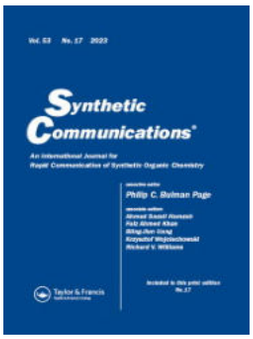

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An International Journal for Rapid Communication of Synthetic Organic Chemistry

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/lsyc20

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To cite this article: Bharti P. Koli, Rambhau P. Gore & Pravin R. Malasane (2023): Green and efficient three-component synthesis of novel isoniazid pyrazoles, molecular docking, antioxidant and antitubercular evaluation, Synthetic Communications, DOI: 10.1080/00397911.2023.2235624

To link to this article: https://doi.org/10.1080/00397911.2023.2235624

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https://doi.org/10.1080/00397911.2023.2235624

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Green and efficient three-component synthesis of novel isoniazid pyrazoles, molecular docking, antioxidant and antitubercular evaluation

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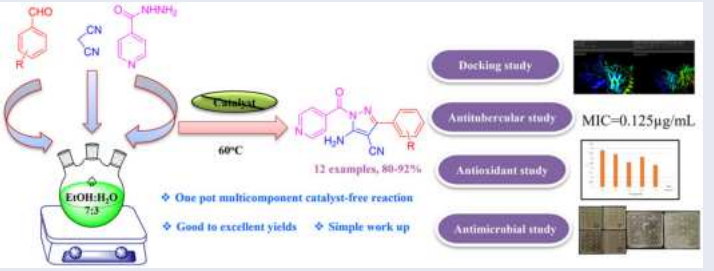
ABSTRACT

A series of novel isoniazid pyrazole derivatives were synthesized by an efficient, multicomponent reaction of various substituted aromatic aldehydes, isoniazid, and malononitrile in aqueous ethanol. The catalyst-free, green, three-component reaction took place well with good to excellent yields of the desired derivatives. The synthesized novel derivatives were screened for antimicrobial, antioxidant, and anti-TB properties along with the molecular docking study. Some of the synthesized compounds exhibit moderate antibacterial and comparable antifungal properties, while all of them display strong antioxidant activity. The compound **4l** demonstrates the most significant activity against *M. tuberculosis* with MIC = 0.125 μg/mL. The various analytical techniques including FT-IR, ^{1}H NMR, ^{13}C NMR, and HRMS were utilized to confirm the structures of the newly synthesized compounds.

GRAPHICAL ABSTRACT



ARTICLE HISTORY
Received 27 April 2023

KEYWORDS
Green synthesis; multicomponent reaction; anti-TB; antioxidant; docking study

Introduction

Tuberculosis (TB) continues a highly infectious disease despite the advancement in diagnosis and treatment regimens, caused by the bacillus *Mycobacterium tuberculosis* (Mtb)[1,2] remains a major cause of high mortality from a single pathogen ranking above HIV/AIDS.[3] The WHO 2022, world tuberculosis report showed around 10.6 million cases were diagnosed in 2021 with an increase of 4.5% from 2020 and 1.6 million

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/00397911.2023.2235624.
This article has been corrected with minor changes. These changes do not impact the academic content of the article.

deaths.[4] Isoniazid is one of the first-line drugs along with rifampicin, ethambutol, and pyrazinamide has been extensively used to treat and control TB infections.[5–7] It is activated by the KatG, mycobacterial catalase-peroxidase enzyme releasing active form an isonicotinoyl radical, which on further reaction with NADH and in turn inactivates InhA.[8,9] This leads to the blocking of mycolic acid biosynthesis crucial for the Mtb cell wall. The mutations in the KatG, InhA gene, and multi-drug resistance [10] and severe side effects of isoniazid hamper its efficacy and potency against the bacilli, known to produce hepatotoxicity and neurotoxicity.[11] These are the major hurdles in the treatment and management of TB and pose public health.[12]

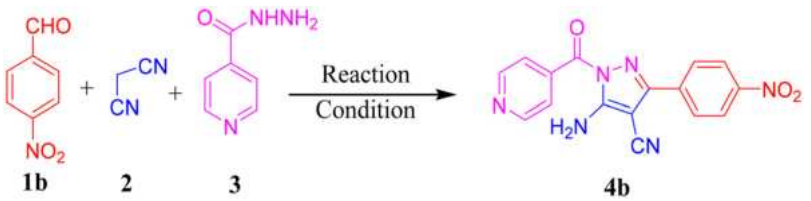
In this situation, to manage the increasing spread and co-infections in patients suffering from HIV/AIDS more reliable, safe, and less expensive drugs need to be introduced in the regimes of treatments. Despite the FDA's approval of bedaquiline and delamanid to treat multi-drug resistance TB,[13] isoniazid remains the first drug of choice for TB since its discovery in 1952.[14] Nowadays, the hypothesis of molecular hybridization is frequently used to produce novel antimicrobial molecules, which allows one to modulate the bioactivity and pharmacodynamic properties of the target molecule.[15] It is known that the free amino group of the isoniazid molecule undergoes enzymatic acetylation by N-acetyltransferase (NATs) [16] which makes it hepatotoxic. Therefore, we thought to synthesize derivatives of isoniazid by masking the amino group to bioactive pyrazole moiety to enhance the anti-mycobacterial benefits of isoniazid. Pyrazole and its derivatives are important heterocyclic compounds found in many natural products like Nostacine A, Withasomnine, and Fluviols (A–E) [17] and are parts of some marketed drugs such as celecoxib, lonazolac, betazole,[18] fomepizole, sildenafil, and cimetidine.[19]

The remarkable biological activities associated with the pyrazole derivatives include antimicrobial,[20] antiinflammatory, anticancer [21,22] antituberculosis,[23–25] antiviral,[26] antidiabetics,[27,28] antioxidants,[29] and angiotensin-converting enzyme (ACE) inhibitors.[30] The pyrazole derivatives which were screened particularly for antituberculosis activity are coumarinyl pyrazole,[31] phenylpyrazolbenzoic acid,[32] 4-aminothiophene-3-carboxylate pyrazole,[18] hispolonpyrazole sulfonamide,[33] nitroso pyrazole, NSC 18725,[34] isoxazole pyrazole.[35] In recent years, the various derivatives of isoniazid have been designed and reported such as pyridyl, dibenzofuran, amidoether, carvone, Schiff bases,[36] and nicotinic acid pyrazole with a wide spectrum of pharmacological activities such as antimicrobial,[37] antileishmanial, anti-cancer, antiinflammatory, antioxidant and anti-tubercular.[38] Due to the broad spectrum of biological activities associated with pyrazole and the wonder anti-tubercular drug, isoniazid prompts us to design and synthesize novel pyrazole-isoniazid derivatives with an anticipation of convincing antimicrobial, particularly anti-tuberculosis activities.

The literature search on the synthesis of isoniazid-pyrazole derivatives revealed that isoniazid is mostly converted to hydrazone,[39,40] hydrazides,[41–43] nicotinic acid pyrazole[44] and tetrahydro pyrimidine[45] which were screened for antimicrobial and antimycobacterial activity. In concern to the objectives of the present research work relevant to green chemistry, this is the first attempt at an eco-friendly, multicomponent synthesis of the pyrazole-isoniazid hybrid and the study of antimycobacterial activity.

Results and discussion

In continuation of our efforts to design green, catalyst-free synthesis of heterocyclic compounds of biological and pharmaceutical benefits, using water, ethanol-water as a solvent initially we stirred 4-nitrobenzaldehyde (**1b**), malononitrile (**2**) and isoniazid (**3**) in an aqueous medium (Scheme 1) at rt for 5 h but only 71% conversion noted (Table 1, Entry 5) while in neat ethanol increased product yield of 80% was isolated in short reaction time (Table 1, Entry 1). Therefore, we chose the co-solvent systems of EtOH: H_2O for the selected model reaction. To examine the EtOH: H_2O system, the proportion of water increases from EtOH: H_2O (1:1) to (8:2) and (7:3). In the EtOH: H_2O (7:3) system the model catalyst-free, a multicomponent reaction took place in 1.15 h. with the highest yield of the product reached (Table 1, Entry 4). This solvent system could have expedited the protonation of nitrile N- atom and thereby the nucleophilic attack of amine on the C-atom of the nitrile group accelerating the ring closure to pyrazole (Scheme 2). The proposed mechanism for the synthesis of 5-amino-1-isonicotinoyl-3-(substituted phenyl)-1H-pyrazole-4-carbonitrile can be explained in Scheme 2. According to the proposed mechanism, olefin (**I**) is easily generated in the reaction mixture through the Knoevenagel condensation of aromatic aldehyde (**1**), malononitrile (**2**), isoniazid (**3**) is capable of readily reacting with olefin (**I**), leading to the formation of intermediate (**II**). A tautomeric proton shift within intermediate (**II**) facilitates the ring closure , resulting in the production of 2,3-dihydroisonicotinoylpyrazole (**IV**). Finally, atmospheric oxygen functions as an oxidizing agent, converting the 2,3-dihydroisonicotinoylpyrazole derivative into the corresponding isonicotinoylpyrazole (**4**). Consequently, it is possible that an alternative mechanism, the reaction between aryl aldehyde (**1**) and isoniazid (**3**) initially forms an imine (**III**). This imine (**III**) then reacts with malononitrile (**2**) resulting in the formation of intermediate (**II**) which ultimately converts to pyrazole (**4**) (Scheme 2).

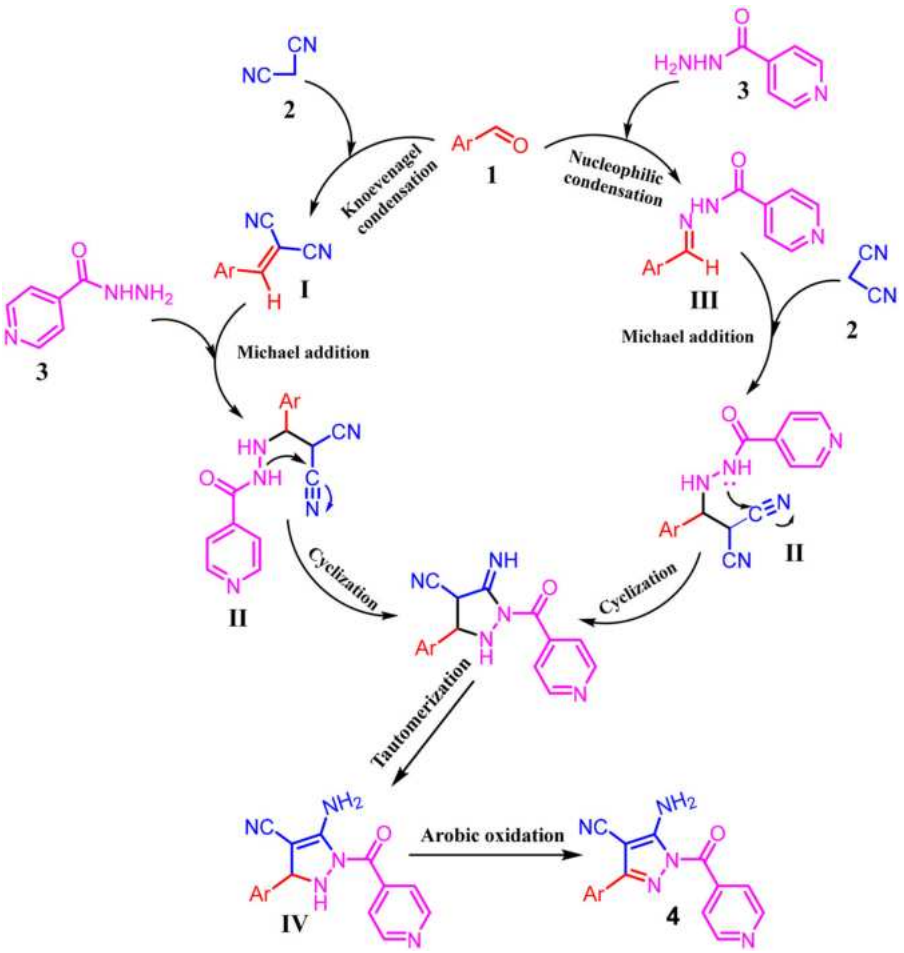


Scheme 1. Model reaction for optimization.

Table 1. Optimization of solvent for the synthesis of 5-amino-1-isonicotinoyl-3-(4-nitrophenyl)-1H-pyrazole-4-carbonitrile.[a]

Entry	Solvent	Time (h)	Yields[b] (%)
1.	EtOH	2	80
2.	EtOH: H_2O (1:1)	2:30	69
3.	EtOH: H_2O (8:2)	3	67
4.	EtOH: H_2O (7:3)	1:15	87
5.	H_2O	5	71

[a]Reaction Conditions: 4-Nitrobenzaldehyde(1 mmol), malononitrile(1 mmol), isoniazid(1 mmol) and solvent (5 ml) at 60 °C
[b]Isolated yield.



Scheme 2. Proposed Mechanism for the synthesis of 5-amino-1-isonicotinoyl-3-(substituted phenyl)-1H-pyrazole-4-carbonitrile.

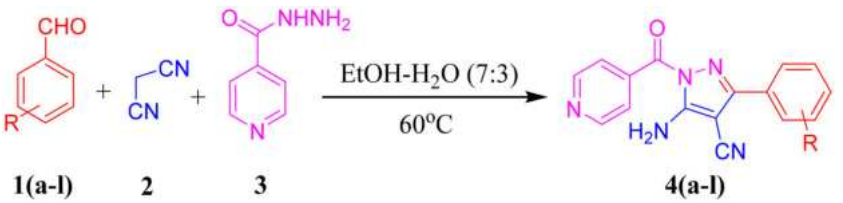
With the optimized co-solvent system in hand, we were interested to know the effect of temperature on the yield and ease of the model reaction. The reaction was executed at a different temperature, at 40 °C the product obtained an increased yield of 86% (Table 2, Entry 2) as compared to the reaction at rt. Accordingly, with the desire for improvement of yield the reaction was run at a temperature of 60 °C to our surprise in a short reaction time, the TLC showed an almost completed reaction with advancement in yield (Table 2, Entry 3). While the further increase of reaction temperature to 80 °C did not enhance the yield of the product.

Following the good results of the model reaction, the methodology was applied to the synthesis of a series of desired novel derivatives, 5-amino-1-isonicotinoyl-3-(substituted phenyl)-1H-pyrazole-4-carbonitrile (**4a–l**) using isoniazid (**3**), malononitrile (**2**) and different substituted aldehydes (**1a–l**) (Scheme 3). A noticeable substitution effect was observed for the reaction (Table 3), the aromatic aldehydes with the electron-withdrawing group reacted efficiently in a short reaction time of 2–3h. with excellent yields while the reaction of electron-releasing substituted aldehydes required a little more time to

Table 2. Study of the effect of temperature on reaction time and yields the for synthesis of 5-amino-1-isonicotinoyl-3-(4-nitrophenyl)-1H-pyrazole-4-carbonitrile.[a]

Entry	Temp (°C)	Time (hr)	Yields[b] (%)
1.	RT	10	81
2.	40	3	86
3.	60	1:15	87
4.	80	1:15	84

[a]Reaction Conditions: 4-Nitrobenzaldehyde(1 mmol), malononitrile(1 mmol), isoniazid(1 mmol) and EtOH-H_2O (7:3) 5 ml
[b]Isolated yield.



Scheme 3. Synthesis of 5-amino-1-isonicotinoyl-3- (substituted phenyl)-1H-pyrazole-4-Carbonitrile, **4a–l.**

complete in comparison. The products in all cases precipitated from the reaction mixture and were recrystallized from ethanol. The progress of the reaction was monitored by TLC plate using solvents ethyl acetate and hexane (8:2, v/v). The newly synthesized derivatives were isolated in excellent yields and were characterized by FT-IR, ^{1}H-NMR, ^{13}C NMR, and HRMS analysis.

Biological evaluation

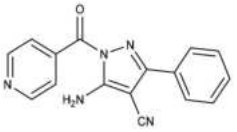
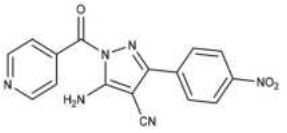
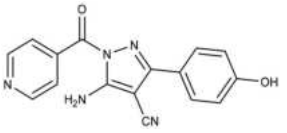
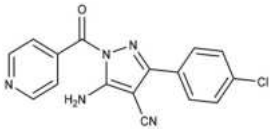
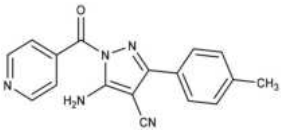
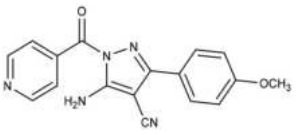
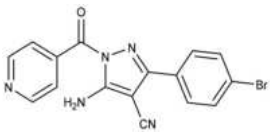
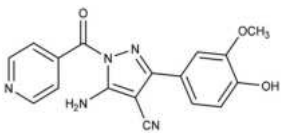
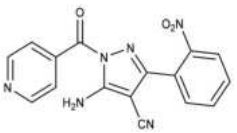
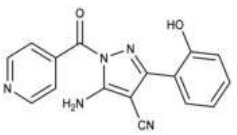
Antimicrobial activity

The synthesized compounds were screened for antibacterial and antifungal activities against various bacterial and fungal strains. The bacterial strains tested included *Staphylococcus aureus* NCIM 2079, *Bacillus subtilis* NCIM 2063 (both gram-positive), *Escherichia coli* NCIM 2109, and *Proteus vulgaris* NCIM 2172 (both gram-negative), while the fungal strains tested included *Candida albicans* NCIM 3471 and *Aspergillus niger* NCIM 1028. The standard drugs used for the antifungal assay were Chloramphenicol and Amphotericin-B. The compounds were tested using the disk diffusion method,[46,47] and the zones of inhibition were measured in millimeters (Table 4). The results showed that compounds **4a**, **4b**, **4d**, **4j**, and **4k** exhibited moderate antibacterial activity, while none of the compounds exhibited significant effects against *P. vulgaris*. All other compounds were inert against the bacterial strains tested. Compounds **4e**, **4f**, **4j**, and **4k** exhibited comparable activity in the antifungal assay.

Antioxidant activity

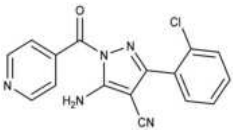
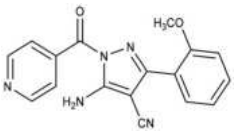
The antioxidant strength of the synthesized sample complexes was measured using the DPPH radical scavenging activity assay.[48,49] This was done by adding 100 μl of

Table 3. Synthesis of 5-amino-1-isonicotinoyl-3-(substituted phenyl)-1H-pyrazole-4- carbonitrile[a] **4a–l**.

Sr. No.	Entry	Product	Time(hr)	Yield[b](%)
1.	**4a**		2:30	81
2.	**4b**		1	87
3.	**4c**		3:30	85
4.	**4d**		3	89
5.	**4e**		3:45	84
6.	**4f**		4	80
7.	**4g**		1:30	92
8.	**4h**		4	88
9.	**4i**		4:15	82
10.	**4j**		4	90

(*continued*)

Table 3. Continued.

Sr. No.	Entry	Product	Time(hr)	Yield[b](%)
11.	**4k**		3	80
12.	**4l**		3:30	89

[a]Reaction Conditions: Substituted benzaldehyde(1 mmol), malononitrile(1 mmol), isoniazid (1 mmol) and EtOH-H_2O (7:3) 5 ml at 60 °C
[b]Isolated yield.

Table 4. Evaluation of antimicrobial activity of 5-amino-1-isonicotinoyl-3-(substituted phenyl)-1H-pyrazole-4- carbonitrile, **4a–l** by the disk diffusion method.

	Inhibition zones diameter/ mm					
	Gram − ve bacteria		Gram + ve bacteria		Fungi	
Compounds	*E. Coli* NCIM 2109	*P. vulgaris* NCIM 2172	*B. subtilis* NCIM 2063	*S. aureus* NCIM 2079	*C. albicans* NCIM 3471	*A. niger* NCIM 1028
4a	9.88	–	–	–	–	–
4b	9.05	–	–	8.86	–	–
4c	–	–	–	–	–	–
4d	–	–	9.55	–	–	–
4e	–	–	–	–	11.48	–
4f	–	–	–	–	7.09	–
4g	–	–	–	–	–	–
4h	–	–	–	–	–	–
4i	–	–	–	–	–	–
4j	11.67	–	16.24	11.32	–	6.58
4k	–	–	7.18	6.44	8.11	–
4l	–	–	–	–	–	–
Chloramphenicol (Standard)	30.08	21.14	35.21	26.67	NA	NA
Amphotericin-B (Standard)	NA	NA	NA	NA	16.69	17.50

Diameter in 'mm' calculated by Vernier Caliper. –:means no zone of inhibition; NA: Not applicable.

an ethanolic solution (0.5 mg/ml) of the sample to each well of a 96-well plate, followed by the addition of 100 μl of ethanolic DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) (0.2 mM). The plates were shaken for 2 min, then covered and incubated at 37 °C for 30 min. The absorbance of the reaction mixture was measured at 517 nm using an ELISA plate reader (Bio-Tek Instruments, USA). The assay mixture was prepared in triplicate and the mean absorbance was calculated. Vitamin C (0.5 mg/ml) was used as an antioxidant standard and positive control. The percentage scavenging (decolorization) of the free radical by the sample was determined using the following equation,

$$\%\ \text{inhibition of DPPH} = \left(\frac{Abs\ control - Abs\ sample}{Abs\ control}\right) \times 100$$

The compounds **4a–l** that were tested exhibited significant antioxidant activity. Compared to the standard, all the compounds showed strong antioxidant activity (Table 5). Specifically, among these compounds, **4f, 4c**, and **4l** demonstrated even more potent activity than the standard (Vitamin C).

Antitubercular activity

The inhibitory activity of compounds **4a–l** against *M. tuberculosis*, *M. abscessus*, *M. fortuitum*, and *M. chelonae* was evaluated using the Microplate Alamar Blue Assay (MABA).[50] The Minimum Inhibitory Concentration (MIC) of each compound was recorded in μg/mL (Table 6). Among the compounds tested, compound **4l** exhibited the highest activity against *M. tuberculosis* compared to Isoniazid and Rifampicin, which were the reference standards. In contrast, compound **4a–k** showed only moderate activity. However, all the compounds (**4a–l**) were inactive against *M. abscessus*, *M. fortuitum*, and *M. chelonae*.

Table 5. Evaluation of antioxidant activity of 5-amino-1-isonicotinoyl-3-(substituted phenyl)-1H-pyrazole-4-carbonitrile, **4a–l** by DPPH assay.

	Mean			
	Absorbance ($A_{517\,nm}$)			
	Concentration of Extract (0.5 mg/ml)			
Compound	T1	T2	T3	Mean
4f	0.386	0.369	0.388	0.381
4c	0.381	0.319	0.323	0.341
4h	0.287	0.275	0.214	0.258
4l	0.303	0.318	0.327	0.316
4j	0.248	0.213	0.226	0.229
Vitamin C	0.012	0.011	0.013	0.012

Table 6. Antitubercular activity (MIC) of 5-amino-1-isonicotinoyl-3-(substituted phenyl)-1H-pyrazole-4- carbonitrile, **4a–l** by MABA assay.

	MIC(μg/mL)			
Compounds	*M. tuberculosis*	*M. abscessus* ATCC 19977	*M. fortuitum* ATCC 6841	*M. chelonae* ATCC 35752
4a	0.25	>64	>64	>64
4b	0.5	>64	>64	>64
4c	0.5	>64	>64	>64
4d	0.25	>64	>64	>64
4e	0.25	>64	>64	>64
4f	0.25	>64	>64	>64
4g	0.25	>64	>64	>64
4h	0.5	>64	>64	>64
4i	0.5	>64	>64	>64
4j	8	>64	>64	>64
4k	0.5	>64	>64	>64
4l	0.125	>64	64	64
Standard drugs				
Isoniazid	0.03	NT	NT	NT
Rifampicin	0.03	NT	NT	NT
Streptomycin	0.5	NT	NT	NT
Ethambutol	1	NT	NT	NT
Levofloxacin	0.6	2	0.06	0.06
Amikacin	0.5	8	0.5	0.5

Table 7. Molecular docking interaction formed between Isoniazid derivatives and Protein 3k1D.

Sr. No	Name of ligand	Binding energy Kcal/mol	Interacting amino acids	Hydrogen bond
1	**4a**	−8.3	ASN 166 TRP 188	2
2	**4b**	−7.4	ASN 169	1
3	**4c**	−7.8	HIS 51 ASN 166 GLU 172	3
4	**4d**	−7.2	ARG 49 TYR 92	2
5	**4e**	−7.6	ASN 166 GLU 172	2
6	**4f**	−7.5	HIS 51 ASN 166 GLU 172	3
7	**4g**	−7.4	ARG 49 TYR 92	2
8	**4h**	−7.6	TYR 525 SER 303 LYS 486	3
9	**4i**	−8.9	ASN 167 TRY 92	2
10	**4j**	−7.9	ASN 166 GLU 172	2
11	**4k**	−7.9	ASN 166 GLN 443	2
12	**4l**	−7.5	ASP 367 HIS 365	2
13	Isoniazid	−5.6	TYR 398 ARG 325 HIS 133	3

Molecular docking study

The molecular docking study of all the synthesized novel derivatives was performed to recognize the binding affinity with the receptor enzyme, glgB from Mtb H37Rv. The inhibition of Mtb cell wall and glycolipid synthesis are the central focus of anti-TB drug research. Recent findings showed that the Mtb cells are surrounded by an α-glucan layer in the form of a capsule, involved in evading and virulence of Mtb by host-pathogen interaction.[51] Regulation of glycogen metabolism involves a complex mechanism, involving several synthase enzymes such as glycogen synthase A (glgA), glycogen branching enzyme (glgB), and catalytic enzyme (glgC). Mtb H37Rv encodes for an −1,4-glucan branching enzyme (MtbGlgB, EC 2.4.1.18, Uniprot entry Q10625). This enzyme belongs to the glycoside hydrolase (GH) family 13 and catalyzes the branching of a linear glucose chain during glycogenesis by cleaving a 1–4 bond and making a new 1–6 bond. In the current study, the isoniazid derivatives were studied by docking them with glycogen branching enzyme (glgB). Glycogen synthetase glgB (3k1D) crystal downloaded from PBD. Isoniazid is used for the treatment of tuberculosis (as part of combination therapy) or latent tuberculosis infection. The emergence of multidrug-resistant strains of *M. tuberculosis* accentuates the need to identify novel drug targets or new drugs for the treatment of tuberculosis, which could act against the tubercular bacilli that persist during prolonged therapy with currently available drugs isoniazid. The ligand-receptor interaction between isoniazid derivatives and the receptor (3k1D)[52] was carried out using a molecular docking technique by employing the Pyrx virtual screening software. The PyRx software [https://pyrx.sourceforge.io], is software used for the execution of virtual screening. PyRx uses AutoDock Vina and AutoDock 4.2 as docking software. Pymol and Protein-ligand interaction profiler was used to visualize and analyzed the docked results (Table 7).

Conclusion

The present research showcases a green, multicomponent method for the synthesis of novel 5-amino-1-isonicotinoyl-3-(substituted phenyl)-1H-pyrazole-4-carbonitrile derivatives using a one-pot, three-component reaction at a temperature of 60 °C without the need for a catalyst in aqueous ethanol. This process has several benefits, in terms of green chemistry views use of easily accessible starting materials, green reaction conditions, short reaction time, and high yields of the derivatives with a straightforward purification process without

column chromatography. All new isoniazid pyrazoles were evaluated for their antimicrobial, antitubercular, and antioxidant properties. Some of the compounds synthesized in the series, **4a–l** show moderate antibacterial and comparable antifungal properties, while all exhibit strong antioxidant activity. The molecular docking results showed that the compounds **4i** and **4a** have the highest binding energy, while all other compounds showed significant binding energy. The compound **4l** displays the most significant anti-TB activity against *M. tuberculosis*, with a MIC value of 0.125 µg/mL.

Experimental

Materials and methods

Sigma-Aldrich and Merck were the sources of all the reagents and solvents used. The reaction progress was monitored by carrying out thin layer chromatography (TLC) using silica gel plates with a 60 F_{254}. The synthesized derivatives melting points were measured using the open capillary method. The FT-IR spectra were recorded using an Alpha II Bruker spectrophotometer. DMSO-d_6 was used as a solvent to obtain ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra on a Bruker Advance Neo 500 MHz spectrometer The mass spectra were obtained using a Maldi-TOF Synapt XS HD Mass spectrometer.

General procedure for synthesis of 5-amino-1-isonicotinoyl-3-(substituted phenyl)-1H-pyrazole-4-carbonitrile (4a–l)

A mixture of aromatic aldehyde (1 mmol), malononitrile (1 mmol), and isoniazid (1 mmol) was stirred in aqueous ethanol (5 ml) at 60 °C. The progress of the reaction was observed using thin-layer chromatography (TLC) with a solvent mixture of ethyl acetate and hexane (8:2,v/v). When the reaction was completed, the mixture was poured into ice-cold water. The resulting precipitate was collected by filtration, washed with water, dried, and recrystallized with ethanol.

Spectral data of 5-amino-1-isonicotinoyl-3-(4-nitrophenyl)-1H-pyrazole-4-carbonitrile (4b). M.P. 250-252 °C; IR(ATR)υ_{max} Cm^{-1}: 3532, 3471 (NH_2), 2810 (CH Ar), 2226 (CN), 1677 (CO Amide),1501 (C = C Ar), 1265,1078 (C-N), 833 (Para disubstituted); ^{1}H NMR(500 MHz, DMSO-d_6): δ 12.35 (s,1H, NH), 8.81 (d, 2H, J = 5.75 Hz, Py-H), 8.5 (s, 1H, NH), 8.32 (d, 2H, J = 8.7 Hz, Ar-H), 8.03(d, 2H, J = 8.7 Hz, Ar-H), 7.84 (d, 2H, J = 5.8 Hz, Py-H); ^{13}C NMR, (125 MHz, DMSO-d_6): δ 161.87, 150.29, 147.96, 146.40, 140.17, 140.02, 136.19, 128.11,123.99, 121.45, 115.41, 112.25; HRMS (ESI) m/z calcd for $C_{16}H_{10}N_6O_3$ $[M+H]^+$: 335.0892, found: 335.0905.

Copies of HRMS ^{1}H, and ^{13}C NMR spectra of the synthesized compounds, along with characterization data are provided in the Supporting Information.

Acknowledgments

The author, Bharti P. Koli acknowledged the financial assistance awarded for the research work from Mahatma Jyotiba Phule Research Fellowship (MJPRF-2021), Nagpur, Maharashtra, and SAP Scheme of Govt. of India for the financial support involved in the research work. R. P. Gore and

P. R. Malasane are thankful to Ms. Deepali S. Ghorpade, Assistant Professor, Government College of Pharmacy, Amravati, Maharashtra for valuable discussion and input regarding docking study and SAIF, Punjab University, Chandigarh for spectral characterization. We are thankful to Dr. Mahesh Patil, Assistant Professor, Department of Microbiology, R. C. Patel A. S. C. College, Shirpur, Maharashtra, for the antimicrobial and antioxidant activities of the synthesized compounds. We are grateful to CSIR-Central Drug Research Institute, Lucknow for antitubercular activity.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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