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Full Paper

Design, Synthesis, and Characterization of Some Hybridized Pyrazolone Pharmacophore Analogs against *Mycobacterium tuberculosis*

Sivakumar Kullampalayam Krishnasamy[1], Vigneshwaran Namasivayam[2], Sincy Mathew[1], Ragavendran S. Eakambaram[1], Ibrahim A. Ibrahim[1], Adhirajan Natarajan[3], and Senthilkumar Palaniappan[1]

[1] Department of Pharmaceutical Chemistry, Medicinal Chemistry Research, KMCH College of Pharmacy, Kovai Estate, Coimbatore, India
[2] PharmaCenter Bonn, Pharmaceutical Chemistry I, Pharmaceutical Institute, Rheinische Friedrich-Wilhelms-University Bonn, Bonn, Germany
[3] Department of Pharmaceutical Biotechnology, KMCH College of Pharmacy, Kovai Estate, Coimbatore, India

Twenty-seven hybridized pyrazolone analogs were designed, docked, synthesized in two series and evaluated for their *in vitro* antimycobacterial properties. In the first series, four Schiff base derivatives, **6b**, **7b**, **7h**, and **7i**, show good antitubercular activity with minimum inhibition concentration (MIC) values in the range of 32.56–42.55 μM. In the second series, two compounds, **8b** and **8c**, possessed significant antitubercular activity with MIC <0.37 and <0.44 μM, respectively; they were even more potent than the standards pyrazinamide (12.99 μM), ciprofloxacin (4.82 μM), and streptomycin (5.36 μM), with a selectivity index of >630. Compounds **8b** and **8c** showed shikimate kinase inhibition activity at 5.84 and 6.93 μM, respectively. The activity and docking results lead to the conclusion that the compounds without double bond in the imine side chain and hydrophobic clashes at the pyrazolone end are necessary for good accommodation in the binding pocket and for imparting flexibility. All the compounds were also tested for antimicrobial activity (antibacterial and antifungal) and show highly significant activities against all the microorganisms tested.

Keywords: Antimycobacterial / *Mycobacterium tuberculosis* / Pyrazolone / Shikimate kinase inhibitor

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Introduction

New and re-emerging microbial infections continue to be a growing global health problem and potential bioterrorism threats around the world [1]. Microbial infection has exacerbated because of the presence of various complicating factors such as emergence of multidrug resistance, uncontrolled and inappropriate use of antibiotics, human immunodeficiency virus (HIV) co-infection, lack of patient compliance, long-term multiple-regimen chemotherapy, variable efficacy of vaccines, and newly discovered micro-organisms [2].

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), is declared as a global emergency by World Health Organization (WHO). Some harbor the bacterium without symptoms (latent TB), but few develop into active disease [3]. Of the new TB cases reported, 95% occur in developing

Correspondence: Dr. Senthilkumar Palaniappan, Department of Pharmaceutical Chemistry, Medicinal Chemistry Research, KMCH College of Pharmacy, Kovai Estate, Kalapatti Road, Coimbatore 641 048, India.
E-mail: drsenthilkumarp@gmail.com
Fax: +91422-2628645

countries every year. Currently, among the infected individuals, approximately eight million develop active TB, and almost two million die from this disease [4]. The recommended standard chemotherapeutic regimen for TB treatment is prescribed under directly observed treatment short-course (DOTS), lasting for minimum 6 months. Poor patient compliance can promote the emergence of drug resistance [5]. New chemical entities with novel mechanism of actions will possess potent sterilizing activity that will lead to shortening of the duration of chemotherapy [6].

Among the different mechanisms targeted for the development of novel TB drugs and other chemotherapeutic drugs, shikimate kinase is one of the potential targets. Shikimate kinase belongs to the family of nucleoside monophosphate (NMP) kinases. The NMP family is an important group of enzymes which catalyzes reversible phosphoryl transfer from nucleoside triphosphate to a specific nucleoside mono/diphosphate, and the product of the reaction is subsequently phosphorylated [7]. Shikimate kinase catalyzes adenosine triphosphate (ATP)-dependent phosphorylation of shikimate to form shikimate-3-phosphate. The shikimate pathway is present only in bacteria, fungi (in cytoplasm), and in plants, to synthesize the common precursors of essential aromatic amino acids (phenylalanine, tyrosine, and tryptophan) and secondary metabolites (phenyl propanoids and alkaloids) [8]. The absence of the pathway in all other genera has rendered the enzyme a useful target for the development of new chemotherapeutic agents.

There are various approaches to develop a new chemotherapeutic agent [9] and to create compound library. Besides the exploitation of new targets, there is another approach of merging two or more pharmacophores into a single molecule. These "merged" pharmacophores may be addressing the active site effectively and offer the possibility to overcome drug resistance. To achieve the target compound library, pyrazolone moiety was selected since pyrazolone derivatives are key structures for the development of new chemical class of chemotherapeutic agents. Keeping in view the antimicrobial potential of pyrazolone derivatives and as part of our ongoing development of new class of antitubercular and antimicrobial agents [10, 11], the pyrazolone was merged with another pharmacophore having potent antimicrobial properties. Literature showed that hydrazone (–NHN=C–) and amide (–CONH–) pharmacophores (compounds **I** and **II**; Fig. 1) demonstrated significant antimicrobial activity against various microorganism at low concentration levels [12, 13]. Consequently, the combination of pyrazolone ring containing aromatic-substituted group through –NH–CO–CH_2–NH–N=C linkage is a promising approach in drug-like molecule design (compound **III**; Fig. 1) in the current study. We report herein docking, synthesis of some pyrazolone derivatives by ecofriendly (microwave irradiation) method, which have been found to possess an interesting profile of antitubercular (TB) and antimicrobial activity as shikimate kinase inhibitor, with significant reduction in their cytotoxicity potency.

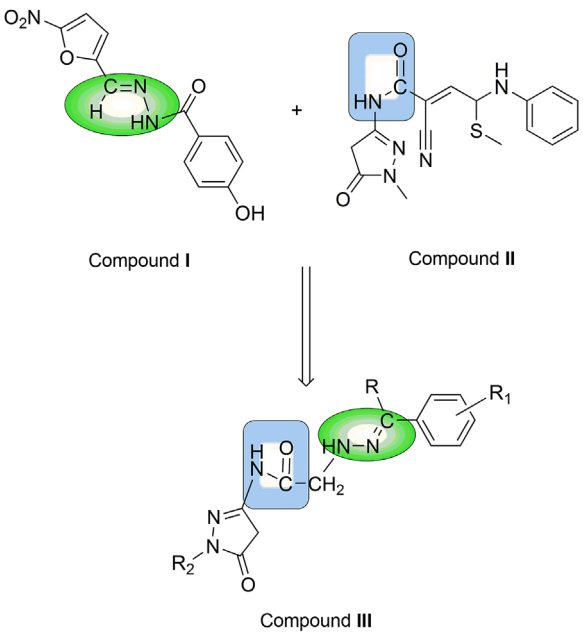


Figure 1. Designed hybridized common pyrazolone structure.

Results and discussion

Library of hybridized pyrazolone compounds was designed based on literature [12, 13] and our past research experience on pyrazolone nucleus [10, 11]. The designed hybridized pyrazolone compounds were screened against shikimate kinase [14, 15]. The mechanism of transferring phosphate group from ATP to shikimate by shikimate kinase is reported as three-dimensional structure in apo and complex state [16]. The shikimate kinase consists of three domains including SB (shikimate binding domain), a core domain containing highly conserved phosphate binding loop (P-loop), and the "lid", a highly flexible domain in open and closed conformation [17]. From the reported structures, the complex with ADP and shikimate was selected for the docking study because the conformations of SB domain in both the states were similar [18]. Among these three domains, the SB is responsible for large conformational changes and the analogs have shown inhibition of shikimate pathway [14, 19]. Shikimate is stabilized within the binding site through H-bond interactions with residues Asp 34, Arg 58, Gly 80, and Arg 136. Furthermore, residues Pro 11, Ile 45, Phe 49, Phe 57, Glu 61, Gly 79, Gly 81, Pro 118, and Leu 119 contributed within to form the binding site for shikimate (Fig. 2). Initial goal of the current study was to discover specific shikimate kinase inhibitors by *in silico* virtual screening of designed compound libraries. A library of designed compounds was docked onto the binding pocket (SB) of the shikimate kinase. The lowest free binding energy values of the designed molecules obtained from the docking studies are given in

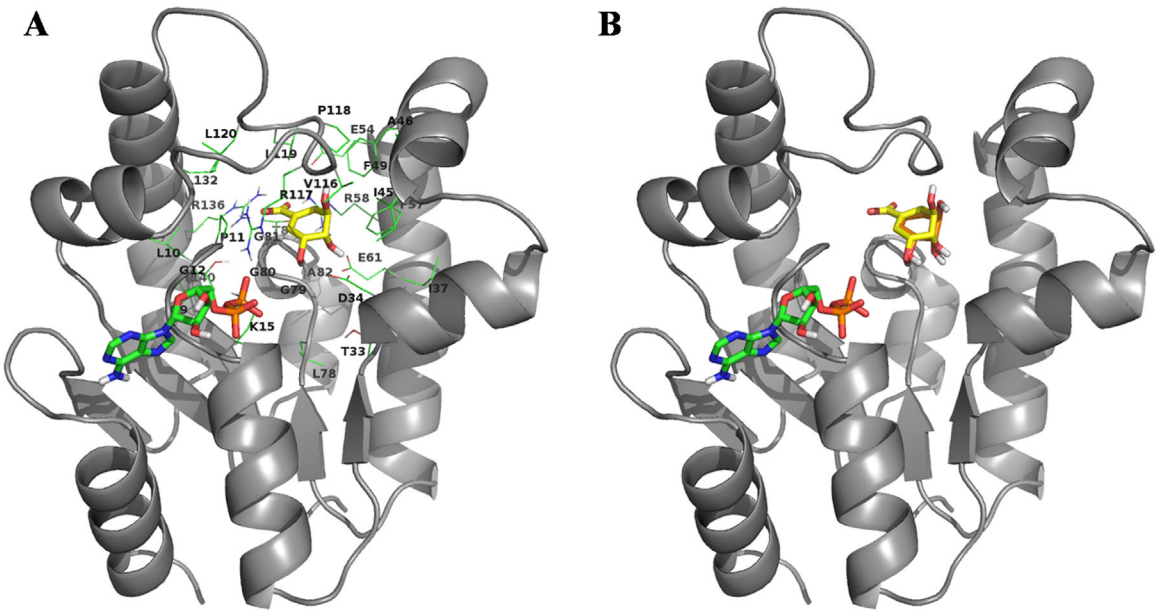


Figure 2. (A) The crystal structure of shikimate on the shikimate kinase along with ADP are shown in stick represenation. The amino acids within the binding pocket are shown in line representation and carbon atoms are colored in green. (B) Binding pose comparison of docked and crystal structure of shikimate on the shikimate kinase along with ADP. The crystal structure of shikimate kinase is represented in ribbon and colored in gray. Carbon atoms of AMP is colored in green, crystal structure of shikimate and docked pose are colored in yellow and orange, respectively. Oxygen atoms are colored in red, nitrogen atoms in blue, hydrogen in silver white, and phosphor atoms in orange.

Supporting Information Table S1. The binding energy of the compounds was found to be in the range from −6.06 to −9.49 kcal/mol. However, the binding poses generated for each compound were individually visualized for their interactions with the amino acids in the binding pocket. After analyzing the binding poses and interactions of the compounds, 22 molecules were selected in the first series for synthesis and biological testing. In addition to the lowest binding energy and interaction with amino acids at the pocket, for initial experiments, the ease of synthesis was also taken into consideration.

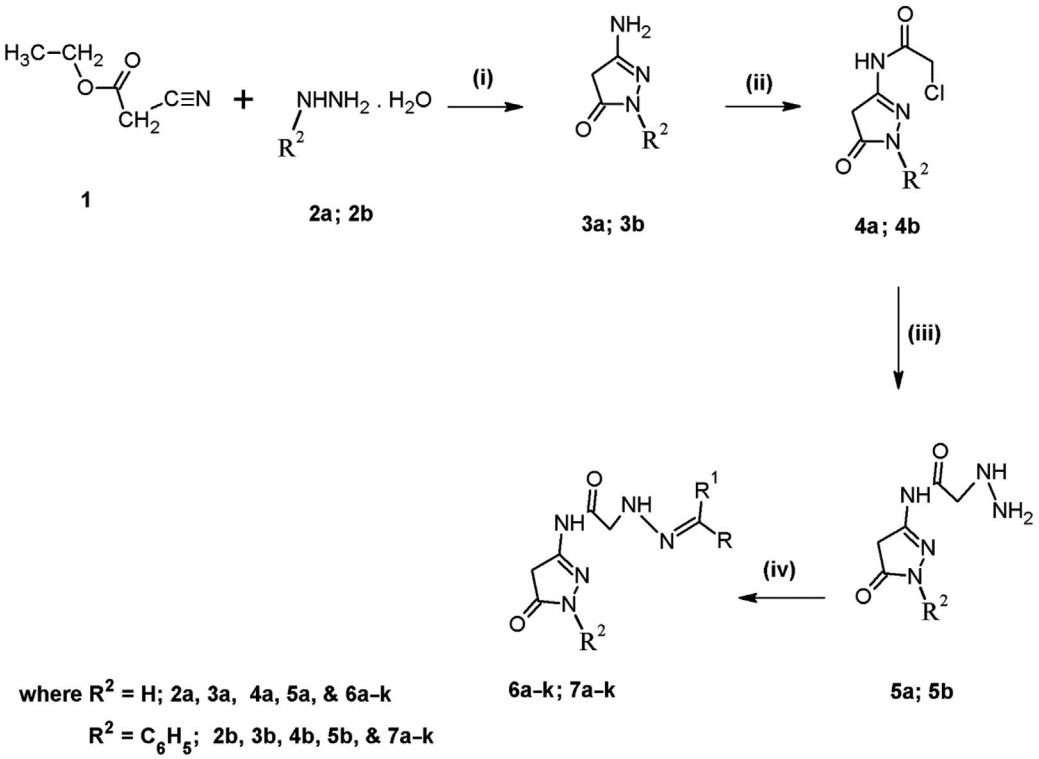
Chemistry

The synthetic pathway for the synthesis of the targeted compounds is illustrated in Schemes 1 and 2. In Scheme 1, the pyrazolones **3a**, **3b** were synthesized by cyclizing ethyl-cyanoacetate (**1**) with hydrazine hydrate (**2a**)/phenyl hydrazine hydrate (**2b**) under microwave-assisted condition. Compounds **3a** and **3b** were acetylated with chloroacetyl-chloride in the presence of triethylamine to yield compounds **4a** and **4b**. The intermediate hydrazide derivatives **5a** and **5b** were synthesized by reacting compounds **4a** and **4b** with hydrazine hydrate in the presence of magnesium sulfate in ethanol at 0–5°C. Schiff bases (**6a–k** and **7a–k**) were synthesized under microwave irradiation of various aromatic carbonyl compounds with intermediate compounds **5a** and **5b** in the presence of ethanol and glacial acetic acid (pH 4–5). A total of 22 compounds (**6a–k** and **7a–k**) were synthesized using Scheme 1. In Scheme 2, the free chlorine group at third position of pyrazolone ring of the intermediate compound **4a** was replaced with various aromatic amine by microwave irradiation in the presence of tetrahydrofuran and potassium carbonate to yield **8a–e**. A total of five compounds (**8a–e)** were synthesized using Scheme 2. Microwave-assisted techniques offer several advantages [10], more effective in perspective of environment, reaction time, high yields, ease of work-up, and isolation of products. The reaction progress and purity of the synthesized compounds was monitored by thin layer chromatography (TLC). The physicochemical properties of the synthesized compounds are tabulated in Table 1. Structures of the synthesized compounds (**6a–k**, **7a–k**, and **8a–e**) were established on the basis of physicochemical, elemental analysis, and spectral data (IR, ^{1}H NMR, and mass). Furthermore, the titled compounds were confirmed by mass spectra (*m/z* values) and elemental analyses (C, H, N) were within ±0.4% of theoretical values (Supporting Information Table S2).

Pharmacology

Antimycobacterial activity and docking

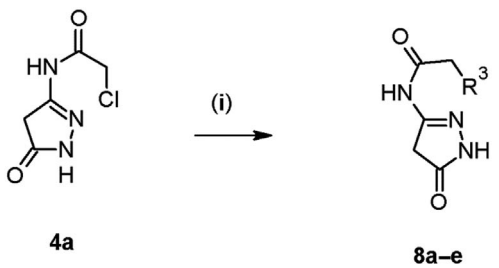
All the 22 compounds in the first series (**6a–k** and **7a–k**) were tested for *in vitro* antitubercular activity by microplate alamar



Reagents and conditions: (i) C_2H_5OH, MWI, 80°C, 5 min; (ii) THF, TEA, $ClCOCH_2Cl$, stirr 5 h;
(iii) $NH_2NH.H_2O$, $MgSO_4$, 0°C, stirr 8 h; (iv) aromatic aldehydes/ketones, C_2H_5OH, CH_3COOH, MWI, 75°C, 5–7 min.

Scheme 1. The synthesis of selected compounds (**6a–k** and **7a–k**).

blue assay (MABA). All the tested compounds inhibited MTB with MIC in the range of 32.56–100.32 μM. Among the compounds, **6b**, **7b**, **7h**, and **7i** inhibited MTB with MIC of 42.55, 33.80, 32.56, and 34.02 μM, respectively. The compounds show inhibition of MTB which is evident from the MIC values (Table 2), but were not much promising. To improve the activity, compounds were further explored with the interactions obtained from docking which revealed that the compounds

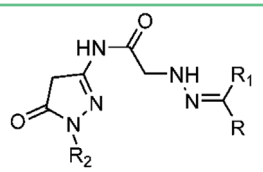


Reagents and conditions: (i) Aromatic amine, K_2CO_3, MWI, 80°C, 2–4 min.

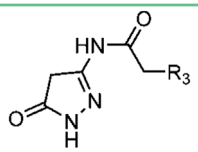
where R^3 = aniline; 4-chloro aniline; piperazine; 4-phenyl piperazine; 4-nitro aniline

Scheme 2. The synthesis of selected compounds (**8a–e**).

Table 1. Physicochemical data of title compounds (6a–k, 7a–k, and 8a–e).



6a–k; 7a–k



8a–e

Code	Structure				Mol. formula	Mol. weight	% Yield	M.p. (°C)	R_f value[a)]
	R	R^1	R^2	R^3					
6a	–H	-C_6H_5	–H	–	$C_{12}H_{13}N_5O_2$	259.22	75	182	0.51
6b	–H	4-ClC_6H_4	–H	–	$C_{12}H_{12}N_5O_2Cl$	293.72	74	201	0.55
6c	–H	2,4-diClC_6H_3	–H	–	$C_{12}H_{11}N_5O_2Cl_2$	328.21	80	214	0.58
6d	–H	-Furyl	–H	–	$C_{10}H_{11}N_5O_3$	249.18	88	160	0.54
6e	–H	2-OHC_6H_4	–H	–	$C_{12}H_{13}N_5O_3$	275.22	79	206	0.52
6f	–H	4-$OCH_3C_6H_4$	–H	–	$C_{13}H_{15}N_5O_3$	289.25	84	141	0.57
6g	–H	2,4-di$OCH_3C_6H_3$	–H	–	$C_{14}H_{17}N_5O_4$	319.27	71	148	0.55
6h	$–CH_3$	4-ClC_6H_4	–H	–	$C_{13}H_{14}N_5O_2Cl$	307.74	68	172	0.63
6i	$–CH_3$	4-FC_6H_4	–H	–	$C_{13}H_{14}N_5O_2F$	291.24	75	209	0.67
6j	$–CH_3$	2-OHC_6H_4	–H	–	$C_{13}H_{15}N_5O_3$	289.25	73	201	0.62
6k	$–CH_3$	4-$NO_2C_6H_4$	–H	–	$C_{13}H_{14}N_6O_4$	318.23	78	240	0.59
7a	–H	-C_6H_5	$–C_6H_5$	–	$C_{18}H_{17}N_5O_2$	335.32	73	189	0.65
7b	–H	4-ClC_6H_4	$–C_6H_5$	–	$C_{18}H_{16}N_5O_2Cl$	369.81	77	190	0.60
7c	–H	2,4-diClC_6H_3	$–C_6H_5$	–	$C_{18}H_{15}N_5O_2Cl_2$	404.31	81	204	0.64
7d	–H	-Furyl	$–C_6H_5$	–	$C_{16}H_{15}N_5O_3$	325.28	83	173	0.66
7e	–H	2-OHC_6H_4	$–C_6H_5$	–	$C_{18}H_{17}N_5O_3$	351.32	76	213	0.57
7f	–H	4-$OCH_3C_6H_4$	$–C_6H_5$	–	$C_{19}H_{19}N_5O_3$	365.34	79	153	0.51
7g	–H	2,4-di$OCH_3C_6H_3$	$–C_6H_5$	–	$C_{20}H_{21}N_5O_4$	395.36	81	158	0.61
7h	$–CH_3$	4-ClC_6H_4	$–C_6H_5$	–	$C_{19}H_{18}N_5O_2Cl$	383.84	77	185	0.68
7i	$–CH_3$	4-FC_6H_4	$–C_6H_5$	–	$C_{19}H_{18}N_5O_2F$	367.34	72	220	0.55
7j	$–CH_3$	2-OHC_6H_4	$–C_6H_5$	–	$C_{19}H_{18}N_5O_3$	364.34	82	207	0.58
7k	$–CH_3$	4-$NO_2C_6H_4$	$–C_6H_5$	–	$C_{19}H_{18}N_6O_4$	393.32	85	232	0.53
8a	–	–	–	Aniline	$C_{11}H_{12}N_4O_2$	232.21	86	102	0.61
8b	–	–	–	4-Chloroaniline	$C_{11}H_{11}N_4O_2Cl$	266.70	89	125	0.54
8c	–	–	–	Piperazine	$C_9H_{15}N_5O_2$	225.20	90	96	0.63
8d	–	–	–	Phenyl piperazine	$C_{15}H_{19}N_5O_2$	301.31	82	143	0.51
8e	–	–	–	4-Nitroaniline	$C_{11}H_{11}N_5O_4$	277.19	92	140	0.59

[a)] Solvent system: EtOAc/hexane (1:1).

are stabilized through hydrogen bonding interactions with the residues Lys 15, Asp 34, Ala 46, Arg 58, Gly 80, Gly 81, Arg 117, and Arg 136. Furthermore, residues Gly 9, Pro 11, Ser 16, Asp 32, Val 35, Glu 38, Ser 44, Ile 45, Phe 57, Arg 58, Glu 61, Gly 79, Gly 80, Ala 82, Val 116, Arg 117, and Leu 119 contribute within to form the binding site for the compounds (Figs 3 and 4). Re-examining closely the binding poses generated for the compounds at the active site exposes that the presence of double bond in the imine side chain and phenyl group at the end could alter the orientation of the compounds. Probably, the double bond imparts rigidity to the compound, reducing the flexibility, and the phenyl group introduces hydrophobic clashes during the binding process of compounds. This might be the reason for low activity of the compounds and the aryl ring at the side chain influences alteration into the interaction and not fitting into the binding pocket properly.

The above examination of the results suggested us to design second series of compounds without the double bond in the side chain and the phenyl group at the end, thereby reducing the rigidity and hydrophobicity to the compound. Subsequently, all the designed compounds were docked into the SB of shikimate kinase. The compounds showed an improvement in the fit onto the active site pocket wherein aryl ring in the side chain sliding perfectly into the loop as shown in Fig. 5, in a much better way than the compounds **6a–k** and **7a–k**. The compounds **8a–e** were stabilized within the binding site through hydrogen bonding with the residues Pro 11, Lys 15, Asp 32, Asp 34, Arg 58, and Arg 136. Furthermore, residues Leu 10, Gly12, Ser 16, Glu 38, Ile 45, Phe 57, Leu 78, Gly 79, Gly 80, Gly 81, Val 116, Arg 117, and Leu 119 contribute within to form the binding site for the compounds.

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Table 2. ***In vitro*** **antitubercular activity of the title compounds (6a–k, 7a–k, and 8a–e).**

Code	MTB MIC		Cytotoxicity CC_{50} (μM)	Selectivity index (SI)
	IC_{50} (μg/mL)	IC_{50} (μM)		
6a	25	96.44	NT	NT
6b	12.5	42.55	>665.25	15.63
6c	25	76.17	NT	NT
6d	25	100.32	NT	NT
6e	25	90.83	NT	NT
6f	25	86.43	NT	NT
6g	25	78.30	NT	NT
6h	25	81.43	NT	NT
6i	25	85.83	NT	NT
6j	25	86.43	NT	NT
6k	25	78.55	NT	NT
7a	25	74.55	NT	NT
7b	12.5	33.80	>653.39	19.33
7c	25	61.83	NT	NT
7d	25	76.85	NT	NT
7e	25	71.16	NT	NT
7f	25	68.42	>725.45	10.60
7g	25	63.23	>695.36	10.99
7h	12.5	32.56	>750.25	23.04
7i	12.5	34.02	>765.14	22.49
7j	25	68.61	>771.54	11.24
7k	25	63.56	>719.45	11.31
8a	12.5	48.22	>280.99	5.83
8b	<0.1	<0.37	>234.34	633.35
8c	<0.1	<0.44	>277.53	630.75
8d	6.25	20.74	>207.64	10.01
8e	3.13	11.29	>225.47	19.97
PYZ	1.6	12.99	>507.66	39.08
CFN	1.6	4.82	>188.62	39.13
SM	3.12	5.36	>107.47	20.05

YZ, pyrazinamide; CFN, ciprofloxacin; SM, streptomycin; NT, not tested.

Second series compounds (**8a–e**) were tested for *in vitro* antitubercular activity using MABA assay. As predicted, the compounds were found to possess excellent activity. The results are given in Table 2. Compounds **8b** and **8c** possessed the maximum activity with IC_{50} values less than 0.1 μg/mL (<0.37 and <0.44 μM, respectively). The compounds **8b** and **8c** were also found to be 35 and 30 times more potent than standard drug pyrazinamide (12.99 μM); 13 and 11 times more potent than ciprofloxacin (4.82 μM); 15 and 12 times more potent than streptomycin (5.36 μM), respectively. Compounds **8e** and **8d** showed moderate activity with IC_{50} values of 3.13 μg/mL (11.29 μM) and 6.25 μg/mL (20.74 μM), respectively. Further, shikimate kinase inhibition activity of the lead compounds **8b** and **8c** showed inhibition at 1.56 μg/mL (5.84 and 6.93 μM, respectively).

The structure–activity relationship (SAR) suggests the presence of electron rich group like Cl, NH_2, etc., at the terminal position of the molecule, essential for the antitubercular activity. Pyrazolone and amide carbonyl groups in the chain of the designed "merged pharmacophore" are necessary for the antitubercular activity and for binding at the active site of the enzyme. The phenyl group of the pyrazolone ring (**7a–k**) does not significantly contribute to the activity as compared with the one without phenyl group (**6a–k** and **8a–e**). In addition, the molecule interaction with the amino acid residues of Lys 15, Asp 34, Arg 58, and Arg 136 are required to stabilize into the binding pocket of SB domain.

Cytotoxicity

Among the 27 newly synthesized compounds, compounds showing ≤70 μM antitubercular activity were selected for *in vitro* cytotoxicity activity and the results are reported in Table 2. All the compounds showed CC_{50} values ranging from 207.64 to 780.99 μM. All the compounds tested were found to be safe up to 62.5 μg/mL, suggesting that their antimicrobial activity was not as a result of cytotoxicity.

Selectivity index [20] ($SI = CC_{50}/MIC$) was calculated (Table 2). According to Orme [21], new antimicrobial agents must have a selectivity index equal or higher than 10, with minimum inhibition concentration (MIC) lower than

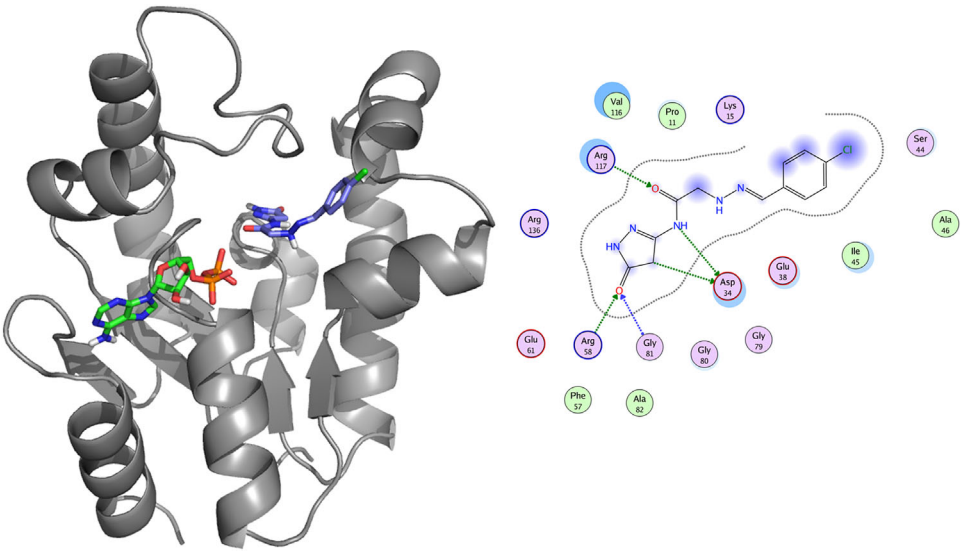

Figure 3. The docked pose and the 2D interaction diagram of compound **6b** at the SB of the shikimate kinase. The binding pose representation and other atoms are colored as described in Fig. 2.

6.25 μg/mL and a low cytotoxicity [21]. SI is used to estimate the therapeutic window of a drug and to identify drug candidates for further studies. In the current study, four pyrazolone derivatives (**8b–e)** with SI values of 633.35, 630.75, 10.01, and 19.97, respectively, display significant MIC, low cytotoxicity with good SI compared with standard first line or second line drugs (pyrazinamide, ciprofloxacin, and streptomycin) commonly used to treat TB. Standard drugs show MIC of 12.99, 4.82, and 5.36 μM; and SI of 39.08, 39.13, and 20.05, respectively. Thus, the identified pyrazolone derivatives are very promising newer antitubercular drug candidates.

Antimicrobial studies

Antimicrobial testing is indicated for any organism that contributes to an infectious disease warranting antimicrobial

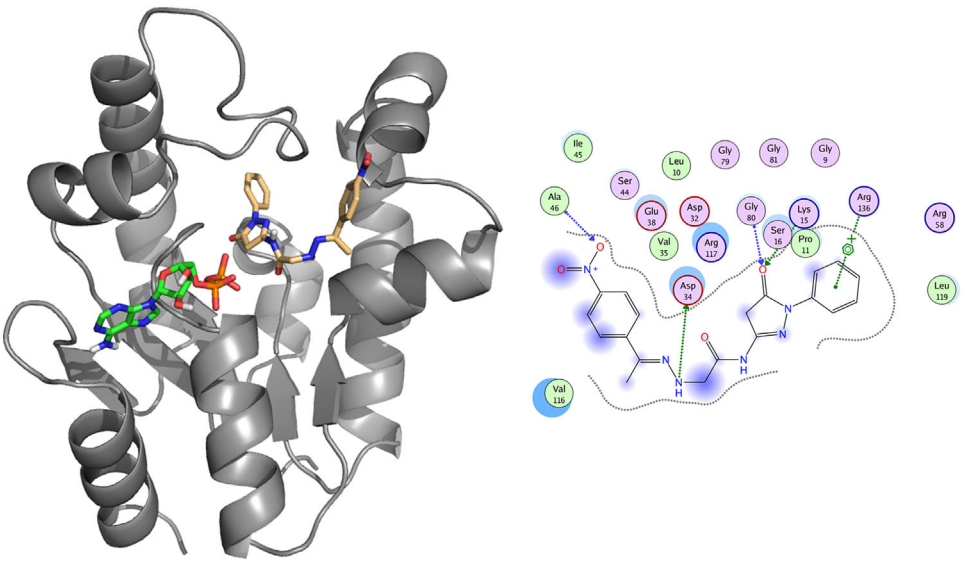

Figure 4. The docked pose and the 2D interaction diagram of compound **7k** at the SB of the shikimate kinase. The binding pose representation and other atoms are colored as described in Fig. 2.

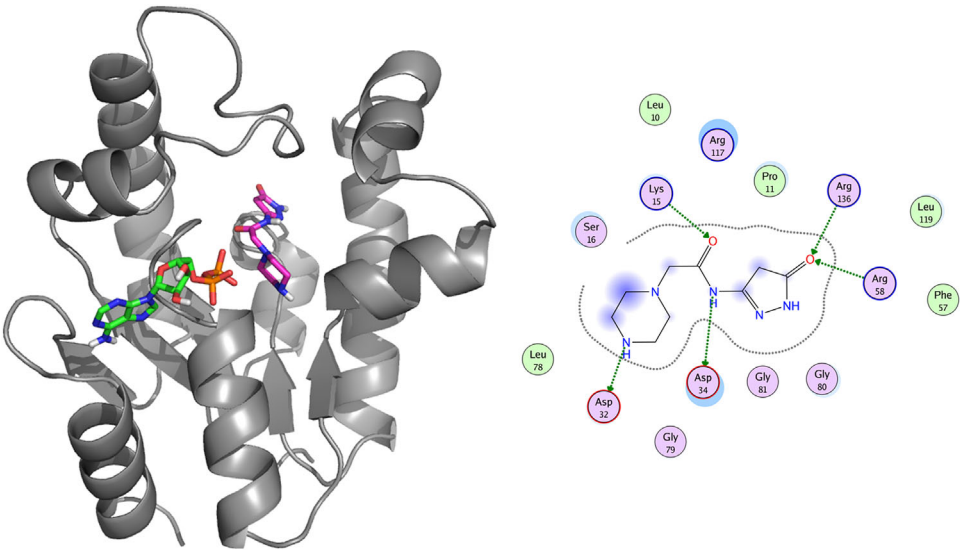

Figure 5. The docked pose and the 2D interaction diagram of compound **8c** at the SB of the shikimate kinase. The binding pose representation and other atoms are colored as described in Fig. 2.

chemotherapy, if its susceptibility cannot be reliably predicted from pre-existing antibiograms. The introduction of a variety of antimicrobials today makes it a necessity to perform antimicrobial susceptibility test, the result of which can guide us to the selection of appropriate antimicrobial drug with unquestionable benefits and least antimicrobial resistance.

All newly synthesized title compounds were screened for their preliminary antibacterial activity against three Gram-positive strains: *Micrococcus luteus*, *Staphylococcus aureus*, and *Staphylococcus albus*; three Gram-negative strains: *Escherichia coli*, *Klebsiella pneumoniae*, and *Vibrio cholerae*; and three fungal strains: *Candida albicans*, *Aspergillus fumigatus*, and *Aspergillus parasiticus* by disc diffusion method. The compounds, which exhibited zone of inhibition ($\geq$8 mm) in the primary screening at 50 μg/disc, were evaluated for MIC at 50–0.1953 μg/mL concentration level by twofold serial dilution method. The values of antimicrobial results are presented in Table 3.

Among the 11 compounds in the series **6a–k**, compound **6a** (unsubstituted aryl ring in the side chain) demonstrated excellent activity against *E. coli* with MIC of 1.56 μg/mL, also inhibits *S. aureus*, *K. pneumoniae*, *V. cholerae*, *A. fumigatus*, and *A. parasiticus* with MIC of 3.12 μg/mL concentration and inhibits *M. luteus*, *S. albus*, and *C. albicans* at 6.25 μg/mL. Among all the 11 compounds of series, compounds **6a**, **6b**, and **6i** possessed MIC of 3.12 μg/mL against *A. fumigatus* and *A. parasiticus*, and when compared with all other compounds in the same series, showed better activity. Compound **6f** demonstrated significant activity against *S. aureus*, *S. albus*, and *V. cholerae* with MIC 6.25 μg/mL. Compounds **6b**, **6d**, and **6k** exhibited MIC of 6.25 μg/mL against *K. pneumoniae* and compounds **6g**, **6i**, and **6k** inhibited *V. cholerae* at MIC 6.25 μg/mL. Overall, compound **6a** demonstrated broad spectrum of activity when compared to all the synthesized compounds in the series.

Among the 11 compounds of series **7a–k**, five compounds (**7a**, **7d**, **7g**, **7i**, and **7j**) inhibited *A. fumigates* at MIC as low as 3.12 μg/mL, four compounds (**7b**, **7e**, **7f**, and **7k**) showed significant antifungal activity against *A. fumigates* at MIC 6.25 μg/mL. Compounds **7b–e** and **7g** demonstrated significant fungal activity against *A. parasiticus* with MIC 6.25 μg/mL. Two of the eleven compounds (**7e** and **7g**) exhibited excellent antifungal activity against *C. albicans* with MIC as low as 1.56 μg/mL. Against *S. albus*, *K. pneumoniae*, and *V. cholerae* strains, five compounds (**7d–f**, **7i**, and **7k**) display significant activity with MIC 6.25 μg/mL. Among all the tested compounds, compound **7e** inhibited almost all the micro-organisms tested.

Compound **8b** demonstrated MIC of 1.56 μg/mL against all the screened Gram-positive and Gram-negative bacteria. On the other hand, compound **8b** demonstrated MIC of 1.56 μg/mL aganist Gram-positive bacteria. Compound **8a** shows significant antifungal activity against *A. fumigates*, *A. parasiticus*, and *C. albicans* at MIC 6.25 μg/mL. Compound **8e** also shows significant antifungal activity against *A. fumigates* and *C. albicans* with MIC 6.25 μg/mL.

The SAR of the molecules shows that the presence of phenyl ring at pyrazolone ring (R^2) has increased the antimicrobial activity of the molecule. The compounds (**7a–k**) with phenyl ring inhibited almost all the microorganisms tested as compared with unsubstituted compounds (**6a–k**). In the individual series, the compounds with electronegative element or molecules possessing electron rich group demonstrated significant activity. The order of activity for the groups attached to phenyl of the pyrazolone side chain can be given as Cl, F > NH, OCH_3 > OH > unsubstituted.

Table 3. ***In vitro*** **antimicrobial evaluation against bacteria and fungi for the synthesized compounds (6a–k, 7a–k, and 8a–e).**

Code	Minimum inhibition concentration (μg/mL)								
	M. luteus	*S. albus*	*S. aureus*	*E. coli*	*K. pneumoniae*	*V. cholerae*	*A. fumigates*	*A. parasiticus*	*C. albicans*
6a	6.25	6.25	3.12	1.56	3.12	3.12	3.12	3.12	6.25
6b	25.0	50.0	12.5	50.0	6.25	12.5	3.12	3.12	50.0
6c	50.0	25.0	25.0	25.0	12.5	25.0	12.5	6.25	25.0
6d	12.5	25.0	50.0	50.0	6.25	25.0	25.0	25.0	50.0
6e	12.5	50.0	50.0	12.5	25.0	12.5	12.5	12.5	25.0
6f	50.0	6.25	6.25	12.5	25.0	6.25	25.0	25.0	25.0
6g	25.0	25.0	12.5	12.5	12.5	6.25	25.0	6.25	50.0
6h	25.0	25.0	25.0	6.25	12.5	12.5	25.0	6.25	25.0
6i	50.0	12.5	12.5	12.5	12.5	6.25	3.12	3.12	25.0
6j	50.0	12.5	12.5	12.5	12.5	12.5	25.0	25.0	12.5
6k	12.5	12.5	25.0	25.0	6.25	6.25	3.12	3.25	12.5
7a	25.0	12.5	25.0	25.0	25.0	25.0	3.12	12.5	12.5
7b	12.5	12.5	12.5	12.5	12.5	12.5	6.25	6.25	6.25
7c	12.5	12.5	25.0	12.5	12.5	12.5	12.5	6.25	12.5
7d	12.5	6.25	12.5	6.25	6.25	6.25	3.12	6.25	50.0
7e	12.5	6.25	6.25	12.5	6.25	6.25	6.25	6.25	1.56
7f	25.0	6.25	6.25	50.0	6.25	6.25	6.25	12.5	50.0
7g	25.0	25.0	6.25	6.25	25.0	6.25	3.12	6.25	1.56
7h	25.0	6.25	25.0	6.25	12.5	25.0	3.12	12.5	6.25
7i	50.0	6.25	6.25	6.25	6.25	6.25	12.5	12.5	12.5
7j	50.0	12.5	12.5	12.5	12.5	12.5	3.12	25.0	12.5
7k	25.0	6.25	25.0	6.25	6.25	25.0	6.25	12.5	12.5
8a	12.5	12.5	12.5	25.0	25.0	12.5	6.25	6.25	6.25
8b	1.56	1.56	1.56	1.56	1.56	1.56	3.13	1.56	3.13
8c	1.56	1.56	1.56	3.13	3.13	3.13	3.13	3.13	3.13
8d	12.5	12.5	25.0	12.5	25.0	3.12	12.5	12.5	6.25
8e	25.0	50.0	25.0	25.0	50.0	25.0	6.25	12.5	6.25
Std	0.50*	0.20*	0.03*	0.05*	2.00*	0.02*	0.12**	0.20**	0.12**
Solvent	–	–	–	–	–	–	–	–	–

Std: *Ampicillin (bacteria), **fluconazole (fungi); solvent: DMSO; –: no inhibition.

Conclusion

All the designed and synthesized compounds showed promising antimycobacterial, antibacterial, and antifungal activities. Compounds **8a–e** were accommodating well into the SB pocket of shikimate kinase and also possessed good interaction with the amino acid residues at the binding pocket. Based on our modifications to the structure of the pyrazolone derivatives (docking interaction studies), thereby significant improvement in the antitubercular activity and by the shikimate kinase enzyme inhibition studies, it can be concluded that the compounds act by the response of the shikimate kinase enzyme inhibition. Further modifications of these lead molecules can be a promising candidate for the treatment of tuberculosis. Against antimicrobials, compounds **7a–k** and **8a–e** showed better antifungal activity compared with compounds **6a–k**.

Experimental

Docking studies

The protein-ligand docking studies were performed on the basis of crystal structure of shikimate kinase in complex with ADP and shikimate (2IYQ.pdb) [16]. The shikimate and solvent molecules were removed from the protein and polar hydrogen atoms were added using Molecular Operating Environment software package (MOE.2013.08). AutoDock 4.2 and AutoDockTools [22, 23] were used for flexible ligand docking into the protein structure and to add atomic partial charges. Three-dimensional scoring grids were computed within a user-specified three dimensional box of $60 \times 60 \times 60$ grid points with a spacing of 0.375 Å centered on the co-crystallized ligand shikimate. Fifty independent docking runs for the ligand molecules were run into the binding site of shikimate kinase using the Lamarckian Genetic Algorithm in AutoDock 4.2. All the other parameters were set to their default values. Docking simulations with Autodock 4.2 successfully reproduced the crystallographic pose of shikimate with an RMSD value of 0.75 Å (Fig. 2). On the basis of visual inspection of their interactions with the enzyme, high-scoring docking poses were selected as putative binding modes for the analysis and further designing of molecules.

Chemistry

Microwave synthesis was carried out using Biotage microwave initiator. The homogeneity of the compounds was monitored by ascending thin layer chromatography (TLC) on silica gel-G (Merck)-coated aluminum plates, visualized by iodine vapor and UV light. Melting points were determined in open end capillary tubes on a Buechi 530 m.p. apparatus and were uncorrected. Infrared (IR) and proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded for the compounds on JASCO FTIR Report 4100 (KBr) and Bruker Avance

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(300 MHz) instruments, respectively. Chemical shifts are reported in parts per million (ppm) using tetramethyl silane (TMS) as an internal standard. All exchangeable protons were confirmed by the addition of D_2O. ^{13}C NMR spectra were recorded on Bruker AC 200/DPX 400 MHz. The mass spectra were recorded on JEOL GCMATE instrument. The mass of the compounds are expressed in *m/z* values. Elemental analyses (C, H, and N) were undertaken with PerkinElmer model 240C analyzer and all analyses were consistent with theoretical values (within ±0.4%) unless indicated.

Please also see the Supporting Information for the InChI codes of the new compounds.

*Synthesis of 5-amino-2,4-dihydro-3H-pyrazol-3-one (**3a**) and 5-amino-2-phenyl-2,4-dihydro-3H-pyrazol-3-one (**3b**)*
Ethyl cyanoacetate (**1**; 0.01 mol) and hydrazine hydrate (**2a**; 0.01 mol)/phenyl hydrazine hydrate (**2b**; 0.01 mol) was taken in an Erlenmeyer flask and mixed thoroughly. The mixture was irradiated under microwave for 5 min at 80°C. The mixture was then poured into ice cold water. The crude product was filtered, dried, and recrystallized from ethanol.

*5-Amino-2,4-dihydro-3H-pyrazol-3-one (**3a**)*
Yield: 82%; m.p.: 85°C; IR (KBr) cm^{-1}: 3432 (broad, NH), 1708 (C=O ring str); ^1H NMR (DMSO-d_6) δ ppm: 10.32 (1H, s, NH–pyrazolone), 9.67 (2H, s, NH_2, D_2O exchangeable), 2.8 (2H, s, $–CH_2$ pyrazolone); Anal. ($C_3H_5N_3O$) C, H, N.

*5-Amino-2-phenyl-2,4-dihydro-3H-pyrazol-3-one (**3b**)*
Yield: 79%; m.p.: 92°C; IR (KBr) cm^{-1}: 3462 (broad, NH), 3116 (Ar–H str), 1692 (C=O ring str); ^1H NMR (DMSO-d_6) δ ppm: 10.12 (2H, s, NH_2, D_2O exchangeable), 6.8–7.6 (5H, m, Ar–H), 2.8 (2H, s, $–CH_2$ pyrazolone); Anal. ($C_9H_9N_3O$) C, H, N.

*Synthesis of 2-chloro-N-(5-oxo-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**4a**) and 2-chloro-N-(5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**4b**)*
To a solution of **3a**/**3b** (0.01 mol) in tetrahydrofuran (10 mL), added triethylamine (0.5 mL). The mixture was stirred properly and then added dropwise chloroacetylchloride (0.02 mol) and stirred for 5 h. Then poured into crushed ice with stirring. The solid separated is filtered and dried to give **4a**/**4b**.

*2-Chloro-N-(5-oxo-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**4a**)*
Yield: 86%; m.p.: 103°C; IR (KBr) cm^{-1}: 3420 (broad, NH), 1695 (C=ONH), 1554 (C=O str), 756 (C–Cl str); ^1H NMR (DMSO-d_6) δ ppm: 10.84 (H, s, NH, D_2O exchangeable), 9.52 (1H, s, NH–pyrazolone), 3.83 (2H, d, $COCH_2$–), 2.6 (2H, s, CH_2 pyrazolone); Anal. ($C_5H_6N_3O_2Cl$) C, H, N.

*2-Chloro-N-(5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)-acetamide (**4b**)*
Yield: 75%; m.p.: 117°C; IR (KBr) cm^{-1}: 3435 (broad, NH), 3114 (Ar C–H str), 1696 (C=ONH), 1557 (C=O str), 759 (C–Cl str); ^1H NMR (DMSO-d_6) δ ppm: 10.12 (2H, s, NH_2, D_2O exchangeable), 6.8–7.6 (5H, m, Ar–H), 4.78 (2H, d, CH_2CO), 2.9 (2H, s, $–CH_2$ pyrazolone); Anal. ($C_{11}H_{10}N_3O_2Cl$) C, H, N.

*Synthesis of 2-hydrazinyl-N-(5-oxo-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**5a**) and 2-hydrazinyl-N-(5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**5b**)*
The solution of product **4a**/**4b** (0.01 mol) with ethanol (10 mL) was added to a mixture of hydrazine hydrate (0.1 mol), magnesium sulfate (0.5 g) and ethanol (10 mL) maintained at 0°C. The reaction mixture was stirred for 8 h. Ethanol is then distilled and added to the crushed ice with stirring and extracted using ethyl acetate. Distilled ethyl acetate to yield compound **5a**/**5b**.

*2-Hydrazinyl-N-(5-oxo-4,5-dihydro-1H-pyrazol-3-yl)-acetamide (**5a**)*
Yield: 71%; m.p.: 121°C; IR (KBr) cm^{-1}: 3420, 3325 (NH str), 1568 (C=ONH), 1695 (C=O str); ^1H NMR (DMSO-d_6) δ ppm: 11.17 (H, s, NH, D_2O exchangeable), 9.08 (3H, s, $NHNH_2$, D_2O exchangeable), 3.61 (2H, d, $COCH_2$), 2.7 (2H, s, CH_2 pyrazolone); ^{13}C NMR (DMSO-d_6) δ ppm: 174.8, 172.1, 156.2, 73.1, 59.6; MS: *m/z* 171.16 $[M]^+$; Anal. ($C_5H_9N_5O_2$) C, H, N.

*2-Hydrazinyl-N-(5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**5b**)*
Yield: 76%; m.p.: 128°C; IR (KBr) cm^{-1}: 3420, 3325 (NH str), 3112 (Ar C–H str), 1568 (C=ONH), 1690 (C=O str); ^1H NMR (DMSO-d_6) δ ppm: 11.27 (H, s, NH, D_2O exchangeable), 9.12 (3H, s, NH–NH_2, D_2O exchangeable), 6.6–7.6 (5H, m, Ar–H), 3.82 (2H, d, $COCH_2$), 2.9 (2H, s, $–CH_2$ pyrazolone); ^{13}C NMR (DMSO-d_6) δ ppm: 174.3, 173.7, 154.9, 141.1, 130.7, 130.2, 125.9, 121.2, 120.9, 71.1, 53.3; MS: *m/z* 247.11 $[M]^+$; Anal. ($C_{11}H_{13}N_5O_2$) C, H, N.

*Synthesis of 2-[2-(substituted)hydrazinyl]-N-(5-oxo-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**6a–k**) and 2-[2-(substituted)hydrazinyl]-N-(5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**7a–k**)*
To a solution of substituted aldehydes/substituted ketones (0.01 mol) in ethanol (10 mL), added compound **5a**/**5b** (0.01 mol). To this added glacial acetic acid (0.2 mL) to maintain the pH between 4 and 5. The reaction mixture was irradiated with microwave for 5–7 min at 75°C. The solid obtained then was filtered and recrystallized from ethanol to give the compounds **6a–k**/**7a–k**.

*2-[2-Benzylidenehydrazinyl]-N-(5-oxo-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**6a**)*
IR (KBr) cm^{-1}: 3520 (broad, NH), 1658 (C=N str), 1567 (C=ONH), 1612 (C=O str); ^1H NMR (DMSO-d_6) δ ppm: 10.67 (2H, bs, 2NH, D_2O exchangeable), 9.12 (1H, s, NH– pyrazolone), 8.5 (s, 1H, =CH), 6.9–7.5 (5H, m, Ar–H), 3.92 (2H, d, $COCH_2$), 2.7 (2H, s, $–CH_2$ pyrazolone); ^{13}C NMR (DMSO-d_6) δ ppm: 175.3, 173.9, 153.2, 141.2, 132.7, 130.2, 128.9, 127.2, 71.1, 53.3; MS: *m/z* 259.26 $[M]^+$; Anal. ($C_{12}H_{13}N_5O_2$) C, H, N.

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*2-[2-(4-Chlorobenzylidene)hydrazinyl]-N-(5-oxo-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**6b**)*
IR (KBr) cm^{-1}: 3675 (broad, NH), 1733 (C=O str), 1684 (C=N str), 1521 (NHCO–R), 1089 (Ar C–Cl str); ^{1}H NMR (DMSO-d_6) δ ppm: 10.52 (2H, bs, 2NH, D_2O exchangeable), 9.15 (1H, s, NH pyrazolone), 8.7 (s, 1H, =CH), 6.9–7.9 (4H, m, Ar–H), 3.89 (2H, d, $COCH_2$), 2.9 (3H, s, CH_2 pyrazolone); ^{13}C NMR (DMSO-d_6) δ ppm: 175.7, 174.1, 153.6, 141.5, 132.4, 129.8, 128.3, 127.5, 72.1, 52.9; MS: *m/z* 293.71 $[M]^+$; Anal. ($C_{12}H_{12}N_5O_2Cl$) C, H, N.

*2-[2-(2,4-Dichlorobenzylidene)hydrazinyl]-N-(5-oxo-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**6c**)*
IR (KBr) cm^{-1}: 3675 (broad, NH), 2923 (Ar C–H str), 1684 (C=N str); 1539 (NHCO); 1090 (Ar C–Cl str); ^{1}H NMR (DMSO-d_6) δ ppm: 10.50 (2H, bs, 2NH, D_2O exchangeable), 9.09 (1H, s, NH pyrazolone), 8.5 (s, 1H, =CH), 6.6–7.8 (3H, m, Ar–H), 3.82 (2H, d, $COCH_2$), 2.8 (2H, s, CH_2 pyrazolone); ^{13}C NMR (DMSO-d_6) δ ppm: 175.5, 173.7, 156.2, 143.2, 138.3, 136.1, 132.9, 131.6, 132.1.2, 127.1, 71.9, 53.2; MS: *m/z* 328.15 $[M]^+$; Anal. ($C_{12}H_{11}N_5O_2Cl_2$) C, H, N.

*2-[2-[1-(Furan-3-yl)ethylidene]hydrazinyl]-N-(5-oxo-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**6d**)*
IR (KBr) cm^{-1}: 3648 (NH str), 3122 (Ar C–H str), 1653 (C=N str), 1539 (NHCO str); ^{1}H NMR (DMSO-d_6) δ ppm: 10.55 (2H, bs, 2NH, D_2O exchangeable), 9.11 (1H, s, NH pyrazolone), 8.7 (s, 1H, =CH), 6.5–7.4 (3H, m, furan), 3.89 (2H, d, $COCH_2$), 2.65 (2H, s, CH_2 pyrazolone); ^{13}C NMR (DMSO-d_6) δ ppm: 174.8, 173.8, 156.1, 150.2, 143.7, 135.2, 110.2, 109.1, 72.1, 53.8; MS: *m/z* 249.23 $[M]^+$; Anal. ($C_{10}H_{11}N_5O_3$) C, H, N.

*2-[2-(2-Hydroxybenzylidene)hydrazinyl]-N-(5-oxo-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**6e**)*
IR (KBr) cm^{-1}: 3648 (NH str), 3132 (OH bend), 1653 (C=N str), 1540 (NHCO str); ^{1}H NMR (DMSO-d_6) δ ppm: 10.61 (2H, bs, 2NH, D_2O exchangeable), 9.13 (1H, s, NH pyrazolone), 8.7 (1H, s, Ar–OH), 8.2 (s, 1H, =CH), 6.4–7.79 (4H, m, Ar–H), 3.79 (2H, d, $COCH_2$), 2.62 (2H, s, CH_2 pyrazolone); ^{13}C NMR (DMSO-d_6) δ ppm: 175.2, 173.5, 161.4, 155.4, 144.1, 133.2, 131.1, 121.9, 119.2, 117.1, 71.7, 53.9; MS: *m/z* 275.26 $[M]^+$; Anal. ($C_{12}H_{13}N_5O_3$) C, H, N.

*2-[2-(4-Methoxybenzylidene)hydrazinyl]-N-(5-oxo-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**6f**)*
IR (KBr) cm^{-1}: 3649 (NH str), 1684 (C=N str), 1603 (C=C str), 1540 (NHCO), 1258 (C–O–C str); ^{1}H NMR (DMSO-d_6) δ ppm: 10.59 (2H, bs, 2NH, D_2O exchangeable), 9.1 (1H, s, NH pyrazolone), 8.8 (s, 1H, =CH), 6.4–7.8 (4H, m, Ar–H), 3.8 (2H, d, $COCH_2$), 3.2 (3H, s, OCH_3), 2.62 (2H, s, CH_2 pyrazolone); ^{13}C NMR (DMSO-d_6) δ ppm: 175.3, 173.9, 163.2, 155.2, 143.3, 130.3, 126.7, 117.2, 71.9, 56.7, 54.3; MS: *m/z* 289.29 $[M]^+$; Anal. ($C_{13}H_{15}N_5O_3$) C, H, N.

*2-[2-(2,4-Dimethoxybenzylidene)hydrazinyl]-N-(5-oxo-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**6g**)*
IR (KBr) cm^{-1}: 3649 (NH str), 1683 (C=N str), 1623 (C=C str), 1558 (NHCO), 1269 (C–O–C str); ^{1}H NMR (DMSO-d_6) δ ppm: 10.61 (2H, bs, 2NH, D_2O exchangeable), 9.07 (1H, s, NH pyrazolone), 8.6 (s, 1H, =CH), 6.7–7.9 (4H, m, Ar–H), 3.9 (2H, d, $COCH_2$), 3.5 (6H, d, OCH_3), 2.62 (2H, s, CH_2 pyrazolone); ^{13}C NMR (DMSO-d_6) δ ppm: 175.3, 173.6, 164.9, 161.8, 155.7, 143.5, 129.9, 108.9, 107.2, 101.9, 71.1, 56.3, 53.3; MS: *m/z* 319.32 $[M]^+$; Anal. ($C_{14}H_{17}N_5O_4$) C, H, N.

*2-{2-[1-(4-Chlorophenyl)ethylidene]hydrazinyl}-N-(5-oxo-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**6h**)*
IR (KBr) cm^{-1}: 3648 (NH str), 1733 (C=O str), 1684 (C=N str), 1601 (C=C str), 1558 (NHCO), 1022 (Ar C–Cl str); ^{1}H NMR (DMSO-d_6) δ ppm: 10.61 (2H, bs, 2NH, D_2O exchangeable), 9.13 (1H, s, NH pyrazolone), 6.6–7.8 (4H, m, Ar–H), 3.82 (2H, d, $COCH_2$), 2.75 (2H, s, CH_2 pyrazolone), 2.2 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 175.3, 173.9, 169.2, 155.2, 137.7, 132.6, 131.9, 129.9, 73.1, 53.1.1, 14.3; MS: *m/z* 307.74 $[M]^+$; Anal. ($C_{13}H_{14}N_5O_2Cl$) C, H, N.

*2-{2-[1-(4-Fluorophenyl)ethylidene]hydrazinyl}-N-(5-oxo-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**6i**)*
IR (KBr) cm^{-1}: 3648 (NH str), 1733 (C=O str), 1683 (C=N str), 1558 (CONH), 1071 (C–F str); ^{1}H NMR (DMSO-d_6) δ ppm: 10.59 (2H, bs, 2NH, D_2O exchangeable), 9.07 (1H, s, NH pyrazolone), 6.4–7.58 (4H, m, Ar–H), 3.89 (2H, d, $COCH_2$), 2.72 (2H, s, CH_2 pyrazolone), 2.1 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 175.3, 173.9, 169.2, 167.5, 155.2, 132.6, 131.9, 129.9, 73.1, 53.1.1, 14.3; MS: *m/z* 291.11 $[M]^+$; Anal. ($C_{13}H_{14}N_5O_2F$) C, H, N.

*2-{2-[1-(2-Hydroxyphenyl)ethylidene]hydrazinyl}-N-(5-oxo-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**6j**)*
IR (KBr) cm^{-1}: 3649 (NH str), 3115 (OH bending), 1733 (C=O str), 1684 (C=N str), 1559 (CONH); ^{1}H NMR (DMSO-d_6) δ ppm: 10.55 (2H, bs, 2NH, D_2O exchangeable), 9.6 (1H, s, NH pyrazolone), 9.2 (1H, s, Ar–OH), 6.5–7.89 (4H, m, Ar–H), 3.79 (2H, d, $COCH_2$), 2.76 (2H, s, CH_2 pyrazolone), 2.2 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 175.6, 173.5, 169.2, 162.2, 155.7, 133.2, 131.6, 126.9, 117.2, 115.1, 73.1, 53.3, 15.2; MS: *m/z* 289.12 $[M]^+$; Anal. ($C_{13}H_{15}N_5O_3$) C, H, N.

*2-{2-[1-(4-Nitrophenyl)ethylidene]hydrazinyl}-N-(5-oxo-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**6k**)*
IR (KBr) cm^{-1}: 3648 (NH str), 1653 (C=N str), 1558 (amide str), 1521 (NO str); ^{1}H NMR (DMSO-d_6) δ ppm: 10.55 (2H, bs, 2NH, D_2O exchangeable), 9.11 (1H, s, NH pyrazolone), 6.8–7.7 (4H, m, Ar–H), 3.89 (2H, d, $COCH_2$), 2.65 (2H, s, CH_2 pyrazolone), 2.3 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 175.7, 173.5, 169.2, 151.2, 155.7, 141.2, 130.9, 121.9, 73.2, 53.3, 14.4; MS: *m/z* 318.11 $[M]^+$; Anal. ($C_{13}H_{14}N_6O_4$) C, H, N.

*2-[2-Benzylidenehydrazinyl]-N-(5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**7a**)*
IR (KBr) cm^{-1}: 3059 (Ar C–H str), 1693 (C=O str), 1522 (CONH); ^{1}H NMR (DMSO-d_6) δ ppm: 10.69 (2H, bs, 2NH, D_2O exchangeable), 8.4 (s, 1H, =CH), 6.9–7.5 (10H, m, Ar–H), 3.89 (2H, d, $COCH_2$), 2.5 (2H, s, CH_2 pyrazolone); ^{13}C NMR (DMSO-d_6) δ ppm: 175.3, 173.2, 153.7, 142.2, 140.7,

132.9, 130.9, 128.9, 128.2, 127.9, 123.8, 121.3, 70.1, 54.3; MS: *m/z* 335.14 $[M]^+$; Anal. ($C_{18}H_{17}N_5O_2$) C, H, N.

*2-[2-(4-Chlorobenzylidene)hydrazinyl]-N-(5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**7b**)*
IR (KBr) cm^{-1}: 3536 (NH str), 2942 (Ar C–H str), 1559 (NH–C=O str), 1089 (C–Cl str); ^1H NMR (DMSO-d_6) δ ppm: 10.61 (2H, bs, 2NH, D_2O exchangeable), 8.72 (s, 1H, =CH), 6.8–8.2 (9H, m, Ar–H), 3.76 (2H, d, $COCH_2$), 2.8 (3H, s, CH_2 pyrazolone); ^{13}C NMR (DMSO-d_6) δ ppm: 175.6, 173.1, 153.9, 143.1, 140.1, 134.4, 129.8, 129.5, 129.3, 128.6, 128.4, 128.3, 128.0, 124.5, 122.4, 122.1, 71.1, 53.9; MS: *m/z* 369.10 $[M]^+$; Anal. ($C_{18}H_{16}ClN_5O_2$) C, H, N.

*2-[2-(2,4-Dichlorobenzylidene)hydrazinyl]-N-(5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**7c**)*
IR (KBr) cm^{-1}: 3588 (NH str), 1601 (C=O str); ^1H NMR (DMSO-d_6) δ ppm: 10.58 (2H, bs, 2NH, D_2O exchangeable), 8.54 (s, 1H, =CH), 6.6–8.1 (8H, m, Ar–H), 3.62 (2H, d, $COCH_2$), 2.6 (2H, s, CH_2 pyrazolone); ^{13}C NMR (DMSO-d_6) δ ppm: 175.4, 172.9, 154.1, 143.3, 140.2, 134.6, 129.8, 129.5, 129.4, 128.7, 128.3, 128.1, 127.9, 125.2, 122.4, 122.1, 71.1, 53.9; MS: *m/z* 403.06 $[M]^+$; Anal. ($C_{18}H_{15}N_5O_2Cl_2$) C, H, N.

*2-{2-[1-(Furan-3-yl)ethylidene]hydrazinyl}-N-(5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**7d**)*
IR (KBr) cm^{-1}: 3406 (NH str), 2923 (Ar C–H str), 1582 (NHCO–R), 1138 (C–O str); ^1H NMR (DMSO-d_6) δ ppm: 10.55 (2H, bs, 2NH, D_2O exchangeable), 8.7 (s, 1H, =CH), 6.2–7.6 (8H, m, 3H furan and 5H Ar), 3.89 (2H, d, $COCH_2$), 2.65 (2H, s, CH_2 pyrazolone); ^{13}C NMR (DMSO-d_6) δ ppm: 175.3, 173.9, 153.2, 148.2, 143.4, 139.7, 135.2, 129.9, 129.6, 124.3, 120.9, 120.5, 111.4, 110.1, 69.5, 54.7; MS: *m/z* 325.12 $[M]^+$; Anal. ($C_{16}H_{15}N_5O_3$) C, H, N.

*2-[2-(2-Hydroxybenzylidene)hydrazinyl]-N-(5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**7e**)*
IR (KBr) cm^{-1}: 3796 (NH str), 3392 (OH bend), 1607 (C=C str), 1507 (C–N str); ^1H NMR (DMSO-d_6) δ ppm: 10.61 (2H, bs, 2NH, D_2O exchangeable), 8.9 (1H, s, Ar–OH), 8.1 (s, 1H, =CH), 6.3–7.7 (9H, m, Ar–H), 3.69 (2H, d, $COCH_2$), 2.72 (2H, s, CH_2 pyrazolone); ^{13}C NMR (DMSO-d_6) δ ppm: 174.4, 172.9, 160.9, 154.3, 143.2, 140.6, 133.8, 129.5, 129.4, 129.2, 124.9, 122.1, 121.9, 121.6, 119.4, 115.1, 69.1, 54.9; MS: *m/z* 351.13 $[M]^+$; Anal. ($C_{18}H_{17}N_5O_3$) C, H, N.

*2-[2-(4-Methoxybenzylidene)hydrazinyl]-N-(5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**7f**)*
IR (KBr) cm^{-1}: 3654 (NH str), 1683 (C=N str), 1567 (NHCO–R), 1253 (C–O–C str); ^1H NMR (DMSO-d_6) δ ppm: 10.68 (2H, bs, 2NH, D_2O exchangeable), 8.6 (s, 1H, =CH), 6.3–8.1 (9H, m, Ar–H), 3.8 (2H, d, $COCH_2$), 3.1 (3H, s, OCH_3), 2.62 (2H, s, CH_2 pyrazolone); ^{13}C NMR (DMSO-d_6) δ ppm: 174.2, 172.6, 162.9, 154.2, 143.1, 140.2, 130.8, 130.5, 129.4, 129.2, 126.0, 123.1, 121.9, 121.6, 115.4, 115.1, 69.1, 56.4, 54.1; MS: *m/z* 365.15 $[M]^+$; Anal. ($C_{19}H_{19}N_5O_3$) C, H, N.

*2-[2-(2,4-Dimethoxybenzylidene)hydrazinyl]-N-(5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**7g**)*
IR (KBr) cm^{-1}: 3437 (NH str), 1653 (C=N str), 1601 (C=C str), 1513 (CONH); ^1H NMR (DMSO-d_6) δ ppm: 10.51 (2H, bs, 2NH, D_2O exchangeable), 8.8 (s, 1H, =CH), 6.4–7.9 (8H, m, Ar–H), 3.9 (2H, d, $COCH_2$), 3.5 (6H, d, OCH_3), 2.81 (2H, s, CH_2 pyrazolone); ^{13}C NMR (DMSO-d_6) δ ppm: 174.2, 172.8, 162.8, 161.2, 154.2, 143.2, 140.3, 130.9, 130.1, 129.8, 124.3, 121.8, 121.1, 110.1, 107.1, 102.4, 69.1, 56.4, 56.1, 54.1; MS: *m/z* 395.16 $[M]^+$; Anal. ($C_{20}H_{21}N_5O_4$) C, H, N.

*2-{2-[1-(4-Chlorophenyl)ethylidene]hydrazinyl}-N-(5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**7h**)*
IR (KBr) cm^{-1}: 3385 (NH str), 2924 (Ar C–H str), 1607 (C=C str), 1091 (Ar C–Cl str); ^1H NMR (DMSO-d_6) δ ppm: 10.65 (2H, bs, 2NH, D_2O exchangeable), 6.7–8.2 (9H, m, Ar–H), 3.86 (2H, d, $COCH_2$), 2.85 (2H, s, CH_2 pyrazolone), 2.1 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 174.4, 172.9, 168.9, 154.3, 141.2, 136.6, 133.1, 129.5, 129.4, 129.1, 128.8, 128.5, 128.3, 124.6, 121.4, 121.1, 69.1, 54.9, 14.8; MS: *m/z* 383.11 $[M]^+$; Anal. ($C_{19}H_{18}N_5O_2Cl$) C, H, N.

*2-{2-[1-(4-Fluorophenyl)ethylidene]hydrazinyl}-N-(5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**7i**)*
IR (KBr) cm^{-1}: 3437 (NH str), 1595 (NHCO–R), 1496 (C=C ring str), 964 (C–F str); ^1H NMR (DMSO-d_6) δ ppm: 10.49 (2H, bs, 2NH, D_2O exchangeable), 6.6–8.1 (9H, m, Ar–H), 3.83 (2H, d, $COCH_2$), 2.76 (2H, s, CH_2 pyrazolone), 2.1 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 174.9, 172.5 167.8, 154.9, 141.1, 136.2, 133.6, 129.8, 129.5, 129.2, 128.8, 128.5, 128.2, 124.5, 121.6, 121.3, 69.5, 55.9, 14.2; MS: *m/z* 367.14 $[M]^+$; Anal. ($C_{19}H_{18}N_5O_2F$) C, H, N.

*2-{2-[1-(2-Hydroxyphenyl)ethylidene]hydrazinyl}-N-(5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**7j**)*
IR (KBr) cm^{-1}: 3400 (NH str), 2923 (OH bend), 1603 (C=C str); ^1H NMR (DMSO-d_6) δ ppm: 10.69 (2H, bs, 2NH, D_2O exchangeable), 9.8 (1H, s, Ar–OH), 6.6–7.8 (4H, m, Ar–H), 3.79 (2H, d, $COCH_2$), 2.76 (2H, s, CH_2 pyrazolone), 2.3 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 174.2, 172.9, 167.2, 162.1, 155.1, 141.0, 133.2, 130.8, 129.9, 129.5, 124.2, 121.8, 121.5, 121.2, 119.5, 116.6, 69.3, 55.6, 14.9; MS: *m/z* 365.15 $[M]^+$; Anal. ($C_{19}H_{19}N_5O_3$) C, H, N.

*2-{2-[1-(4-Nitrophenyl)ethylidene]hydrazinyl}-N-(5-oxo-1-phenyl-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**7k**)*
IR (KBr) cm^{-1}: 3675 (NH str), 1684 (C=N str), 1558 (NHCO–R str), 1521 (NO str); ^1H NMR (DMSO-d_6) δ ppm: 10.76 (2H, bs, 2NH, D_2O exchangeable), 6.8–8.2 (4H, m, Ar–H), 3.75 (2H, d, $COCH_2$), 2.65 (2H, s, CH_2 pyrazolone), 2.1 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 174.4, 172.3, 168.4, 155.6, 151.1, 141.0, 140.2, 130.4, 129.9, 129.6, 129.0, 124.5, 121.6, 121.1, 120.8, 120.7, 69.5, 55.2, 14.6; MS: *m/z* 394.14 $[M]^+$; Anal. ($C_{19}H_{18}N_6O_4$) C, H, N.

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*Synthesis of 2-[(substituted)]-N-(5-oxo-4,5-dihydro-1H-pyrazol-3-yl)acetamide (**8a–e**)*

To a solution of compound **4a** (0.01 mol) in tetrahydrofuran (10 mL), various substituted aromatic amines (0.01 mol) and potassium carbonate (0.1 g) are added. The reaction mixture was irradiated with microwave at 80°C for 2–4 min. The resultant mixture was poured into cold water with stirring. The solid separated was filtered, washed with water (3 × 20 mL), and dried. The crude compound was recrystallized using ethanol to give **8a–e**.

*N-(4,5-Dihydro-5-oxo-1H-pyrazol-3-yl)-2-(phenylamino)-acetamide (**8a**)*

IR (KBr) cm^{-1}: 3337 (pyrazolone N–H str), 3029 (Ar C–H str), 1694 (C=O str), 1618 (C=N str), 1588 (Ar C=C ring str), 1520 (–NHCOCH2–); ^{1}H NMR (DMSO-d_6) δ ppm: 11.12 (1H, s, NH pyrazolone), 10.4 (1H, s, NHCO, D_2O exchangeable), 9.2 (1H, s, Ar–NH, D_2O exchangeable), 6.8–7.5 (5H, m, Ar–H), 3.75 (2H, d, $COCH_2$), 2.65 (2H, s, CH_2 pyrazolone); ^{13}C NMR (DMSO-d_6) δ ppm: 174.4, 172.3, 156.1, 148.1, 131.4, 131.2, 118.8, 114.5, 114.2, 73.8, 55.2; MS: *m/z* 232.24 $[M]^+$; Anal. ($C_{11}H_{12}N_4O_2$) C, H, N.

*2-(4-Chlorophenylamino)-N-(4,5-dihydro-5-oxo-1H-pyrazol-3-yl)acetamide (**8b**)*

IR (KBr) cm^{-1}: 3332 (pyrazolone N–H str), 3022 (Ar C–H str), 1691 (C=O str), 1615 (C=N str), 1586 (Ar C=C str), 889 (Ar C–Cl str); ^{1}H NMR (DMSO-d_6) δ ppm: 11.16 (1H, s, NH pyrazolone), 10.2 (1H, s, NHCO, D_2O exchangeable), 9.3 (1H, s, Ar–NH, D_2O exchangeable), 6.9–7.8 (4H, m, Ar–H), 3.68 (2H, d, $COCH_2$), 2.62 (2H, s, CH_2 pyrazolone); ^{13}C NMR (DMSO-d_6) δ ppm: 174.3, 172.5, 155.9, 146.5, 130.6, 130.1, 125.1, 114.8, 114.5, 73.5, 55.6; MS: *m/z* 266.68 $[M]^+$; Anal. ($C_{11}H_{11}N_4O_2Cl$) C, H, N.

*N-(4,5-Dihydro-5-oxo-1H-pyrazol-3-yl)-2-(piperazin-1-yl)-acetamide (**8c**)*

IR (KBr) cm^{-1}: 3339 (pyrazolone N–H str), 3021 (Ar C–H str), 1689 (C=O str), 1612 (C=N str), 1574 (Ar C=C str); ^{1}H NMR (DMSO-d_6) δ ppm: 11.16 (1H, s, NH pyrazolone), 10.9 (1H, s, NHCO, D_2O exchangeable), 3.72 (2H, d, $COCH_2$), 2.96 (4H, t, CH_2 piperazine), 2.68 (2H, s, CH_2 pyrazolone), 2.34 (2H, t, CH_2 piperazine), 2.12 (2H, t, CH_2 piperazine); ^{13}C NMR (DMSO-d_6) δ ppm: 174.5, 172.3, 155.1, 73.5, 57.3, 55.8, 55.3, 46.1, 45.9; MS: *m/z* 225.25 $[M]^+$; Anal. ($C_9H_{15}N_5O_2$) C, H, N.

*N-(4,5-Dihydro-5-oxo-1H-pyrazol-3-yl)-2-(4-phenylpiperazin-1-yl)acetamide (**8d**)*

IR (KBr) cm^{-1}: 3334 (pyrazolone N–H str), 3026 (Ar C–H str), 1691 (C=O str), 1616 (C=N str), 1596 (Ar C=C str); ^{1}H NMR (DMSO-d_6) δ ppm: 10.84 (1H, s, NH pyrazolone), 10.2 (1H, s, NHCO, D_2O exchangeable), 6.5–7.8 (5H, m, Ar–H), 3.78 (2H, d, $COCH_2$), 3.36 (4H, t, CH_2 piperazine), 2.68 (2H, s, CH_2 pyrazolone), 2.41 (2H, t, CH_2 piperazine), 2.25 (2H, t, CH_2 piperazine); ^{13}C NMR (DMSO-d_6) δ ppm: 174.9, 173.3, 156.4, 150.6, 131.9, 131.1, 119.2, 115.4, 115.2, 73.2, 59.1, 53.9, 53.6, 50.7, 50.2; MS: *m/z* 301.34 $[M]^+$; Anal. ($C_{15}H_{19}N_5O_2$) C, H, N.

*2-(4-Nitrophenylamino)-N-(4,5-dihydro-5-oxo-1H-pyrazol-3-yl)acetamide (**8e**)*

IR (KBr) cm^{-1}: 3339 (pyrazolone N–H str), 3026 (Ar C–H str), 1689 (C=O str), 1620 (C=N str), 1593 (Ar C=C str), 1550 (Ar N=O str); ^{1}H NMR (DMSO-d_6) δ ppm: 11.12 (1H, s, NH pyrazolone), 10.3 (1H, s, NHCO, D_2O exchangeable), 9.6 (1H, s, Ar–NH, D_2O exchangeable), 6.5–7.9 (4H, m, Ar–H), 3.72 (2H, d, $COCH_2$), 2.58 (2H, s, CH_2 pyrazolone); ^{13}C NMR (DMSO-d_6) δ ppm: 174.4, 172.3, 155.6, 154.1, 138.0, 122.9, 122.4, 116.2, 115.8, 113.9, 113.5, 73.5, 56.2; MS: *m/z* 277.24 $[M]^+$; Anal. ($C_{11}H_{11}N_5O_4$) C, H, N.

Pharmacology

Antimycobacterial activity: Microplate Alamar Blue assay (MABA)

The antimycobacterial activity of compounds was assessed against MTB using MABA [24]. This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with proportional and BACTEC radiometric method. Briefly, 200 μL of sterile-deionized water was added to all outer perimeter wells of sterile 96 well plate to minimize evaporation of medium in the test wells during incubation. The 96 well plate received 100 μL of the Middlebrook 7H9 broth (HiMedia, Mumbai) and serial dilution of compounds. The final drug concentrations tested were 100–0.2 μg/mL. A 100 μL of *M. tuberculosis* inoculum (2 × 10^5 cfu/mL) was added to the wells. Plates were covered and sealed with parafilm and incubated at 37°C for 5 days. Then 50 μL of freshly prepared 1:1 mixture of Alamar Blue reagent and 10% Tween 80 was added to the plate and incubated for 24 h. A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth. The MIC was the lowest drug concentration which prevented the color change from blue to pink. In addition, a control and a blank, excluding the test compounds, were also performed with and without the organisms, respectively. The MIC is defined as the minimum concentration of compound required to give complete inhibition of bacterial growth.

Antimicrobial activity: Determination of zone of inhibition

A standard inoculum (1.5 × 10^8 cfu/mL 0.5 OD McFarland standards) was introduced onto the surface of sterile agar plates and a sterile cotton swab was used for even distribution of the inoculum. The discs measuring 6.25 mm in diameter were prepared from Whatman filter paper no. 1 and sterilized by dry heat at 140°C for 1 h. The sterile discs previously soaked in a known concentration (50 μg/10 μL) of the test compounds were placed on top of the nutrient agar medium. The plates were kept for diffusion at 4°C for 15 min. Then the plates were inverted and incubated for 24 h at 37 ± 1°C for bacteria and 72–96 h at 27 ± 1°C for fungi. After the incubation zone of inhibition was measured. The media used was nutrient agar medium and Sabouraud dextrose medium for antibacterial

and antifungal activities, respectively. Ampicillin (5 μg/disc) and fluconazole (5 μg/disc) were used as standard drugs for antibacterial and antifungal activities, respectively. Triplicates were maintained for all tested strains. Activity was determined by measuring the diameter (in mm) of the zone showing complete inhibition [25].

Antimicrobial activity: Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) in μg/mL of the title compounds was carried out by twofold serial dilution method. The test compounds were dissolved in dimethyl sulfoxide (DMSO) to obtain 0.5 mg/mL stock solution. Seeded broth (containing microbial spores) was prepared in nutrient broth (NB) from 24 h old bacterial cultures on nutrient agar at 37 ± 1°C while fungal spores from 1 to 7 days old Sabouraud agar slant cultures were suspended in Sabouraud dextrose broth (SDB). The colony forming units (cfu) of the seeded broth was determined by plating technique and adjusted in the range of 10^4–10^5 cfu/mL. The final inoculums size was 10^5 cfu/mL for antibacterial assay and $1.1–1.5 \times 10^2$ spores/mL for antifungal assay. Testing was performed at pH 7.4 ± 0.2 for bacteria and at a pH 5.6 for fungi. Exactly 0.4 mL of the solution of test compound was added to 1.6 mL of seeded broth to form the first dilution. One milliliter of this was diluted with a further 1 mL of seeded broth to give the second dilution and so on till eight of such dilutions are obtained. A set of assay tubes containing only seeded broth was kept as control. The tubes were incubated in BOD incubators at 37 ± 1°C for bacteria and 28 ± 1°C for fungi. The MICs were recorded through visual observations after 24 h (for bacteria) and 72–96 h (for fungi) of incubation. Ampicillin was used as standard for bacterial studies and fluconazole was used as standard for fungal studies. The lowest concentration at which there was no visible growth was taken as MIC [25, 26].

Shikimate kinase enzyme inhibition studies

The shikimate kinase enzyme inhibition studies were performed using the method described by Simithy et al. [14], with a slight modification. Test compounds at different concentrations viz., 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.10 μg/mL, and 15 nM of shikimate kinase were pre-incubated for 15 min in a micro-centrifuge tube containing 455 μL of assay buffer (100 mM Tris-HCl pH = 7.5, 50 mM KCl, and 5 mM $MgCl_2$) at 25°C. The reaction was initiated by the addition of an aqueous solution of shikimic acid and ATP (2 and 0.2 mM, final concentrations, respectively) and quenched after 5 min by the addition of 2 μL of 100% formic acid. The total volume of the reaction mixture was 500 μL. The presence of shikimate-3-phosphate after incubation indicates enzyme activity. The shikimate-3-phosphate in the final solution was determined by using HPLC (Supporting Information S1.1). Inhibitory concentration is the conc. at which the compounds inhibit the enzyme and the absence or decrease in the amount of shikimate-3-phosphate in the final solution.

Negative control experiments were performed in a similar manner, adding DMSO instead of test compounds. The concentration of DMSO in the functional assay was kept lower than 2% (v/v), a concentration that was found not to influence the enzymatic activity of mycobacterial shikimate kinase (*Mt*SK). In order to ensure reproducibility, all functional assays were performed in duplicate and analyzed twice. The inhibition curves were plotted and the IC_{50} value of each compound was calculated by fitting the data to a sigmoidal dose–response curve.

Cytotoxicity

Some compounds were further examined for toxicity (CC_{50}) in mammalian Vero cell line till 62.5 μg/mL concentration. After 72 h of exposure, viability was assessed on the basis of cellular conversion of MTT into a formazan product. MTT is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate dehydrogenase, cleaves the tetrazolium ring, converting MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells [27, 28]. The % cell inhibition was determined using the following formula:

$$\% \text{ Cell inhibition} = 100 - [\text{Abs(sample)}/\text{Abs(control)} \times 100]$$

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