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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis of novel 2-alkyl-4-substituted-amino-pyrazolo [3,4-*d*]pyrimidines as new leads for anti-bacterial and anti-cancer activity

Murty Devarakonda · Raghava Doonaboina · Sreedher Vanga · Jahnavi Vemu · Sathoshini Boni · Raghu Prasad Mailavaram

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Abstract Treatment of 6-alkyl-1-phenyl-4-chloro-(1*H*)-pyrazolo[3,4-*d*]pyrimidines, with different amines afforded a series of compounds whose identity and purity were confirmed by spectral and analytical means. The compounds were tested for antibacterial activity against four organisms viz. *Staphylococcus aureus* (Gram positive), *S. epidermidis* (Gram positive), *Bacillus subtilis* (Gram positive), *Escherichia coli* (Gram negative) using amoxicillin as standard control. Compounds **4d**, **6b**, **6c** have shown best antibacterial activity in the series. The antiproliferative activity was tested against human skin cancer cell line G361. The compounds **3d**, **4d**, **5b**, **5d**, **5e**, **6c**, **7a** were found to be the best of the series and showed the activity at micromolar concentration.

Keywords Antibacterial activity · Anticancer activity · G361 cell line · Skin cancer · Sulforhodamine B assay

Introduction

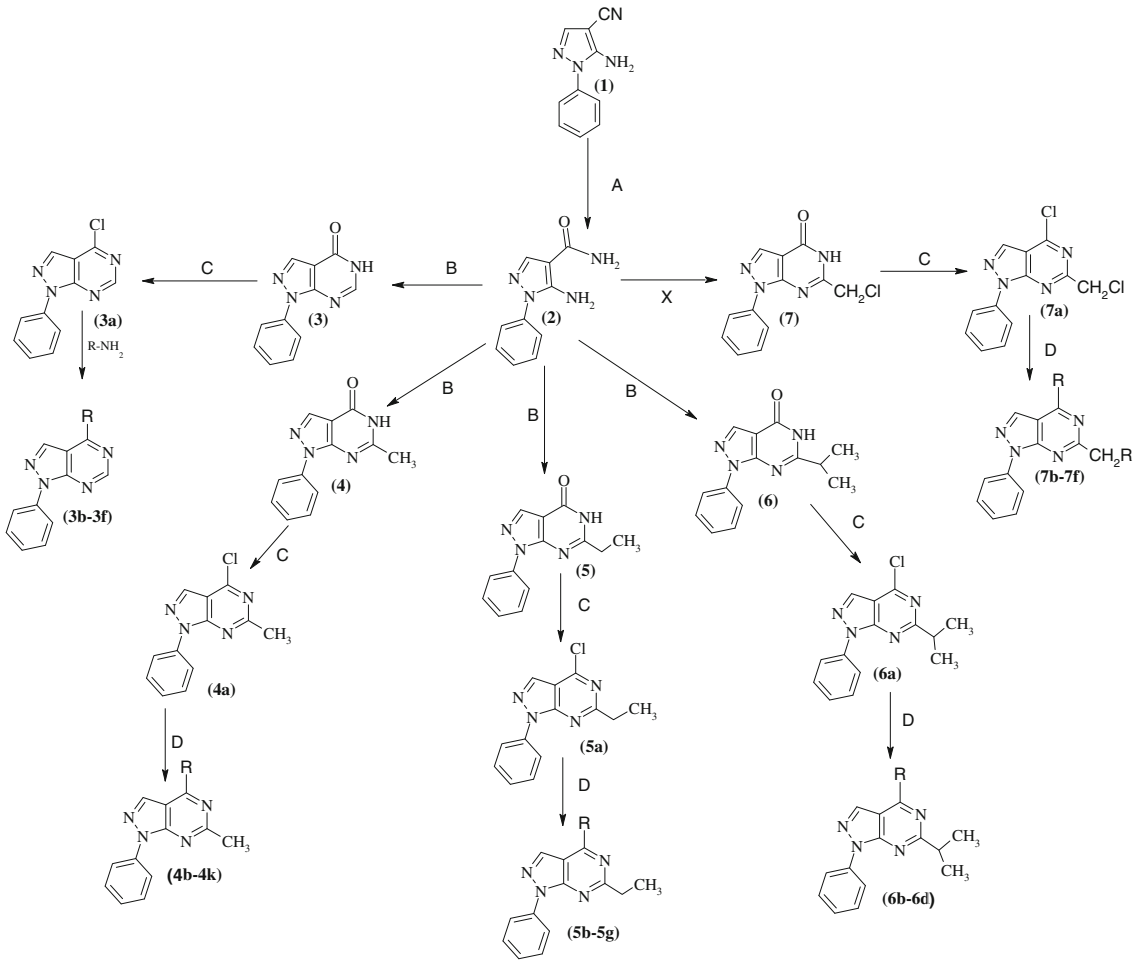
The synthetic heterocyclic chemistry has provided means for the invention of various drugs and pyrazolopyrimidines are one such. These derivatives, being purine analogs, are of considerable chemical and pharmacological importance. Literature survey reveals that pyrazolopyrimidines have antibacterial (Bakavoli *et al*., 2010; Bazgir *et al*., 2008), antiproliferative (Ahmed *et al*., 2009; Krystof *et al*., 2006), CNS modulating (Neustadt *et al*., 2007), antiviral (Chern *et al*., 2004), antifungal (Holla *et al*., 2006), anticancer (Abd Razik and Abdel Wahab, 2011) and anti-inflammatory (Quintela *et al*., 2003) activities. These molecules have shown affinity towards adenosine receptors (Quinn and Poulsen, 1996), 5HT-6 and CRF-1 (Gilligan *et al*., 2000) receptors. Further, it has been revealed that the replacement of 1H of pyrazole of the pyrazolo[3, 4-*d*]pyrimidine ring system with other bioactive moiety shows good pharmacological properties probably by increasing the π staking (Holla *et al*., 2006). In the present study, proton at position 1 on pyrazole ring has replaced with phenyl group. In view of developing molecules with potent antibacterial and anticancer activities, we aimed at the synthesis of 6-alkyl-1-phenyl-4-substituted-amino-(1*H*)-pyrazolo[3,4-*d*]pyrimidines. As a part of our research, we synthesized the title compounds, with phenyl group at 1H position and amine substitution at 4H position of 6-alkyl pyrazolo[3,4-*d*]pyrimidines. The compounds were tested for antibacterial activity against four organisms viz. *Staphylococcus aureus*, *S. epidermidis*, *Bacillus subtilis*, *Escherichia coli.* The antiproliferative activity was tested against human skin cancer cell line G361.

Results and discussion

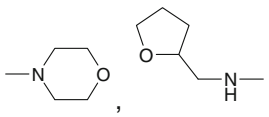
Chemistry

The *general procedure for synthesis of* 4-chloro-6-alkyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidine (**3a**–**7a**) were synthesized by route shown in Scheme 1. The starting material, 1-phenyl-4-cyano-5-aminopyrazole **1** was synthesized as described in the literature (Das *et al*., 2008). The key intermediate 5-amino-1-phenyl-1*H*-pyrazole-4-carboxamide **2** was obtained by acid hydrolysis of 1-phenyl-4-cyano-

M. Devarakonda · R. Doonaboina · S. Vanga · J. Vemu · S. Boni · R. P. Mailavaram (✉)
Department of Pharmaceutical Chemistry, Shri Vishnu College of Pharmacy (Affiliated To Andhra University, Visakhapatnam), Vishnupur, Bhimavaram 534202, Andhra Pradesh, India
e-mail: raghumrp@mailcity.com; raghumrp@rediffmail.com



Scheme 1 Synthesis of 6-alkyl-1-phenyl-4-substituted-(1*H*)-pyrazolo[3,4-*d*]pyrimidin-amines. *A* H_2SO_4/room temp/1 h, *B* sodium ethoxide/ethanol reflux/MWI, 100 W, 80 °C, 45 s, 20 bar, *X* sodium ethoxide/ethanol/room temp/1 h, *C* phosphorous oxychloride/MWI, 100 W, 120 °C, 10 min 30 bar; *D* toluene/RH/room temp/18–36 h, *R* $–NHCH_2CH_2CH_3$, $–NHCH(CH_3)_2$, $–NHCH_2CH_2CH_2CH_3$,



5-aminopyrazole **1**. The amide derivative **2** was reacted with various esters by irradiating in a *CEM Discover* microwave oven at 80 °C to give pyrazolo[3,4-*d*]pyrimidin-ones **3**–**7** (Miyashita *et al*., 1990; Daniels *et al*., 2008). These compounds were chlorinated with phosphorous oxychloride (Jung *et al*., 2005) by irradiating in a microwave oven to give **3a**–**7a** and finally treated with various amines at room temperature in the presence of dry toluene (Schenone *et al*., 2004) to give the target compounds. The intermediate compounds **3**–**6**, **3a**–**6a** were synthesized both by conventional and microwave irradiations. By microwave irradiation, the reaction times drastically reduced and compound yields were also improved. Table 1 gives comparison data of reaction time and compound yields by conventional of synthesis and microwave irradiation methods. The synthetic route has been outlined in Scheme 1. The newly synthesized compounds were analysed and their structures were confirmed by IR, ^{1}H NMR, mass spectral studies and elemental analysis.

The condensation of 5-amino-1-phenyl-1*H*-pyrazole-4-carboxamide **2** with methyl chloro acetate afforded 6-chloromethyl-1-phenyl-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one **7**. The ^{1}H NMR of **7** showed the signal at δ 4.63 ppm as singlet corresponding to two protons indicating the presence of chloro methyl ($–CH_2Cl$) group at sixth position of pyrazolopyrimidines. The compounds **3**–**7** were chlorinated with phosphorous oxychloride, and preceded to the next step immediately without any purification as they

Table 1 Results obtained by conventional and microwave methods

Compound code	MWI (conditions)	Conv (time, h)	MWI (time)	Conv (yield %)	MWI (yield %)
3	B	14	45 s	80	91
4	B	14	45 s	78	96
5	B	14	45 s	80	92
6	B	14	45 s	82	93
3a	C	12	5 min	40	44
4a	C	12	5 min	35	45
5a	C	12	7 min	37	42
6a	C	12	6 min	33	43
7a	C	12	6 min	35	41

B sodium ethoxide/ethanol reflux/MWI, 100 W, 80 °C, 45 s, 20 bar, *C* phosphorous oxychloride/MWI, 100 W, 120 °C, 10 min 30 bar, *Conv* conventional, *MWI* microwave irradiation

were unstable. The chlorinated compounds were treated with various amines, in dry toluene to produce the title compounds. The obtained compounds showed a prominent peak at 3,500–3,200 cm^{-1} which is a characteristic of secondary amino group in IR spectrum, which indicated formation of the expected product. The ^1H NMR of compounds **3b**, **4b**, **5b**, **7b** showed signal at δ 0.94–0.98 ppm, a triplet, integrating for three protons indicating the terminal protons of the propyl amine substituent. It also showed signals at δ 1.62–1.69 ppm, a sextet and a peak at δ 3.47–3.52, a quartet integrating the protons of the second and first carbon of the propyl amine substituent at fourth position of pyrazolopyrimidines. The compounds **3c**, **4c**, **5c**, **6c**, **7c** showed a heptet at δ 4.43–4.47 ppm confirms the isopropyl group. The compounds **3e**, **4e**, **5e** showed multiplet at δ 7.40 ppm and doublet at δ 7.48–7.52 ppm confirming the fourth and third,

Table 2 Antibacterial activity data of **3b–3f**, **4b–4k**, **5b–5g**, **6b–6d**, **7b–7f** at 20 μg/mL

Code	X	R	*E. coli*	*B. s*	*S. a*	*S. e*
3b	H	Propyl	13.2 ± 0.060	13.2 ± 0.080	12.7 ± 0.040	13.3 ± 0.050
3c	H	Isopropyl	13.3 ± 0.050	13.3 ± 0.050	13.6 ± 0.050	13.6 ± 0.050
3d	H	Butyl	13.6 ± 0.050	13.0 ± 0.050	13.3 ± 0.050	13.3 ± 0.050
3e	H	Furfuryl	12.8 ± 0.060	13.6 ± 0.050	13.0 ± 0.086	13.6 ± 0.050
3f	H	Morpholine	13.3 ± 0.050	12.8 ± 0.060	13.6 ± 0.050	12.7 ± 0.083
4b	CH_3	Propyl	12.7 ± 0.062	13.0 ± 0.707	13.6 ± 0.050	13.6 ± 0.050
4c	CH_3	Isopropyl	13.0 ± 0.070	13.6 ± 0.707	13.3 ± 0.050	13.7 ± 0.092
4d	CH_3	Butyl	14.4 ± 0.101	13.8 ± 0.120	14.6 ± 0.120	14.5 ± 0.087
4e	CH_3	Furfuryl	13.6 ± 0.050	12.5 ± 1.052	12.7 ± 0.060	13.3 ± 0.050
4f	CH_3	Morpholine	12.7 ± 0.044	13.6 ± 0.050	13.3 ± 0.050	13.6 ± 0.050
4g	CH_3	Aniline	13.3 ± 0.050	13.6 ± 0.050	13.4 ± 0.072	13.6 ± 0.050
4h	CH_3	Diethyl	13.6 ± 0.100	13.1 ± 0.060	13.7 ± 0.044	13.3 ± 0.050
4i	CH_3	Benzyl	12.6 ± 0.050	13.3 ± 0.050	13.1 ± 0.060	12.7 ± 0.066
4j	CH_3	Ethyl	13.4 ± 0.050	13.3 ± 0.050	13.3 ± 0.050	13.3 ± 0.050
4k	CH_3	*o*-Anisidine	13.5 ± 0.052	13.4 ± 0.057	13.3 ± 0.050	13.6 ± 0.050
5b	C_2H_5	Propyl	13.6 ± 0.050	13.0 ± 0.00	13.3 ± 0.050	13.3 ± 0.050
5c	C_2H_5	Isopropyl	13.3 ± 0.050	13.3 ± 0.050	13.6 ± 0.050	13.6 ± 0.050
5d	C_2H_5	Butyl	12.7 ± 0.066	12.8 ± 0.060	13.3 ± 0.050	13.0 ± 0.00
5e	C_2H_5	Furfuryl	12.7 ± 0.044	13.6 ± 0.050	13.3 ± 0.050	13.6 ± 0.050
5f	C_2H_5	Morpholine	13.0 ± 0.00	13.3 ± 0.050	13.0 ± 0.00	13.0 ± 0.00
5g	C_2H_5	Aniline	13.3 ± 0.050	13.3 ± 0.050	13.6 ± 0.050	13.6 ± 0.050
6b	i-C_3H_7	Butyl	13.3 ± 0.050	12.8 ± 0.060	14.4 ± 0.101	14.1 ± 0.105
6c	i-C_3H_7	Morpholine	14.6 ± 0.111	14.4 ± 0.113	14.2 ± 0.109	14.3 ± 0.111
6d	i-C_3H_7	Aniline	12.8 ± 0.078	13.5 ± 0.050	13.3 ± 0.050	13.3 ± 0.050
7b	CH_2Cl	Propyl	13.3 ± 0.050	13.6 ± 0.050	1.33 ± 0.050	1.33 ± 0.050
7c	CH_2Cl	Isopropyl	1.30 ± 0.00	13.6 ± 0.050	1.30 ± 0.000	1.33 ± 0.050
7d	CH_2Cl	Ethyl	13.3 ± 0.050	12.7 ± 0.083	12.6 ± 0.050	13.6 ± 0.050
7e	CH_2Cl	Morpholine	13.6 ± 0.050	1.30 ± 0.00	13.6 ± 0.050	13.3 ± 0.050
7f	CH_2Cl	Aniline	13.3 ± 0.050	13.6 ± 0.050	13.2 ± 0.044	13.0 ± 0.000
AMOX	–	–	18.7 ± 0.066	19.0 ± 0.086	17.3 ± 0.132	16.6 ± 0.05

Zone of inhibition in millimeters (average ± SEM) ($n = 9$)

E. coli Escherichia coli (NCIM-2803), *B. s Bacillus subtilis* (NCIM-2545), *S. a Staphylococcus aureus* (NCIM-5021), *S. e* Staphylococcus epidermidis (NCIM-2493)

Table 3 Antibacterial activity selected compounds at 10 μg/mL

Compound	X	R	*E. coli*	*B. s*	*S. a*	*S. e*
4d	CH_3	Butyl	11.0 ± 0.382	0.0	11.3 ± 0.050	11.1 ± 0.033
6b	i-C_3H_7	Butyl	0.0	0.0	11.0 ± 0.0	11.0 ± 0.0
6c	i-C_3H_7	Morpholine	10.6 ± 0.050	11.3 ± 0.050	10.33 ± 0.10	11.0 ± 0.0
AMOX	–	–	15.5 ± 0.072	16.3 ± 0.05	16.6 ± 0.05	13.0 ± 0.30

Zone of inhibition in millimeters (average ± SEM) ($n = 9$)

E. coli Escherichia coli (NCIM-2803), *B. s Bacillus subtilis* (NCIM-2545), *S. a Staphylococcus aureus* (NCIM-5021), *S. e* Staphylococcus epidermidis (NCIM-2493), *i-C_3H_7* isopropyl

Table 4 Results of in vitro anticancer activity of testing of **3b–3f**, **4b–4k**, **5b–5g**, **6b–6d**, **7b–7f**

Drug code	Human skin cancer cell line G361			
	% Growth[a]			
	Molar drug concentration			
3b	100.0	100.0	99.7	64.8
3d	100.0	90.8	43.4	−4.5
3e	100.0	97.5	92.3	51.7
3f	100.0	100.0	100.0	61.5
4b	100.0	100.0	100.0	25.3
4c	100.0	100.0	100.0	13.5
4d	100.0	100.0	100.0	−30.0
4e	100.0	100.0	100.0	30.1
4f	100.0	100.0	99.8	43.9
4g	100.0	100.0	100.0	65.6
4h	100.0	100.0	100.0	61.8
4i	100.0	100.0	100.0	45.5
4j	100.0	100.0	99.6	29.2
5b	100.0	94.7	84.7	−34.7
5c	100.0	96.4	83.3	40.0
5d	100.0	96.5	84.8	−62.5
5e	100.0	96.5	86.3	−61.0
5f	100.0	85.5	65.9	30.9
6b	100.0	100.0	100.0	45.4
6c	100.0	92.5	77.6	6.9
6d	100.0	100.0	91.9	19.2
7a	100.0	100.0	90.9	−59.3
7b	100.0	99.6	100.0	75.1
7c	100.0	100.0	99.7	56.5
7d	100.0	100.0	100.0	87.7
7e	99.7	99.6	94.3	47.6
7f	100.0	100.0	99.7	48.5
ADR	−24.0	−62.5	−64.2	−70.7

[a] Average value of 3 experiments

Table 5 Parameters of anticancer activity calculated from graph

Drug code	Molar drug concentration		
	LC_{50}	TGI	GI_{50}
3b	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
3d	$>10^{-4}$	3.7×10^{-5}	2.1×10^{-6}
3e	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
3f	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
4b	$>10^{-4}$	$>10^{-4}$	3.6×10^{-5}
4c	$>10^{-4}$	$>10^{-4}$	3.2×10^{-5}
4d	$>10^{-4}$	$>10^{-4}$	2.4×10^{-6}
4e	$>10^{-4}$	$>10^{-4}$	3.9×10^{-5}
4f	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
4g	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
4h	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
4i	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
4j	$>10^{-4}$	$>10^{-4}$	3.87×10^{-5}
5b	$>10^{-4}$	3.7×10^{-5}	2.2×10^{-6}
5c	$>10^{-4}$	$>10^{-4}$	3.8×10^{-5}
5d	$>10^{-4}$	3.2×10^{-5}	2.02×10^{-6}
5e	$>10^{-4}$	3.2×10^{-5}	2.04×10^{-6}
5f	$>10^{-4}$	$>10^{-4}$	2.8×10^{-5}
6b	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
6c	$>10^{-4}$	$>10^{-4}$	2.6×10^{-6}
6d	$>10^{-4}$	$>10^{-4}$	3.2×10^{-5}
7	$>10^{-4}$	3.32×10^{-5}	2.08×10^{-6}
7b	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
7c	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
7d	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
7e	$>>10^{-4}$	$>10^{-4}$	$>10^{-4}$
7f	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
ADR	1.7×10^{-7}	$<10^{-7}$	$<10^{-7}$

fifth proving of furfuryl group. The compounds **3f**, **4f**, **5f**, **6c**, **7e** showed triplets at δ 3.77–3.79 ppm and δ 3.96–3.98 ppm which indicates the protons of morpholine group. The benzyl group is confirmed by the doublet signals of two protons CH_2 at δ 4.78–4.79 ppm, thus differing from aniline substituent. The mass spectrum showed molecular ion peak [M^+] as the base peak and fragmentation pattern characteristic to its structure. The elemental analyses of all the newly synthesized compounds confirmed their formation.

Antibacterial activity

The compounds **3b–3f**, **4b–4k**, **5b–5g**, **6b–6d**, **7b–7f** were screened for their antibacterial activity against Gram positive [*B. subtilis* (NCIM-2545), *S. epidermidis* (NCIM-2493), *S. aureus* (NCIM-5021)] and Gram negative [*E. coli* (NCIM-2803)] bacteria by agar diffusion method and the results are summarized in Tables 2 and 3. All the compounds were found to be active against all the four bacterial strains at 20 μg/mL concentration. The activity of these compounds varied with the kind of organism. The compounds **4d**, **6b**, **6c** showed higher zone of inhibitions than the rest thus being the best of the series. The above studies also showed that 6-isopropyl-4-(morpholino-4-yl)-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidine **6c** was the best of the series, showed activity against all the four organisms, at a concentration of 10 μg/mL. The compound *N*-butyl-6-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine **4d** exhibited activity against *E. coli*, *S. aureus* and *S. epidermidis* at a concentration of 10 μg/mL. The compound *N*-butyl-6-isopropyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine **6b** showed activity against *S. aureus* and *S. epidermidis.*

Anticancer activity

The compounds were **3b–3f**, **4b–4k**, **5b–5g**, **6b–6d**, **7b–7f** screened for their anticancer activity against Human Skin Cancer Cell Line G361 by sulforhodamine B (SRB) assay and the results are summarized in Tables 4 and 5. The compounds **3d**, **4d**, **5b**, **5d**, **5e**, **6c**, **7a** showed antiproliferative activity. The % growth inhibition was found to be considerable at a concentration of 10^{-4} M, while the reference drug doxorubicin has its effect at 10^{-7} M. TGI_{50} (growth inhibition of 50 % cells, calculated from drug concentration resulting in a 50 % reduction in the net protein increase) values are in the range 2.08–2.8 $\times$ 10^{-6}.

Based on structural features and the activity shown by the synthesized compounds, structure and activity relationship may be obtained. As the SAR is concerned, the isopropyl amine group at sixth position of pyrazolopyrimidine ring of compounds **6b**, **6c** might be contributing to the best antibacterial activity. Similarly, the presence of butyl amine group at fourth position in **4d**, **6b** might be the reason for the antibacterial activity at low concentration. The presence of butyl amine group at fourth position may be responsible for the anticancer activity for **3d**, **4d**, **5d**, **6b**. The presence of bioactive groups such as furfuryl amine and morpholine groups at fourth position may be the reason for anticancer activity in **5e**, **6c** compounds. The presence of ethyl group at sixth position may contribute to anticancer activity as that series produced greater number of compounds **5b**, **5d**, **5e**, **5f** with anticancer property.

Conclusions

We have successfully synthesized 6-alkyl-1-phenyl-4-substituted-1*H*-pyrazolo[3,4-*d*]pyrimidines. Basically pyrazolo[3,4-*d*]pyrimidines were reported to be good antibacterial agents, and in our study we have replaced the 1H atom of pyrazole ring with phenyl group as an active group compared to the non substituent group. And the final compounds which are substituted with various amines were found to be more active towards the tested organisms. The antibacterial activity data showed that **4d**, **6b**, **6c** are the compounds found to have best antibacterial activity of the series. The compounds **7**, **3d**, **4d**, **5b**, **5d**, **5e**, **5f**, **6c** showed antiproliferative activity against human skin cancer cell lines. The presence of ethyl group at sixth position was found to be responsible for anticancer property. The presence of isopropyl group at sixth position in **6b**, **6c** and the presence of butyl amine group at fourth position in **4d**, **6b** might be the reason for both antibacterial and anticancer activities. The replacement of aliphatic amines at fourth position with bioactive groups such as furfuryl amine and morpholine groups showed anticancer activity.

Experimental

General techniques

Melting points were determined on VEEGO-programmable melting point apparatus and are uncorrected. The microwave-assisted synthesis was carried out on microwave (CEM Discover). The IR spectra (KBr pellet method) were recorded on a Bruker-Alpha IR spectrophotometer. ^{1}HNMR spectra were recorded on Bruker NMR spectrophotometer 400 MHz in DMSO-d_6, $CDCl_3$ as solvents. Chemical shift values are reported in δ ppm relative to tetramethylsilane (TMS) as relative standard. The mass spectra [EI–MS] of the compounds were recorded on APEX instrument. Elemental analyses for C, H, N and O were performed using a Carlo Erba 1108 elemental analyser and the % of C, H, N was found to be within ±0.4 % of the theoretical values. The purity of compounds was checked by thin layer chromatography (TLC, Merck, silica F_{254}) plates using chloroform:ethyl acetate (7:3) as mobile phase. All the compounds were characterized by the above techniques. Amoxicillin and doxorubicin were obtained commercially.

*General procedure for synthesis of 6-alkyl-1-phenyl-1, 5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-ones (**3–6**)*

Conventional synthesis Sodium ethoxide was prepared by dissolving sodium metal (4 g, 100 mmol) in ethanol (150 mL) under nitrogen atmosphere. The compound

5-amino-1-phenyl-1*H*-pyrazole-4-carboxamide **2** (3.4 g, 20 mmol) obtained in the previous step and various esters (200 mmol) were added to the prepared sodium ethoxide and heated to reflux at 80 °C for 14 h to get the products pyrazolo[3,4-*d*]pyrimidin-ones **3–6**.

The compound 5-amino-1-phenyl-1*H*-pyrazole-4-carboxamide **2** (3.4 g, 20 mmol) obtained in the previous step and various esters (200 mmol) were added to the prepared sodium ethoxide and stirred the reaction mixture in a round-bottomed flask at room temperature for 1 h to get the compounds 6-chloromethyl-1-phenyl-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one **7**.

Microwave method of synthesis The compound 5-amino-1-phenyl-1*H*-pyrazole-4-carboxamide **2** (3.4 g, 20 mmol) obtained in the previous step and various esters (200 mmol) were added to the prepared sodium ethoxide and irradiated at 80 °C in CEM microwave for 45 s at 100 W, 20 bar for compounds pyrazolo[3,4-*d*]pyrimidin-ones **3–6**.

Reaction workup The reaction mixture was added into cold water and neutralized with dropwise addition of concentrated hydrochloric acid. The precipitate formed was filtered, dried and recrystallized from chloroform to give compounds **3–7**.

*6-Chloromethyl-1-phenyl-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (**7**)*

Yield 45.89 %, mp 230 °C. IR (cm^{-1}) (KBr): 3442.7 (–NH, 2° amide), 2971.9 (–CH_2), 1693.4 (–C=O), 1594.1 (C=C, aromatic), 779.1 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.63 (s, 2H, –C<u>H</u>$_2$ –Cl), 7.41–7.44 (t, $J = 7.4$ Hz, 1H, H-4′ Ph), 7.56–7.60 (t, $J = 7.8$ Hz, 2H, H-2′ and 6′ Ph), 8.03–8.05 (d, $J = 8.0$ Hz, 2H, H-3′ and 5′ Ph), 8.35 (s, 1H, H-3). MS *m/z* 260 (M+1), 261 (M+2). Anal. Calc. for $C_{12}H_9ClN_4O$: C 55.29, H 3.48, Cl 13.60, N 21.49, O 6.14. Found: C 55.09, H 3.28, Cl 13.10, N 21.09, O 6.04.

*General procedure for synthesis of 4-chloro-6-alkyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidines (**3a–7a**)*

Conventional synthesis Compounds **3–7** and phosphorous oxychloride in 1 g:10 mL ratio were taken in the reaction vessel and heated to reflux for 10 min at 120 °C for 12 h to get the product.

Microwave method of synthesis Compounds **3–7** and phosphorous oxychloride in 1 g:10 mL ratio were taken in the reaction vessel and irradiated in microwave at 120 °C for 5–7 min at 100 W, 30 bar to get the products.

Reaction workup Excess phosphorous oxychloride was distilled off then crushed ice was added to the reaction mixture and neutralized acid using sodium bicarbonate solution to precipitate the product. The product obtained was filtered, dried and taken to next step immediately without further purification as they were unstable (**3a–7a**).

*General procedure for synthesis of 4-substituted-amino-6-alkyl/aryl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidines (**3b–3f, 4b–4k, 5b–5g, 6b–6g, 7b–7f**)*

The series of compounds prepared in the previous step (**3a–7a**) (20 mmol) was dissolved in anhydrous toluene (10 mL) and corresponding amines (30 mmol) was added and the reaction mixture was stirred at room temperature for 18–36 h. The reaction mixture was washed with water (10 mL), and the organic phase was dried under reduced pressure. The oil residue was crystallized by absolute ethanol to give the title compounds.

*N-Propyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**3b**)* Yield 58.65 %, mp 188 °C. IR (KBr, cm^{-1}): 3444.54 (–NH), 3218.10 (=C–H, aromatic), 1589.49 (C=C, aromatic), 770.88 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.94–0.98 (t, $J = 7.4$ Hz, 3H, –CH_2CH_2C<u>H</u>$_3$), 1.62–1.69 (sextet, $J = 7.2$ Hz, 2H, –CH_2C<u>H</u>$_2CH_3$), 3.47–3.52 (q, $J = 6.6$ Hz, 2H, –C<u>H</u>$_2CH_2CH_3$), 7.32–7.36 (t, $J = 8.0$ Hz, 1H, H-4′ Ph), 7.52–7.56 (d, $J = 8.0$ Hz, 2H, H-2′ and 6′ Ph), 8.19–8.21 (d, $J = 7.6$ Hz, 2H, H-3′ and 5′ Ph), 8.37 (s, 1H, H-3), 8.4 (s, 1H, H-6). MS *m/z* 253 (M+1), 254 (M+2). Anal. Calc. for $C_{14}H_{15}N_5$: C 66.38, H 5.97, N 27.65. Found: C 65.38, H 4.89, N 26.65.

*N-Isopropyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**3c**)* Yield 69.93 %, mp 137 °C. IR (KBr, cm^{-1}): 3446.29 (–NH), 3087.90 (=C–H, aromatic), 2970.40 (–CH), 1585.79 (C=C, aromatic), 747.31 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.26–1.28 (d, $J = 6.8$ Hz, 6H, –CH–[C<u>H</u>$_3$]$_2$), 4.43–4.47 (heptet, $J = 5.2$ Hz, 1H, –C<u>H</u>–[CH_3]$_2$), 7.32–7.35 (t, $J = 7.4$ Hz, 1H, H-4′ Ph), 7.52–7.56 (d, $J = 8.0$ Hz, 2H, H-2′ and 6′ Ph), 8.21–8.23 (d, $J = 8.0$ Hz, 2H, H-3′ and 5′ Ph), 8.37 (s, 1H, H-3), 8.4 (s, 1H, H-6). MS *m/z* 253 (M+1), 254 (M+2). Anal. Calc. for $C_{14}H_{15}N_5$: C 66.38, H 5.97, N 27.65. Found: C 65.38, H 4.97, N 26.65.

*N-Butyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**3d**)* Yield 60.34 %, mp 102 °C. IR (KBr, Cm^{-1}): 3446.21 (–NH), 3077.47 (=C–H, aromatic), 2931.03 (–CH_2), 1591.60 (C=C, aromatic), 750.70 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.97–1.01 (t, $J = 7.4$ Hz, 3H, –CH_2–CH_2–CH_2–C<u>H</u>$_3$), 1.40–1.49 (sextet, $J = 7.3$ Hz, 2H, –CH_2–CH_2–C<u>H</u>$_2$–CH_3), 1.63–1.71 (pentet, $J = 7.2$ Hz, 2H, –CH_2–C<u>H</u>$_2$–CH_2–CH_3), 3.56–3.61 (q, $J = 6.5$ Hz, 2H, –C<u>H</u>$_2$–CH_2–CH_2–CH_3), 7.37–7.41 (t, $J = 7.4$ Hz, 1H, H-4′

Ph), 7.57–7.61 (d, $J = 7.8$ Hz, 2H, H-2′ and 6′ Ph), 8.24–8.26 (d, $J = 7.6$ Hz, 2H, H-3′ and 5′ Ph), 8.42 (s, 1H, H-3), 8.44 (s, 1H, H-6). MS *m/z* 267 (M+1), 268 (M+2). Anal. Calc. for $C_{15}H_{17}N_5$: C 67.39, H 6.41, N 26.20. Found: C 66.05, H 5.31, N 25.20.

*N-(Furan-2-yl-methyl)-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**3e**)* Yield 55.37 %, mp 160 °C. IR (KBr, cm^{-1}): 3451.14 (–NH); 3071.76 (=C–H, aromatic), 2911.84 (–CH_2), 1591.64 (C=C, aromatic), 739.76 (–CH oop); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.86–4.87 (d, $J = 5.2$ Hz, 2H, –NH–C$\underline{H}_2$–), 5.85 (s, 1H, N$\underline{H}$), 6.34–6.36 (d, $J = 3.2$ Hz, 2H, H-2′ and 6′ Ph), 7.30–7.34 (t, $J = 7.4$ Hz, 1H, H-4′ Ph), 7.40 (m, 1H, H-4″), 7.48–7.52 (d, $J = 8.0$ Hz, 2H, H-3″ and 5″), 8.04 (s, 1H, H-3), 8.13–8.15 (d, $J = 8.0$ Hz, 2H, H-3′ and 5′ Ph), 8.51 (s, 1H, H-6). MS *m/z* 291 (M+1), 292 (M+2). Anal. Calc. for $C_{16}H_{13}N_5O$: C 65.97, H 4.50, N 24.04, O 5.49. Found: C 64.35, H 3.50, N 22.69, O 4.51.

*4-(Morpholin-4-yl)-1-phenyl-1H-pyrazolo[3,4-d]pyrimidine (**3f**)* Yield 51.21 %, mp 156 °C. IR (KBr, cm^{-1}): 3018.2 (=C–H, aromatic), 2917.65 (–CH_2), 1570.75 (C=C, aromatic), 771.58 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.77–3.79 (t, $J = 4.8$ Hz, 4H, H-2″ and 6″ morph), 3.96–3.98 (t, $J = 4.8$ Hz, 4H, H-3″ and 5″ morph), 7.35–7.39 (t, $J = 7.4$ Hz, 1H, H-4′ Ph), 7.54–7.58 (d, $J = 7.6$ Hz, 2H, H-2′ and 6′ Ph), 8.17–8.19 (d, $J = 8.4$ Hz, 2H, H-3′ and 5′ Ph), 8.41 (s, 1H, H-3), 8.60 (s, 1H, H-6). MS *m/z* 281 (M+1), 282 (M+2). Anal. Calc. for $C_{15}H_{15}N_5O$: C 64.04, H 5.37, N 24.90, O 5.69. Found: C 63.35, H 5.00, N 22.69, O 4.51.

*N-Propyl-6-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**4b**)* Yield 70.68 %, mp 113 °C. IR (KBr, cm^{-1}): 3447.9 (–NH), 2955.7, 2922.8 (–CH_3), 1606.2 (C=C, aromatic), 784.9 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.94–0.98 (t, $J = 7.4$ Hz, 3H, –$CH_2CH_2C\underline{H}_3$), 1.60–1.69 (sextet, $J = 7.2$ Hz, 2H, –$CH_2C\underline{H}_2CH_3$), 2.48 (s, 3H, –C$\underline{H}_3$), 3.46–3.51 (q, $J = 6.5$ Hz, 2H, –C$\underline{H}_2$–CH_2–CH_3), 7.30–7.34 (t, $J = 7.4$ Hz, 1H, H-4′ Ph), 7.52–7.56 (t, $J = 7.8$ Hz, 2H, H-2′ and 6′ Ph), 8.20–8.22 (dd, $J = 8.0$ Hz, 2H, H-3′ and 5′ Ph), 8.33 (s, 1H, H-3). MS *m/z* 267 (M+1), 268 (M+2). Anal. Calc. for $C_{15}H_{17}N_5$: C 67.39, H 6.41, N 26.20. Found: C, 66.39, H 5.41, N 25.20.

*N-Isopropyl-6-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**4c**)* Yield 80.2 %, mp 114 °C. IR (KBr, cm^{-1}): 3382.9 (–NH), 2981.0, 2966.8, 2925.2 (–CH_3), 1597.1 (C=C, aromatic), 787.2 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.25–1.27 (d, $J = 6.8$ Hz, 6H, –CH–[C$\underline{H}_3$]$_2$), 2.49–2.51 (dd, $J = 8.0$ Hz, 3H, –C$\underline{H}_3$), 4.46–4.48 (d, $J = 7.2$ Hz, 1H, –C$\underline{H}$–[CH_3]$_2$), 7.31–7.34 (t, $J = 7.4$ Hz, 1H, H-4′ Ph), 7.52–7.56 (d, $J = 7.8$ Hz, 2H, H-2′ and 6′ Ph), 8.20–8.22 (d, $J = 8.0$ Hz, 2H, H-3′ and 5′ Ph), 8.35 (s, 1H, H-3). MS *m/z* 267 (M+1), 268 (M+2). Anal. Calc. for $C_{15}H_{17}N_5$: C 67.39, H 6.41, N 26.20. Found: C 66.13, H 5.21, N 25.10.

*N-Butyl-6-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**4d**)* Yield 76.25 %, mp 110 °C. IR (KBr, cm^{-1}): 3382.9 (–NH), 2981.0, 2966.8, 2925.2 (–CH_3), 1597.1 (C=C, aromatic), 787.2 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.93–0.96 (t, $J = 7.4$ Hz, 3H, –CH_2–CH_2–CH_2–C$\underline{H}_3$), 1.36–1.43 (pentet, $J = 7.4$ Hz, 2H,–CH_2–CH_2–C$\underline{H}_2$–CH_3), 1.58–1.65 (pentet, $J = 7.1$ Hz, 2H, –CH_2–C$\underline{H}_2$–CH_2–CH_3), 2.48 (s, 3H, –C$\underline{H}_3$), 3.50–3.55 (q, $J = 6.5$ Hz, 2H, –C$\underline{H}_2$–CH_2–CH_2–CH_3), 7.30–7.34 (t, $J = 7.4$ Hz, 1H, H-4′ Ph), 7.52–7.56 (t, $J = 8.0$ Hz, 2H, H-2′ and 6′ Ph), 8.20–8.22 (d, $J = 7.6$ Hz, 2H, H-3′ and 5′ Ph), 8.33 (s, 1H, H-3). MS *m/z* 281 (M+1), 282 (M+2). Anal. Calc. for $C_{16}H_{19}N_5$: C 68.30, H 6.81, N 24.89. Found: C 67.05, H 5.51, N 23.69.

*N-(Furan-2-ylmethyl)-6-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**4e**)* Yield 73.29 %, mp 116 °C. IR (KBr, cm^{-1}): 3241.1 (–NH), 2957.3 (–CH_2), 1580.4 (C=C, aromatic), 784.0 (–CH oop); ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.32 (s, 3H, –C$\underline{H}_3$) 4.77–4.78 (d, $J = 4.8$ Hz, 2H, –NH–C$\underline{H}_2$–), 6.39–6.44 (dd, $J = 8.0$ Hz, 2H, H-3″ and 5″), 7.32–7.35 (t, $J = 7.2$ Hz, 1H, H-4′ Ph), 7.53–7.57 (t, $J = 7.8$ Hz, 2H, H-2′ and 6′ Ph), 7.63 (s, 1H, H-4″), 8.20–8.22 (d, $J = 8.0$ Hz, 2H, H-3′ and 5′ Ph), 8.37 (s, 1H, H-3). MS *m/z* 305 (M+1), 306 (M+2). Anal. Calc. for $C_{17}H_{15}N_5O$: C 66.87, H 4.95, N 22.94, O 5.24. Found: C 65.47, H 3.55, N 21.34, O 4.04.

*6-Methyl-4-(morpholin-4-yl)-1-phenyl-1H-pyrazolo[3,4-d]pyrimidine (**4f**)* Yield 85.5 %, mp 116 °C. IR (KBr, cm^{-1}): 2960.7 (–CH_3), 1590.6 (C=C, aromatic), 787.6 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.68 (s, 3H, –C$\underline{H}_3$), 3.76–3.78 (t, $J = 4.0$ Hz, 4H, H-2″ and 6″ morph), 3.94–3.95 (d, $J = 4.4$ Hz, 4H, H-3″ and 5″ morph), 7.34–7.37 (t, $J = 7.2$ Hz, 1H, H-4′ Ph), 7.54–7.58 (t, $J = 7.4$ Hz, 2H, H-2′ and 6′ Ph), 8.18–8.20 (d, $J = 8.0$ Hz, 2H, H-3′ and 5′ Ph), 8.52 (s, 1H, H-3). MS *m/z* 295 (M+1), 296 (M+2). Anal. Calc. for $C_{16}H_{17}N_5O$: C 65.07 H 5.80, N 23.71, O 5.42. Found: C 64.07, H 4.27, N 22.41, O 4.16.

*6-Methyl-N,1-diphenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**4g**)* Yield 75.5 %, mp 115 °C. IR (KBr, cm^{-1}): 3449.7 (–NH), 2909.3 (–CH_2), 1596.5 (C=C, aromatic), 784.49 (–CH oop);^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.51–2.58 (d, $J = 3.0$ Hz, 3H, –C$\underline{H}_3$), 7.05–7.14 (dd, $J = 4.4$ Hz, 2H, H-4′ and 4″ Ph), 7.31–7.42 (m, $J = 6.8$ Hz, 4H, H-2″· 3″, 5″

and 6″ Ph), 7.91–7.92 (d, $J = 6.0$ Hz, 2H, H-2′ and 6′ Ph), 8.21–8.23 (d, $J = 7.2$ Hz, 2H, H-3′ and 5′ Ph), 8.48 (s, 1H, H-3). MS *m/z* 301 (M+1), 302 (M+2); Anal. Calc. for $C_{18}H_{15}N_5$: C 71.74, H 5.02, N 23.24. Found: C 70.34, H 4.13, N 22.04.

*N,N-Diethyl-6-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**4h**)* Yield 79.04 %, mp 145 °C. IR (KBr, cm^{-1}): 2982.0 (–CH_2), 1592.4 (C=C, aromatic), 780.8 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.16–1.21 (t, $J = 7.6$ Hz, 3H, –N (–CH_2–C$\underline{H}_3$)$_2$), 2.51 (s, 3H, –C$\underline{H}_3$), 2.86–2.91 (q, $J = 7.2$ Hz, 2H, –N (–C$\underline{H}_2$–CH_3)$_2$), 7.30–7.34 (t, $J = 7.4$ Hz, 1H, H-4′ Ph), 7.52–7.56 (t, $J = 7.8$ Hz, 2H, H-2′ and 6′ Ph), 8.20–8.22 (d, $J = 8.0$ Hz, 2H, H-3′ and 5′ Ph), 8.31 (s, 1H, H-3). MS *m/z* 281 (M+1), 282 (M+2). Anal. Calc. for $C_{16}H_{19}N_5$: C 68.30, H 6.81, N 24.89. Found: C 67.38, H 5.61, N 23.59.

*N-Benzyl-6-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**4i**)* Yield 56.78 %, mp 139 °C. IR (KBr, cm^{-1}): 3449.7 (–NH), 2909.3 (–CH_2), 1596.5 (C=C, aromatic), 784.49 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.50–2.51 (m, $J = 1.7$ Hz, 3H, –C$\underline{H}_3$), 4.78–4.79 (d, $J = 5.6$ Hz, 2H, –NH–C$\underline{H}_2$–C_6H_5), 7.28–7.31 (t, $J = 6.8$ Hz, 1H, H-4′ Ph), 7.33–7.41 (m, $J = 7.2$ Hz, 5H, H-2″, 3″, 5″, 6″ and 4′ Ph), 7.52–7.56 (t, $J = 7.8$ Hz, 2H, H-2′ and 6′ Ph), 8.20–8.22 (d, $J = 8.0$ Hz, 2H, H-3′ and 5′ Ph), 8.37 (s, 1H, H-3). MS *m/z* 315 (M+1), 316 (M+2). Anal. Calc. for $C_{19}H_{17}N_5$: C 72.36, H 5.43, N 22.21. Found: C 71.06, H 4.13, N 21.01.

*N-Ethyl-6-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**4j**)* Yield 56.89 %, mp 140 °C. IR (KBr, cm^{-1}): 3274.1 (–NH), 2957.5 (–CH_2), 1616.6 (C=C, aromatic), 786.6 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.22–1.25 (t, $J = 7.2$ Hz, 3H, –NH–CH_2–C$\underline{H}_3$), 2.49–2.51 (d, $J = 6.8$ Hz, 2H, –C$\underline{H}_3$), 3.52–3.59 (pentet, $J = 6.6$ Hz, 2H, –NH–C$\underline{H}_2$–CH_3), 7.30–7.34 (t, $J = 7.4$ Hz, 1H, H-4′ Ph), 7.52–7.56 (t, $J = 7.8$ Hz, 2H, H-2′ and 6′ Ph), 8.20–8.22 (d, $J = 8.0$ Hz, 2H, H-3′ and 5′ Ph), 8.31 (s, 1H, H-3). MS *m/z* 253 (M+1), 254 (M+2). Anal. Calc. for $C_{14}H_{15}N_5$: C 66.38, H 5.97, N 27.65. Found: C 65.38, H 4.27, N 26.65.

*N-(2-Methoxyphenyl)-6-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**4k**)* Yield 62.65 %, mp 150 °C. IR (KBr, cm^{-1}): 3423.2 (–NH), 2909.3 (–CH_2), 1628.3 (C=C, aromatic), 746.3 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.89 (s, 3H, –C$\underline{H}_3$), 3.90 (s, 3H, –O–C$\underline{H}_3$), 7.31–7.42 (m, $J = 6.8$ Hz, 4H, H-3″, 4″, 5″ and 6″ Ph), 7.91–7.92 (d, $J = 6.0$ Hz, 2H, H-2′ and 6′ Ph), 8.21–8.23 (d, $J = 7.2$ Hz, 2H, H-3′ and 5′ Ph), 8.48 (s, 1H, H-3), 9.90 (s, 1H, –N$\underline{H}$). MS *m/z* 331 (M+1), 332 (M+2); Anal. Calc. for $C_{19}H_{17}N_5O$: C 68.87, H 5.17, N 21.13, O 4.83. Found: C 68.87, H 4.13, N 21.13, O 4.83.

*N-Propyl-6-ethyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**5b**)* Yield 75.21 %, mp 132 °C. IR (KBr, cm^{-1}): 3422.2 (–NH), 2958.9, 2924.8 (–CH_2), 1596.2 (C=C, aromatic), 757.6 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.94–0.98 (t, $J = 7.4$ Hz, 3H, –NH–CH_2–CH_2–C$\underline{H}_3$), 1.27–1.31 (t, $J = 7.4$ Hz, 3H, –CH_2–C$\underline{H}_3$), 1.63–1.70 (pentet, $J = 7.1$ Hz, 2H, –NH–CH_2–C$\underline{H}_2$–CH_3), 2.72–2.78 (q, $J = 7.3$ Hz, 2H, –C$\underline{H}_2$–CH_3), 3.47–3.52 (q, $J = 6.6$ Hz, 2H, –NH–C$\underline{H}_2$–CH_2–CH_3), 7.30–7.33 (t, $J = 7.4$ Hz, 1H, H-4′ Ph), 7.52–7.56 (t, $J = 7.8$ Hz, 2H, H-2′ and 6′ Ph), 8.24–8.25 (d, $J = 7.6$ Hz, 2H, H-3′ and 5′ Ph), 8.32 (s, 1H, H-3). MS *m/z* 281 (M+1), 282 (M+2). Anal. Calc. for $C_{16}H_{19}N_5$: C 68.30, H 6.81, N 24.89. Found: C 67.30, H 5.81, N 23.89.

*N-Isopropyl-6-ethyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**5c**)* Yield 72.9 %, mp 122 °C. IR (KBr, cm^{-1}): 3397.1 (–NH), 2948.2 (–CH_2), 1566.6 (C=C, aromatic), 784.9 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.26–1.31 (q, $J = 6.6$ Hz, 9H, –CH–(C$\underline{H}_3$)$_2$ and –CH_2–C$\underline{H}_3$), 2.73–2.78 (q, $J = 7.1$ Hz, 2H, –C$\underline{H}_2$–CH_3), 4.47 (s, 1H, –N$\underline{H}$), 7.30–7.33 (t, $J = 7.2$ Hz, 1H, H-4′ Ph), 7.52–7.56 (t, $J = 7.8$ Hz, 2H, H-2′ and 6′ Ph), 8.23–8.25 (d, $J = 7.6$ Hz, 2H, H-3′ and 5′ Ph), 8.35 (s, 1H, H-3). MS *m/z* 281 (M+1), 282 (M+2). Anal. Calc. for $C_{16}H_{19}N_5$: C 68.30, H 6.81, N 24.89. Found: C 67.30, H 5.81, N 23.89.

*N-Butyl-6-ethyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**5d**)* Yield 65.7 %, mp 126 °C. IR (KBr, cm^{-1}): 3442.7 (–NH), 2961.6, 2926.5 (–CH_2), 1583.5 (C=C, aromatic), 753.6 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.92–0.96 (t, $J = 7.4$ Hz, 3H,–NH–CH_2–CH_2–CH_2–C$\underline{H}_3$), 1.27–1.31 (t, $J = 7.4$ Hz, 3H, –CH_2–C$\underline{H}_3$), 1.37–1.44 (pentet, $J = 7.2$ Hz, 2H,–NH–CH_2–CH_2–C$\underline{H}_2$–CH_3), 1.59–1.66 (pentet, $J = 6.8$ Hz, 2H, –NH–CH_2–C$\underline{H}_2$–CH_2–CH_3), 2.72–2.78 (q, $J = 7.3$ Hz, 2H, –C$\underline{H}_2$–CH_3), 3.52–3.57 (q, $J = 6.2$ Hz, 2H, –NH–C$\underline{H}_2$–CH_2–CH_2–CH_3), 7.30–7.33 (t, $J = 7.4$ Hz, 1H, H-4′ Ph), 7.52–7.56 (t, $J = 7.8$ Hz, 2H, H-2′ and 6′ Ph), 8.24–8.26 (d, $J = 8.0$ Hz, 2H, H-3′ and 5′ Ph), 8.32 (s, 1H, H-3). MS *m/z* 295 (M+1), 296 (M+2). Anal. Calc. for $C_{17}H_{21}N_5$: C 69.12, H 7.17, N 23.71. Found: C 68.12, H 6.17, N 22.31.

*N-Furfuryl-6-ethyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**5e**)* Yield 56.7 %, mp 124 °C. IR (KBr, cm^{-1}): 3442.2 (–NH), 2975.5, 2923.7 (–CH_2), 1583.6 (C=C, aromatic), 784.2 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.29–1.33 (t, $J = 7.6$ Hz, 3H, –CH_2–C$\underline{H}_3$), 2.76–2.81 (q, $J = 7.4$ Hz, 2H, –C$\underline{H}_2$–CH_3), 4.77–4.78 (d, $J = 5.6$ Hz,

2H, –NH–C<u>H</u>$_2$–), 6.39–6.43 (dd, $J = 8.0$ Hz, 2H, H-3″ and 5″furfuryl), 7.31–7.34 (t, $J = 7.2$ Hz, 1H, H-4′ Ph), 7.53–7.57 (t, $J = 8.0$ Hz, 2H, H-2′ and 6′ Ph), 7.62 (s, 1H, H-4″furfuryl), 8.23–8.25 (d, $J = 8.0$ Hz, 2H, H-3′ and 5′ Ph), 8.36 (s, 1H, H-3). MS *m/z* 319 (M+1), 320 (M+2). Anal. Calc. for $C_{18}H_{17}N_5O$: C 67.70, H 5.37, N 21.93, O 5.01. Found: C 66.47, H 4.21, N 20.53, O 4.01.

*6-Ethyl-4-(morpholin-4-yl)-1-phenyl-1H-pyrazolo[3,4-d]pyrimidine (**5f**)* Yield 75.8 %, mp 123 °C. IR (KBr, cm^{-1}): 2946.4 (–CH_2), 1566.3 (C=C, aromatic), 784.2 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.27–1.31 (t, $J =$ 7.4 Hz, 3H, –CH_2–C<u>H</u>$_3$), 2.74–2.79 (q, $J = 7.4$ Hz, 2H, –C<u>H</u>$_2$–CH_3), 3.76–3.78 (t, $J = 4.8$ Hz, 4H, H-2″ and 6″ morph), 3.93–3.96 (t, $J = 4.8$ Hz, 4H, H-3″ and 5″ morph), 7.32–7.36 (t, $J = 7.4$ Hz, 1H, H-4′ Ph), 7.53–7.57 (t, $J = 7.8$ Hz, 2H, H-2′ and 6′ Ph), 8.22–8.24 (d, $J = 8.0$ Hz, 2H, H-3′ and 5′ Ph), 8.50 (s, 1H, H-3). MS *m/z* 309 (M+1), 310 (M+2). Anal. Calc. for $C_{17}H_{19}N_5O$: C 66.00, H 6.19, N 22.64, O 5.17. Found: C 65.35, H 5.10, N 21.24, O 4.11.

*6-Ethyl-N,1-diphenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**5g**)* Yield 74.6 %, mp 125 °C. IR (KBr, cm^{-1}): 3423.2 (–NH), 2935.2 (–CH_2), 1577.9 (C=C, aromatic), 758.5 (–CH oop). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.33–1.37 (t, $J = 7.4$ Hz, 3H, –CH_2–C<u>H</u>$_3$), 2.84–2.89 (q, $J = 6.9$ Hz, 2H, –C<u>H</u>$_2$–CH_3), 7.12–7.15 (t, $J = 7.4$ Hz, 1H, H-4″ Ph), 7.34–7.38 (t, $J = 7.4$ Hz, 1H, H-4′ Ph), 7.40–7.44 (t, $J = 7.0$ Hz, 2H, H-2″ and 6″ Ph), 7.56–7.59 (t, $J = 7.0$ Hz, 2H, H-2′ and 6′ Ph), 7.92–7.94 (d, $J = 8.0$ Hz, 2H, H-3″ and 5″ Ph), 8.25–8.27 (d, $J = 7.6$ Hz, 2H, H-3′ and 5′ Ph), 8.47 (s, 1H, H-3). MS *m/z* 315 (M+1), 316 (M+2). Anal. Calc. for $C_{19}H_{17}N_5$: C 72.36, H 5.43, N 22.21. Found: C 71.16, H 4.13, N 21.04.

*N-Butyl-6-isopropyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**6b**)* Yield 65.4 %, mp 125 °C. IR (KBr, cm^{-1}): 3315.5 (–NH), 2962.7, 2929.0 (–CH_3), 1594.6 (C=C, aromatic), 753.3 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.93–0.97 (t, $J = 7.0$ Hz, 3H,–NH–CH_2–CH_2–CH_2–C<u>H</u>$_3$), 1.29–1.31 (d, $J = 6.8$ Hz, 6H, –CH–(C<u>H</u>$_3$)$_2$), 1.34–1.36 (d, $J = 6.0$ Hz, 1H, –C<u>H</u>–$(CH_3)_2$), 1.38–1.43 (q, $J = 7.3$ Hz, 2H, –NH–CH_2–CH_2–C<u>H</u>$_2$–CH_3), 1.63–1.66 (t, $J = 7.0$ Hz, 2H, –NH–CH_2–C<u>H</u>$_2$–CH_2–CH_3), 3.59–3.60 (d, $J = 4.8$ Hz, 2H, –NH–C<u>H</u>$_2$–CH_2–CH_2–CH_3), 3.69 (s, 1H, –N<u>H</u>), 7.33–7.36 (t, $J = 7.0$ Hz, 1H, H-4′ Ph), 7.54–7.58 (t, $J = 7.6$ Hz, 2H, H-2′ and 6′ Ph), 8.21–8.23 (d, $J = 7.2$ Hz, 2H, H-3′ and 5′ Ph), 8.44 (s, 1H, H-3). MS *m/z* 309 (M+1), 310 (M+2). Anal. Calc. for $C_{18}H_{23}N_5$: C 69.87, H 7.49, N 22.63. Found: C 68.12, H 6.17, N 21.31.

*6-Isopropyl-4-(morpholino-4-yl)-1-phenyl-1H-pyrazolo[3,4-d]pyrimidine (**6c**)* Yield 69.9 %, mp 129 °C. IR (KBr, cm^{-1}): 2959.8, 2927.0 (–CH_3), 1592.6 (C=C, aromatic), 756.7 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.28–1.30 (d, $J = 6.8$ Hz, 6H, –CH–(C<u>H</u>$_3$)$_2$), 2.94–3.05 (heptet, $J = 6.8$ Hz, 1H, –C<u>H</u>–$(CH_3)_2$), 3.76–3.79 (t, $J = 4.8$ Hz, 4H, H-2″ and 6″ morph), 3.94–3.97 (t, $J = 4.6$ Hz, 4H, H-3″ and 5″ morph), 7.32–7.36 (t, $J = 7.4$ Hz, 1H, H-4′ Ph), 7.54–7.58 (t, $J = 8.0$ Hz, 2H, H-2′ and 6′ Ph), 8.24–8.26 (d, $J = 7.6$ Hz, 2H, H-3′ and 5′ Ph), 8.51 (s, 1H, H-3). MS *m/z* 323 (M+1), 324 (M+2). Anal. Calc. for $C_{18}H_{21}N_5O$: C 66.85, H 6.55, N 21.66, O 4.95. Found: C 65.35, H 5.10, N 20.24, O 3.56.

*6-Isopropyl-N,1-diphenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**6d**)* Yield 72.3 %, mp 127 °C. IR (KBr, cm^{-1}): 3370.1 (–NH), 2966.3, 2925.1 (–CH_3), 1597.5 (C=C, aromatic), 754.1 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.34–1.36 (d, $J = 6.8$ Hz, 6H, –CH_2–(C<u>H</u>$_3$)$_2$), 3.06–3.13 (pentet, $J = 6.9$ Hz, 1H, –C<u>H</u>–$(CH_3)_2$), 7.11–7.15 (t, $J = 7.4$ Hz, 1H, H-4″ Ph), 7.34–7.37 (t, $J = 7.4$ Hz, 1H, H-4′ Ph), 7.40–7.44 (t, $J = 7.8$ Hz, 2H, H-2″ and 6″ Ph), 7.56–7.60 (t, $J = 8.0$ Hz, 2H, H-2′ and 6′ Ph), 7.94–7.96 (d, $J = 8.0$ Hz, 2H, H-3″ and 5″ Ph), 8.27–8.29 (d, $J = 8.0$ Hz, 2H, H-3′ and 5′ Ph), 8.48 (s, 1H, H-3), 10.10 (s, 1H, –N<u>H</u>), MS *m/z* 329 (M+1), 330 (M+2), Anal. Calc. for $C_{20}H_{19}N_5$: C 72.93, H 5.81, N 21.26. Found: C 71.16, H 4.13, N 20.04.

*N-Propyl-6-[(propylamino) methyl]-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (**7b**)* Yield 67.7 %, mp 113 °C. IR (KBr, cm^{-1}): 3449.1 (–NH), 2959.6, 2924.7 (–CH_2), 1592.4 (C=C, aromatic), 758.4 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.48–0.51 (t, $J = 4.4$ Hz, 3H, –NH–CH_2–CH_2–C<u>H</u>$_3$), 0.94–0.98 (t, $J = 7.4$ Hz, 3H, –CH_2–NH–CH_2–CH_2–C<u>H</u>$_3$), 1.60–1.69 (sextet, $J = 7.2$ Hz, 2H, –NH–CH_2–C<u>H</u>$_2$–CH_3), 1.89–1.99 (pentet, $J = 7.8$ Hz, 2H, –CH_2–NH–CH_2–C<u>H</u>$_2$–CH_3), 3.47–3.52 (q, $J = 6.6$ Hz, 2H, –NH–C<u>H</u>$_2$–CH_2–CH_3), 3.96–4.05 (q, $J = 6.2$ Hz, 2H, –CH_2–NH–C<u>H</u>$_2$–CH_2–CH_3), 4.48 (s, 2H, –C<u>H</u>$_2$–NH–CH_2–CH_2–CH_3), 7.30–7.34 (t, $J = 7.4$ Hz, 1H, H-4′ Ph), 7.52–7.56 (t, $J = 7.8$ Hz, 2H, H-2′ and 6′ Ph), 8.20–8.22 (d, $J = 8.0$ Hz, 2H, H-3′ and 5′ Ph), 8.31 (s, 1H, H-3). MS *m/z* 324 (M+1), 325 (M+2). Anal. Calc. for $C_{18}H_{24}N_6$: C 66.64, H 7.46, N 25.90. Found: C 65.43, H 6.21, N 24.60.

*N-Isopropyl-6-[(isopropylamino) methyl]-1-phenyl-1H-pyrazolo [3,4-d]pyrimidin-4-amine (**7c**)* Yield 72.7 %, mp 115 °C. IR (KBr, cm^{-1}): 3447.8 (–NH), 2961.4, 2925.0 (–CH_2), 1593.5 (C=C, aromatic), 759.2 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.26–1.28 (d, $J = 6.8$ Hz, 6H, –NH–CH–(C<u>H</u>$_3$)$_2$), 1.48–1.50 (d, $J = 6.4$ Hz, 6H, CH_2–NH–CH–(C<u>H</u>$_3$)$_2$), 3.04–3.11 (q, $J = 8.0$ Hz, 1H,

CH_2–NH–C<u>H</u>–$(CH_3)_2$), 4.43–4.47 (q, $J = 6.7$ Hz, 6H, –NH–C<u>H</u>–$(CH_3)_2$), 4.62 (s, 2H, –C<u>H_2</u>–NH–CH–$(CH_3)_2$), 7.32–7.35 (t, $J = 7.4$ Hz, 1H, H-4′ Ph), 7.52–7.56 (t, $J = 7.8$ Hz, 2H, H-2′ and 6′ Ph), 8.21–8.24 (d, $J = 8.0$ Hz, 2H, H-3′ and 5′ Ph), 8.37 (s, 1H, H-3). MS *m/z* 324 (M+1), 325 (M+2). Anal. Calc. for $C_{18}H_{24}N_6$: C 66.64, H 7.46, N 25.90. Found: C 65.43, H 6.21, N 24.60.

*N-Ethyl-6-[(ethylamino) methyl]-1-phenyl-1H-pyrazolo[3,4-d] pyrimidin-4-amine (7**d**)* Yield 69.9 %, mp 116 °C. IR (KBr, cm^{-1}): 3441.5 (–NH), 2966.3, 2924.3 (–CH_2), 1631.4 (C=C, aromatic), 758.9 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.69–0.73 (t, $J = 7.4$ Hz, 3H, –CH_2–NH–CH_2–C<u>H_3</u>), 1.22–1.25 (t, $J = 7.2$ Hz, 3H, –NH–CH_2–C<u>H_3</u>), 2.67–2.73 (pentet, $J = 6.8$ Hz, 2H, –CH_2–NH–C<u>H_2</u>–CH_3), 3.52–3.59 (pentet, $J = 6.8$ Hz, 2H, –CH_2–NH–CH_2–C<u>H_2</u>–CH_3), 3.47–3.52 (q, $J = 6.6$ Hz, 2H, –NH–C<u>H_2</u>–CH_2–CH_3), 3.96–4.05 (q, $J = 6.2$ Hz, 2H, –CH_2–NH–C<u>H_2</u> –CH_3), 4.47 (s, 2H, –C<u>H_2</u>–NH–CH_2–CH_3), 7.30–7.34 (t, $J = 7.4$ Hz, 1H, H-4′ Ph), 7.52–7.56 (t, $J = 7.8$ Hz, 2H, H-2′ and 6′ Ph), 8.20–8.22 (d, $J = 8.0$ Hz, 2H, H-3′ and 5′ Ph), 8.31 (s, 1H, H-3). MS *m/z* 296 (M+1), 297 (M+2); Anal. Calc. for $C_{16}H_{20}N_6$: C 64.84, H 6.80, N 28.36. Found: C 63.54, H 5.52, N 27.15.

*6-(Morpholin-4-ylmethyl)-4-(morpholino-4-yl)-1-phenyl-1H-pyrazolo[3,4-d]pyrimidine (**7e**)* Yield 59.8 %, mp 120 °C. IR (KBr, cm^{-1}): 2966.3, 2924.3 (–CH_2), 1631.4 (C=C, aromatic), 758.9 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.32–2.34 (t, $J = 5.2$ Hz, 4H, H-2‴ and 6‴ morph), 3.53–3.55 (t, $J = 4.8$ Hz, 4H, H-3‴ and 5‴ morph), 3.77–3.79 (t, $J = 4.8$ Hz, 4H, H-2″ and 6″ morph), 3.96–3.98 (t, $J = 4.8$ Hz, 4H, H-3″ and 5″ morph), 4.47 (s, 2H, –C<u>H_2</u>–), 7.35–7.39 (t, $J = 7.4$ Hz, 1H, H-4′ Ph), 7.54–7.58 (t, $J = 7.6$ Hz, 2H, H-2′ and 6′ Ph), 8.17–8.19 (d, $J = 8.0$ Hz, 2H, H-3′ and 5′ Ph), 8.41 (s, 1H, H-3). MS *m/z* 380 (M+1), 381 (M+2). Anal. Calc. for $C_{20}H_{24}N_6O_2$: C 63.14, H 6.36, N 22.09, O 8.41. Found: C 62.01, H 5.16, N 21.00, O 7.32.

*N,1-Diphenyl-6-[(phenylamino)methyl]-1H-pyrazolo[3,4-d] pyrimidin-4-amine (**7f**)* Yield 72.4 %, mp 158 °C. IR (KBr, cm^{-1}): 3397.2 (–NH), 2926.1 (–CH_2), 1575.7 (C=C, aromatic), 787.1 (–CH oop); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.47 (s, 2H, –C<u>H_2</u>–NH–C_6H_5), 6.58–6.62 (t, $J = 7.0$ Hz, 1H, H-4‴ Ph), 6.76–6.62 (d, $J = 7.6$ Hz, 2H, H-2‴ and 6‴ Ph), 7.10–7.14 (t, $J = 7.2$ Hz, 3H, H-3‴,5‴ and 4″ Ph), 7.34–7.38 (t, $J = 6.8$ Hz, 3H, H-2″,6″ and 4′ Ph), 7.50–7.54 (t, $J = 7.8$ Hz, 2H, H-3″ and 5″ Ph), 7.83–7.85 (d, $J = 7.6$ Hz, 2H, H-2′ and 6′ Ph), 8.17–8.19 (d, $J = 8.0$ Hz, 2H, H-3′ and 5′ Ph), 8.51 (s, 1H, H-3), MS *m/z* 392 (M+1), 393 (M+2), Anal. Calc. for $C_{24}H_{20}N_6$: C 73.45, H 5.14, N 21.41. Found: C 72.17, H 4.13, N 20.01.

Antibacterial testing

Test microorganisms

The newly synthesized compounds were screened for their antibacterial activity against Gram positive [*B. subtilis* (NCIM-2545), *S. epidermidis* (NCIM-2493), *S. aureus* (NCIM-5021)], and Gram negative [*E. coli* (NCIM-2803)] bacteria. All the strains were maintained by weekly subculturing on nutrient agar slant stored at 4 °C after previous 24 h incubation at 37 °C. Before each experiment, the organism was activated by successive subculturing and incubation.

Standardization of test microorganisms

To standardize the inoculums density for a susceptibility test, a $BaSO_4$, turbidity standard, equivalent to a 0.5 McFarland standard is used. The $BaSO_4$, Mcfarland 0.5 standard is prepared as follows. A 0.5 mL of 1.175 % w/v of $BaCl_2{\cdot}2H_2O$ is added to 99.5 mL of 1 % w/v of H_2SO_4 with constant stirring to maintain suspension. The correct density of the turbidity standard is verified using a UV spectrophotometer by determining the absorbance. The absorbance at 625 nm is 0.08–0.10 for this standard. This suspension is used to standardize the inoculums density. A 10-mL volume of sterile water was added to the agar slant containing a 24-h-old culture of purified test microorganism and shaken carefully to harvest the organism. Subsequently, dilutions were carried out to get microbial population of 10^5 cfu/mL by comparing with $BaSO_4$, equivalent to Mcfarland 0.5 standard.

Preparation of stock solution and determination of zones of inhibition

All the compounds to be tested were dissolved in DMSO to obtain a stock concentration of 1 mg/mL. The final concentrations were made from the stock solution and the zones of inhibition measured. Inoculums of 100-μL solution from the standardized bacterial suspension was added to the Muller Hinton broth and plated. The cups for each compound were made in three Petri dishes so as to make ($n = 9$) and the bacterial suspension used is a 24-h culture. Cups were made in the agar plates, which were filled with the specific concentration (20 μg/mL) of the prepared drug solution. The plates were incubated for 24 h at 35 °C in an ambient air incubator. Solvents and growth controls were kept and the zones of inhibition were noted. These results were compared with amoxicillin as standard and are given in Table 1. The above studies showed that **6c** was best of the series of compounds, showed activity against all the four organisms. The compound **4d**, exhibited against

E. coli, *S. aureus* and *S. epidermidis*. The compound **6b** showed activity against *S. aureus* and *S. epidermidis.* The remaining compounds were found to have antibacterial activity against the tested organisms.

Anticancer activity

Cells

The anticancer activity was performed on Human Skin Cancer cell Line G36, with 5×10^3 cells/well. The cell lines were obtained from NCCS, Pune. The vehicle used is dimethyl sulfoxide. The anticancer activity of the synthesized compounds was evaluated by SRB assay. The % growth of cells after treatment with the compounds is reported in Table 3. From the graphs drawn, the parameters (LC_{50}, TGI and GI_{50}) were calculated. GI_{50} is the growth inhibition of 50 % (GI_{50}) calculated from drug concentration resulting in a 50 % reduction in the net protein increase. TGI is the drug concentration resulting in total growth inhibition. LC_{50} is the concentration of drug resulting in a 50 % reduction in the measured protein at the end of the drug treatment (concentration of drug causing lethality to 50 % of the cells as compared to that of the beginning) indicating a net loss of cells following treatment.

SRB assay

The SRB assay is used for cell density determination, based on the measurement of cellular protein content. The method used here has been optimized for the toxicity screening of compounds to adherent cells in a 96-well format. After an incubation period, cell monolayers are fixed with 10 % (w/v) trichloroacetic acid and stained for 30 min, after which the excess dye is removed by washing repeatedly with 1 % (v/v) acetic acid. The protein-bound dye is dissolved in 10-mM Tris base solution for OD determination at 510 nm using a microplate reader. The results are linear over a 20-fold range of cell numbers and the sensitivity is comparable to those of fluorometric methods. The method not only allows a large number of samples to be tested within a few days but also requires only simple equipment and inexpensive reagents. The SRB assay is, therefore, an efficient and highly cost-effective method for screening (Vacha and Kanyawim 2006).

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