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Gluconic acid aqueous solution: A bio-compatible media for one-pot multicomponent synthesis of dihydropyrano [2,3-*c*] pyrazoles

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Abstract: A new facile, green and efficient protocol was developed for synthesis of Dihydropyrano [2,3-*c*] pyrazoles using gluconic acid aqueous solution (GAAS), as catalyst and solvent under environmentally friendly conditions. The advantageous features of this methodology are the environmentally benign character, operational simplicity, high yield processing (91-96%), easy handling; reaction medium can be recycled and reused several times without significant loss of its efficiency. The synthesized compounds were evaluated for antimicrobial and antioxidant activity and also analyzed for ADME properties. The structure of compounds has been confirmed by IR, 1H NMR, 13C NMR, Mass spectrometry and elemental analysis.

Keywords: Dihydropyrano [2,3-*c*] pyrazoles; gluconic acid aqueous solution (GAAS); green media; antimicrobial; antioxidant; ADME.

1. Introduction

Gluconic acid is an organic compound with molecular formula $C_6H_{12}O_7$ and it is abundantly available in plants, fruits, and other foodstuffs, such as rice, meat, dairy products, wine, honey, and vinegar. In particular, the nonhazardous nature of gluconic acid also allows its use in the formulation of food, pharmaceutical and hygienic products.[1] Gluconic acid aqueous solution (GAAS) is a weak acidic aqueous solution. It is noncorrosive, nonvolatile, stable, inexpensive industrial product and largely available in the market. GAAS is a effective promoting medium for organic reactions, such as the Michael addition of indoles to α,β-unsaturated ketones, the electrophilic ring-opening reaction of 3,4-dihydropyran with indoles and Friedel-Crafts alkylation of electron-rich aromatics with benzyl alcohols.[2] Whereas in our present work we have demonstrated, the use of GAAS as a green reaction media for the bicyclic ring closure reaction using one pot four component system.

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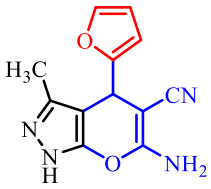
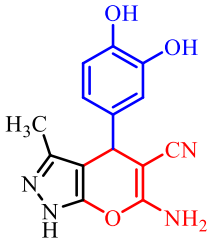
In medicinal chemistry and drug designing, Dihydropyrano [2,3-*c*]pyrazoles is became the first choice of researchers and scientist due to its potential biological activity. Therefore it becomes an interesting template for medicinal chemistry research. Most of these compounds, are well known for antioxidant,[3] antimicrobial,[4] insecticidal,[5] molluscicidal,[6] analgesic,[7] anti-inflammatory agents[8] and some of their analogues act as vasodilators, hypotensive,[9] hypoglycemic and anticancer agents.[10] They are also potential inhibitors of human Checkpoint kinase-1 (Chk1)[11] (Figure 1). Furthermore, they play a significant role as crucial synthetic intermediates.[12]



Molluscicide

6-amino-4-(furan-2-yl)-1,4-dihydro-3-methylpyrano[2,3-*c*]pyrazole-5-carbonitrile



Inhibitors of human Chk1 kinase

6-amino-1,4-dihydro-4-(3,4-dihydroxyphenyl) 3-methylpyrano[2,3-*c*]pyrazole-5-carbonitrile

Figure 1. Biologically active dihydropyrano [2,3-*c*]-pyrazoles

Thus considering the different potential therapeutic activity of pyrano [2,3-*c*]pyrazoles heterocyclic compounds, various methodologies for synthesis of Dihydropyrano [2,3-*c*]pyrazoles have been reported in the literature. These methodologies have shown many good results in many instances. However, some of the synthetic strategies have limitation in term of using metal catalyst, expensive reagents, long reaction time, environmental hazard, harsh reaction conditions, tedious workup procedure, unsatisfactory yield and use of homogeneous catalyst. Which are difficult in separation from reaction mixture inspite of many reported methods for the synthesis Dihydropyrano [2,3-*c*]pyrazoles derivatives. The development of new synthetic strategy using easily accessible catalyst and mild sustainable alternative reaction condition still demand a lot of researcher's attention.

Recently, One pot four-component reactions of aldehydes, 1,3-dicarbonyl compounds, malononitrile, and hydrazine have been developed for the synthesis of pyranopyrazoles using triphenylphosphine,[13] urea,[14] ionic liquid,[15] water containing a catalytic amount of piperidine,[16] CTACl,[17] heteropolyacids,[18] microwave,[19] piperazine,[20] *N*-methylmorpholine,[21] L-proline,[22] alumina,[23] per-6-amino-β-cyclodextrin,[24] sodium benzoate,[25] amberlyst A21,[26] glycine,[27] imidazole,[28] and I_2.[29] Although these methods are quite satisfactory, some of them suffer from the absence of green chemistry and have been associated with several shortcomings, such as the use of volatile and hazardous organic solvents, low yields, extended reaction time, high temperature and tedious procedure for the preparation of catalysts. Considering the significance of Dihydropyrano [2,3-*c*]pyrazoles derivatives in pharmaceutical and medicinal fields, and in continuation of earlier work,[30] the development of general, economically and environmentally benign synthetic methodologies for these heterocyclic is highly desirable.

2. Experimental

2.1. Chemical Material and Apparatus

All the reagents and solvents used for the synthesis were purchased from Sigma Aldrich, Spectrochem and Molychem and were used as such without further purification. The melting points of all compounds were determined on a Toshniwal apparatus and are uncorrected. IR spectra were recorded on a Shimadzu FTIR-8400S spectrophotometer using KBr pellets. ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 using TMS as an internal standard on a Bruker spectrophotometer,

respectively. Mass spectra of representative compounds were recorded on JEOL SX-102 spectrometer at 70 eV. Elemental microanalyses were carried out on a Carlo Erba1108 CHN analyzer. Thin layer chromatography was performed on pre-coated silica gel 60 F_{254} aluminium sheets (E. Merck, Germany) using various solvents systems and spots were identified by UV light and Iodine.

2.2. Biological Activity

2.2.1. Antibacterial Activity

Minimum inhibitory concentration (MIC) values for bacteria determined according to the two-fold broth micro-dilution method using Muller-Hinton broth in 96-well micro-test plates recommended by National Committee for Clinical Laboratory Standards (NCCLS) guidelines.[31a,b] The antimicrobial susceptibility testing of newly synthesized compounds was performed In Vitro against bacterial strains viz., Gram-positive Staphylococcus Aureus (ATCC No. 29737), Micrococcus Luteus (ATCC No. 398) and Gram negative Escherichia Coli (NCIM No. 2256) and Pseudomonas Fluorescens (NCIM No. 2173), respectively, to find out minimum inhibitory concentration (MIC). The MIC was defined as the lowest concentrations of compound that completely inhibit the growth of each strain. Serial twofold dilutions of all samples were prepared in triplicate in micro titer plates and inoculated with suitably prepared cell suspension to achieve the required initial concentration. Serial dilutions were prepared for screening. Dimethylsulfoxide (DMSO) was used as solvent control. Ampicilin & kanamycin were used as a standard antibacterial drug. The concentration range of tested compounds and standard was 128-0.5 µg/mL. The plates were incubated at 37 °C for all micro-organisms; absorbance at 595 nm was recorded to assess the inhibition of cell growth after 24 h. The compounds which are showing promising antibacterial activity were selected for MIC studies. The MIC was determined by assaying at 128, 64, 32, 16, 8, 4, 2, 1 and 0.5 µg/mL concentrations along with standards at the same concentrations.

2.2.2. Antifungal Activity

The antifungal activity was evaluated against five human pathogenic fungal strains, such as Candida albicans (NCIM 3471), Fusarium oxysporum (NCIM 1332) and Aspergillus flavus (NCIM 539), which are often encountered clinically and were compared with standard drug fluconazole & miconazole. Minimum inhibitory concentration (MIC) values were determined using standard agar method as per CLSI (formerly, NCCLS) guidelines (Approved Standard M7-A6, vol. 23. 2003).[32a,b] The standards used in the study were dissolved in a suitable solvent. The primary solutions were further diluted to the final strength using test medium. The medium yeast nitrogen base (Himedia, India) was dissolved in Phosphate buffer pH 7 and it was autoclaved at 110 °C for 10 minutes. The suitable concentration of standards was incorporated in the medium. The fungal strains were freshly subcultered on to Sabouraud dextrose agar (SDA) and incubated at 25 °C for 72 h. The fungal cells were suspended in sterile distilled water and diluted to get 105 cells/mL. 10 µL of standardized suspension was inoculated onto the control plates and the media incorporated with the antifungal agents. The inoculated plates were incubated at 25 °C for 48 h. The readings were taken at the end of 48 and 72 h.

2.2.3 Antioxidant Activity

Antioxidant activities of the synthesized compounds **5a-q** were measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay.[33] The hydrogen atom or electron donation ability of some compounds were measured from the bleaching of the purple colored methanol solution of DPPH. The spectrophotometric assay uses the stable radical DPPH as a reagent. 1 mL of various concentrations of the test compounds (5, 10, 25, 50 and 100 µg/mL) in methanol was added to 4 mL of 0.004% (w/v) methanol solution of DPPH. The reaction mixture was incubated at 37 °C. The scavenging activity on DPPH was determined by measuring the absorbance at 517 nm after 30 min. All tests were performed in triplicate and the mean values were entered. The percent of inhibition (I %) of free radical production from DPPH was calculated by the following equation % of scavenging =

[(Acontrol – Asample)/(Asample × 100)] Where, Acontrol is the absorbance of the control (DPPH radical without test sample) Asample is the absorbance of the test sample (DPPH radical with test sample). The control contains all reagents except the test samples. A lower IC50 value indicates the greater antioxidant activity. The IC50 (concentration required to scavenge 50% of the radicals) were calculated to evaluate the potential antioxidant activities. Butylated hydroxytoluene (BHT) has been used as a standard drug for the comparison of antioxidant activity and the observed results are summarized in Table 3.

2.2.4. Computational Study

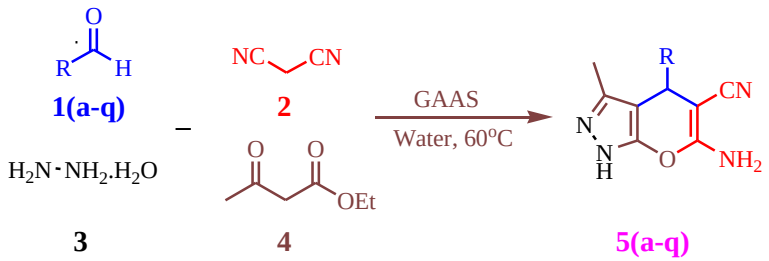
2.2.4.1. ADME Properties

The success of a drug is determined not only by good efficacy but also by an acceptable ADME (absorption, distribution, metabolism and excretion) profile. In the present study, we have calculated molecular volume (MV), molecular weight (MW), logarithm of partition coefficient (miLog P), number of hydrogen bond acceptors (n-ON), number of hydrogen bonds donors (n-OHNH), topological polar surface area (TPSA), number of rotatable bonds (n-ROTB) and Lipinski's rule of five[34] using Molinspiration online property calculation toolkit.[35] Absorption (% ABS) was calculated by: % ABS = 109-(0.345×TPSA)[36] Drug-likeness model score (a collective property of physic-chemical properties, pharmacokinetics and pharmacodynamics of a compound is represented by a numerical value) was computed by MolSoft[37] software.

3. Result and discussion

3.1. Chemistry

A facile, economic, green and environmentally being protocol, was developed for one-pot MCR of aldehyde, malononitrile, hydrazine hydrate and ethyl acetoacetate (Scheme 1). Successful implementation of GAAS reaction medium for an efficient and rapid synthesis of pyrano [2,3-*c*]pyrazole derivatives has been described. Higher product yields with shorter reaction time, reusable and economical catalytic system, and consistent performance on large scale make this synthetic strategy an attractive one (Scheme 1).



Scheme 1. Synthesis of pyrano[2,3-*c*]pyrazole derivatives.

In order to optimize the reaction conditions, initially we carried out the reaction between benzaldehyde (**1**) (1 mmol), malononitrile (**2**) (1 mmol), hydrazine hydrate (**3a**) (1 mmol) and ethyl acetoacetate (**4**) (1 mmol) as a model reaction. Before proceeding towards the actual experimental part, a thorough analysis of the mechanistic path leading to the formation of the desired pyrano [2, 3-*c*] pyrazole system was performed. This detailed study revealed that the first two steps involved in the reaction path *i.e.* formation of Knoevenagel condensation product **A** and pyrazolone **B** can be achieved either under solvent-free condition or using water as a reaction medium, that even in the absence of catalyst. The only challenge was to achieve the desired product **C** by cycloaddition of **A** and **B** (Figure 2).

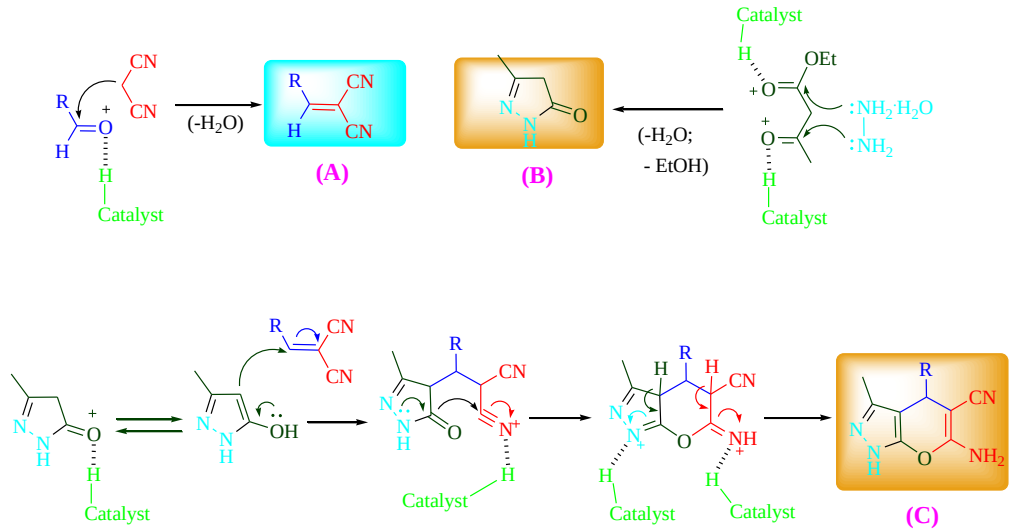


Figure 2. Proposed mechanism for GAAS catalyzed synthesis of pyrano [2,3-*c*] pyrazoles.

Considering the significance of green chemistry concept, model reaction was carried out initially under solvent-free and catalyst-free conditions at RT and higher temperature (100 °C) for 60 min. But, formation of the desired product was not observed (Table 1, entries 1-2). During the study, model reaction was performed using water as a reaction medium at different temperatures. To our surprise, reaction in aqueous media at reflux conditions proceeds towards the desired product in 40 % yield (Table 1, entry 4). Similarly, reaction carried out in different solvents like acetonitrile, THF, DMSO and methanol; trace amount of products were detected (Table 1, Entry 5-8).

Table 1. Screening of solvent and GAAS at different conditions[a]

Entry	Solvent	Temp (°C)	Time (h)	Yield[b] (%)
1	Neat	RT	3	Trace
2	Neat	100	3	Trace
3	Water	RT	2	Trace
4	Water	Reflux	2	40
5	CH_3CN	Reflux	3	Trace
6	THF	Reflux	3	Trace
7	DMSO	Reflux	3	Trace
8	Methanol	Reflux	3	Trace
09	Acetic acid	Reflux	3	48
10	Citric acid-DMU (40:60)	Reflux	2	54
11	L-(+)-Tartaric acid-DMU(30:70)	Reflux	2	58
12	D-(-)-Fructose-DMU(70:30)	Reflux	2	55
13	GAAS	RT	2	30
14	GAAS	40	60 min	70
15	GAAS	60	30 min	95
16	GAAS	80	30 min	95

[a]*Reaction conditions:* **1a** (1 mmol), **2** (1 mmol), **3** (1 mmol), **4** (1 mmol) and solvent (5 mL)
[b]Isolated Yields.

Therefore, to improve the yield, it was thought that intervention of catalyst is necessary. Acetic acid was examined and displayed less efficiency (Table 1, Entry 9). When the reaction proceeded in low melting mixtures, such as citric acid-dimethylurea (DMU), L-(+)-tartaric acid-DMU, and D-(-)-Fructose-DMU slightly improved yields obtained (Table 1, Entry 10-12). With these results in hand, our next objective was to increase the product yield obtained in the earlier study. The only way in mind was the addition of a suitable catalyst which could enhance efficiency of the present method in terms of product yield as well as reaction time. It was decided to utilize GAAS system for our reaction. When the model reaction was performed in this reaction medium, at RT, 40, 60 and

80°C; we were delighted to know that the reaction was completing within only 30 min at 60 and 80°C affording the product in good yield (95%) (Table 1, Entry 15-16). Considering the effective catalytic activity, GAAS was preferred as a catalyst as well as solvent of choice for subsequent optimization studies.

Reason behind the success of GAAS bringing the reaction in its favor may be hydrophobic interactions which induce favorable aggregation of organic substrates in water. Due to this the organic substrates aggregate and result in their increased concentration which leads to fast collisions of the reactants thus leading to the formation of desired product in very short reaction times. Furthermore, exceptional performance of gluconic acid as an effective organic reaction medium in water could be attributed to the fact that the organic substrates get adsorbed to the gluconic acid by hydrophobic interactions between the surface of the gluconic acid and the organic molecule. In addition, the surface area available for a reaction (surface area of the gluconic acid) in such a system is quite large compared with that of the interface in a conventional liquid-liquid biphasic system.

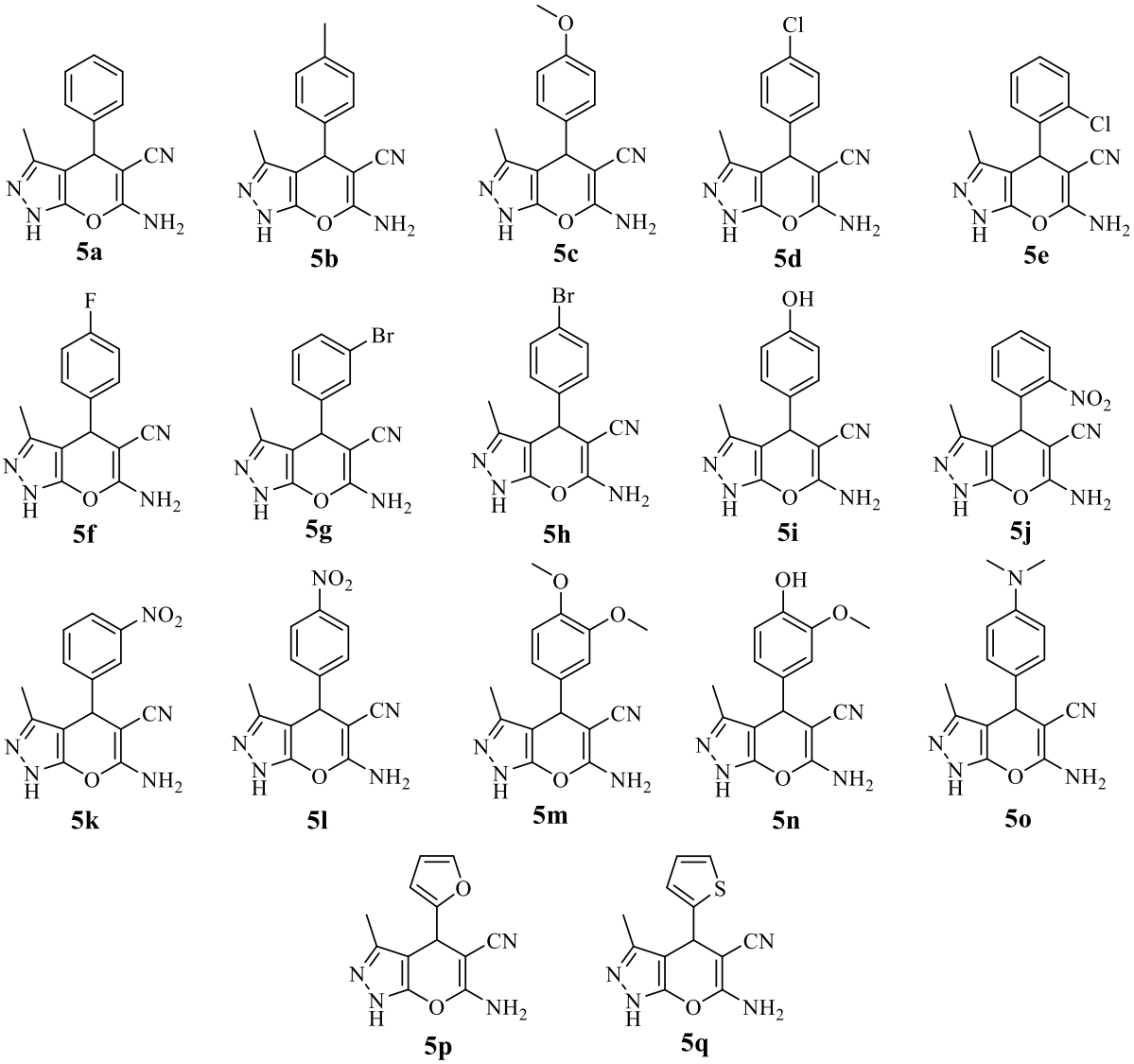


Figure 3. Structures of Dihydropyrano [2,3-*c*]pyrazole derivatives **5(a-q)**

It should be noted that, because of the immiscibility of GAAS with non-polar organic solvent, the formed product could be easily extracted from the GAAS phase with an appropriate organic solvent. After completion of the reaction, the reaction mixture was cooled to room temperature and extracted with ethyl acetate. The recovered gluconic acid aqueous solution was then subjected to the next run in the model reaction and could be reused at least four times without significant loss of activity (Table 2).

Table 2. Results for the recyclability of the GAAS

Entry	Cycle	Isolated Yield%	Recovered GAAS (w/w %)
1	1	95	98
2	2	93	96
3	3	92	95
4	4	90	93
5	6	90	90

To demonstrate the efficiency and the applicability of the developed method, reaction was performed with variety of electronically divergent aryl aldehydes under optimized reaction conditions and no obvious electronic effects of the substituent present on the aromatic ring of aldehyde was observed, affording the products in each case with excellent yields (*Supporting Information*). Structures of all the synthesized compounds shown in Figure 3.

The synthesized compound may exist in 1*H* or 2*H* forms (**5a** or **5b**). Structure **5a** is established through inspection of result by NOE experiments. Thus upon irradiation into the methyl signal a strong effect for the NH proton was revealed. This is a proof for the major position of the NH proton on N-2. A fast equilibrium with **5b** as minor components cannot be excluded at room temperature (Figure 4).[38]

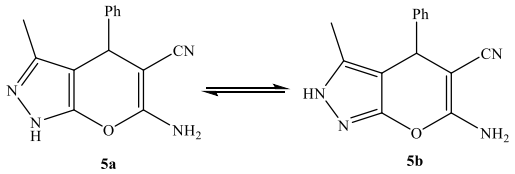


Figure 4. The synthesized compound may exist in 1*H* or 2*H* tautomeric forms (**5a** or **5b**)

*3.2. General Procedure for the Synthesis Dihydropyrano [2,3-c]pyrazoles **5(a-q)***

A mixture of aromatic aldehyde **1**(**a-q**) (1 mmol), malononitrile **(2)** (1 mmol), hydrazine hydrate (**3**) (1 mmol) and ethyl acetoacetate (**4**) (1 mmol) in gluconic acid aqueous solution (GAAS) (5 mL) were taken in a 50 mL round-bottomed flask. The resulting mixture was stirred at 60°C for a period as indicated in Table 3. After completion of the reaction (monitored by TLC), the solid obtained was collected by simple filtration and washed successively with water. The crude product was purified by crystallization from ethanol. The products **5**(**a-q**) were confirmed by comparing the physical and spectral data with those of the reported compounds.

*6-Amino-3-methyl-4-phenyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**5a**)*: Time: 30 min; yield: 95%; Melting point: 241-243°C;[15] FTIR (KBr,cm^{-1}): υ = 1654 (C=C), 2193 (C≡N), 3398 (NH_2). ^{1}H NMR (400 MHz, DMSO-d_6) *δ ppm*: 2.06 (s, 3H, CH_3), 4.59 (s, 1H, CH), 5.48 (s, 1H, NH), 7.23-7.47 (m, 5H, Ar-H), 10.48-11.6 (bs, 2H, NH_2); ^{13}C NMR (400 MHz, DMSO-d_6) *δ ppm*: 8.82, 34.74, 57.77, 94.70, 96.54, 119.71, 127.38, 134.69, 134.85, 153.85, 157.02, 159.50; Mass (LC-MS) *m/z*: 251.2 (M^-); Elemental analysis calculated for $C_{14}H_{12}N_4O$: C (66.65 %), H (4.79 %), N (22.21 %). Found: C (66.57 %), H (4.63 %), N (22.13 %).

*6-Amino-1,4-dihydro-3-methyl-4-p-tolylpyrano[2,3-c]pyrazole-5-carbonitrile (**5b**)*: Time: 30 min; yield: 91%; Melting point: 208-210°C;[17] FTIR (KBr,cm^{-1}): υ = 1579.37 (C=C), 1624.91 (C=N), 2188.78 (CN), 3353.51 (NH_2), 3448.78 (NH); ^{1}H NMR (400 MHz, DMSO d6): δ = 1.8 (s, 3H, CH_3), 2.28 (s, 3H, Ar-CH_3), 4.52 (s, 1H, C-4 pyran), 6.85 (d, 2H, p-tolyl-H), 7.00 (d, 2H, p-tolyl-H), 7.18 (s, 2H, NH_2), 12.09 (s, 1H, NH); ^{13}C NMR (75 MHz, DMSO-d6): δ = 9.9, 20.7, 36.7, 40.1, 78.3, 97.4,

120.7, 127.2, 128.6, 135.4, 140.9, 154.7, 160.6; Elemental analysis calculated for $C_{15}H_{14}N_4O$: C (67.65 %), H (5.30 %), N (21.04 %). Found: C (67.15 %), H (5.17%), N (21.00%).

6-Amino-4-(4-methoxyphenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (***5c***)**:** Time: 35 min; yield: 94%; Melting point: 210-212°C;[15] ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.76 (s, 3H, -CH_3), 3.71 (s, 3H, -OCH_3), 4.51 (s, 1H), 6.79 (s, 2H, -NH_2), 6.84 (d, 2H, *J* = 8.0 Hz), 7.04 (d, 2H, *J* = 8.0 Hz), 12.04 (s, 1H, -NH). ^{13}C NMR (50 MHz, DMSO-d_6+$CDCl_3$) δ. 8.8, 34.7, 53.8, 57.7, 96.5, 112.5, 119.7, 127.4, 134.7, 134.8, 153.8, 157.0, 159.5. Mass (ES-MS) *m/z* 283.2 (M^+).

*6-Amino-4-(4-chlorophenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**5d**)*: Time: 35 min; yield: 93%; Melting point: 231-233°C;[15] Yellow solid, FTIR (KBr,cm^{-1}): υ = 2228.22, 3231.27, 3346.76, 3484.11; ^{1}H NMR (300 MHz, DMSO): δ 1.77 (s, 3H, CH_3), 4.67 (s, 1H, -CH), 6.55 (bs, 2H, -NH_2), 7.35-7.37 (dd, 2H, Ar-H, *J* = 6.0 Hz), 8.09-8.12 (dd, 2H, Ar-H, *J* = 9.0 Hz), 11.95 (s, 1H, -NH),^{13}C NMR (75 MHz,$CDCl_3$): δ 10.21, 36.33, 97.37, 120.99, 128.67, 129.54, 131.96, 135.99, 143.47, 155.19, 161.27; Elemental analysis calculated for $C_{14}H_{11}ClN_4O$: C (55.39%); H, (4.62%); N (23.09%). Found: C (55.36%), H (4.65%), N (23.06%).

*6-Amino-4-(2-chlorophenyl)-1,4-dihydro-3-methylpyrano[2,3-c]pyrazole-5-carbonitrile (**5e**)*: Time: 30 min; yield: 89%; Melting point: 144-146°C.[27b] FTIR (KBr,cm^{-1}): υ = 1218, 1407, 1600, 1651, 2187, 3313, 3413; ^{1}H NMR (DMSO-d_6, δ ppm, *J* Hz): 1.8 (s, 3H, CH_3); 4.6 (s, 1H, CH); 7.08 (s, 2H, NH_2); 7.32 (d, 1H, *J* = 7.7, Ar-H); 7.47 (t, 1H, *J* = 7.5, Ar-H); 7.54 (t, 1H, *J* = 7.5, Ar-H); 7.83 (d, 1H, *J* = 7.4, Ar-H); 12.25 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ ppm): 9.63, 31.57, 56.16, 96.50, 120.46, 123.37, 124.91, 127.99, 132.25, 134.07, 135.93, 148.24, 155.09, 161.33.

*6-Amino-4-(4-fluorophenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**5f**)*: Time: 30 min; yield: 93%; Melting point: 244-246°C;[15] White powder; FTIR (KBr, cm^{-1}): υ = 1395, 1491, 1591, 2198, 3090, 3226; ^{1}H NMR (300 MHz, DMSO-d_6): δ= 1.79 (s, 3H), 4.64 (s, 1H), 6.92 (s, 2H), 7.10-7.30 (m, 4H), 12.13 (s, 1H); ^{13}C NMR (75 MHz, DMSO-dd): δ= 9.78, 35.47, 38.69, 38.96, 39.24, 45.01, 49.29, 57.07, 97.53, 115.07, 115.36, 120.77, 129.33, 129.43, 135.67, 140.68, 140.72, 154.72, 159.37, 160.85; MS (ESI): *m/z*= 271.1 $(M+H)^+$.

*6-Amino-4-(3-bromophenyl)-1,4-dihydro-3-methylpyrano[2,3-c]pyrazole-5-carbonitrile (**5g**)*: Time: 40 min; yield: 87%; Melting point: 219-221°C.[15] FTIR (KBr,cm^{-1}): υ =1600, 1610, 1645, 2195 (C =N), 3260 (NH), 3320, 3400 (C=C, C=N); ^{1}H NMR: 2.3 (s, 3H, CH_3), 4.6 (s, 1H, pyran 4-H), 6.8 (s, 2H, NH), 7.1 (m, 4H, phenyl protons) and 11.8 (s, 1H, NH). Elemental analysis calculated for $C_{14}H_{11}BrN_4O$ C (50.77%), H (3.35%), N (16.92%), Br. Found C (50.70%), H (3.50%), (N17.10%).

*6-Amino-4-(4-bromophenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**5h**)*: Time: 40 min; yield: 90%; Melting point: 176-178°C;[15] Yellow solid, FTIR (KBr,cm^{-1}): υ = 2193.51, 3271.99, 3431.74, 3486.37; ^{1}H NMR (200 MHz, DMSO): δ 1.82 (s, 3H, -CH_3), 4.56 (s, 1H, CH), 6.57 (bs, 2H, -NH_2), 7.10-7.14 (dd, 2H, Ar-H, *J* = 12.0 Hz), 7.42-7.46 (dd, 2H, Ar-H, J = 12.0 Hz), 11.96 (s, 1H, -NH), ^{13}C NMR (50MHz, DMSO): δ =9.75, 36.93, 57.07, 96.75, 119.85, 120.52, 120.52, 129.39, 131.08, 136.52, 143.33, 154.71 and 160.77; Elemental analysis calculated for $C_{14}H_{11}BrN_4O$: C (50.76%), H (3.38%), N (16.95%). Found C (50.77%), H (3.35%), N (16.92%).

*6-Amino-4-(4-hydroxyphenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**5i**)*: Time: 35 min; yield: 92%; Melting point: 222-224°C;[17] Yellow solid; FTIR (KBr,cm^{-1}): υ = 2223.29, 3126.13, 3253.86, 3459.99; ^{1}H NMR (200 MHz, DMSO): δ 1.81 (s, 3H, -CH_3), 4.44 (s, 1H, -CH), 6.48 (bs, 2H, -NH_2), 6.71 (dd, 2H, Ar-H), 6.94 (dd, 2H, Ar-H), 9.06 (bs, 1H, -OH), 11.88 (s, 1H, -NH), Elemental analysis calculated for $C_{14}H_{12}N_4O_2$: C (58.92%), H (5.31%), N (24.58%). Found C, (58.94%), H (5.30%), N (24.55%).

*6-Amino-1,4-dihydro-3-methyl-4-(2-nitrophenyl)pyrano[2,3-c]pyrazole-5-carbonitrile (**5j**)*: Time: 35 min; yield: 93%; Melting point: 220-222°C;[27b] yellow solid. FTIR (KBr,cm^{-1}): υ = 733, 805, 1349,

1400, 1491, 1525, 1652, 2195, 3117, 3224, 3473; ^{1}H NMR (300, $CDCl_3$): δ 1.82 (s, 3H), 4.82 (s,1H), 6.92 (s, 2H), 7.59-7.81 (m, H), 8.01 (s, 1H), 8.09 (d, *J* = 8.4 Hz, 1H), 12.13 (s, 1H).

*6-Amino-1,4-dihydro-3-methyl-4-(3-nitrophenyl)pyrano[2,3-c]pyrazole-5-carbonitrile (**5k**)*: Time: 30 min; yield: 89%; Melting point: 194-196°C;[15] FTIR (KBr,cm^{-1}): υ =2193.51, 3271.99, 3431.74, 3486.37; ^{1}H NMR (200 MHz, DMSO): δ 1.82 (s, 3H, -CH_3), 4.56 (s, 1H, -CH), 6.57 (bs, 2H, -NH2), 7.10-7.14 (dd, 2H, Ar-H, *J* = 12.0 Hz), 7.42-7.46 (dd, 2H, Ar-H, *J* = 12.0 Hz), 11.96 (s, 1H, -NH), ^{13}C NMR (100 MHz, DMSO): δ 9.75, 36.93, 57.07, 96.75, 119.85, 120.52, 120.52, 129.39, 131.08, 136.52, 143.33, 154.71, 160.77; Elemental analysis calculated for $C_{14}H_{11}BrN_4O$: C (50.76%), H (3.38%), N (16.95%). Found C (50.77%), H (3.35%), N (16.92%).

*6-Amino-3-methyl-4-(4-nitrophenyl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**5l**)*: Time: 40 min; yield: 90%; Melting point: 249-251°C;[15] Yellow solid; FTIR (KBr,cm^{-1}): υ =1643.37, 2189.52, 3177.13, 3307.11, 3386.6; ^{1}H NMR (300 MHz, DMSO): δ 1.76 (s,3H, -CH_3), 4.65 (s, 1H, -CH), 6.35 (bs, 2H, -NH_2), 7.33-7.36 (dd, 2H, Ar-H, *J* = 9.0 Hz), 8.08-8.11 (dd, 2H, Ar-H *J* = 9.0 Hz), 11.90 (s, 1H, -NH), ^{13}C NMR (75 MHz,$CDCl_3$): δ 10.16, 36.73, 58.01, 96.39, 120.49, 123.88, 128.73, 136.43, 146.88, 151.22, 155.18, 161.17; Elemental analysis calculated for $C_{14}H_{11}N_5O_3$: C (53.48%), H (4.52%), N (26.75%). Found C (53.50%), H (4.49%), N (26.74%).

*6-Amino-4-(3,4-dimethoxyphenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**5m**)*: Time: 40 min; yield: 84%; Melting point: 192-194°C;[27b] Yellow solid, FTIR (KBr,cm^{-1}): υ =2186.78, 3176.29, 3350.11, 3413.28; ^{1}H NMR (300 MHz, DMSO): δ 1.76 (s, 3H, -CH_3), 3.71 (s, 6H, $(OCH_3)_2$), 4.45 (s, 1H, -CH), 6.37 (bs, 2H, -NH_2), 6.75-6.78 (dd, 2H, Ar-H, *J* = 9.0 Hz), 7.02-7.04 (dd, 2H, Ar-H), 11.84 (s, 1H, -NH); Elemental analysis calculated for $C_{16}H_{16}N_4O_3$: C (58.37%), H (5.84%), N (21.25%). Found C (58.35%), H (5.81%), N (21.26%).

*6-Amino-4-(4-hydroxy-3-methoxyphenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**5n**)*: Time: 45 min; yield: 88%; Melting point: 236-238°C;[15] Yellow solid, FTIR (KBr,cm^{-1}): υ= 2195.64, 3275.81, 3413.72, 3490.79; ^{1}H NMR (200 MHz, DMSO): δ 1.85 (s, 3H, -CH_3), 3.79 (s, 3H,-OCH_3), 4.47 (s, 1H, -CH), 6.18 (bs, 2H, -NH_2), 6.66 (m, 2H, Ar-H), 7.86 (s, 1H, Ar-H), 8.46 (bs, 1H,-OH), 11.82 (s, 1H, -NH),^{13}C NMR (50MHz, DMSO):δ = 9.78, 26.15, 53.37, 86.18, 97.46, 114.92, 119.74, 134.91, 140.13, 145.01, 154.79, 180.42, 186.13, 196.73, 211.13; Elemental analysis calculated for $C_{15}H_{14}N_4O_3$: C (57.16%), H (5.42%), N (22.19%). Found C (57.13%), H (5.43%), N (22.21%).

*6-Amino-4-(4-(dimethylamino)phenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**5o**)*: Time: 40 min; yield: 92%; Melting point: 165-167°C;[27b] Yellow solid, FTIR (KBr,cm^{-1}): υ =3441.70, 3142.41, 2173.69; ^{1}H NMR (300 MHz, DMSO): δ 1.78 (s,3H, -CH_3), 2.86 (s, 6H, -$(N(CH_3)_2)$, 4.41 (s, 1H, -CH), 6.01-6.62 (m, 4H, Ar-H, -NH_2), 6.94-6.97 (dd, 2H, Ar-H, *J* = 9.0 Hz), 8.08-8.11 (dd, 2H, Ar-H, *J*= 9.0 Hz), 11.91 (s, 1H, NH), ^{13}C NMR (75 MHz, $CDCl_3$)(Fig. 4.13): δ 10.22, 35.97, 58.83, 98.43, 112.61, 121.34, 128.37, 132.30, 135.91, 149.56, 155.26, 160.95; Elemental analysis calculated for $C_{16}H_{17}N_5O$: C (61.55%), H (6.43%), N (26.88%). Found C (61.52%), H (6.45%), N, (26.90%).

*6-Amino-4-(furan-2-yl)-1,4-dihydro-3-methylpyrano[2,3-c]pyrazole-5-carbonitrile (**5p**)*: Time: 30 min; yield: 90%; Melting point: 235-238°C.[27b]

*6-Amino-1,4-dihydro-3-methyl-4-(thiophen-2-yl)pyrano[2,3-c]pyrazole-5-carbonitrile (**5q**)*: Time: 40 min; yield: 90%; Melting point: 234-237°C.[27b]

3.4. Biological Assay

3.4.1. Antibacterial Activity

For bacterial strain *S. aureus*, it can be seen that, the compounds **5d**, **5e**, **5f** and **5j** showed excellent inhibitory activity with MIC value 4 μg/mL, which is equivalent to ampicilin (MIC 4

µg/mL). For bacterial strain *M. luteus*, compounds **5c**, **5f**, **5h**, **5i**, **5k** and **5l** exhibited four-fold antibacterial activity with MIC value 4 µg/mL and compounds **5d**, **5j**, **5m** and **5p** with MIC value 8 µg/mL exhibited two-fold more activity as compared to the clinical drug ampicilin (MIC 16 µg/mL). For bacterial strain *E. coli* and *P. fluorescens*, all the synthesized compounds exhibited moderate antibacterial activity compared to the standard drug (Table 3).

3.4.2. Antifungal Activity

Compounds **5b**, **5c**, **5h** and **5k** with MIC value 4 µg/mL exhibited four-fold more activity compared with the standard drug miconazole and compounds **5d**, **5f**, **5l**, **5m**, **5o** and **5p** with MIC value 8 µg/mL exhibited two-fold more activity compared to the miconazole against the fungicidal strain *C. albicans*. Compounds **5a**, **5e**, **5g**, **5i**, **5j**, **5n** and **5q** with MIC value 16 µg/mL exhibited equivalent activity compared with the standard drug miconazole. Compounds **5c** and **5f** with MIC value 4 µg/mL exhibited four-fold more activity compared with the standard drug miconazole and compounds **5b**, **5h**, **5j**, **5k**, **5l** and **5p** with MIC value 8 µg/mL exhibited two-fold more activity compared to the miconazole for the fungicidal strain *F. oxysporum*. Compounds **5a**, **5d**, **5e**, **5g**, **5m**, **5n**, **5o** and **5q** with MIC value 16 µg/mL exhibited equivalent activity compared with the standard drug miconazole. Compounds **5k** with MIC value 4 µg/mL exhibited four-fold more activity compared with the standard drug miconazole and compounds **5b**, **5c**, **5d**, **5f**, **5h**, **5j**, **5l** and **5m** with MIC value 8 µg/mL exhibited two-fold more activity compared to the miconazole against the fungicidal strain *A. flavus*. Compounds **5a**, **5e**, **5i**, **5n,** **5o**, **5p** and **5q** with MIC value 16 µg/mL exhibited equivalent activity compared with the standard drug miconazole (Table 3).

Table 3. *In vitro* antimicrobial and antioxidant activities of compounds **5a-q** (µg/mL)

Compounds	Gram positive bacteria IC_{50}		Gram negative bacteria IC_{50}		Antifungal activity IC_{50}			DPPH IC_{50}
	SA	**ML**	**EC**	**PF**	**CA**	**FO**	**AF**	
5a	32	32	32	16	16	16	16	22.1
5b	16	16	16	16	4	8	8	27.3
5c	16	4	8	8	4	4	8	23.1
5d	4	8	8	8	8	16	8	20.1
5e	4	32	8	8	16	16	16	18.3
5f	4	4	8	8	8	4	8	14.1
5g	16	16	8	8	16	16	32	18.1
5h	16	4	16	16	4	8	8	15.3
5i	8	4	16	16	16	32	16	10.3
5j	4	8	16	16	16	8	8	21.3
5k	16	4	8	4	4	8	4	19.2
5l	8	4	8	16	8	8	8	21.3
5m	8	8	8	16	8	16	8	24.1
5n	16	16	16	8	16	16	16	10.2
5o	32	16	8	8	8	16	16	24.6
5p	16	8	8	16	8	8	16	28.8
5q	16	16	16	8	16	16	16	29.3
Ampicilin	4	16	4	2	NA	NA	NA	NA
Kanamycin	2	2	2	2	-	NA	NA	NA
Miconazole	NA	NA	NA	NA	16	16	16	NA
Fluconazole	NA	NA	NA	NA	2	2	4	NA
BHT	NA	NA	NA	NA	NA	NA	NA	16.5

SA: *Staphylococcus aureus*; ML: *Micrococcus luteus*; EC: *Escherichia coli*; PF: *Pseudomonas fluorescens*; CA: *Candida albicans*; FO: *Fusarium oxysporum*; AF: *Aspergillus flavus;* BHT: *Butylated hydroxy toluene*; NA*: Not applicable*

3.4.3. Antioxidant Activity

All the synthesized compounds **5a-q** shows good to moderate antioxidant activity as compared to the standard drug BHT (Table 3). The compounds **5i** and **5n** with -OH substituent on phenyl ring have shown excellent activity as compared to standard drug. Again, the compound **5f** (14.1 µg/mL) with *fluoro*- group and **5h** (15.3 µg/mL) with *bromo*- group showed excellent antioxidant activity as

compared to the BHT. Remaining compounds exhibit well to moderate antioxidant activity as compared to standard drug BHT (Table 3).

3.5. Computational Chemistry

3.5.1. In silico ADME Prediction

It is observed that, the compounds exhibited a good % ABS (% absorption) ranging from 62.92 to 78.73% (Table 4). Furthermore, none of the compounds violated Lipinski's rule of five (miLog $P \leq 5$). A molecule likely to be developed as an orally active drug candidate should show no more than one violation of the following four criteria: miLog *P* (octanol-water partition coefficient) ≤ 5, molecular weight ≤ 500, number of hydrogen bond acceptors ≤ 10 and number of hydrogen bond donors ≤ 5.[39] The larger the value of the drug likeness model score, the higher is also probability that the particular molecule will be active. All the tested compounds followed the criteria for orally active drug and therefore, these compounds may have a good potential for eventual development as oral agents.

Table 4. Pharmacokinetic parameters important for good oral bioavailability

Comp.	ABS %	TPSA (A^2)	n-ROTB	MV	MW	miLog *P*	n-ON	n-OHNH	Lipinski violation	Drug-likeness model score
Rule	-	-	-	-	< 500	≤ 5	< 10	< 5	≤ 1	-
5a	78.73	87.73	1	223.38	252.28	1.44	5	3	0	-0.16
5b	78.73	87.73	1	239.94	266.30	1.89	5	3	0	-0.26
5c	75.54	96.97	2	248.92	282.30	1.50	6	3	0	0.05
5d	78.73	87.73	1	236.91	286.72	2.12	5	3	0	0.29
5e	78.73	87.73	1	236.91	286.72	2.07	5	3	0	0.19
5f	78.73	87.73	1	228.31	270.27	1.61	5	3	0	0.13
5g	78.73	87.73	1	241.26	331.17	2.23	5	3	0	-0.23
5h	78.73	87.73	1	241.26	331.17	2.25	5	3	0	-0.06
5i	71.75	107.96	1	231.40	268.28	0.96	6	4	0	0.24
5j	62.92	133.56	2	246.71	297.27	1.35	8	3	0	-0.22
5k	62.92	133.56	2	246.71	297.27	1.38	8	3	0	-0.11
5l	62.92	133.56	2	246.71	297.27	1.40	8	3	0	-0.18
5m	72.36	106.20	3	274.47	312.33	1.09	7	3	0	0.43
5n	68.56	117.19	2	256.94	298.30	0.78	7	4	0	0.51
5o	77.62	90.97	2	269.28	295.35	1.55	6	3	0	-0.25
5p	74.20	100.87	1	204.95	242.24	0.70	6	3	0	-0.23
5q	78.73	87.73	1	214.09	258.31	1.34	5	3	0	-0.11

4. Conclusion

A facile, eco-friendly and green protocol developed for one-pot multicomponent cyclo-condensation of aldehydes, malononitrile, hydrazine hydrate and ethyl acetoacetate is established. Application of gluconic acid aqueous solution (GAAS) as a reaction medium for the synthesis of pyrano [2,3-*c*] pyrazoles has been exploited first time. The reaction conditions are mild accepting several functional groups present in the molecules and all reactions proceed under essentially neutral conditions, thus reducing the possibility of many unwanted side reactions. In addition, present method offers marked improvements with regard to product yield, reaction time, and greenness of procedure, avoiding hazardous organic solvents/toxic catalysts and provides a better, clean and practical alternative route of synthesis to the existing protocols. The synthesized Dihydropyrano [2,3-*c*] pyrazoles were evaluated for antimicrobial and antioxidant activity and also analyzed for ADME properties.

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Supporting Information

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