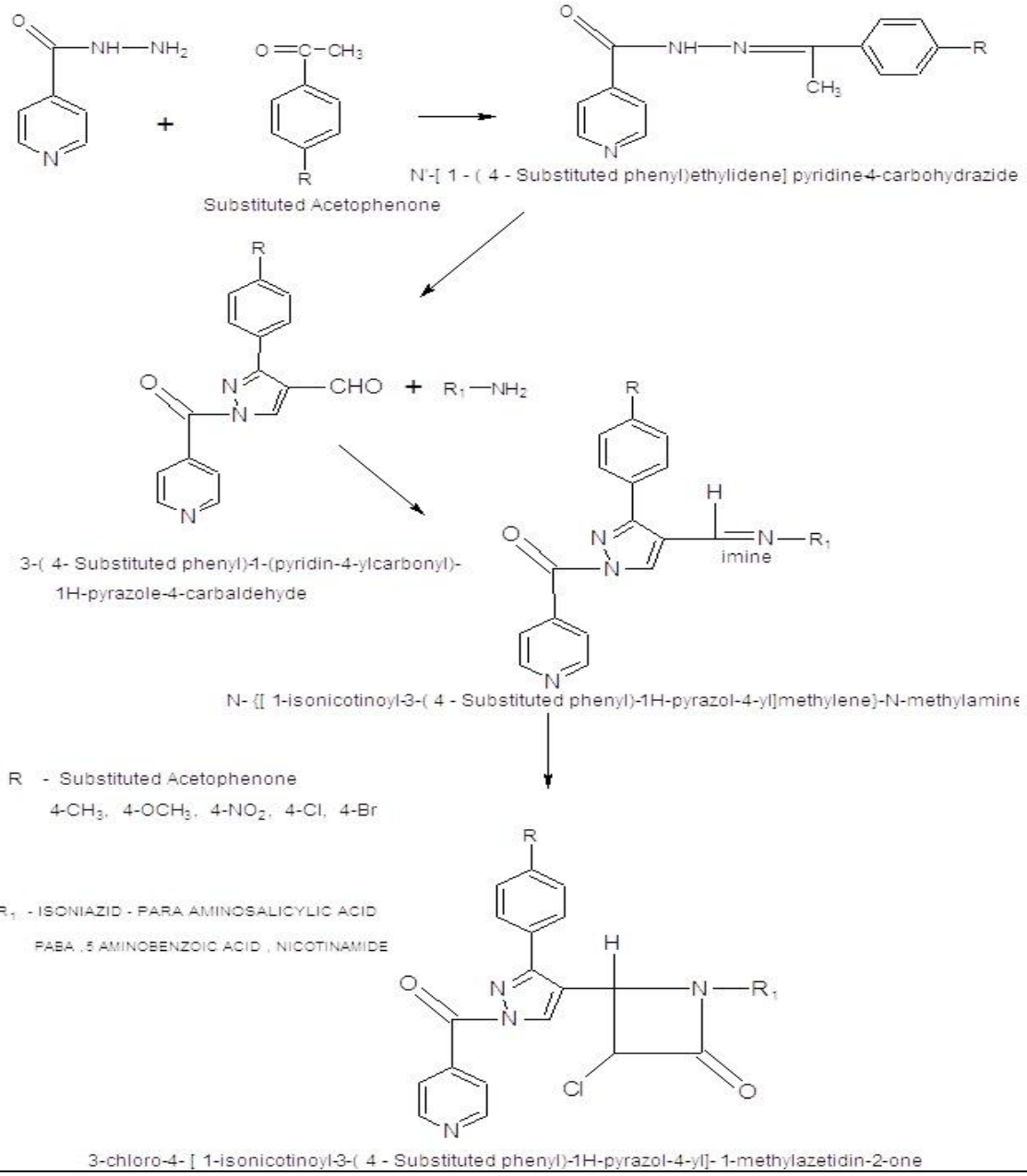
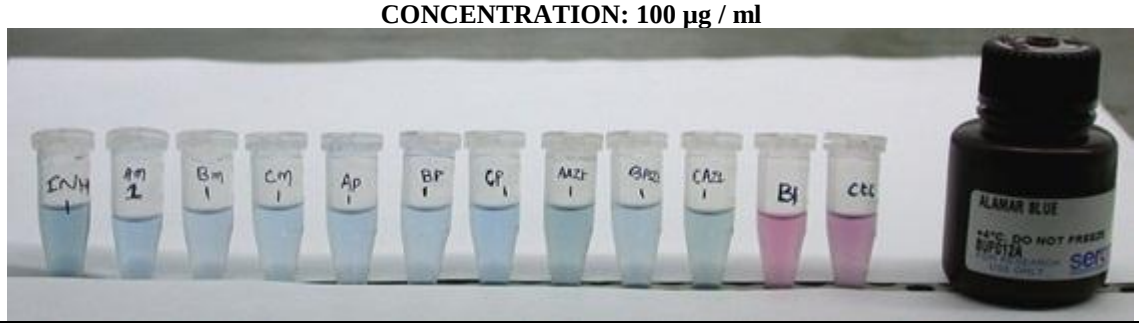
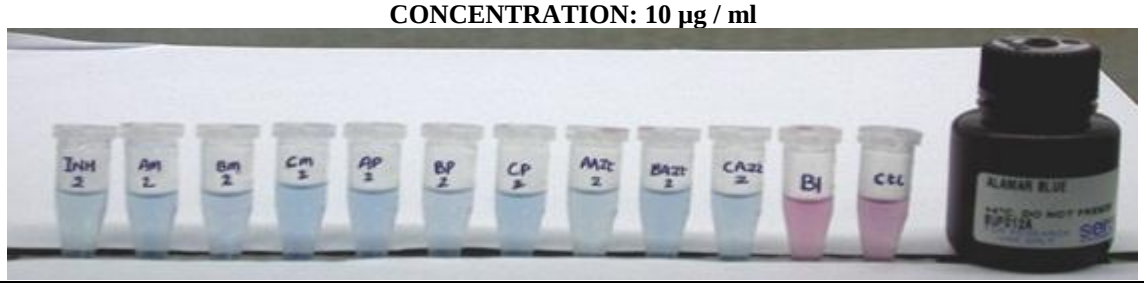


Fig 1. Showing Antitubercular Activity by Alamar Blue Assay Method

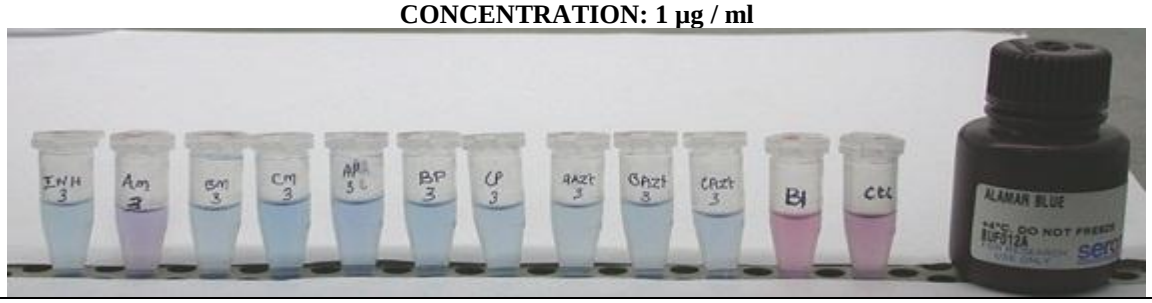
CONCENTRATION: 100 µg / ml



CONCENTRATION: 10 µg / ml



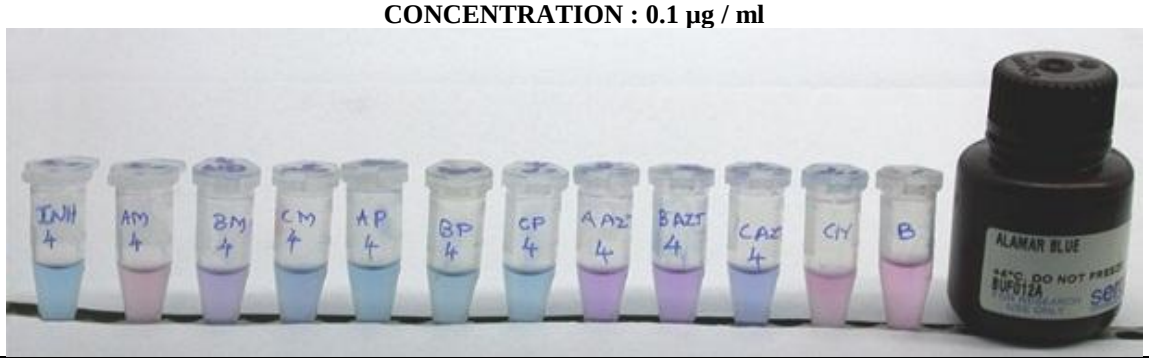
CONCENTRATION: 1 µg / ml



STRAINED USED: Mycobacterium Tuberculosis H_{37} RV

BLUE – Sensitive **PINK- Resistant**

CONCENTRATION : 0.1 µg / ml



CONCENTRATION: 0.05 µg / ml

CONCENTRATION: 0.01 µg / ml

STRAINED USED: Mycobacterium Tuberculosis H_{37} RV

BLUE – Sensitive **PINK- Resistant**

ACKNOWLEDGEMENT

My sincere thanks to Thiru. D.N.C.Manivannan, Chairman, Sri Vijay Vidyalaya College of Pharmacy, Nallampalli, Dharmapuri, Tamilnadu-636807 and Thiru K. Shanmugham, Chairman, Sri Shanmugha College of Pharmacy, Sankari (Tk), Morur (Po), Salem District, 637 304, Tamilnadu for providing very excellent facilities for the completion of my research work.

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Cite this article:

Rathinavel G and Senthilkumar KL. Synthesis of certain azetidin-2-one derivatives for anti-tubercular activities by alamar blue assay method. ***International Journal of Biological & Pharmaceutical Research***. 2018; 9(3):90-97.

DOI: http://dx.doi.org/10.21276/ijbpr.2018.9.3.6