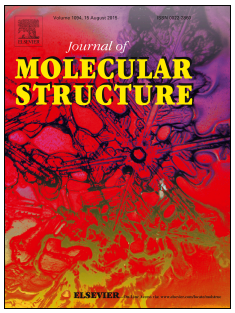

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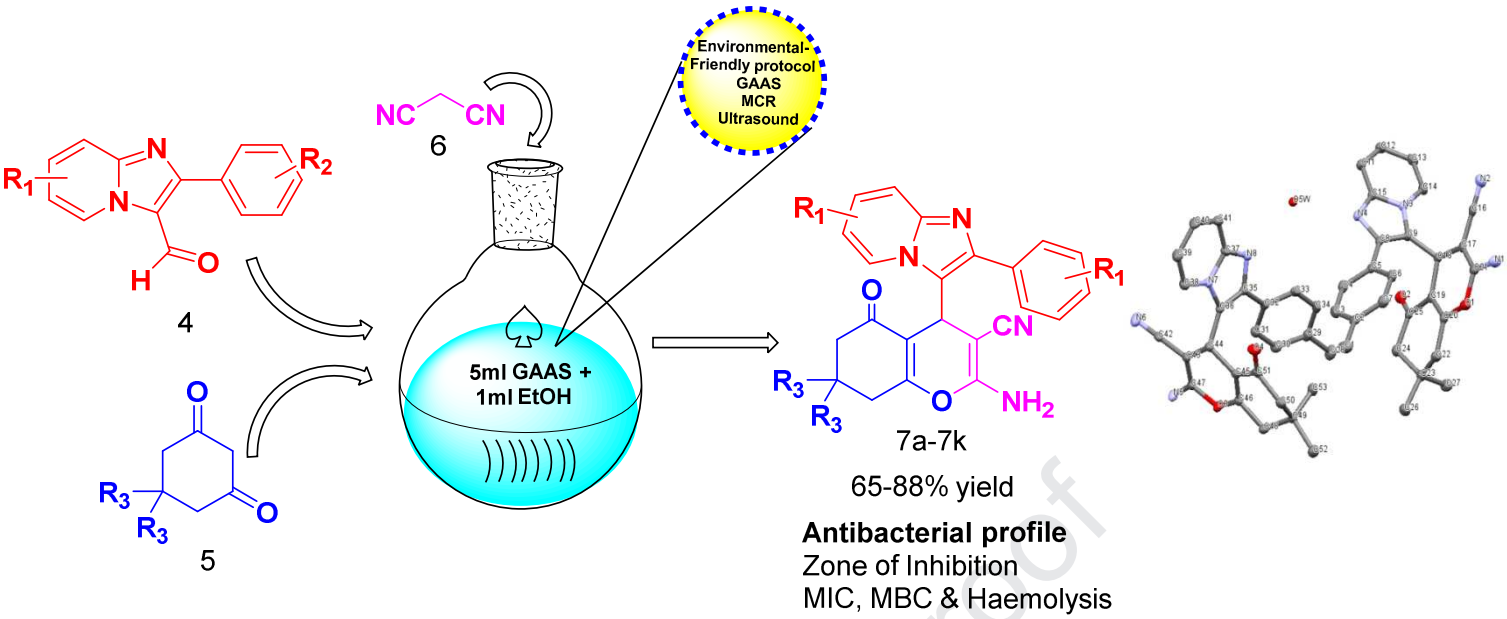
Authors	Role
Ashima Thakur	**Synthesis and characterization of molecules under conventional condition**
Gavin Pereira	**Synthesis and characterization of molecules in ultrasound irradiation**
Chetananda Patel	**Synthesis and characterization of molecules under conventional condition**
Vinita Chauhan	**Biological activity**
Ram Kumar Dhaked	**Biological activity**
Abha Sharma	**Conceptualization**



Design, one-pot green synthesis and antimicrobial evaluation of novel imidazopyridine bearing pyran bis-heterocycles

Ashima Thakur[1], Gavin Pereira[1], Chetananda Patel[1], Vinita Chauhan[2], Ram Kumar Dhaked[2,] and Abha Sharma[1]*

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Abstract

Herein, we report design, one pot synthesis and antibacterial evaluation of novel imidazopyridine bearing pyran bis-heterocycles. The compounds were synthesized in an aqueous solution of gluconic acid under both conventional heating and ultrasound irradiation. The target compounds were obtained in good to moderate yields with yield of 65 to 88% in 20-60 minutes under ultrasonic irradiation. The compounds were characterized by spectroscopic methods IR, ^{1}H-NMR, ^{13}C-NMR, MS and HRMS. X-ray single crystal structure of 7i was also determined. The compounds were evaluated for antibacterial activity by measuring zone of inhibition using disk diffusion method thatrevealed that some compounds were inhibiting the growth of Gram +ve and Gram -vebacteria. Result of minimum inhibitory concentration (MIC) showed that 7a, 7h &7k from a series 7a-7k inhibited the growth of *S. aureus*. The minimum bactericidal concentration (MBC) value was determined for 7a, 7h &7k. MBC/MIC ratio of the derivatives 7a, 7k, 7h suggest former two derivatives act as bactericidal agent & later act as bacteriostatic agents against Gram-positive bacteria. Haemolysis results showed that compounds are non-cytotoxic to erythrocytes.

1. Introduction

Bis-heterocycles are compounds comprising two or more heterocyclic moieties linked via a spacer or fused together. There is a growing interest in the synthesis of bis-heterocycles due to the fact that they exhibit potent biological activities [1-3]. Figure 1 represents some drugs and bioactive molecules consist of bis-heterocycles[1, 2, 4]. A large number of reports referring to synthesis of bioactive bis-heterocycles such as sulfone linked pyrrolyl-oxadiazoles/thiadiazoles/triazoles and pyrazolyl oxadiazoles/thiadiazoles/triazoles as antimicr

obial[5], 2-mercapto benzothiazole linked with 1,2,3-triazoles as anti-inflammatory &anti-nociceptive activity[6], bis-sulfonamide Schiff bases as carbonic anhydrase inhibitors[7], bis-isatin derivatives as anti-mycobacterial agent[8] spirochromenes[9] via multistep or one-pot reaction have been reported. Inspired by literature reports on bis-heterocycles we aim to synthesize novel bis-heterocyclic scaffold.

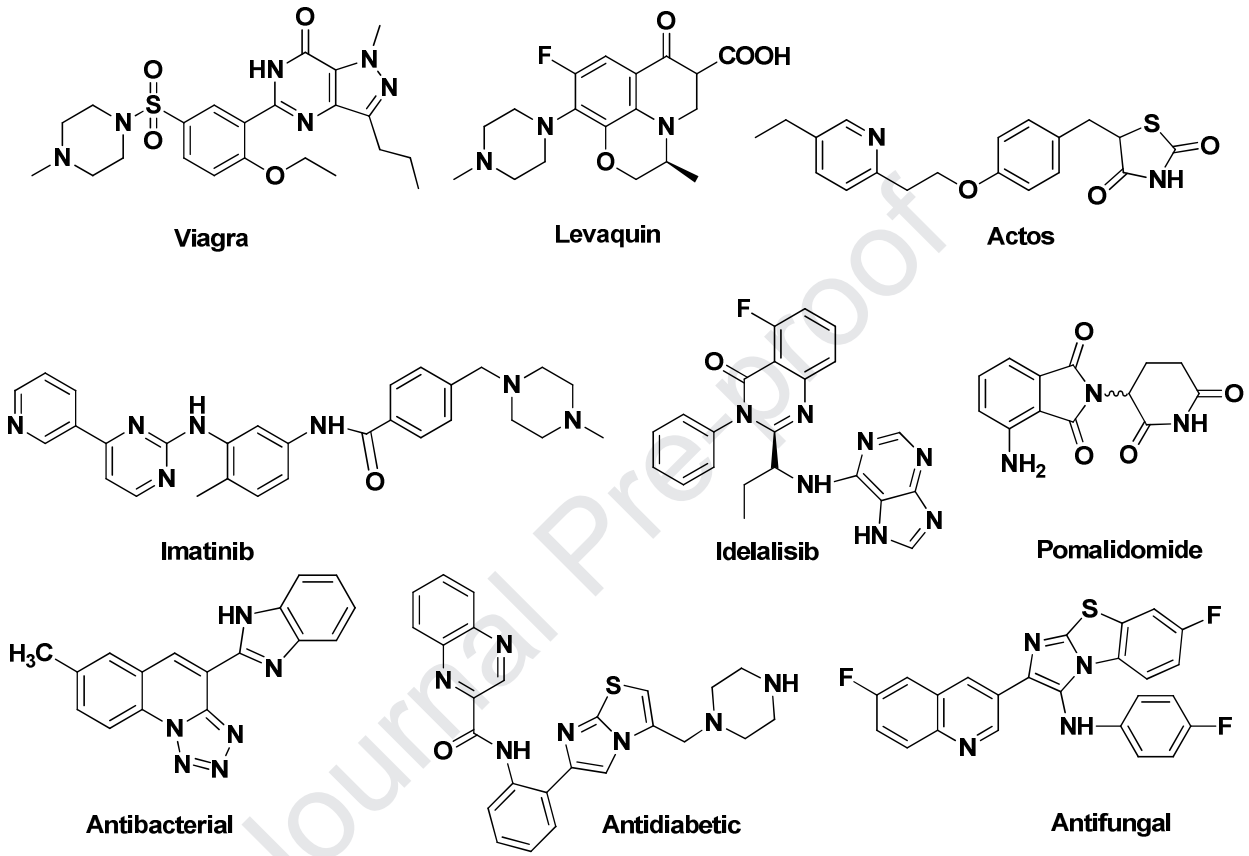


Figure 1: Drugs and Bioactive Bis-heterocyclic Molecules

Imidazo[1,2-a]pyridine is an important fused heterocycle possessing various biological activities such as antibacterial, anticancer, anti-inflammatory, antihypertensive, antiviral, antiosteoporotic, antiparasitic, and neurodegenerative diseases [10-15]. Some of the compounds with imidazopyridinescaffold are also used in psychiatry and autoimmune disorders[16]. Other than this, pyran is an important heterocycle exhibiting a wide variety of biological activities. For example, pyran demonstrates activities like anticancer, antioxidant, antimicrobial, antiproliferative, and calcium channel antagonists(Figure 2)[17, 18]. Due to the importance of these heterocyclic moieties, we planned to combine both pyran and imidazopyridine moiety in a single structure.

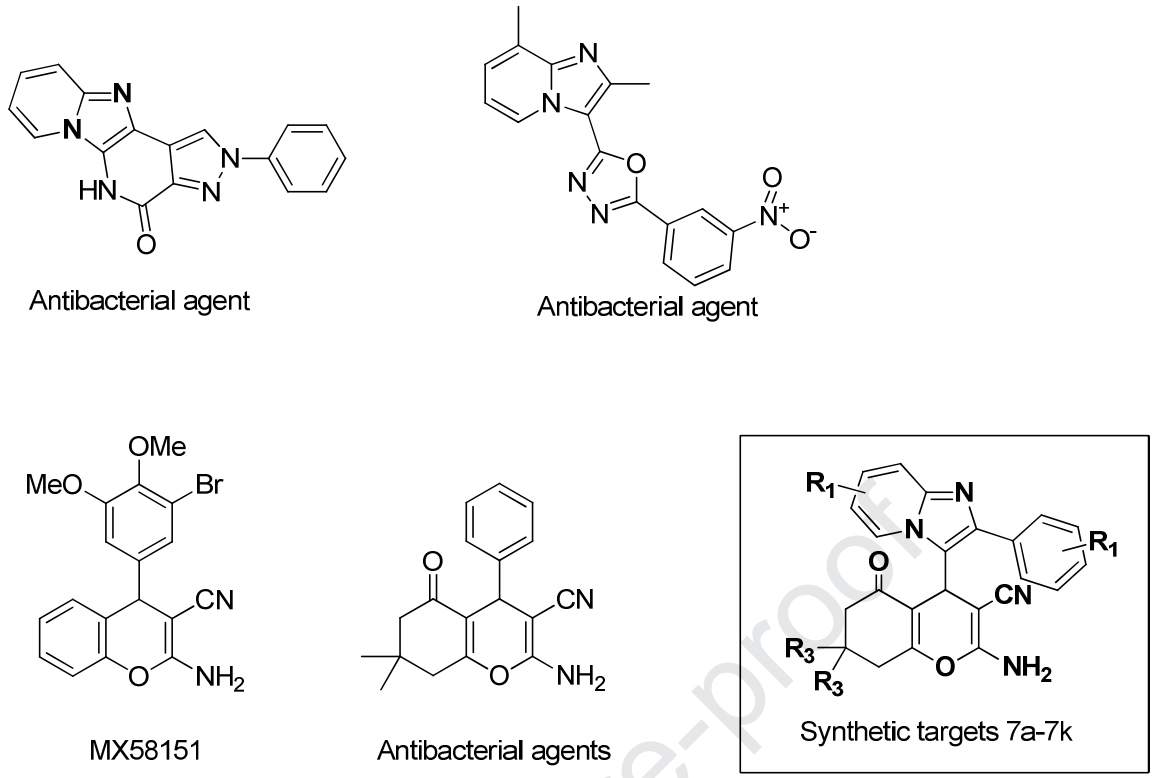


Figure 2: Biologically active imidazopyridine, 2-Amino-3-cyano-4H-pyrans derivatives, and synthetic targets

Now, we thought about the protocol which can be used to combine imidazo[1,2-a] pyridine and pyran scaffold. In this direction, gluconic acid aqueous solution (50 wt %, GAAS), introduced by Gu et al. as a promoting medium and catalyst utilized for many organic transformations was initially tested for the synthesis of target compound [19]. Gluconic acid (GA) is a bio-based substance, naturally present in plants, fruits, and other foodstuffs and commercially available in the market as a 50% aqueous solution. GAAS is a nonvolatile, noncorrosive, stable, inexpensive, recyclable and biodegradable organic acid. Under normal condition, GAAS exist in an equilibrium, in which about 5% of glucono- δ-lactone is present. In particular, when a proton dissociates from GA molecule, it results in the aqueous solution which makes the solution slightly acidic. This allows the use of GAAS for promoting organic reactions that need the assistance of a weak acid [20-22]. Thus, such media may constitute an alternative solvent for the development of organic compounds. Meanwhile, multicomponent reactions (MCRs) are known to combine more than two components for the construction of novel and structurally complex molecules in a single pot. It is performed to ensure high atom-economy, good yields, and to avoid expensive purification processes. It has been established

that MCRs are generally much more environmentally friendly and has emerged as a powerful synthetic methodology used for the synthesis of drugs and bioactive compounds[23].

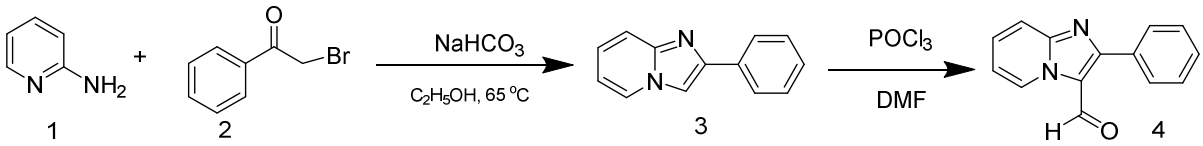
The combination of multicomponent reactions, an environmentally benign form of catalyst and a green solvent has become a promising frontier field of research, which enables simultaneous growth of both MCRs and green solvents toward ideal organic synthesis[24-28]. Apart from this, the use of ultrasound irradiation in organic synthesis is now becoming an emerging powerful technique. The prominent features of the ultrasound-assisted synthesis are the time required for completion of the reaction is less and products obtained in high yield [29-31]. A variety of biologically important scaffolds like pyrazoles[32], benzoxanthenes[30], dihydroquinazolinones[33], coumarins[34], gem dichloroaziridine[35], tetrazolopyrimidine[36] have been synthesized with & without catalyst under ultrasound irradiation. In the view of the above points and as a continuation of our ongoing work towards the design and development of novel and environmental benign synthetic methodologies[37-39], herein, we report one-pot synthesis of novel imidazopyridine bearing pyran moiety via three component condensation reaction of malononitrile, 2-phenylimidazo [1,2-a]pyridine-3-carbaldehyde and cyclohexanedione using GAAS as an efficient and reusable promoting medium under conventional and ultrasonication method. In addition, we have investigated molecules against microbes as microbial infections are causing various serious diseases such as bacteremia, pneumonia, intra-abdominal & urinary tract infection. Gram-negative & Gram-positive bacterium's like *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa&Staphylococcus aureus* are associated with various types of infections[40]. According to the World Health Organization and Centre for Disease Control & Prevention data show that death due to bacterial infection is highper year [41, 42]. The situation becomes dangerous by the emergence of multi, extensive & pan drug resistance bacterial strains[43, 44]. For example, a nosocomial infection is caused by methicilin resistance *Staphylococcus aureus*[45, 46].

2. Results and Discussion

2.1 Chemistry

We started work with the synthesis of precursor 2-phenylimidazo [1,2-a]pyridine-3-carbaldehyde (4) using reported protocols (Scheme 1). The precursor 2-phenylimidazo[1,2 a] pyridine carbaldehyde (4) was synthesized in two steps by reacting 2-amino pyridine (1) with

phenacyl bromide (2) first to form 2-phenylimidazo[1,2 a] pyridine (3). Then product (4) was obtained by Vilsmeier Haack reaction performed on 3.

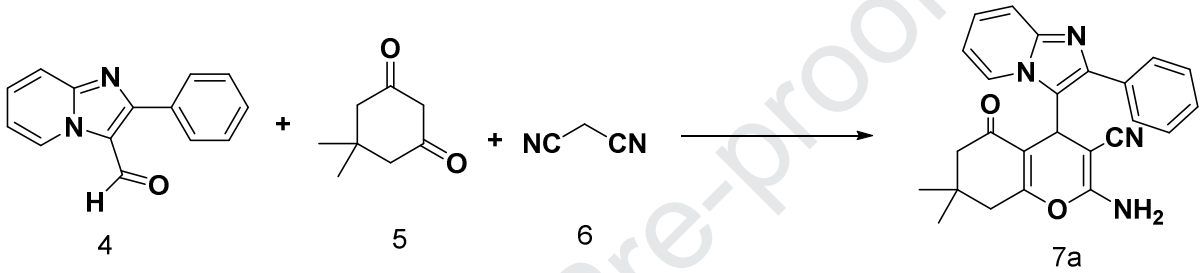


Scheme 1: Synthesis of 2-phenylimidazo [1,2-a]pyridine-3-carbaldehyde

After the synthesis of precursor (4), we investigated the synthesis of imidazopyridine fused pyran moiety via MCR in neat at rt using reactants 1(0.2 g,1 mmol), 2 (0.14 g, 1mmol) & 3 (0.063 ml, 1 mmol) as depicted in Table 1 and reaction was monitored via TLC at various intervals of time for 12 h. Visualization of TLC under UV showed no new spot formation, indicating no product formation (Table 1, entry 1). Then, the reactions were carried out in different solvents like water, ethanol, methanol, PEG-400, glycerol, and GAAS at rt for 12 h. The expected product was not obtained in all tested solvents expect in GAAS, a new spot was visible on TLC (Table 1, entries 2-7). When the reaction was performed in GAAS at temperature 60, 80, & 100 ºC, a light spot which was visible on TLC of reaction carried out at rt (Table 1, entry 7) now appeared as an intense spot on TLC under heating conditions (Table 1, entries 8-10). The maximum yield of product was obtained in GAAS (Table 1, entry 10), prompted us to check the combination of GAAS with other solvents at 100 ºC. In all the reaction conditions, a product was formed (Table 1, entries 11-15) but the maximum yield was obtained in a combination of GAAS with EtOH (Table 1, entry 11). After obtaining promising results, we increased the amount of GAAS from 1ml to 7ml with 1ml EtOH, with every time an increment of 1ml GAAS was made (Table 1, entries 16-21). The product yield was increased till 5ml GAAS and remained the same in the remaining two conditions i.e., 6 and 7ml (Table 1, entries 20 & 21). Therefore, the optimum reaction condition required for the synthesis of fused pyran moiety with imidazopyridine was GAAS 5ml + EtOH 1ml (Table 1, Entry 19). Then, the optimum condition was checked for substrate scope. Different substituted 2-phenylimidazo[1,2-a]pyridine-3-carbaldehyde and cyclohexanedione along with malononitrile were tested for the synthesis of target compounds. Owing to this we have synthesized 11 derivatives (7a-7k) in good yield (Table 3). The model reaction was completed in 2 h. Initially, time was fixed for the reactions but it was found that in some cases some reactants remained in the reaction mixture so we decided to monitor the reaction till completion. Thus, different derivatives took different time to give the maximum yield. Particularly, when dimedone was used for the synthesis of compounds, a solid product was

obtained. The formation of a solid product made the work-up process easier. Different colored solid products were isolated by filtration and washed with hexane. Then the filtrate was reduced to ultimately recover GAAS for reuse and finally, the crude products were crystallized from ethanol. A different observation perceived in the case of 1,3-cyclohexanedione that no solid product was obtained therefore extracted with ethyl acetate and washed with water. The product collected in the ethyl acetate layer which on concentrated under reduced pressure over rotary evaporator gave a solid product. The crude product was further purified by silica gel column chromatography if needed.

Table 1: Optimization of reaction conditions in conventional method[a]



Entry	Solvent	Quantity	Temperature (^{o}C)	Time (h)	Yield[b] (%)
					7a
1.	No solvent	-	rt	12	-
2.	Water	1 ml	rt	12	-
3.	Ethanol	1 ml	rt	12	-
4.	Methanol	1 ml	rt	12	-
5.	PEG-400	1 ml	rt	12	-
6.	Glycerol	1 ml	rt	12	-
7.	GAAS	1 ml	rt	12	Trace
8.	GAAS	1 ml	60	12	Trace
9.	GAAS	1 ml	80	12	Trace
10.	GAAS	1 ml	100	12	25
11.	GAAS + EtOH	1 ml each	100	4	34
12.	GAAS+ Water	1 ml + 1ml	100	4	20
13.	GAAS+ MeOH	1 ml + 1ml	100	4	28
14.	GAAS+PEG-400	1 ml + 1ml	100	4	28
15.	GAAS+ Glycerol	1 ml + 1ml	100	4	24
16.	GAAS+ EtOH	2 ml + 1ml	100	4	43
17.	GAAS+ EtOH	3 ml + 1ml	100	4	54
18.	GAAS+ EtOH	4 ml + 1ml	100	4	78
19.	**GAAS+ EtOH**	**5 ml + 1ml**	**100**	**2**	**88**
20.	GAAS+ EtOH	6 ml + 1ml	100	2	88
21.	GAAS+ EtOH	7 ml + 1ml	100	2	88
22.	[c]GAAS+ EtOH	5 ml + 1ml	100	2	83
23.	[d]GAAS+ EtOH	5 ml + 1ml	100	2	87

[a]Reaction Condition: 1a (1.0 mmol, 2a (1.0 mmol) and 3 (1.0mmol).

[b]Yield: Isolated yield after silica gel chromatography.
[C]: Recovered GAAS third run
[d]: 20 mmol scale

GAAS was recovered by concentrating water layer under reduced pressure. The recovered GAAS was further tested for its catalytic potential for the same reactions. The yield obtained in the recovered GAAS is given in table 1. Furthermore, on scaling the reactions upto 20 mmol produced a product in almost no significant difference in the yield, indicating an effective methodology for practical synthesis of target molecules.

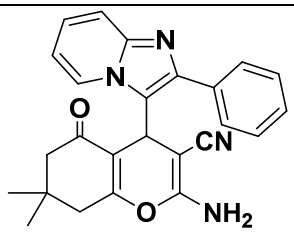
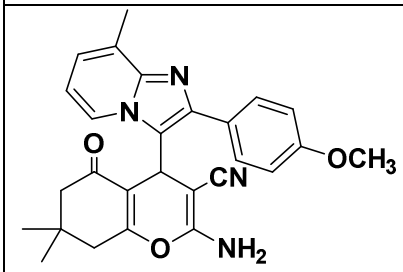
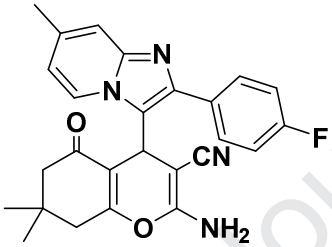
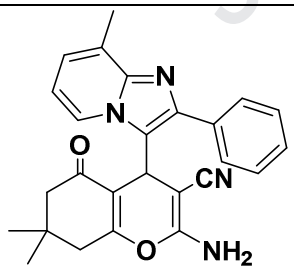
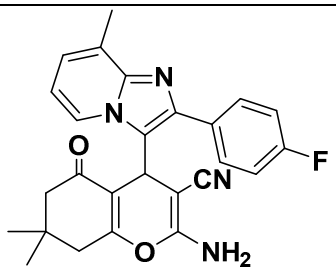
Subsequently, this MCR reaction was performed under ultrasonic irradiation using ultrasonicator. The reactions were performed in optimum solvent conditions (5ml GAAS: 1ml EtOH) as established from table 1. Initially, the reaction was performed at 80^{o}C and monitored for completion via TLC at various time intervals. Surprisingly, the reaction was completed in 20 minutes (Table 2, entry 1) which indicates better efficiency of the ultrasonication method than the conventional method. Furthermore, to determine the effect of temperature on the reaction time, the reactions were performed at 70, 60, 50 and 40ºC and were monitored at different intervals for completion via TLC. Notably, the time for completion of the reaction was found to decrease with increase in temperature (Table 2, entries 2-5). Hence the optimum conditions were established to be 5ml GAAS: 1ml EtOH at 80ºC for the synthesis of fused pyran moiety with imidazopyridine via ultrasonication method (Table 2, entry 1). Owing to this the optimized conditions were tested for substrate scope, so different substituted 2-phenylimidazo[1,2-a]pyridine-3-carbaldehyde and cyclohexandione along with malononitrile were tested for the synthesis of target compounds. With this, we have synthesized 11 derivatives (7a-7k) in less time with good yield compared to the conventional method (Table 3). Initially, 20 minutes time was fixed for the reactions as the model reaction was completed in this time but it was found that in some cases some reactants remained in the reaction mixture so we decided to monitor the reaction till completion. Thus, different derivatives took different time to give the maximum yield.

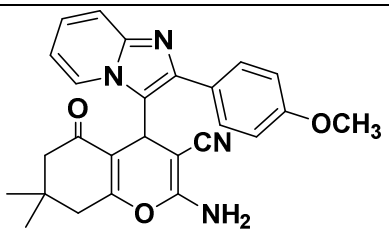
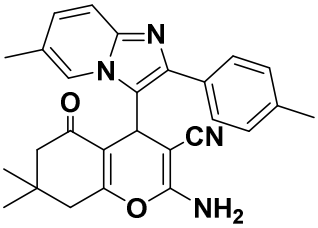
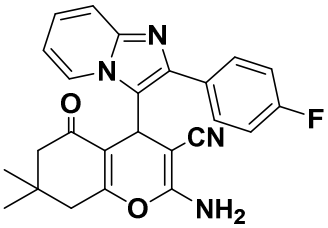
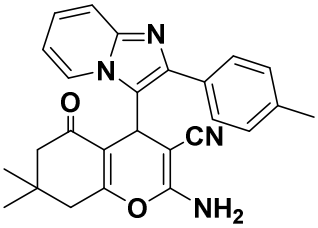
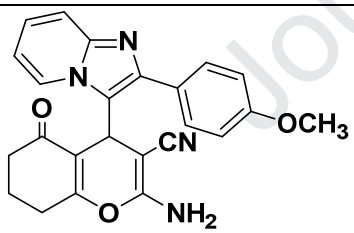
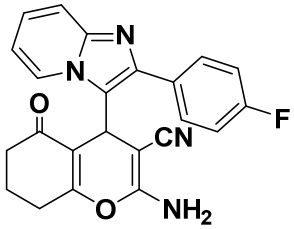
Table 2: Optimization of reaction in Ultrasonicator

Entry	Solvent	Quantity	Temperature (^{o}C)	Time (h)	Yield[b] (%)
					7a
1.	GAAS+ EtOH	5 ml + 1ml	80	0.33	88
2.	GAAS+ EtOH	5 ml + 1ml	70	0.66	86
3.	GAAS+ EtOH	5 ml + 1ml	60	1.5	85
4.	GAAS+ EtOH	5 ml + 1ml	50	3.1	84

5.	GAAS+ EtOH	5 ml + 1ml	40	3.5	86

Table 3: Synthesis of 7a-7k by conventional and ultrasound method.

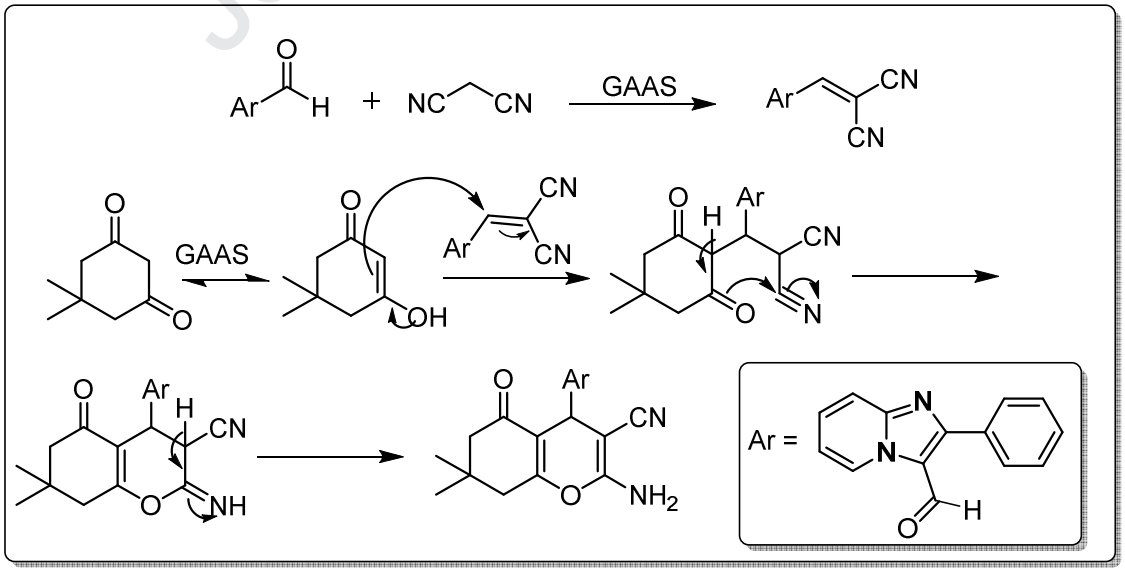
Compound	Conventional method		Ultrasonication method	
	Time (h)	Yield (%)	Time (h)	Yield (%)
 7a	2	80	0.33	88
 7b	5	78	0.66	82
 7c	10	62	0.5	65
 7d	5	77	0.66	83
 7e	9	64	1	66

 7f	5	72	0.50	78
 7g	11	76	0.50	78
 7h	10	62	1	66
 7i	10	74	0.66	82
 7j	6	80	0.66	84
 7k	11	62	1	65

All novel compounds (7a-7k) showed variable colors such as pale yellowish, off-white & white and were characterized by spectroscopic techniques(MS, IR, ^{1}H NMR and ^{13}C NMR spectra).Single Crystal X-Ray diffraction studies have been done for the characterization of Compound 7i which was crystallized by using ethanol under slow evaporation.The crystallography data indicated that the crystallized compound is $C_{26}H_{24}N_4O_2$. The pyran ring is fused with the imidazopyridine group. TheX-ray crystallographic data of 7i compound showed monoclinic shape (Figure 3).

Figure 3: a) Crystals of 7i compound b) ORTEP diagram of 7i (CCDC 1865906)

The reaction mechanism of product formation as depicted in scheme 2 involved two name reactions Knoevenagel condensation and Michael addition followed by cyclization and tautomerization (37).



Scheme 2: Proposed reaction mechanism of Imidazopyridine fused pyran ring.

2.2 Biological Evaluation

The *in vitro* antibacterial profile of imidazopyridine fused pyran derivatives 7a-7k was evaluated by measuring the inhibition zone against Gram-positive (*Staphylococcus aureus,*ATCC 25923*)* and Gram-negative *(Escherichia coli*, ATCC 25922 and *Salmonella typhi*, MTCC 734*)* bacteria via disk diffusion method.[20]Among the tested compounds 7a, 7b, 7e, & 7g were found active against *S. aureus,* 7e against *E.coli* and 7g and 7h against *S. typhi*with inhibition zone in the range of 8-20mmin different patterns(Table 4). The compounds 7h & 7k inhibition zone were in the range of 17±1mm & 20±2 mm respectively, compared to standard vancomycin, 15±1. No substituent is present on imidazopyridine moiety of 4a derivative showed inhibition zone of 14±1 mm. The other derivatives are substituted showed different inhibition zone value that indicated that activity is depending on type & position of the substituent. In 7h derivative fluorine is present on phenyl ring of imidazopyridine with 7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4H-chromene-3-carbonitrile, found less active than 7k but more active than 7a. When only fluorine substituent is attached to phenyl ring of imidazopyridine ring (7k), the activity was found better than derivatives 7a and 7h.

Table 4. Antimicrobial activity evaluation of 7a to 7k derivatives against Gram-positive and Gram-negative bacterial strains using a disc diffusion test

Inhibition zone (mm)			
Compound	*S. aureus*(ATCC 25923)	*E.coli*(ATCC 25922)	*S.typhi*(MTCC 734)
7a	14 ± 1.0[#]	0	0
7b	11 ± 2.0	0	0
7c	NA	NA	NA
7d	0	0	0
7e	11 ± 2.0	11 ± 3 .0	0
7f	0	0	0
7g	12 ± 1.0	0	8.00 ± 2
7h	17 ± 1.0[#]	0	9.00 ± 1
7i	0	0	0
7j	NA	NA	NA
7k	20 ± 2.0[#]	0	0
Vancomycin	15 ± 1.0	-	-

Ciprofloxacin	-	22±1	20 ±1

Ciprofloxacin: positive control used for Gram-negative strains. Vancomycin: positive control used for Gram-positive strains. NA: not active concentrations. Mean diameters of inhibition zones of the test samples were calculated from three independent experiments. (#) $p < 0.05$ compared to control.

After measuring the inhibition zone, minimum inhibitory concentrations (MIC) assay was conducted for the determination of the lowest concentration of compounds require to inhibit the visible bacterial growth. The compounds 7a, 7h, and 7k inhibited the growth of Gram-positive (*S. aureus*) (Table 5). The substituent fluorine is present on the para position of the phenyl ring of imidazopyridine of 7h & 7k derivatives showed that fluorine was contributing for activity. 4h was found better than 7k in inhibiting bacterial growth, the former has dimethyl substituent on cyclohexanone ring which was absent in 7k derivative.

Table 5. Minimum inhibitory concentrations (MICs) for a series of compounds 7a-7k

Compound	*S. aureus*	*E.coli*	*S. typhi*
7a	125.00	NA	NA
7b	NA	NA	NA
7c	NA	NA	NA
7d	NA	NA	NA
7e	NA	NA	NA
7f	NA	NA	NA
7g	NA	NA	NA
7h	7.8	NA	NA
7i	NA	NA	NA
7j	NA	NA	NA
7k	31.25	NA	NA
Vancomycin	3.00	-	-
Ciprofloxacin	-	0.80	0.80

Ciprofloxacin: positive control used for Gram-negative strains. Vancomycin: positive control used for Gram-positive strains. NA: not active at concentrations up to 400 µg/ml

The minimal bactericidal concentration (MBC) values were also determined for 7a, 7h & 7k. MBC is the lowest concentration of compounds required for microbial death (Table 6). The ratio of MBC/MIC indicates the type of antimicrobial agent i.e., bactericidal or bacteriostatic.

The ratio of MBC/MIC ≤2 indicate bactericidal effect whereas a ratio of MBC/MIC ≥4 show bacteriostatic effect [21].

Interestingly, the MBC/MIC ratio of the active derivatives 7a and 7k were ≤2 against Gram-positive (*S. aureus*), suggesting that the molecules can be classified as a bactericidal agent against Gram-positive bacteria. Whereas the MBC/MIC ratio of the active derivative 7h was ≥4 against Gram-positive (*S. aureus*), suggesting that the compounds can be classified as a bacteriostatic agent against Gram-positive bacteria.

Table 6. Minimal bactericidal concentration (MBC) of the active compounds 7a, 7h, and 7k

MBC (µg/m1)			
Compound	*S. aureus*	*E.coli*	*S.typhi*
7a	250.00	NA	NA
7h	31.25	NA	NA
7k	62.50	NA	NA

Hemolysis tests on humanerythrocytes performed to a series of compounds '7a-7k'. No hemolytic effect was observed for the compounds after 3 hours of incubation. As hemolysis rate is less than 10% indicate that compounds are non-cytotoxic to erythrocytes according to literature report (Figure 4)[22].The result of the study showed that there is a need of modification in derivatives with respect to type & position of the substituent for improving antibacterial profile them. These antibacterial results show that there is a scope and need for structural modification of compounds to find more active derivatives against bacterial strains.

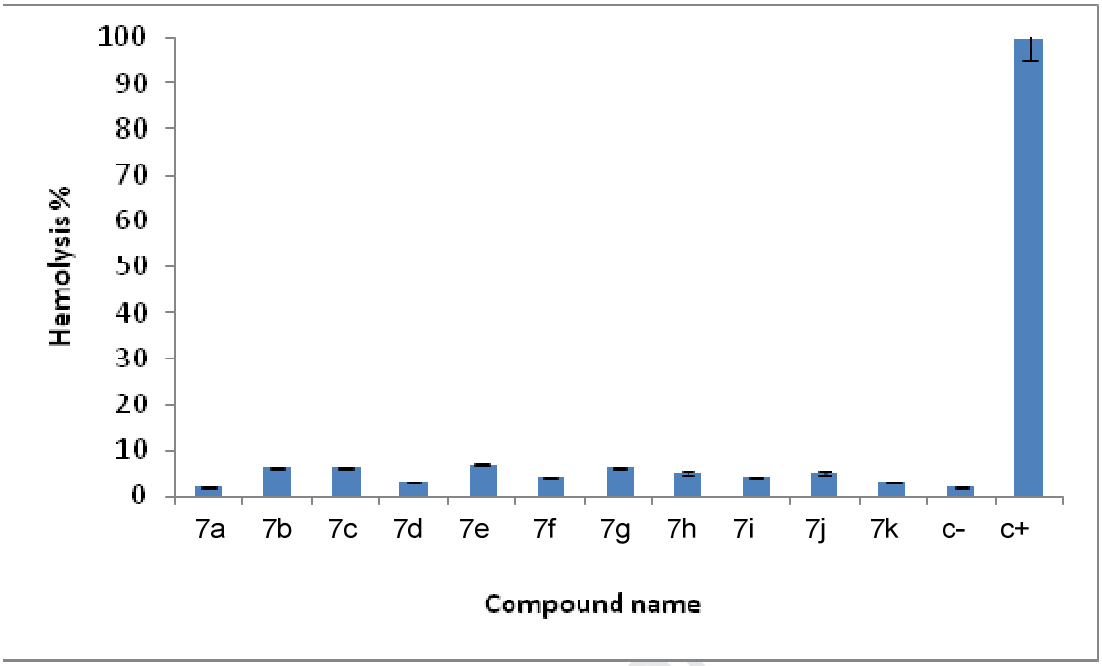


Figure 4: Haemolytic profile of compounds (7a to 7k) through haemolysis assay. The hemolysis obtained from Triton-X 100 was taken as 100% hemolysis and that with respective concentration of DMSO was taken as negative control. The haemolytic percentage was observed to be below 10% for all the compounds.

3. Experimental Section

3.1 Materials and methods

All chemicals and reagents were purchased from commercial sources and were used for the experiments without purification. Reactions were monitored by silica gel coated aluminum plate containing 60 F_{254}TLC. Visualization was achieved by UV light and 2,4-dinitrophenyl hydrazine (for aldehyde). Ultrasonication was achieved using Digital Ultrasonic cleaner with a frequency of 40 kHz & a nominal power of 150 W. Column chromatography was performed with silica gel (60-120 mesh). ^{1}H were recorded on Bruker DRX 300 and 400 Spectrometers at 300, 400 MHz (^{1}H) and ^{13}C NMR was recorded on Joel 500 NMR spectrometer at 125 MHz. Experiments were recorded in $CDCl_3$ and DMSO-d_6. Chemical shifts (d) are reported in ppm downfield from an internal TMS standard. Splitting patterns are described as singlet (s), doublet (d), triplet (t) and multiplet (m). J values are given in Hertz. ESI mass spectra were recorded on ThermoLcq Advantage Max spectrometer. IR spectra

were recorded on a BRUKER IR spectrophotometer. Melting points were recorded on lab melting point apparatus and are uncorrected.

3.2 General procedure for synthesis of 2-phenylimidazo[1,2-a]pyridine-3-carbaldehyde (4):[47]

2-Aminopyridine (0.094 g, 1mmol) was dissolved in ethanol and sodium bicarbonate (0.126 g, 1.5mmol) was added to above solution with stirring at room temperature for 5min. After 5min phenacyl bromide (0.023 g, 1.2mmol) was added. The reaction mixture was heated at 65^{o} for 4-5 h. Completion of the reaction was monitored by TLC. After completion of the reaction, the reaction mixture was cooled and ethanol was evaporated by rotavapor and then quenched with water (10ml). The precipitate formed was filtered using Whatman paper. The precipitate was washed with ice-cold water and recrystallized from ethanol to obtain pure 2-Phenyl-imidazo[1,2-α]pyridine derivatives.

After this formylation of the 2-Phenyl-imidazo[1,2-α]pyridine was performed using Vilsmeier reagent. It was prepared at 0–4 ^{o}C by dropping $POCl_3$ (0.37 ml, 4mmol)into a stirred solution of DMF(0.62 ml, 8mmol). 2-Phenylimidazo [1, 2-α] Pyridine derivatives (0.194g, 1mmol)in DMF was added to Vilsmeier reagent maintaining stirring and cooling. The reaction mixture was kept at RT for 2h and then the reaction mixture was refluxed at 90^{o}C for 24 h. After completion of the reaction (monitored by TLC with using DNP), the reaction mixture was cooled and poured onto ice. The crude product was collected by filtration and crystallized from EtOH (5 mL) in order to obtain the pure product.

3.3 General procedure for the synthesis of 7a-7k

Conventional method

1,3-Cyclohexanedione/5,5-Dimethyl-1,3-cyclohexanedione (0.14 g, 1mmol) was dissolved inethanol: GAAS (1ml:5ml).Then, 2-Phenyl–imidazo [1,2-α] pyridine-3-carbaldehyde (0.2 g,1 mmol) and malononitrile (0.063 ml, 1 mmol) was added to the above reaction mixture. The reaction mixture was stirred at 100 ºC under reflux conditions in a round-bottomed flask fitted with a reflux condenser. After completion of the reaction (the progress of the reaction was monitored by TLC using n-hexane: ethylacetate as eluent), the reaction mixture was cooled and then extracted with water and ethyl acetate. The ethyl acetate layer was dried over anhydrous sodium sulfate and evaporated on a rotavapor to get the crude product. The crude product was purified by silica gel (60-120 mesh) column chromatography using n-hexane and ethyl acetate as eluent to afford the pure product.

Ultrasonication Method

1,3-Cyclohexanedione/5,5-Dimethyl-1,3-cyclohexanedione (0.14 g, 1mmol) was dissolved inethanol:GAAS (1ml:5ml).Then, 2-Phenyl–imidazo [1,2-α] pyridine-3-carbaldehyde (0.2 g,1 mmol) and malononitrile (0.063 ml, 1 mmol) was added to the above reaction mixture. The reaction mixture was kept at 80 ºC in a pressure tube under ultrasonication. After completion of the reaction (the progress of the reaction was monitored by TLC using n-hexane: ethylacetate as eluent), the reaction mixture was cooled and then extracted with water and ethyl acetate. The ethyl acetate layer was dried over anhydrous sodium sulfate and evaporated on a rotavapour to get the crude product. The crude product was purified by silica gel (60-120 mesh) column chromatography using n-hexane and ethyl acetate as eluent to afford the pure product.

3.4 Antimicrobial Assays

Determination of Diameters of Inhibition Zones

Inhibition zone diameters were determined by the disk diffusion method as described by Tamokou and co-workers (2011)[48]. Stock solutions of compounds were prepared in 100 % dimethylsulfoxide (DMSO) solution (Sigma-Aldrich) at a concentration of 10 mg/ml. Bacterial strains used for the antimicrobial study were *Staphylococcus aureus*(ATCC 25923) and *Escherichia coli*(ATCC 25922) and *Salmonella typhi*(MTCC 734). The bacterialsuspension (1.0 x 10^6CFU m/l)) wasinoculated on a Petri dish containing 1.5 % Nutrient agar, after drying; two concentrations of (400 and 200µg) of the compound were applied ontothem. After 24 h at 37 °C, the inhibition zone formed that was measured in mm.Vancomycin and Ciprofloxacin (Thermo Scientific™) were used as positive controls for bacteria. Dimethylsulfoxide solution (100%) was used as a negative control. The data were expressed as the mean ± SEM of three independent experiments. Statistical comparisons between samples were performed using one-way ANOVA followed student's *t* test. Differences with $p < 0.05$ were taken as significant.

Minimum inhibitory concentration (MIC)

All compounds were subjected to MIC analyses by the microdilution methodfor determining the minimum concentration of compounds that inhibit bacterial growth. A 100ml volume of two-fold dilutions (400–1.625mg m/l) was taken in each well of 96-well microplate. No detectable effect on bacterial growth was observed at 5% DMSO concentration. Then, 100 ml

ofbacterial inoculum containing 10^6 CFU m/l was added. Negative control was prepared using the inoculum without compound, whereas vancomycin and ciprofloxacin were used aspositive controls. The microplates were incubated at 37 °C for 24 h and the result was determined by visual reading of thelowest concentration with no detectable turbidity (growth)[49].

Minimal bactericidal concentration (MBC)

To determine MBC value, the culture medium showing no visible growth transfer to agar plate, incubated for 24h at 37 °C. MBC is defined as theminimum concentration of compound that showed a 99.9%reduction of the original growth. The MBC was determined in the well treated with thelowest compound concentration[49].

3.5 Haemolysis assay

Sample erythrocytes were obtained from a healthy human subject, washed 3times with PBS (pH 7.4) by centrifugation and suspended in thesame buffer according to Sathler and collaborator (2014). 200µg/ml of the concentration of the compound was incubated with the erythrocyte suspension for 3 h at 37°C. The optical density of supernatant at 540 nm was determined to calculate the erythrocytes lysis. 1% Triton X-100 was taken as a positive control. A value of less than 10% indicates that compounds are nontoxic to erythrocyte membrane hence, haemocompatible [50]. Assay was performed in triplicates. The percent haemolysis is calculated using the following formula:

Percent haemolysis =100× [(A_{540} sample - A_{540} negative control) ÷ (A_{540} positive control- A_{540} negative control)]

3.6 Spectroscopic data of all compounds

2-amino-7,7-dimethyl-5-oxo-4-(2-phenylimidazo[1,2-a]pyridin-3-yl)-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile(7a)

Off white solid (Yield 88%); mp=148-150^{o}C; FT-IR(cm^{-1}) 3617, 2332, 2224, 1668, 1525,1147, 1036; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.85- 0.89 (m, 6 H) 1.23-1.34 (m, 2 H) 2.10-2.18 (m, 1H) 2.19 - 2.20 (m, 1 H) 5.18 (s, 1 H) 6.96–6.99 (m, 2 H) 7.25 - 7.29 (m, 2 H)7.38 - 7.47 (m, 2 H) 7.71 (br s, 1H) 7.71-7.72 (br s, 2 H) 7.84 - 8.10 (m, 1 H); ^{13}C-NMR (DMSO-d6, 125 MHz): δ= 196.37, 159.77, 158.91, 144.50, 143.76, 135.61, 129.47, 128.70, 128.26, 127.93, 125.50, 124.54, 123.81, 119.38, 118.05, 117.14, 113.16, 112.16, 53.43,

50.48, 31.97, 31.70, 27.72, 27.34, 25.70; MS-ESI calculated for $C_{25}H_{22}N_4O_2$ $(M+H)^+$: 411.18, found 411; HRMS (ESI) *m/z* $[M+H]^+$calculated for $C_{25}H_{22}N_4O_2$; 411.1822 found 411.1812.

2-amino-4-(2-(4-methoxyphenyl)-8-methylimidazo[1,2-a]pyridin-3-yl)-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile(7b)

Yellow solid (Yield 80%); mp=156-158^{o}C; FT-IR(cm^{-1}) 3369, 2351, 2189, 1669, 1492,1160, 1030; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.82 - 0.98 (m, 5 H) 0.98 - 1.11 (m, 2 H) 1.99 (br s, 1 H) 2.02 - 2.12 (m, 1 H) 2.13 - 2.36 (m, 1 H) 2.46 - 2.54 (m, 5 H) 2.54 - 2.74 (m, 1 H) 3.35 (s, 7 H) 3.82 (s, 3 H) 5.11 (s, 1 H) 6.85 - 6.89 (m, 1 H) 6.89 – 6.97 (m, 1 H) 7.00 - 7.04 (m, 3 H) 7.06 (br s, 2 H) 7.23 - 7.88 (m, 1 H) 7.88 - 7.90 (m, 1 H); ^{13}C-NMR (DMSO-d6, 125 MHz): δ= 196.47, 168.38, 167.22, 159.79, 158.87, 141.57, 139.50, 131.04, 127.69, 118.83, 118.03, 114.75, 114.33, 111.12, 108.49, 101.97, 55.77, 50.48, 46.56, 33.05, 32.06, 28.23, 22.60, 17.10; MS-ESI calculated for $C_{27}H_{26}N_4O_2$ $(M+H)^+$: 455.20, found 455; HRMS (ESI) *m/z* $[M+H]^+$calculated for $C_{27}H_{26}N_4O_2$; 455.2084 found 455.2074.

2-amino-4-(2-(4-fluorophenyl)-7-methylimidazo[1,2-a]pyridin-3-yl)-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile(7c)

Yellow solid (Yield 62%); mp=158-160^{o}C; FT-IR(cm^{-1}) 3319, 2950, 2530, 2142, 1675, 1432,1116, 1030; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.98 (s, 6 H) 2.18 (s, 2 H) 2.38 - 2.40 (m, 2H) 5.32 (br s, 2 H) 5.78 (s, 1 H) 6.95 (dd, *J*=6.91, 1.53 Hz, 1 H) 7.29 - 7.37 (m, 2 H) 7.93 - 8.01 (m, 2 H) 8.40 (s, 1 H) 8.51 (d, *J*=6.97 Hz, 1 H); ^{13}C-NMR (DMSO-d6, 125 MHz): δ= 196.77, 167.22, 165.86, 165.44, 156.63, 155.41, 144.72, 135.26, 132.06, 131.30, 117.88, 116.25, 115.62, 115.47, 115.26, 115.00, 111.11, 110.90, 50.48, 50.26, 32.28, 32.06, 29.60, 27.48, 27.22, 21.18; MS-ESI calculated $C_{26}H_{23}FN_4O_2$ $(M+H)^+$: 443.18, found 443; HRMS (ESI) *m/z* $[M+H]^+$calculated for $C_{26}H_{23}FN_4O_2$; 443.1884 found 443.1852.

2-amino-7,7-dimethyl-4-(8-methyl-2-phenylimidazo[1,2-a]pyridin-3-yl)-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile(7d)

White solid (Yield 82%); mp=172-174^{o}C; FT-IR(cm^{-1}) 3335, 2945, 2832, 2183, 1681, 1416,1113, 1021; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.82 – 1.02 (m, 6 H) 1.23 - 1.35 (m, 3 H) 2.09 (br s, 1 H) 2.19 - 2.21 (m, 1 H) 2.51 - 2.54 (m, 1 H)) 2.54 - 2.58 (m, 1 H) 5.14 (s, 1 H) 6.87 - 6.91 (m, 2 H) 6.91 (s, 1H) 7.08-7.10 (m, 1H) 7.25-7.39 (m, 3 H) 7.59 (br s, 1 H) 7.62 - 7.80 (m, 1 H) 7.80 - 7.95 (m, 1 H); ^{13}C-NMR (DMSO-d6, 125 MHz): δ= 196.33, 159.73, 158.66, 144.77, 143.95, 135.77, 129.57, 128.64, 128.23, 127.85, 123.11, 121.58, 120.26, 113.06, 112.16, 108.83, 53.52, 50.50, 31.99, 28.81, 27.62, 27.32, 25.78, 17.17; MS-

ESI calculated $C_{26}H_{24}N_4O_2$ $(M+H)^+$: 425.19, found 425; HRMS (ESI) *m/z* $[M+H]^+$calculated for $C_{26}H_{24}N_4O_2$; 425.1978 found 425.1972.

2-amino-4-(2-(4-fluorophenyl)-8-methylimidazo[1,2-a]pyridin-3-yl)-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile(7e)

Off white solid (Yield 64%); mp=159-161^{o}C; FT-IR(cm^{-1}) 3318, 2943, 2522, 2043, 1659, 1449, 1114, 1021; ^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 1.63-1.87 (m, 7 H) 1.95 - 2.21 (m, 3 H) 2.85 - 3.12 (m, 3 H) 3.24 - 3.35 (m, 5 H) 4.19 (br s, 3 H) 5.92 (s, 1 H) 7.69 - 7.85 (m, 2 H) 7.86 - 8.01 (m, 1H) 8.01 - 8.47 (m, 4 H) 8.47 - 8.52 (m, 1 H) 8.80 (s, 1 H); ^{13}C-NMR (DMSO-d6, 125 MHz): δ= 196.56, 163.33, 161.51, 151.20, 144.26, 141.77, 131.75, 131.69, 125.66, 124.41, 121.86, 121.86, 120.46, 119.93, 117.83, 117.09, 115.56, 113.87, 101.27, 50.47, 33.02, 31.97, 28.09, 27.39, 25.77, 17.17; MS-ESI calculated $C_{26}H_{23}$ FN_4O_2 $(M+H)^+$: 443.18, found 443; HRMS (ESI) *m/z* $[M+H]^+$calculated for $C_{26}H_{23}$ FN_4O_2; 443.1884 found 443.1852.

2-amino-4-(2-(4-methoxyphenyl)imidazo[1,2-a]pyridin-3-yl)-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile(7f)

Off white solid (Yield 78%); mp=154-156^{o}C; FT-IR(cm^{-1}) 3323, 2948, 2562, 2178, 1678, 1442, 1113, 1021; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.81 - 0.98 (m, 6 H) 0.98 - 1.15 (m, 2 H) 2.04–2.12 (m, 2 H) 5.16 (s, 1 H) 6.96 - 7.04 (m, 4 H) 7.25 - 7.29 (m, 3 H) 7.60 – 7.70 (m, 2 H) 7.89 (s, 1H); ^{13}C-NMR (DMSO-d6, 125 MHz): δ= 196.40, 164.44, 159.75, 159.32, 158.73, 144.26, 143.21, 130.70, 127.86, 125.54, 124.40, 123.71, 119.38, 117.83, 116.85, 114.20, 113.77, 113.07, 55.67, 50.55, 32.00, 31.22, 28.73, 27.33, 25.77, 21.59; MS-ESI calculated $C_{26}H_{24}N_4O_3$$(M+H)^+$: 441.19, found 441.

2-amino-7,7-dimethyl-4-(6-methyl-2-(p-tolyl)imidazo[1,2-a]pyridin-3-yl)-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile(7g)

Pale Yellow solid (Yield 76%); mp=152-154^{o}C; FT-IR(cm^{-1}) 3421, 3204, 2960, 2358, 2194, 1656, 1499, 1144, 1035; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.83 – 1.03 (m, 6 H) 1.89 – 2.08 (m, 2 H) 2.27 - 2.50 (m, 2 H) 5.13 (s, 1 H) 6.99 (br s, 1 H) 7.07 - 7.22 (m, 2H) 7.22 - 7.37 (m, 3 H) 7.38 - 7.52 (m, 2 H) 7.83 (br s, 1 H); ^{13}C-NMR (DMSO-d6, 125 MHz): δ= 196.44, 159.85, 158.82, 142.23, 139.94, 138.68, 131.69, 129.52, 129.38, 129.20, 124.10, 118.50, 117.72, 114.20, 112.96, 101.73, 50.28, 46.18, 33.04, 32.17, 28.18, 24.99, 21.43, 18.40; MS-ESI calculated $C_{27}H_{26}$ N_4O_2 $(M+H)^+$: 439.21, found 439; HRMS (ESI) *m/z* $[M+H]^+$calculated for$C_{27}H_{26}$ N_4O_2; 439.2135 found 439.2108.

2-amino-4-(2-(4-fluorophenyl)imidazo[1,2-a]pyridin-3-yl)-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile(7h)

Off white solid (Yield 62%); mp=156-158^{o}C; FT-IR(cm^{-1}) 3313, 2943, 2521, 2223, 2044, 1655, 1448, 1114, 1021; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 0.86 - 1.02 (m, 6 H) 1.15-1.35 (m, 2 H) 2.09-2.12 (m, 2 H) 5.15 (s, 1 H) 6.97-7.01 (m, 2 H) 7.27-7.31 (m, 5 H) 7.62-7.71 (m, 2 H) 7.99 (m, 1 H); ^{13}C-NMR (DMSO-d6, 125 MHz): δ= 196.54, 163.18, 161.29, 159.68, 144.41, 143.48, 131.41, 125.69, 124.69, 123.82, 119.81, 119.34, 118.07, 117.05, 115.50, 113.23, 112.19, 108.64, 53.36, 50.50, 31.98, 28.68, 27.95, 27.40, 25.70; MS-ESI calculated for $C_{25}H_{21}FN_4O_2(M+H)^+$: 429.17, found 429; HRMS (ESI) *m/z* $[M+H]^+$calculated for$C_{25}H_{21}FN_4O_2$; 429.1728 found 429.1725.

2-amino-7,7-dimethyl-5-oxo-4-(2-(p-tolyl)imidazo[1,2-a]pyridin-3-yl)-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile(7i)

Pale Yellow (Yield 74%); mp=150-152^{o}C; FT-IR(cm^{-1}) 3319, 2950, 2530, 2142, 1675, 1432, 1116, 1030; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 0.62 - 1.08 (m, 6 H) 1.83 - 2.12 (m, 2 H) 2.17 (br s, 1 H) 2.27 - 2.41 (m, 3 H) 2.51 (m, 1 H) 5.15 (s, 1 H) 6.96 - 7.11 (m, 1 H) 7.12 - 7.39 (m, 5 H) 7.48 - 7.61 (m, 1 H) 7.82-7.85 (m, 1H) 8.72 - 8.84 (m, 1 H)); ^{13}C-NMR (DMSO-d6, 125 MHz): δ= 196.34, 164.42, 159.75, 144.53, 137.15, 132.75, 129.34, 128.77, 125.52, 124.39, 123.74, 119.35, 118.00, 113.08, 112.08, 108.77, 53.43, 50.52, 31.99, 28.78, 28.15, 27.82, 27.31, 21.39; MS-ESI calculated for $C_{26}H_{24}N_4O_2(M+H)^+$: 425.19, found 425; HRMS (ESI) *m/z* $[M+H]^+$calculated for$C_{26}H_{24}N_4O_2$; 425.1978 found 425.1975.

2-amino-4-(2-(4-methoxyphenyl)imidazo[1,2-a]pyridin-3-yl)-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile(7j)

White solid (Yield 82%); mp=164-166^{o}C; FT-IR(cm^{-1}) 3350, 2946, 2521, 2187, 1631, 1416, 1119, 1030; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 2.28 (s, 2 H) 2.41 - 2.44 (m, 4 H) 3.86 (s, 3 H) 6.04 (s, 1 H) 7.18 (d, *J*=8.80 Hz, 2 H) 7.47 (ddd, *J*=6.63, 5.23, 2.93 Hz, 1 H) 7.85 - 7.90 (m, 5 H) 8.67 (s, 1 H) 8.84 (d, *J*=6.72 Hz, 1 H); ^{13}C-NMR (DMSO-d6, 125 MHz): δ= 196.37, 157.52, 156.85, 144.18, 137.10, 135.47, 133.18, 129.31, 128.97, 125.42, 124.41, 122.32, 121.46, 119.83, 117.93, 117.36, 113.60, 112.81, 54.15, 50.82, 36.69, 27.08, 21.41, 20.05; MS-ESI calculated for $C_{24}H_{20}N_4O_3(M+H)^+$: 413.16, found 413.

2-amino-4-(2-(4-fluorophenyl)imidazo[1,2-a]pyridin-3-yl)-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile(7k)

Yellow solid (Yield 65%); mp=165-167^{o}C; FT-IR(cm^{-1}) 3321, 2943, 2529, 2043, 1630, 1449, 1114, 1021; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.24 - 1.40 (m, 1 H) 1.88 - 2.21 (m, 4 H)

2.52 - 2.70 (m, 1 H) 5.12 (s, 1 H) 6.99 (td, *J*=6.85, 1.10 Hz, 3 H) 7.09 - 7.29 (m, 4 H) 7.30 (ddd, *J*=8.99, 6.72, 1.04 Hz, 2 H) 7.58 -7.89 (m, 1 H); ^{13}C-NMR (DMSO-d6, 125 MHz): δ= 196.58, 163.18, 161.24, 143.31, 132.38, 131.43, 131.37, 124.66, 119.83, 117.53, 115.41, 115.41, 115.25, 112.78, 60.80, 36.72, 30.14, 27.06, 20.12; MS-ESI calculated for $C_{23}H_{17}FN_4O_2(M+H)^+$: 401.14, found 401; HRMS (ESI) *m/z* $[M+H]^+$calculated for$C_{23}H_{17}FN_4O_2$; 401.1414 found 401.1411.

4. Conclusions

In summary, here we have reported a simple and efficient one-potsyntesis of novel fused bisheterocyclic compounds (7a-7k) in an aqueous solution of gluconic acid. The protocol developed is greener, easy to operate, and cost effective. The experiment was performed using both conventional and ultrasonication methodology in which the later has proven more efficient for the synthesis. The broad substrate scope, high yield of compounds and solvent reusability makes this a promising method for synthesis of imidazopyridine bearing pyran derivatives.Furthermore, the compounds were evaluated *in vitro* for their antimicrobial activity. Some compounds of 7a-7kseries were found active in inhibiting the growth of bacteria. In addition, MIC & MBC values were also determined along with the hemocompatibility test. Further, the study would be carried out for structural variation of derivatives in order to enhance antimicrobial activity.

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- One pot synthesis of novel imidazopyridine bearing pyran bis-heterocycles.
- X-ray single crystal structure of 7i.
- MIC showed that 7a, 7h & 7k from a series 7a-7k inhibited the growth of *S. aureus*.
- 7a, 7k derivatives are bactericidal and 7h is bacteriostatic.
- Haemolysis results showed that compounds are non-cytotoxic to erythrocytes.

Declaration of interests

The authors declare that they have no known competing financialinterestsor personal relationships that could have appeared to influence the work reported in this paper.