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SYNTHESIS CHARACTERIZATION OFNOVEL SERIES OF PYRROLYL PYRANOPYRAZOLE AS ANTIMICROBIAL AGENTS

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ABSTRACT

New series of pyrrolylpyranopyrazole were synthesized by reacting with (one pot synthesis) ethyl acetoacetate in aqueous ethanol **(1),** hydrazine hydrate **(2)**, substituted aldehydes **(3a-g)**, malononitrile **(4)** and triethylamine's base stirring for 5hr the solid precipitate was obtained, it has been filtered and washed with cold water to give 6-amino-3-methyl-4-substituted phenyl-1,3a,4,7a-tetrahydropyrano [2,3-c] pyrazole-5-carbonitrile **(5a-g)**. Further it is reacted with 4-pyrrol-1-yl benzoic acid **(6)** with substituted pyranopyrazoline presence of HBTU and DIEA which resulting in formation of substituted N-5-cyano-3-methyl-4-substituted phenyl 1,3a,47a-tetrahydropyrano [2,3-c] pyrazol-6-yl)-4-(1H-pyrrol-1-yl) benzamides **(7a-g)** was obtained. All newly synthesized compounds were confirmed by TLC and melting point. The structure of the all newly synthesized compounds were confirmed by spectral study such as IR, ^{1}H NMR, ^{13}C NMR and Mass spectroscopy. The newly synthesized compounds were screened for their antibacterial and antitubercular activities. Compounds exhibited antitubercular activity in the range of 1.6 to 25 µg/ml (MIC). Compounds showed antibacterial activity in the range of 0.8 to 100 µg/ml (MIC). It was indeed very much encouraging to note that most of the compounds have shown better and significant antibacterial and antitubercular activities.

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INTRODUCTION

It is an agent that kills micro-organisms or stops their growth. Antimicrobial medicines can be grouped according to the micro-organisms they act primarily against. For example, antibiotics are used against bacteria and antifungals are used against fungi. They can also be classified according to their function. Agents that kill microbes are called microbicidal, while those that merely inhibit their growth are called biostatic. The use of antimicrobial medicines to treat infection is known as antimicrobial chemotherapy, while the use of antimicrobial medicines to prevent infection is known as antimicrobial prophylaxis.

The main classes of antimicrobial agents are disinfectants ("nonselective antimicrobials" such as bleach), which kill a wide range of microbes on non-living surfaces to prevent the spread of illness, antiseptics (which are applied to living tissue and help reduce infection during surgery), and antibiotics (which destroy microorganisms within the body). The term "antibiotic" originally described only those formulations derived from living microorganisms but is now also applied to synthetic antimicrobials, such as the sulfonamides, or fluoroquinolones. The term also used to be restricted to anti-bacterial (and is often used as a synonym for them by medical professionals and in medical literature), but its context has broadened to include all antimicrobials. Antibacterial agents can be further subdivided into bactericidal agents, who kill bacteria, and bacteriostatic agents, which slow down or stall bacterial growth. In response, further advancements in antimicrobial technologies have resulted in solutions that can go beyond simply inhibiting microbial growth. Instead, certain types of porous media have been developed to kill microbes on contact.[1] Infection diseases such as bacterial and fungal infections have been reported to increase dramatically worldwide in recent times and one of the major causes is suppressed immunity.[2]

TB is one of the most important chronic communicable bacterial diseases caused by *Mycobacterium tuberculosis.* Present anti-tubercular drugs are quite effective in the treatment of TB but resistance to these drugs by *M. tuberculosis* strains is on the rise which is a growing concern among scientific community worldwide. New anti-tubercular compound/structures are urgently needed in the present situation because very few agents belonging to first line (e.g., rifabutin) or second line drugs (e.g., capreomycin, levofloxacin) have become available for the treatment of this disease since the introduction of rifampicin in 1966.[3]

It is a form of tuberculosis (TB) infection caused by bacteria that are resistant to treatment with at least two of the most powerful first-lineanti-TB medications (drugs), isoniazid and rifampin. Some forms of TB are also resistant to second-line medications, and are called extensively drug-resistant TB (XDR-TB).[4]

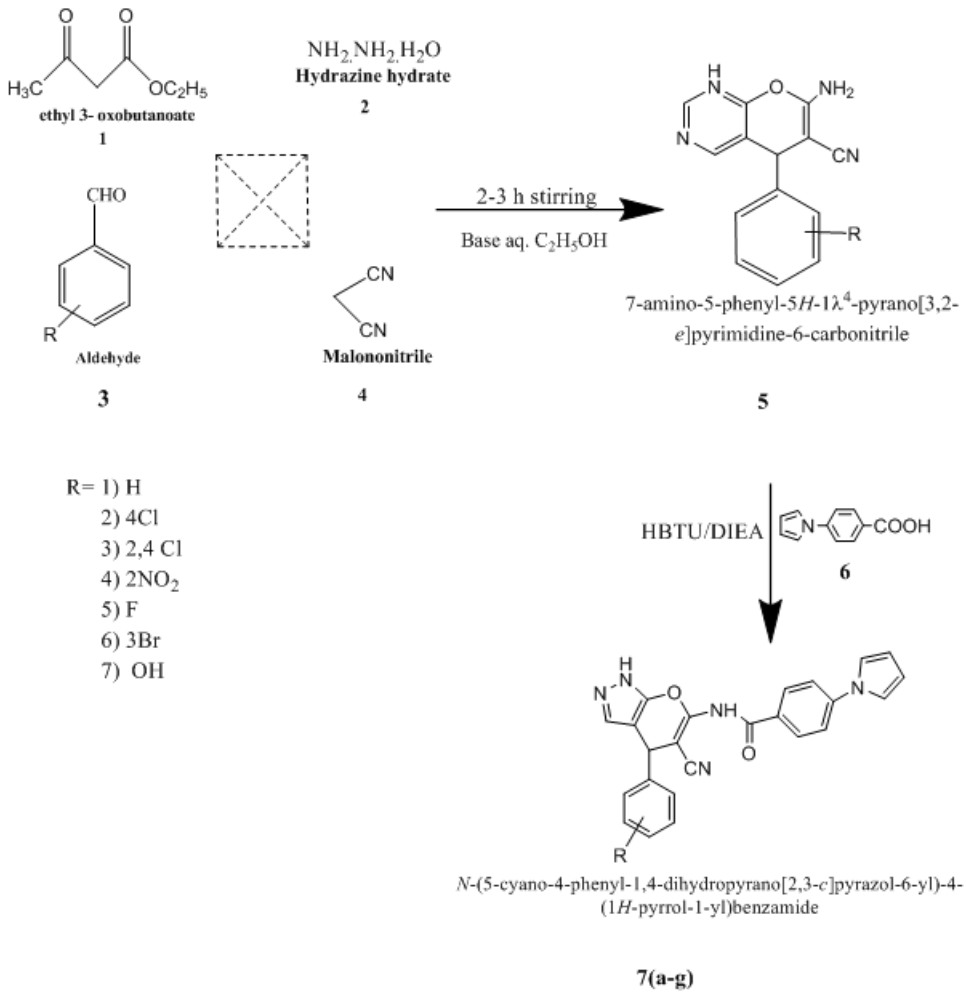
Tuberculosis is caused by infection with the bacteria Mycobacterium tuberculosis. Almost one in four people in the world are infected with TB bacteria. Only when the bacteria become active do people become ill with TB. Bacteria become active as a result of anything that can reduce the person's immunity, such as HIV, advancing age, diabetes or other immune compromising illnesses. TB can usually be treated with a course of four standard, or first-line, anti-TB drugs (i.e., isoniazid, rifampin and any fluoroquinolone).[5]

Pyrroles are found to be as the fundamental structural motifs in various classes of natural and biologically important molecules such as porphyrins, bile pigments, coenzymes and alkaloids. This moiety is also present in several synthetic pharmaceuticals as well as electrically conducting polymers.[6]

Pyrrole and its derivatives have shown to possess biological activities such as antibacterial,[7] antitumor,[8] analgesics [9] antitubercular [10,11], anti-inflammatory, and antiallergic [12]. Several macromolecular antibiotics having pyrrole structure were isolated from biological sources and their activities were defined [13,14].

As per the above findings, we came to know that pyrrole and pyrazoline containing heterocyclic compounds are useful chemical entities because of their wide range of biological applications. Keeping in view, we thought of combining pyrrole and pyrazoline moieties and analyzing their antitubercular and antimicrobial activity.

SCHEME:



Experimental:

All the melting points and boiling points of synthesized compounds were determined by capillary method in a paraffin bath/digital melting point apparatus. FT-IR spectra were recorded on Bruker spectrophotometer by using KBr pellets and values are expressed in cm^{-1}. The ^{1}H and ^{13}CNMR spectra were recorded on Bruker AVANCE II 400/100 MHz instrument using chloroform ($CDCl_3$) and dimethyl sulfoxide (DMSO-d_6) as a solvent and TMS as an internal standard. The chemical shifts are expressed as δ values (ppm) and the splitting of the NMR spectra are termed as singlet (s), doublet (d), triplet (t), quartet (q) and multiplet (m). Mass spectra (MS) were recorded on Waters-Q-Tof Premier-HAB213, all the compounds' spectra showed the data in consistent with the projected structure and analytical thin-layer chromatography (TLC) was performed on the precoated TLC sheets of silica gel $60F_{254}$ (Merck, Darmstadt, Germany) visualized by long- and short-wavelength ultraviolet lamps.

The purity of the compound was characterized by melting point and TLC. The structure of newly synthesized compound was confirmed by analytical and spectral data such as (IR, ^{1}H NMR and mass spectra). All the synthesized compounds were screened for anti-tubercular activity using MicroplateAlmar Blue Assay (MABA) method and antibacterial activity against *S. aureus*, and *E. coli*usingbroth micro dilution assay method.

General procedure for the synthesis of pyran[2,3-c] pyrazoles (one pot synthesis)[71] (1-4):

The mixture of hydrazine hydrate 96% **(2)** (1ml, 1mol) and ethyl acetoacetate **(1)** (2.6ml,1mol) in aqueous ethanol (20%) was stirred for 5-10 min. Substituted aldehydes**(3a-g)** (1mol), malononitrile **(4)** (1ml,1mol) and triethylamine as base (2ml,5mol) were added to it successively at room temperature under open atmosphere with vigorous stirring for 5 hr. The progress of the reaction was monitored by TLC. The precipitated solid was filtered and washed with water, to afforded pure product 6-amino-3-methyl-4-substituted phenyl-1,3a,4,7a-tetrahydropyrano [2,3-c] pyrazole-5-carbonitrile**(5a-g) further compounds** dried to get a good yield of compound, no further purification is required MP: 240-242^{0}C.

General procedure for the synthesis of 4-(-1*H*-pyrrol-1-yl) benzoic acid (6):

A mixture of 4-aminobenzoic acid (13.72gm,0.1mol) in dried acetic acid (100 ml) and 2,5-dimethoxytetra hydrofuran (16gm,0.12mol) is reflux for 60 minutes. Then the reaction mixture was poured onto crushed ice crude crystals are obtained, the separated solid was filtered and recrystallized from ethanol MP: 280-282^{0}C.

General procedure for the synthesis of *N*'-(5-cyano-3-methyl-4-substitutedphenyl-1,3a,4,7a-tetrahydropyrano[2,3-c] pyrazol-6-yl)-4-(-1*H*-pyrrol-1-yl)benzamide (7a-g):

Mixer of 4-(-1*H*-pyrrol-1-yl) benzoic acid **(6)** (0.16gm, 0.001mol), with substituted pyranopyrazoles **(1-4)** (0.0019 mol) were dissolved in dry DMF (20ml), HBTU (0.87gm, 0.0023 mol) and DIEA (0.93ml) and stirred for 36hr at 23^0C.The solutionof mixture was quenched by brine. The resulting mixture was extracted with ethyl acetate 50ml for 3 times each. The total ethyl acetate layer washed with 1N HClthan saturated $NaHCO_3$ followed by brine. Then evaporate the solvent to obtain the crystals.

Spectral data of *N*-(3-methyl-4-phenyl-1,3a,4,7a-tetrahydropyrano[2,3-c] pyrazol-6-yl)-4-(-1*H*-pyrrol-1-yl) benzamide (7a-g):
***N*-(5-cyano-4-phenyl-1,4-dihydropyrano[2,3-*c*] pyrazole-6-yl)-4-(1*H*-pyrrol-1-yl) benzamide(7a):**

IR Spectrum (KBr,Cm^{-1}), 3373.80(-NH), 2924.43(Ar), 2193.88(-CN), 1694.92(C=O), 1331.21(-CH_3), 1183.13(C-O-C).^{1}H-NMR Spectrum (400 MHz, DMSO)(δ-*ppm*): 8.01(s, 1H benzamide NH -H), 8.01-7.99(d, 2H bridging phenyl Ar-C_2, C_6 -H), 7.98-7.70(d, 2H bridging phenyl Ar-C_3, C_5 -H), 7.42-7.39(t, 3H phenylAr-C^1_3,C^1_5,C^1_4-H), 7.25-7.24(d, 2H pyrrole-C_2, C_5 -H),7.22-7.20(d, 2H phenyl Ar-C^1_2, C^1_6 -H),6.33-6.335(d, 2H pyrrole-C_3, C_4 -H),2.05(s, 3H CH_3-H).

***N*-(5-cyano-4-(2,3,4,5-tetrachlorophenyl)-1,4-dihydropyrano[2,3-c] pyrazol-6-yl)-4-(1*H*-pyrrol-1-yl) benzamide(7b):**

IR Spectrum (KBr,Cm^{-1}), 3145.33(-NH), 2924.73(Ar), 2188.27(-CN),1601.01(C=O), 1483.88(-CH_3), 1184.08(C-O-C), 726.73(-Cl).^{1}H-NMR Spectrum (400 MHz, DMSO) (δ-*ppm*): 12.17(s, 1H pyran pyrazole NH-H),8.72(s,1H benzamide NH -H -CONH), 8.01-7.99(d, 2H bridging phenyl Ar-C_2, C_6 -H), 7.91-7.89(d, 2H bridging phenyl Ar-C_3, C_5 -H), 7.39-7.37(d, 2H phenyl Ar-C^1_2, C^1_6 -H), 7.32-7.30(d, 2H phenyl Ar-C^1_3, C^1_5-H),7.25(d, 2H pyrrole-C_2, C_5 -H),6.32-6.29(d, 2H pyrrole-C_3, C_4 -H),1.80(s, 3H CH_3-H).^{13}C NMR (100 MHz,DMSO-d_6) (δ-*ppm*): 160.88, 143.45, 130.99, 129.34, 126.58, 118.98, 110.86.

***N*-(5-cyano-4-(2,4,-dichlorophenyl)-1,4-dihydropyrano[2,3-c] pyrazol-6-yl)-4-(1*H*-pyrrol-1-yl) benzamide(7c):**

IR Spectrum (KBr,Cm^{-1}), 3320.22(-NH), 2920.91(Ar), 2189.68(-CN),1602.19(C=O),1330.41(-CH_3), 1181.87(C-O-C), 763.84(-Cl).^{1}H-NMR Spectrum (100 MHz,DMSO-d_6) (δ-*ppm*):12.705(s, 1H pyranopyrazole NH-H), 8.02(s, 1H benzamide NH -H), 8.01-8.00(d, 2H bridging phenyl Ar-C_2, C_6 -H), 7.99-7.98(d, 2H bridging phenyl Ar-C_3, C_5 -H), 7.71(s, 1H phenyl Ar-C^1_3 -H), 7.39(d, 2H pyrrole-C_2, C_5 -H), 7.32(s, 1H phenyl Ar-C^1_6 -H),7.03(s, 1H phenyl Ar-C^1_5 -H), 6.33(d, 2H pyrrole-C_3, C_4 -H), 1.98(s, 3HCH_3-H).^{13}C NMR (100 MHz,DMSO-d_6) (δ-*ppm*): 161.31, 143.22, 142.88, 140.09, 135.45, 132.81, 132.10, 130.82, 128.82, 128.02, 126.08, 120.29, 117.80, 110.78, 60.67, 20.72.

***N*-(5-cyano-4-(2, -dichlorophenol)-1,4-dihydropyrano[2,3-c] pyrazol-6-yl)-4-(1*H*-pyrrol-1-yl) benzamide(7d)::**

IR Spectrum (KBr,Cm^{-1}), 3257.98(-NH), 2923.73(Ar), 2193.82(-CN), 1520.23(C=O), 1376.68(-CH_3), 1183.60(C-O-C), 1330.00(-NO_2).^{1}H-NMR Spectrum (400 MHz, $CDCl_3$) (δ-*ppm*): 12.62(s, 1H pyran pyrazole NH-H), 8.10(s, 1H benzamide NH -H), 8.08-8.04(d, 2H bridging phenyl Ar-C_2, C_6 -H), 7.90-7.88(d, 2H bridging phenyl Ar-C_3, C_5 -H), 7.965-7.962(d, 2H phenyl Ar-C^1_2-H), 7.71-7.70(d, 2H phenyl Ar-C^1_5 -H),7.68-7.66(t, 3H phenyl Ar-C^1_4 -H),7.62-7.58(t, 3H phenyl Ar-C^1_3 -H),7.33-7.31(d, 2H pyrrole-C_2, C_5 -H),6.31-6.30(d, 2H pyrrole-C_3, C_4 -H),2.24(s, 3H CH_3-H).

***N*-(5-cyano-4-(4-fluorophenyl)-1,4-dihydropyrano[2,3-c] pyrazol-6-yl)-4-(1*H*-pyrrol-1-yl) benzamide(7e):**

IR Spectrum (KBr,Cm^{-1}), 3332.38(-NH), 2921.40(Ar), 2187.59(-CN), 1606.62(C=O), 1331.58(-CH_3), 1188.03(C-O-C), 1015.88(-F).^{1}H-NMR Spectrum (400 MHz, $CDCl_3$) (δ-*ppm*): 12.70(s, 1H pyran pyrazole NH-H), 8.024(s, 1H benzamide NH -H), 8.01-8.00(d, 2H bridging phenyl Ar-C_2, C_6 -H), 7.94-7.90(d, 2H bridging phenyl Ar-C_3, C_5 -H), 7.49-748(d, 2H phenyl Ar-C^1_2, C^1_6 -H),6.74(d, 2H pyrrole-C_2, C_5 -H),6.33-6.32(d, 2H pyrrole-C_3, C_4 -H),1.95(s, 3H CH_3-H).Mass Spectrum: =440.55[M+1], Calcd. =439.45

***N*-(5-cyano-4-(4-fluorophenyl)-1,4-dihydropyrano[2,3-c] pyrazol-6-yl)-4-(1*H*-pyrrol-1-yl) benzamide(7f):**

IR Spectrum (KBr, Cm^{-1}), 3320.20(-NH), 2921.34(Ar), 2188.33(-CN), 1604.76(C=O), 1386.99(-CH_3), 1182.49(C-O-C), 763.70(-Br).^{1}H-NMR Spectrum (400 MHz, $CDCl_3$) (δ-*ppm*): 8.023(s, 1H benzamide NH –H), 8.002-8.001(d, 2H bridging phenyl Ar-C_2, C_6 –H), 7.96-7.95(d, 2H bridging phenyl Ar-C_3, C_5 –H), 7.53(s, 1H phenyl Ar-C^1_2-H),.08(d, 2H pyrrole-C_2, C_5 –H),7.01(s, 1H phenyl Ar-C^1_6–H),6.337-6.332(d, 2H pyrrole-C_3, C_4–H), 2.23(s, 3H CH_3 –H).

***N*-(5-cyano-4-(4-hydroxyphenyl)-1,4-dihydropyrano[2,3-c]pyrazol-6-yl)-4-(1*H*-pyrrol-1-yl)benzamide(7g):**

IR Spectrum (KBr, Cm^{-1}),3311.02(-NH), 2924.58(Ar), 2188.36(-CN), 1602.85(C=O), 1331.82(-CH_3), 1179.80(C-O-C), 3165.88(OH).^{1}H-NMR Spectrum (400 MHz, $CDCl_3$) (δ-*ppm*): 8.19(s, 1H benzamide NH –H), 8.09-8.04(d, 2H bridging phenyl Ar-C_2, C_6 –H), 7.99(s, 2H bridging phenyl Ar-C_3, C_5 –H), 7.28-7.26(d, 2H phenyl Ar-C^1_2,C^1_6 –H),7.26(d, 2H pyrrole-C_2, C_5 –H),6.68(d, 2H phenyl Ar-C^1_3, C^1_5 –H), 6.33(d, 2H pyrrole-C_3, C_4 –H), 1.98(s, 3H CH_3 –H).

RESULTS AND DISCUSSION

Antibacterial activity

The MIC determination of the tested compounds was carried out simultaneously in comparison with ciprofloxacin, norfloxacin against Gram-positive (*Staphylococcus aureus and Bacillus subtilis*). Gram-negative bacteria (*Vibrio cholerae and Escherichia coli*) by broth micro dilution method [15, 16]. Serial dilutions of the test compounds and reference drugs were prepared in Mueller-Hinton broth. Drugs (10 mg) were dissolved in dimethyl sulfoxide (DMSO, 1 mL). Further progressive dilutions were done to obtain final concentrations of 0.2, 0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25, 50 and 100 µg mL^{-1}. The tubes were inoculated with 10^5cfu mL^{-1}(colony forming unit/mL) and incubated at 37 °C for 18 h. The MIC was the lowest concentration of the tested compound that yield no visible growth on the plate. To ensure that the solvent had no effect on the bacterial growth, a control was performed with the test medium supplemented with DMSO at the same dilutions as used in the experiments and DMSO had no effect on the microorganisms in the concentrations studied. The MIC values are given in µg/ml. Ciprofloxacin and norfloxacin were used as standard drugs. The preliminary results of antibacterial activities are depicted in Table 1. Compounds showed antibacterial activity between MIC of 100-3.12µg/ml.

Compounds 7f, 7g, shows good MIC value of 3.12µg/ml against gram negative bacteria such as *V. cholera, E. coli* and gram-positive bacteria such as *S. aureus* and *B. subtilis*, while other compounds7a, 7c,7e, showed moderate MIC value of 6.25µg/ml, so the combination of both heterocycles (pyrrole and pyranopyrazole) can be considered as potential candidates for antibacterial activity against both Gram-positive and Gram-negative organisms after structural modification.

Table 1: Antibacterial activities by broth microdilution assay method (MIC µg/ml).

Comp	R	S. aureus	E. coli
7a	-H	100	6.25
7b	-4Cl	100	12.5
7c	-2,4Cl	100	6.25
7d	-2NO_2	25	12.5
7e	-F	25	6.25
7f	-3Br	100	3.12
7g	-OH	25	3.12

MIC: Minimum Inhibitory Concentration in µg/ml.
Standard Drugs: *Ciprofloxacin 3.125*µg/ml.

Antitubercular activity

MIC values were determined for the newly synthesized compounds against *M. tuberculosis* strain H_{37}Rv using the Microplate Alamar Blue assay (MABA) [17] using isoniazid as the standard drug. The 96 wells plate received 100 µl of Middlebrook 7H9 broth and serial dilution of compounds were made directly on the plate with drug concentrations of 0.2, 0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25, 50 and 100 µg/ml. Plates were covered and sealed with parafilm and incubated at 37ºC for 5 days. Then, 25 µl of freshly prepared 1:1 mixture of almar blue reagent and 10% Tween 80 was added to the plate and incubated for 24 h. A blue color in the well was interpreted as no bacterial growth and pink color was scored as growth. Compound 7g showed MIC value of 0.8 µg/ml and compound 7c, 7e showed MIC value at 6.25 µg/ml against *M. tuberculosis*, based on screening results, it can be concluded that combination of pyrrole and pyranopyrazole with different substituents like hydroxy, chloro and 2,4-dichloro helps to improve antitubercular activity of synthesized compounds.

Table 2: Anti-tubercular screening by Micro plate Alamar Blue Assay (MABA) method (MIC µg/ml):

Comp	R	Minimum inhibitory concentration (MIC) value. µg/ml
7a	-H	12.5
7b	-4Cl	12.5
7c	-2,4Cl	6.25
7d	-2NO_2	12.5
7e	-F	6.25
7f	-3Br	12.5
7g	-OH	0.8

Standard Drugs: Pyrazinamide (3.125 µg/ml), Streptomycin (6.25 µg/ml).

CONCLUSIONS

Novel series of pyrrolyl pyranopyrazole derivatives were designed and synthesized with an approach to reduce the growing anti-tubercular resistance and to develop more potent and less side effects having anti-tubercular agents. We synthesized pyrrolyl pyran pyrazole by reacting with (one pot synthesis) of ethyl acetoacetate in aqueous ethanol **(1),** hydrazine hydrate **(2)**, substituted aldehyde **(3a-g)**, malononitrile **(4)** with triethylamine base for 5hrs stirring, the precipitate solid has been filtered and washed with cold water to give 6-amino-3-methyl-4-substituted phenyl-1,3a,4,7a-tetrahydropyrano [2,3-c] pyrazole-5-carbonitrile **(5a-g)**. New series were synthesized by reacting with 4- pyrrol-1-yl benzoic acid **(6)** with substituted pyran pyrazole in presence of HBTU and DIEA which resulting in formation of substituted N-5-cyano-3-methyl-4-substituted phenyl 1,3a,47a-tetrahydropyrano [2,3-c] pyrazol-6-yl)-4-(1H-pyrrol-1-yl) benzamide **(7a-g)**. The synthesized compound showed MIC against gram negative bacteria and gram-positive bacteria ranging from 0.8 to 50 µg/ml. Compounds 7f, 7g showed good MIC value of 3.12µg/ml. While other compounds in the series showed moderate antibacterial activity.

The synthesized compound showed MIC range of antitubercular activity extend from 0.8 to 12.5 µg/ml against *M. tuberculosis* strain H_{37}Rv.Compound 7g showed highly significant MIC value at 0.8µg/ml, based on screening results, it can be concluded that synthesized compounds can be considered for further modification with the molecular modelling and *in-silico* studies to get more potent antitubercular and antibacterial agents.

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Authors' agreements

Authors hereby declare that there is no conflict of interest for the publication.

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