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# Green synthesis of pyrazolo[4,3-*d*]isoxazol derivatives and their antimicrobial, antimalarial and antituberculosis evaluation

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This article deals with the synthesis of new pyrazolo[4,3-*d*]isoxazol derivatives by utilizing 3,4-disubstituted isoxazol-5(4*H*)-ones and thiosemicarbazide catalyzed by triethylamine in PEG-400 under MWI at 300 W as well as under thermal heating at 90°C. All the synthesized compounds have been characterized by various spectroscopic techniques such as $^{1}$H and $^{13}$C NMR, IR, ESI-MS and elemental analysis. All the synthesized compounds have been screened for antimicrobial, antimalarial and antituberculosis activities.

**Keywords**: Pyrazolo[4,3-*d*]isoxazol, isoxazol-5(4*H*)-ones, green synthesis, antimicrobial, antimalarial, antituberculosis

The green synthesis of heterocyclic compounds as well as pharmaceutical intermediates in the field of bio-organic chemistry can be considered as an attractive research opportunity, because of waste reduction, energy savings, atom economy, easy work-up processes and avoiding the use of hazardous chemicals are advanced features associated with these syntheses[1,2]. The expansion of a green, clean and eco-friendly methodologies for the synthesis of highly potent bio-active molecules is an exciting and to be discovered area of medicinal chemistry[3,4].

The development of effective antimicrobial compoundsbecomes one of the most important areas of antibacterial research today, because of their resistance to the existing antibacterial and antifungal drugs[5-7]. Thus new antimicrobial agents are urgently essential to survive this situation. This is the goal why it seems crucial in order to examine for novel antimicrobial molecules with new mechanisms of action, to overcome antimicrobial resistance[8].

Pyrazole derivatives are one of asignificant class of heterocyclic compounds in medicinal chemistry that attracted much consideration due to their wide range of biological activities, such as antiviral[9,10], anticancer[11,12], antifungal[13], antiinflammatory[14-16], antidepressant[17], analgesic[18], antimicrobial[19], antidiabetic[20] andanticonvulsant[21].

Due to this reason the development of pyrazolo[4,3-*d*]isoxazole derivativesgained attention recently. Recently, Gomhaand coworkers have synthesised 4-(4-chlorophenyl)-3-phenyl-4*H*-pyrazolo [4,3-*d*]isoxazole-5(6*H*)-carbothioamide from the reaction of 4-(4-chlorobenzylidene)-3-phenylisoxazol-5(4*H*)-one and thiosemicarbazidein ethanol in the presence of catalytic amount of triethylamineunder reflux condition[22]. However, this reaction is performed in ethanol at reflux temperatures for prolonger time giving only moderate yields; therefore, this synthetic methodology does not meet the requirement of green chemistry. Also, this methodology do not provide the possibility of synthesizing a wide range of derivatives of imidazo[2,1-*b*]thiazole derivatives.

Regulatory pressure is increasingly focusing on the use, manufacture, and disposal of organic solvents because these solvents in an industrial process may cause occupational health hazards as well as they are harmful to the environment. The unique solvent properties, higher solubilizing power, and cation coordination ability of polyethylene glycol (PEG) solutions make them useful as green solvents and phase transfer catalysts in organic synthesis[23]. PEGs are non-ionic, non-toxic, inexpensive, thermally stable and recoverable reaction media by phase separation methods. In addition PEG-400 is non-

volatile, biodegradable, and have low flammability. PEGs have been found to be stable to acid, base, and high temperature[24,25]. PEGs exhibit different solubility in organic solvents; this property can enable the precipitation of PEGs from reaction mixtures in organic solvents for purification. Therefore, polyethylene glycol (PEG) is found to be an exciting reaction media in recent years for many organic synthesis[26-40]. The importance of green chemistry in organic synthesis has encouraged scientists to explore the use of microwave irradiation for the organic synthesis. Over the last few years, microwave irradiation (MWI) has emerged a great energy source for the wide range of organic transformation with short reaction time and high yield of the products with high purity[41]. The combined use of microwaves (MW) with PEGs as reaction media can speed up organic reactions by the selective absorption of microwave energy by polar molecules.

Herein, we report PEG-400 is an extremely effective catalytic system for the synthesis of pyrazolo[4,3-*d*]isoxazole derivatives from the reaction of isoxazol-5(4*H*)-one derivatives and thiosemicarbazide in the presence of catalytic amount of triethylamine in PEG-400 under reflux at the 90°C or irradiated under microwave at the 300 W (Scheme I).

## Experimental Section

All chemicals of the highest purity available were purchased from commercial sources and used as received. The progress of the reaction was monitored by thin layer chromatography (TLC) analysis on Merck pre-coated silica gel 60 F254 aluminum sheets, visualized by UV light. Various isoxazol-5(4*H*)-one derivatives were synthesized as per green protocol[42].

The reactions were performed under CEM discover microwave system as well as Samsung modified microwave oven. Melting points were measured on an Optimelt MPA 100 melting point apparatus and are uncorrected. Fourier transform infrared spectroscopy (FT-IR) spectra were recorded on a Perkin-Elmer FT-IR 377 spectrometer using KBr. $^1$H NMR spectra were recorded on Bruker AV 400 MHz spectrometer using DMSO-$d_6$ as solvent and TMS as the internal reference. $^{13}$C NMR was recorded on a Bruker AV 100 MHz spectrometer using DMSO-$d_6$ as solvent. Mass spectra were recorded at Advion Expression CMS, USA. Acetone was used as the mobile phase, and electron spray ionization (ESI) was used as the ion source. Elemental analysis was performed on a CHNS elemental analyzer.

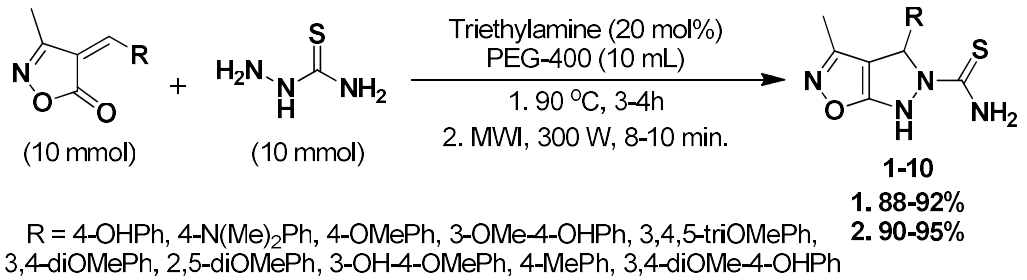
### General procedure for the synthesis of pyrazolo-[4,3-*d*]isoxazole derivatives, 1-10

To a mixture of isoxazol-5(4*H*)-ones (10 mmol) and thiosemicarbazide (10 mmol) in PEG-400 (10 mL), a catalytic amount of triethylamine (20 mol%) was added, then heated under reflux at 90°C or irradiated under microwave at 300 W, until the reaction was completed. The progress of the reaction mixture was monitored by TLC analysis. The reaction mixture was cooled to RT and water added. The resulting solid was collected, washed with ethanol and recrystallized from acetic acid to give pure product.

## Microbiology

#### Antibacterial and antifungal activity

Mueller-Hinton broth and Sabouraud's broth were used as nutrient medium to grow bacteria and fungi, respectively. Inoculum size for the test strain was adjusted to $10^6$ colony-forming unit (CFU) per milliliter by comparing the turbidity. Serial dilutions were prepared in primary and secondary screening. The control tube containing no antibiotic was immediately subcultured (before inoculation) by spreading a loopful evenly over a quarter of plate of medium suitable for the growth of test organism and incubated at 37°C for bacteria and 22°C for fungi overnight. The MIC of the control organism was read to check the accuracy of the drug concentrations. The lowest concentration inhibiting growth of the organism was recorded as the MIC. Each test




Scheme I — General reaction scheme for the preparation of pyrazolo[4,3-*d*]isoxazole derivatives **1-10**

compound was diluted, obtaining 2,000 µg/mL concentration, as a stock solution. In primary screening 1000, 500, 250 and 125 µg/mL concentrations of the test compounds were taken. The active synthesized compounds found in this primary screening were further tested in a second set of dilution against all organisms. The drugs found active in primary screening were similarly diluted to obtain 100, 50, 62.5, 25, 12.5 and 6.25 µg/mL concentrations. The highest dilution showing at least 99% inhibition was taken as MIC.

All newly synthesized pyrazolo[4,3-*d*]isoxazole derivatives (**1-10**) were examined for antimicrobial activity against two gram-positive bacterial strains (*Staphylococcus aureus* MTCC 96, *Streptococcus pyogenes* MTCC 442), two gram-negative bacterial strains (*Escherichia coli* MTCC 443, *Pseudomonas aeruginosa* MTCC 1688) as well as three fungal strains (*Aspergillus clavatus* MTCC 1323, *Candida albicans* MTCC 227 and *Aspergillus niger* MTCC 282) using the agar dilution method[43]. Ampicillin, Ciprofloxacin and Chloramphenicol were used as standard control drugs for antibacterial activity, whereas Nystatin and Greseofulvin were used as standard control drugs for antifungal activity.

**Anti-malarial activity**

A stock solution of 5 mg/mL of each of the test samples as well as standards was prepared in DMSO and subsequent dilutions were prepared with the culture medium. The diluted samples in 20 µL volume were added to the test wells so as to obtain final concentrations (at five-fold dilutions) ranging between 0.4 and 100 µg/mL in duplicate well containing parasitized cell preparation. The *in vitro* antimalarial assay was carried out in 96 well plates according to the micro assay protocol of Reickmann and co-workers with minor modifications[44]. The cultures of *P. falciparum* strain were maintained in medium RPMI 1640 supplemented with 25 mM HEPES, 1% D-glucose, 0.23% sodium bicarbonate and 10% heat inactivated human serum. The asynchronous parasites of *P. falciparum* were synchronized after 5% D-sorbitol treatment to obtain only the ring stage parasitized cells. For carrying out the assay, an initial ring stage parasitaemia of 0.8-1.5% at 3% haematocrit in a total volume of 200 µL of medium RPMI-1640 was determined by Jaswant Singh Bhattacharya (JSB) staining[45] to assess the percent parasitaemia (rings) and uniformly maintained with 50% RBCs ($O^{+ve}$). The culture plates were incubated at 37°C in a candle jar. After 36-40 h incubation, thin blood smears from each well were preparedand stained with JSB stain. The slides were microscopically observed to record maturation of the ring stage parasites into trophozoites and schizonts in the presence of different concentrations of the test agents. The test concentration which inhibited the complete maturation into schizonts was recorded as the minimum inhibitory concentration (MIC). Chloroquine and Quinine were used as the reference drugs.

**Anti-tuberculosis activity**

MIC of the test compounds against *M. tuberculosis* $H_{37}Rv$ was determined by L. J. agar (MIC) method[46,47] where primary 1,000, 500 and 250 µg/mL and secondary 200, 100, 50, 25, 12.5, 6.250 and 3.125 µg/mL dilutions of each test compound were added to liquid L. J. medium and then media were sterilized by inspissation method. A culture of *M. tuberculosis* $H_{37}Rv$ growing on L. J. medium was harvested in 0.85% saline in bijou bottles. For all test compounds, first a stock solution of 2,000 µg/mL concentration was prepared in DMSO. These tubes were then incubated at 37°C for 24 h followed by streaking of *M. tuberculosis* $H_{37}Rv$ ($5\times10^4$ bacilli per mL). These tubes were then incubated at 37±1°C. Growth of bacilli was seen after 12, 22 and finally 28 days of incubation. Tubes having the compounds were compared with control tubes where medium alone was incubated with *M. tuberculosis* H37Rv. The concentration at which no development of colonies occurred or less than 20 colonies was taken as MIC of test compound. The standard strain *M. tuberculosis* $H_{37}Rv$ was also tested with known drug Rifampicin.

**Results and Discussion**

After successful synthesis of various isoxazol-5(4*H*)-one derivatives *via* green methodology, we have planned to develop an efficient and green protocol for the preparation of pyrazolo[4,3-*d*]isoxazole derivatives **1-10**. To recognize the optimization of the reaction conditions, the reaction was studied by employing several solvents with the hope to maximize the product yield in short reaction times (Table I). In a model reaction, we used 4-benzylidene-3-methylisoxazol-5(4*H*)-one (10 mmol) with thiosemicarbazide (10 mmol) as reactants in the presence of triethylamine as a catalyst in ethanol at 90°C and found that pyrazolo[4,3-*d*]isoxazole **1** could be produced in 64% yield in 4 h (Table I, Entry 1).

Table I — Optimization of the reaction conditions for the synthesis of compound **1**

| Entry | Solvent (10 mL) | Catalysts (mol %) | Conditions | Time | Yield[a] (%) |
|---|---|---|---|---|---|
| 1 | Ethanol | TEA (20) | 80°C | 4 h | 64 |
| 2 | Butanol | TEA (20) | 100°C | 4 h | 70 |
| 3 | Propen-2-ol | TEA (20) | 100°C | 4 h | 68 |
| 4 | Water | TEA (20) | Reflux | 4 h | 34 |
| 5 | Methanol | TEA (20) | Reflux | 4 h | 52 |
| 6 | PEG-400 | TEA (20) | 90°C | 3 h | 90 |
| 7 | PEG-400 | TEA (20) | 100°C | 3 h | 90 |
| 8 | PEG-400 | TEA (20) | 300 W (MWI)[b] | 9 min | 94 |
| 9 | PEG-400 | TEA (20) | 450 W (MWI)[b] | 9 min | 93 |
| 10 | PEG-400 | TEA (10) | 300 W (MWI)[b] | 9 min | 79 |
| 11 | PEG-400 | TEA (15) | 300 W (MWI)[b] | 9 min | 86 |
| 12 | PEG-400 | TEA (25) | 300 W (MWI)[b] | 9 min | 94 |

[a] = Isolated yield.
[b] = Microwave Irradiation.

xTo improve the yield and to optimize the reaction conditions, the same reaction was carried out in the presence of solvents like butanol and propane-2-ol at 100°C for 4 h, although they afforded products in moderate yield (Table I, entries 2, 3). We have also tested water and methanol for this reaction. However, they gave poor results as compared to other solvents (Table I, entries 4, 5). Thereafter, the same reaction was performed utilizing PEG-400 as a green and eco-friendly reaction medium. Surprisingly, improved result was observed in the yield of compound **1** after stirring the mixture at 90°C for 3 h (Table I, entry 6). Increasing the temperature of the reaction from 90°C to 100°C led to no significant change in the reaction time as well as in product yield (Table I, entry 7). Finally, we achieved optimized reaction conditions, when we performed this cyclocondensation in PEG-400 as a green and bio-degradable solvent. After that, we performed this reaction under microwave irradiation (MWI) instead of thermal heating. Surprisingly, we achieved greatest yield (94%) of compound **1** within 9 min, when reaction was accomplished by utilizing PEG-400 in the presence of triethylamine as a catalysts under microwave irradiation 300 W (Table I, entry 8). In addition, it is also observed that high microwave irradiation power upto 450 W, resulted in high yield (Table I, entry 9). Compared to conventional heating, reaction under microwave irradiation shows higher efficiency in terms of product yields and reaction times. Variation of the amount of triethylamine (TEA) like 10 mol; 15 mol% and 25 mol% led to product **1** in 79; 86% and 94% yield, respectively (Table I, entries 10-12). These results indicate that 20 mol% of triethylamine gives high yield of the product within shorter period of time.

The optimal conditions found for the compound **1** was successfully applied to the reactions of various isoxazol-5(4*H*)-one derivatives with thiosemicarbazide in the presence of catalytic amount of triethylamine in PEG-400 under reflux at 90°C or irradiated under microwave at 300 W, which afforded corresponding pyrazolo[4,3-*d*]isoxazole derivatives **1**-**10** in excellent yields. The reaction proceeded smoothly and provided excellent yields in all cases (Table II). Both electron-withdrawing and electron-donating substituents bearing isoxazol-5(4*H*)-ones reacted smoothly under this protocol to afford excellent yields of the products. Furthermore, all synthesized products were easily purified by recrystallization from acetic acid, thus avoiding extraction steps and chromatographic separations. The homogeneity of the synthesized compounds was confirmed by TLC. Elemental analysis of all compounds was also carried out. The structures of the final products were well characterized by using spectral (IR, MS, $^{1}$H and $^{13}$C NMR) techniques.

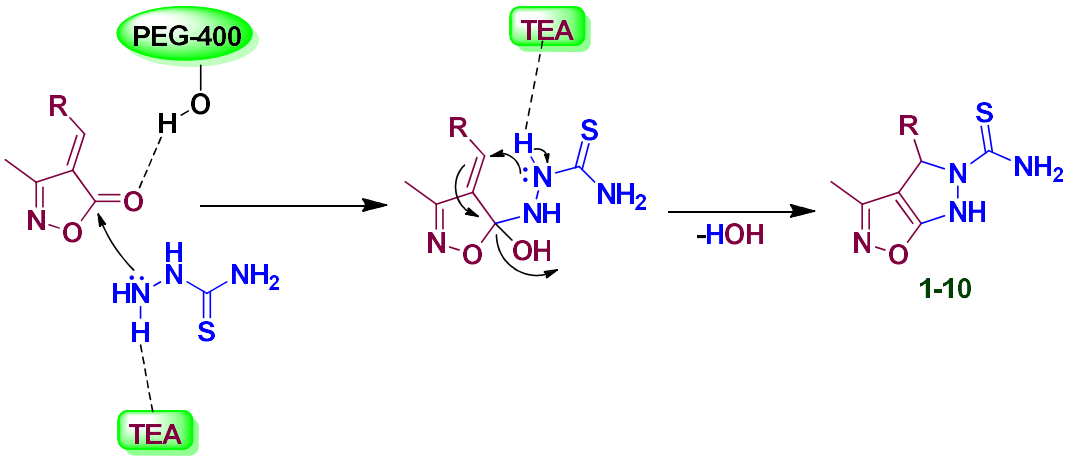
**Proposed reaction mechanism**

We have also described possible reaction mechanism of formation of pyrazolo[4,3-*d*]isoxazole derivatives (1-10) (Scheme II). According to that, initially, etheral oxygen of PEG-400 forms hydrogen bond with $-NH_2$ group of thiosemicarbazide, which makes $-NH-$ bond weaker enhancing the nucleophilicity of nitrogen for nucleophilic addition to electron deficient carbonyl carbon of isoxazol-5(4*H*)-one derivatives. Simultaneously, terminal -OH group of PEG-400

Table II — Preparation of pyrazolo[4,3-*d*]isoxazole derivatives **1-10** using PEG-400 as a reaction media

| Compd | R | Thermal Heating (90°C) | | Microwave Irradiation (300 W) | | m.p. (°C) |
|---|---|---|---|---|---|---|
| | | Time (h) | Yield[a] (%) | Time (min) | Yield[a] (%) | |
| **1** | 4-OH-$C_6H_4$ | 3 | 90 | 9 | 94 | 220-22 |
| **2** | 4-N(Me)$_2$-$C_6H_4$ | 4 | 89 | 10 | 92 | 215-16 |
| **3** | 4-OMe-$C_6H_4$ | 3 | 88 | 8 | 95 | 204-206 |
| **4** | 3-OMe-4-OH-$C_6H_3$ | 3 | 92 | 9 | 94 | 176-78 |
| **5** | 3,4,5-triOMe-$C_6H_2$ | 3 | 88 | 10 | 91 | 154-56 |
| **6** | 3,4-diOMe-$C_6H_3$ | 3 | 90 | 10 | 92 | 197-99 |
| **7** | 2,5-diOMe-$C_6H_3$ | 4 | 91 | 8 | 90 | 226-28 |
| **8** | 3-OH-4-OMe-$C_6H_3$ | 3 | 90 | 8 | 94 | 211-13 |
| **9** | 4-Me-$C_6H_4$ | 4 | 88 | 10 | 90 | 190-92 |
| **10** | 3,5-diOMe-4-OH-$C_6H_2$ | 4 | 92 | 9 | 95 | 183-85 |

[a] = Isolated yields




Scheme II — Proposed reaction mechanism

forms hydrogen bond with the electron deficient carbonyl carbon of isoxazol-5(4*H*)-one derivatives. Finally pyrazolo[4,3-*d*]isoxazole derivatives **1-10** form *via* intramolecular cyclization and hydrogen transfer followed by removal of $H_2O$ molecule.

**Spectroscopic characterization of pyrazolo[4,3-*d*]isoxazole derivatives, 1-10**

IR spectra shows characteristic C=S stretching peaks near 1530 and 1360 cm$^{-1}$ which corresponds to $CSNH_2$ group and two −NH stretching peaks between 3200-3400 cm$^{-1}$ corresponding to $NH_2$ group. The $^1$H NMR spectrum exhibits a singlet at δ 4.8, which indicates a proton of the pyrazole nucleus, while a singlet at δ 11.2, indicates a proton of the −NH group of pyrazole nucleus. In addition, peaks between δ 6.6 and 8.8 can be observed for respective aromatic protons. Furthermore, singlet at δ 2.24 indicates protons of $CH_3$ group of the isoxazole. The $^{13}$C NMR spectrum exhibits peak at δ 177, which indicates C=S group. In addition, peaks at δ 11-12 indicate −$CH_3$ group of the isoxazole. The ESI-MS spectra of compounds **1-10** show corresponding $(M+1)^+$ peak as well as $(M+2)^+$ peak. In all spectra $(M+2)^+$peak can be observed due to presence of sulphur atom of pyrazolo[4,3-*d*]isoxazole derivatives.

**Spectral data of synthesized compounds**

**4-(4-Hydroxyphenyl)-3-methyl-4*H*-pyrazolo[4,3-d]isoxazole-5(6*H*)-carbothioamide, 1**: Off-white solid. IR (KBr): 3360.4, 3297.7, 2970.8, 1600.12, 1540.9, 1329.4, 1374.2, 1267.1, 809.8 cm$^{-1}$; $^1$H NMR (400 MHz, DMSO-$d_6$): δ 2.25 (s, 3H), 4.76 (s, 1H) 6.76-6.78 (d, 2H, $J$ = 8.8), 7.02-7.04, (d, 2H, $J$ = 9.2), 8.76 (s, 2H), 10.77 (s, 1H), 11.21 (s, 1H); $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 11.34, 61.96, 111.77, 115.17, 123.78, 131.25, 156.18, 158.69, 169.83, 177.22; ESI-MS: *m/z* for (276.31): 277.3 $(M+1)^+$, 278.3 $(M+2)^+$ . Anal. Calcd for $C_{12}H_{12}N_4O_2S$ (276.07): C, 52.16; H, 4.38; N, 20.28; S, 11.60. Found: C, 52.15; H, 4.39; N, 20.27; S, 11.61%.

**4-(4-(Dimethylamino)phenyl)-3-methyl-4*H*-pyrazolo[4,3-d]isoxazole-5(6*H*)-carbothioamide, 2**: Pale yellow solid. IR (KBr): 3357.3, 3262.6, 2968.7, 1612.00, 1537.7, 1368.6, 1269.6, 811.3 $cm^{-1}$; $^1H$ NMR (400 MHz, DMSO-$d_6$): δ 2.22 (s, 3H), 3.14 (s, 6H), 4.78 (s, 1H) 6.69-6.70 (d, 2H, $J$ = 8.8), 6.85-6.88, (d, 2H, $J$ = 9.2), 8.48 (s, 2H), 11.19 (s, 1H); $^{13}C$ NMR (100 MHz, DMSO-$d_6$): δ 11.20, 48.55, 88.17, 108.96, 111.62, 128.57, 137.55, 151.32, 162.11, 169.81, 176.92; ESI-MS: *m/z* for (303.38): 304.4 $(M+1)^+$, 305.4 $(M+2)^+$. Anal. Calcd for $C_{14}H_{17}N_5OS$ (303.12): C, 55.42; H, 5.65; N, 23.08; S, 10.57. Found: C, 55.40; H, 5.63; N, 23.10; S, 10.55%.

**4-(4-Methoxyphenyl)-3-methyl-4*H*-pyrazolo[4,3-d]isoxazole-5(6*H*)-carbothioamide, 3**: Off-white solid. IR (KBr): 3355.2, 3261.7, 2962.2, 1632.2, 1534.1, 1198.6, 1271.3, 809.8. $cm^{-1}$; $^1H$ NMR (400 MHz, DMSO-$d_6$): δ 2.24 (s, 3H), 3.80 (s, 3H), 4.76 (s, 1H) 6.88-6.90 (d, 2H, $J$ = 8.4), 7.16-7.18, (d, 2H, $J$ = 8.2), 8.88 (s, 2H), 11.20 (s, 1H); $^{13}C$ NMR (100 MHz, DMSO-$d_6$): δ 10.48, 56.04, 61.95, 111.62, 114.03, 125.42, 130.74, 156.18, 159.28, 170.22, 176.90; ESI-MS: *m/z* for (290.34): 291.3 $(M+1)^+$, 292.3 $(M+2)^+$. Anal. Calcd for $C_{13}H_{14}N_4O_2S$ (290.08): C, 53.78; H, 4.86; N, 19.30; S, 11.04. Found: C, 53.75; H, 4.87; N, 19.31; S, 11.05%.

**4-(4-Hydroxy-3-methoxyphenyl)-3-methyl-4*H*-pyrazolo[4,3-d]isoxazole-5(6*H*)-carbothioamide, 4**: White solid. IR (KBr): 3362.6, 3198.2, 2899.4, 1597.9, 1521.3, 1322.8, 1202.3, 1245.4, 815.4, 769.4 $cm^{-1}$; $^1H$ NMR (400 MHz, DMSO-$d_6$): δ 2.21 (s, 3H), 3.74 (s, 3H), 4.81 (s, 1H), 6.55 (s, 1H), 6.77-6.79 (d, 1H, $J$ = 8.2), 7.99-8.01, (d, 1H, $J$ = 8.4), 8.74 (s, 2H), 10.95 (s, 1H), 11.04 (s, 1H); $^{13}C$ NMR (100 MHz, DMSO-$d_6$): δ 10.34, 56.79, 62.02, 110.69, 111.77, 115.22, 121.28, 124.62, 147.76, 146.14 156.20, 160.20, 180.10; ESI-MS: *m/z* for (306.34): 307.4 $(M+1)^+$, 308.4 $(M+2)^+$. Anal. Calcd for $C_{13}H_{14}N_4O_3S$ (306.08): C, 50.97; H, 4.61; N, 18.29; S, 10.47. Found: C 50.99; H 4.60; N 18.28; S 10.50%.

**3-Methyl-4-(3,4,5-trimethoxyphenyl)-4*H*-pyrazolo[4,3-d]isoxazole-5(6*H*)-carbothioamide, 5**: Yellow solid. IR (KBr): 3347.5, 3302.7, 2958.6, 1628.6, 1521.3, 1164.2, 1251.7, 813.2, 775.3 $cm^{-1}$; $^1H$ NMR (400 MHz, DMSO-$d_6$): δ 2.32 (s, 3H), 3.79 (s, 9H), 4.78 (s, 1H), 6.23 (s, 1H), 6.49 (s, 1H), 8.88 (s, 2H), 11.12 (s, 1H); $^{13}C$ NMR (100 MHz, DMSO-$d_6$): δ 10.45, 56.79, 80.88, 104.81, 111.69, 127.50, 139.87, 151.77, 156.18, 161.03, 179.32; ESI-MS: *m/z* for (350.39): 351.4 $(M+1)^+$, 352.4 $(M+2)^+$. Anal. Calcd for $C_{15}H_{18}N_4O_4S$ (350.10): C, 51.42; H, 5.18; N, 15.99; S, 9.15. Found: C, 51.45; H, 5.19; N, 16.00; S, 9.16%.

**4-(3,4-Dimethoxyphenyl)-3-methyl-4*H*-pyrazolo[4,3-d]isoxazole-5(6*H*)-carbothioamide, 6**: Light yellow solid. IR (KBr): IR (KBr): 3354.3, 3245.7, 2968.1, 1621.4, 1541.6, 1276.3, 801.1, 776.3 $cm^{-1}$; $^1H$ NMR (400 MHz, DMSO-$d_6$): δ 2.25 (s, 3H), 3.80 (s, 6H), 4.76 (s, 1H), 6.69 (s, 1H), 6.90-6.92 (d, 1H, $J$ = 8.2), 6.94-6.96 (d, 1H, $J$ = 8.6), 8.78 (s, 2H), 11.23 (s, 1H); $^{13}C$ NMR (100 MHz, DMSO-$d_6$): δ 10.65, 56.75, 62.21, 111.23, 111.77, 115.12, 120.94, 125.55, 149.64, 150.16, 156.18, 160.19, 181.03; ESI-MS: *m/z* for (320.37): 321.4 $(M+1)^+$, 322.4 $(M+2)^+$. Anal. Calcd for $C_{14}H_{16}N_4O_3S$ (320.09): C, 52.49; H, 5.03; N, 17.49; S, 10.01. Found: C, 52.50; H, 5.02; N, 17.47; S, 10.03%.

**4-(2,4-Dimethoxyphenyl)-3-methyl-4*H*-pyrazolo[4,3-d]isoxazole-5(6*H*)-carbothioamide, 7**: Pale yellow solid. IR (KBr): 3327.1, 3264.3, 3008.4, 1591.4, 1491.2, 1301.3, 811.8, 748.1 $cm^{-1}$; $^1H$ NMR (400 MHz, DMSO-$d_6$): δ 2.23 (s, 3H), 3.79 (s, 6H), 4.72 (s, 1H), 6.52-6.54 (d, 1H, $J$ = 8.2), 6.60 (s, 1H), 7.03-7.05 (d, 1H, $J$ = 8.4), 8.72 (s, 2H), 11.35 (s, 1H); $^{13}C$ NMR (100 MHz, DMSO-$d_6$): δ 10.36, 56.04, 56.79, 58.87, 99.46, 107.74, 111.27, 115.24, 132.69, 156.12, 160.05, 160.76, 162.17, 178.96; ESI-MS: *m/z* for (320.37): 321.4 $(M+1)^+$, 322.4 $(M+2)^+$. Anal. Calcd for $C_{14}H_{16}N_4O_3S$ (320.09): C, 52.49; H, 5.03; N, 17.49; S, 10.01. Found: C, 52.52; H, 5.04; N, 17.47; S, 9.99%.

**4-(3-Hydroxy-4-methoxyphenyl)-3-methyl-4*H*-pyrazolo[4,3-d]isoxazole-5(6*H*)-carbothioamide, 8**: White solid. IR (KBr): 3327.1, 3232.4, 2960.1, 1624.4, 1537.4, 1297.4, 1214.5, 999.9, 812.5, 773.1 $cm^{-1}$; $^1H$ NMR (400 MHz, DMSO-$d_6$): δ 2.26 (s, 3H), 3.76 (s, 3H), 4.39 (s, 1H), 6.70 (s, 1H), 6.75-6.77 (d, 1H, $J$ = 8.2), 6.82-6.84, (d, 1H, $J$ = 8.6), 8.70 (s, 2H), 10.80 (s, 1H), 11.31 (s, 1H); $^{13}C$ NMR (100 MHz, DMSO-$d_6$): δ 10.41, 56.78, 62.02, 111.74, 114.92, 116.16, 120.06, 124.31, 146.99, 147.55, 156.18, 160.23, 176.89; ESI-MS: *m/z* for (306.34): 307.3 $(M+1)^+$, 308.3 $(M+2)^+$. Anal. Calcd for $C_{13}H_{14}N_4O_3S$ (306.08): C, 50.97; H, 4.61; N, 18.29; S, 10.47. Found: C, 50.98; H, 4.62; N, 18.30; S, 10.44%.

**3-Methyl-4-(p-tolyl)-4*H*-pyrazolo[4,3-d]isoxazole-5(6*H*)-carbothioamide, 9**: Off-white solid. IR (KBr): 3270.1, 3260.4, 2970.1, 1623.4, 1536.4, 1285.4, 1211.5, 1002.7, 808.2 $cm^{-1}$; $^1H$ NMR (400 MHz, DMSO-$d_6$): δ 2.30 (s, 3H), 2.72 (s, 3H), 4.77 (s, 1H),

7.13-7.15 (d, 4H, $J$ = 8.8), 8.77 (s, 2H), 11.22 (s, 1H); $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 10.22, 21.13, 61.96, 111.67, 129.55, 130.50, 134.80, 138.04, 156.18, 160.31, 177.93; ESI-MS: *m/z* for (274.34): 275.3 $(M+1)^+$, 276.3 $(M+2)^+$. Anal. Calcd for $C_{13}H_{14}N_4OS$ (274.09): C, 56.91; H, 5.14; N, 20.42; S, 11.69. Found: C, 56.94; H, 5.13; N, 20.41; S, 11.68%.

**4-(4-Hydroxy-3,5-dimethoxyphenyl)-3-methyl-4*H*-pyrazolo[4,3-d]isoxazole-5(6*H*)-carbothioamide, 10**: Dark yellow solid. IR (KBr): 3330.4, 3262.7, 2996.0, 1614.7, 1540.1, 1301.9, 1217.8, 1111.9, 817.5, 774.8 cm$^{-1}$; $^1$H NMR (400 MHz, DMSO-$d_6$): δ 2.28 (s, 3H), 3.81 (s, 6H), 4.75 (s, 1H), 6.28 (s, 1H), 6.37 (s, 1H), 8.90 (s, 2H), 10.80 (s, 1H), 11.12 (s, 1H); $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 10.23, 56.75, 62.61, 104.12, 111.74, 125.55, 136.40, 146.36, 156.18, 160.32, 178.45; ESI-MS: *m/z* for (336.37): 337.8 $(M+1)^+$, 338.8 $(M+2)^+$. Anal. Calcd for $C_{14}H_{16}N_4O_4S$ (336.09): C, 49.99; H, 4.79; N, 16.66; S, 9.53. Found: C, 49.98; H, 4.80; N, 16.67; S, 9.54%.

## Pharmacology

### Antimicrobial activity

#### *In vitro* antibacterial activity

All novel synthesized scaffolds were investigated for their *in vitro* antibacterial activity (Table III). The bioassay results demonstrated that pyrazolo[4,3-*d*] isoxazole derivatives **1-10** succeeded to indicate remarkably activity against the mentioned microorganisms as compared to standard drugs. In general, most of the tested compounds exhibited better activity against the Gram-positive as well as the Gram-negative bacteria.

Among the Gram-positive bacterial strain, *Staphylococcus aureus* showed relatively higher sensitivity towards the tested compounds. In this view, compound **10** bearing an electron donating -OMe group at 3 and 5 positions of the phenyl ring as well as –OH group at 4 position of the phenyl ring, was found to be the most active compound that inhibits the gram positive *S. aureus* bacterial growth at the lowest minimum inhibitory concentration (MIC) value of 125 µg/mL then Ampicillin (MIC 250 µg/mL). Furthermore, compounds **1**, **5**, **7** and **8** displayed excellent effectiveness with MIC value 200 µg/mL against *S. aureus* as compared to standard drug Ampicillin, but 50% less active than Chloramphenicol (MIC 50 µg/mL) and Ciprofloxacin (MIC 50 µg/mL). With regard to the activity against *S. pyogenus*, the best activity was exhibited by compounds **5** and **6** (MIC 100 µg/mL) having electron donating −OMe group on phenyl ring, which is equally potent as Ampicillin (MIC 100 µg/mL) but 50% less active than Chloramphenicol (MIC 50 µg/mL) and Ciprofloxacin (MIC 50 µg/mL).

On the other hand, investigation of antibacterial activity of all newly synthesized analogues against the two tested Gram-negative strains revealed that analogs **5** and **7** (MIC 62.5 µg/mL) displayed excellent activity against *E. coli* as compared to standard drug Ampicillin (MIC 100 µg/mL). Furthermore, analogs **2** and **6** (MIC 100 µg/mL) were equipotent to Ampicillin (MIC 100 µg/mL) against *E. coli*, but 50% less active than Chloramphenicol (MIC 50 µg/mL). Moreover, compounds **1**, **5**, **6** and **8** were able to inhibit the gram negative *P. aeruginosa* bacterial growth at the lowest minimum inhibitory concentration (MIC) value of 100 µg/mL equipotent to ampicillin (MIC 100 µg/mL).

Table III — *In vitro* antibacterial activity of compounds **1-10**

| Compd | MIC [µg/mL] | | | |
|---|---|---|---|---|
| | *E.coli* MTCC 443 | *P. aeruginosa* MTCC 1688 | *S. aureus* MTCC 96 | *S. pyogenus* MTCC 442 |
| **1** | 500 | 100 | 200 | 500 |
| **2** | 100 | 200 | 500 | 500 |
| **3** | 200 | 200 | 1000 | 1000 |
| **4** | 500 | 500 | 500 | 500 |
| **5** | 62.5 | 100 | 200 | 100 |
| **6** | 100 | 100 | 500 | 100 |
| **7** | 62.5 | 200 | 200 | 200 |
| **8** | 125 | 100 | 200 | 250 |
| **9** | 200 | 250 | 500 | 200 |
| **10** | 200 | 200 | 125 | 250 |
| Ampicillin | 100 | 100 | 250 | 100 |
| Ciprofloxacin | 25 | 25 | 50 | 50 |
| Chloramphenicol | 50 | 50 | 50 | 50 |

Table IV — *In vitro* antifungal activity of compounds **1-10**

| Compd | MIC [μg/mL] | | |
|---|---|---|---|
| | *C. albicans* MTCC 227 | A. *niger* MTCC 282 | A. *clavatus* MTCC 1323 |
| **1** | 250 | >1000 | >1000 |
| **2** | 500 | 1000 | 1000 |
| **3** | 1000 | 250 | 500 |
| **4** | 1000 | 500 | 500 |
| **5** | >1000 | 250 | 250 |
| **6** | >1000 | 500 | 500 |
| **7** | 250 | 1000 | 1000 |
| **8** | 1000 | >1000 | >1000 |
| **9** | 500 | 1000 | >1000 |
| **10** | 1000 | >1000 | >1000 |
| Nystatin | 100 | 100 | 100 |
| Greseofulvin | 500 | 100 | 100 |

Table V — *In vitro* antimalarial activity of compounds **1-10**

| Compd | Mean IC50 values (μg/mL) |
|---|---|
| **1** | 1.05 |
| **2** | 0.91 |
| **3** | 1.12 |
| **4** | 1.14 |
| **5** | 0.34 |
| **6** | 0.68 |
| **7** | 0.85 |
| **8** | 0.76 |
| **9** | 1.11 |
| **10** | 1.03 |
| Quinine | 0.268 |
| Chloroquine | 0.02 |

### *In vitro* antifungal activity

Concerning the antifungal activity of tested compounds, only one strain, *i.e*., *C. albicans* showed certain sensitivity against some of the tested compounds, whereas rest of the other two fungal strain were insensitive to the same compounds (Table IV).

Antifungal activity data showed that among the analogues **1-10**, compounds **1** and **7** displayed excellent activity with MIC 250 μg/mL against *C. albicans* as compared to Greseofulvin (MIC 500 μg/mL). Furthermore, compounds **2** and **9** showed excellent activity with MIC 500 μg/mL against *C. albicans* equipotent to Greseofulvin (MIC 500 μg/mL). Moreover, compounds **1-10** exerted moderate inhibitory efficiency against *A. niger* and *A. clavatus*.

### *In vitro* antimalarial activity

The synthesized compounds were also screened for *in vitro* antimalarial activity against *Plasmodium falciparum* 3D7-chloroquine-sensitive strain (Microcare Laboratory and TRC, Surat, Gujarat, India). All experiments were performed in duplicate and a mean IC50 value is mentioned in Table V. All compounds exhibited only moderate antimalarial activity (IC50 = 0.68-1.14 μg/mL).

Table VI — *In vitro* antimalarial activity of compounds **1-10**

| Compd | MIC (μg/mL) |
|---|---|
| **1** | 62.5 |
| **2** | 50 |
| **3** | 250 |
| **4** | 500 |
| **5** | 50 |
| **6** | 62.5 |
| **7** | 62.5 |
| **8** | 100 |
| **9** | 250 |
| **10** | 50 |
| Isoniazid | 0.20 |
| Rifampicin | 0.25 |

### *In vitro* antituberculosis activity

The preliminary screening of the title compounds **1-10** for their *in vitro* anti-tuberculosis activity against *Mycobacterium tuberculosis* $H_{37}$Rv strain was determined. The observed MIC values of these compounds are presented in Table VI. Among the screened analogs, **2**, **5** and **10** showed the best activity (50 mg/mL), followed by compounds **1**, **6** and **7** (62.5 mg/mL). Isoniazid and rifampicin were used as the standard drugs.

## Conclusions

In summary, PEG-400 is utilized as an extremely effective catalytic system for the one-pot synthesis of pyrazolo[4,3-*d*]isoxazole derivatives from the reaction of isoxazol-5(4*H*)-one derivatives and thiosemicarbazide in the presence of catalytic amount of triethylamine under reflux at the 90°C or irradiated under microwave at the 300 W. The major advantages of the present protocol comprises non-volatile and biodegradable PEG-400 as substitute reaction media, excellent product yield, short reaction time and simple work-up procedure. Moreover, by this methodology, highly pure products are obtained and there is no need for column purification. From the bioassays it is clear that the pyrazolo[4,3-*d*]isoxazole derivatives lead to more active antimicrobial activity. In the present study, compounds **5** and **7** exhibit highly potent activity against most of the tested bacteria.

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## References


1 Kumar A, Gupta M K & Kumar M, *Green Chem*, 14 (2012) 290.
2 Anastas P T & Kirchhoff M M, *Acc Chem Res*, 35 (2002) 686.
3 Vekariya R H & Patel H D, *Arkivoc*, 1 (2015) 136.
4 Vekariya R H & Patel H D, *Arkivoc*, 1 (2015) 70.
5 Hafez H N & El-Gazzar A B A, *Acta Pharm*, 65 (2015) 215.
6 Hafez H N, Hussein H A R & El-Gazzar A B A, *Eur J Med Chem*, 45 (2010) 4026.
7 Grare M, Mourer M, Fontanay S, Regnouf-de-Vains J B, Finance C & Duval R E, *J Antimicrob Chemother*, 60 (2007) 575.
8 Coleman K, *Drug Discov Today Ther Strateg*, 37 (2004) 455.
9 Storer R, Ashton C J, Baxter A D, Hann M M, Marr C L P, Mason A M, Mo C L, Myers P L, Noble S A, Penn C R, Weir N G, Woods J M & Coe P L, *Nucleosides Nucleotides Nucleic Acids*, 18 (1999) 203.
10 Genin M J, Biles C, Keiser B J, Poppe S M, Swaney S M, Tarpley W G, Yagi Y & Romero D L, *J Med Chem*, 43 (2003) 1034.
11 Brough P A, Aherne W, Barril X, Borgognoni J, Boxall K, Cansfield J E, Cheung K M J, Collins I, Davies N G, Drysdale M J & Dymock B, *J Med Chem*, 51 (2008) 196.
12 Vera Di Vaio M A F, Freitas A C C, Castro H C A, de Albuquerque S, Cabral L M, Rodrigues C R, Albuquerque M G, Martins R C A, Henriques M G & Dias L R S, *Bioorg Med Chem*, 17 (2009) 295.
13 Prakash O, Kumar R & Parkash V, *Eur J Med Chem*, 43 (2008) 435.
14 Cheng H, DeMello K M L, Li J, Sakya S M, Ando K, Kawamura K, Kato T, Rafka R J, Jaynes B H, Ziegler C B & Stevens R, *Bioorg Med Chem Lett*, 16 (2006) 2076.
15 Bekhit A A, Ashour H M A, Ghany Y S A, Bekhit A E A & Baraka A, *Eur J Med Chem*, 43 (2008) 456.
16 Ahlstrom M M, Ridderstrom M, Zamora I & Luthman K, *J Med Chem*, 50 (2007) 4444.
17 Bailey D M, Hansen P E, Hlavac A G, Baizman E R, Pearl J, Defelice A & Feigenson M E, *J Med Chem*, 28 (1985) 256.
18 Gursoy A, Demirayak S, Capen G, Erol K & Vural K, *Eur J Med Chem*, 35 (2000) 359.
19 Pimerova E V & Voronina E V, *J Pharm Chem*, 35 (2001) 18.
20 Cottineau B, Toto P, Marot C, Pipaud A & Chenault J, *Bioorg Med Chem Lett*, 12 (2002) 2105.
21 Ozdemir Z, Kandilici H B, Gumusel B, Calis U & Bilgin A A, *Eur J Med Chem*, 42 (2007) 373.
22 Gomha S M, Badrey M G, Abdalla M M & Arafa R K, *Med Chem Comm*, 5, (2014) 1685.
23 Chen J, Spear S K, Huddleston J G & Rogers R D, *Green Chem*, 7 (2005) 64.
24 Chen J, Spear S K, Huddleston J G, Holbrey J H & Rogers R D, *Ind Eng Chem Res*, 43 (2004) 5358.
25 Gou Z, Li M, Willauer H D, Huddleston J G, April G C & Rogers R D, *Ind Eng Chem Res*, 41 (2002) 2535.
26 Merrill E W, 'Poly(ethy1ene oxide) and Blood Contact: A Chronicle of One Laboratory', in *Biomedical Applications of Polyethylene Glycol Chemistry*, Chapter 16, edited by Harris J M (Plenum, New York) (1991).
27 Harris J M & Zalipsky S, *Chemistry and Biological Applications of Polyethylene Glycol* (ACS Books: Washington DC) 1 (1997) 2.
28 Mao J, Guo J, Fang F & Ji S J, *Tetrahedron*, 64 (2008) 3905.
29 Mukhopadhyay C & Tapaswi P K, *Tetrahedron Lett*, 49 (2008) 6237.
30 Kouznetsov V V, Merchan Arenas D R & Bohorquez A R R, *Tetrahedron Lett*, 49 (2008) 3097.
31 Sindhu J, Singh H, Khurana J, Sharma C & Aneja K, *Chin Chem Lett*, 26 (2015) 50.
32 Zeng H, Li Y & Shao H, *Synth Commun*, 42 (2012) 25.
33 Rajanarendar E, Govardhan Reddy K, Nagi Reddy M, Raju S & Rama Murthy K, *Green Chem Lett Rev*, 4 (2011) 257.
34 Kidwai M, Jahan A & Bhatnagar D, *J Sulfur Chem*, 31 (2010) 161.
35 Dawane B S, Shaikh B M, Khandare N T, Kamble V T, Chobe S S & Konda S G, *Green Chem Lett Rev*, 3 (2010) 205.
36 Kidwai M, Bhatnagar D & Chauhan R, *J Hetero Chem*, 50 (2013)234.
37 Zhong X, Dou G & Wang D, *Molecules*, 18 (2013) 13139.
38 Rajeswari M, Sindhu J, Singh H & Khurana J M, *RSC Adv*, 5 (2015) 39686.
39 Kidwai M & Bhatnagar D, *Chemical Papers*, 64 (2010) 825.
40 Wang Z G & Lu M, *RSC Adv*, 4 (2014) 1234.
41 Vekariya R H, Panchal S N, Patel K D & Patel H D, *Curr Microwave Chem*, 2 (2015) 61.
42 Vekariya R H, Patel K D & Patel H D, *Res Chem Intermed*, 42(2016) 7559.
43 Hawkey P & Lewis D, *Medical Bacteriology*: *A Practical Approach* (OUP: Oxford) (2003) 265.
44 Reickmann K H, Campbell G H, Sax L J & Mrema J E, *Lancet*, 1 (1978) 221.
45 Singh J J S B, *Indian J Malariol*, 10 (1956) 117.
46 Desai N C, Shukla H K & Tahker K A, *J Indian Chem Soc*, 61 (1984) 239.
47 Anargyros P, Astill D J S & Lim I S L, *J Clin Microbiol*, X 28 (1990) 1288.