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(RESEARCH ARTICLE)

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Synthesis, characterization and *in vitro* anti mycobacterial activity of some novel benzothiazole derivatives

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Abstract

World Health Organization considers Mycobacterium tuberculosis is the most dangerous chronic transmittable disease. Development of resistance by Mycobacterium tuberculosis is a common problem world Wide. To combat these problems there could be a solution that we should search newer molecules with different mechanism of action to fight against such diseases. A series of some novel benzothiazole derivatives (I-X) have been synthesized and evaluated for their in vitro antimycobacterial activity against Mycobacterium tuberculosis H37Rv using Microplate Alamar Blue Assay Method (MABA). Structures of the synthesized compounds were supported by means of IR, NMR and MASS spectral studies. Among the synthesized compounds, compound [IX], 2-[5-(4-Bromophenyl)-2-furylcarboxamido]-6-methoxy-l,3-benzothiazole exhibited a significant activity when compared with the standard drug Isoniazid. This could be the remarkable starting point to develop new lead molecules with potential antitubercular activity.

Keywords: Mycobacterium tuberculosis H37Rv; Benzothiazole; Antitubercular activity; Isoniazid; MABA

1. Introduction

Tuberculosis (TB) is a pulmonary illness caused by Mycobacterium tuberculosis characterized by symptoms which include coughing with thick mucus, fever, fatigue, and chest pain [1]. Around one third of the world's populations are infected, resulting in some two million deaths per year [2]. The dramatic increase in TB observed in the recent years is a result of two major factors. Firstly, the increased susceptibility of people infected with acquired immunodeficiency syndrome (AIDS) to TB, which augments the risk of developing the disease 100 fold [3]. Secondly, the increase in resistant strains of the disease [4] with some shows cross-resistance to as many as drugs. Today, first line drugs used in short-course treatment involve different combinations of Isoniazid, Rifampicin, Pyrazinamide, Ethambutol, and Streptomycin. However, due to inappropriate dosage, duration prescribed, and poor patient compliance, drug resistant strains started to emerge [1]. Multi-Drug Resistant TB is a form of TB that fails to respond to first-line drugs. In 2009, 3.3% of all new TB cases were estimated to be multi-drug resistant [5]. New drugs to treat TB are thus urgently required, specifically those which involve shorter treatment regimen than first-line drugs.

Heterocyclic compounds are of immense importance due to their wide spectrum of pharmacodynamics applications. These compounds have attracted the attention of chemists and biologists due to their varied nature of physicochemical and pharmacological activities. It is equally interesting for its theoretical implication for the diversity of its synthetic procedure and for the physiological and industrial significances. Synthetic heterocyclic chemistry has influenced almost every place of human life and the heterocyclic compounds have found their application in diverse field as medicine, agriculture, polymer, and various industries.

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Various natural drugs such as papaverine, theophylline, procaine, reserpine, quinine, atropine, emetine, morphine and codeine are heterocyclic in nature. Most of the synthetic drugs such as isoniazid (INH), diazepam, metronidazole, chlorpromazine, barbiturates, captopril, methotrexate, azidothymidine and antipyrine are also heterocyclic compounds. Heterocyclic compounds occupy a central position among those molecules that make life possible. Heterocycles have been explored for developing pharmaceutically important molecules.

Heterocycles bearing nitrogen atoms constitute the core structure of a number of important physiologically active molecules and play a major role in the metabolism of living cells. Their practical applications range from clinical use to fields as diverse as agriculture, biocide formulation, photography and polymer sciences. In recent decades there has been constant interest in the chemistry of azoles because more than hundred azole derivatives are used today as drugs [6]. Azoles are heterocyclic compounds characterized by a five-membered ring which contains an atom of nitrogen and at least one other non-carbon atom, nitrogen, sulfur or oxygen. These compounds are aromatic and have two double bonds. Azoles include pyrazole; imidazole; triazole; tetrazole; thiazole; thiadiazole; isothiazole; oxazole and oxadiazole nucleus.

The most widely studied application of heterocycles in the preparations of biologically active and medicinally important molecules. Modern drug discovery focuses on the synthesis of specific bimolecular targets, which invariably contain a heterocyclic component. A key challenge in the synthesis of such targets continues to be the development of new pathways and improvement of existing pathways.

2. Material and methods

2.1. Chemistry

All the chemical substances used were laboratory grade & provided by E. Merck (Germany) and S.D. Fine Chemicals (India). Melting points of all the synthesized compounds were determined by Open Tube Capillary Method and are uncorrected. Thin layer chromatography plates were prepared by Silica Gel G was used to monitor the reactions as well as to confirm the purity of the compounds synthesized and to verify the purity of the commercial reagents.

Two different solvent systems: toluene: ethyl acetate: formic acid (5:4:1) & petroleum ether: toluene: acetic acid (5:4:1) were used to run the Thin layer chromatography. The spots were visualized under Iodine Vapor / UV light. The IR spectra were recorded on a Perkin Elmer 1720 FT-IR spectrophotometer using KBr pellets. NMR spectra were obtained on Bruker AC 400 MHz in general using TMS as internal standard in $CDCl_3$ / DMSO-d_6. The FAB Mass spectra were obtained on a JEOL SX 102/DA 6000 Mass Spectrophotometer.

2.1.1. General procedure for synthesis of 5-(4-substituted phenyl)-2-furoic acid (I-III)

Suitably substituted anilines were diazotized at 0°C to get respective diazonium salts I, II, III(Products were not separated due to unstability), after 20 minutes it was directly filtered into a solution of furoic acid in acetone. The reaction mixture was maintained at 25±2°C for 48h with occasional stirring. The compound so separated was filtered and washed with plenty of water and recrystallized to get (IV).

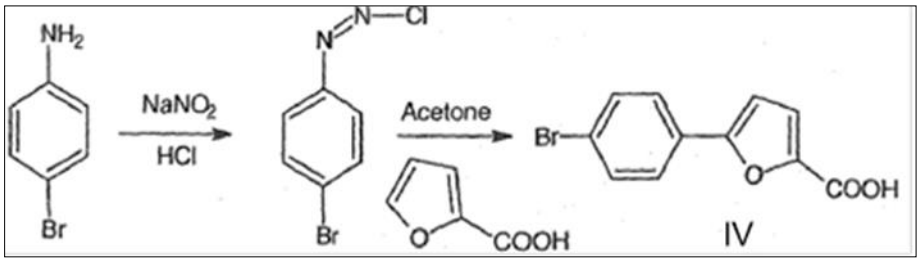


Figure 1 5-(4-Fluorophenyl)-2-furoic acid (IV)

IR(KBr, cm^{-1}): 3186 (COOH), 1684 (C=0), 1541 (C=C). ^{1}HNMR (300 MHz, $CDCl_3$, δ, ppm): 6.84 (1-H. d, J=: 3.6Hz, furyl-H), 6.92 (1-H, d, J = 3.6 Hz, furyl-H), 7.52-7.65 (4H, m, Ar-H), (1H, s,-COOH Missing).

2.1.2. General Procedure for Synthesis of Compounds (V-X)

The compound IV with 6-substituted-2-amino benzothiazole was stirred at 5±2°C for 5h in dry pyridine with catalytic amount of phosphorous oxychloride. After completion of the reaction the compounds were poured onto the crushed ice. A solid compound, so separated were filtered and washed with plenty of water and recrystallized from ethanol.

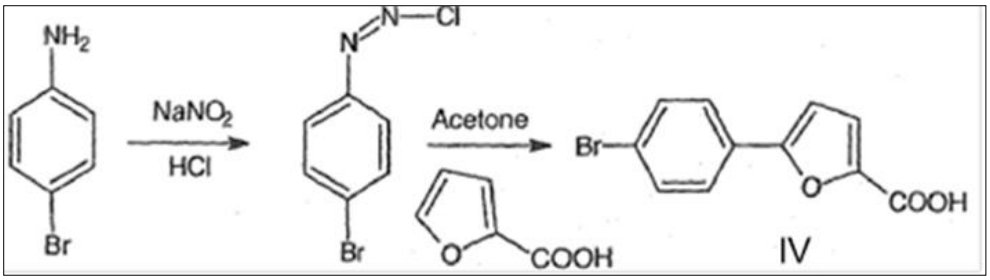


Figure 2 2-[5-(4-Bromophenyl)-2-furylcarboxamido]-l, 3-benzothiazole (V)

IR (KBr, cm^{-1}): 3232 (CONH), 1651 (C=0), 1394 (Benzothiazole). ^{1}HNMR (DMSO, d_6, δ, ppm): 7.26-7.42 (3-H, m, Ar-H), 7.64 (1-H, d, J= 3.6 Hz, furyl-H), 7.71 (1-H, d, J=4.0Hz, furyl-H), 7.75-7.92 (5-H, m, Ar-H), 12.22 (1-H, s, Amide-H). Anal.Calcd.for. $C_{18}H_{11}BrN_20_2S$: C, 54.15; H, 2.78; N, 7.02. Found. C, 54.10; H, 2.77; N, 7.00 %.

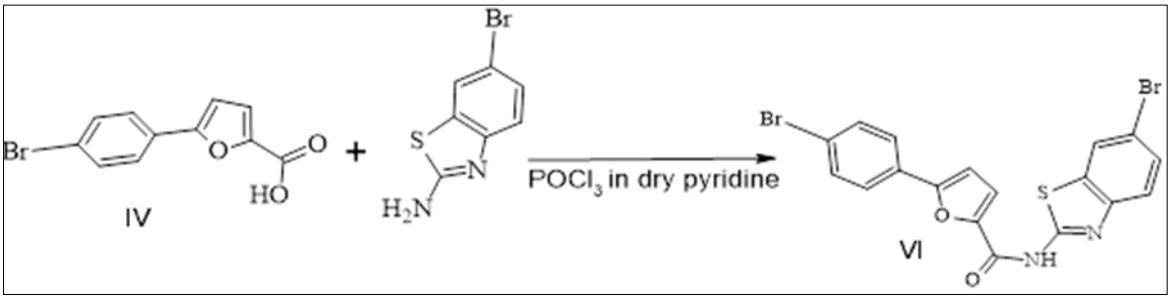


Figure 3 6-Bromo-2-[5-(4-bromophenyl)-2-furylcarboxamido]-1,3-benzothiazole (VI)

IR (KBr, cm^{-1}): 3252 (CONH), 1710 (C=0), 1411 (Benzothiazole). ^{1}HNMR (DMSO, d_6, δ, ppm): 7.19 (1-H, d, J=4.0Hz, furyl-H), 7.28 (1-H, d, J=3.6Hz, furyl-H), 7.51-7.68 (3-H, m, Ar-H), 7.72-7.8 (2-H, m, Ar-H)), 7.92 (2-H, d, J = 8Hz, Ar-H), 13.01(1-H, s, Amide-H). Anal.Calcd.for $C_{18}H_{10}Br_2N_2O_2S$: C, 45.21; H, 11; N, 5.86. Found: C, 45.20; H, 2.10; N, 5.89%.

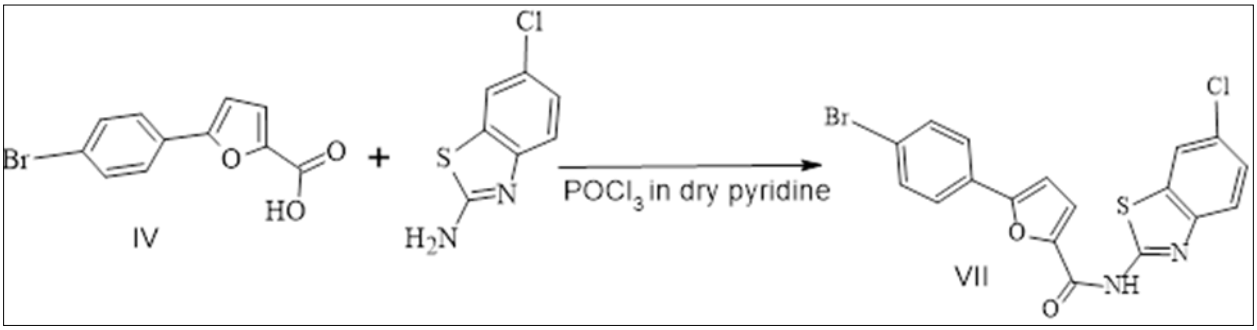


Figure 4 2-[5-(4-Bromophenyl)-2-furylcarboxamido]-6-Chloro-l, 3-benzothiazole (VII)

IR (KBr, cm^{-1}); 3243 (CONH), 1658 (C=0), 1398 (Benzothiazole). ^{1}HNMR (DMSO, d_6, δ, ppm): 7.18 (1-H, d, J= 4.0Hz, furyl-H), 7.31 (1-H, d, J=4.0Hz, furyl-H), 7.47 (2-H, d, J = 4.8Hz, Ar-H), 7.67 (3-H, m, Ar-H), 7.78 (2-H, d, J=5.2Hz, Ar-H), 13.12 (1-H, s, Amide-H). Anal.Calcd.for $C_{18}H_{10}BrClN_2O_2S$: C, 49.85; H, 2.32; N, 6.46. Found. C, 49; H, 2.32; N, 6.45%.

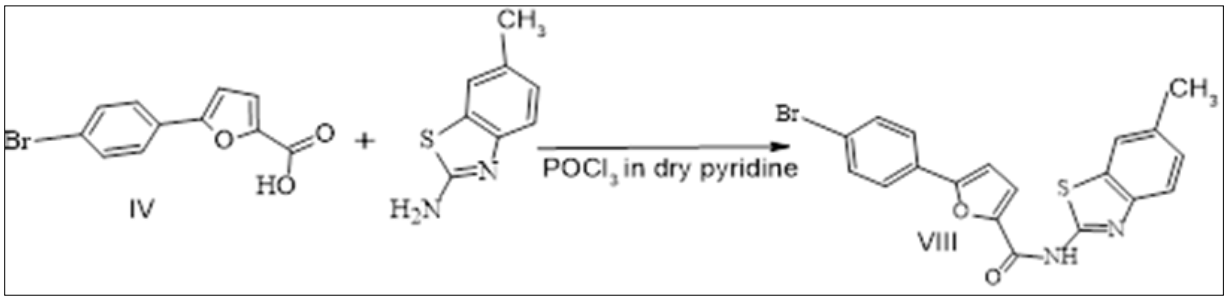


Figure 5 2-[5-(4-Bromophenyl)-2-furylcarboxamido]-6-methyl-1, 3-benzothiazole (VIII)

IR (KBr, cm^{-1}): 3234 (CONH), 1662 (C=0), 1414 (Benzothiazole). ^{1}H-NMR ($CDCl_3$, δ, ppm):2450 (3-H, s, $-CH_3$), 6.81 (1-H, d, J=3.6Hz, furyl-H), 7.19 (1-H, d, Ar-H, J= 8.4Hz), 7.45 (1-H, d, J=3.6 furyl-H), 7.51-7.61 (7H, m, 6-Ar-H + 1 Amide-H). Anal.Calcd.for $C_{19}H_{13}BrN_20_2S$: C, 55.22; H, 3.17; N, 6.78. Found. C, 55.02; H, 3.16; N, 6.79%.

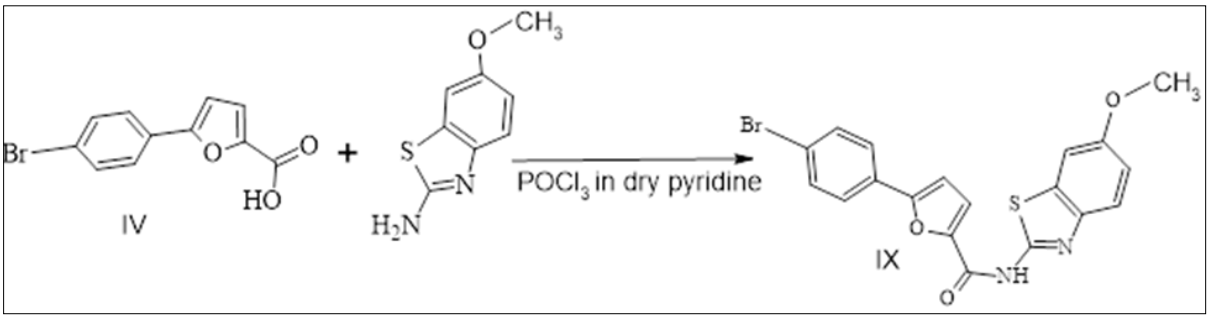


Figure 6 2-[5-(4-bromophenyl)-2-furylcarboxamido]-6-methoxy-l,3-benzothiazole(IX)

IR (KBr, cm^{-1}): 3235 (CONH), 1650 (C=0), 1408 (Benzothiazole). ^{1}HNMR (DMSO, d_6, δ, ppm): 3.84(3-H, s, $-OCH_3$), 6.69-8.00 (9-H, m, 2-furyl-H+7-Ar-H), 10.64 (1-H, s, Amide-H). Anal.Calcd.for $C_{19}H_{13}BrN_2O_3S$: C, 53.16; H, 3.05; N, 6.53. Found. C, 53.22; H, 3.04; N, 6.53%.

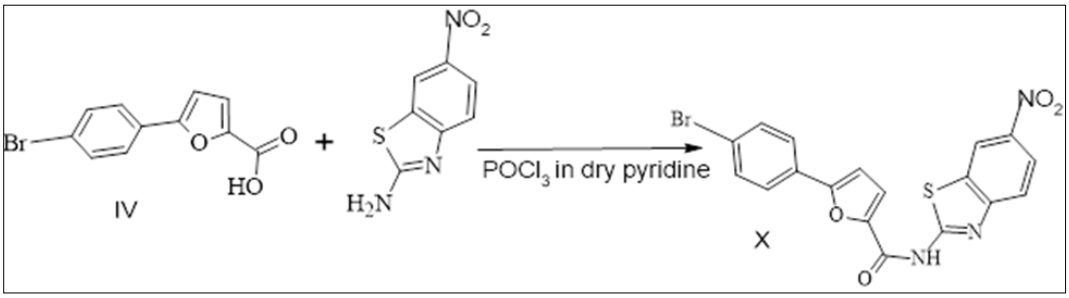


Figure 7 2-[5-(4-bromophenyl)-2-furylcarboxamido]-6-nitro-l, 3-benzothiazole (X)

IR (KBr, cm^{-1}): 3244 (CONH), 1647 (C=0), 1314 (Benzothiazole). ^{1}H-NMR (DMSO, d_6, δ, ppm): 7.32 (1-H, d, J = 3.6Hz, furyl-H), 7.44-7.48 (2-H, m, Ar-H), 7.65 (1-H, d, J=3.2Hz, furyl-H), 7.79-7.84 (2-H, m, Ar-H), 7.98-8.20 (3-H, m, Ar-H), 11.61 (1-H, s, Amide-H). Anal.Calcd.for $C_{18}H_{10}BrN_30_4S$: C, 48.66; H, 2.27; N, 9.46. Found. C, 48.70; H, 2.27; N, 9.50%.

2.2. Antitubercular Activity

Antimycobacterial assay was done by Microplate Alamar Blue Susceptibility Test (MABA) [7]. It was performed in black, clear bottomed, 96-well microplates (black view plates; Packard Instrument Company, Meriden, Conn) in order to minimize background fluorescence. Outer perimeter wells were filled with sterile water to prevent dehydration in experimental wells. Initial drug dilutions were prepared in either DMSO or distilled deionized water and subsequent 2 fold dilutions were performed in 0.1ml of 7H9GC (no Tween 80) in the microplates. BACTEC 12B passaged inocula were initially diluted 1:2 in 7H9GC and 0.1ml was added to wells. Subsequent determination of bacterial titers yielded 1×10^6, 2.5×10^6 and 3.5×10^5 CFU/ml in plate wells for M.tuberculosis H37RV. Frozen inocula were initially diluted 1:20 in

BACTEC 12B medium followed by 1:50 dilution in 7H9GC. Addition of 1/10ml to wells resulted in final bacterial titers of 2.0×10^5 and 5×10^4 CFU/ml for M.tuberculosis H37RV. Wells containing drugs only were used to detect auto fluorescence of compounds. Additional control wells consisted of bacteria only (B) and medium only (M). Plates were incubated at 37°C. Starting at day 4 of incubation, 20µl of 10× Alamar Blue solution (Alamar Biosciences/Accumed, Westlake, Ohio) and 12.5µl of 20% Tween 80 were added to one B well and one M well and plates were incubated at 37°C. Wells were observed at 12 and 24hrs for a color change from blue to pink and for a reading of ≥50,000 fluorescence units (FU). Fluorescence was measured in a Cytofluor II Microplate Flourometer (Perseptive Biosystems, Framingham, MA) in bottom reading mode with excitation at 530nm and emission at 590nm. If the B wells became pink by 24hr, reagent was added to the entire plate. If the well remained blue or ≤50000 FU was measured, additional M and B wells were tested daily until a color change occurred, at which time reagents were added to all remaining wells. Plates were then incubated at 37°C and the results were recorded at 24hrs post-reagent addition. Visual Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of drug that prevented a color change. For flourometric MICs, a background subtraction was performed on all wells with a mean of triplicate M wells. Percent inhibition was defines as 1-(test well FU/mean FU of triplicate B wells) × 100. The lowest drug concentration effecting an inhibition of ≥ 90% was considered the MIC.

2.3. Cell Cytotoxicity (CC_{50})

The VERO cell cytotoxicity assay was performed for active compounds only. After 72 hrs exposure viability was assessed by using Promega's Cell Titer Glo Luminescent Cell Viability Assay, a homogeneous method of determining the number of viable cells in culture based on quantitation of the ATP present. Cytotoxicity was determined from the dose response curve as the CC_{50} using a curve fitting program. Ultimately the CC_{50} was divided by the IC_{90} to calculate SI (selectivity index) value. The compounds with SI values of ≥10 considered for further testing.

3. Results and discussion

3.1. Synthesis of 5-(4-substituted phenyl)-2-furoic acid (IV)

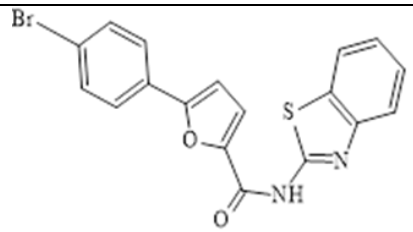
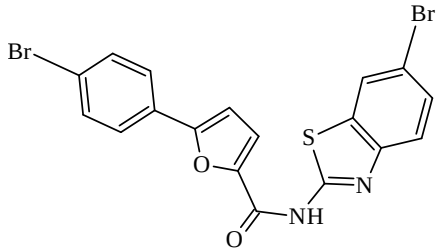
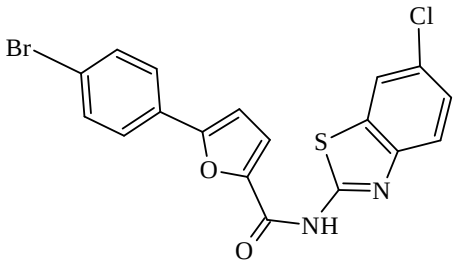
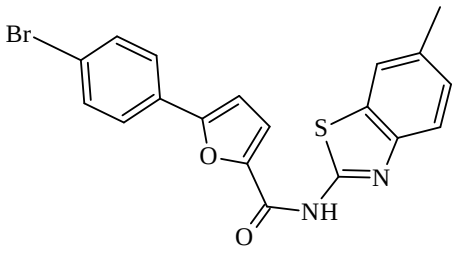
p-Substituted aniline was diazotized with $NaNO_2$ in presence of conc. HCl and maintained at 0°C for further 20 minutes. Thereafter, the diazotized compound in solution was directly filtered into a solution of acetone containing 2 -furoic acid, $CuCl_2$ and water. The evolution of nitrogen insured the progress of the reaction and its cessation confirmed the completion of the reaction. During the reaction, a solid separated was filtered & washed with plenty of water. From the crude reddish material desired product was extracted by means of sodium bicarbonate solution followed by its neutralization with dilute HCI. The structure of the compounds was determined by ^{1}HNMR and IR spectra through functional group and proton environment respectively. In the IR the characteristic C=O absorption was very prominent, a broad absorption due to COOH in IR at ≃3186 cm^{-1} was considered as final.

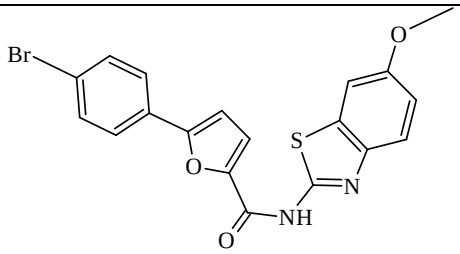
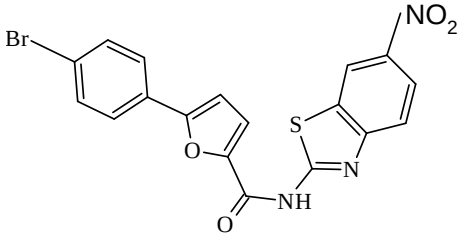
^{1}H-NMR spectra defines two furyl protons with J values of 3.6Hz which also indicates that the point of attachment for aryl ring is at 5th position of 2-furoic acid. The spectrum explained all the four protons of aryl ring with usual splitting whereas acidic (COOH) proton was missing. The mass spectrum also supports the structure of the compounds.

3.2. Condensation of 5-(4-substituted phenyl)-2-furoic acid with 2-amino-6-substituted benzothiazoles (V-X)

5-(4-substituted phenyl)-2-furoic acid (IV) was condensed separately with 2- amino-6-substituted benzothiazole in dry pyridine using $POCl_3$ in catalytic amount. During the reaction the temperature was maintained between 0-5°C. After completion and usual work of the reaction the elemental analysis was performed and found satisfactory. The formation of product was also confirmed by negative sodium bicarbonate effervescence test. In ^{1}HNMR spectrum, the signals of the respective protons of the compounds were verified on the basis of their chemical shifts, multiplicities and their coupling constants. In most of the spectra 2 doublet were usual for furyl protons with J values ranging from 3.2 to 4.0Hz. Apart from multiplets due to aromatic protons, a prominent singlet in the down field region was attributed to amide proton. The physical data are presented in Table 1.

Table 1 Physical Properties of the Synthesized Compounds

Compound	Structure & IUPAC Name	Mol. Formula	Mol. Weight	% Yield	M.P °C
V	 **2-[5-(4-Bromophenyl)-2-furylcarboxamido]-l, 3-benzothiazole**	$C_{18}H_{11}BrN_2O_2S$	399	67	110-14
VI	 **6-Bromo-2-[5-(4-bromophenyl)-2-furylcarboxamido]-1,3-benzothiazole**	$C_{18}H_{10}Br_2N_2O_2S$	478	76	170-72
VII	 **2-[5-(4-Bromophenyl)-2-furylcarboxamido]-6-Chloro-l, 3-benzothiazole**	$C_{18}H_{10}BrClN_2O_2S$	433	63	220-22
VIII	 **2-[5-(4-Bromophenyl)-2-furylcarboxamido]-6-methyl-1, 3-benzothiazole**	$C_{19}H_{13}BrN_2O_2S$	413	67	140-42

IX	 **2-[5-(4-bromophenyl)-2-furylcarboxamido]-6-methoxy-l,3-benzothiazole**	C_{19} H13BrN_2O_3S	429	58	98-100
X	 **2-[5-(4-bromophenyl)-2-furylcarboxamido]-6-nitro-l, 3-benzothiazole**	$C_{18}H_{10}BrN_3O_4S$	444	62	142-44

3.3. Antimycobacterial Activity

Table 2 Antimycobacterial activity against H37Rv strain of Mycobacterium tuberculosis

Compound	MABA: H37Rv Data		CTG:Vero cell	SI (CC_{50}/IC_{90})
	IC_{50} (µg/ml)	IC_{90} (µg/ml)	CC_{50}	
V	13.202	29.654	-	-
VI	7.315	>50	-	-
VII	4.752	11.175	-	-
VIII	2.289	5.3	8.739	1.648
IX	1.169	3.386	>40	>11.8
X	0.794	1.724	6.64	3.831

MABA-Micro plate Alamar Blue Assay; H37Rv strain of Mycobacterium tuberculosis used in the study; IC_{50}, IC_{90}- concentration that causes 50% and 90% microbial growth inhibition respectively; CTG-cytotoxicity in Vero cell culture to determine 50% cytotoxic concentration (CC_{50}); SI-Selectivity Index, should be more than or equal to 10 for further studies; -Not applicable.

The newly synthesized compounds were evaluated in vitro for anti-tubercular activity on Mycobacterium tuberculosis H37Rv strain (ATCC27294) by Micro plate Alamar Blue Assay Method. Isoniazid is reported to have MIC at 0.05 µg/ml with selectivity index of >1250 by MABA [8]. For each compound both IC_{50} and IC_{90} were determined. The IC_{50} value (inhibitory concentration 50%) corresponds to the concentration of the compound that inhibits 50% of bacterial growth. The IC_{90} or MIC referred to the concentration of the compound required to inhibit 90% bacterial growth. Compounds which exhibited 90% growth inhibition at a concentration of ≤ 10 µg/ml were evaluated for cytotoxicity. The cytotoxicity studies were performed on Vero cell lines and CC_{50} values were determined. CC_{50} value (50% cytotoxic concentration) corresponds to the concentration of the compound which affects 50% of the cells. The values of CC_{50} were divided by IC_{90} of respective compounds to determine Selectivity Index (SI). Only those compounds which had SI ratio ≥10 were selected for further evaluation.

Total six compounds (V-X) of benzothiazole series were evaluated for antitubercular activity by MABA and most of the compounds of the series were showed weak to highly significant activity.

3 compounds of the series (VIII, IX, X*)* were found to have antitubercular activity at less than 10 µg/mL concentration. The compounds VIII, IX & X found to have IC_{90} at 5.3, 3.386 and 1.724 µg/mL respectively. These compounds were put on to VERO cell culture to estimate cytotoxic concentration 50% (CC_{50}). The CC_{50} concentration was recorded to be 8.739, >40 and 6.64 µg/mL respectively. On the basis of MIC the most active compound of the series was found to be X with IC_{90} value of 1.724 µg/mL. whereas on overall basis compound IX with *p*-bromo substitution on the phenyl ring of arylfuran and methoxy substitution on 6th position of benzothiazole have some optimum correlation which have also been calculated to have selectivity Index (SI) >11.8. The compound IX is under investigation at higher levels of study. (Table 2).

4. Conclusion

Total six compounds were screened for antitubercular activity, out of which 3 compounds were considered as active with IC_{90} value ranging between 1.724-5.3 µg/mL. The CC_{50} values were determined for all the active compounds and used to calculate the SI. The selectivity index of all the 3 compounds was found less than 10, except one compound i.e. 2-[5-(4-Bromophenyl)-2-furylcarboxamido]-6-methoxy-l,3-benzothiazole (IX) have selectivity index greater than 11.8 and was considered the best compound and is under higher levels of screening with a hope to get a turning point in the fight against tuberculosis.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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