

Isoxazole clubbed 1-carbothioamido-4,5-dihydro-1H-pyrazoles: Design, Synthesis, Characterization And Antitubercular Evaluation

Abstract: Mycobacterium tuberculosis causes tuberculosis (TB) and this disease remains a global problem despite the availability of drugs due to this issues like multi-drug resistance and toxicities. As a result, there is a pressing need for new antitubercular medicines to be developed. In the present investigation we designed and synthesized a series of Isoxazole clubbed 1-carbothioamido-4,5-dihydro-1H-pyrazoles (16-30) in considerable yields. Further these compounds were purified by recrystallization and characterized by spectral techniques-Mass, FT-IR and ¹H NMR and then evaluated for their antitubercular activity against *Mycobacterium tuberculosis* H37Rv strain. Among the tested compounds, the analogues 24 bearing 3,5-dimethoxyphenyl scaffold at the 5th position of 4,5-dihydro-1H-pyrazole ring showed superior activity than isoniazid (MIC = 0.25 µg/mL) with MIC value 0.1 µg/mL whereas the compound 25 containing 2,3,4-trimethoxyphenyl had equal potency as that of isoniazid. Additionally, 24 and 25 were found to be less selective towards the human normal liver cell lines-LO2 in their cytotoxicity assays. Hence, these two compounds are safe and useful lead candidates for the development of novel antitubercular drugs.

KEYWORDS: Isoxazole; 1-carbothioamido-4,5-dihydro-1H-pyrazoles; Antitubercular activity ; *Mycobacterium tuberculosis* H37Rv; MABA assay; Cytotoxicity assay.

1. Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis*, which is the leading cause of single pathogen infection-related deaths worldwide. Tuberculosis is found worldwide. South-East Asia, with 44% of new TB cases, had the greatest number of new TB cases in 2019, followed by Africa, with 25%, and the Western Pacific, with 18%. 87% of new TB cases in the 30 countries with the greatest TB burden in 2019 Indonesia, China, the Philippines, Pakistan, Nigeria, Bangladesh, and South Africa account for two-thirds of new TB cases. Despite WHO's intention of reducing TB cases by 20% between 2015 and 2020, this target was not reached owing to MDR-TB and Rifampicin Resistant-TB (RR-TB). A total of 206,362 cases of MDR/RR-TB were recorded in 2019, a 10% rise from 186,883 in 2018. Multidrug-resistant tuberculosis affects mainly three countries: India, China, and Russia. *Mycobacterium tuberculosis* with drug-resistant isoniazid and rifampicin bacteria is a kind of MDR-TB. TB may be treated and cured. Second-line treatment choices are limited and need expensive and need intensive chemotherapy. Sometimes this may lead to severe drug resistance. TB which is not responsive to a second-line therapy, leaves patients with no further therapeutic choices [1]. To succeed, new antitubercular therapies are required to overcome this global issue.

Five membered heterocyclic scaffolds constitute an important class of heterocyclics with potential bioactivities. Among them isoxazole and dihydropyrazole were of special interest to medicinal chemists, owing to their useful biological actions. Both these rings are not only the part of many drug molecules but their derivatives were reported with significant activities

including antitubercular [2-9], antioxidant [10-11], anticancer [12-13], antibacterial [14-15], antifungal [16-17] etc. Hence combining these two rings into a single molecule has greater propensity of improving the activity of new analogues. In the previous study Kishor et al., synthesized isoxazole appended dihydropyrazole-1-carboxamides considering the presence of carbohydrazide portion of isoniazid and isoxazolidine ring of cycloserine and arrived with potential antitubercular lead molecules [18]. Ethionamide and thioacetazone are two drugs bearing carbothioamide ($S=C-NH_2$) functionality which is crucial for their potential antitubercular activity. In view of the significant antitubercular activity of isoxazole appended dihydropyrazole-1-carboxamides and the importance of carbothioamide group for antitubercular activity, here we synthesized and screened isoxazole clubbed 1-carbothioamido-4,5-dihydro-1H-pyrazoles as prospective antitubercular agents (Figure 1).

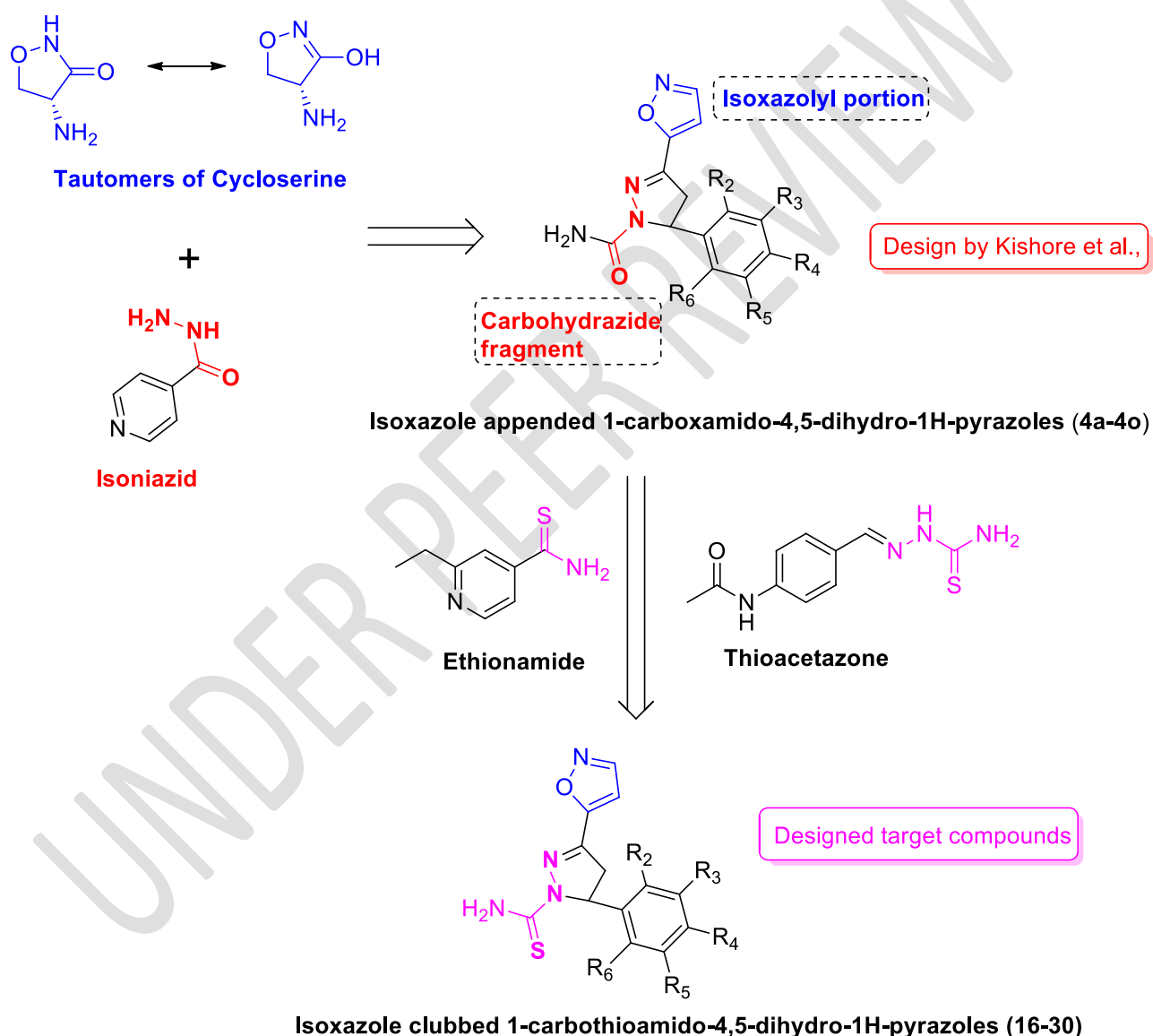


Figure 1. Design of isoxazole clubbed 1-carbothioamido-4,5-dihydro-1H-pyrazoles.

2. Materials and Methods

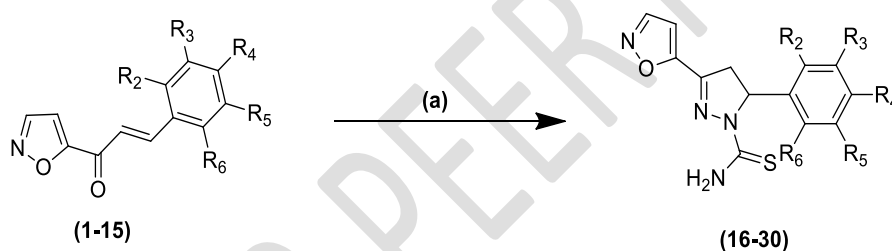
2.1 General

Isoxazolyl chalcones were used for the synthesis whereas thiosemicarbazide was purchased from Sigma Aldrich Chemical Co. (Milwaukee, Wisconsin, USA). Merck grade silica gel-GF was used as the adsorbent for TLC to monitor the reactions. Boetius melting point apparatus was used to determine the melting points in open capillaries and the values are expressed in °C and are uncorrected. Mass spectra were recorded on Agilent LC-MS spectrometer whereas the FT-IR spectra were recorded on Bruker Vertex 80v spectrometer using potassium bromide disks and the wave numbers of the absorption bands are expressed in cm^{-1} . ^1H spectra were recorded on Bruker AMX 400 MHz NMR spectrophotometer using TMS as an internal standard and the chemical shifts (δ) are expressed in ppm.

2.2 Chemistry

General method of synthesis of isoxazole clubbed 1-carbothioamido-4,5-dihydro-1H-pyrazoles

The intermediate (E)-1-(isoxazole-5-yl)-3-(aryl substituted)prop-2-en-1-one derivatives (1-15) were synthesized by using the protocol prescribed in the literature [19]. (E)-1-(isoxazole-5-yl)-3-(aryl substituted)prop-2-en-1-one derivatives (1-15) (1 mmol) and thiosemicarbazide (1 mmol) were refluxed for 8-11 h in 15-20 ml glacial acetic acid. After the completion of the reaction, excess acetic acid was removed under decreased pressure and then the reaction mixture was transferred into the crushed ice. The target isoxazole clubbed 1-carbothioamido-4,5-dihydro-1H-pyrazoles (16-30) were obtained by filtering, drying, and recrystallizing the solid mass using ethanol [20] (Scheme 1).



16: $\text{R}_2=\text{H}$; $\text{R}_3=\text{H}$; $\text{R}_4=\text{OCH}_3$; $\text{R}_5=\text{H}$; $\text{R}_6=\text{H}$

17: $\text{R}_2=\text{H}$; $\text{R}_3=\text{OCH}_3$; $\text{R}_4=\text{H}$; $\text{R}_5=\text{H}$; $\text{R}_6=\text{H}$

18: $\text{R}_2=\text{OCH}_3$; $\text{R}_3=\text{H}$; $\text{R}_4=\text{H}$; $\text{R}_5=\text{H}$; $\text{R}_6=\text{H}$

19: $\text{R}_2=\text{OCH}_3$; $\text{R}_3=\text{OCH}_3$; $\text{R}_4=\text{H}$; $\text{R}_5=\text{H}$; $\text{R}_6=\text{H}$

20: $\text{R}_2=\text{OCH}_3$; $\text{R}_3=\text{H}$; $\text{R}_4=\text{OCH}_3$; $\text{R}_5=\text{H}$; $\text{R}_6=\text{H}$

21: $\text{R}_2=\text{OCH}_3$; $\text{R}_3=\text{H}$; $\text{R}_4=\text{H}$; $\text{R}_5=\text{OCH}_3$; $\text{R}_6=\text{H}$

22: $\text{R}_2=\text{OCH}_3$; $\text{R}_3=\text{H}$; $\text{R}_4=\text{H}$; $\text{R}_5=\text{H}$; $\text{R}_6=\text{OCH}_3$

23: $\text{R}_2=\text{H}$; $\text{R}_3=\text{OCH}_3$; $\text{R}_4=\text{OCH}_3$; $\text{R}_5=\text{H}$; $\text{R}_6=\text{H}$

24: $\text{R}_2=\text{H}$; $\text{R}_3=\text{OCH}_3$; $\text{R}_4=\text{H}$; $\text{R}_5=\text{OCH}_3$; $\text{R}_6=\text{H}$

25: $\text{R}_2=\text{OCH}_3$; $\text{R}_3=\text{OCH}_3$; $\text{R}_4=\text{OCH}_3$; $\text{R}_5=\text{H}$; $\text{R}_6=\text{H}$

26: $\text{R}_2=\text{OCH}_3$; $\text{R}_3=\text{H}$; $\text{R}_4=\text{OCH}_3$; $\text{R}_5=\text{OCH}_3$; $\text{R}_6=\text{H}$

27: $\text{R}_2=\text{OCH}_3$; $\text{R}_3=\text{H}$; $\text{R}_4=\text{OCH}_3$; $\text{R}_5=\text{H}$; $\text{R}_6=\text{OCH}_3$

28: $\text{R}_2=\text{H}$; $\text{R}_3=\text{OCH}_3$; $\text{R}_4=\text{OCH}_3$; $\text{R}_5=\text{OCH}_3$; $\text{R}_6=\text{H}$

29: $\text{R}_2=\text{F}$; $\text{R}_3=\text{OCH}_3$; $\text{R}_4=\text{OCH}_3$; $\text{R}_5=\text{H}$; $\text{R}_6=\text{H}$

30: $\text{R}_2=\text{Cl}$; $\text{R}_3=\text{H}$; $\text{R}_4=\text{OCH}_3$; $\text{R}_5=\text{H}$; $\text{R}_6=\text{OCH}_3$

Scheme 1. Synthetic strategy employed for the preparation of Isoxazole clubbed 1-carbothioamido-4,5-dihydro-1H-pyrazoles (16-30); (a) glacial acetic acid, reflux.

3-(isoxazol-5-yl)-5-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbothioamide (16): Yield 62%; Molecular Weight: 302.35; **m.p.** 95-97 °C; **FT-IR** (KBr, cm^{-1}): 1589 (C=N), 1239 (C=S), 3359 (-NH₂); **^1H NMR** (400 MHz, CDCl₃, ppm): δ 3.05 (1H, H_A, dd, J_{AX} = 3.6Hz, dd, J_{AB}=16 Hz), 3.65 (1H, H_B, dd, J_{AB} = 16Hz, dd, J_{BX}= 12 Hz), 5.05 (1H, H_X, dd, J_{AX} = 3.6 Hz, dd, J_{BX} = 12 Hz), 9.65 (2H, s, NH₂, D₂O exchangeable), 3.54 (3H, s, Ar-OCH₃), 6.06-8.11 (6H, Ar-H); **MS** (*m/z*, %): 303.25 (M+1, 98.59).

3-(isoxazol-5-yl)-5-(3-methoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbothioamide (17): Yield 43%; Molecular Weight: 302.35; **m.p.** 88-89 °C; **FT-IR** (KBr, cm^{-1}): 1591 (C=N), 1233 (C=S), 3366 (-NH₂); **^1H NMR** (400 MHz, CDCl₃, ppm): δ 3.08 (1H, H_A, dd, J_{AX} = 3.6

Hz, dd, $J_{AB} = 16$ Hz), 3.66 (1H, H_B , dd, $J_{AB} = 16$ Hz, dd, $J_{BX} = 12$ Hz), 5.11 (1H, H_X , dd, $J_{AX} = 3.6$ Hz, dd, $J_{BX} = 12$ Hz), 9.82 (2H, s, NH_2 , D_2O exchangeable), 3.77 (3H, s, Ar-OCH₃), 6.06-7.95 (6H, Ar-H); **MS** (m/z , %): 303.25 (M+1, 98.56).

3-(isoxazol-5-yl)-5-(2-methoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbothioamide (18): Yield 49%; Molecular Weight: 302.35; **m.p.** 92-94 °C; **FT-IR** (KBr, cm^{-1}): 1588 (C=N), 1245 (C=S), 3312 (-NH₂); **¹H NMR** (400 MHz, CDCl₃, ppm): δ 3.09 (1H, H_A , dd, $J_{AX} = 3.6$ Hz, dd, $J_{AB} = 16$ Hz), 3.74 (1H, H_B , dd, $J_{AB} = 16$ Hz, dd, $J_{BX} = 12$ Hz), 5.13 (1H, H_X , dd, $J_{AX} = 3.6$ Hz, dd, $J_{BX} = 12$ Hz), 9.66 (2H, s, NH_2 , D_2O exchangeable), 3.62 (3H, s, Ar-OCH₃), 6.11-8.11 (6H, Ar-H); **MS** (m/z , %): 303.25 (M+1, 99.85).

3-(isoxazol-5-yl)-5-(2,3-dimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbothioamide (19): Yield 44%; Molecular Weight: 332.38; **m.p.** 115-117 °C; **FT-IR** (KBr, cm^{-1}): 1573 (C=N), 1241 (C=S), 3321 (-NH₂); **¹H NMR** (400 MHz, CDCl₃, ppm): δ 3.11 (1H, H_A , dd, $J_{AX} = 3.6$ Hz, dd, $J_{AB} = 16$ Hz), 3.76 (1H, H_B , dd, $J_{AB} = 16$ Hz, dd, $J_{BX} = 12$ Hz), 5.14 (1H, H_X , dd, $J_{AX} = 3.6$ Hz, dd, $J_{BX} = 12$ Hz), 9.79 (2H, s, NH_2 , D_2O exchangeable), 3.51 (3H, s, Ar-OCH₃), 3.64 (3H, s, Ar-OCH₃), 6.15-8.15 (5H, Ar-H); **MS** (m/z , %): 333.38 (M+1, 99.51).

3-(isoxazol-5-yl)-5-(2,4-dimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbothioamide (20): Yield 65%; Molecular Weight: 332.38; **m.p.** 132-134 °C; **FT-IR** (KBr, cm^{-1}): 1566 (C=N), 1244 (C=S), 3322 (-NH₂); **¹H NMR** (400 MHz, CDCl₃, ppm): δ 3.12 (1H, H_A , dd, $J_{AX} = 3.6$ Hz, dd, $J_{AB} = 16$ Hz), 3.78 (1H, H_B , dd, $J_{AB} = 16$ Hz, dd, $J_{BX} = 12$ Hz), 5.09 (1H, H_X , dd, $J_{AX} = 3.6$ Hz, dd, $J_{BX} = 12$ Hz), 9.41 (2H, s, NH_2 , D_2O exchangeable), 3.46 (3H, s, Ar-OCH₃), 3.52 (3H, s, Ar-OCH₃), 6.12-8.05 (5H, Ar-H); **MS** (m/z , %): 333.38 (M+1, 99.12).

3-(isoxazol-5-yl)-5-(2,5-dimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbothioamide (21): Yield 66%; Molecular Weight: 332.38; **m.p.** 141-143 °C; **FT-IR** (KBr, cm^{-1}): 1568 (C=N), 1230 (C=S), 3312 (-NH₂); **¹H NMR** (400 MHz, CDCl₃, ppm): δ 3.06 (1H, H_A , dd, $J_{AX} = 3.6$ Hz, dd, $J_{AB} = 16$ Hz), 3.79 (1H, H_B , dd, $J_{AB} = 16$ Hz, dd, $J_{BX} = 12$ Hz), 5.16 (1H, H_X , dd, $J_{AX} = 3.6$ Hz, dd, $J_{BX} = 12$ Hz), 9.22 (2H, s, NH_2 , D_2O exchangeable), 3.59 (3H, s, Ar-OCH₃), 3.68 (3H, s, Ar-OCH₃), 6.01-7.98 (5H, Ar-H); **MS** (m/z , %): 333.38 (M+1, 99.31).

3-(isoxazol-5-yl)-5-(2,6-dimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbothioamide (22): Yield 51%; Molecular Weight: 332.38; **m.p.** 119-121 °C; **FT-IR** (KBr, cm^{-1}): 1571 (C=N), 1232 (C=S), 3319 (-NH₂); **¹H NMR** (400 MHz, CDCl₃, ppm): δ 3.09 (1H, H_A , dd, $J_{AX} = 3.6$ Hz, dd, $J_{AB} = 16$ Hz), 3.81 (1H, H_B , dd, $J_{AB} = 16$ Hz, dd, $J_{BX} = 12$ Hz), 5.22 (1H, H_X , dd, $J_{AX} = 3.6$ Hz, dd, $J_{BX} = 12$ Hz), 9.54 (2H, s, NH_2 , D_2O exchangeable), 3.55 (3H, s, Ar-OCH₃), 3.73 (3H, s, Ar-OCH₃), 6.02-8.15 (5H, Ar-H); **MS** (m/z , %): 333.38 (M+1, 99.31).

3-(isoxazol-5-yl)-5-(3,4-dimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbothioamide (23): Yield 55%; Molecular Weight: 332.38; **m.p.** 164-168 °C; **FT-IR** (KBr, cm^{-1}): 1574 (C=N), 1241 (C=S), 3331 (-NH₂); **¹H NMR** (400 MHz, CDCl₃, ppm): δ 3.16 (1H, H_A , dd, $J_{AX} = 3.6$ Hz, dd, $J_{AB} = 16$ Hz), 3.72 (1H, H_B , dd, $J_{AB} = 16$ Hz, dd, $J_{BX} = 12$ Hz), 5.24 (1H, H_X , dd, $J_{AX} = 3.6$ Hz, dd, $J_{BX} = 12$ Hz), 9.41 (2H, s, NH_2 , D_2O exchangeable), 3.58 (3H, s, Ar-OCH₃), 3.81 (3H, s, Ar-OCH₃), 6.19-8.38 (5H, Ar-H); **MS** (m/z , %): 333.38 (M+1, 99.31).

3-(isoxazol-5-yl)-5-(3,5-dimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbothioamide (24): Yield 69%; Molecular Weight: 332.38; **m.p.** 155-157 °C; **FT-IR** (KBr, cm^{-1}): 1577 (C=N), 1242 (C=S), 3329 (-NH₂); **¹H NMR** (400 MHz, CDCl₃, ppm): δ 3.17 (1H, H_A , dd, $J_{AX} = 3.6$ Hz, dd, $J_{AB} = 16$ Hz), 3.78 (1H, H_B , dd, $J_{AB} = 16$ Hz, dd, $J_{BX} = 12$ Hz), 5.28 (1H, H_X , dd, $J_{AX} = 3.6$ Hz, dd, $J_{BX} = 12$ Hz), 9.58 (2H, s, NH_2 , D_2O exchangeable), 3.48 (3H, s, Ar-OCH₃), 3.94 (3H, s, Ar-OCH₃), 6.29-8.58 (5H, Ar-H); **MS** (m/z , %): 333.38 (M+1, 99.31).

3-(isoxazol-5-yl)-5-(2,3,4-trimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbothioamide (25): Yield 75%; Molecular Weight: 362.10; m.p. 191-193 °C; FT-IR (KBr, cm⁻¹): 1578 (C=N), 1238 (C=S), 3338 (-NH₂); ¹H NMR (400 MHz, CDCl₃, ppm): δ 3.06 (1H, H_A, dd, J_{AX} = 3.6 Hz, dd, J_{AB} = 16 Hz), 3.77 (1H, H_B, dd, J_{AB} = 16 Hz, dd, J_{BX} = 12 Hz), 5.34 (1H, H_X, dd, J_{AX} = 3.6 Hz, dd, J_{BX} = 12 Hz), 9.48 (2H, s, NH₂, D₂O exchangeable), 3.61 (3H, s, Ar-OCH₃), 3.94 (6H, s, 2x Ar-OCH₃), 6.32-8.65 (4H, Ar-H); MS (*m/z*, %): 363.10 (M+1, 99.56).

3-(isoxazol-5-yl)-5-(2,4,5-trimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbothioamide (26): Yield 71%; Molecular Weight: 362.10; m.p. 186--188 °C; FT-IR (KBr, cm⁻¹): 1574 (C=N), 1239 (C=S), 3328 (-NH₂); ¹H NMR (400 MHz, CDCl₃, ppm): δ 3.08 (1H, H_A, dd, J_{AX} = 3.6 Hz, dd, J_{AB} = 16 Hz), 3.68 (1H, H_B, dd, J_{AB} = 16 Hz, dd, J_{BX} = 12 Hz), 5.38 (1H, H_X, dd, J_{AX} = 3.6 Hz, dd, J_{BX} = 12 Hz), 9.51 (2H, s, NH₂, D₂O exchangeable), 3.76 (3H, s, Ar-OCH₃), 3.92 (6H, s, 2x Ar-OCH₃), 6.46-8.75 (4H, Ar-H); MS (*m/z*, %): 363.10 (M+1, 99.88).

3-(isoxazol-5-yl)-5-(2,4,6-trimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbothioamide (27): Yield 77%; Molecular Weight: 362.10; m.p. 165-167 °C; FT-IR (KBr, cm⁻¹): 1579 (C=N), 1238 (C=S), 3341 (-NH₂); ¹H NMR (400 MHz, CDCl₃, ppm): δ 3.09 (1H, H_A, dd, J_{AX} = 3.6 Hz, dd, J_{AB} = 16 Hz), 3.81 (1H, H_B, dd, J_{AB} = 16 Hz, dd, J_{BX} = 12 Hz), 5.25 (1H, H_X, dd, J_{AX} = 3.6 Hz, dd, J_{BX} = 12 Hz), 9.29 (2H, s, NH₂, D₂O exchangeable), 3.72 (3H, s, Ar-OCH₃), 3.95 (6H, s, 2x Ar-OCH₃), 6.44-8.58 (4H, Ar-H); MS (*m/z*, %): 363.10 (M+1, 99.45).

3-(isoxazol-5-yl)-5-(3,4,5-trimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbothioamide (28): Yield 71%; Molecular Weight: 362.10; m.p. 181-183 °C; FT-IR (KBr, cm⁻¹): 1578 (C=N), 1243 (C=S), 3344 (-NH₂); ¹H NMR (400 MHz, CDCl₃, ppm): δ 3.10 (1H, H_A, dd, J_{AX} = 3.6 Hz, dd, J_{AB} = 16 Hz), 3.76 (1H, H_B, dd, J_{AB} = 16 Hz, dd, J_{BX} = 12 Hz), 5.18 (1H, H_X, dd, J_{AX} = 3.6 Hz, dd, J_{BX} = 12 Hz), 9.38 (2H, s, NH₂, D₂O exchangeable), 3.66 (3H, s, Ar-OCH₃), 3.97 (6H, s, 2x Ar-OCH₃), 6.22-8.44 (4H, Ar-H); MS (*m/z*, %): 363.10 (M+1, 99.71).

3-(isoxazol-5-yl)-5-(2-fluoro-3,4-dimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbothioamide (29): Yield 76%; Molecular Weight: 350.37; m.p. 136-138 °C; FT-IR (KBr, cm⁻¹): 1584 (C=N), 1248 (C=S), 3349 (-NH₂); ¹H NMR (400 MHz, CDCl₃, ppm): δ 3.11 (1H, H_A, dd, J_{AX} = 3.6 Hz, dd, J_{AB} = 16 Hz), 3.82 (1H, H_B, dd, J_{AB} = 16 Hz, dd, J_{BX} = 12 Hz), 5.35 (1H, H_X, dd, J_{AX} = 3.6 Hz, dd, J_{BX} = 12 Hz), 9.56 (2H, s, NH₂, D₂O exchangeable), 3.65 (3H, s, Ar-OCH₃), 3.91 (3H, s, 2x Ar-OCH₃), 6.48-8.65 (4H, Ar-H); MS (*m/z*, %): 351.37 (M+1, 99.16).

3-(isoxazol-5-yl)-5-(2-chloro-4,6-dimethoxyphenyl)-4,5-dihydro-1H-pyrazole-1-carbothioamide (30): Yield 78%; Molecular Weight 366.82; m.p. 172-174 °C; FT-IR (KBr, cm⁻¹): 1588 (C=N), 1246 (C=S), 3351 (-NH₂); ¹H NMR (400 MHz, CDCl₃, ppm): δ 3.08 (1H, H_A, dd, J_{AX} = 3.6 Hz, dd, J_{AB} = 16 Hz), 3.81 (1H, H_B, dd, J_{AB} = 16 Hz, dd, J_{BX} = 12 Hz), 5.36 (1H, H_X, dd, J_{AX} = 3.6 Hz, dd, J_{BX} = 12 Hz), 9.52 (2H, s, NH₂, D₂O exchangeable), 3.48 (3H, s, Ar-OCH₃), 3.94 (3H, s, 2x Ar-OCH₃), 6.38-8.25 (4H, Ar-H); MS (*m/z*, %): 367.82 (M+1, 99.38); MS (*m/z*, %): 369.82 (M+1, 33.13).

2.3 In vitro antitubercular activity

The target isoxazole clubbed 1-carbothioamido-4,5-dihydro-1H-pyrazoles were screened for their antimycobacterial activity against *M. tuberculosis* H37Rv strain. The minimum inhibitory concentration (MIC) of all the compounds was obtained using a broth dilution assay [28-29] and is defined as the lowest concentration of the drug that inhibits ≤ 99% of the bacteria present at the start of the assay. The MIC of the test compounds were compared with that of the standard Isoniazid. The culture was thawed and diluted in broth to 10⁵ cfu mL⁻¹ (colony forming unit/mL) dilutions using Middlebrook 7H9 broth enriched with

10% albumin-dextrose-catalase and 0.2 percent glycerol. All the target compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted twice in broth to achieve the appropriate concentration. The assay medium contained 1.3 percent DMSO at its final concentration. Following that, each U-tube was injected with 0.05 mL of standardised culture and cultured for 21 days at 37°C. The appearance of the growth in the U-tubes was observed and compared in contrast to isoniazid (positive control) and ioculum without drug (negative control) [21-23].

2.4 Cytotoxicity studies

The target compounds with the highest activity 24 and 25 were evaluated for their cytotoxic properties using Mosmanns MTT assay (Mosmann T *et al.*, 1983) against normal human liver cell lines-LO2. In MTT assay, mitochondrial reductase of living cells converts soluble MTT (0.5 mg mL⁻¹, 100 µL), into a formazan (bluish-purple) product. Cells used in cytotoxicity assays were grown in RPMI 1640 media supplemented with 10% foetal calf serum, penicillin, and streptomycin at 37 °C and 5% CO₂. Twenty-four hours after seeding, the cells were transferred to 96-well plates at 100 µL per well and allowed to adhere overnight before treatment with the compounds in DMSO solution (10⁻⁵, 10⁻⁶ and 10⁻⁷ mol/L final concentration). Three times, the same treatment was provided. After continuous compound exposure with MTT, cell viability was measured after 96 hours. 150 µL of DMSO solution was applied to each well. The plates were mechanically mixed until the colour response was homogeneous and the OD₅₇₀ was measured using a micro plate reader. The IC₅₀ was determined as the concentration that decreased the absorbance of the untreated wells by 50% relative to vehicle in the MTT test. Triplicate assays were conducted and the reproducibility of the findings was excellent with standard errors below 10% [24-25].

3. Results and Discussion

3.1 Chemistry

The synthesis of new isoxazole linked 1-carbothioamido-4,5-dihydro-1H-pyrazole derivatives was achieved by the condensation of isoxazolyl chalcones (1-15) with thiosemicarbazide using glacial acetic acid. All the compounds were purified by recrystallization. Mass spectrometry, FT-IR and ¹H NMR techniques enabled to elucidate the structures of the purified compounds. The compounds showed M+1 peak corresponding to their molecular weights in their positive ion mass spectrum. Additionally, the compound 30 also displayed a satellite peak due to the ³⁷Cl isotope at m/z value 369.82 (M+1, 33.13). In their FT-IR spectra, the compounds exhibited three diagnostic absorption bands corresponding to C=S, C=N and NH₂ around wave numbers 1230-1248 cm⁻¹, 1566-1588 cm⁻¹ and 3312-3359 cm⁻¹ respectively. The three diagnostic peaks of 2-pyrazoline scaffold in the ¹H NMR spectra of target compounds corresponding to the ABX system was observed at chemical shift values 3.05-3.17 ppm (1H, H_A, dd, J_{AX} = 3.6 Hz, dd, J_{AB} = 16 Hz), 3.65-3.82 ppm (1H, H_B, dd, J_{AB} = 16 Hz, dd, J_{BX} = 12 Hz), and 5.05-5.38 ppm (1H, H_X, dd, J_{AX} = 3.6 Hz, dd, J_{BX} = 12 Hz) respectively. Furthermore, the other characteristic peaks corresponding to the amino group (9-10 ppm), aromatic protons (Ar-H, 6-8.5 ppm) and methoxyl groups (2-4 ppm) in their ¹H NMR spectrum had confirmed the structures of the target compounds.

3.2 Antitubercular activity and SAR

The antimycobacterial activity of all the target compounds (16-30) was evaluated against H37Rv strain of *Mycobacterium tuberculosis*. Some of the compounds were more active

than isoniazid and previously investigated carboxamide derivatives (4a-4o) by Kishor et al []. In the 4a-4o series, the compounds with methoxy group at the meta-positions were poorly active. However, in our case compounds substituted with meta-methoxyl groups had contributed positively for the activity. For instance, compound 24 containing 3,5-dimethoxy groups at meta positions was the most active analogue with an MIC value of 0.1 $\mu\text{g/mL}$. Its activity was similar to compounds 4n and 4o containing 2-fluoro-3,4-dimethoxy and 2-chloro-3,4-dimethoxy rings respectively in the carboxamide series. The compound 25 containing 2,3,4-trimethoxy substituents was equipotent to isoniazid (MIC, 0.25 $\mu\text{g/mL}$). In a similar way the compounds-26 and 28 containing 2,4,5-trimethoxy and 3,4,5-trimethoxy groups showed more activity than the carboxamide counters but less activity than isoniazid with MIC 1 and 2 $\mu\text{g/mL}$ respectively. The other compounds elicited activity ranging between 4-128 $\mu\text{g/mL}$.

Table 1. Antitubercular activity of isoxazole linked dihydropyrazole-1-carboxamides (4a-4o) Vs isoxazole clubbed dihydropyrazole-1-carbothioamides (16-30).

Compound code	MIC values ($\mu\text{g/mL}$) of <i>M. tuberculosis</i> H ₃₇ Rv	Compound code	MIC values ($\mu\text{g/mL}$) of <i>M. tuberculosis</i> H ₃₇ Rv
4a	32	16	128
4b	62.5	17	2
4c	126	18	64
4d	62.5	19	32
4e	0.5	20	128
4f	32	21	32
4g	16	22	128
4h	32	23	32
4i	126	24	0.1
4j	8	25	0.25
4k	8	26	1
4l	0.25	27	128
4m	8	28	2
4n	0.1	29	4
4o	0.1	30	8
Isoniazid	0.25		0.25

3.3 Cytotoxicity studies

The MTT assay result of compounds 24 and 25 for their cytotoxicity against L02 (human normal cell line) showed that the compounds were less selective against the human cells as their IC₅₀ values were more than 70 $\mu\text{g/mL}$ (Table 2) indicating the usefulness of these analogues for further studies.

Table 2. Cytotoxicity of compounds 24 and 25 against human normal cells (IC₅₀ \pm SD, $\mu\text{g/mL}$)^{a,b}.

S.No	Compounds	Human liver normal cells (L02)
1	24	>70
2	25	>70

^a Mean value \pm SD (standard deviation from three experiments).

^b Boldface: IC₅₀ \leq the control, (IC₅₀, $\mu\text{g mL}^{-1}$)

4. Conclusions

A novel series of isoxazole clubbed dihydropyrazole-1-carbothioamides (16-30) were synthesized, characterized and screened for their antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv strain. The compounds 24 and 25 bearing methoxyl groups at the meta positions were found to be the most promising lead molecules considering their potential antitubercular activity with MIC values 0.1 and 0.25 µg/mL respectively. In addition the less selectivity of these compounds against the normal human liver cells suggests the significance of these compounds in the further discovery and development of novel antitubercular drugs. Further studies are under process in order to elucidate the plausible mode of action for the proposed activity.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

References

1. WHO Global tuberculosis report 2019. <https://www.who.int/news-room/fact-sheets/detail/tuberculosis> (accessed on March 15, 2021).
2. Ganesh, N.; Singh, M.; Chandrashekar, V.M.; Pujar, G.V. Antitubercular Potential of Novel Isoxazole Encompassed 1, 2, 4-Triazoles: Design, Synthesis, Molecular Docking Study and Evaluation of Antitubercular Activity. *Anti-Infective Agents*. **2021**, *19*, 147-61. <https://doi.org/10.2174/2211352518999200711163714>
3. Abdullah, M.M.; Siddiqui, N.A.; Mothana, R.A.; Nasr, F.A.; Al-Rehaily, A.J.; Almarfadi, O.M.; Karim, S.; Haider, K.; Haider, M.R.; Yar, M.S. Design, in-silico study and biological evaluation of newly synthesized 3-chlorobenzofuran congeners as antitubercular agents. *Arabian Journal of Chemistry*. **2021**, *14*, 103034. <https://doi.org/10.1016/j.arabjc.2021.103034>
4. Yan M, Xu L, Wang Y, Wan J, Liu T, Liu W, Wan Y, Zhang B, Wang R, Li Q. Opportunities and challenges of using five-membered ring compounds as promising antitubercular agents. *Drug development research*. **2020**, *81*, 402-18. <https://doi.org/10.1002/ddr.21638>
5. Shaik AB, Bhandare RR, Nissankararao S, Edis Z, Tangirala NR, Shahanaaz S, Rahman MM. Design, facile synthesis and characterization of dichloro substituted chalcones and dihydropyrazole derivatives for their antifungal, antitubercular and antiproliferative activities. *Molecules*. **2020** Jan;25(14):3188. <https://dx.doi.org/10.3390%2Fmolecules25143188>

6. Lokesh, B.V.; Prasad, Y.R.; Shaik, A.B. Synthesis, Biological evaluation and molecular docking studies of new pyrazolines as an antitubercular and cytotoxic agents. *Infectious Disorders-Drug Targets (Formerly Current Drug Targets-Infectious Disorders)*. **2019**, 19, 310-21. <https://doi.org/10.2174/1871526519666181217120626>
7. Pappula, N.; Sharabu, R. Synthesis, Characterization, Anti-Mycobacterial Evaluation and In-Silico Molecular Docking of Novel Isoxazole Clubbed Pyrimidine Derivatives. *Journal of Pharmaceutical Research International*. **2021**, 28, 69-79. <https://doi.org/10.9734/jpri/2021/v33i26B31484>
8. Venugopal, M.; Sundararajan, R.; Choppala, A.D. Synthesis, Characterization, Antitubercular Activity and Docking Studies of 2-(benzo [d] oxazol-2-ylthio)-1-(3-(4-fluoro-3-methylphenyl)-5-(substituted aryl)-4, 5-dihydro-1H-pyrazol-1-yl) ethanone. *Journal of Young Pharmacists*. **2020**, 12, s1-s9. <http://dx.doi.org/10.5530/jyp.2020.12s.39>
9. Zala, M.; Vora, J.J.; Patel, H.B. Synthesis, Characterization, and Comparative Study of Some Heterocyclic Compounds Containing Isoniazid and Nicotinic Acid Hydrazone Moieties. *Russian Journal of Organic Chemistry*. **2020**, 56, 1795-800. <https://doi.org/10.1134/S1070428020100218>
10. Rana, M.; Arif, R.; Khan, F.I.; Maurya, V.; Singh, R.; Faizan, M.I.; Yasmeen, S.; Dar, S.H.; Alam, R.; Sahu, A.; Ahmad, T.; Rahisuddin. Pyrazoline analogs as potential anticancer agents and their apoptosis, molecular docking, MD simulation, DNA binding and antioxidant studies. *Bioorganic Chemistry*. **2021**, 108, 104665.
11. Eid, A.M.; Hawash, M.; Amer, J.; Jarrar, A.; Qadri, S.; Alnimer, I.; Sharaf, A.; Zalmoot, R.; Hammoudie, O.; Hameedi, S.; Mousa, A. Synthesis and Biological Evaluation of Novel Isoxazole-Amide Analogues as Anticancer and Antioxidant Agents. *BioMed Research International*. **2021**, 2021, 1-9. <https://doi.org/10.1155/2021/6633297>
12. Ravula, S.; Bobbala, R.R.; Kolli, B. Synthesis of novel isoxazole functionalized pyrazolo [3, 4-b] pyridine derivatives; their anticancer activity. *Journal of Heterocyclic Chemistry*. **2020**, 57, 2535-8. <https://doi.org/10.1002/jhet.3968>
13. Meleddu, R.; Petrikaite, V.; Distinto, S.; Arridu, A.; Angius, R.; Serusi, L.; Škarnulytė, L.; Endriulaitytė, U.; Paškevičiūtė, M.; Cottiglia, F.; Gaspari, M. Investigating the anticancer activity of isatin/dihydropyrazole hybrids. *ACS medicinal chemistry letters*. **2018**, 10, 571-6. <https://doi.org/10.1021/acsmedchemlett.8b00596>
14. Aarjane, M.; Slassi, S.; Tazi, B.; Amine, A. Synthesis and biological evaluation of novel isoxazole derivatives from acridone. *Archiv der Pharmazie*. **2021**, 354, 2000261. <https://doi.org/10.1002/ardp.202000261>
15. Murugavel, S.; Deepa, S.; Ravikumar, C.; Ranganathan, R.; Alagusundaram, P. Synthesis, structural, spectral and antibacterial activity of 3, 3a, 4, 5-tetrahydro-2H-benzo [g] indazole fused carbothioamide derivatives as antibacterial agents. *Journal of Molecular Structure*. **2020**, 1222, 128961. <https://doi.org/10.1016/j.molstruc.2020.128961>
16. Xie, F.; Ni, T.; Ding, Z.; Hao, Y.; Wang, R.; Wang, R.; Wang, T.; Chai, X.; Yu, S.; Jin, Y.; Jiang, Y. Design, synthesis, and in vitro evaluation of novel triazole analogues featuring isoxazole moieties as antifungal agents. *Bioorganic Chemistry*. **2020**, 101, 103982. <https://doi.org/10.1016/j.bioorg.2020.103982>
17. Sever, B.; Altintop, M.D.; Özdemir, A. Synthesis of New Bis-pyrazolines Endowed with Potent Antifungal Activity against *Candida albicans* and *Aspergillus niger*. *Letters in Drug Design & Discovery*. **2021**, 18, 3-15. <https://doi.org/10.2174/1570180817999201008155247>
18. Palleapati, K.; Kancharlapalli, V.R.; Shaik, A.B. Synthesis, characterization and antitubercular evaluation of some new isoxazole appended 1-carboxamido-4, 5-dihydro-1H-pyrazoles. *J. Res. Pharm*. **2019**, 23, 156-63. <https://doi.org/10.12991/jrp.2019.120>
19. Kishor P, Ramana KV, Shaik AB. Antitubercular evaluation of isoxazolyl chalcones. *Research Journal of Pharmaceutical Biological and Chemical Sciences*. **2017**, 8, 730-5.

-
20. Ahsan, M.J.; Saini, V. Design and synthesis of 3-(4-aminophenyl)-5-(4-methoxyphenyl)-4, 5-dihydro-1H-pyrazole-1-carboxamide/carbothioamide analogues as antitubercular agents. *Beni-Suef University Journal of Basic and Applied Sciences*. **2015**, 4, 41-6. <https://doi.org/10.1016/j.bjbas.2015.02.006>
 21. Afzal, B.S.; Lohitha, S.V.; Puttagunta, S.B.; Shaik, A.; Supraja, K.; Sai, H.K. Synthesis and screening of novel lipophilic diarylpropeones as prospective antitubercular, antibacterial and antifungal agents. *Biointerface Res. Appl. Chem.* **2019**, 9, 3912-8. <https://doi.org/10.33263/BRIAC93.912918>
 22. Lokesh, B.V.; Prasad, Y.R.; Shaik, A.B. Novel pyrimidine derivatives from 2, 5-dichloro-3-acetylthienyl chalcones as antifungal, antitubercular and cytotoxic agents: Design, synthesis, biological activity and docking study. *Asian J. Chem.* **2019**;19, 310-21. <https://doi.org/10.14233/ajchem.2019.21747>
 23. Lagu, S.B.; Rajendra, P.Y.; Srinath, N.; Afzal, B.S. Synthesis. antibacterial, antifungal antitubercular activities and molecular docking studies of nitrophenyl derivatives. *Int. J. Life Sci. Pharma Res.* **2019**, 9, 54-64. <http://dx.doi.org/10.22376/ijpbs/lpr.2019.9.1.P54-64>
 24. Lokesh, B.V.; Prasad, Y.R.; Shaik, A.B. Synthesis and biological activity of novel 2, 5-dichloro-3-acetylthiophene chalcone derivatives. *Indian J Pharm Educ Res.* **2017**, 51, s679-90. DOI: 10.5530/ijper.51.4s.99
 25. Shaik, A.B.; Bhandare, R.R.; Nissankararao, S.; Lokesh, B.V.; Shahanaaz, S.; Rahman, M.M. Synthesis, and biological screening of chloropyrazine conjugated benzothiazepine derivatives as potential antimicrobial, antitubercular and cytotoxic agents. *Arabian Journal of Chemistry*. **2021**, 14, 102915. <https://doi.org/10.1016/j.arabjc.2020.102915>