# Research Paper

# Design and Synthesis of New Bioactive 1,2,4-Triazoles, Potential Antitubercular and Antimicrobial Agents

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Singh, *et al.*: Design and Synthesis of New Bioactive 1,2,4-Triazoles

A New series of 1,2,4-triazole derivatives were synthesized using appropriate synthetic route and structures were confirmed by infrared spectroscopy, proton nuclear magnetic resonance, carbon-13 nuclear magnetic resonance, mass spectroscopy and elemental analysis. Antimycobacterial activity of the synthesized compounds (1-12) was carried out and percent reduction in relative light units was calculated using luciferase reporter phage assay. Percent reduction in relative light units for isoniazid was also calculated. The test compounds showed significant antitubercular potential against *Mycobacterium tuberculosis* H37Rv and clinical isolates, S, H, R and E resistant *M. tuberculosis*, when tested *in vitro*. Compound 8 and 12 showed better antitubercular activity compared to reference isoniazid against *M. tuberculosis* H37Rv strain while compounds 5, 8 and 12 found superior to isoniazid against clinical isolates: S, H, R and E resistant *M. tuberculosis*. Synthesized compounds were also tested *in vitro* against representative bacterial and fungal strains. Tested compounds showed better antibacterial activities (minimum inhibitory concentration) against Gram-positive bacteria compared to Gram-negative. Compound 5 showed better antibacterial activity than ampicillin against *B. subtilis*. Compound 12 in the series displayed most potent antifungal activity, which was comparable to reference fluconazole against both the fungal strains.

Key words: 1,2,4-Triazole, antibacterial, antimicrobial activity, Schiff base, isoniazid, LRP assay, *Mycobacterium tuberculosis*, thiol group

Although antibiotics are among the most prescribed drugs in the world today and their development and commercialization have saved countless lives, the unmet need of efficacious drugs against bacteria is still high. Several bacterial infections such as diarrhoea, food poisoning, rheumatic fever, extra intestinal and intestinal wall infections are caused by multidrug resistant bacteria[1,2]. This rapid rise in bacterial resistance towards available antibiotics is becoming a major threat to human health. Therefore, design of new class of compounds, with novel and distinct mode of action, from those of well-known classes, is of prime interest. Fungal infections pose a continuous and serious threat to human life. The severity of infection ranges from minor irritations such as athlete's foot to life-threatening systemic infections caused by *Aspergillus fumigates*[3]. Hence the development of a potent, safe and selective antifungal agent is of prime importance for medicinal chemist in the quest for effective chemotherapeutic treatment for fungal diseases[4].

Tuberculosis is the leading cause of mortality from a single infectious agent and is responsible for more than three million deaths worldwide every year[5]. Despite half a century of antitubercular chemotherapy, there are still 8-10 million new cases of active tuberculosis each year and nearly two billion individuals are believed to harbour latent tuberculosis. In 2011, there were an estimated 8.7 million new cases of tuberculosis (13 % co-infected with HIV) and 1.4 million people died from tuberculosis[6]. One third of the world's population is currently infected and more than 5000 people die from tuberculosis everyday[7]. The synergy between tuberculosis and the AIDS epidemic as well as the surge of multidrug resistant isolates of *Mycobacterium tuberculosis* has reaffirmed tuberculosis as a primary

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public health threat. The current threat in TB treatment lies in the emergence of strains resistant to two of the best antitubercular drugs, isoniazid (INH) and rifampicin (RIF).

Triazole heterocycles is a building block of great value in drug candidates[8,9] and a large number of ring systems containing this heterocyclic core have been incorporated into a wide variety of therapeutically interesting drug compounds including antiinflammatory, CNS stimulants, sedatives, antianxiety[10,11] and antimycotic agents such as fluconazole, itraconazole and voriconazole[12,13]. The 1,2,4-triazole, as well found to exhibit antiasthmatic[14], antiviral (ribavirin)[15], antifungal (fluconazole)[16], antimicrobial[17,18], antibacterial[19-21], insecticidal[22], hypnotic[23], cytotoxic[24], antitubercular[25] and hypotensive[26,27] activities. This moiety was also found in potent agonist and antagonist receptor ligands[28,29] in HIV-1 protease inhibitors[30] and in thrombin inhibitors[31].

Various isonicotinic acid derivatives like isonicotinyl hydrazides[32], isonicotinamide derivatives[33], 1,2,4-triazoles from isonicotinic acid hydrazide[34], novel nalidixic acid derived 1,2,4-triazole[35], 5-mercapto-1,2,4-triazole, novel 4H-1,2,4-triazol-3-yl-cycloalkanols[36], new imidazole and 1,2,4-triazole substituted fluorobenzimidazoles[37], and diphenylamine containing 1,2,4-triazoles[38], have been reported to possess diverse activity including antimicrobial and antitubercular. The therapeutic potential of 1,2,4-triazole prompted us to synthesize new compounds in which substituent could be arranged in a pharmacophoric pattern to display high order of antimycobacterial activity.

Driven by the findings of above documented literature, an attempt was made to incorporate various structural components to derive a novel pharmacologically active core so as to explore its potential further for antitubercular effect. The current study is such an attempt, in which 1,2,4-triazoles were synthesized and evaluated as antitubercular agents. The hypothesis was to join pyridine component of INH and Schiff base structure with the emerging antitubercular 1,2,4-triazole framework in these target compounds. The thiol group (-SH) has been introduced in the target compounds with the view that it could help binding to the cationic functional group or metal ion in the active site of the target. The compounds attempted to synthesize were given in fig. 1. Synthesized compounds were screened against sensitive and resistant *M. tuberculosis* strains. Additionally, these synthesized compounds were also

tested *in vitro* for antibacterial, antifungal activities against various strains.

## MATERIALS AND METHODS

The chemicals used for the experimental work were of synthetic grade (CDH, HiMedia, Rankem) and were used without further purification. Reactions were monitored by thin layer chromatography (TLC) on precoated silica gel-G glass plates using methanol and ethyl acetate in 3:7 ratio as mobile phase and the spots were visualized by iodine vapour. Melting points of synthesized compounds were determined by open glass capillary method and were uncorrected. Infrared (IR) spectra were recorded on Shimadzu FT-IR and Perkin-Elmer FT-IR spectrophotometer as KBr pellet and values are expressed as $\upsilon_{max}$ cm$^{-1}$. $^1$H and $^{13}$C nuclear magnetic resonance spectroscopy (NMR) spectral analysis of the synthesized compounds were recorded on a Bruker Avance III 500 MHz (AV 500) in deuterated chloroform using tetramethylsilane as internal standard. The chemical shift values were recorded on δ scale. Elemental analysis was undertaken with Elemental Vario EL III Carlo Erba 1108 analyser. Syntheses of the target compounds (1-12) were performed according to fig. 2, through following steps.

### Synthesis of potassium dithiocarbazinate (B)[39]:

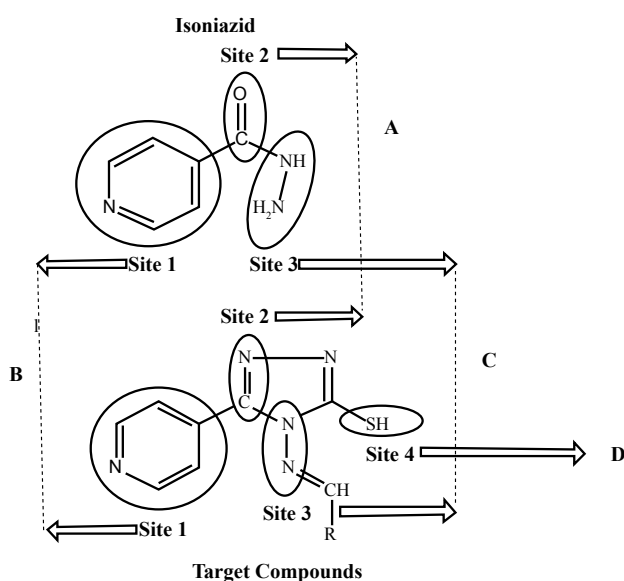Isonicotinic acid hydrazide (A; 14 g, 0.10 mol) was added to a solution of potassium hydroxide (8.4 g,



**Fig. 1: Pictorial design strategy and comparison of isoniazid and test compounds (1-12)**
**A: Isocratic fragment of C=O of isoniazid; B: pyridine component as in isoniazid; C: C–N–N chain resembles C–NH–NH$_2$ of isoniazid; D: help in binding to the cationic functional group or metal ion in the active site of the target**
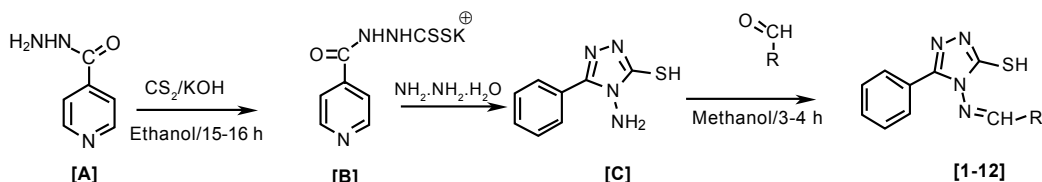
**Fig. 2: Synthesis of target compounds (1-12)**
1 (-CH₃), 2 (-CH₂CH₃), 3 (CH₂CH₂CH₃), 4 (C₆H₅), 5 (4-F-C₆H₄-), 6 (4-Cl-C₆H₄-), 7 (4-Br-C₆H₄-), 8 (4-CH₃-C₆H₄-), 9 (4-OCH₃-C₆H₄-), 10 (-4-NO₂-C₆H₄), 11 (-3,4,5-(OCH₃)₃-C₆H₂-), 12 (CH=CH-C₆H₅-)

0.15 mol) in absolute ethanol (100 ml) and resulting content was stirred for 10-15 min at room temperature. After this, carbon disulphide (8 ml, 0.15 mol) and ethanol (50 ml) were added to the above mixture and stirred further for 15-16 h at room temperature till the complete precipitation of potassium dithiocarbazinate (B). Precipitate thus obtained was filtered. After evaporating to dryness under reduced pressure, the residue was purified by column chromatography, eluting with methanol:ethyl acetate (30:70 v/v).

**Synthesis of 4-amino-5-pyridin-4-yl-1,2,4-triazole-3-thiol (C)[40]:**

Potassium dithiocarbazinate (B; 21 g, 0.079 mol), hydrazine hydrate (12 ml, 0.24 mol) and distilled water (50 ml) were placed in a round bottom flask and refluxed for 4 h, after which the reaction mixture was cooled to room temperature, diluted with cold distilled water (50 ml) and acidified with dilute acetic acid to obtain a light yellow precipitate, which, was then filtered, washed with cold distilled water, dried and recrystallized with methanol to get white crystals of 4-amino-5-pyridin-4-yl-1,2,4-triazole-3-thiol (C).

**Synthesis of target compounds (1-12)[41]:**

A mixture of compound (C; 1g, 0.005 mol) and equimolar quantity of respective aldehydes in methanol (50-70 ml) was heated until a clear solution was obtained. This solution in presence of a few drops of concentrated hydrochloric acid was then refluxed for 3-4 h. After this reaction mixture was kept overnight at room temperature to obtain the corresponding product, which was then filtered. Evaporation and purification on silica-gel column (methanol/methylene dichloride 12:88 v/v) yielded compounds 1-12.

**4-Ethylideneamino-5-pyridin-4-yl-4H-[1,2,4] triazole- 3-thiol (1):**

Yield 61 % as off white solid, melting point 213-215°, IR (KBr, cm⁻¹) $\upsilon_{max}$: 2733 (S-H), 1617 (C=N), 1121 (C-O), 702 (C-S); ¹H NMR (300 MHz, CDCl₃): 7.63 (m, 2H of 4-pyridine), 8.66 (m, 2H of 4-pyridine),

3.08 (s, 1H of aromatic C-SH), 7.5 (s, 1H of CH of aldimine), 1.0 (d, 3H of CH₃); ¹³C NMR (300 MHz, CDCl₃): 143.4, 122.1, 150.4, 150.4, 122.1 (C of 4-pyridine), 148.2 (C₃ and C₅ of triazole), 163.8 (C of N=CH), 9.33 (C of CH₃); Anal. Calcd for C₉H₉N₅S: C, 49.30; H, 4.14; N, 31.94; S, 14.62. Found: C, 49.31; H, 4.14; N, 31.96; S, 14.6. Mass (ES+) spectra of compound exhibited molecular ion peak at m/z 219 (M⁺).

**4-Propylideneamino-5-pyridin-4-yl-4H-[1,2,4] triazole-3-thiol (2):**

Yield 55 % as mud white fluffy solid, melting point 219-21°, IR (KBr, cm⁻¹) $\upsilon_{max}$: 2725 (S-H), 1607 (C=N), 1126 (C-O), 699 (C-S); ¹H NMR (300 MHz, CDCl₃): 7.65 (m, 2H of 4-pyridine), 8.6 (m, 2H of 4-pyridine), 3.05 (s, 1H of aromatic C-SH), 7.53 (s, 1H of CH of aldimine), 1.4 (m, 2H of CH₂), 1.1 (t, 3H of CH₃); ¹³C NMR (300 MHz, CDCl₃): 143.5, 122.5, 150.1, 150.1, 122.5 (C of 4-pyridine), 148.0 (C₃ and C₅ of triazole), 163.5 (C of N=CH), 18.54 (C of CH₂), 10.33 (C of CH₃); Anal. Calcd for C₁₀H₁₁N₅S: C, 51.48; H, 4.75; N, 30.02; S, 13.74. Found: C, 51.47; H, 4.76; N, 30.04; S, 13.74. Mass (ES+) spectra of compound exhibited molecular ion peak at m/z 233 (M⁺).

**4-Butylideneamino-5-pyridin-4-yl-4H-[1,2,4] triazole -3-thiol (3):**

Yield 63 % as off white solid, melting point >229°, IR (KBr, cm⁻¹) $\upsilon_{max}$: 2734 (S-H), 1611 (C=N), 1125 (C-O), 695 (C-S); ¹H NMR (300 MHz, CDCl₃): 7.61 (m, 2H of 4-pyridine), 8.66 (m, 2H of 4-pyridine), 3.02 (s, 1H of aromatic C-SH), 7.55 (s, 1H of CH of aldimine), 1.34 (m, 2H of CH₂), 1.42 (m, 2H of CH₂), 1.0 (t, 3H of CH₃); ¹³C NMR (300 MHz, CDCl₃): 143.2, 122.5, 150.2, 150.2, 122.5 (C of 4-pyridine), 148.2 and 148.1 (C₃ and C₅ of triazole), 163.7 (C of N=CH), 29 (C of CH₂), 19.3 (C of CH₂), 14.1 (C of CH₃); Anal. Calcd for C₁₁H₁₃N₅S: C, 53.42; H, 5.30; N, 28.32; S, 12.97. Found: C, 53.46; H, 5.32; N, 28.29; S, 12.92. Mass (ES+) spectra of compound exhibited molecular ion peak at m/z 247 (M⁺).

## 4-(Benzylidene-amino)-5-pyridin-4-yl-4H-[1,2,4]triazole-3-thiol (4):

Yield 58 % as brown solid, melting point 236-237°, IR (KBr, cm$^{-1}$) $\upsilon_{max}$: 2720 (S-H), 1615 (C=N), 1120 (C-O), 696 (C-S); $^1$H NMR (300 MHz, CDCl$_3$): 7.61 (m, 2H of 4-pyridine), 8.63 (m, 2H of 4-pyridine), 3.02 (s, 1H of aromatic C-SH), 8.09 (s, 1H of CH of N=CH), 7.60 (m, 2H of 4-aromatic CH), 7.28 (m, 3H of 4-aromatic CH); $^{13}$C NMR (300 MHz, CDCl$_3$): 143.8, 122.5, 150.1, 150.1, 122.5 (C of 4-pyridine), 148.1 (C$_3$ and C$_5$ of triazole), 163.8 (C of N=CH), 131.1, 129.2, 128.5, 130.3, 128.5 and 129.2 (C of 4-aryl); Anal. Calcd for C$_{14}$H$_{11}$N$_5$S: C, 59.77; H, 3.94; N, 24.89; S, 11.40. Found: C, 59.75; H, 3.94; N, 24.9; S, 11.40. Mass (ES+) spectra of compound exhibited molecular ion peak at m/z 281 (M$^+$).

## 4-[(4-Fluoro-benzylidene)-amino]-5-pyridin-4-yl-4H-[1,2,4]triazole-3-thiol (5):

Yield 70 % as orange red solid, melting point 240-242°, IR (KBr, cm$^{-1}$) $\upsilon_{max}$: 2737 (S-H), 1611 (C=N), 1122 (C-O), 690 (C-S); $^1$H NMR (300 MHz, CDCl$_3$): 7.64 (m, 2H of 4-pyridine), 8.63 (m, 2H of 4-pyridine), 3.0 (s, 1H of aromatic C-SH), 8.08 (s, 1H of CH of N=CH), 7.62 (m, 2H of 4-aromatic CH), 7.01 (m, 2H of 4-aromatic CH); $^{13}$C NMR (300 MHz, CDCl$_3$): 143.3, 122.9, 150.4, 150.4, 122.9 (C of 4-pyridine), 148.2 and 148.1 (C$_3$ and C$_5$ of triazole), 163.5 (C of N=CH), 126.7, 131.1, 116.2, 165.3, 116.2 and 131.1 (C of 4-aryl); Anal. Calcd for C$_{14}$H$_{10}$FN$_5$S: C, 56.18; H, 3.37; F, 6.35; N, 23.40; S, 10.71. Found: C, 56.19; H, 3.37; F, 6.37; N, 23.40; S, 10.73. Mass (ES+) spectra of compound exhibited molecular ion peak at m/z 299 (M$^+$).

## 4-[(4-Chloro-benzylidene)-amino]-5-pyridin-4-yl-4H-[1,2,4]triazole-3-thiol (6):

Yield 78 % as orange crystalline solid, melting point 237-238°, IR (KBr, cm$^{-1}$) $\upsilon_{max}$: 2731 (S-H), 1610 (C=N), 1114 (C-O), 694 (C-S); $^1$H NMR (300 MHz, CDCl$_3$): 7.6 (m, 2H of 4-pyridine), 8.7 (m, 2H of 4-pyridine), 3.05 (s, 1H of aromatic C-SH), 8.05 (s, 1H of CH of N=CH), 7.63 (m, 2H of 4-aromatic CH), 7.29 (m, 2H of 4-aromatic CH); $^{13}$C NMR (300 MHz, CDCl$_3$): 143.5, 122.5, 150.4, 150.4, 122.5 (C of 4-pyridine), 148.2 (C$_3$ and C$_5$ of triazole), 163.8 (C of N=CH), 130.1, 131, 130, 135.3, 130 and 131 (C of 4-aryl); Anal. Calcd for C$_{14}$H$_{10}$ClN$_5$S: C, 53.25; H, 3.19; Cl, 11.23; N, 22.18; S, 10.15. Found: C, 53.22; H, 3.19; Cl, 11.25; N, 22.18; S, 10.13. Mass (ES+) spectra of compound exhibited molecular ion peak at m/z 315 (M$^+$), 317 (M+1).

## 4-[(4-Bromo-benzylidene)-amino]-5-pyridin-4-yl-4H-[1,2,4]triazole-3-thiol (7):

Yield 66 % as yellow crystalline solid, melting point 204-205°, IR (KBr, cm$^{-1}$) $\upsilon_{max}$: 2730 (S-H), 1616 (C=N), 1136 (C-O), 691 (C-S); $^1$H NMR (300 MHz, CDCl$_3$): 7.58 (m, 2H of 4-pyridine), 8.71 (m, 2H of 4-pyridine), 3.1 (s, 1H of aromatic C-SH), 8.0 (s, 1H of CH of N=CH), 7.53 (m, 2H of 4-aromatic CH), 7.49 (m, 2H of 4-aromatic CH); $^{13}$C NMR (300 MHz, CDCl$_3$): 143.5, 122.3, 150.4, 150.4, 122.3 (C of 4-pyridine), 148, 148.2 (C$_3$ and C$_5$ of triazole), 163.6 (C of N=CH), 130.4, 131.1, 132.1, 125.3, 132.1 and 131.1 (C of 4-aryl); Anal. Calcd for C$_{14}$H$_{10}$BrN$_5$S: C, 46.68; H, 2.80; Br, 22.18; N, 19.44; S, 8.90. Found: C, 46.69; H, 2.81; Br, 22.17; N, 19.44; S, 8.90. Mass (ES+) spectra of compound exhibited molecular ion peak at m/z 361 (M$^+$), 359 (M+2).

## 4-[(4-Methyl-benzylidene)-amino]-5-pyridin-4-yl-4H-[1,2,4]triazole-3-thiol (8):

Yield 75 % as yellow crystalline solid, melting point 160-162°, IR (KBr, cm$^{-1}$) $\upsilon_{max}$: 2740 (S-H), 1620 (C=N), 1132 (C-O), 705 (C-S); $^1$H NMR (300 MHz, CDCl$_3$): 7.62 (m, 2H of 4-pyridine), 8.65 (m, 2H of 4-pyridine), 3.15 (s, 1H of aromatic C-SH), 8.15 (s, 1H of CH of N=CH), 7.55 (m, 2H of 4-aromatic CH), 7.17 (m, 2H of 4-aromatic CH), 2.35 (s, 3H of CH$_3$); $^{13}$C NMR (300 MHz, CDCl$_3$): 143.3, 122.3, 150.1, 150.1, 122.3 (C of 4-pyridine), 148.2 (C$_3$ and C$_5$ of triazole), 163.8 (C of N=CH), 128.4, 129.3, 129.1, 140.1, 129.1 and 129.3 (C of 4-aryl); Anal. Calcd for C$_{15}$H$_{13}$N$_5$S: C, 61.00; H, 4.44; N, 23.71; S, 10.86. Found: C, 61.03; H, 4.44; N, 23.71; S, 10.83. Mass (ES+) spectra of compound exhibited molecular ion peak at m/z 295 (M$^+$).

## 4-[(4-Methoxy-benzylidene)-amino]-5-pyridin-4-yl-4H-[1,2,4]triazole-3-thiol (9):

Yield 69 % as off white crystalline solid, melting point 208-210°, IR (KBr, cm$^{-1}$) $\upsilon_{max}$: 2742 (S-H), 1623 (C=N), 1133 (C-O), 702 (C-S); $^1$H NMR (300 MHz, CDCl$_3$): 7.61 (m, 2H of 4-pyridine), 8.67 (m, 2H of 4-pyridine), 3.08 (s, 1H of aromatic C-SH), 7.9 (s, 1H of CH of N=CH), 7.53 (m, 2H of 4-aromatic CH), 6.84 (m, 2H of 4-aromatic CH), 3.75 (s, 3H of OCH$_3$); $^{13}$C NMR (300 MHz, CDCl$_3$): 143.7, 122.1, 150.3, 150.3, 122.1 (C of 4-pyridine), 148, 148.1 (C$_3$ and C$_5$ of triazole), 163.5 (C of N=CH), 123.4, 130.1, 114.2, 164.5, 114.2 and 130.1 (C of 4-aryl), 56.1 (C of OCH$_3$); Anal. Calcd for C$_{15}$H$_{13}$N$_5$OS: C, 57.86; H, 4.21; N, 22.49; O, 5.14; S, 10.30. Found: C, 57.85; H, 4.21; N, 22.47; O, 5.11;

S, 10.31. Mass (ES+) spectra of compound exhibited molecular ion peak at m/z 311 ($M^+$).

## 4-[(4-Nitro-benzylidene)-amino]-5-pyridin-4-yl-4H-[1,2,4]triazole-3-thiol (10):

Yield 58 % as reddish brown powder, melting point 256-258°, IR (KBr, cm$^{-1}$) $\upsilon_{max}$: 2732 (S-H), 1628 (C=N), 1136 (C-O), 692 (C-S); $^1$H NMR (300 MHz, CDCl$_3$): 7.6 (m, 2H of 4-pyridine), 8.6 (m, 2H of 4-pyridine), 3.02 (s, 1H of aromatic C-SH), 8.03 (s, 1H of CH of N=CH), 7.92 (m, 2H of 4-aromatic CH), 8.31 (m, 2H of 4-aromatic CH); $^{13}$C NMR (300 MHz, CDCl$_3$): 143.4, 122.5, 150.7, 150.7, 122.5 (C of 4-pyridine), 148.4 (C$_3$ and C$_5$ of triazole), 163.1 (C of N=CH), 137.1, 130, 123.5, 150.3, 123.5 and 130 (C of 4-aryl); Anal. Calcd for C$_{14}$H$_{10}$N$_6$O$_2$S: C, 51.53; H, 3.09; N, 25.75; O, 9.81; S, 9.83. Found: C, 51.55; H, 3.1; N, 25.75; O, 9.83; S, 9.83. Mass (ES+) spectra of compound exhibited molecular ion peak at m/z 326 ($M^+$).

## 5-Pyridin-4-yl-4-[(3,4,5-trimethoxy-benzylidene)-amino]-4H-[1,2,4]triazole-3-thiol (11):

Yield 74 % as lemon yellow fluffy crystals, melting point 261-262°, IR (KBr, cm$^{-1}$) $\upsilon_{max}$: 2737 (S-H), 1614 (C=N), 1136 (C-O), 697 (C-S); $^1$H NMR (300 MHz, CDCl$_3$): 7.65 (m, 2H of 4-pyridine), 8.6 (m, 2H of 4-pyridine), 3.03 (s, 1H of aromatic C-SH), 8.0 (s, 1H of CH of N=CH), 6.53 (m, 2H of 4-aromatic CH), 3.76 (s, 9H of OCH$_3$); $^{13}$C NMR (300 MHz, CDCl$_3$): 143.5, 122.1, 150.5, 150.5, 122.1 (C of 4-pyridine), 148, 148.2 (C$_3$ and C$_5$ of triazole), 163.5 (C of N=CH), 125.4, 107.1, 148.2, 134.5, 148.2 and 107.1 (C of 4-aryl), 56.5 (C of OCH$_3$); Anal. Calcd for C$_{17}$H$_{17}$N$_5$O$_3$S: C, 54.97; H, 4.61; N, 18.86; O, 12.92; S, 8.63. Found: C, 54.99; H, 4.63; N, 18.82; O, 12.92; S, 8.63. Mass (ES+) spectra of compound exhibited molecular ion peak at m/z 371 ($M^+$).

## 4-(3-Phenyl-allylideneamino)-5-pyridin-4-yl-4H-[1,2,4]triazole-3-thiol (12):

Yield 66 % as brownish yellow crystals, melting point >243°, IR (KBr, cm$^{-1}$) $\upsilon_{max}$: 2733 (S-H), 1605 (C=N), 1124 (C-O), 699 (C-S); $^1$H NMR (300 MHz, CDCl$_3$): 7.63 (m, 2H of 4-pyridine), 8.62 (m, 2H of 4-pyridine), 3.0 (s, 1H of aromatic C-SH), 7.54 (s, 1H of CH of N=CH), 5.56 (d, 1H of CH=CH), 6.52 (d, 1H of CH=CH), 7.32 (m, 2H of benzene), 7.23 (m, 2H of benzene), 7.15 (m, 1H of benzene); $^{13}$C NMR (300 MHz, CDCl$_3$): 143.6, 122.3, 150.3, 150.3, 122.3 (C of 4-pyridine), 148.2, 148.1 (C$_3$ and C$_5$ of triazole), 163.6 (C of N=CH), 112.2 and 136.1 (C of CH=CH), 134.4, 126.1, 128.7, 127.5, 128.7 and 126.1 (C of 4-aryl); Anal. Calcd for C$_{16}$H$_{13}$N$_5$S: C, 62.52; H, 4.26; N, 22.78; S, 10.43. Found: C, 62.51; H, 4.24; N, 22.77; S, 10.43. Mass (ES+) spectra of compound exhibited molecular ion peak at m/z 307 ($M^+$).

## Biological evaluation:

All the newly synthesized 1,2,4-triazoles were assayed *in vitro* for antitubercular activity against *M. tuberculosis* H37Rv and clinical isolates: S, H, R and E resistant *M. tuberculosis*. In case of antimycobacterial activity, percentage reduction in relative light units (RLU) was calculated using luciferase reporter phage (LRP) assay using INH as a reference standard. Synthesized compounds were also tested against a representative panel of bacterial and fungal pathogens by the broth microdilution minimal inhibitory concentration (MIC) method.

## LRP assay[42,43]:

Fifty-microliter bacterial suspension equivalent to McFarland's No.2 standard was added to 400 ml of G7H9 with and without the test compound. For each sample, two drug-free controls and two drug concentrations were prepared and this setup was incubated for 72 h at 37°. After incubation 50 ml of the high titer LRP (phAE129) and 400 ml of 0.1 M CaCl$_2$ were added to all the vials and this setup was incubated at 37° for 4 h. After incubation, 100 ml of the mixture was taken from each tube into a luminometer cuvette and equal amount of working D-luciferin (0.3 mM in 0.05 M sodium citrate buffer, pH 4.5) solution was added. The RLU was measured after 10 s of integration in the luminometer (Monolight 2010). Duplicate readings were recorded for each sample and the mean was calculated. The percentage reduction in the RLU was calculated for each test sample and compared with the control. The experiment was repeated when the mean RLU of the control was less than 1000.

## Antibacterial and antifungal screening:

The *in vitro* antimicrobial activity of all the compounds and standard drugs were assessed against two representatives of Gram-positive bacteria *viz*. *S. aureus* (MTCC 96), *B. subtilis* (MTCC 441), two Gram-negative bacteria *viz*. *E. coli* (MTCC 1687), *P. aerugenosa* (MTCC 1688) and two fungi *viz*. *C. albicans* (MTCC 227), *A. niger* (MTCC 1344) by the broth microdilution MIC method. Mueller Hinton broth and Sabouraud dextrose broth were used as a nutrient medium to grow and dilute the compound

suspension for the test bacteria and fungi, respectively. Ampicillin and norfloxacin were used as standard antibacterial drugs, whereas fluconazole was used as standard antifungal drug.

Primary inoculation of bacteria was done into Mueller-Hinton agar for overnight growth to produce a number of colonies, which were then directly suspended in saline solution until the turbidity matched the turbidity of the McFarland standard ($10^8$ CFU ml$^{-1}$), i.e., inoculum size for test strain was adjusted to $10^8$ colony forming unit (CFU)/ml per well by comparing the turbidity (turbidimetric method). Similar procedure was adopted for fungi with Sabouraud dextrose broth. Dimethyl sulfoxide (DMSO) was used as diluents to get desired concentration of the synthesized compounds and standard drugs. Each compound and standard drugs were diluted to obtain 500 µg/ml concentrations, as a stock solution. Stock solution was further progressively diluted with the test medium and required concentrations were obtained for primary and secondary screening. In primary screening 500, 250 and 125 µg/ml concentrations of the synthesized compounds were tested. The active compounds found in this primary screening were further diluted and tested against the corresponding microorganism. Each test tube was then put for incubation at 37° for 24 h for bacteria and 48 h for fungi. Growth or a lack of growth in the tubes containing the antimicrobial agent was determined by comparison with the growth control, indicated by turbidity. The lowest concentration that completely inhibited visible growth of the organism was recorded as the MIC (µg/ml). A set of tubes containing only seeded broth and the DMSO controls were maintained under identical conditions so as to make sure that the solvent had no influence on strain growth. The interpretation of the results was based on fluconazole for the fungi and also on ampicillin and norfloxacin for bacterial pathogens. The results (MIC, µg/ml) obtained are summarized in Table 2.
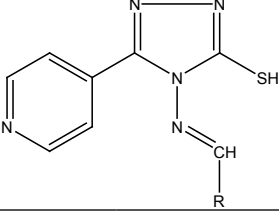
## RESULTS AND DISCUSSION

INH (A) was first reacted with potassium hydroxide in absolute ethanol by simple stirring the mixture at room temperature. Carbon disulphide and ethanol were then added to the above mixture and resulting content was stirred further for 15-16 h at room temperature to obtain potassium dithiocarbazinate (B). After this a mixture of potassium dithiocarbazinate (B), hydrazine hydrate and distilled water was refluxed for approximately 4 h and then cooled to room temperature, diluted with

cold distilled water and acidified with dilute acetic acid to obtain a light-yellow precipitate, which appeared as white crystals of 4-amino-5-pyridin-4-yl-1,2,4-triazole-3-thiol (C) after filtration, washing with cold water, drying and recrystallization. Finally, a mix of compound (C) and equimolar quantity of respective aldehyde in methanol was then refluxed for 3-4 h in presence of concentrated hydrochloric acid and kept overnight to obtain the corresponding products (1-12).

Structures of compounds 1-12 were confirmed by IR, NMR spectroscopy data as well as their distinct $R_f$ values in TLC analysis. As the desired target compounds (1-12) are formed, strong band of -NH$_2$ stretch (single band in the range 3350-3500 cm$^{-1}$) is disappeared and –C=N signal appear in the ranges 1605-1630 cm$^{-1}$ in their corresponding IR spectra. Similarly characteristic proton NMR signal near 7.5 for 1H of CH of aldimine further confirm the formation of the target compounds. Number of signals in $^{13}$C NMR spectra of compounds 1-12 was measured with their corresponding chemical shift values to characterize the formation of compounds. Compound 1 provided nine $^{13}$C NMR signals with characteristics 163.8 for C of N=CH and 9.33 for C of CH$_3$ attached to aldimine carbon. Similarly, compound 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 given 10, 11, 14, 14, 14, 14, 15, 15, 14, 17 and 16 $^{13}$C NMR signals along with characteristic range of aldimine C signal, which is given with individual compounds in experimental section.

All the newly synthesized compounds were assayed *in vitro* for antitubercular activity against *M. tuberculosis* H37Rv and clinical isolates: S, H, R and E resistant *M. tuberculosis*. In case of antimycobacterial activity, percent reduction in RLU was calculated using LRP assay at two different concentrations (50 and 100 µg/ml) using INH as a reference standard and the observed percent inhibition has been tabulated in Table 1. Compound is considered to be an antimycobacterial, if 50 % reduction in the RLU was observed when compared to the control using a luminometer.

At 50 µg/ml concentration, compounds 4, 5, 6, 8, 10 and 12 were found to be active, while at higher concentration (100 µg/ml), these compounds showed higher activities against *M. tuberculosis* H37Rv. Among these, compound 8 and 12 at 100 µg/ml exhibited greater reduction in RLU than the reference standard INH, while compound 5 at 100 µg/ml displayed reduction in RLU comparable to that of the INH. Most active compounds 8, 12 and 15 of this series

**TABLE 1: PERCENT REDUCTION IN RELATIVE LIGHT UNITS (RLU) AGAINST *M. TUBERCULOSIS* H37RV AND CLINICAL ISOLATES, S, H, R, AND E RESISTANT *M. TUBERCULOSIS***



| Compound | R | Percent reduction in RLU | | | |
|---|---|---|---|---|---|
| | | *M. tuberculosis* $H_{37}Rv$ | | *Clinical isolates, S, H, R and E resistant *M. tuberculosis* | |
| | | 50 µg/ml | 100 µg/ml | 50 µg/ml | 100 µg/ml |
| 1 | $-CH_3$ | 32.13 | 39.76 | 26.43 | 31.3 |
| 2 | $-CH_2CH_3$ | 35.3 | 43.28 | 25.0 | 32.53 |
| 3 | $-CH_2CH_2CH_3$ | 39.53 | 48.71 | 29.86 | 35.51 |
| 4 | $-C_6H_5$ | 56.11 | 68.36 | 39.64 | 44.32 |
| 5 | $4-F-C_6H_4-$ | 61.14 | 80.50 | 45.97 | 50.72 |
| 6 | $4-Cl-C_6H_4-$ | 52.16 | 77.33 | 35.55 | 39.85 |
| 7 | $4-Br-C_6H_4-$ | 20.93 | 35.90 | 11.02 | 36.64 |
| 8 | $4-CH_3-C_6H_4-$ | 80.54 | 82.01 | 37.36 | 52.79 |
| 9 | $4-OCH_3-C_6H_4-$ | 9.72 | 25.73 | 22.79 | 41.20 |
| 10 | $4-NO_2-C_6H_4-$ | 51.77 | 67.65 | 27.40 | 37.6 |
| 11 | $3,4,5-(OCH_3)_3-C_6H_2-$ | 10.70 | 46.08 | 6.25 | 22.74 |
| 12 | $CH=CH-C_6H_5$ | 74.07 | 83.49 | 38.07 | 52.47 |
| Isoniazid | - | 81.91 | | 34.44 | |

Percent reduction in relative light units (RLU) was calculated using luciferase reporter phage (LRP) assay at two different concentrations (50 and 100 µg/ml) using INH as a reference standard. Compound is considered to be an antimycobacterial, if 50 % reduction in the relative light units (RLU) is observed when compared to the control using a luminometer

bear $4-CH_3-C_6H_4-$, $CH=CH-C_6H_5-$ and $4-F-C_6H_4-$ substituents, respectively at 4th position in the triazole ring.

In case of clinical isolates: S, H, R, and E resistant *M. tuberculosis*, <50 % reduction in RLU was only seen with compounds 8, 12 and 5 at 100 µg/ml concentration, however, compounds 4, 5, 8, 12 at 50 µg/ml and 3, 4, 6, 7, 9 at 100 µg/ml showed higher reduction in RLU than INH and hence exhibited better antitubercular activity than the standard drug. Three compounds, 8, 12 and 5 found to be most potent among the series against H37Rv as well as S, H, R and E resistant *M. tuberculosis* strains. Higher potencies of these compounds suggest that substituents at 4th position contributing pharmacokinetically toward antitubercular activity. This has been stated because compounds 12 and 8 seems to be highly lipophilic due to contribution by styryl and tolyl components respectively, while compound 5 bears $4-F-C_6H_4-$ at 4th position that is comparatively polar in nature.

All the newly synthesized compounds were assayed *in vitro* for antibacterial and antifungal activity against a representative panel of bacterial and fungal pathogens. In primary screening 500, 250 and 125 µg/ml concentrations of the synthesized compounds were tested. The active compounds found in this primary screening were further diluted and tested against the corresponding microorganism to obtain their MICs, where the lowest concentration that completely inhibited visible growth of the organism was recorded as MIC (µg/ml).

The obtained results (Table 2) revealed that all of the synthesized compounds exhibited potent antibacterial activities against both Gram-positive strains. In case of *Staphylococcus aureus*, compound 5 (MIC=12.5 µM), 6 (MIC=14.1 µM), 9 (MIC=18.75 µM), 10 (MIC= 13.3 µM) and 11 (MIC=15.6 µM) exhibited MICs as good as ampicillin (MIC=10.2 µM). Interestingly, the same compounds i.e. 5, 6, 9, 10 and 11 were shown to be most active against *Bacillus subtilis*, where compound 5 gave better anti*B. subtilis* activity (MIC=10.2 µM) than ampicillin (MIC=10.9 µM). Antibacterial activities of the compounds improve as the electron withdrawing or donating capacity of the substituent on phenyl ring at fourth position of 1,2,4-triazole increase.

Prepared compounds showed lower antibacterial potencies against Gram-negative bacteria as compared to Gram-positive bacterial strains. Compounds

displayed MICs range from 14.1 to 106.3 μM against *Pseudomonas aeruginosa* and from 15.6 to 137.5 μM against *Escherichia coli*. This may be due to the polar nature of the substituents on phenyl ring at fourth position of 1,2,4-triazole which finally turn into the lesser lipophilic compounds.

The obtained results for antifungal activities as depicted in Table 2 revealed that most of compounds could effectively inhibit the growth of the tested fungal strains, however, none of them shown to be superior to the reference drug fluconazole. Compound 12 displayed most potent antifungal activities against both fungus. Compared to the reference fluconazole (*A. niger*: MIC=9.4 μM; *Candida albicans*: MIC= 10.2 μM), compound 12 exhibited comparable (*A. niger*: MIC=11.7 μM; *C. albicans*: MIC=10.9 μM) activities. Apart from this compound 8 (*A. niger*: MIC=15.6 μM; *C. albicans*: MIC=14.1 μM) and compound 1 (*A. niger*: MIC=28.1 μM; *C. albicans*: MIC=18.8 μM) were also found to be potent antifungal.

Many significant facts were observed on analysing the effect of the substituents on the antibacterial activity of the synthesized compounds. Antibacterial activity of the compounds has been improved by the substitution of the more electron withdrawing group on the phenyl ring at the fourth position of 1,2,4-triazoles. The polar nature of the substituent on the phenyl ring at the fourth position converted the compounds into less lipophilic character. The addition of the benzyl substitution on the 1,2,4-triazoles potentiates antibacterial activity. Substitution of –F on the phenyl group potentiate

the activity as compared to the ampicillin. Table 2 represent all the data of antibacterial and antifungal activity correlated with this conclusion. Substitution of methyl, ethyl, propyl and phenyl at the fourth position diminished the antibacterial activity. As far as antitubercular activity is concern substitution of more electron withdrawing group at the fourth position on the phenyl ring of the 1,2,4-triazoles is favourable for the activity. Compound 5 with 4-fluorophenyl group at position 4 of 1,2,4-triazoles exhibited better activity than phenyl or chlorophenyl substitutions. This is due to more electronegativity of fluorine than chloro. Substitution of methyl on 4th position of phenyl ring as in compound 8 has shown better activity profile as compared to electron donating group with less steric hindrance effect. The group contributes to positive inductive effect to the benzyl electron cloud leading more active electron cloud system, which has led to a positive denotable effect. Compounds with methoxy (compound 9), tri-methoxy (compound 11) or nitro (compound 10) group on phenyl ring exhibited lesser activity may be due to high steric and moderate electron withdrawing effect. Higher potencies of these compounds suggest that substituents at 4th position also contributes pharmacokinetically toward antitubercular activity because compounds 12 and 8 seems to be highly lipophilic due to contribution of styryl and tolyl components, respectively, while compound 5 bears 4-fluorophenyl at 4th position is comparatively polar in nature.

In the present investigation, 12 different 1,2,4-triazoles were synthesized and evaluated for

**TABLE 2: *IN VITRO* ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY RESULTS OF COMPOUNDS 1-12**

| Compound | R | Minimum inhibitory concentration (MIC, μg/ml) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Gram-positive bacteria | | Gram-negative bacteria | | Fungi | |
| | | *S. aureus* | *B. subtilis* | *P. aeruginosa* | *E. coli* | *A. niger* | *C. albicans* |
| 1 | -CH$_3$ | 62.5 | 68.8 | 34.4 | 40.6 | 28.1 | 18.8 |
| 2 | -CH$_2$CH$_3$ | 75 | 62.5 | 62.5 | 68.8 | 162.5 | 150 |
| 3 | -CH$_2$CH$_2$CH$_3$ | 93.8 | 87.5 | 21.9 | 25 | 112.5 | 137.5 |
| 4 | -C$_6$H$_5$ | 31.3 | 28.1 | 14.1 | 15.6 | 17.2 | 15.6 |
| 5 | 4-F-C$_6$H$_4$- | 12.5 | 10.2 | 106.3 | 100 | 125 | 106.3 |
| 6 | 4-Cl-C$_6$H$_4$- | 14.1 | 15.6 | 87.5 | 106.3 | 118.8 | 112.5 |
| 7 | 4-Br-C$_6$H$_4$- | 25 | 23.4 | 75 | 62.5 | 150 | 125 |
| 8 | 4-CH$_3$-C$_6$H$_4$- | 46.9 | 50 | 37.5 | 31.3 | 15.6 | 14.1 |
| 9 | 4-OCH$_3$-C$_6$H$_4$- | 18.75 | 17.2 | 50 | 56.3 | 40.6 | 50 |
| 10 | 4-NO$_2$-C$_6$H$_4$- | 13.3 | 12.5 | 125 | 137.5 | 100 | 87.5 |
| 11 | 3,4,5-(OCH$_3$)$_3$- C$_6$H$_2$- | 15.6 | 20.3 | 81.3 | 75 | 34.4 | 43.8 |
| 12 | -CH=CH-C$_6$H$_5$ | 75 | 81.3 | 93.8 | 100 | 11.7 | 10.9 |
| Ampicillin | - | 10.2 | 10.9 | - | - | - | - |
| Norfloxacin | - | 11.7 | 14.1 | 10.2 | 7.8 | - | - |
| Fluconazole | - | - | - | - | - | 9.4 | 10.2 |

their antimycobacterial activity. Most compounds exhibited significant antimycobacterial activity. A remarkable activity against *M. tuberculosis* H37Rv was found in compound 8, 12 and 5 carrying 4-CH$_3$-C$_6$H$_4$-, CH=CH-C$_6$H$_5$- and 4-F-C$_6$H$_4$- substituent, respectively. Compounds 8, 12 and 5 showed more than 50 % reduction in RLU against clinical isolates, S, H, R and E resistant *M. tuberculosis* at 100 µg/ml concentration, however, compounds 4, 5, 8, 12 at 50 µg/ml and 3, 4, 6, 7, 9 at 100 µg/ml exhibited more reduction in RLU than INH. Overall, compounds 8, 12 and 5 found to be most potent among the series against H37Rv as well as S, H, R and E resistant *M. tuberculosis* strains. Synthesized compounds were found to be more active against Gram-positive bacteria as compared to Gram-negative bacteria, which might be due to highly polar substitutions in most of the compounds. Antibacterial activities against Gram-positive bacteria were found to improve with both electron withdrawing and donating substituent. Compound 5 with 4-F-C$_6$H$_4$-substitution showed better antibacterial activity (MIC= 10.2 µM) than ampicillin (MIC=10.9 µM) against *B. subtilis*. Compound 12 in the series displayed most potent antifungal activities (comparable to reference fluconazole) against both fungal strains.

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## Conflict of interest:

The authors have declared no conflict of interest.

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