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Research Article

Synthesis of Pyrazolo [4,3-e] Pyrimido [1,2-a] Pyrimidin-3-Amine Derivatives and their Antimicrobial Activity

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ABSTRACT
The present work demonstrated the synthesis of some new pyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amine derivatives by sequential course. The reaction of 4- chloro benzaldehydes (**1**) with ethylcynoacetate (**2**) and guanidine hydrochloride (**3**) gives the intermediate product (**4**), which on treated with substituted chalcones (**5a-j**) gives the intermediate product (**6a-j**) which on treated with hydrazine hydrate **(7)** gives the final pyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amine derivatives (**8a-j**). All the synthesized derivatives were characterized and screened for their antimicrobial activity has been carried out. Most of the synthesized compounds exhibited intensive antimicrobial activity.

Keyword: Pyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin; bleaching earth clay (pH 12.5); green Synthesis; polyethylene glycol (PEG-400); antimicrobial activity

INTRODUCTION

Natural antibiotic agents have become essential to the ongoing health care system, supporting and complementing the natural immune system against the microbial organisms. As conventional antibiotics are often abused to treat microbial infections, some microorganisms have developed tolerance to these antibiotics. Because of the appearance of antibiotic resistant strains, the steady development of novel and efficient antibiotic agents is more crucial than ever [1]. So, the medical community deals with severe crisis against infections caused by the pathogenic bacteria and needs an effective therapy and search for novel antimicrobial agents. Synthetic organic chemistry has always been a vital part of highly integrated and multidisciplinary process of various drug developments.

Pyrazoles are well known class of heterocyclic compound with two adjacent nitrogen atoms and have great scientific interest in medicinal [2], pharmaceutical [3] and agrochemical field [4]. pyrazoles play an important role in medicinal chemistry as synthetic building blocks with wide range of application, such as antimicrobial [5], anticancer[6], antifungal [7], anti-inflammatory [8], antiviral [9] and anticonvulsant [10]. The ring of pyrazole has a main framework in agricultural field for transition metal cross coupling and polymerization reaction. Apart from this, pyrazole derivatives have been utilized for the development of crop protecting agents [11]. These compounds were also applicable for have demonstrate to possess

various applications in technological impact like photo protecting agents and ultraviolet stabilizer [12].

Pyrazolopyrimidines and related fused heterocycles are of gaining enough concern as potential bioactive molecules. The heterocyclic fusion of pyrimidine and pyrazole nucleus resulted in the formation of pyrazolopyrimidines. The pyrazolopyrimidine is a facile scaffold for the synthesis of potential drugs or molecular tools.

The pyrazolo[3,4-*d*]pyrimidine scaffold is an obvious and allowanced structural motif with pharmaceutical consequences in discrete therapeutic areas. They are biologically effective isomeric purine correspondent and have convenient properties as antimetabolites in purine biochemical reactions [13-15]. Pyrazolopyrimidine and related heterocycles are found to possess wide applications in the field of medicine and agriculture. They exhibit diversified pharmacological activities like antibacterial [16], antifungal [17], CNS-depressant [18], antiviral [19], anticancer [20] and tuberculostatic [21]. An array of biological activities such as antiphlogistic, antitumor [22], inhibitor kinases [23], adenosine antagonist [24], glutamate modulator [25] and herbicidal [26] exhibited by the pyrazolopyrimidines.

With this background and to broaden the scope of our ongoing research on developing new and novel heterocycles by green approach [27-33]. In the present research work we demonstrate the synthesize and investigate the antimicrobial potentiality of a series of pyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amine derivatives (**8a-j**) via three steps, in PEG-400 as green solvent. All the newly synthesized derivatives were estimated for their inherent antimicrobial activity.

MATERIAL AND METHODS

Chemistry

All the melting points were uncorrected and determined in an open capillary tube. The chemicals and solvents used were of laboratory grade and purified. The compound **4** prepared by the reported method [34, 35] and the compounds (**5a-j)** were prepared by the method previously reported by us [32, 33]. Completion of the reaction was monitored by thin layer chromatography on percolated sheets of silica gel-G (merck, Germany) using UV lamp for detection. IR spectra were recorded in KBr pellets on a FTIR Schimadzu spectrophotometer. ^{1}H NMR (400 MHz) spectra were recorded in (DMSO)-d6 with an Avance spectrophotometer (Brukar, Germany) at a 400-MHzfrequency using TMS as an internal standard. Chemical shift are reported in parts per million and coupling constant in hertz (Hz). Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), m (multiplet). Mass spectra were recorded on an El-Shimadzu QP 2010 PLUS GC-MS system (Shimadzu, Japan). Elemental analysis was performed on a Carlo Erba 106 Perkin-Elmer model 240 analyzer (Perkin-Elmer, USA).

General procedure for the synthesis of 2-amino-4-(4-chlorophenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (4)

An equimolar mixture of 4-chlorobenzaldehyde **1** (1.00 mmol), ethylcyanoacetate **2** (1.00 mmol), guanidine hydrochlodide **3** (1.00 mmol) were stirred in presence of sodium methoxide at 70-80°C in PEG-400 for 1-2 hrs. The reaction progress was monitored by TLC method. After completion of reaction, reaction mixture was cool at room temperature and pour in ice cold water. The solid separate out was filtered, wash, dried and recrystallized by aq. acetic acid.

2-amino-4-(4-chlorophenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (4)

IR (KBr, cm^{-1}): 3416 (-NH_2), 3240 (-NH), 2219 (-C≡N), 1675 (>C=O, amidic); ^{1}H NMR (400 MHz, DMSO-d6, TMS, δ, ppm): 13.40 (s, 2H, -NH_2), 13.18 (s, 1H, -NH), 7.68 (m, 4H,Ar-H); EIMS: 246 [M+], 248 [M+2]; MF: $C_{11}H_7ClN_4O$.

General procedure for the synthesis of 2-(4-chlorophenyl)-4-oxo-6,8-substituted diphenyl-4H-pyrimido[1,2-a]pyrimidine-3-carbonitrile (6 a-j)

An equimolar mixture of compound **4** (1.00 mmol) and substituted **Chalcones (α, β-unsaturated ketones)** (5a-j) (1.00 mmol) were stirred in the presence of BEC (PEG-400) at 80°C for 2-3 hrs. After completion of reaction (monitored by TLC), cool the reaction mixture and poured in ice cold water. The separated solid product was filtered, dried and recrystallized from ethanol.

Spectral data of selected compounds

2,6,8-tris(4-chlorophenyl)-4-oxo-4H-pyrimido[1,2-a]pyrimidine-3-carbonitrile 6a : IR (KBr, cm^{-1}): 3427, 3343 (-NH_2), 3076 (Ar C-H), 2230 (C≡N), 1628 (C=N); ^{1}H NMR (400 MHz, DMSO-d6, TMS, δ, ppm): 7.82-7.65 (m, 13H, Ar-H); EIMS: 503 [M+], 506 [M+2]; MF: $C_{26}H_{13}Cl_3N_4O$.

8-(4-bromophenyl)-2,6-bis(4-chlorophenyl)-4-oxo-4H-pyrimido[1,2-a]pyrimidine-3-carbonitrile 6b: IR (KBr, cm^{-1}): 3417, 3323 (-NH_2), 3066 (Ar C-H), 2220 (C≡N), 1618 (C=N); ^{1}H NMR (400 MHz, DMSO-d6, TMS, δ, ppm): 7.89-7.67 (m, 13H, Ar-H); EIMS: 545 [M+], 547 [M+2]; MF: $C_{26}H_{13}Cl_2BrN_4O$.

General procedure for the synthesis of pyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amine (8a-j)

A mixture of compound **6** (1.00 mmol) and excess amount of hydrazine hydrate 90% (2.00 mmol) and catalytic amount of acetic acid (2-3 drops) were stirred in PEG-400 for 2-3 hrs at 90ºC. Progress of the reaction was monitored by TLC method. After completion of reaction monitored by TLC, the reaction mixture was left to cool and poured in ice cold water. The separated solid product was filtered off, washed with water, dried, and recrystallized from ethanol to obtain the targeted compounds.

4,7,9-tris(4-chlorophenyl)pyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amine 8a: IR (KBr, cm^{-1}): 3417, 3353 (-NH_2), 3086 (Ar C-H), 1618 (C=N); ^{1}H NMR (400 MHz, DMSO-d6, TMS, δ, ppm): 12.40 (s, 2H, -NH_2), 7.91 (s, 1H, Het Ar-H), 7.82-7.65 (m, 12H, Ar-H); EIMS: 516 [M+], 518 [M+2]; MF: $C_{26}H_{15}Cl_3N_6$.

7-(4-bromophenyl)-4,9-bis(4-chlorophenyl)pyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amine 8b: IR (KBr, cm^{-1}): 3415, 3363 (-NH_2), 3086 (Ar C-H), 1617 (C=N); ^{1}H NMR (400 MHz, DMSO-d6, TMS, δ, ppm): 12.23 (s, 2H, -NH_2), 7.90 (s, 1H, Het Ar-H), 7.79-7.23 (m, 12H, Ar-H); EIMS: 561 [M+]; MF: $C_{26}H_{15}BrCl_2N_6$.

***4,9-bis(4-chlorophenyl)-7-phenylpyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amine* 8c:** IR (KBr, cm^{-1}): 3410, 3361 (-NH_2), 3081 (Ar C-H), 1610 (C=N); ^{1}H NMR (400 MHz, DMSO-d6, TMS, δ, ppm): 12.13 (s, 2H, -NH_2), 7.92 (s, 1H, Het Ar-H), 7.89-7.34 (m, 13H, Ar-H); EIMS: 482 [M+]; MF: $C_{26}H_{16}Cl_2N_6$.

***4,9-bis(4-chlorophenyl)-7-(4-nitrophenyl)pyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amine* 8d:** IR (KBr, cm^{-1}): 3419, 3368 (-NH_2), 3096 (Ar C-H), 1612 (C=N); ^{1}H NMR (400 MHz, DMSO-d6, TMS, δ, ppm): 12.20 (s, 2H, -NH_2), 7.97 (s, 1H, Het Ar-H), 7.99-7.62 (m, 12H, Ar-H); EIMS: 527 [M+]; MF: $C_{26}H_{15}Cl_2N_7O_2$.

***4,9-bis(4-chlorophenyl)-7-(4-methoxyphenyl)pyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amine* 8e:** IR (KBr, cm^{-1}): 3413, 3360 (-NH_2), 3080 (Ar C-H), 1615 (C=N); ^{1}H NMR (400 MHz, DMSO-d6, TMS, δ, ppm): 12.07 (s, 2H, -NH_2), 7.88 (s, 1H, Het Ar-H), 7.69-7.53 (m, 12H, Ar-H), 3.43 (s, 3H); EIMS: 513 [M+]; MF: $C_{27}H_{18}Cl_2N_6O$.

***4-(4-chlorophenyl)-7,9-bis(4-nitrophenyl)pyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amine* 8f:** IR (KBr, cm^{-1}): 3405, 3353 (-NH_2), 3090 (Ar C-H), 1620 (C=N); ^{1}H NMR (400 MHz, DMSO-d6, TMS, δ, ppm): 12.24 (s, 2H, -NH_2), 7.96 (s, 1H, Het Ar-H), 7.70-7.31 (m, 12H, Ar-H); EIMS: 561 [M+]; MF: $C_{26}H_{15}ClN_8O_4$.

4,7-bis(4-chlorophenyl)-9-(4-nitrophenyl)pyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amine 8g :IR (KBr, cm^{-1}): 3409, 3360 (-NH_2), 3086 (Ar C-H), 1618 (C=N); ^{1}H NMR (400 MHz, DMSO-d6, TMS, δ, ppm): 12.23 (s, 2H, -NH_2), 7.91 (s, 1H, Het Ar-H), 7.90-7.72 (m, 12H, Ar-H); EIMS: 527 [M+]; MF: $C_{26}H_{15}Cl_2N_7O_2$

7-(4-bromophenyl)-4-(4-chlorophenyl)-9-(4-nitrophenyl)pyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amine 8h :IR (KBr, cm^{-1}): 3411, 3354 (-NH_2), 3082 (Ar C-H), 1623 (C=N); ^{1}H NMR (400 MHz, DMSO-d6, TMS, δ, ppm): 12.20 (s, 2H, -NH_2), 7.93 (s, 1H, Het Ar-H), 7.77-7.29 (m, 12H, Ar-H); EIMS: 573 [M+]; MF: $C_{26}H_{15}BrClN_7O_2$.

4-(4-chlorophenyl)-7-(4-methoxyphenyl)-9-(4-nitrophenyl)pyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amine 8i : IR (KBr, cm^{-1}): 3413, 3369 (-NH_2), 3095 (Ar C-H), 1612 (C=N); ^{1}H

NMR (400 MHz, DMSO-d6, TMS, δ, ppm): 12.08 (s, 2H, -NH_2), 7.83 (s, 1H, Het Ar-H), 7.67-7.45 (m, 12H, Ar-H), 3.41 (s, 3H); EIMS: 561 [M+]; MF: $C_{27}H_{18}ClN_7O_3$.

4-(4-chlorophenyl)-7-(4-methoxyphenyl)-9-phenylpyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amine 8j : IR (KBr, cm^{-1}): 3419, 3353 (-NH_2), 3089 (Ar C-H), 1609 (C=N); ^1H NMR (400 MHz, DMSO-d6, TMS, δ, ppm): 12.18 (s, 2H, -NH_2), 7.87 (s, 1H, Het Ar-H), 7.61-7.50 (m, 13H, Ar-H), 3.44 (s, 3H); EIMS: 561 [M+]; MF: $C_{27}H_{19}ClN_6O$.

Antimicrobial Activity

The well plate agar diffusion method

The solutions of different compounds under test at a concentration of 1-2 mg/mL of DMSO solvent were poured in the well of bacteria seeded agar plates. These plates were incubated at 37°C for 24 hrs for various bacteria. The activity was reported by measuring the diameter of zone of inhibition in mm and MICs (µg/mL). The standard antibiotic was used as penicillin. The solution without compound i.e. only DMSO was used as control [34, 35]. The results are already given in Tables 1.

Table 1: Antibacterial activity of pyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amine Derivatives (8a-j)

Sl.no	Compound code	Zone of inhibition in mm (MIC in µg/mL)			
		Ec	***Sa***	***Bs***	***Pv***
1	8a	*19(30)*	*18(30)*	*17(30)*	*18(30)*
2	8b	*17(30)*	*19(25)*	*16(50)*	*12(100)*
3	8c	*13(30)*	*12(50)*	*15(50)*	*22(25)*
4	8d	*21(25)*	*22(25)*	*20(25)*	*19(30)*
5	8e	*12(100)*	*10(100)*	*13(100)*	*23(25)*
6	8f	*22(25)*	*20(25)*	*20(25)*	*14(50)*
7	8g	*23(25)*	*21(25)*	*20(25)*	*13(100)*
8	8h	*18(30)*	*17(30)*	*19(25)*	*19(30)*
9	8i	*14(50)*	*14(50)*	*12(50)*	*21(25)*
10	8j	*15(50)*	*12(50)*	*16(50)*	*20(25)*
11	Penicilin	*30(25)*	*28(25)*	*30(25)*	*32(25)*

Ec-Escherichia coli; Pv-Proteus vulgaris; Bs-Bacillus subtilis; Sa-Staphylococcus aureus; Zones of inhibition measured in mm; MIC values (µg/ml) are given in parentheses

Experimental procedure

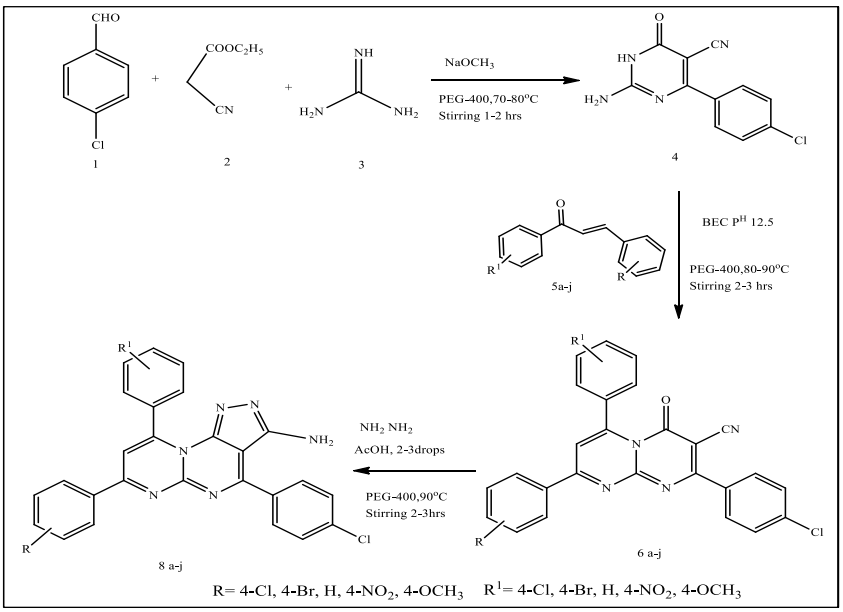
All the culture strains were obtained from IMTech Chandigarh and maintained on nutrient agar slant. For antibacterial activity, loop full of culture was transferred in 25 mL of nutrient broth and activated at 37°C for 24 hrs and cell density was adjusted at 6 x 106 cfu/mL. The nutrient agar plates with base (2.5% agar) were prepared. 0.1 mL of each activated culture transferred in 10 mL of nutrient agar (2%) to prepared seed agar and then poured over it (base) after solidification. The control DMSO solvent and standard Penicillin antibiotic of concentration 25 µg/disc was used. The wells were equipped and filled with 0.1 mL of each solution of test compound. Plates were kept in freeze for diffusion for 15 minutes and then incubated at 37 °C for 24 hrs.

After incubation zone of inhibition was measured in terms of mm along with the standard. For the MIC value determination of each compound of concentration 25µg/mL, 30µg/mL, 50µg/mL and 100µg/mL were screened. MIC value was obtained in the range of 25µg/mL-100 µg/mL for test compound for antibacterial activity. All compound dilutions were made in DMSO solvents.

RESULTS AND DISCUSSION

Chemistry

The synthetic approach endorsed to acquire the targeted pyrazolo [4,3-e] pyrimido [1,2-a]pyrimidin-3-amine derivatives. (**8a-j**) is outlined in Scheme 1:

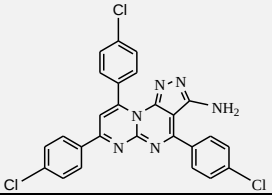


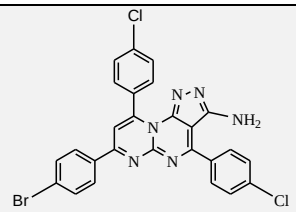
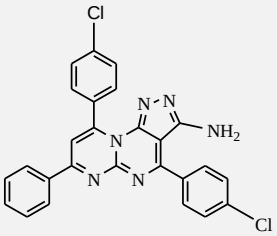
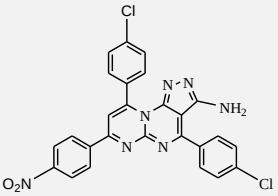
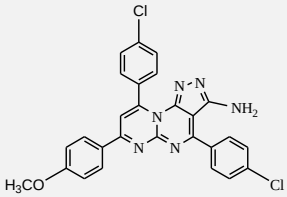
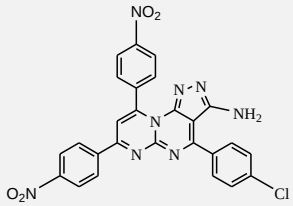
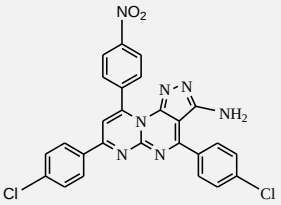
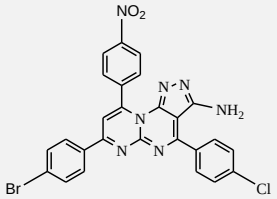
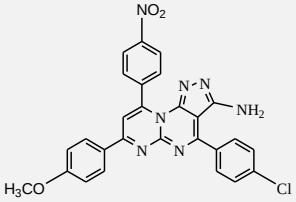
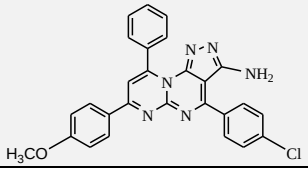
Scheme 1: Synthetic route of pyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amine derivatives

The study driven with synthesis of essential building block **(4)** according to the literature procedure [34, 35] consisting the one pot three component coupling of the 4-chlorobenzaldehyde (**1**), ethyl cyanoacetate **(2)** and guanidine hydrochlodide **(3)** in the presence of sodium methoxide and PEG-400 with continuous stirring for 1-2 hrs at 70-80 °C gives the corresponding 2-amino-4-(4-chlorophenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (**4**). After while the compound (**4**) was allowed to react with substituted **Chalcones (α, β-unsaturated ketones)** (5a-j) in BEC and PEG-400 with continuous stirring at 80-90 °C for 2-3 hrs to give the another intermediate 8-(4-substituted phenyl)-2-(4-chlorophenyl)-6-(4-substituted phenyl)-4-oxo-4H-pyrimido[1,2-a] pyrimidine-3-carbonitrile (**6a-j**). In further reaction, the intermediate (**6a-j**) which on finally treated with excess amount of hydrazine hydrate**(7)** (99%) in the presence of catalytic amount of acetic acid (2-3 drops) and PEG-400 with continuous stirring at 90°C afford the targeted pyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amin derivatives (**8a-j**) in good to excellent yields

The structure validation of the synthesized pyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amine derivatives (8a-j) were analyzed by spectral data (IR, ^{1}H NMR, and Mass spectra). While examined their antimicrobial activity against different bacterial strains. The physico-chemical data of synthesized derivatives (**8a-j**) is depicted in Table 2.

Table 2. Physicochemical data of pyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amine derivatives

Compound code	Compounds	Yield[a] in %	M.P.(°C)
8a		87	169-171

8b		79	155-157
8c		81	174-176
8d		83	162-164
8e		77	150-152
8f		80	185-187
8g		75	158-160
8h		89	170-172
8i		78	188-190
8j		80	166-169

[a]*All the yields on the isolated basis*

The IR spectra of intermediate compound **4** exhibited characteristic absorption band at 3416 cm^{-1} and 3240 cm^{-1} due to the $–NH_2$ and -NH respectively. An absorption band observed at 2219 cm^{-1} due to -C≡N group and an amidic carbonyl stretching observed at 1675 cm^{-1}. ^1H NMR spectrum of compound **4** shows two singlets at δ13.40 ppm and δ13.18 ppm for $–NH_2$ and –NH protons respectively. And all the aromatic protons were observed in their respective aromatic region. Furthermore, the IR spectrum another intermediate compound **6b** showed the absence of - NH stretch at 3345 cm^{-1} and the band at 2220 cm^{-1} indicate presence of -C≡N stretch. The carbonyl stretching's observed at 1696 cm^{-1} and 1665 cm^{-1} for amidic stretch. In the ^1H NMR spectrum of intermediate compound **6b** displayed the absence of characteristic $–NH_2$ and -NH singlet which confirms the intermediate **4** underwent the process of cyclization. And all the aromatic protons were observed in their respective aromatic region.

The IR spectrum of final **8b** compound, showed the disappearance of specific bands, like nitriles (-C≡N) and carbonyls which confirms the formation of cyclized final product. The presence of characteristic bands at 3415 cm^{-1} and 3363 cm^{-1} confirms the presence of $-NH_2$ functional group. The aromatic -C-H stretching observed at 3086 cm^{-1}. The ^1H NMR spectrum of compound **8b** showed $–NH_2$ singlet at δ 12.23 ppm. The remaining aromatic protons were observed in the aromatic region.

Antibacterial Activity

All the newly prepared compounds of the series were screened for their antibacterial activity by Agar-diffusion method [35]. The results of antibacterial studies in particulars of zone of inhibition (ZOI) and minimum inhibitory concentrations (MICs) are summarized in **Table 2**. In comparison with Penicillin as standard antibacterial drug, the compounds 8**d**, **8f** and 8**g** exhibited maximum antibacterial activity, whereas compounds **8a**, **8b** and 8**h** exhibited good antibacterial activity against the bacterial strains *Escherichia Coli* (MTCC- 443), *Bacillus subtilis* (MTCC-441), and *Staphylocous aureus* (MTCC-96) and the remaining **8c**, **8e**, **8i**, and **8j** compounds possesses moderate antibacterial activity. In case of *Proteus vulgaris* (MTCC 426), the compounds, **8c**, **8e**, **8i** and **8j** displayed significant antibacterial activity and the compounds **8a**, **8d** and **8h** exhibited good antibacterial activity. However, the remaining compounds **8b**, **8f**, and **8g** were less active against *Proteus vulgaris* (MTCC-426), a bacterial strain. Overall the SAR studies indicates that the presence of chloro and nitro substitution on aromatic ring seem to be important for the superior antibacterial activity.

CONCLUSION

A new series of substituted pyrazolo[4,3-e]pyrimido[1,2-a]pyrimidin-3-amine derivatives **(8a-j)** were prepared and screened for their antimicrobial activity. The antibacterial data given for the compounds presented in this chapter allowed us to state that the variation of antibacterial activity may be associated with the nature of tested micro-organisms and due to the chemical structure of the tested compounds. From the obtained results it is clear that pharmacophoric entities affect the activity of compounds in different series. Also, the presence of one or more halogen atom in the structure has considerable increases the antibacterial activity of the molecules. The best antibacterial effect has been shown by the compounds **8d**, **8f**, **8g, 8a**, **8b** and **8h** against the bacterial strains *Escherichia coli, Bacillus subtilis,* and *Staphylocous aureus* while the compounds **8c**, **8e**, **8i** and **8j** displayed significant antibacterial activity against *Proteus vulgaris*. And all the remaining derivatives exhibited good to moderate antibacterial activity against the same bacterial strains.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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