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Research Article

SYNTHESIS AND BIOLOGICAL EVALUATION OF PYRIMIDINE ANALOGS AS POTENTIAL ANTIMICROBIAL AGENTS

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ABSTRACT

Amino and halogenated pyrimidines were synthesized and screened for biological activity. All the compounds have shown broad spectrum of activity against *Staphylococcus epidermidis*, tested fungal species and moderate activity towards other tested species. The compound **3d** was the most potent with good efficacy against *S. epidermidis* and 2d against fungal species.

Keywords: Amino pyrimidines, Halogenated pyrimidines, Antibacterial activity and Antifungal activity.

INTRODUCTION

Pyrimidines occupy a unique and distinctive role biologically and medicinally. The analogs of pyrimidine are used as antibacterial[1-4], anticonvulsant[5], antiprotozoal[6], antiviral[7], antihypertensive[8], antihistaminic[9], and antifungal10 and anti-inflammatory[11] agents. Numerous drugs were developed involving pyrimidine ring[12]. Some drugs have either modified pyrimidine ring (flucytosine, Idoxuridine)[13] or pyrimidine ring in fused form with other heterocycles (methotrexate, prazocin)[14] and in some drugs, pyrimidines are commonly employed as substituent on lead moieties (sulphonamides)[15]. Halogenated pyrimidines and amino pyrimidines are the most commonly entities attached to the lead molecules[16,17]. Bacterial and fungal diseases are the most common all over the world. Though, many antibiotics are currently marketed, they have a tendency of becoming resistant and are prone to severe adverse effects after long term use[18]. Hence, there is a never lasting demand in synthesis of novel antimicrobial agents having good potency, efficacy with lesser side effects. The current article is aimed at synthesis of halogenated and amino pyrimidine analogs that are active against common pathogenic bacteria and fungi. These pyrimidine analogs can be synthesized at ease and can also be employed as substituents to lead moiety for further enhancement of potency and efficacy.

MATERIALS AND METHODS

All the melting points were recorded on Fischer-Johns melting point apparatus and were uncorrected. Mass spectra were recorded on a Micromass VG Autospec-M and Micromass Quattro LC-MS. Mass spectra were obtained under electrospray ionization (ESI) and liquid secondary ion mass spectrometric techniques. ^{1}H NMR spectra were recorded on Varian Gemini 200, Varian Inova 500 using TMS as internal standard and $CDCl_3$ as solvent and the chemical signals are represented as δ, ppm. IR spectra were recorded using KBr pellets on Perkin-Elmer 683. Progress of reactions and purity of compounds were tested using TLC on silica gel.

2-amino-6-hydroxy-*3H*-pyrimidin-4-one (1a)

Guanidine nitrate (2.2g, 0.037moles) was added to the freshly prepared sodium methoxide (0.062moles, 1.5g of sodium in 20 mL methanol) solution and stirred for 20min at room temperature and then it was filtered. The filtrate was added to diethylmalonate (5g, 0.031moles) and stirred for 30min. After completion of reaction, methanol was distilled off to give **1a** in 98% yield as a white solid. m.p. 189 °C; IR(KBr): 3630 (-OH), 3500 (-NH), 3150 (-NH), 1650 cm^{-1} (>C=O); ^{1}H NMR ($CDCl_3$): δ 7.7 (bs, 2H, -NH), 6.8 (s, 1H, aromatic); MS: *m/z* 127 (M$^+$). Anal. Calcd. for $C_4H_5N_3O_2$: C, 37.80; H, 3.97; N, 33.06. Found: C, 37.92; H, 3.88; N, 33.24 %.

4,6-dichloropyrimidin-2-amine (1b)

To a stirred solution of **1a** (1g, 0.078moles) in toluene (10mL), $POCl_3$ (4mL/g) was added drop wise at 0ºC. The reaction mixture was refluxed at 120ºC for 12hr. After completion of reaction, ice (100g) was added and the reaction mixture was adjusted to pH 7.5-8 with sodium bicarbonate solution and extracted with EtOAc. The organic layer was dried ($NaSO_4$), evaporated and the residue was purified by column chromatography (Silica gel, 60% EtOAc in petroleum ether) to give **1b** in 58% yield as a solid. m.p.59 °C; IR (KBr): 3360 (-NH), 1570 (C=C), 1560 cm^{-1} (>C=N); ^{1}H NMR ($CDCl_3$): 7.7 (bs, 2H, -NH), 6.8 (s, 1H, aromatic); MS: *m/z* 164 (M$^+$). Anal. Calcd. for $C_4H_3N_3Cl_2$: C, 29.30; H, 1.84; N, 25.62. Found: C, 29.74; H, 1.68; N, 25.88 %.

2-amino-pyrimidine (1c)

A solution of **1b** (1g, 0.006mole) in methanol (10mL) was treated with 10% Pd-C (0.08g) and stirred at room temperature under hydrogen atmosphere for 12hrs. The reaction mixture was filtered, the solvent was evaporated under reduced pressure and residue was purified by column chromatography (Silica gel, 60% EtOAc in petroleum ether) to give **1c** in 65% yield as a white solid. m.p.126°C; IR(KBr): 3450 (-NH), 1580 (>C=N), 1510 (C=C)cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 8.3 (d, 2H, aromatic), 6.8 (s, 1H, aromatic), 5.0 (bs, 2H, -NH); MS: *m/z* 95 (M$^+$). Anal. Calcd. for $C_4H_5N_3$: C, 50.52; H, 5.30; N, 44.18. Found: C, 50.86; H, 5.88; N, 45.04 %.

5-bromo-2-amino-pyrimidine (1d)

To a stirred solution of **1c** (0.5g, 0.005moles) in acetic acid (8mL) bromine solution (0.55mL, 0.005moles) was added at 0ºC and stirred for 2hrs at room temperature. The reaction mixture was adjusted to pH 7.5-8 with sodium bicarbonate solution and extracted with EtOAc. The organic layer was dried (Na_2SO_4), evaporated and purified by column chromatography (Silica gel, 50% EtOAc in petroleum ether) to give acid **1d** in 58% yield as a white solid. m.p. 245°C; IR(KBr): 3360 cm^{-1} (-NH), 1550 (>C=N); ^{1}H NMR ($CDCl_3$): δ 8.3 (d, 2H, aromatic), 6.8 (bs, 2H, -NH); MS: *m/z* 174 (M$^+$). Anal. Calcd. for $C_4H_4N_3Br$: C, 27.61; H, 2.32; N, 24.15. Found: C, 27.42; H, 2.58; N, 23.94 %.

Diethyl 2-(ethoxymethylene) malonate (2a)

To a stirred solution of triethyl orthoformate (2mL, 0.018moles) in acetic anhydride (10mL), diethyl malonate (2g, 0.012moles) and $ZnCl_2$ (0.05g, catalytic amount) were added. The reaction mixture was stirred at 100ºC for 2hrs, then at 160ºC for 6hrs. The reaction mixture was fractional distilled at 105°C for separation of acetic acid and acetic anhydride mixture and at 130°C, 2a was collected in 85% yield as a syrup. b.p. 280°C; IR(KBr): 1770 (>C=O), 1640 cm^{-1} (C=C); ^{1}H NMR ($CDCl_3$): δ 5.3 (d, 1H, -C=CH), 4.3 (m, 4H, $-OCH_2$), 4.2 (q, 2H, $-OCH_2$), 1.45 (t, 3H, $-CH_3$), 1.3 (t, 6H, $-CH_3$); MS: m/z 216 (M$^+$). Anal. Calcd. for $C_{10}H_{16}O_5$: C, 55.55; H, 7.46; N, 37.00. Found: C, 55.32; H, 7.56; N, 37.14 %.

Ethyl 1, 6-dihydro-6-oxopyrimidine-5-carboxylate (2b)

Formimidine acetate (0.45g, 0.01 moles) was added to the freshly prepared sodium methoxide (0.032moles, 0.75g of sodium in 10mL methanol) solution and stirred for 20min at room temperature and then it was filtered. The filtrate was added to **2a** (1.5g, 0.069moles) and refluxed for 2hrs. The solvent was removed under reduced pressure, the residue was dissolved in dichloromethane (20mL), washed with water (10mL) and brine (10mL). The organic layer was dried ($NaSO_4$), evaporated and purified by column chromatography (Silica gel, 40% EtOAc in petroleum ether) to give **2b** in 56% yield as a solid. m.p.186°C; IR (KBr): 3350 (-NH), 1720 (>C=O), 1680 (>C=O), 1570 cm^{-1} (>C=N); ^{1}H NMR ($CDCl_3$): δ 8.1 (s, 2H, aromatic), 4.2 (q, 2H, OCH_2), 1.45 (t, 3H, CH_3); MS: *m/z* 168 (M$^+$). Anal. Calcd. for $C_7H_8N_2O_3$: C, 50.0; H, 4.80; N, 16.66. Found: C, 50.12; H, 4.88; N, 16.22 %.

Ethyl 4-chloropyrimidine-5-carboxylate (2c)

To a stirred solution of **2b** (2g, 0.011moles) in toluene (20mL), $POCl_3$ (8mL, 4mL/g) was added dropwise at 0ºC. The reaction mixture was refluxed at 120ºC for 4hrs. After completion of reaction, ice (100g) was added and the reaction mixture was adjusted to pH 7.5-8 with sodium bicarbonate solution and extracted with EtOAc. The organic layer was dried ($NaSO_4$), evaporated and purified by column chromatography (Silica gel, 45% EtOAc in petroleum ether) to give **2c** in 85% yield as a solid. m.p. 155°C; IR (KBr): 1720 (>C=O), 1620 (C=C), 1570 cm^{-1} (>C=N); ^{1}H NMR ($CDCl_3$): δ 9.0-9.1 (s, 2H, aromatic), 4.5 (q, 2H, $-OCH_2$), 1.45 (t, 3H, $-CH_3$); MS: *m/z* 187, 189 (1:3) isomeric peaks. Anal. Calcd. for $C_7H_7N_2O_2Cl$: C, 45.06; H, 3.78; N, 15.01. Found: C, 37.72; H, 3.68; N, 15.04 %.

4-chloropyrimidine-5-carboxylic acid (2d)

A solution of ester **2c** (2g, 0.01moles) in 10% methanolic KOH (20mL) was stirred for 2hrs at reflux temperature. The reaction mixture was adjusted to pH 2-3 with aq. 1N HCl and extracted with EtOAc. The organic layer was dried (Na_2SO_4), evaporated and purified by column chromatography (Silica gel, 60% EtOAc in petroleum ether) to give **2d** in 80% yield as a solid. m.p.180°C; IR(KBr): 2900 (-OH), 1720 (>C=O), 1620 (C=C), 1570 cm^{-1} (>C=N); ^{1}H NMR ($CDCl_3$): δ 9.0-9.1 (s, 2H, aromatic); MS: *m/z* 160 (M+H). Anal. Calcd. for $C_5H_3N_2O_2Cl$: C, 37.88; H, 1.91; N, 17.67. Found: C, 37.98; H, 1.87; N, 17.28 %.

2-amino-6-mercapto-*3H*-pyrimidin-4-one (3a)

Ethyl cyanoacetate (0.95ml, 0.884moles, d-1.047g/ml,) and thiourea (0.805g, 0.106moles) were added to the freshly prepared sodium methoxide (0.064moles, 1.5g of sodium in 20mL methanol) solution and stirred for 20min at room temperature and then it was reflux for 2hrs, cooled and filtered. The solid was dissolved in potassium hydroxide solution and re-precipitated by addition of glacial acetic acid, then filtered to give **3a** in 95% yield as a solid. m.p. 330°C; IR(KBr): 3423 (-NH), 3320 (-NH), 2550 (-SH), 1630 cm^{-1} (>C=O); ^{1}H NMR (DMSO d_6): δ 11.5 (bs, 1H, -SH), 6.4 (s, 2H, $-NH_2$), 4.7 (s, 1H, -CH); MS: m/z 144 (M$^+$). Anal. Calcd. for $C_4H_5N_3OS$: C, 33.56; H, 3.52; N, 29.35. Found: C, 33.52; H, 3.51; N, 29.53 %.

2-amino-*3H*-pyrimidin-4-one (3b)

A solution of **3a** (1g, 0.007moles) in methanol (20mL) was treated with raney nickel (0.1g, catalytic amount) and stirred at reflux temperature for 12hrs. The reaction mixture was filtered, the solvent was evaporated under reduced pressure and the residue was purified by column chromatography (Silica gel, 60% EtOAc in petroleum ether) to give **3b** in 92% yield as a white solid. m.p. 206°C; IR(KBr): 3300 (-NH), 1710 (>C=O), 1580 cm^{-1} (>C=N); ^{1}H NMR (DMSO d_6): δ 7.8 (s, 1H, aromatic), 6.4 (s, 2H, NH_2), 5.0 (s, 1H, NH); MS m/z: 112 (M+H). Anal. Calcd. for $C_4H_5N_3O$: C, 43.24; H, 4.54; N, 37.82. Found: C, 43.12; H, 4.63; N, 37.65 %.

4-chloropyrimidin-2-amine (3c)

To a stirred solution of **3b** (1g, 9.09moles) in toluene (20mL), $POCl_3$ (4mL, 4mL/g) was added drop wise at 0ºC. The reaction mixture was refluxed at 120ºC for 5hrs. After completion of reaction, ice (100g) was added and the reaction mixture was adjusted to pH 7.5-8 with sodium bicarbonate solution and extracted with EtOAc. The organic layer was dried ($NaSO_4$), evaporated and purified by column chromatography (Silica gel, 60% EtOAc in petroleum ether) to give **3c** in 82% yield as a solid. m.p. 212°C; IR (KBr): 3450 (-NH), 1550 cm^{-1} (>C=N); ^{1}H NMR (DMSO d_6): δ 8.2 (s, 1H, -CH), 7.2 (s, 2H, $-NH_2$), 6.4 (s, 1H, -CH); MS m/z : 130, 132 (1:3) isomeric peaks. Anal. Calcd. for $C_4H_4N_3Cl$: C, 37.09; H, 3.11; N, 32.44. Found: C, 37.02; H, 3.18; N, 33.04 %.

4-bromopyrimidin-2-amine (3d)

To a stirred solution of **3c** (1g, 0.007moles) in acetic acid (8mL), bromine solution (0.55mL, 0.005moles) was added at 0ºC and stirred for 6hrs at reflux temperature. The reaction mixture was adjusted to pH 7.5-8 with sodium bicarbonate solution and extracted with EtOAc. The organic layer was dried (Na_2SO_4), evaporated and purified the residue by column chromatography (Silica gel, 50% EtOAc in petroleum ether) to give acid **3d** in 70% yield as a white solid. m.p. 174°C; IR (KBr): 3500 (-NH), 1580 cm^{-1} (>C=N); ^{1}H NMR (DMSO d_6): δ 8.1 (s, 2H, aromatic), 6.3 (s, 2H, $-NH_2$); MS: m/z 174, 176 (1:1) isomeric peaks. Anal. Calcd. for $C_4H_4N_3Br$: C, 27.61; H, 2.32; N, 24.15. Found: C, 27.68; H, 2.24; N, 24.34 %.

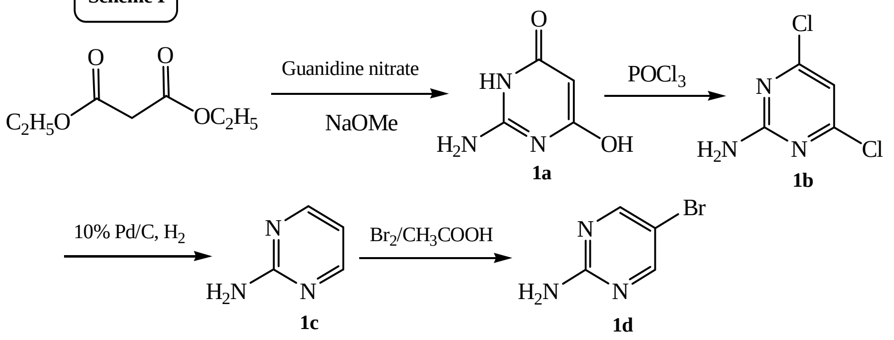
Reaction Scheme

The present study reports the synthesis of pyrimidine analogs using different synthetic methods and screened for their *in vitro* antibacterial and antifungal activities. The condensation of diethylmalonate and guanidine nitrate in the presence of NaOMe in methanol resulted in the formation of 2-amino-6-hydroxy-*3H*-pyrimidin-4-one **1a** with 98% yield. (**Scheme I**). Chlorination of **1a** with $POCl_3$ afforded 4,6-dichloropyrimidin-2-amine **1b** respectively, which on further reduction using 10% Pd/C in the presence of hydrogen gave 2-aminopyrimidine **1c** with 65% yield. Bromination of **1c** in the presence of bromine in acetic acid afforded 5-bromo-2-amino pyrimidine **1d** in 58% yield. Diethyl malonate was treated with triethylorthoformate in the presence of Ac_2O and $ZnCl_2$ to give diethyl 2-(ethoxymethylene) malonate **2a** with 85% yield (**Scheme II**). Further, **2a** on reaction with formimidine acetate in the presence of sodium methoxide gave ethyl 1,6-dihydro-6-oxopyrimidine-5-carboxylate **2b** with 56% yield, which on chlorination using $POCl_3$ gave ethyl 4-chloropyrimidine 5-carboxylate **2c** at 85% yield. The ester **2c** on alkaline hydrolysis produced 4-chloropyrimidine 5-carboxilic acid **2d** with 80% yield. The reaction of ethyl cyanoacetate with thiourea in the presence of sodium methoxide produced 2-amino-6-mercapto-*3H*-pyrimidin-4-one **3a** with 95% yield (**Scheme III**). The compound **3a** on reduction with raney nickel in the presence of hydrogen gave 2-amino-*3H*-pyrimidin-4-one **3b** with 92% yield, which on further reaction with $POCl_3$ afforded 4-chloropyrimidin-2-amine **3c** with 82% yield. The compound **3c** was treated with bromine in acetic acid to give 4-bromopyrimidin-2-amine **3d** with 70% yield. All the chemical structures of the synthesized compounds were confirmed by their IR, ^{1}H NMR and mass spectral data.

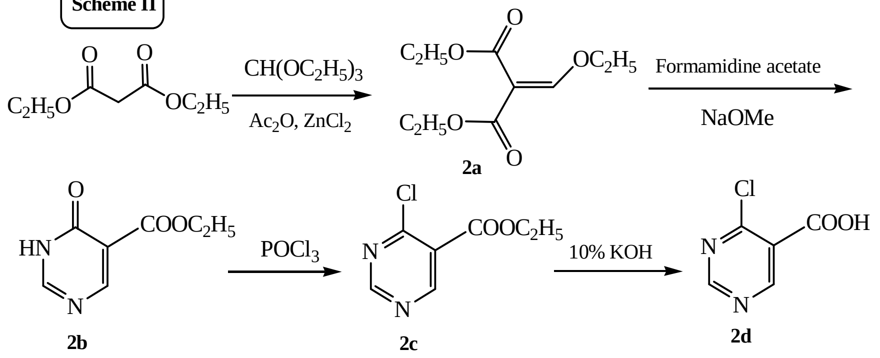
Determination of Minimum Inhibitory Concentration (MIC)

Antibacterial activity was carried out by broth dilution method[19,20]. The compounds **1b**, **1d**, **2b**, **2c**, **2d**, **3a**, **3c** and **3d** were screened for antibacterial activity against *Staphylococcus epidermidis* and *Bacillus subtilis* (Gram positive bacteria), *Escherichia coli* and *Klebsiella pneumonia* (Gram negative bacteria) at concentrations of 1000, 500, 200 and 100μg/mL. The same compounds were tested against *Candida albicans* and *Aspergillus niger* (fungi) at concentrations of 1000, 500, 200 and 100μg/mL. The compounds active at 100 μg/ mL were further tested by diluting the stock to obtain concentrations of 50, 20 and 10μg/mL. Further, the compounds active at 10μg/ mL were subsequently tested at concentrations of 5, 2, 1 and 0.5 μg/mL. The test mixture contained 10^8 organisms/mL. 10μL from each well was further inoculated on appropriate media and growth was noted after 24 and 48hrs. The lowest concentration which showed no growth after subculturing was considered as Minimum Inhibitory Concentration (MIC) for each tested compound. The standard drug used in the present study was ciprofloxacin which showed MIC at 1, 0.5, 0.5 and 2μg/mL against *S.epidermidis, E.coli*, *B.subtilis*, *K.pnuemoniae* respectively for antibacterial activity and fluconazole which showed MIC at 0.5 and 1μg/mL against *C.albicans* and *A.niger* for antifungal activity.

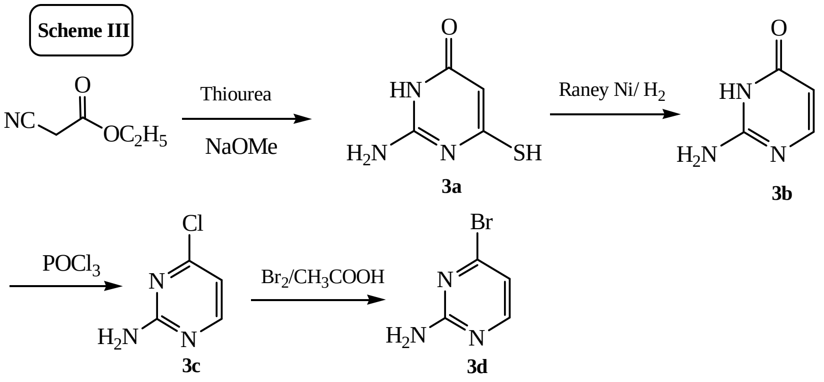
Scheme I



Scheme II



Scheme III



Determination of Zone of Inhibition

Since the synthesized compounds were highly potent against the *S.epidermidis*, the efficacy was determined by zone of inhibition values using disk diffusion technique[21]. To each petriplate, 20 mL of sterilized medium was added. After the agar had set, 10% of inoculum (*S.epidermidis* culture) was added to each petriplate and spread thoroughly. Sterilized Wh atmann no. 1 filter paper discs (diameter 6mm) were thoroughly moistened with the synthesized compounds of various concentrations- 8, 4, 2 and 1 µg/ mL in DMSO and placed on seeded agar plates. Paper discs moistened with DMSO were considered as control. Discs saturated with ciprofloxacin at various concentrations (8, 4, 2 and 1µg/mL) were taken as standard. The plates were incubated at 37°C for 24hrs. The clear zone of inhibition around paper dics demonstrated the relative susceptibility towards the synthesized derivatives.

Table 1: Minimum Inhibitory Concentration (MIC in µg/ mL) values of the reported compounds against various strains of bacteria and fungi.

S. No.	Comp.	*S.epidermidis*[a]	*E.coli*[a]	*K.pneumoniae*[a]	*B.subtilis*[a]	*C.albicans*[b]	*A.niger*[b]
1	1b	1.0	200	500	200	50	20
2	1d	1.0	500	1000	100	10	50
3	2b	2.0	200	500	>1000	100	200
4	2c	2.0	>1000	>1000	500	100	100
5	2d	1.0	500	200	200	10	10
6	3a	0.5	500	500	200	20	10
7	3c	2.0	1000	>1000	500	100	20
8	3d	0.5	200	500	500	50	50

[a] antibacterial activity, [b] antifungal activity

RESULTS AND DISCUSSION

All the synthesized compounds were confirmed by the spectral data. Then, the synthesized compounds were screened for their *in vitro* antibacterial and antifungal activities. The prepared compounds were tested against the standard strains: *S.epidermidis, B.subtilis* (gram positive), *E.coli, K.pneumoniae* (gram negative), *C.albicans, A.niger* (fungi). All the tested compounds showed moderate antimicrobial activity; however, the potency towards fungal species was better than tested bacterial species except *Staphylococcus epidermidis*. The tested compounds were very potent against the growth of *Staphylococcus epidermidis* at MIC values between 0.5-2μg/mL. The tested compounds showed antifungal activity with a range of MICs between 25-100μg/mL. The results of antibacterial and antifingal activity evaluation are presented in Table 1. Microbiological results showed that the synthesized compounds possessed broad spectrum of activity against *S.epidermidis* and fungal species (*C.albicans* and *A.niger*). Since the synthesized compounds were highly potent against *S.epidermidis*, zone of inhibition values were determined at various concentrations 2, 4, 6, 8μg/mL (Figure 1). The potency and efficacy of these compounds was comparable with that of standard drug, Ciprofloxacin (1μg/mL).

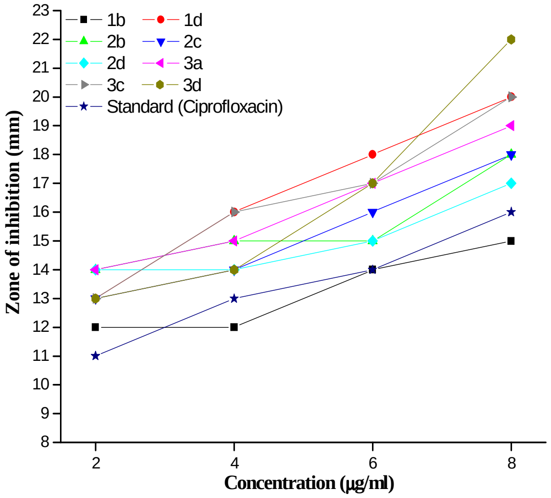


Fig. 1: Comparison of zone of inhibition values of the reported compounds on *S. epidermidis* with reference standard.

Most of the synthesized compounds possessed antibacterial activity against the other Gram-negative and Gram-positive bacteria showing MIC values between 100 and 1000 μg/mL. Of the compounds tested, **3d** was the potent molecule with good efficacy against *S. epidermidis* and **2d** was most potent against fungal species. Figure 1 shows the comparison of antibacterial activity against *S. epidermidis* of reported compounds with that of reference standard. The results of the present investigation encourage us to develop more moieties and test them for wide range of biological activities

CONCLUSION

Our present study has achieved very good activity against *Staphylococcus epidermidis*, fungal species and moderate activity towards other tested species. The compound **3d** was the most potent with good efficacy against *S.epidermidis* and **2d** against fungal species. These compounds were synthesized by simple and economical methods. These small molecules can also be employed as an adjunct moieties enhancing the activity on lead compound.

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