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Original article

A regioselective synthesis of some new pyrazol-1′-ylpyrazolo[1,5-*a*]pyrimidines in aqueous medium and their evaluation as antimicrobial agents

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ABSTRACT

An efficient and environmental benign regioselective synthesis of some new pyrazol-1′-ylpyrazolo[1,5-*a*] pyrimidines (**7b**–**h**) has been accomplished *via* treatment of 3(5)-amino-5(3)-hydrazinopyrazole dihydrochloride (**5**) with several unsymmetrical 1,3-diketones (**6b**–**h**) using water as a solvent without any catalysts or additives. The structure of **7b**–**h** was established on the basis of rigorous analysis of ^{1}H, ^{13}C NMR, IR spectral data and MS. Eight compounds (**7a**–**h**) were screened for their antibacterial activity against two gram-positive and two gram-negative bacteria and compounds (**7a**, **b**, **d** and **e**) for antifungal activity against four phytopathogenic fungi. Compounds **7c** and **7e** manifest rather broad antibacterial activity than standard antibiotics. One lead compound, **7a** (10 mg/ml and 200 mg/ml) exhibited equipotent or more potent antifungal activity against all tested microorganisms than standard drug.

1. Introduction

Pyrazolo[1,5-*a*]pyrimidines are purine analogues and are used as antimetabolites in purine biochemical reactions [1]. They act as potent inhibitors of enzymes – 3′,5′-cyclic AMP phosphodiesterase [2], cyclooxygenase-2 (COX-2) [3], cyclin-dependent kinase 2 (CDK2) [4] and c-Src kinase [5]. They also possess affinity for estrogen [6], GABA, $GABA_A$/peripheral benzodiazepine receptors [7] besides being corticotropin-releasing factor (CRF) receptor antagonists [8]. The recent discovery of *N*-[3-(3-cyanopyrazolo[1,5-*a*]pyrimidin-7-yl) phenyl-*N*-ethylacetamide, Zaleplon, as an ideal hypnotic drug has stimulated further interest in the pyrazolo[1,5-*a*]pyrimidine chemistry [9]. A large number of pyrazolo[1,5-*a*]pyrimidine derivatives are reported to exhibit a broad spectrum of biological activities such as antitumor [10], anxiolytics [11] and antimicrobial [12].

The most versatile approach available for the synthesis of pyrazolo[1,5-*a*]pyrimidines consists of the condensation of bifunctional nucleophile 3-amino-1*H*-pyrazoles with bifunctional electrophiles [13] such as 1,3-diketones [6,14]. However, condensation of various symmetrical β-diketones (**2**) with a series of 5-amino-1*H*-pyrazoles (**1**) has been reported to result in the formation of pyrazolo[1,5-*a*] pyrimidines (**3**) and/or their structural isomers 1*H*-pyrazolo[3,4-*b*] pyridines (**4**) (Scheme 1) [15,16] depending upon the nature of substituents on β-diketones [15]. Solvent also plays a crucial role in controlling the structure of the product. For instance, heating **1** and **2** in acetic acid leads to the formation of a mixture of **3** and **4** whereas pyrazolopyrimidine **3** was formed as the only product when DMSO was used as solvent and reaction in ethanol in presence of triethylamine (TEA) afforded pyrazolopyridine **4** as the predominant product [16].

Very recently, we have reported the reaction of 3(5)-amino-5(3)-hydrazinopyrazole dihydrochloride (**5**) with pentane-2,4-dione (**6a**) in aqueous system which resulted in the exclusive formation of 2-(3′,5′-dimethylpyrazol-1′-yl)-5,7-dimethylpyrazolo[1,5-*a*]pyrimidine (**7a**) without a trace of 3-(3′,5′-dimethylpyrazol-1′-yl)-4,6-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine isomer (**8**) (Scheme 2) [17]. The structure of **7a** was unambiguously established on the basis of elemental analysis and spectral data (IR, high resolution ^{1}H NMR, MS). The characteristic chemical shifts of the methyl substituents at C_5, C_7 of pyrazolopyrimidine ring and $C_{3'}$ and $C_{5'}$ of pyrazole ring were established utilizing (^{1}H–^{13}C) HMQC as well as (^{1}H–^{13}C) and (^{1}H–^{15}N) HMBC measurements, thus solving the discrepancy existing in the literature about the position of methyl protons on pyrazolopyrimidine ring [2,3a].

Encouraged by these observations and in continuation with the work related to the synthesis, spectral studies and biological properties of pyrazolo[1,5-*a*]pyrimidines [18], we report herein the regioselective synthesis of some new substituted pyrazol-

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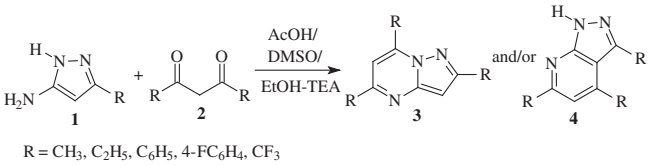


Scheme 1. Synthesis of compounds **3** and/or **4** in different solvents.

1′-ylpyrazolo[1,5-*a*]pyrimidines (**7b**–**h**) based on cyclocondensation of 3(5)-amino-5(3)-hydrazinopyrazole dihydrochloride (**5**) with unsymmetrical 1,3-diketones (**6b**–**h**) in water, with an aim to find more potent antimicrobial agents.

2. Chemistry

2.1. Synthesis

The starting compound, 3(5)-amino-5(3)-hydrazinopyrazole dihydrochloride (**5**) obtained by the condensation of hydrazine hydrate and malononitrile [19], is quite polar and encountered solubility problem in most organic solvents such as ethanol, chloroform, dichloromethane, tetrahydrofuran and acetonitrile. Attempts were made to react **5** with phenyl-1,3-butanedione (**6b**) in THF, CH_3CN and EtOH with or without NaOAc but no product could be isolated even when the reaction time was extended to 48 h and the starting material was recovered as such. Recently, aqueous mediated reactions have received considerable attention in organic synthesis due to environmental safety reasons. Keeping this in view, a series of experiments was accomplished with an objective to ascertain the reaction conditions: temperature, ratio of β-diketone to hydrazine dihydrochloride, amount of NaOAc and the solvent system (water as a solvent (entry 7) or co-solvent in conjunction with other organic solvents, e.g., THF, CH_3CN and EtOH (entries 1–6)) with phenyl-1,3-butanedione **6b** as a model compound (Table 1). TLC and ^1H NMR examination of the crude reaction mixture indicated the formation of three products namely: fused pyrazol-1′-ylpyrazolopyrimidine (**7b**), 3(5)-methyl-5(3)-phenyl-1*H*-pyrazole (**9b**) obtained by the C–N bond cleavage of **5** and benzoic acid (**10b**).

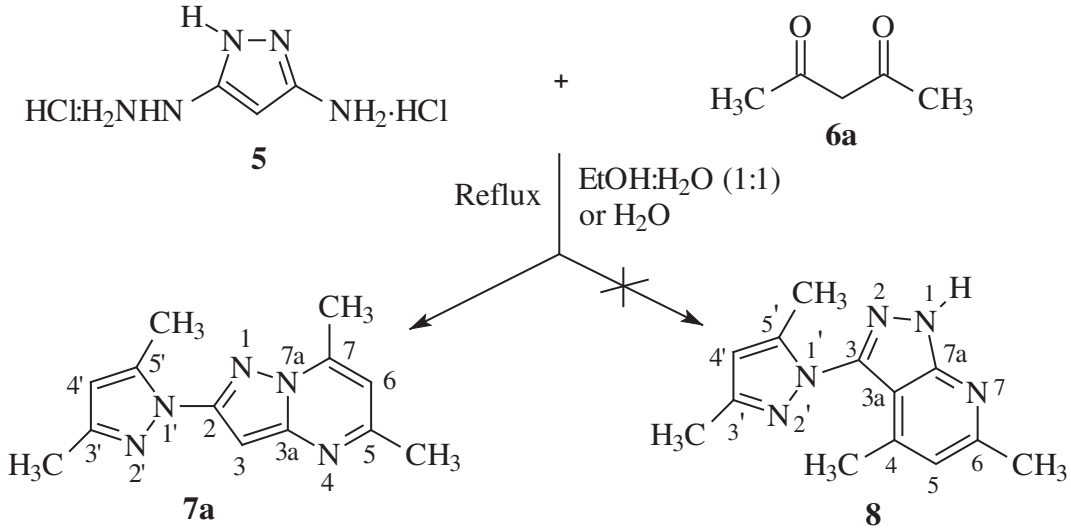
As evident from the Table 1, the optimum condition for the formation of fused pyrazol-1′-ylpyrazolopyrimidine (**7b**) was established as refluxing equimolar amounts of phenyl-1,3-butanedione with **5** in H_2O. Other β-diketones were also made to react under similar conditions to obtain corresponding pyrazolo[1,5-*a*]pyrimidines (**7b**–**f**) in excellent yields. It is worthy to note that C–N bond cleavage could be suppressed significantly when water was employed as the solvent (**9b** only 10%) and a minute amount of substituted benzoic acids (**10b**–**f**) (3–5%) was observed (Scheme 3). Thus, this route provides an effective, economic and environmentally friendly method to synthesize these fused pyrazoles.

Table 1
Optimization of reaction conditions for the reaction of 3(5)-amino-5(3)-hydrazinopyrazole dihydrochloride (**5**) with phenyl-1,3-butanedione (**6b**).[a]

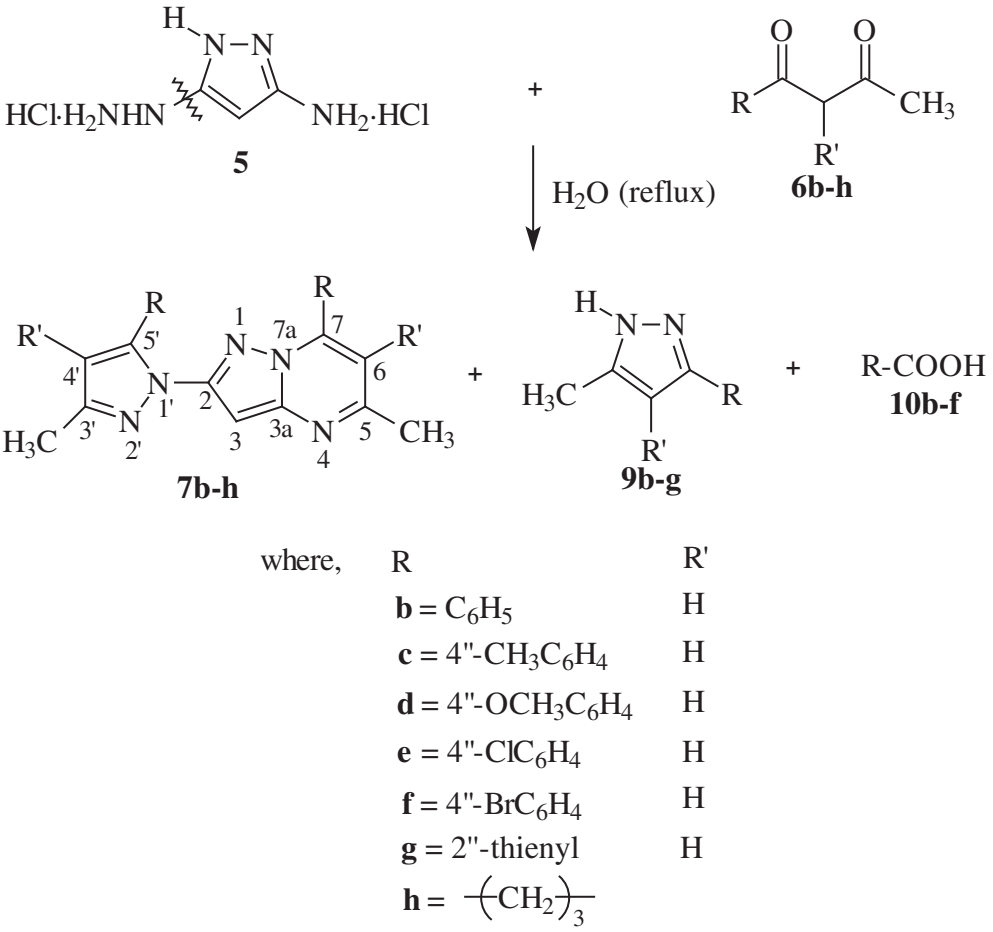
Entry	Solvent	**5** (equiv)	**6b** (equiv)	NaOAc (equiv)	Yield of **7b** (%)[b]	Yield of **9b** (%)[b]	Yield of **10b** (%)[b]
1	Acetonitrile/H_2O	1	1	–	9	91	[c]
2	THF/H_2O	1	1	–	8	82	10
3	EtOH/H_2O (3:2 v/v)	1	1	–	35	65	[c]
4	EtOH/H_2O (3:2 v/v)	1	1	1	35	65	[c]
5	EtOH/H_2O (3:2 v/v)	1	1	2	35	65	[c]
6	EtOH/H_2O (3:2 v/v)	1	2	2	35	65	[c]
7	H_2O	1	1	–	85	10	5
8	H_2O + acetone[d] (2:5 v/v)	1	1	–	8[d]	32[d]	[c]

[a] Reaction conditions: reflux for 4 h.
[b] Analysis of crude reaction product based on ^1H NMR.
[c] Yield not determined.
[d] Hydrazone of acetone is obtained in 60% yield as another product of this reaction through C–N bond cleavage.

All the products were purified by column chromatography using petroleum ether/chloroform (0–50% gradient) as eluent and ratio of different products was measured by ^1H NMR spectroscopy of the crude reaction mixture. To further widen the scope of this regioselective synthesis, 1-(2″-thienyl)-1,3-butanedione (**6g**) and 2-acetylcyclopentanone (**6h**) were made to react with **5** under the same experimental conditions. While the reaction with **6g** afforded 2-(3′-methyl-5′-(thien-2″-yl)pyrazol-1′-yl)-5-methyl-7-(thien-2″-yl)pyrazolo[1,5-*a*]pyrimidine (**7g**) and 3-methyl-5-(2-thienyl)-1*H*-pyrazole (**9g**); reaction with **6h** afforded 2-(3′-methyl-5′,6′-dihydro-4*H*-cyclopenta[*d*]pyrazol-1′-yl)-5-methyl-7,8-dihydro-6*H*-cyclopenta[*g*]pyrazolo[1,5-*a*]pyrimidine (**7h**) as an exclusive product without any trace of cleaved pyrazole and carboxylic acid (Scheme 3).



Scheme 2. Synthesis of **7a**.



Scheme 3. Synthesis of title compounds **7b**–**h**.

2.2. Results and discussion

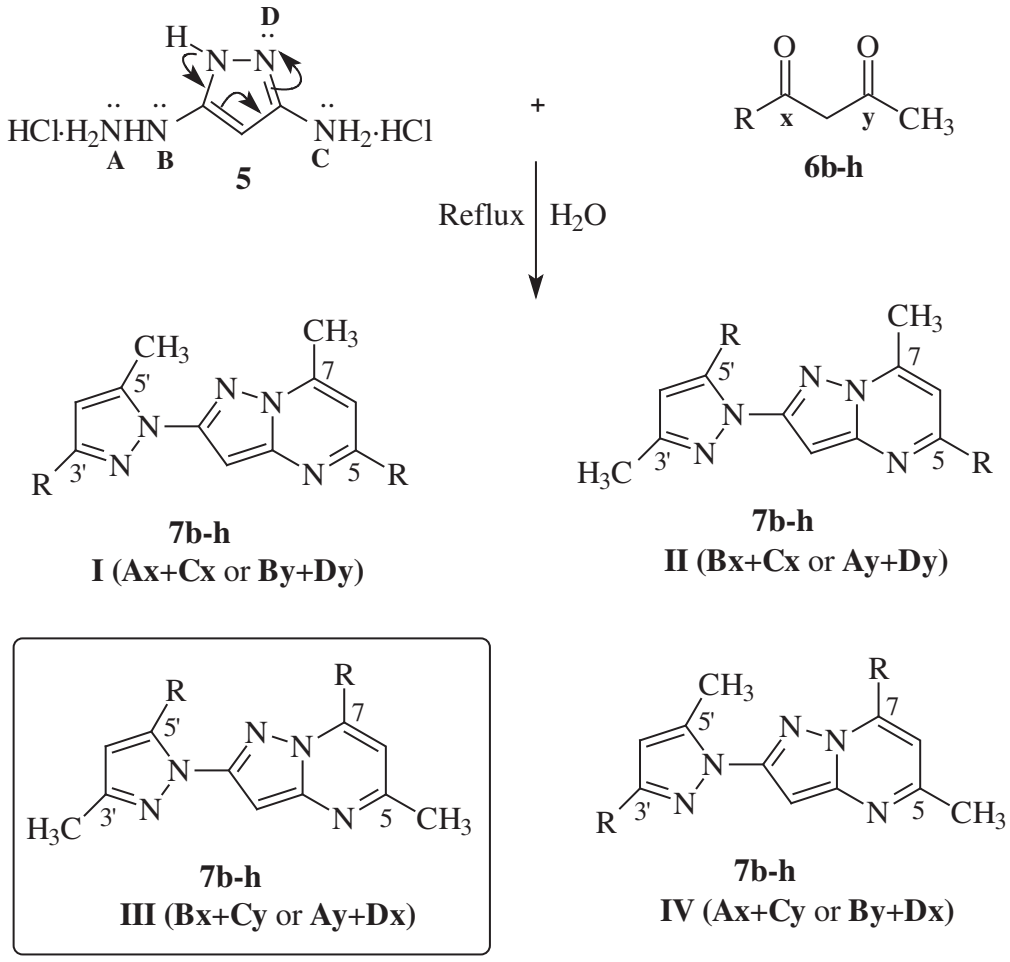
In principle, the reaction of 3(5)-amino-5(3)-hydrazinopyrazole dihydrochloride (**5**) with unsymmetrical β-diketones (**6b**–**h**) might result in the formation of four regioisomeric pyrazol-1′-ylpyrazolo [1,5-*a*]pyrimidines – (3′-(*R*)-5′-methylpyrazol-1′-yl-5-(*R*)-7-methyl-) (**I**), (3′-methyl-5′-(*R*)pyrazol-1′-yl-5-(*R*)-7-methyl-) (**II**), (3′-methyl-5′-(*R*)pyrazol-1′-yl-5-methyl-7-(*R*)-) (**III**) and (3′-(*R*)-5′-methylpyrazol-1′-yl-5-methyl-7-(*R*)-) (**IV**) due to competitive reactivities of different nucleophilic sites (**A**, **B**, **C** and **D**) and electrophilic centers (**x** and **y**) through the pathways depicted in Scheme 4, however, only one isomer was obtained during the present investigation.

The fused product was unambiguously characterized as structure **III** by a combined application of ^{1}H and ^{13}C NMR spectral data. High resolution ^{1}H NMR spectra of the isolated compounds **7b**–**h** exhibited two sharp singlets of three protons intensity at δ 2.2–2.4 and 2.46–2.64 ppm corresponding to C_3'–CH_3 and 5-CH_3 and three sharp singlets signals of one proton intensity in the range δ 6.1–6.4 ppm, 6.4–6.7 ppm and δ 6.6–7.0 ppm due to H-4′ [20], H-3 [20] and H-6 [20], respectively. Chemical shift of C_3'–CH_3 at δ 2.2–2.4 eliminates the possibility of structures **I** and **IV** as it is well established by us [21] and the other workers [22] that the methyl group located at position-5 of pyrazole moiety resonates downfield (~2.7 ppm) as compared to 3-CH_3 (δ 2.3) in case of 2-(pyrazol-1-yl) heterocycles. Moreover, had the structure been **I**, **II** and **IV** then C_4'–H and/or C_6–H and C_5'–CH_3 and/or 7-CH_3 signals would have splitted into quartet ^{4}J = 1.0 Hz (CH_3-7, H-6) [23] and doublet ^{4}J = 0.8 Hz (CH_3-5′, H-4′) [24], respectively, due to allylic coupling between them as it has been observed in the case of **7a** [17]. On the contrary, the methyl groups at position-3′ and position-5 does not show coupling to H-4′ and H-6, respectively.

Furthermore, the presence of methyl groups at position-3′ and position-5 finds support by an inspection of ^{13}C NMR spectra of **7b**–**h** which display CH_3-3′ signal at δ 11.4–13.7 and CH_3-5 at δ 22.0–24.8 ppm in close agreement with **7a** spectral results [17]. Also, the location of phenyl group at position-7 of the pyrimidine ring in regioisomer **7b** was determined on the basis of the ^{13}C NMR data, taking into account characteristic chemical shift of *ipso* atom in phenyl ring 7-C*i* at δ 131.3 ppm (C_7–C*ipso* at δ 131 ppm Refs. [14a.b]). Had the phenyl group been located at position-5, signal for 5-C*i* would have appeared at δ 136.1 ppm (Ref. [18]). Compounds **7b**–**h** displayed C-3′ and C-5′ signals of pyrazole ring resonated at δ 148.5–151.2 and δ 138.1–145.0 ppm, respectively, in complete agreement with literature data [21,25]. The complete assignment of the signals in ^{13}C NMR spectra of the compounds is given in Table 2.

It is noteworthy to mention that in ^{13}C NMR spectra of **7b**–**f**, the C-5′ appeared at δ 143.0–145.0 ppm instead of 140.9 ppm [17] in case of **7a.** This downfield shift of signal of C-5′ by about 3–5 ppm may be due to the replacement of methyl by an aryl group [21].

Finally, a conclusive evidence for the proposed structure **III** was received through (^{1}H–^{13}C) HMQC, (^{1}H–^{13}C) HMBC and (^{1}H–^{15}N) HMBC experiments on **7b** as a representative example. Based on these 2D correlations, a complete and unambiguous assignment of ^{1}H, ^{13}C, ^{15}N of **7b** are given in our paper in Journal of Molecular Structure [17].



Scheme 4. Synthesis of regioisomers (framed the real structure).

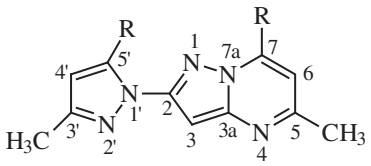
Besides the expected substituted pyrazol-1′-ylpyrazolo[1,5-*a*] pyrimidines (**7b**–**g**), an unexpected product 3(5)-methyl-5(3)-aryl-1*H*-pyrazole (**9b**–**g**) was also separated from the reaction mixture by fractional crystallization or column chromatography. The structure was confirmed by comparing its physical and spectral data (m.p., mixed m.p., TLC, IR, NMR and mass spectra) with an authentic sample [26–28] which was independently synthesized *via* reacting equimolar amounts of hydrazine hydrate with **6b**–**g** in ethanol under reflux.

Formation of 3(5)-methyl-5(3)-substitutedphenyl pyrazoles (**9**) suggests an unusual C–N pivot bond cleavage [29] either after the synthesis of pyrazol-1′-ylpyrazolo[1,5-*a*]pyrimidines (**7**) or through the self decomposition of hydrazine (**5**) under the reaction conditions. The former possibility was ruled out by the fact that **7** remains largely unchanged under reflux for 4–5 h. However, refluxing **5** in EtOH/H_2O followed by the addition of aldehydes/ketone to the reaction mixture led to the formation of corresponding hydrazones or azines (**11**) [30a] in 50–60% yield (Scheme 5), thus indicating the formation of hydrazine as a result of self decomposition of **5**. This observation finds further support by the isolation of hydrazone of acetone [30b] (Table 1, entry 8) when the reaction of phenyl-1,3-butanedione (**6b**) was carried with 3(5)-amino-5(3)-hydrazinopyrazole dihydrochloride (**5**) in H_2O having excess of acetone. This indicates that the lesser reactivity of aryl-1,3-diketones towards hydrazine permits the self decomposition of **5** to afford hydrazone of acetone. In the absence of acetone, reaction of aryl-1,3-diketones with **5** as well as its decomposition product (hydrazine) proceeds in a competitive manner to give pyrazol-1′-ylpyrazolo[1,5-*a*]pyrimidines (**7**) and cleaved pyrazoles **9**.

Formation of **10b**–**f** may be rationalized by the aqueous acidic hydrolysis of 1,3-diketones. Such acidic hydrolysis and the relation of structure to the proportion of cleavage products and to the mode of cleavage (water, alcohol and hydrogen) of 1,3-diketones under a variety of conditions has already been reported previously [31].

The reaction was highly regioselective and only the pyrazolo [1,5-*a*]pyrimidine isomer **III** was obtained, where methyl substituents was attached to C5 in contrast with previous studies [16]. The high regioselectivity of the reaction could be attributed to the primary attack of more nucleophilic NH_2 group (site **C**) of **5** rather than ring nitrogen (site **D**) of the pyrazole ring (hard) on the more electrophilic and less hindered carbonyl carbon [32] of acetyl group (site **y**) rather than the hindered and electronically disfavored carbonyl carbon (hard) attached to the aryl/heteroaryl group (site **x**) to afford pyrimidine ring. Also the unsubstituted nitrogen of heteroarylhydrazine **5** (site **A**) preferentially reacts with the carbonyl carbon of acetyl group (site **y**) to afford pyrazole ring in conformity with our earlier observation [21].

Table 2
^{13}C NMR chemical shifts of pyrazol-1′-ylpyrazolo[1,5-*a*]pyrimidines **7b**–**h** (in ppm).



7b-h

Carbon	7b	7c	7d	7e	7f	7g	7h
C-2	152.83	150.68	152.87	152.94	153.0	152.98	150.35
C-3	89.2	86.95	88.76	88.75	88.74	89.14	83.45
C-5	159.42	157.06	159.21	159.48	159.43	158.15	156.52
C-6	108.43	105.78	107.44	108.20	108.17	104.67	121.71
C-7	145.74	143.90	145.43	144.96	145.27	140.58	145.49
C-3a	149.6	147.16	149.67	148.88	148.65	147.56	148.78
5-CH_3	24.82	22.48	24.79	24.48	24.39	23.81	22.02
C-3′	150.86	148.59	150.73	151.11	151.22	151.15	149.32
C-4′	108.88	106.50	108.57	109.40	109.50	110.39	128.23
C-5′	145.02	143.03	144.76	143.61	143.69	138.11	142.03
3′-CH_3	13.7	11.49	13.7	13.59	13.59	13.62	12.90
Others C*i*-7	131.3	126.72	123.76	129.72	130.15	Th-2 133.33/133.05	Cyclopentyl
C*i*-5′	130.4	126.11	122.63	128.98	128.78	Th-4 131.07/130.70	Carbons 22.47,
C*o*	130.86	125.31	130.91	130.52	130.83	Th-3128.41/128.14	26.38, 29.59, 29.69,
C*o*, 2C*m*, 2C*p*	129.11/129.06/	123.67/127.10/	130.44/113.78/	130.42/128.75/	130.60/131.84/	Th-5 127.24/126.88	29.86, 30.70
	128.4/128.16/	126.86/139.26/	113.63161.65/	128.35/137.40/	131.36/126.01/		
	128.06	135.85	159.60	134.34	122.63		
OCH_3	–	–	55.27, 55.36	–	–	–	–
CH_3	–	19.18, 19.38	–	–	–	–	–

3. Biological results and discussion

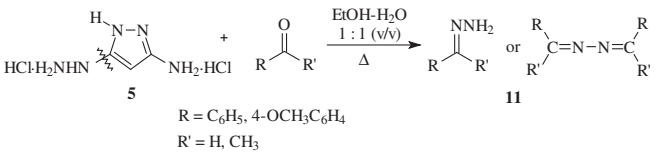
3.1. Antibacterial activity

Eight chemically synthesized compounds (**7a**–**h**) were assayed *in vitro* for their antibacterial activity against two gram-negative bacteria (*Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 25668) and two gram-positive bacteria (*Staphylococcus aureus* ATCC 9144 and *Bacillus cereus* ATCC 11778).

3.1.1. Results

For evaluating antibacterial activity Gentamicin and Linezolid were used as the standard drugs. The results of antibacterial activity have been summarized in Table 3. The observed minimum inhibitory concentrations (MIC) presented in Table 4 were in accordance with the results obtained in the primary screening. Compound **7e** exhibited excellent activity against Gram-positive bacteria *B. cereus* (MIC 50 μg/ml), while exhibited high activity against Gram-negative bacteria *E. coli* (MIC 25 μg/ml) as compared with the reference drugs (Gentamicin and Linezolid). Thus, this compound could be used as lead structure in pharmaceutical industry if it is nontoxic to the human system. Compound **7c** exhibited good activity towards Gram-negative bacteria *E. coli*, but compounds **7a**, **7b**, **7d** and **7f**–**h** exhibited slight activity towards Gram-negative and Gram-positive bacteria. As it can be seen in Table 4, MICs for compounds **7c** and **7e** are lower than positive control Linezolid employed in this study against *E. coli*, *P. aeruginosa* and *B. cereus*.

The antibacterial activity is considerably affected by substituents at 5′ and 7-positions of pyrazol-1′-ylpyrazolo[1,5-*a*]pyrimidines. The results suggests that substitution of 5′ and 7-positions of pyrazol-1′-ylpyrazolo[1,5-*a*]pyrimidines with *para*-substituted phenyl ring either with CH_3 or Cl group (**7c** and **7e**) increased their antibacterial activities against all or some tested bacteria like *E. coli* (**7c** and **7e**) and *B. cereus* (**7e**) in comparison to their unsubstituted analogoue **7b**. Similarly, replacement of 4-substituent of the phenyl ring and phenyl ring of pyrazol-1′-ylpyrazolo[1,5-*a*]pyrimidines **7** with OCH_3, Br group and 2-thienyl group (**7d**, **f** and **g**), respectively, diminished their activities. The significant loss in potency was observed when phenyl moiety attached to 5′ and 7-positions of pyrazol-1′-ylpyrazolo[1,5-*a*]pyrimidines is replaced with alkyl moieties (**7a** and **7h**).



Scheme 5. Isolation of hydrazones or azines **11**: indication of C–N bond cleavage.

Table 3
In vitro antibacterial activity of **7a**–**h** by using agar diffusion assay technique.

Compound	Diameter of zone of growth inhibition (mm)[a]			
	Gram −ve bacteria		Gram +ve bacteria	
	E. coli	*P. aeruginosa*	*S. aureus*	*B. cereus*
7a	7	7	7	7
7b	8	7.5	8	8
7c	10.5	8	8	8.5
7d	8	8	8	8
7e	13	10	9	13
7f	8	8	8	8
7g	8	8	8	8
7h	7	7	7	7
Gentamicin	24	12	14	14
Linezolid	12	8.5	18	10
Control	–	–	–	–

Inhibition zone, 6–10 mm slight activity, 11–15 mm moderate activity, more than 15 mm high activity.
[a] Mean of three replicates.

Table 4
MIC (μg/ml) values of compounds **7a**–**h** and reference drugs against the respective microorganisms.

Organism	7a	7b	7c	7d	7e	7f	7g	7h	Gentamicin	Linezolid
E. coli	>400	200	50	>100	25	>100	200	>400	4	>100
P. aeruginosa	>400	>200	100	>100	100	>100	200	>400	2	>100
S. aureus	400	200	100	>100	100	>100	200	400	2	2
B. cereus	>400	200	100	>100	50	>100	>200	400	4	>100

3.2. Antifungal activity

Four organic compounds (**7a**, **7b**, **7d** and **7e**) were screened for their antifungal activity *in vitro* against phytopathogenic *Aspergillus terrus*, *Alternaria alternata*, *Fusarium oxysporum* and *Helminthosporium* sp. by poisoned food technique [33].

3.2.1. Results

All these compounds were found to be active and inhibit the growth of pathogenic fungi significantly as compared to control and reference drug Mancozeb at different concentrations (Table 5). In case of compounds **7a**, maximum inhibition was seen at 200 μg/ml on *Helminthosporium* sp. (79.2 ± 0.06), *A. terrus* (73.2 ± 0.45), *A. alternata* (73.1 ± 0.06) followed by *F. oxysporum* (71.9 ± 0.02). Similarly, compound **7d** exhibited maximum mycelial inhibition in *Helminthosporium* sp. (62.2 ± 0.02) at 200 μg/ml followed by *F. oxysporum* (26.2 ± 0.18) at 100 μg/ml. However, there was no effect of compounds **7b** and **7e** at any concentration on *Helminthosporium sp.* and *A. alternata*, respectively. Compound **7e** showed maximum inhibition at 200 μg/ml with *Helminthosporium* sp. and *F. oxysporum*, respectively (Table 5). It seems that these nitrogen containing heterocyclic compounds are the reservoir of effective chemotherapeutants that can be used as pesticides.

The data reported in Table 5 and Fig. 1 revealed that compound **7a** is the most active compound as compared to other compounds and standard drug against all pathogenic fungal strains tested. To probe the structure–activity relationship of pyrazol-1′-ylpyrazolo [1,5-*a*]pyrimidines versus fungi, the preferred substitution of the position-5′ of pyrazole and position-7 of pyrimidine ring is a methyl group which is the most potent inhibitor (**7a**). Results indicate that **7a** with over 2.0, 2.6 and 3.4-fold potency than Mancozeb at 10 μg/ml against *F. oxysporum*, *A. alternata* and *Helminthosporium* sp., respectively, could be a promising antifungal agent. Replacement of CH_3 group with phenyl ring in **7b** causes huge loss of antifungal activity against all pathogenic fungal strains tested. Inclusion of a para-substituted phenyl ring (either electron-donating OCH_3 group **7d** or electron-withdrawing Cl **7e**) regardless of the size, electronic nature or difference in lipophilicity of substituent, potency further seems to reduce the antifungal activity.

Table 5
Antifungal activity (% inhibition) of pyrazol-1′-ylpyrazolo[1,5-*a*]pyrimidines.

Compound	Control	10 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml
Aspergillus terreus					
7a	0[a] (±0.20)	9.87 (±0.20)	33.7 (±0.44)	68.5 (±0.27)	73.2 (±0.45)
7b	0[a] (±0.20)	7.4 (±0.15)	13.5 (±0.05)	19.2 (±0.53)	19.7 (±0.27)
7d	0[a] (±0)	2.5 (±0.02)	6.25 (±0.02)	7.5 (±0.02)	11.3 (±0.05)
7e	0[a] (±0)	0 (±0)	0 (±0)	2.5 (±0.02)	12.5 (±0.02)
Mancozeb	0[a] (±0)	13.48	32.58	48.31	76.40
Alternaria alternata					
7a	0[a] (±0.37)	47.5 (±0.09)	69.5 (±0.05)	71.6 (±0.07)	73.1 (±0.06)
7b	0[a] (±0.37)	18.2 (±0.35)	20.7 (±0.33)	19.5 (±0.17)	20.7 (±0.53)
7d	0[a] (±0)	0 (±0)	0 (±0)	11.1 (±0)	11.1 (±0)
7e	0[a] (±0)	0 (±0)	0 (±0)	0 (±0)	0 (±0)
Mancozeb	0[a] (±0)	18.18	29.24	45.45	65.90
Fusarium oxysporum					
7a	0[a] (±0.12)	56.0 (±0.11)	67.0 (±0.03)	67.0 (±0.04)	71.9 (±0.02)
7b	0[a] (±0.12)	8.53 (±0.02)	6.09 (±0.31)	3.65 (±0.04)	2.40 (±0.10)
7d	0[a] (±0)	6.55 (±0.05)	3.27 (±0.08)	26.2 (±0.18)	20.0 (±0.04)
7e	0[a] (±0)	1.63 (±0.04)	3.27 (±0.02)	9.8 (±0.02)	16.3 (±0.04)
Mancozeb	0[a] (±0)	27.77	42.22	62.22	81.11
Helminthosporium sp.					
7a	0[a] (±0.02)	42.2 (±0.17)	66.6 (±0.29)	79.2 (±0.16)	79.2 (±0.06)
7b	0[a] (±0.02)	0 (±0)	0 (±0)	0 (±0)	0 (±0)
7d	0[a] (±0)	0 (±0)	0 (±0)	27.7 (±0.25)	62.2 (±0.02)
7e	0[a] (±0)	11.1 (±0.04)	15.5 (±0.06)	17.7 (±0.02)	36.6 (±0.21)
Mancozeb	0[a] (±0)	12.22	24.44	44.44	70.00

'±' SEM.
[a] Average of three replications each.

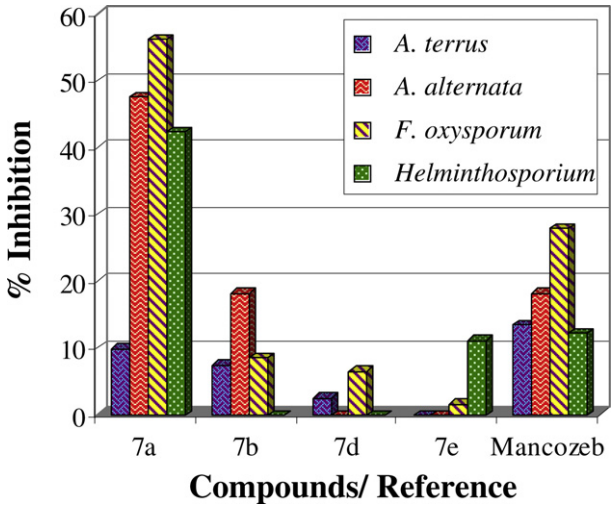


Fig. 1. *In vitro* antifungal assay of test compounds/reference at concentration 10 μg/ml.

4. Conclusion

In summary, a series of new pyrazol-1′-ylpyrazolo[1,5-*a*]pyrimidines was synthesized regioselectively in an efficient and environmental benign manner using water as solvent without any additives or catalysts and their antimicrobial properties were evaluated. Structure–activity studies showed that the antimicrobial potency was mainly influenced by the substituents at 5′ and 7-positions of pyrazol-1′-ylpyrazolo[1,5-*a*]pyrimidines. The results suggest that compound **7a** exhibited more potent and pronounced antifungal activity *in vitro* at the concentration of 10 μg/ml with respect to reference drug Mancozeb and it may be considered as a promising lead for further design and development of new agricultural fungicides. Compounds **7c** and **7e** exhibited promising antimicrobial activity/lower MIC values comparable to commercial antibiotics Gentamicin and Linezolid.

5. Experimental

Melting points were determined in open capillaries and are uncorrected. IR spectra were recorded on a Buck Scientific IR M-500 spectrophotometer in KBr pellets (ν_{max} in cm^{-1}), ^1H and ^{13}C NMR

spectra for analytical purpose were recorded in $CDCl_3$ on a Bruker instrument at 300 MHz and 75 MHz, respectively. High resolution NMR spectra were recorded on a Bruker DRX 400 spectrometer at 400.13 MHz for ^1H, 100.62 MHz for ^{13}C. Chemical shifts (δ in ppm) are given from internal solvent, $CDCl_3$ 7.26 for ^1H and 77.0 for ^{13}C. Coupling constants (J) are given in hertz (Hz). High resolution mass spectra (HRMS) were measured in EI mode on a Kratos MS-50 spectrometer. Yields are calculated assuming 1 equiv of **6b**–**h** is reacting with 1 equiv of **5**.

2-Acetylcyclopentanone (**6h**) is commercially available. Other aryl/heteroaryl-1,3-diketones (**6b**–**g**) [34] and 3(5)-amino-5(3)-hydrazinopyrazole dihydrochloride (**5**) [19] were prepared according to the literature procedures.

5.1. Synthesis of 2-(3′,5′-disubstituted pyrazol-1′-yl)-5,7-disubstituted pyrazolo[1,5-a]pyrimidines (**7**)

5.1.1. 2-(3′-Methyl-5′-phenylpyrazol-1′-yl)-5-methyl-7-phenylpyrazolo[1,5-a]pyrimidine (**7b**)

To H_2O (20 ml) were added 3(5)-amino-5(3)-hydrazinopyrazole dihydrochloride (**5**) (0.93 g, 0.005 mol) and $CH_3COCH_2COC_6H_5$ (**6b**) (0.81 g, 0.005 mol). This reaction mixture was found to have pH 0.8 at 25 °C. Then the reaction mixture was refluxed for 4 h. After completion of the reaction (monitored by TLC), the reaction mixture was cooled to room temperature and extracted using 2 × 30 ml portions of ethyl acetate. The combined organic layers were successively washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated to give a residual mass. The residue obtained on cooling was found to be a mixture of three products as predicted by TLC and ^1H NMR spectrum. The residue was column chromatographed over silica gel (100–200 mesh) using petroleum ether followed by petroleum ether/$CHCl_3$ of increasing polarity as eluent to yield three compounds– benzoic acid **10b** m.p. 120–121 °C (lit. [35] m.p. 121–123 °C) in the first fraction, followed by 3-methyl-5-phenyl-1*H*-pyrazole (**9b**) m.p. 129–130 °C; (lit. [26–28] m.p. 128 °C); yield 8% and finally 2-(3′-methyl-5′-phenylpyrazol-1′-yl)-5-methyl-7-phenylpyrazolo[1,5-*a*] pyrimidine (**7b**).

5.1.2. 2-(3′-Methyl-5′-phenylpyrazol-1′-yl)-5-methyl-7-phenylpyrazolo[1,5-a]pyrimidine (**7b**)

M.p. 179–180 °C; yield 76%. IR (cm^{-1}): 3038, 2924, 1616, 1519, 1414. ^1H NMR ($CDCl_3$, δ): 2.39 (s, 3H, C_3'–CH_3), 2.61 (s, 3H, C_5–CH_3), 6.28 (s, 1H, C_4'–H), 6.61 (s, 1H, C_3–H), 6.75 (s, 1H, C_6–H), 7.31–7.46 (m, 8H, Ph–H), 7.63 (m, 2H, Ph–H*o*). MS (*m*/*z*): 365 (M^+). Anal. Calcd. for $C_{23}H_{19}N_5$: C, 75.59; H, 5.24; N, 19.16. Found: C, 75.82; H, 5.57; N, 19.51.

All other compounds (**7c**–**h**) were synthesized according to the procedure mentioned for **7b** using 3-amino-5-hydrazinopyrazole dihydrochloride (**5**) and different β-diketones (**6c**-**h**). After the reaction was complete (from TLC), the mixture was cooled and extracted with EtOAc (2 × 30 ml). The combined organic layers were dried over anhydrous Na_2SO4. The solvent was evaporated and the crude product was purified by recrystallization or column chromatography. The characterization data for **7c**–**h** and **9c**–**g** are presented below:

5.1.3. 2-(3′-Methyl-5′-(4″-methylphenyl)pyrazol-1′-yl)-5-methyl-7-(4″-methylphenyl)pyrazolo[1,5-a]pyrimidine (**7c**)

M.p. 171–172 °C; yield 60%. IR (cm^{-1}): 3038, 2924, 1615, 1518. ^1H NMR ($CDCl_3$, δ): 2.31 (s, 3H, C_3'–CH_3), 2.32 (s, 3H, CH_3), 2.33 (s, 3H, CH_3), 2.57 (s, 3H, C_5–CH_3), 6.17 (s, 1H, C_4'–H), 6.48 (s, 1H, C_3–H), 6.67 (s, 1H, C_6–H), 7.05–7.13 (m, 4H, Ph–H), 7.16–7.19 (d, 2H, J_o = 8.1 Hz, Ph–H), 7.51–7.54 (d, 2H, J_o = 8.4 Hz, Ph–H). MS (*m*/*z*): 393 (M^+). Anal. Calcd. for $C_{25}H_{23}N_5$: C, 76.31; H, 5.89; N, 17.80. Found: C, 76.76; H, 5.69; N, 17.67.

5.1.4. 3-Methyl-5-(4-methylphenyl)-1H-pyrazole (**9c**)

M.p. 130–132 °C (lit. [26,27] m.p. 118–119 °C); yield 28%.

5.1.5. 2-(3′-Methyl-5′-(4″-methoxyphenyl)pyrazol-1′-yl)-5-methyl-7-(4″-methoxyphenyl)pyrazolo[1,5-a]pyrimidine (**7d**)

M.p. 172–174 °C; yield 62%. IR (cm^{-1}): 3131, 2931, 1613, 1508, 1250 (OCH_3). ^1H NMR ($CDCl_3$, δ): 2.31 (s, 3H, C_3'–CH_3), 2.52 (s, 3H, C_5–CH_3), 3.73 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 6.16 (s, 1H, C_4'–H), 6.48 (s, 1H, C_3–H), 6.66 (s, 1H, C_6–H), 6.78–6.84 (m, 4H, Ph–H), 7.21–7.24 (dd, 2H, J_o = 8.4 Hz, J_m = 2.1 Hz, Ph–H), 7.63–7.66 (dd, 2H, J_o = 8.1 Hz, J_m = 2.1 Hz, Ph–H). HRMS (*m*/*z*): 426.1921 $(M+1)^+$ ($C_{25}H_{23}N_5O_2$ $(M+1)^+$ requires 426.193). Anal. Calcd. for $C_{25}H_{23}N_5O_2$: C, 70.57; H, 5.45; N, 16.46. Found: C, 70.36; H, 5.76; N, 16.21.

5.1.6. 3-Methyl-5-(4-methoxyphenyl)-1H-pyrazole (**9d**)

M.p. 112–114 °C (lit. [26,27] m.p. 70–73 °C, [36] 115–116 °C, [37] 111 °C); yield 25%.

5.1.7. 2-(3′-Methyl-5′-(4″-chlorophenyl)pyrazol-1′-yl)-5-methyl-7-(4″-chlorophenyl)pyrazolo[1,5-a]pyrimidine (**7e**)

M.p. 170–172 °C; yield 55%. IR (cm^{-1}): 3150, 2925, 1615, 1540, 1488, 1091. ^1H NMR ($CDCl_3$, δ): 2.32 (s, 3H, C_3'–CH_3), 2.57 (s, 3H, C_5–CH_3), 6.19 (s, 1H, C_4'–H), 6.66 (s, 1H, C_3–H), 6.69 (s, 1H, C_6–H), 7.19–7.22 (d, 2H, J_o = 8.7 Hz, Ph–H), 7.23–7.26 (d, 2H, J_o = 8.7 Hz, Ph–H), 7.29–7.32 (d, 2H, J_o = 8.4 Hz, Ph–H), 7.50–7.53 (dd, 2H, J_o = 8.7 Hz, J_m = 1.8 Hz, Ph–H). HRMS (*m*/*z*): 434.0945 $(M+1)^+$ ($C_{23}H_{17}N_5Cl_2$ $(M+1)^+$ requires 434.0939). Anal. Calcd. for $C_{23}H_{17}N_5Cl_2$: C, 63.60; H, 3.95; N, 16.12. Found: C, 63.32; H, 3.66; N, 16.27.

5.1.8. 3-Methyl-5-(4-chlorophenyl)-1H-pyrazole (**9e**)

M.p. 150–160 °C (Lit. [28] m.p. 147–148 °C); yield 32%.

5.1.9. 2-(3′-Methyl-5′-(4″-bromophenyl)pyrazol-1′-yl)-5-methyl-7-(4″-bromophenyl)pyrazolo[1,5-a]pyrimidine (**7f**)

M.p. 194 °C; yield 50%. IR (cm^{-1}): 3147, 2918, 1617, 1523, 1486. ^1H NMR ($CDCl_3$, δ): 2.32 (s, 3H, C_3'–CH_3), 2.59 (s, 3H, C_5–CH_3), 6.20 (s, 1H, C_4'–H), 6.68 (s, 1H, C_3–H), 6.69 (s, 1H, C_6–H), 7.14–7.17 (d, 2H, J_o = 8.4 Hz, Ph–H), 7.41–7.52 (m, 6H, Ph–H). MS (*m*/*z*): 521 (M^+). Anal. Calcd. for $C_{23}H_{17}N_5Br_2$: C, 52.80; H, 3.27; N, 13.39. Found: C, 52.42; H, 3.72; N, 13.61.

5.1.10. 3-Methyl-5-(4-bromophenyl)-1H-pyrazole (**9f**)

M.p. 132–134 °C; yield 34%. IR (cm^{-1}): 3183, 3101, 2979, 1587, 1445. ^1H NMR ($CDCl_3$, δ): 2.26 (s, 3H, CH_3), 6.26 (s, 1H, C_4–H), 7.41–7.44 (d, 2H, J_o = 8.7 Hz, Ph-2′, 6′-H), 7.51–7.54 (d, 2H, J_o = 8.7 Hz, Ph-3′, 5′-H). ^{13}C NMR ($CDCl_3$, δ): 11.43 (CH_3), 102.05 (C4), 121.69 (C-4′), 127.16 (C-2′, C-6′), 128.89 (C-1′), 131.77 (C-3′, C-5′), 142.22 (C5), 149.33 (C3).

5.1.11. 2-(3′-Methyl-5′-(thien-2″-yl)pyrazol-1′-yl)-5-methyl-7-(thien-2″-yl)pyrazolo[1,5-a]pyrimidine (**7g**)

M.p. 149–150 °C; yield 58%. IR (cm^{-1}): 2930, 2852, 1687, 1603, 1426. ^1H NMR ($CDCl_3$, δ): 2.40 (s, 3H, C_3'–CH_3), 2.64 (s, 3H, C_5–CH_3), 6.40 (s, 1H, C_4'–H), 6.63 (s, 1H, C_3–H), 6.98–7.02 (dt, 1H, J_o = 5.1 Hz, Th–H), 7.09 (s, 1H, C_6–H), 7.12–7.14 (dd, 1H, J_o = 3.6 Hz, J_o = 1.2 Hz, Th–H), 7.15–7.18 (dt, 1H, J_o = 5.1 Hz, Th–H), 7.31–7.33 (dd, 1H, J_o = 5.1 Hz, J_o = 0.9 Hz, Th–H), 7.59–7.61 (dd, 1H, J_o = 5.1 Hz, Th–H), 8.15–8.17 (dd, 1H, J_o = 3.9 Hz, J_o = 0.9 Hz, Th–H). HRMS (*m*/*z*): 378.085 $(M+1)^+$ ($C_{19}H_{15}N_5S_2$ $(M+1)^+$ requires 378.0847). Anal. Calcd. for $C_{19}H_{15}N_5S_2$: C, 60.45; H, 4.01; N, 18.55. Found: C, 63.32; H, 3.71; N, 18.27.

*5.1.12. 3-Methyl-5-(2-thienyl)-1H-pyrazole (**9g**)*

M.p. 139–140 °C; yield 35%. IR (cm^{-1}): 3179, 3083, 2932, 1584, 1423. ^1H NMR ($CDCl_3$, δ): 2.28 (s, 3H, CH_3), 6.25 (s, 1H, C_4–H), 7.01–7.04 (m, 1H, Th–H), 7.21–7.28 (m, 2H, Th–H). MS (*m*/*z*): 164 (M^+).

*5.1.13. 2-(3′-Methyl-5′,6′-dihydro-4H-cyclopenta[d]pyrazol-1′-yl)-5-methyl-7,8-dihydro-6H-cyclopenta[g]pyrazolo[1,5-a]pyrimidine (**7h**)*

M.p. 250 °C; yield 75%. IR (cm^{-1}): 3113, 2938, 1627, 1552, 1366. ^1H NMR ($CDCl_3$, δ): 2.24 (s, 3H, C_3'–CH_3), 2.46 (s, 3H, C_5–CH_3), 2.52–2.62 (m, 5H, Cyclopentyl–H), 2.89–2.98 (m, 5H, Cyclopentyl–H), 3.29–3.34 (m, 2H, Cyclopentyl–H), 6.50 (s, 1H, C_3–H). HRMS (*m*/*z*): 294.1714 $(M+1)^+$ ($C_{17}H_{19}N_5$ $(M+1)^+$ requires 294.1719). Anal. Calcd. for $C_{17}H_{19}N_5$: C, 69.60; H, 6.53, N, 23.87. Found: C, 69.93; H, 6.77; N, 24.12.

6. Biological activity

6.1. Evaluation of antibacterial assay: preliminary screening

The bacterial isolates representing Gram-negative and Gram-positive bacteria were maintained and stored at 5–8 °C on Brain Heart Infusion Agar (HI-media). The screening of eight compounds **7a**–**h** was done *in vitro* using the agar-well diffusion method [38]. The stock solutions (2 mg/ml) of the test compounds were prepared by dissolving 2 mg of test compound in 1 ml of dimethyl sulfoxide (DMSO). All samples were sterilized through a 0.2 μm membrane filter and stored at 4 °C until further use. Bacterial inoculums were prepared from 24 h old cultures and turbidity was adjusted equivalent to 0.5 McFarland turbidity standard, i.e. 1×10^6 CFU/ml [39].

By inoculating 100 μl of each test bacterial culture in 20 ml of warm, melted, autoclaved Mueller Hinton Agar (HI-media) seed layers were prepared (separate flasks were used for each bacterial culture). After mixing, it was poured in sterilized and labeled Petri plates (150 mm × 20 mm). The wells of 6 mm were punched in the solidified Petri plates with the help of a sterile steel borer. With the help of micropipette, 100 μl of each test compound (stock 2 mg/ml) was added aseptically to the individual wells. The loaded plates were incubated in upright position at 37 ± 1 °C for 24 h. The diameter of the zone of growth inhibition around each well after incubation was measured in mm using a Vernier Caliper (Table 3). Gentamicin (4 μg/ml) and Linezolid (1 mg/ml in water) were used as standard antibiotic and DMSO as a negative control under similar conditions for comparison.

6.1.1. Determination of minimum inhibitory concentration (MIC)

MICs were determined by the broth microdilution method according to the National Committee for Clinical Laboratory Standards (NCCLS) recommendations [40]. Mueller Hinton broth (HI-media) was used as the test medium. Testing was performed at pH 7.4 ± 0.1. The inoculums were prepared using a 16 h broth culture of each bacterial strains adjusted to a turbidity equivalent to a 0.5 McFarland standard, diluted in Mueller Hinton Agar broth media to give concentration of 1×10^6 CFU/ml for bacteria. For antibacterial assay the final inoculum size was 10^5 CFU/ml. The test compounds were dissolved in DMSO to obtain 4 mg/ml stock solutions. Serial twofold dilutions of the test compounds in distilled water in concentration ranging from 800 to 12.5 μg/ml and extra dilutions (100–0.2 μg/ml) for antibiotic standards (Gentamicin; Linezolid) were prepared. To ensure that the solvent had no effect on bacterial growth, a control test was performed with test medium supplemented with DMSO at the same dilution as used in the experiments. A positive control (containing inoculum but no compound) and a negative control (medium only, without inoculum) were also prepared. Appropriately diluted each test sample was added to 20 ml of warm, melted, autoclaved MHA (separate flasks were taken for each dilution). After thorough mixing, medium was poured in sterilized Petri plates. Bacterial inoculums were spotted in a predefined pattern by aseptically transferring 100 μl of each bacterial culture on the surface of presolidified agar plates. The plates were incubated in at 37 ± 1 °C and the MICs were recorded by visual observations after 24 h. MICs values were the lowest concentration of compounds inhibiting visible growth of organism and are listed in Table 4.

6.2. Evaluation of antifungal activity

The pathogenic fungi were isolated from the soil of Kurukshetra. Potato dextrose agar (PDA) medium was autoclaved at 15 psi for 15 min. All the fungal isolates were inoculated on PDA plates in triplicates and incubated at 28 ± 1 °C for 6 days to obtain young, actively growing colonies of molds. Each of the test compound first prepared as concentrated suspension or solution (stock solution 1 mg/ml in DMSO) was added to 15–20 ml of molten agar media at 40 ± 2 °C into 9.0 cm diameter sterile Petri plates to achieve desired concentrations (10, 50, 100 and 200 μg/ml) and allowed the media to solidify. A mycelial disc of diameter 6 mm, cut from the periphery of 6 day old culture, was aseptically inoculated at the center of each Petri plate. For each treatment three replicates were maintained. Mancozeb was used as a standard and PDA medium without the test compound or with DMSO were served as the control. The inoculated Petri dishes were incubated in a biochemical oxygen demand (BOD) incubator at 28 ± 1 °C for the growth of pathogen. The radial growth of the colony was measured in mm and recorded after 7 days of incubation. Percentage of mycelial growth inhibition was calculated by using the formula:

$$\text{Percent inhibition} = C - T \times 100/C$$

where, C = Average increase in mycelial growth in control, and T = average increase in mycelial growth in the presence of test compound.

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