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Original article

Synthesis, characterization and *in vitro* biological studies of novel cyano derivatives of *N*-alkyl and *N*-aryl piperazine

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Abstract

Cyano derivatives of *N*-alkyl and *N*-aryl piperazine have been synthesized and screened for antibacterial and antifungal activities. All the synthesized compounds showed the antibacterial activity against pathogenic strains of *Staphylococcus aureus* (MTCCB 737), *Pseudomonas aeruginosa* (MTCCB 741), *Streptomyces epidermidis* (MTCCB 1824) and *Escherichia coli* (MTCCB 1652) and antifungal activity against pathogenic strains of *Aspergillus fumigatus* (ITCC 4517), *Aspergillus flavus* (ITCC 5192) and *Aspergillus niger* (ITCC 5405). All compounds showed mild to moderate antimicrobial activity. However, compounds **3c**, **4a** and **6** showed potent antibacterial activity against pathogenic strains used in the study. Compounds **3a**, **3b**, **4b**, and **4d** showed mild to moderate antifungal activity against *Aspergillus* pathogenic strains. The compounds reported in this study were assessed for there cytotoxicity using MTT colorimetric assay on Hela cells. All the compounds showed cell viability more than the control drug gentamicin, with compound **2** having highest i.e. 95% cell viability.

Keywords: Piperazine; Cyano; Pathogenic strain; Cytotoxicity; Antibacterial; Antifungal activity; Antibiotics

1. Introduction

In recent decades, the problems of multi-drug resistant microorganisms have reached on alarming level in many countries around the world [1–3]. Several antibiotics have been prescribed and found to be effective on various infectious disorders. For the treatment of these intractable infections, new anti-infectious agents are needed. Quinolone antibiotics are widely prescribed drugs because of their safety, good tolerance, broad antibacterial spectrum and less resistance [4–7]. Macrolide antibiotics are an important [1] therapeutic class against Gram-positive organisms [8]. Oxazolidinone's antibacterial agents are newer class of synthetic antibacterial agents with activity against Gram-positive bacteria [9]. Cyano derivatives of piperazine have been known for their uses in the synthesis of pharmaceutical intermediates, peptide analogues, antibiotics and other biologically active molecules and drugs [10–12].

The present investigation describes the synthesis of a series of cyano derivatives of *N*-alkyl and *N*-aryl piperazine. The derivatives so prepared were characterized by employing various spectroscopic techniques such as ^{1}H NMR, ^{13}C NMR, mass spectroscopy etc. The derivatives were assessed for their *in vitro* antimicrobial activity (zone of inhibition and minimum inhibitory concentration (MIC) activity) against a number of bacterial strains and anti-*Aspergillus* activity (minimum inhibitory concentration (MIC) activity). The compounds were further assessed for their *in vitro* cytotoxicity on Hela cells using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay.

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2. Results and discussion

2.1. Chemistry

Herein, we report the synthesis, characterization and *in vitro* antimicrobial activity of cyano derivatives of *N*-alkyl and *N*-aryl piperazine. Cyano derivatives of *N*-alkyl and *N*-aryl substituted piperazine **3a–e** have been prepared by substituting benzotriazolyl group with cyano anion from the key benzotriazolyl intermediates **2a–e** (Scheme 1). The condensation of 1-methylpiperazine **1a**, 1-phenylpiperazine **1b**, 1-benzylpiperazine **1c**, 1-(2-methoxyphenyl) piperazine **1d**, and 1-(4-nitrophenyl) piperazine **1e** with 1 equiv of benzotriazole and 1 equiv of formaldehyde (37% aqueous solution) in MeOH/H_2O (9:1) at 25 °C gave benzotriazolyl intermediate **2a–e** in 87–94% yields as a sole bt[1] isomer [13].

Treatment of benzotriazolyl intermediate **2a–e** with 1.5 equiv of sodium cyanide in DMSO at room temperature replaced the benzotriazole group with cyanide group to afford (4-methylpiperazin-1-yl)-acetonitrile **3a**, (4-phenylpiperazin-1-yl)-acetonitrile **3b**, (4-benzylpiperazin-1-yl)-acetonitrile **3c**, [4-(2-methoxyphenyl)-piperazin-1-yl]-acetonitrile **3d** and [4-(4-nitrophenyl)-piperazin-1-yl]-acetonitrile **3e** in 76–88% yields (Scheme 1). The reactive C–N bond of the key benzotriazolyl intermediates **2a–e** allows easy replacement of benzotriazolyl group with cyano anion to afford cyano derivatives **3a–e** in good to excellent yields via nucleophilic substitutions [13].

Cyano ethyl derivatives of *N*-alkyl and *N*-aryl piperazine **4a–f** (Scheme 2) and **6** (Scheme 3) have been prepared by aza-Michael reaction of *N*-alkyl and *N*-aryl piperazine with acrylonitrile using 10 mol% copper-nanoparticles (14–17 nm) as a catalyst [14]. The spectral data of all synthesized compounds were in consistency with the reported spectral data.

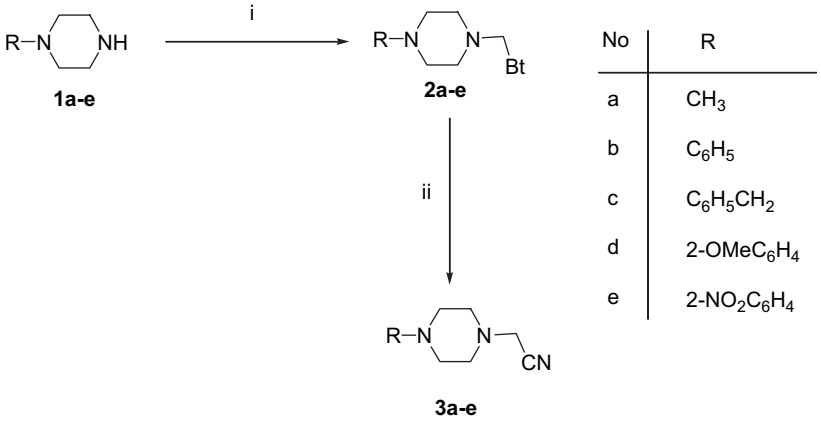
2.2. In vitro antibacterial activity

The *in vitro* antibacterial activity was tested by disc diffusion method [15] and microbroth dilution technique [16] using pathogenic strains of *Staphylococcus aureus* (MTCCB 737), *Pseudomonas aeruginosa* (MTCCB 741), *Streptomyces epidermidis* (MTCCB 1824) and *Escherichia coli* (MTCCB 1652). The experimental result of antibacterial activity indicated variable degree of efficacy of the compounds against different strains of bacteria (Table 1). The zone of inhibition and MIC's values of piperazine derivatives were determined by disc diffusion method [15] and microbroth dilution technique [16].

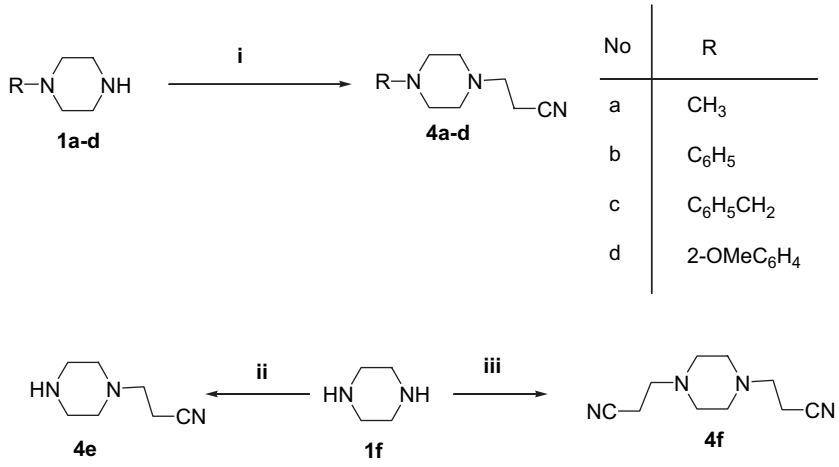
We have synthesized these cyano derivatives of *N*-alkyl and *N*-aryl piperazine for antimicrobial activity. (4-Benzyl-piperazin-1-yl)-acetonitrile **3c** shows potent antibacterial activity against *S. aureus* with MIC value at 19.5 μg/ml and zone of inhibition at 18 mm, however, it did not show significant effect on other strains of bacteria used in experiment. Similarly 3-(4-methyl-piperazin-1-yl)-propanitrile **4a** was effective against *P. aeruginosa*, *E. coli* only, the MIC being 19.5 μg/ml and 39.06 μg/ml with zone of inhibition of 19 mm and 18 mm, respectively (Table 1). 3-[4-(2-Hydroxy-ethyl)-piperazin-1-yl]-acetonitrile **6** shows potent antibacterial activity against *S. aureus* with MIC value 19.5 μg/ml and zone of inhibition 18 mm (Table 1), but moderate activity against *P. aeruginosa*, *E. coli* with MIC at 39.1 μg/ml (Table 1). Compounds **3c** and **4a** showing better activity in comparison to other compounds used in study might be due to the presence of benzyl and methyl groups at 4-position of piperazine nucleus. Enhanced activity of compound **6** might owe to the presence of hydroxyethyl group at 4-position of piperazine nucleus. Other compounds appeared as broad spectrum, as they show mild to moderate effect on most of the strains used in the experiment.

2.3. In vitro antifungal activity

Nine cyano derivatives of *N*-alkyl and *N*-aryl piperazine have also been examined for antifungal activity against pathogenic strains of *Aspergillus fumigatus* (ITCC 4517), *Aspergillus flavus* (ITCC 5192) and *Aspergillus niger* (ITCC 5405). The anti-*Aspergillus* activity of all the synthesized have been evaluated by the disc diffusion (DDA), microbroth dilution (MDA) [17] and percentage spore germination inhibition (PSGI) assay [18]; the results are given in Table 2.



Scheme 1. Reagent and conditions: (i) CH_2O/Benzotriazole (1:1), MeOH/H_2O (9:1), 25 °C; (ii) NaCN, DMSO, 25 °C.



Scheme 2. Reagent and conditions: (i) and (ii) 1.5 equiv acrylonitrile, 10 mol% Cu-nanoparticles (14–17 nm), THF, 25 °C; (iii) 2.8 equiv acrylonitrile, 15 mol% Cu-nanoparticles (14–17 nm), THF, 25 °C.

Result of *in vitro* anti-*Aspergillus* activity demonstrates that **3a**, **3b**, **4b** and **4d** show mild to moderate anti-*Aspergillus* activity against pathogenic strains. Compound (4-methylpiperazin-1-yl)-acetonitrile **3a** has MIC_{90} at 93.8 μg/disc in DDA assay against *A. flavus* and *A. niger*. The next most active compound is 3-(4-phenyl-piperazin-1-yl)-propanitrile **4b**, which exhibited activity at 93.8 μg/disc in DDA against *A. fumigatus*. The structure of compound **3a** has methyl group at 1-position of piperazine nucleus and cyano group at 2-position of side chain at 4-position of piperazine nucleus. Compound **4b** has phenyl group at 1-position of piperazine nucleus and cyano group at 3-position of 4-position of piperazine nucleus.

2.4. In vitro cytotoxicity assay

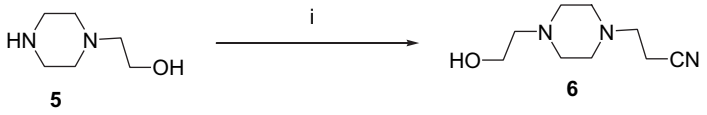
The *in vitro* cytotoxicity of synthesized compounds (1-phenyl-piperazin-4-yl)-acetonitrile **3b**, [4-(4-nitro-phenyl)-piperazin-1-yl]-acetonitrile **3e**, 3-(4-phenyl-piperazin-1-yl)-propanitrile **4b**, 3-[4-(2-methoxy-phenyl)-piperazin-1-yl]-propanitrile **4d**, 3-[4-(2-cyano-ethyl)-piperazin-1-yl]-propanitrile **4f** and 3-[4-(2-hydroxy-ethyl)-piperazin-1-yl]-propanitrile **6** was estimated by MTT colorimetric assay [20]. The Hela cells were incubated with increasing concentration of compounds **3b**, **3e**, **4b**, **4d**, **4f**, **6** and gentamicin as control drug. Compounds **3e**, **4b** and **6** have shown ~60% cell viability at 30 μg, which was comparable to gentamicin at the same concentration. Whereas other compounds **3b**, **4d** and **4f** showed cell viability more than gentamicin at all the concentrations used for the assay. At a concentration of 30 μg of compound (1-phenyl-piperazin-4-yl)-acetonitrile **3b** gave more than 95% cell viability (Fig. 1). From this investigation it can be inferred that the compounds **3b**, **3e**, **4b**, **4d**, **4f**, and **6** prepared in this study have cell viability more than standard drug gentamicin.

3. Conclusion

Series of cyano derivatives of *N*-alkyl and *N*-aryl piperazine were synthesized and their antimicrobial activities were evaluated. Antibacterial activities against antibiotic susceptible standard and clinically isolated pathogenic both Gram-positive and Gram-negative strains (*S. aureus*, *P. aeruginosa*, *S. epidermidis*, *E. coli*) and antifungal activities against *A. fumigatus*, *A. flavus* and *A. niger* were used in the study for performing various experiments. Compounds **3c**, **4a** and **6** were considered to be potent antibacterial due to significant antibacterial activity against bacteria used in the study. Compounds **3a**, **3b**, **4b** and **4d** show mild to moderate anti-*Aspergillus* activity against pathogenic strains. The *in vitro* cytotoxicity performed on Hela cells showed that the compound (1-phenyl-piperazin-4-yl)-acetonitrile **3b** synthesized in this study has 95% cell viability. The compounds **3b**, **3e**, **4b**, **4d**, **4f**, and **6** were found to have low cytotoxicity as compared to control drug gentamicin, with cell viability in the range of 60–95%.

4. Experimental

All reagents used were of AR grade. THF was distilled from sodium/benzophenone prior to use. Melting points were determined using a Thomas Hoover melting point apparatus and are uncorrected. ^1H (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Bruker 300 NMR spectrometer in $CDCl_3$ (with TMS for ^1H and chloroform-*d* for ^{13}C



Scheme 3. Reagent and conditions: 2.5 equiv acrylonitrile, 10 mol% Cu-nanoparticles (14–17 nm), THF, 25 °C.

Table 1
Antibacterial activities of cyano derivatives of *N*-alkyl and *N*-aryl piperazine showing zone of inhibition (mm) and MIC values (μg/ml) against selected pathogenic strains

Compound	*S. aureus*	*S. epidermidis*	*P. aeruginosa*	*E. coli*
3a	12/310.5	8/625.0	9/625.0	12/310.5
3b	10/310.5	12/156.3	13/156.3	9/310.5
3c	18/19.5	10/310.5	13/156.3	9/625.0
3d	9/625.0	8/625.0	8/625.0	10/310.5
3e	–	–	–	
4a	10/310.5	11/156.3	19/19.5	18/39.1
4b	13/156.3	12/156.3	9/625.0	12/156.3
4c	–	–	–	
4d	12/156.3	9/625.0	9/625.0	11/156.3
4e	–	–	–	
4f	11/310.5	9/625.0	11/156.3	10/625.0
6	18/19.5	11/310.5	15/39.1	15/39.1
Gentamicin	20/9.8	18/19.5	19/19.5	18/19.5

– Means no activity.

as internal references) unless otherwise stated. Mass spectrum was recorded on Hybrid Quadrupole-TOF LC/MS/MS mass spectrometer (Q. Star XL). Column chromatography was performed on silica gel (230–400 mesh). Microanalyses were obtained with an Elemental Analysensysteme GmbH VarioEL V3.00 element analyser. The reactions were monitored by thin layer chromatography (TLC) using aluminium sheets with silica gel 60 F_{254} (Merck). All of the reactions were carried out under nitrogen atmosphere.

*4.1. General procedure for the synthesis of benzotriazolyl derivatives of N-alkyl and N-aryl piperazines (**2a–d**)*

To a solution of *N*-alkyl and *N*-aryl piperazines **1a–d**, (0.35 g, 2 mmol) and benzotriazole (0.2148 g, 2 mmol) in CH_3OH/H_2O (9:1, 10 ml) was added formaldehyde (37% aqueous solution, 4 mmol). The mixture was stirred at room temperature for 6–8 h. The precipitate formed was filtered and washed with cold Et_2O to gave pure product 1-[(4-methylpiperazin-1-yl) methyl]-1*H*-1,2,3-benzotriazole **2a**, 1-[(4-phenylpiperazin-1-yl) methyl]-1*H*-1,2,3-benzotriazole **2b**, 1-[(4-benzylpiperazin-1-yl) methyl]-1*H*-1,2,3-benzotriazole **2c** and 1-{[4-(2-methoxyphenyl) piperazin-1-yl] methyl}-1*H*-1,2,3-benzotriazole **2d** in 87–94% yields as a sole bt[1] isomer, which was directly used for subsequent reactions. For microanalysis purposes, the precipitate was recrystallized from $CHCl_3$/hexanes (1:1).

Spectral data of the synthesized compounds **2a–d** were consistent with the reported values.

*4.2. General procedure for the replacement of benzotriazole group from **2a–d** with sodium cyanide*

A solution of benzotriazolyl derivative of *N*-alkyl and *N*-aryl piperazines **2a–e** (1.0 mmol) and NaCN (2.0 mmol) was stirred in DMSO (10 ml) for 8–12 h, after removal of the solvent *in vacuo*, the residue was diluted with EtOAc. The mixture was washed with brine and dried over Na_2SO_4. Evaporation of the solvent was done *in vacuo* and the residue was purified by column chromatography with hexanes/EtOAc (6:1 to 3:1) as an eluent gave **3a–e** in 76–88% yields.

Spectral data of the synthesized compounds **3a–d** were consistent with the reported values.

*4.2.1. (4-Methyl-piperazin-1-yl)-acetonitrile **3a***

The general synthetic method described above affords **3a** as semisolid, yield 88–97%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.38 (s, 2H), 2.27 (s, 3H), 1.50 (m, 4H), 1.35 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 113.5, 54.9, 50.05, 40.23, 30.02; LCMS *m/z* found 140.3 (M + 1), 113, 138.

*4.2.2. (4-Phenyl-piperazin-1-yl)-acetonitrile **3b***

The general synthetic method described above affords **3b** as dirty white solid, yield 88%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.31–7.25 (m, 2H), 6.95–6.86 (m, 3H), 3.58 (s, 2H), 3.24 (t, $J = 4.8$ Hz, 4H), 2.76 (t, $J = 4.9$ Hz, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 150.9, 129.1, 120.1, 116.3, 114.5, 51.8, 48.9, 45.9; LCMS *m/z* found 202.3 (M + 1).

*4.2.3. (4-Benzylpiperazin-1-yl)-acetonitrile **3c***

The general synthetic method described above affords **3d** as creamy white solid, yield; 88%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.19–7.12 (m, 5H), 3.39 (s, 2H), 3.34 (s, 2H), 2.47–2.46

Table 2
Antifungal activities of cyano derivatives of *N*-alkyl and *N*-aryl piperazine

Compound	MIC								
	DDA (μg/disc)			MDA (μg/ml)			PSGI (μg/ml)		
	A. flavus	*A. niger*	*A. fumigatus*	*A. flavus*	*A. niger*	*A. fumigatus*	*A. flavus*	*A. niger*	*A. fumigatus*
3a	93.8	93.7	187.5	500	500	500	250	250	500
3b	187.5	187.5	187.5	500	500	500	500	250	250
3c	–	–		–	–	–	–	–	
4a	–	–	–	–	–	–	–	–	–
4b	187.8	187.5	93.8	500	500	250	250	250	250
4d	375.5	–	187.5	1000	–	500	500	–	500
4f	–	–	–	–	–	–	–	–	
4g	–	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–	–	–
Amphotericin B	2.5	2.5	2.5	5	5	5	5	5	5

– Means no activity.

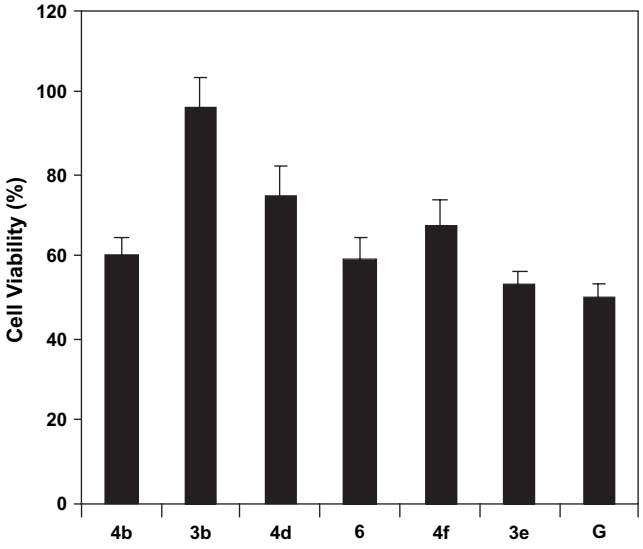


Fig. 1. *In vitro* cytotoxicity assay. The compounds are as follows: (1-phenyl-piperazin-4-yl)-acetonitrile **3b**, [4-(2-methoxy-phenyl)-piperazin-1-yl]-acetonitrile **3e**, 3-(4-phenyl-piperazin-1-yl)-propanitrile **4b**, 3-[4-(2-methoxy-phenyl)-piperazin-1-yl]-propanitrile **4d**, 3-[4-(2-cyano-ethyl)-piperazin-1-yl]-propanitrile **4f**, 3-[4-(2-hydroxy-ethyl)-piperazin-1-yl]-propanitrile **6** and gentamicin **G**.

(m, 4H), 2.39–2.37 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 137.6, 128.6, 127.9, 126.8, 114.5, 62.3, 52.1, 51.4, 45.5; LCMS m/z found 229.3 (M + 1).

4.2.4. [4-(2-Methoxyphenyl)piprazin-1-yl]-acetonitrile ***3d***

The general synthetic method described above affords **3d** as pale solid, yield: 89%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.89–7.86 (m, 2H), 6.87–6.89 (m, 2H), 3.869 (s, 3H), 3.57 (s, 2H), 3.14 (m, 4H), 2.81 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 140.8, 125.9, 123.2, 121.0, 118.3, 114.8, 77.0, 55.4, 52.1, 45.9; LCMS *m/z* found 217.2 (M + 1).

4.2.5. [4-(4-Nitro-phenyl)-piperazin-1-yl]-acetonitrile ***3e***

The general synthetic method described above affords **3e** as yellow crystals, yield 93%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.38–7.24 (m, 5H), 3.57–3.42 (m, 6H), 2.59–2.61 (m, 5H), 2.53–2.48 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 137.9, 132.9, 129.2, 128.1, 125.2, 77.0, 52.8, 52.0, 41.4, 29.7, 3.5; LCMS *m/z* found: 247.3 (M + 1) peak.

4.3. General procedure for the aza-Michael reaction of N-alkyl and N-aryl piperazines (Scheme 2)

To a stirred solution of amine (1.0 equiv) and acrylonitrile (1.2 equiv) in THF (30 ml), Cu-nanoparticles (14–17 nm, 10 mol%) were added at room temperature and stirring was continued for 8–16 h under nitrogen. After completion of the reaction (TLC), THF was removed *in vacuo*, the reaction mixture was treated with water and extracted with ethyl acetate. The organic layer was dried over Na_2SO_4 and after the removal of the solvent *in vacuo*, the residue was purified by column chromatography (silica gel 250–400 mesh size).

4.4. Disc diffusion method

The *in vitro* antibacterial activity was tested by disc diffusion method using pathogenic strains of *S. aureus* (MTCCB 737), *P. aeruginosa* (MTCCB 741), *S. epidermidis* (MTCCB 1824) and *E. coli* (MTCCB 1652) [15]. Compounds with 30 μg/disc concentration were impregnated on the discs. These discs were placed on the surface of the agar plates already inoculated with pathogenic bacteria. The plates were incubated at 37 °C and examined at 48 h for zone of inhibition, if any, around the discs. Gentamicin was used in assay as a standard control drug. An additional control disc without any sample but impregnated with equivalent amount of solvent (DMSO) was also used in the assay. The result of antibacterial activity indicated that some of the compound exhibited mild to moderate activity.

4.5. Broth dilution method

The test was performed in 96-well culture plates (Tarsons). MIC was determined by microbroth dilutions technique using Mueller–Hinton broth and 96-well plates for bacteria [16]. Serial nine-fold dilutions ranging from 1250 to 4.8 mg ml^{-1} were prepared in media. The inoculum was prepared using a 4–6 h broth culture of each bacterial strains adjusted to a turbidity equivalent to a 0.5 McFarland standard, diluted in broth media to give a final concentration of 3×10^6 cfu ml^{-1} for bacteria. The 96-well plates containing Mueller–Hinton broth were incubated at 35 °C for 18–20 h. The MIC was defined as the lowest concentration of compound giving complete inhibition of visible growth.

4.6. Antifungal activity assay

The anti-*Aspergillus* activity of all the compounds was studied by disc diffusion, microbroth dilution and percentage spore germination inhibition assays [17]. The pathogenic strains of *A. fumigatus* (ITCC 4517), *A. flavus* (ITCC 5192) and *A. niger* (ITCC 5405) were used in the study for performing various experiments.

4.7. Microbroth dilution

The test was performed in 96-well culture plates (Nunc, Nunclon). Various concentrations of synthetic compounds in the range of 1000–7.81 μg/ml were prepared in the wells by two-fold dilution method. Assay was performed as per standard method described earlier [17].

4.8. Disc diffusion

The disc diffusion assay was performed in radiation sterilized Petri plates of 10.0 cm diameter (Tarsons) as described [17]. Different concentrations in the range of 750–1.46 μg of the test compounds were impregnated on the sterilized discs (5.0 mm in diameter) of Whatman filter paper no 1. The discs were placed on the surface of the agar plates already

inoculated with *Aspergillus* spores. The plates were incubated at 37 °C and examined at 24, 48 and 96 h for zone of inhibition, if any, around the discs. The concentration, which developed the zone of inhibition of at least 6.0 mm diameter, was considered as minimum inhibitory concentration (MIC).

4.9. Percentage spore germination inhibition

Various concentrations of the test compounds in 90.0 μl of culture medium were prepared in 96-well culture plates (Nunc, Nunclon) by double dilution method [18]. The wells were prepared in triplicates for each concentration. Each well was then inoculated with 10.0 μl of spore suspension containing 100 ± 5 spores. The plates were incubated at 37 °C for 16 h and then examined for spore germination with an inverted microscope (Nikon, Diphot). The number of germinated and non-germinated spores was counted. The lowest concentration of the compound, which resulted in >90% inhibition of germination of spores in the wells, was considered as MIC_{90}.

4.10. In vitro cytotoxicity

The toxicity of compounds (1-phenyl-piperazin-4-yl)-acetonitrile **3b**, [4-(2-methoxy-phenyl)-piperazin-1-yl]-acetonitrile **3d**, 3-(4-phenyl-piperazin-1-yl)-propanitrile **4b**, 3-[4-(2-methoxy-phenyl)-piperazin-1-yl]-propanitrile **4d**, 3-[4-(2-cyano-ethyl)-piperazin-1-yl]-propanitrile **4f** and 3-[4-(2-hydroxy-ethyl)-piperazin-1-yl]-propanitrile **6** was studied by using MTT colorimetric assay [19]. Hela cells were maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100 U/ml of streptomycin–penicillin. The cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C. Hela cells were seeded onto 96-well plates at a density of 8×10^3 cells/well and incubated for 16 h for adherence. Afterwards, the media was aspirated from the wells and the cells were washed once with DMEM without FBS. The compounds **3b**, **3e**, **4b**, **4d**, **4f**, and **6** and gentamicin each increasing in concentration from 10 to 30 μg were diluted with DMEM without FBS to a final volume of 75 μl and this media was added to separate wells, followed by incubation at 37 °C in humidified 5% CO_2 atmosphere for 4 h. Then the media containing the compounds was replaced with 200 μl of normal growth medium and cells were further incubated for 48 h under same conditions. After 48 h media was replaced by 200 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (0.5 mg dissolved in 1.0 ml of DMEM) and further incubated for 2 h. Afterwards, the supernatant was aspirated, and the formazan crystals so formed were suspended in 100 μl *iso*-propanol containing 0.06 M HCl and 0.5% SDS. Aliquots were drawn from each well and the intensity of colour was measured spectrophotometrically in an ELISA plate reader at 540 nm. Untreated cells were taken as control with 100% viability and cells without addition of MTT were used as blank to calibrate the spectrophotometer to zero absorbance. The relative cell viability (%) compared to control cells was calculated by $[abs]_{sample}/[abs]_{control} \times 100$.

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