ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 12, Issue 3, 2019

Online - 2455-3891
Print - 0974-2441

Research Article

ANTIMICROBIAL INVESTIGATION AND BINDING MODE ANALYSIS OF SOME NEWLY SYNTHESIZED 4-AMINO-5-((ARYL SUBSTITUTED)-4H-1, 2, 4-TRIAZOLE-3-YL)-THIO LINKED HYDROXAMIC ACID DERIVATIVES

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Received: 22 November 2018, Revised and Accepted: 04 January 2018

ABSTRACT

Objective: A series of 5-substituted-4-amino-1, 2, 4-triazole-linked hydroxamic acid derivatives have been synthesized and explored *in vitro* to evaluate antibacterial and antifungal activities.

Methods: Different 5-phenyl group substituted-4-amino-1,2,4-triazole-3-thiol reacted with chlorine substituted hydroxamic acid to produce the desired compounds and characterized spectroscopically. Minimum inhibitory concentration (MIC), zone of inhibition (ZOI), growth kinetic studies, and scanning electron microscopy (SEM) were employed to elicit the antimicrobial efficacy of synthesized compounds against a wide range of bacterial and fungal strains.

Results: Compounds 6a, 6b, 6d, and 6k (MIC of 25 µg/ml) have been found to be more potent against *Klebsiella pneumoniae*, *Bacillus cereus, Bacillus pumilus*, *Micrococcus luteus,* and *Pseudomonas aeruginosa*, compounds 6a-6d, 6k, and 6l (MIC of 25–50 µg/ml) have shown potent antibacterial efficacy against *Klebsiella pneumonia*, *P. aeruginosa,* and *Vibrio cholera* compare to the standard drug amoxicillin (MIC of 60 µg/ml, 65 µg/ml, and 25 µg/ml, respectively). Screening for the antifungal activity revealed that the compounds were found to be most active against *Candida albicans* (6a, 6b, and 6l), *Candida tropicalis* (6b and 6d), and *Aspergillus niger* (6a, 6b, 6d, and 6j) with MIC of 15–25 µg/ml. Bacteriostatic and fungistatic effect of titled compounds was revealed from growth kinetics study.

Conclusion: Electron donating group at the 5-position of the 5-substituted-1,2,4-triazole-linked hydroxamic acid derivatives conferred the biological effectiveness of the synthesized compounds and also offer a therapeutically effective prototypical structure for further development of new chemical entities with superior antimicrobial activity.

Keywords: 1,2,4-triazole, Hydroxamic acid, Antibacterial, Antifungal.

 DOI: http://dx.doi.org/10.22159/ajpcr.2019.v12i3.30882

INTRODUCTION

The microbes are getting resistant toward the existing chemotherapeutics in alarming rate which is not only a major concern for public health but also a challenge for the scientific community globally, and the number of cases of multidrug-resistant bacterial infections is increasing nowadays [1]. In clinical practice, the infections caused by the Gram-positive bacteria are very common. However, the infection caused by them is sometime severe. *Bacillus cereus*, *Staphylococcus aureus*, *Bordetella bronchiseptica*, *Micrococcus luteus*, *Bacillus pumilus*, *Bacillus subtilis,* and *Enterococcus faecalis* are some important Gram-positive bacteria causing serious infection in the community, whereas *Salmonella typhi*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella typhimurium* are very common Gram-negative bacteria. Among them, *E. coli* is the most common one. The mortality is higher for infections caused by Gram-negative bacteria than the Gram-positive one. To overcome various microbial infections specifically fungal infection, a large number of triazole drugs have been successfully developed [2]. Azole compounds showed the effect by inhibiting lanosterol 14α-demethylase (CYP51) which catalyzes the oxidative removal of the 14α-methyl group of lanosterol to give $\Delta^{14,15}$-desaturated intermediates in ergosterol biosynthesis [3] resulting in accumulation of toxic methyl-sterols in membranes leading to fungistatic or fungicidal effect [4]. Some important azoles derivatives such as voriconazole, itraconazole, posaconazole, and fluconazole (Fluc) are used for the treatment of fast-growing fungal infections, which are the leading cause of mortality and morbidity in immunocompromised patients [5]. However, Fluc is not effective against aspergillosis due to its severe drug resistance [6,7].

These scenarios are highlighting the urgent need for novel, efficacious, less toxic, and safe drug candidates in the pipeline. The heterocyclic compounds containing nitrogen, sulfur, and oxygen have an enormous significance in the field of medicinal chemistry [8]. To address the concern, we develop a series of novel 5-substituted-4-amino-1,2,4-triazole-linked hydroxamic acid derivatives. Assembling the different pharmacophores in a single frame always leads to compounds having fascinating biological profile [9]. Triazole is one of the pharmacophores in the synthesized compounds. Chemical compounds possess triazole as a core; always play a significant role in the field of medicinal chemistry. Like triazole, imidazole moiety has widely used to synthesize compounds to treat diseases causing by different microorganisms. Even though imidazole and triazole have a similar mode of action, triazoles have several advantages over imidazole, like, oral bioavailability, slow metabolic rate and have minimal effect on sterol synthesis in humans. For these, use of triazole moiety increases rather than the imidazole [10]. Triazole is well known for its antimicrobial property and widely used to treat microorganisms associated diseases [11]. Besides, the antibacterial [12-15] and antifungal [16-19] activity; 1,2,4-triazole possess, anti-inflammatory [20], as well as antioxidants properties [21]. Various well-known drug commercialized in the market possesses 1,2,4-triazole moiety such as Fluc, terconazole,

itraconazole, [22,23] triazolam [24], and ribavirin [25]. Different S and N-bridged heterocycles can be synthesized by 1,2,4-triazoles as it possesses nucleophilic centers. Triazolothiazines, triazolothiadiazoles, triazolothiazepines, triazolothiadiazines, and thiazolotriazoles can be synthesized from triazoles, having versatile therapeutic potentials. Another moiety in our synthetically designed compounds is hydroxamic acid. It is another interesting moiety possess antibacterial, antifungal [26,27], anti-inflammatory [28,29], antitumor [30], and anticancer agents [31]. Hydroxamic acid and its derivatives can exert versatile activity by inhibiting several enzymes, such as urease [32], matrix metalloproteinases [33,34], ribonucleotid reductase [35], and 5-lipoxygenase [36]. Hydroxamic acid has the ability to chelate with metals. Few ion exchange resins are built on hydroxamic acids [37]. A number of hydroxamic acid derivatives are used as chemotherapeutic agents such as ibuproxam, hydroxycarbamide, desferrioxamine B, oxametacin, adrafinil, and bufexamac [38]. Hence, considering the above fact we have designed and synthesized some novel 5-substituted-4-amino-1,2,4-triazole-linked hydroxamic acid derivatives and evaluate their activity against several Gram-positive, Gram-negative bacteria, and a number of fungal strains.

METHODS

All the chemicals were procured from Sigma-Aldrich, India, Spectrochem, Merck India Pvt. Ltd., and the media were of purchased from HiMedia. Dichloromethane and dimethyl sulfoxide (DMSO) were procured from Merck, India; all other chemicals used were of analytical grade. In process monitoring of reaction was done on activated silica gel coated plates and the solvent system used was n-hexane:ethylacetate. The melting points of the synthesized compounds were measured by the capillary method and are reported uncorrected. The Fourier-transform infrared (FT-IR) spectra were measured in Nicolet iS10 FT-IR Spectrometer (Thermo Fisher Scientific, USA), ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on AV300 Digital FT NMR Spectrometer, Bruker at 300 MHz using DMSO-d_6 as the solvent and tetramethylsilane as an internal standard, ^{13}C NMR spectra were obtained at 500 MHz (Bruker, Germany), DMSO-d_6 as the solvent. Mass spectra (m/z) of the compounds were recorded on JEOL-JMS 700 spectrometer using electron ionization technique. Scanning electron microscopy (SEM) study of the synthesized compounds was analyzed using SEM JEOL, Tokyo, Japan.

SYNTHETIC PROCEDURE

General procedure for the synthesis of first intermediate compound (1a-1f)

Substituted-aryl-acid was taken in a 250 ml rbf. 40 ml of ethanol was added to it along with 22.5 ml of conc. sulfuric acid. The mixture was refluxed for 2 h on a steam bath. After that, the mixture was cooled and poured into crushed ice. The mixture was made strongly alkaline by adding sodium carbonate. The mixture was extracted with ether (3×50 ml). The combined ether fraction was collected and kept overnight with a small amount of activated anhydrous sodium sulfate. The ester in the form of oil was collected by means of distilling out the ether.

General procedure for the synthesis of Arylhydrazide (2a-2f)

Esterified compounds (1a-1f) (equimolar) were dissolved in 30 ml of ethanol, and hydrazine hydrate (equimolar) was added dropwise in the mixture with continuous stirring. The resulting mixture was refluxed for 6 h. The solvent was removed by distillation, and the residue was cooled to room temperature. The precipitate formed was filtered and washed subsequently with water, dried, and recrystallized from dehydrated ethanol to yield white crystals.

General procedure for the synthesis of potassium 2-*substituted*-hydrazine-1-carbodithioate (3a-3f)

Potassium hydroxide (equimolar) was dissolved in 75 ml of absolute alcohol, and arylhydrazide (2a-2f) (equimolar) was added to the above solution. The mixture was then cooled in an ice-bath. To this mixture carbon disulfide was added (equimolar) in small portions with continuous stirring. The reaction mixture was continuously agitated for 15 h. Finally, the mixture was diluted with 100 ml of anhydrous ether. The residue was filtered, washed with ether (3×25 ml), dried and used as such for the next reaction.

General procedure for synthesis of 4-amino-5-(*substituted*-phenyl)-4H-1,2,4-triazole-3-thiol (4a-4f)

A suspension of potassium 2-*substituted* hydrazine-1-carbodithioate (3a-3f) in 20 ml of water and hydrazine hydrate was refluxed for 6 h with occasional shaking. Next, the hot mixture was cooled to room temperature and diluted with 100 ml of water. Concentrated hydrochloric acid was added dropwise to make the reaction mixture strongly acidic. The precipitate obtained was filtered, washed thoroughly with cold water and dried. The dried product was further recrystallized from dehydrated ethanol to get white crystals.

General procedure for the synthesis of *substituted*-chloro-N-hydroxyacetamide (5a-5b)

0.01 mol of chloroacetylchloride (to synthesize 5a)/3-chloro propionylchloride (to synthesize 5b) was dissolved in 30 ml of methanol. To the reaction mixture, hydroxylamine stock solution (0.04 mol of hydroxylamine hydrochloride) was dissolved in 12 ml of hot methanol. The mixture was stirred for 5 min and to it, a solution of 0.06 mol potassium hydroxide in 10 ml of methanol was added dropwise. The resulting solution was cooled at room temperature and filtered to obtain 2 mol of stock solution was added dropwise and the mixture was stirred for 2 h. The precipitate thus obtained was washed with ether and recrystallized from methanol.

General procedure for the synthesis of title compounds (6a-6l)

To an equimolar mixture of 4-amino-5-(*substituted*-phenyl)-4H-1,2,4-triazole-3-thiol and *substituted*-chloro-N-hydroxypropanamide in acetonitrile, triethylamine was added dropwise and refluxed for 4 h. After cooling down the reaction mixture to the room temperature, 20 ml water was added and extracted with chloroform thrice; aqueous layer was collected and evaporated to produce solid residue, which was further washed with acetone twice and recrystallized from 50% ethanolic solution to produce white crystals.

Antimicrobial activity

Microbial strains

In vitro antimicrobial activity was evaluated against 15 different pathogenic bacteria include both Gram-negative such as *S. typhi* 62, *K. pneumoniae* ATCC 10031, *Vibrio cholerae* VC 20, *P. aeruginosa* 25619, *Shigella sonnei* NK 4010, *E. coli* ATCC 25923, and *S. typhimurium* NTCC 74, and Gram-positive such as *Bacillus polymyxa* 4747, *B. cereus* 479, *Staphylococcus aureus* ATCC 29737, *E. faecalis* 28, *Bordetella bronchiseptica* 4617, *Micrococcus luteus* 10240, *B. pumilus* 148884, and *Bacillus subtilis* 6673, respectively, and seven different fungi such as *Candida albicans* MTCC 183, *Candida tropicalis* MTCC 2795, *Cryptococcus neoformans*, *Aspergillus niger* MTCC 281, *Microsporum gypseum*, *Penicillium chrysogenum*, and *Cladosporium sp*. All these microbial strains were collected from Division of Microbiology and Biotechnology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

Preparation of inoculums

Nutrient broth was used to grow the bacterial strains at 37°C for 24 h whereas Czapek Dox media were used to culture the fungal strains at 25–30°C for 48–72 h. Afterward, both the bacterial and fungal strains were compared to 0.5 McFarland standards [39] and made the dilution with sterile normal saline was so that produced an initial suspension of 2×10^6 CFU/ml for further use.

Determination of minimum inhibitory concentration (MIC)

To determine the MIC of the synthesized compounds agar dilution method was employed as per the National Committee for Clinical Laboratory Standards (NCCLS) 2006 protocol [40]. The synthesized compounds were initially dissolved in a suitable solvent and then serial

dilution was made to obtain the concentrations 10, 25, 50, 75, 100, 150, 200, 250, 350, and 400 µg/ml. On the separate agar plates with different drug concentrations inocula (2×10^6 CFU/mL) were spotted and incubated for 24 h at 37°C (for bacterial strains). Amoxicillin (Amx) was used as a reference standard. Whereas, for fungal strains, unlike agar plates, agar media having different drug concentrations were placed as a slant in separate test tubes, and the fungal inocula (2×10^6 CFU/mL) were streaked and incubated further for 48–72 h at 28°C. Fluc was used as a reference standard. MIC was recorded for any lowest concentration where complete growth inhibition of microorganisms was observed visually.

Determination of zone of inhibition (ZOI)

The well-diffusion method was used to determine the ZOI. For that method, NCCLS 2004 guideline was followed. Briefly, agar plates were prepared, and 100µl of bacterial/fungal cell suspension (2×10^6CFU/mL) was spread on solid agar plates using a sterile spreader. Sterile borer was used to make the wells, and the tested compounds at their respective MIC was added to the wells and incubated for 24 h at 37°C (for bacteria) and 48–72 h at 28°C (for fungus). Fluc and Amx were used as a reference standard to compare antifungal and antibacterial activity, respectively, of the tested compounds. The zone diameters were measured, and the experiment was performed in triplicates.

Growth kinetic studies

Growth kinetics studies were carried out to those compounds which have shown higher ZOI at their respective MIC as well as for those have comparable and significant MIC values against the MIC of standard drugs for any bacterial and fungal strains used in the experimental design. Thus, selected tested compounds at their concentration of 2× MIC were exposed to Gram-positive and Gram-bacteria to carry out the study. For any given bacterial strain, 1 ml overnight cultured bacterial suspension was taken in two separate test tubes containing 4 ml fresh nutrient broth media and incubated for 2–3 h at 37°C. After that in one tube, tested compound was added and another tube was used as control (without treatment). At each predetermined time point, 100µl of bacterial suspension was taken from the tubes, diluted in sterile water to achieve the bacterial concentration of 2×10^6 CFU/mL. From that diluted suspension, 100µl was taken to spread on a solid agar plate and incubated for 24 h at 37°C. Afterward, a number of the colony was counted for both test and control [41].

For fungal strains, fungal spores at a concentration of 1×10^5 CFU/mL were inoculated 1 mL of Czapek Dox medium and incubated with or without tested compounds for 48 h with shaking (50 rpm) at 28°C. 100µl from each medium was taken to dilute in sterile water and spread onto Czapek Dox agar plate and incubated at 28°C for 48–72 h, numbers of the colony-forming unit were counted for test and control [42].

By calculating the viable counts at each time point, a 24 h time-kill curve was plotted by plotting log CFU/mL against time for bacterial strains and for fungal strains 96 h time-kill curve were plotted. The experiment was performed in triplicates and expressed as mean ± SD.

SEM

Each bacterial and fungal strain was inoculated in two sets, one was treated as test and another was control. After 2 h tested, compounds were inoculated in tube marked as test. After a sufficient incubation period, cultures were centrifuged at 3000 rpm for 10 min, and the supernatant was discarded to obtain cells pellet. Cells were resuspended in media specific for bacteria and fungus and kept for 10 min and again centrifuged at 3000 rpm for 10 min. Fixation of cells was done with 4% glutaraldehyde in 0.1 M phosphate buffer and kept for 2–3 h and again centrifuged at 3000 rpm for 10 min and the supernatant was discarded. Cells were washed with sterile water and subjected to dehydration by a series of alcohol washing with increasing strength of 30%, 50%, 70%, and 100%, respectively. A drop was put on clean glass slide and dried. Platinum coating was done and the sample was examined under SEM [43].

In silico target binding study

AutoDock Vina [44] using the Lamarckian genetic algorithm for the prediction of binding affinity and searching for the optimum binding site together with the AutoDock Tools (ADT) was employed to set up and perform blind docking calculations of the triazole derivatives binding to substrate binding site of respective enzymes. The membrane protein of *S. typhi* (protein data bank [PDB] id: 4KR4); chimeric CYP51 (PDB id: chimeric 1EA1); and crystal structure of cytochrome P450 14-alpha-sterol demethylase (Cyp51) from *Mycobacterium tuberculosis* in complex with azole inhibitors [45] were obtained from the PDB (www.rcsb.org/pdb) [46] at a resolution of 1.60 Å was constructed using AutoDock4 package to study the target binding interaction of all the compounds under study. The coordinates of tested compounds were taken from their two-dimensional structures sketched from ACD ChemSketch Freeware and converted to the PDB format using Open Babel [47].

The target (respective proteins) and the ligand (triazoles) files were prepared using ADT. The water molecules were deleted, and polar hydrogen atoms and Gasteiger charges were added to both the target molecule and the ligands. All other bonds were made rotatable. The target was saved in pdbqt format, and a configuration file was created. Later docking was run. AutoDock generated nine possible binding conformations for each ligand, and the root-mean-square cluster tolerance was set to 2.0 Å in each run. The binding mode and interactions were analyzed for the significant conformers of the compounds under study. All calculations were performed in a Dell system (3.4 GHz processor, 2GB RAM, 320 GB Hard disk operating system). For each of the docking cases, the lowest energy docked conformation, according to the AutoDock scoring function, was selected as the binding mode. Visualization of the docked pose was done using PyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrodinger, LLC) molecular graphics program.

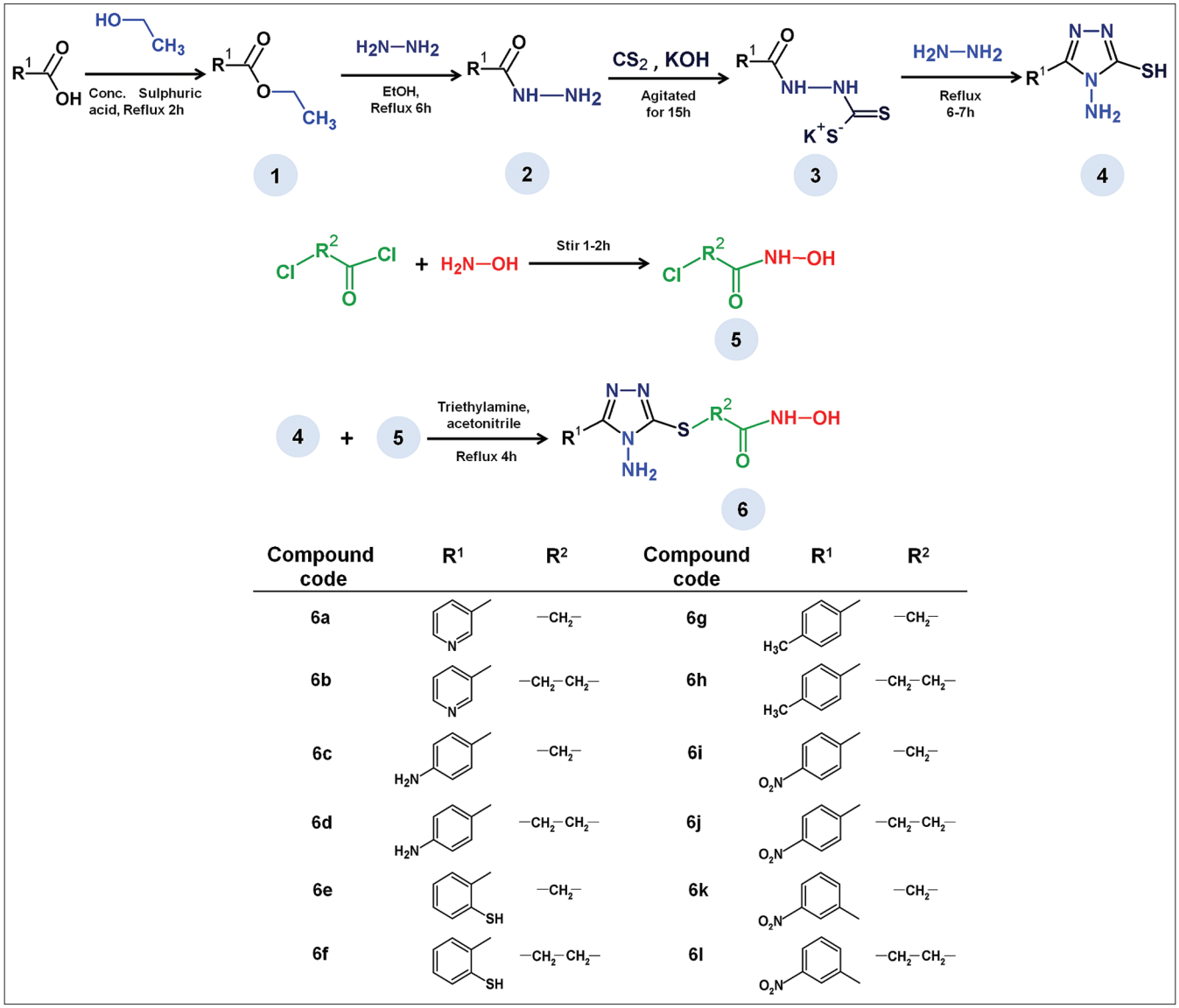
Docking was processed with setting of the grid sizes for 4KR4; 30, 26, and 24 and for chimeric 1EA1 40, 34, and 24 along the X-, Y-, and Z-axes with 1Å spacing which covered all the active residues. In a similar way, the grid center of each target was set for 4KR4; 34.52, 21.86, and 44.96 and for chimeric 1EA1 12.04, 2.87, and 62.50 Å for the protein.

Statistical analysis

Experimental data were presented as mean standard deviation. One-way analysis of variance was used to determine the statistical significance. $p<0.01$ was considered as statistically significant whereas $p>0.05$ was considered to be statistically not significant.

RESULTS AND DISCUSSION

The synthetic route of the proposed scaffold was depicted in Scheme 1. A series of novel 2-((4-amino-5-substituted-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide and 3-((4-amino-5-substituted-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide(6a-6l) compounds were synthesized by reacting 4-amino-5-substituted-4H-1,2,4-triazole-3-thiol with the respective 2-chloro-N-hydroxy acetamides/3-chloro-N-hydroxypropanamide (Scheme 1). Briefly, the reaction was carried out in three parts, i.e., synthesis of 4-amino-5-substituted-4H-1,2,4-triazole-3-thiol and synthesis of 2-chloro-N-hydroxy acetamides/3-chloro-N-hydroxypropanamide then both were coupled to synthesis of the final compounds. 4-amino-5-substituted-4H-1,2,4-triazole-3-thiol were synthesized by Reid-Heindal method [48], i.e., aryl acids were taken as a starting material and refluxed for 2 h with ethanol and concentrated sulfuric acid to produce the corresponding aryl esters. The aryl esters were further refluxed with hydrazine hydrate to yield corresponding aryl hydrazides. Then, in the presence of ethanolic potassium hydroxide, the aryl hydrazides were condensed with carbon-di-sulfide to produce potassium-3-aroyldithiocarbazates. These aroyldithiocarbazates were cyclized to produce corresponding aryl substituted 1,2,4-triazole. In another part, chloroacetylchloride or chloropropionylchloride reacted with hydroxyl amine hydrochloride to yield 2-chloro-N-hydroxy



Compound code	R^1	R^2	Compound code	R^1	R^2
6a		$-CH_2-$	6g		$-CH_2-$
6b		$-CH_2-CH_2-$	6h		$-CH_2-CH_2-$
6c		$-CH_2-$	6i		$-CH_2-$
6d		$-CH_2-CH_2-$	6j		$-CH_2-CH_2-$
6e		$-CH_2-$	6k		$-CH_2-$
6f		$-CH_2-CH_2-$	6l		$-CH_2-CH_2-$

Scheme 1: Synthetic route for target compounds 6a–6l

acetamides or 3-chloro-N-hydroxypropanamide, respectively. In the final step, 4-amino-5-(substituted phenyl)-4H-1,2,4-triazole-3-thiol and 2-chloro-N-hydroxy acetamides or 3-chloro-N-hydroxypropanamide were condensed in the presence of triethylamine to produce the final compound, i.e., 2-((4-amino-5-substituted-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide or 3-((4-amino-5-substituted-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide, respectively. All the final compounds (6a-6l) were characterized by FTIR, ^{1}H NMR, ^{13}C NMR, and mass spectral data.

2-((4-amino-5-(pyridin-3-yl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide (6a)

Yield: 1.2 g (70%), mp: 200–202°C, FTIR (KBR) (cm^{-1}): 3420.39 (N-H stretching), 3028.37 (aromatic C-H stretching), 2981.27 (aliphatic C-H stretching), 1431.09 (aromatic C=C stretching), 1618.65 (C=O stretching), 1530.36 (C=N stretching), 1303.91 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.0370 (s; NH), 9.1331 (s; OH), 8.3740–7.5855 (m; Ar-H), 5.7858 (s; NH_2), 1.0373 (s; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 152.4713 (CH), 147.5985 (CH), 124.2174 (CH), 133.6890 (C), 148.3817 (C), 30.6083 (CH2), 167.2465 (C). Mass: [EI+] ($C_9H_{10}N_6O_2S$) 266 Da; calculated for $C_9H_{10}N_6O_2S$, C, 40.60; H, 3.79; N, 31.56; O, 12.02; S, 12.04 found: C, 40.72; H, 3.73; N, 31.60; O, 11.96; S, 11.98.

3-((4-amino-5-(pyridin-3-yl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (6b)

Yield: 1 g (73%), mp: 197–200°C, FTIR (KBR) (cm^{-1}): 3412.17 (N-H stretching), 3026.40 (aromatic C-H stretching), 2930.53 (aliphatic C-H stretching), 1431.34 (aromatic C=C stretching), 1618.84 (C=O stretching), 1530.61 (C=N stretching), 1304.29 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.0378 (s; NH), 9.1305 (s; OH), 8.3704–7.5807 (m; Ar-H), 5.7834 (s; NH_2), 1.1560–1.0098 (m; CH_2-CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 152.4718 (CH), 147.5995 (CH), 124.2179 (CH), 133.6898 (C), 148.3825 (C), 30.6088 (CH_2), 25.7294 (CH_2), 167.2482 (C). Mass: [EI+] ($C_{10}H_{12}N_6O_2S$) calc. 280.07 Da, Found: 280 Da; calculated for $C_{10}H_{12}N_6O_2S$, C, 42.85; H, 4.32; N, 29.98; O, 11.42; S, 11.44 found: C, 42.92; H, 4.36; N, 29.93; O, 11.39; S, 11.4.

2-((4-amino-5-(4-aminophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide (6c)

Yield: 720 mg (768%), mp: 212–215°C, FTIR (KBR) (cm^{-1}): 3372.34 (N-H stretching), 3038.50 (aromatic C-H stretching), 2918.46 (aliphatic C-H stretching), 1499.91 (aromatic C=C stretching), 1693.64 (C=O stretching), 1536.51 (C=N stretching), 1334.46 (N-N=C stretching).^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 12.80011 (s; NH), 9.32056 (s; OH), 7.62590–6.52428 (m; Ar- H), 5.91882 (s; NH_2), 1.24555 (m; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 116.8490 (CH), 120.9804

(CH), 128.4892 (CH), 147.9976 (C), 148.7785 (C), 30.6102 (CH_2), 167.2496 (C). Mass: [EI+] ($C_{10}H_{12}N_6O_2S$) calc. 280.07 Da, Found: 280 Da; calculated for $C_{10}H_{12}N_6O_2S$, C, 42.85; H, 4.32; N, 29.98; O, 11.42; S, 11.44 found: C, 42.53; H, 4.42; N, 29.82; O, 11.26; S, 11.57.

3-((4-amino-5-(4-aminophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (6d)

Yield: 850 mg (71%), mp: 210–214°C, FTIR (KBR) (cm^{-1}): 3341.13 (N-H stretching), 3054.69 (aromatic C-H stretching), 2899.90 (aliphatic C-H stretching), 1442.34 (aromatic C=C stretching), 1678.97 (C=O stretching), 1574.30 (C=N stretching), 1271.78 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 12.7077 (s; NH), 9.4001 (s; OH), 7.7603–7.2099 (m; Ar- H), 5.2626 (s; NH_2), 1.2561 (m; CH_2-CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 116.8498 (CH), 128.4889 (CH), 120.9809 (C), 147.9981 (C), 148.7789 (C), 30.6109 (CH_2), 25.3169 (CH_2), 167.2498 (C). Mass: [EI+] ($C_{11}H_{14}N_6O_2S$) calc. 294.09 Da, Found: 294 Da; calculated for $C_{11}H_{14}N_6O_2S$, C, 44.89; H, 4.79; N, 28.55; O, 10.87; S, 10.89 found: C, 45.05; H, 4.36; N, 28.76; O, 11.09; S, 10.74.

2-((4-amino-5-(2-mercaptophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide (6e)

Yield: 1.12g (70%), mp: 105–109°C, FTIR (KBR) (cm^{-1}): 3367.57 (N-H stretching), 3082.03 (aromatic C-H stretching), 2980.41 (aliphatic C-H stretching), 1458.46 (aromatic C=C stretching), 1696.7 (C=O stretching), 1563.15 (C=N stretching), 1287.18 (N-N=C stretching).^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 13.98857 (s; NH), 9.58518 (s; OH), 8.04105–7.36677 (m; Ar- H), 5.31007 (s; NH_2), 1.36065 (s; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm, 125.1776 (CH), 126.1872 (CH), 127.2028 (CH), 131.2604 (C), 133.5519 (C), 138.5647 (CH), 148.3655 (C), 30.6009 (CH_2), 165.5395 (C). Mass: [EI+] ($C_{10}H_{11}N_5O_2S_2$) calc. 297.04Da, Found: 297 Da; calculated for $C_{10}H_{11}N_5O_2S_2$, C, 40.39; H, 3.73; N, 23.55; O, 10.76; S, 21.57 found: C, 40.45; H, 4.34; N, 29.94; O, 11.21; S, 11.52.

3-((4-amino-5-(2-mercaptophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (6f)

Yield: 1.3 g (74%), mp: 100–103°C, FTIR (KBR) (cm^{-1}): 3385.76 (N-H stretching), 3093.54 (aromatic C-H stretching), 2980.07 (aliphatic C-H stretching), 1458.33 (aromatic C=C stretching), 1695.47 (C=O stretching), 1562.97 (C=N stretching), 1286.84 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.00011 (s; NH), 9.51002 (s; OH), 8.03405–7.33288 (m; Ar- H), 5.30011 (s; NH_2), 1.37415–1.32802 (m; CH_2-CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm, 125.1786 (CH), 126.1852 (CH), 127.2035 (CH), 131.2606 (C), 133.5505 (C), 138.5682 (CH), 148.4652 (C), 30.6089 (CH_2), 25.8079 (CH_2), 165.5396 (C). Mass: [EI+] ($C_{11}H_{13}N_5O_2S_2$) calc. 311.05Da, Found: 311 Da; calculated for $C_{11}H_{13}N_5O_2S_2$, C, 42.43; H, 4.21; N, 22.49; O, 10.28; S, 20.60 found: C, 41.99; H, 4.28; N, 30.06; O, 11.39; S, 11.72.

2-((4-amino-5-(4-nitrophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide (6g)

Yield: 1.8 g (80%), mp: 138–140°C, FTIR (KBR) (cm^{-1}): 3343.96 (N-H stretching), 3048.17 (aromatic C-H stretching), 2917.92 (aliphatic C-H stretching), 1443.74 (aromatic C=C stretching), 1667.23 (C=O stretching), 1514.60 (C=N stretching), 1269.08 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 13.64368 (s; NH), 9.30012 (s; OH), 7.82233–7.25141 (m; Ar- H), 5.60085 (s; NH_2), 1.13647 (m; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm, 129.0487 (CH), 129.2014 (CH), 129.2701 (CH), 127.9722 (C), 142.9518 (C), 129.3370 (CH), 148.1818 (C), 30.4883 (CH_2), 21.0502 (CH_2), 167.2505 (C). Mass: [EI+] ($C_{11}H_{13}N_5O_2S$) calc. 279.08 Da, found: 279 Da; calculated for $C_{11}H_{13}N_5O_2S$, C, 47.30; H, 4.69; N, 25.07; O, 11.46; S, 11.48 found: C, 48.10; H, 4.18; N, 29.86; O, 11.14; S, 11.57.

3-((4-amino-5-(p-tolyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (6h)

Yield: 1.4 g (73%), mp: 148–150°C, FTIR (KBR) (cm^{-1}): 3342.98 (N-H stretching), 3071.72 (aromatic C-H stretching), 2918.91 (aliphatic C-H stretching), 1444.07 (aromatic C=C stretching), 1667.44 (C=O stretching), 1515.06 (C=N stretching), 1281.37 (N-N=C stretching).^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 13.67881 (s; NH), 9.37850 (s; OH), 7.82335–7.25908 (m; Ar- H), 5.59985 (s; NH_2), 1.16979–1.12207 (m; CH_2-CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 129.0512 (CH), 129.2045 (CH), 129.2723 (CH), 127.9750 (C), 142.9543 (C), 129.3390 (CH), 148.1827 (C), 30.4859 (CH_2), 25.8094 (CH_2), 21.0517 (CH_3), 167.2530 (C). Mass: [EI+] ($C_{12}H_{15}N_5O_2S$) calc. 293.09 Da, found: 293 Da; calculated for $C_{12}H_{15}N_5O_2S$, C, 49.13; H, 5.15; N, 23.87; O, 10.91; S, 10.93 found: C, 48.98; H, 5.10; N, 29.95; O, 10.97; S, 10.99.

Table 1: MIC values of synthesized compounds (6a-6l)

Organisms	**MIC (µg/ml)**												
Bacterial strains	**6a**	**6b**	**6c**	**6d**	**6e**	**6f**	**6g**	**6h**	**6i**	**6j**	**6k**	**6l**	**Amx**
Salmonella typhi	25	25	50	50	-	-	-	-	-	-	50	100	10
Klebsiella pneumoniae	25	50	25	25	75	100	-	-	-	-	-	-	60
Bacillus polymyxa	100	100	-	-	50	50	-	-	-	-	100	100	32
Bacillus cereus	50	50	50	25	-	-	-	-	50	75	75	50	12
Vibrio cholerae	50	75	-	-	-	-	75	100	-	-	50	25	25
Staphylococcus aureus	100	150	100	50	-	-	-	-	-	-	-	-	16
Enterococcus faecalis	-	-	-	-	-	-	-	-	200	200	150	100	25
Bordetella bronchiseptica	50	50	-	-	50	50	-	-	50	100	-	-	25
Micrococcus luteus	-	-	-	-	-	-	100	150	-	-	25	25	0.5
Pseudomonas aeruginosa	25	25	50	50	-	-	-	-	-	-	25	25	65
Shigella sonnei	100	100	-	-	-	-	-	-	-	-	-	-	32
Escherichia coli	-	-	100	100	-	-	100	100	-	-	100	100	10
Bacillus pumilus	25	25	50	25	-	-	-	-	50	50	50	75	2
Salmonella typhimurium	-	-	100	200	200	200	-	-	-	-	150	100	25
Bacillus subtilis	25	25	25	50	-	-	100	100	-	-	100	150	8
Fungal stains	**6a**	**6b**	**6c**	**6d**	**6e**	**6f**	**6g**	**6h**	**6i**	**6j**	**6k**	**6l**	**Fluc**
Candida albicans	15	25	50	25	-	-	100	50	50	100	50	20	12.5
Candida tropicalis	50	20	100	20	-	-	200	100	-	-	-	-	10
Candida neoformans	50	50	-	-	-	-	100	200	200	50	50	50	15
Aspergillus niger	20	20	50	25	300	200	100	50	50	25	50	100	18
Microsporum gypseum	-	-	300	200	-	-	500	350	500	300	50	50	16
Penicillium chrysogenum	-	-	300	250	-	-	400	400	-	-	-	-	56
Cladosporium	250	300	-	-	-	-	400	200	250	300	-	-	32

Amx: Amoxicillin, Fluc: Fluconazole

Table 2: ZOI of tested compounds (6a-6l)

Organisms	Zone of Inhibition (mm)												
Bacterial strains	**6a**	**6b**	**6c**	**6d**	**6e**	**6f**	**6g**	**6h**	**6i**	**6j**	**6k**	**6l**	**Amx**
Salmonella typhi	18.5±0.2	16.5±0.5	15.7±0.5	15.1±0.2	_	_	_	_	_	_	15.1±0.2	11.2±=0.1	21.0±0.9
Klebsiella pneumoniae	16.0±0.4	11.5±0.3	9.3±0.3	13.8±0.1	11.2±0.4	10.1±0.4	_	_	_	_	_	_	13.5±0.9
Bacillus polymyxa	13.3±0.3	16.4±0.4	_	_	15.4±0.1	12.2±0.1	_	_	_	_	11.5±0.4	10.6±0.2	18.7±0.2
Bacillus cereus	14.0±0.1	12.1±0.2	13.6±0.2	15.5±0.1	_	_	_	_	15.2±0.2	13.5±0.4	11.7±0.4	13.2±0.1	17.3±0.2
Vibrio cholerae	11.0±0.9	12.0±0.1	_	_	_	_	14.1±0.2	12.6±0.4	_	_	11.4±0.4	13.6±0.2	13.5±0.5
Staphylococcus aureus	15.3±0.2	12.5±0.4	12.0±0.6	14.7±0.2	_	_	_	_	_	_	_	_	16.5±0.9
Enterococcus faecalis	_	_	_	_	_	_	_	_	11.0±0.6	10.2±0.2	11.2±0.4	11.1±0.2	15.2±0.4
Bordetella bronchiseptica	10.4±0.4	10.5±0.5	_	_	11.1±0.2	10.0±0.1	_	_	11.0±0.6	12.9±0.2	_	_	15.3±0.2
Micrococcus luteus	_	_	_	_	_	_	14.2±0.1	12.9±0.4	_	_	11.4±0.4	9.5±0.2	18.7±0.6
Pseudomonas aeruginosa	14.1±0.2	13.6±0.2	13.1±0.3	11.5±0.3	_	_	_	_	_	_	13.5±0.4	12.6±0.2	16.9±0.2
Shigella sonnei	10.0±0.3	9.6±0.3	_	_	_	_	_	_	_	_	12.0±0.3	11.2±0.2	15.9±0.3
Escherichia coli	_	_	10.8±0.3	10.1±0.2	_	_	10.7±0.3	10.3±0.3	_	_	10.7±0.1	10.1±0.2	17.3±0.2
Bacillus pumilus	13.3±0.3	11.5±0.3	12.0±0.3	11.3±0.3	_	_	_	_	9.4±0.2	9.1±0.4	11.9±0.1	11.2±0.3	14.4±0.1
Salmonella typhimurium	_	_	11.0±0.1	10.1±0.2	12.1±0.2	11.1±0.3	_	_	_	_	13.7±0.2	12.5±0.3	16.9±0.2
Bacillus subtilis	13.3±0.2	11.4±0.2	12.1±0.3	11.1±0.2	_	_	12.4±0.2	11.9±0.3	_	_	12.8±0.2	11.8±0.3	13.9±0.3
Fungal strains	**6a**	**6b**	**6c**	**6d**	**6e**	**6f**	**6g**	**6h**	**6i**	**6j**	**6k**	**6l**	**Fluc**
Candida albicans	11.9±0.7	10.2±0.6	10.8±0.8	7.8 ±0.7	_	_	7.9 ±0.5	9.6 ±0.8	7.6 ±0.8	11.0±0.3	12.2±0.3	11.1±0.3	16.0±0.9
Candida tropicalis	9.4 ±0.8	9.2 ±0.9	8.0 ±0.6	9.2 ±0.8	_	_	9.5 ±0.7	9.4 ±1.2	_	_	_	_	16.3±0.4
Candida neoformans	7.3 ±0.5	8.4 ±0.6	_	_	_	_	8.7 ±0.6	7.5 ±0.7	8.4 ±0.7	10.3±0.3	12.9±0.3	12.6±0.3	14.2±0.6
Aspergillus niger	11.2 ±0.6	11.6 ±0.9	10.2 ±0.6	9.8 ±0.7	11.3±0.9	8.9±0.7	9.6 ±0.7	11.3 ±0.7	7.43 ±0.6	9.8 ±0.1	14.1±0.1	9.9 ±0.1	17.6±0.3
Microsporum gypseum	_	_	7.6 ±0.7	6.8 ±0.7	_	_	6.8 ±0.8	8.7 ±0.7	6.8 ±0.7	10.7±0.4	11.9±0.4	11.1±0.3	16.4±0.9
Penicillium chrysogenum	_	_	6.6 ±0.8	8.5 ±0.8	_	_	7.7 ±0.6	7.3 ±0.5	_	_	_	_	16.3±0.6
Cladosporium	7.5 ±0.6	7.2 ±1.0	_	_	_	_	5.8 ±0.6	8.3 ±0.9	6.5 ±0.8	10.1±0.4	_	_	15.2±0.9

Amx: Amoxicillin, Fluc: Fluconazole, experimental data expressed as mean±SD, n=3, SD: Standard deviation, ZOI: Zone of inhibition

2-((4-amino-5-(4-nitrophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide (6i)

Yield: 1.0 g (67%), mp: 223–225°C, FTIR (KBR) (cm^{-1}): 3351.84 (N-H stretching), 3070.84 (aromatic C-H stretching), 2939.00 (aliphatic C-H stretching), 1451.03 (aromatic C=C stretching), 1698.61 (C=O stretching), 1572.72 (C=N stretching), 1288.70 (N-N=C stretching).^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.0948 (s; NH), 9.48836 (s; OH), 8.31648–7.97074 (m; Ar- H), 5.49898 (s; NH_2), 1.19998 (m; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 122.0874 (CH), 128.4884 (CH), 136.8090 (C), 147.9985 (C), 148.7815 (C), 30.6092 (CH_2), 167.2478 (C). Mass: [EI+] ($C_{10}H_{10}N_6O_4S$) calc. 310.05 Da, Found: 310 Da; calculated for $C_{10}H_{10}N_6O_4S$, C, 38.71; H, 3.25; N, 27.08; O, 20.63; S, 10.33 found: C, 39.76; H, 2.95; N, 26.73; O, 20.48; S, 10.13

3-((4-amino-5-(4-nitrophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (6j)

Yield: 1.2 g (71%), mp: 212–215°C, FTIR (KBR) (cm^{-1}): 3365.73 (N-H stretching), 3076.11 (aromatic C-H stretching), 2857.26 (aliphatic C-H stretching), 1475.39 (aromatic C=C stretching), 1681.59 (C=O stretching), 1519.24 (C=N stretching), 1311.25 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.1975 (s; NH), 9.15008 (s; OH), 8.32100–7.98191 (m; Ar- H), 5.48758 (s; NH_2), 1.20831–1.16040 (m; CH_2-CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 122.0878 (CH), 128.4887 (CH), 136.8098 (C), 147.9989 (C), 148.7818 (C), 30.6095 (CH_2), 25.8370 (CH_2), 167.2482 (C). Mass: [EI+] ($C_{11}H_{12}N_6O_4S$) calc. 324.06 Da, found: 324 Da; calculated for $C_{11}H_{12}N_6O_4S$, C, 40.74; H, 3.73; N, 25.91; O, 19.73; S, 9.89 found: C, 41.14; H, 3.58; N, 26.06; O, 19.95; S, 10.07.

2-((4-amino-5-(3-nitrophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide (6k)

Yield: 1g (70%), mp: 220–224°C, FTIR (KBR) (cm^{-1}): 3308.80 (N-H stretching), 3082.67 (aromatic C-H stretching), 2926.24 (aliphatic C-H stretching), 1481.03 (aromatic C=C stretching), 1700.90 (C=O stretching), 1518.48 (C=N stretching), 1296.15 (N-N=C stretching). ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.1101 (s; NH), 9.4886 (s; OH), 8.9514–7.8044 (m; Ar- H), 5.8517 (s; NH_2), 1.2327 (s; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 121.0683 (CH), 131.2639 (CH), 132.0087 (C), 133.0016 (CH), 148.0011 (C), 148.9088 (C), 31.8313 (CH_2), 165.7338 (C). Mass: [EI+] ($C_{10}H_{10}N_6O_4S$) calc. 310.05 Da, found: 310 Da; calculated for $C_{10}H_{10}N_6O_4S$, C, 38.71; H, 3.25; N, 27.08; O, 20.63; S, 10.33 found: C, 38.80; H, 3.29; N, 26.98; O, 20.61; S, 10.30.

3-((4-amino-5-(3-nitrophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxypropanamide (6l)

Yield: 1.1 g (70%), mp: 210–212°C, FTIR (KBR) (cm^{-1}):. ^{1}H-NMR (DMSO-d6, 300 MHz) δ ppm: 14.1199 (s; NH), 9.4883 (s; OH), 8.9488–7.1773 (m; Ar- H), 5.8455 (s; NH_2), 2.2546 (s; CH_2), 1.2186 (s; CH_2). ^{13}C-NMR (DMSO-d_6) δ ppm: 121.0678 (CH), 131.2635 (CH), 132.0088 (C), 133.0010 (CH), 148.0003 (C), 148.9085 (C), 31.8309 (CH_2), 25.9689 (CH2), 165.7335 (C). Mass: [EI+] ($C_{11}H_{12}N_6O_4S$) calc. 324.06 Da, found: 324 Da; calculated for $C_{11}H_{12}N_6O_4S$, C, 40.74; H, 3.73; N, 25.91; O, 19.73; S, 9.89 found: C, 40.64; H, 3.67; N, 25.99; O, 19.79; S, 9.92.

The synthesized compounds (6a-6l) were exposed to 15 different bacterial strains including both Gram-negative and Gram-positive bacteria and found to be efficacious at a range of MIC values between 25 and 200 µg/ml. Amx was used as a reference standard to compare the antibacterial activity of the synthesized compounds. The comparative MIC values of the compounds were tabulated in Table 1. The results showed that majority of the synthesized compounds elicited variable degrees of inhibition against the tested microorganisms. Compounds 6a, 6b, 6c, 6d, 6k, and 6l exhibited potent antibacterial activity against *K. pneumonia*, *B. cereus, P. aeruginosa, B. pumilus, S. typhi, B. subtilis,* and *V. cholerae* at a MIC of 25 µg/ml whereas compound 6e (75 µg/ml) has shown moderate activity compared to the standard having the MIC of 60 µg/ml. Compound 6f (100 µg/ml) has poor efficacy against the same strain where other compounds have shown no activity. Against *P. aeruginosa*, compounds 6a- 6d, 6k, and 6l (MIC of 25–50 µg/ml) have found to be more potent than standard compound (65 µg/ml) where other compounds have found to be no activity. Against the other bacterial strain compounds have shown moderate to less activity compared to the standard like, compound 6e, 6f (50 µg/ml) against *B. polymyxa*; compounds 6a, 6k, and 6l (50 µg/ml) against *V. cholerae*; compounds 6a, 6b, 6e, 6f, and 6i (50 µg/ml) against *B. bronchiseptica* were moderately active where other compounds have mild bioactivity against the tested bacterial strains, however, compared to the standard they have very less to no activity.

In vitro, antifungal activity was evaluated against seven different fungal strains and the result showed that the synthesized compounds were active against all tested fungi to some extent (Table 1). Among the tested compounds, compound 6a showed potent activity against *Candida albicans* at MIC 15 µg/ml which is nearer to standard Fluc (12.5 µg/ml), whereas compound 6l (20 µg/ml) was found to be moderately active against *Candida albicans*. Against *A. niger* compounds 6a and 6b (20 µg/ml) were potent whereas compounds 6d and 6j (25 µg/ml) were moderately active compared to the standard (18 µg/ml). On the other hand, compounds 6b and 6d (20 µg/ml) elicited moderate activity against *Candida tropicalis*.

The ZOI of the compounds which showed inhibitory efficacy against individual strains of bacteria and fungus, was calculated at their respective MIC and tabulated in Table 2 like, against *S. typhi* compounds 6a and 6b showed the ZOI of 18.5±0.2 mm (at MIC of 25 µg/ml) and 16.5±0.5 mm (at MIC of 25 µg/ml), respectively. Against *K. pneumoniae* compounds 6a, 6c, and 6d showed ZOI of 16.0±0.4 mm, 9.3±0.3 mm, and 13.8±0.1 mm (at MIC of 25 µg/ml), respectively. Compound 6e showed ZOI of 15.4±0.1mm (MIC = 50 µg/ml) against *B. polymyxa*. Compound 6l have a ZOI of 13.6±0.2 mm (MIC = 25 µg/ml) against *V. cholerae*. ZOI of 14.7±0.2 mm (MIC = 50 µg/ml) for compound 6d against *S. aureus*. 11.4±0.4 mm and 9.5±0.2 mm (MIC = 25 µg/ml) for compounds 6k and 6l against *M. luteus*. For compounds 6a, 6b, 6k, and 6l, ZOI was almost 13mm against *P. aeruginosa* at MIC of 25 µg/ml. ZOI of 13.3±0.3 mm and 13.3±0.2 mm were higher for compound 6a against *B. pumilus* and *B. subtilis*, respectively, at MIC of 25 µg/ml.

For fungal strains, it was observed that compound 6a has the ZOI of 11.9±0.7 mm (MIC = 15 µg/ml) for *C. albicans*; compound 6b showed 9.2±0.9 mm (MIC = 20µg/ml) for *C. tropicalis,* and against *A. niger* compounds 6a and 6b have shown almost similar ZOI of 11.2 ±0.6mm and 11.6 ±0.9 mm, respectively, at the MIC of 20µg/ml.

Compounds 6a, 6b, and 6l were most active against the fungal strain *C. albicans*. It was found very interesting that all of the synthesized compounds were active against *A. niger* even compounds 6e and 6f which were not effective against the any of the fungal strain (between the tested concentration to determine MIC) used for this experimental design, were active against *A. niger* although the MIC values were high enough, i.e., 300µg/ml and 200µg/ml, respectively, compared to the standard drug Fluc (18 µg/ml). However, compounds 6b and 6a showed potent antifungal activity against *A. niger* with ZOI of 11.6 ±0.9 mm and 11.2 ±0.6 mm at the MIC of 20 µg/ml.

SEM was carried out to assess morphological changes in bacterial cells following the exposure of most active compounds against selective bacterial as well as fungal strains; like, for bacterial strains, *B. pumilus* was treated with compound 6a and *P. aeruginosa* was treated with compound 6b whereas for fungal strains, *C. tropicalis* was treated with compound 6b and *C. albicans* was treated with compound 6a. The SEM analysis showed clear morphological changes in bacterial cells when treated with tested compounds. Fig. 1k and l depict the SEM image of control and treated *B. pumilus*, respectively. SEM micrographs of *B. pumilus* revealed that when the bacterium was exposed to the tested compound, the cells disrupted following alterations in the cell integrity. *P. aeruginosa* when treated with the tested compound (Fig. 1n), appeared to swell with loss of its normal morphology as depicted by the control (Fig. 1m). Moreover, based on the pathogenic mechanisms of *B. pumilus* and *P. aeruginosa*, an alternative strategy for the treatment

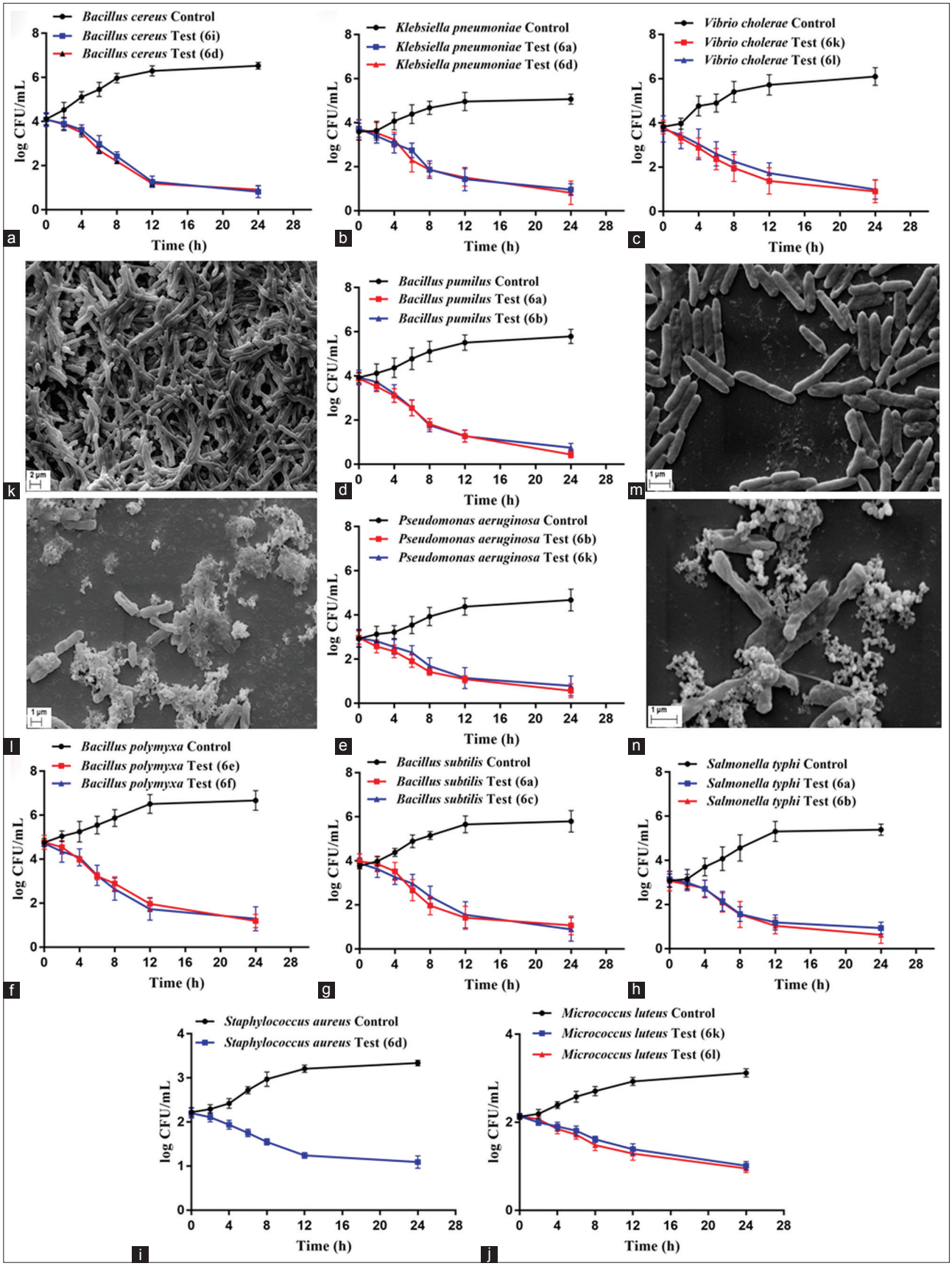


Fig. 1: Time-dependent *in vitro* growth curve of *Bacillus cereus* (a), *Klebsiella pneumoniae* (b), *Vibrio cholerae* (c), *Bacillus pumilus* (d), *Pseudomonas aeruginosa* (e), *Bacillus polymyxa* (f), *Bacillus subtilis* (g), *Salmonella typhi* (h), *Staphylococcus aureus* (i), *Micrococcus luteus* (j) at their 2×MIC values against test compounds and scanning electron micrograph of *B. pumilus* (k) before treatment, (l) after treatment; *P. aeruginosa* (m) before treatment, (n) after treatment

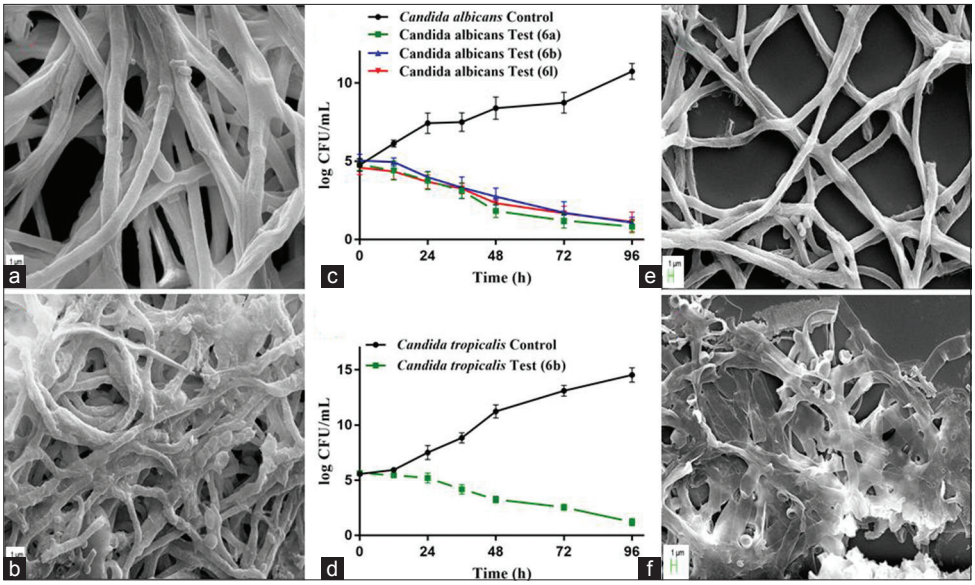


Fig. 2: Scanning electron micrograph of *Candida tropicalis* (a) before treatment, (b) after treatment*; Candida albicans* (e) before treatment, (f) after treatment and time-dependent *in vitro* growth curve of *C. albicans* (c) and *C. tropicalis* (d) at their ×2 MIC values against test compounds

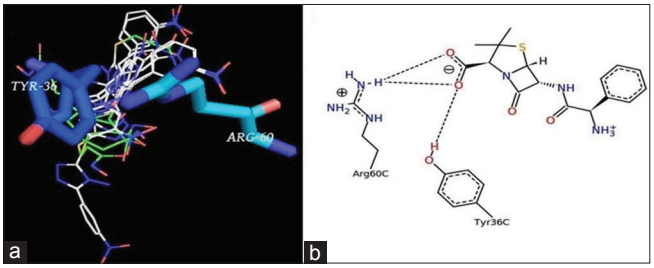


Fig. 3: (a) Cluster of conformers within the active site of the target protein of *Salmonella typhi* (pdb id: 4kr4), (b) PoseView of standard ampicillin within the active site of 4kr4

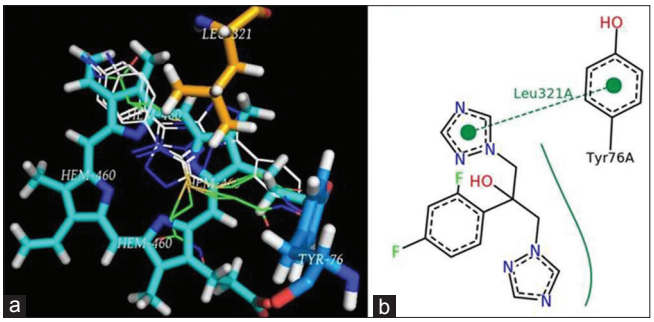


Fig. 4: (a) Cluster of conformers within the active site of the target enzyme of *Candida albicans* (pdb id: Chimeric 1ea1), (b) PoseView of standard fluconazole within the active site of 4kr4

of the infections caused by these organisms is of supreme importance in the current scenario.

Fig. 2a and b are the SEM images of control and treated *Candida tropicalis*, respectively, whereas, Fig. 2e and f show the SEM images of control and treated *Candida tropicalis*, respectively. In both the cases, structural changes in the SEM studies appeared progressively due to cohesion, diffusibility, agglomeration, and ultimate structure distortion and disintegration.

Growth kinetic study was done against the bacterial strains such as *B. cereus*, *K. pneumoniae*, *V. cholerae*, *B. pumilus*, *P. aeruginosa*, *B. polymyxa*, *B. subtilis*, *S. typhi*, *S. aureus,* and *M. luteus* for the tested compounds which showed higher ZOI at their respective MIC (Fig. 1a-J, respectively). Whereas, for fugal strains, compounds 6a, 6b, and 6l were studied for the growth kinetics evaluation against *C. albicans* and compound 6b for *C. tropicalis* displayed in Fig. 2c and d, respectively. Although almost all synthesized compounds have shown antifungal activity against *A. niger*, the growth kinetic study could not be performed as the *A. niger* strains forming very dark brown spores which could not be counted visually. From the growth kinetics data of bacteria as well as fungus from Figs. 1 and 2, respectively, it could be observed that the all best active compounds showed bacteriostatic and fungistatic activity.

It could be observed from the antimicrobial data that synthesized aryl substituted 1,2,4- triazole-linked hydroxamic acid derivatives showed moderate to high activity against tested bacterial as well as fungal strains. The antifungal enzyme, lanosterol-14α-demethylase is one of the primary targets of antifungal drug discovery. The molecular mechanism of triazole as an antifungal has already been established. All the compounds considered for the study possessed 1,2,4-triazole ring in it; therefore, all the compounds exhibited affinity toward the enzyme. The intensity of binding was high in compounds 6a, 6b, and 6l, which may be due to the ring nitrogen and the electronegative nitro group present in the aryl group attached to the central triazole, respectively. Triazole is well known for its antimicrobial activity. However, electron-rich aryl substitutions at 5- position of the 1,2,4-triazole could be an added benefit along with the linked hydroxamic acid. For many decades, the antimicrobial activity of different hydroxamic acid derivatives was evaluated. It was reported that hydroxamic acid is potent as well as a specific inhibitor of bacterial urease [49]. Hence, the antimicrobial activity of the synthesized compounds might be due to the partial effect of hindered urea metabolism by the hydroxamic acid part. It was also reported that the presence of -CONHOH group is very much necessary for the inhibition of microbial urease. In addition, alkyl group present in the hydroxamic acid part of the synthesized compounds influenced the effectiveness.

The binding mode of the highest active test compound exhibiting appreciable antimicrobial activity was investigated by docking studies. Crystal structure of *Salmonella typhi* was obtained from protein repository. On the other hand, no experimental structural information on the active site of the target enzyme *Candida* P450DM is available. It is observed from an extensive literature search that high homology exists

between the mycobacterium P450DM and *Candida* P450DM. Following the method of Rossello *et al.* [50], the chimeric enzyme for the *Candida albicans* (CACYP51) was developed from that of mycobacterium P450DM (MT CYP51) extracted from the PDB (entry code 1EA1). The residues that were arranged in a range of 7Å from Fluc were substituted with those of Candida P450DM. Substitutions were made by replacement of the residues Pro77, Phe78, Met79, Arg96, Met99, Leu100, Phe255, Ala256, His258, Ile322, Ile323, and Leu324 by Lys77, His78, Leu79, Leu96, Lys99, Phe100, Met255, Gly256, Gln258, His322, Ser323, and Ile324, which were thought to be necessary for the ligand-receptor interaction. Compound 6k, 2-((4-amino-5-(3-nitrophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide was within the active site of 4kr4. The active site composition was procured from rcsb and the binding pattern of ampicillin in Fig. 3b was set as standard. Almost all the docked conformers occupied the proximal vicinity of the active site which is clearly understood from Fig. 3a.

Based on the fact that azoles exhibit antifungal activity by inhibition of a cytochrome P450 enzyme, lanosterol-14-α-demethylase during the biosynthesis of ergosterol leading to accumulation of 14-α-sterols in the fungal cell membrane causing destabilization and membrane degradation, it was thought worthwhile to investigate the binding mode of the highest active test compound, 6c, 2-((4-amino-5-(4-aminophenyl)-4H-1,2,4-triazol-3-yl)thio)-N-hydroxyacetamide in the active site of chimeric enzyme of cytochrome P450-14-α-sterol demethylase from *Candida albicans* (*Candida* P450DM) (chimeric 1EA1).

Binding mode analysis of test compound 6c in the active site of chimeric 1EA1 (Fig. 4a) showed that the test compound was very close to the porphyrin ring, HEM 470, similar as that of the cocrystallized ligand Fluc. Docking poses of the test compound suggest the presence of active site residues leu321, tyr76 as in case of Fluc (Fig. 4b).

CONCLUSION

A series of twelve 5-substituted-1,2,4-triazole-linked hydroxamic acid derivatives (6a-6l) were synthesized and evaluated for their *in vitro* antimicrobial potency and found to be potent to moderately active in inhibiting the pathogenic growth. The significant antimicrobial property may be attributed to the putative substructure of triazole (which itself can form ionic interaction with the various microbial target; the most acceptable is lanosterol-14α-demethylase in *Candida albicans*) and the salient feature of the hydroxamic group. The aryl group with its substituent leaves a mixed opinion with respect to the biological interaction. The electron donating ability of methyl, mercapto, amino group strengthen the binding affinity of the aryl group with the active site of the target might be one of the strong supporting evidence for their potency, but in the same ground how this nitro group being a strong electron group helps in lifting the chemotherapeutic index is difficult to establish. Therefore, the series requires to be well extended. Thus, it could be concluded that the synthesized 1,2,4-triazole-linked hydroxamic acid derivatives with electron donating groups at 5-position of the 1,2,4-triazole moiety may provide a therapeutically effective chemical framework from which potential antimicrobial agents may be developed further. Therefore, further optimizations of this prototypical molecular framework with some diversified molecular fragments may generate new drug entities having potent antimicrobial activity.

ACKNOWLEDGMENTS

This experimental work was supported by the Indian Council of Medical Research (ICMR), India. Mousumi Das is a senior research fellow of ICMR (No. 45/9/13-PHA/BMS). The authors are grateful to UPE Phase - II, University Grants Commission, Government of India, for providing instrumental support.

AUTHORS' CONTRIBUTIONS

All authors have made considerable contributions to the work reported in the manuscript.

CONFLICTS OF INTEREST

The authors have no conflicts of interest.

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