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Antimicrobial Activity of Novel Piperazine Molecules

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A B S T R A C T

A series of twenty two novel piperazine derivatives were screened for their antimicrobial activities by disc diffusion method and micro dilution method against the ATCC strains of certain selected Gram- positive (MRSA, *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae*, *Citrobacter*, *Shigella flexineri*, *Pseudomonas aeruginosa*) with the standard drugs amongst the synthesized novel compounds RL-308, RL-327and RL-328 showed potent anti bactericidal activities. Hence, they were subjected to higher assays with different concentrations and were compared to the respective standard drugs. Then Minimum inhibitory concentration (MIC) assay, Minimum bactericidal concentration (MBC) assay were performed to those three compounds. Minimum concentration of RL-308 was analyzed by using MIC assay which displayed a potent results against *Shigella flexineri, S.aureus*, MRSA & *Shigella dysentriae* at the concentration of 2ug, 4ug, 16ug &128 ug concentration. It proved to be very effective against *Shigella flexineri* at the concentration of 2ug. Where in *RL-328* showed MIC at the concentration of 128ug. The MBC of the compound RL-308 was estimated to be 4ug, 8ug, 32ug against the same pathogenic bacterial strains. Hence, the piperazine compound RL-308 proved to be very effective antibacterial activity against human pathogens and can be used as an effective antibacterial agent to cure diseases caused by *Shigella flexineri*, *S. aureus*, Methicillin resistant *S. aureus* & *Shigella dysentriae*.

Introduction

Multi drug resistance (MDR) is the most fatal challenge for public health which has to be addressed very effectively in a present scenario (Mousumi Saha and Agniswar Sarkar, 2021; David van Duin and David L. Paterson, 2020). These drug resistance bacteria are responsible for many disease and mortality (Mohsen Naghavi, 2022). Misuse of antibiotics is the biggest problem which poses the bacterial resistance, antibiotics use in India is been huge and it's been raising from 3.2 billion daily dose to 6.5 billion daily dose from 2000 to 2015 (ICMR survey). Antimicrobial resistance is a big threat to India and also becoming a global threat which

has to be addressed with new class of drugs which can combat the MDR strain bacteria.

Many synthetic molecules have been used as antibacterial agents since many decades (Peter M. Wright *et al.,* 2014). The main draw-back is that, they gradually developed the resistance against pathogens. Piperazines are important class of small molecules which have been used in many FDA approved drugs (Ciprofloxacin, Norfloxacin, Levofloxacin). These heterocyclic ring molecules are important pharmacophore in many antimicrobial drugs (Chattopadhyay *et al.,* 2015**;** D'Costa *et al.,* 2011)**,** in cancer drugs (She and Hao, 2013)**,** anti-inflammatory drugs (Silva *et al.,* 2015)**,** anti-anxiety drugs and antifungal drugs (da Silva *et al.,* 2018)**.** With our effort we have synthesised piperazine based potent chemical library of molecules which displayed potent results against many screened pathogenic bacteria.

Even though human body has natural defence mechanism of skin, tears, earwax, stomach acid and mucous membrane, some of the bacteria can bypass these defences. The next step in infection is the contact and adherence. The contact occurs either by passive adsorption when bacteria comes close to the host cell surface or by active response when motile bacteria move towards the host cell. Bacteria use certain virulence factors like fimbriae, pilli and adhesins to attach to the host cells. Another bacterial adaptation to promote colonization is by the formation of biofilm. Bacteria multiply and survive inside a biofilm.

Materials and Methods

Muller Hinton Agar, Sodium hydrogen diphosphate, Disodium hydrogen phosphate, vancomycin, Gentamycin, Gentamycin, Amphicillin, Cefepime, 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide), sodium citrate, dextrose, dimethyl sulfoxide were purchased from Himedia, India Pvt. Ltd. MTCC grade Microbial strains were kindly donated by Department of Microbiology, Adichunchanagiri Institute for Medical Sciences (AIMS), BG Nagara, Mandya, Karnataka. All other reagents & organic solvents were of analytical grade.

Preparation of piperazine sample

Piperazine compounds stock solutions were freshly prepared at the concentration of 10mM in PBS (pH 7.4, 10mM- Na2HPO4, 137mM of NaCl, 10 mM, 1.8mM KH2PO4 and 2.7mM of KCl). Stored them at 4^0C till use.

Antimicrobial activity of Piperazine compounds

Bacterial strains

The antibacterial ability of piperazine compound was evaluated using six bacterial strains causing various types of diseases in includes both Gram positive & Gram negative bacteria such as - *Escherichia coli, Klebsiella pneumoniae*, *Shigella flexineri,* Methicillin Resistant *Staphylococcus aureus, Staphylococcus aureus*. The bacterial strains were provided from the culture collection Department of Microbiology, Adichunchanagiri Institute of Micro biology, BG Nagara, Adichunchanagiri University, Mandya, Karnataka.

Inoculum preparation

Bacterial strains were sub cultured in peptone water in aseptic condition and incubated overnight at 37˚c. Then concentration of each microbial strain was adjusted to 10^8 cells/ml using 0.5 McFarland standard (Nayan *et al.,* 2011).

Antimicrobial activity of piperazine compounds by disc diffusion method

The antimicrobial activity of piperazine molecules were evaluated by disk diffusion method (Alhusadi *et al.,* 2020). In brief 15ml of melted autoclaved Muller Hinton agar media was dispensed into sterile plates in aseptic condition and kept for solidification under ultraviolet radiation. Fresh cultures were taken swabbed at the intervals of 60˚angle covering the entire plate uniformly on the MH agar plates with a sterile cotton swab to obtain a lawn culture.

Six sterile discs (9mm in diameter) were placed at 3 cm distance from each other in a circular manner. 10mM concentration of each piperazine drug molecules were redissolved in 0.1% 10ul dimethylsulfoxide (DMSO) and loaded over a sterile filter paper disc and let it absorb the compounds for 2 minutes with a standard drug placed at the centre. And one disc was taken as negative control where 0.1% DMSO was incorporated. The plates were incubated at 37˚c for 24 hours. Antibacterial activities were then determined by measuring the clear zone of inhibition to the nearest millimetre (mm) ± S.E.M.

Determination of minimum inhibitory concentrations (MIC's) of Piperazine compounds

Minimum Inhibitory Concentrations are the lowest concentration of an antimicrobial which inhibits the visible growth of a micro-organism after overnight incubation. It is used to determine the in vitro activity of antimicrobials. The most effective piperazine compounds- RL308, RL327, RL328 and RL336 which showing very effective antimicrobial activity at 10mg/ml were used to determine their MIC. MRSA and *S.aureus* loop full overnight culture were inoculated into 2ml Muller Hinton broth and got optical density of 0.2 and 0.1respectively after 2 hours of incubation at 37°C in order to obtain the cells in logarithm phase of cell growth. 2 hours culture of MRSA and *S.aureus* were diluted in 10ml of MH broth with 5µl and 10µl of inoculums respectively to get McFarland standard. Inoculation protocols were followed as per the protocols.

Minimum Bactericidal Concentration (MBC) assay

Minimum Bactericidal Concentrations are the lowest concentration of antimicrobials those are responsible for killing of the target organism. The determination of MBC by the Dilution Method involves the inoculation of an indicator bacterium into various concentrations of an antibiotic, incubating for 18 to 24 h and thereafter testing for bacterial viability by subculturing on agar media prepared without the antibiotic. Generally for MBC determinations, agar medium is prepared and inoculated with samples from tubes which show no turbidity or growth.

10ul of the sample were taken from Two wells before to the seizure of visible growth and two wells next to the MIC and media control were taken from the well for about 10µl and spotted over Muller Hinton agar plates. The spots were let the spots to dry and kept for incubation at 37 for 24 h in inverted position. Then examined for bacterial growth in corresponding piperazine compounds. Piperazine compounds did not exhibiting any bacterial growth on the freshly inoculated agar plates was considered as the MBC of the respective compounds. According to table 3, the concentrations where the organisms are killed are mentioned below with the respective compounds and the organism. MIC for MRSA is 2µg/ml in compound 308, 2 µg/ml in compound 327, 2 µg/ml in compound 328 and 8 µg/ml in compound 336. MIC for *S.aureus* 2µg/ml in compound 308, 4µg/ml in compound 327, 2 µg/ml in compound 328 and 32µg/ml in compound 336.

Results and Discussion

Antibacterial activity of piperazine compounds

Thirty piperazine compounds were used to assess their antibacterial activity against five pathogenic bacteria including both Gram positive (MRSA, *Staphylococcus aureus*) and Gram negative bacteria (*Escherichia coli, Klebsiella pneumoniae*, *Citrobacter*, *Shigella flixineri*, *Pseudomonas aeruginosa*) by disc diffusion method. Their antibacterial activity of was documented in Table-1 and illustrated in Fig. 1 The results revealed that among 30 piperazine compounds five compounds have shown antibacterial activity against six pathogenic bacteria at variable strength as shown in Table-1.

All the five piperazine compounds RL308, RL317, RL318, RL327 & RL328 shown very effective inhibition against S. aureus and Methicillin Resistant S. aureus. RL 327 was very effective against the *Sigella flexineri, K. pneumoniae* & *E. coli* compared to all other piperazine compounds.

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of RL-308

Minimum concentration of RL-308 required for complete bacteriostatic activity was analyzed by using MIC assay which displayed a potent result against Methicillin resistant *Staphylococcus aureus* and *Staphylococcus aureus* with inhibitory concentration of 0.5 µg and 2µg/ ml respectively and the visible growth well the bacterial samples were plated and analyzed for bactericidal activity of RL-308 on MRSA and SA which displayed a potent result against both bacteria with MBC 0.5 and 2 µg/ml.

Bacteriostatic effect of hit molecules against *S. aureus*

Bacteriostatic activity of the identified hit molecules was unveiled using Minimum inhibitory concentration, Figure 8 *Staphylococcus aureus*, Figure 2 MRSA, Lane A, B, C, D, E, F treated with different hit compounds with different concentration. Each experiment repeated for three times.

In figure 2, the plates were incubated overnight at 37 the wells

A4, A5, A7 and A9
B2, B4, B6, B8 and B9
C4, C5, C6, C7, C8 and C9
D5, D6, D7, D8 and D9 showed the visible growth. The wells with clear broth represent the absence of microbial growth where in the turbid wells shows the bacterial load which might or might not be viable. This results that the growth of bacterial inoculum *S. aureus* were treated with drugs which were serially diluted are inhibited by the antibacterial activity of the chemical compounds.

Bacteriostatic effect of hit molecules against MRSA

Bacteriostatic activity of the identified hit molecules was unveiled using Minimum inhibitory concentration, Figure 9 Methicillin Resistant *Staphylococcus aureus*, Figure 9 MRSA, Lane A, B, C, D, E, F treated with different hit compounds with different concentration. Each experiment repeated for three times.

In Figure 3, the plates were incubated overnight at 37 the wells.

A4, A5, A7 and A9
B2, B4, B6, B8 and B9
C4, C5, C6, C7 and C8
D5, D6, D7, D8 and D9 showed the visible growth. The wells with clear broth represents the absence of microbial growth where in the turbid wells shows the bacterial load which might or might not be viable. This results that the growth of bacterial inoculum Methicillin Resistant *S. aureus* were treated with drugs which were serially diluted are inhibited by the antibacterial activity of the chemical compounds.

Bactericidal effect of hit molecules against MRSA

Bactericidal activity of the identified hit molecules was unveiled using Minimum bactericidal concentration, Figure 10 MRSA. Each experiment repeated for three times. In Figure 4, the plate is spotted with the culture available in the wells of mictrotiter plate after performing MIC assay of different concentrations on the test organism MRSA against the drugs 308, 327, 328 and 336 respectively. Basically the controls were taken for 10 µl and spotted over the labelled MH plate at the top.

Then the wells 6, 7, 8, and 9 were chosen for compounds 308, 327and 328. The wells 4, 5, 6 and 7 were chosen for compound 336 due to varied turbid nature of the culture in the well. The wells 4 contains 16 mg/ml, 5 contains 8 mg/ml, 6 contains 4 mg/ml, 7 contains 2 mg/ml, 8 contains 1 mg/ml and 9 contains 0.5 mg/ml concentration of drugs. Hence the reduction of bacterial populations can be visible on the agar plate and so we can take a proof for bactericidal activity of the drugs on the test organism.

Bactericidal effect of hit molecules against *S. aureus*

Bactericidal activity of the identified hit molecules was unveiled using Minimum bactericidal concentration, Figure 5 *Staphylococcus aureus*. Each experiment repeated for three times.

In Figure 4, the plate is spotted with the culture available in the wells of microtiter plate after performing MIC assay of different concentrations on the test organism MRSA against the drugs 308, 327, 328 and 336 respectively. Basically the controls were taken for 10 µl and spotted over the labelled MH plate at the top.

Then the wells 6, 7, 8, and 9 were chosen for compounds 308 and 328. The wells 3, 4, 5 and 6 were chosen for compound 327 and 336 due to varied turbid nature of the culture in the well.

The wells 3 contains 32 mg/ml 4 contains 16 mg/ml, 5 contains 8 mg/ml, 6 contains 4 mg/ml, 7 contains 2 mg/ml, 8 contains 1 mg/ml and 9 contains 0.5 mg/ml concentration of drugs. Hence the reduction of bacterial populations can be visible on the agar plate and so we can take a proof for bactericidal activity of the drugs on the test organism.

Antibacterial activity of the synthesized piperazine compounds were assayed against various bacterial pathogens (*E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Shigella flexneri,* Methicillin resistant *Staphylococcus aureus* and *Staphylococcus aureus*). *Staphylococcus aureus* and MRSA both were very sensitive to RL-308. Antibacterial activity of RL308 was further assessed by zone inhibition assay.

Table.1 Antimicrobial activity of piperazine compounds

Sl. No.	Microorganisms	Inhibition zone (in mm)				
		RL308	RL317	RL318	RL327	RL328
01	*S.aureus*	1.8	1.7	1.8	1.8	1.0
02	MRSA	1.7	1.7	1.8	1.7	1.0
03	*Shigella flexineri*	0.7	0.7	0.8	1.0	0.8
04	*Pseudomonas aeruginosa*	0.8	0.8	0.8	0.7	0.8
05	*K.pneumoniae*	0.6	0.8	0.8	0.9	0.8
06	*E.coli*	0.8	0.8	0.8	0.9	0.8

Figure.1 Antimicrobial activity of piperazine compounds against *E. coli, K. pneumoniae, P. aeruginosa, S. flexineri*, MRSA & *S. aureus.*

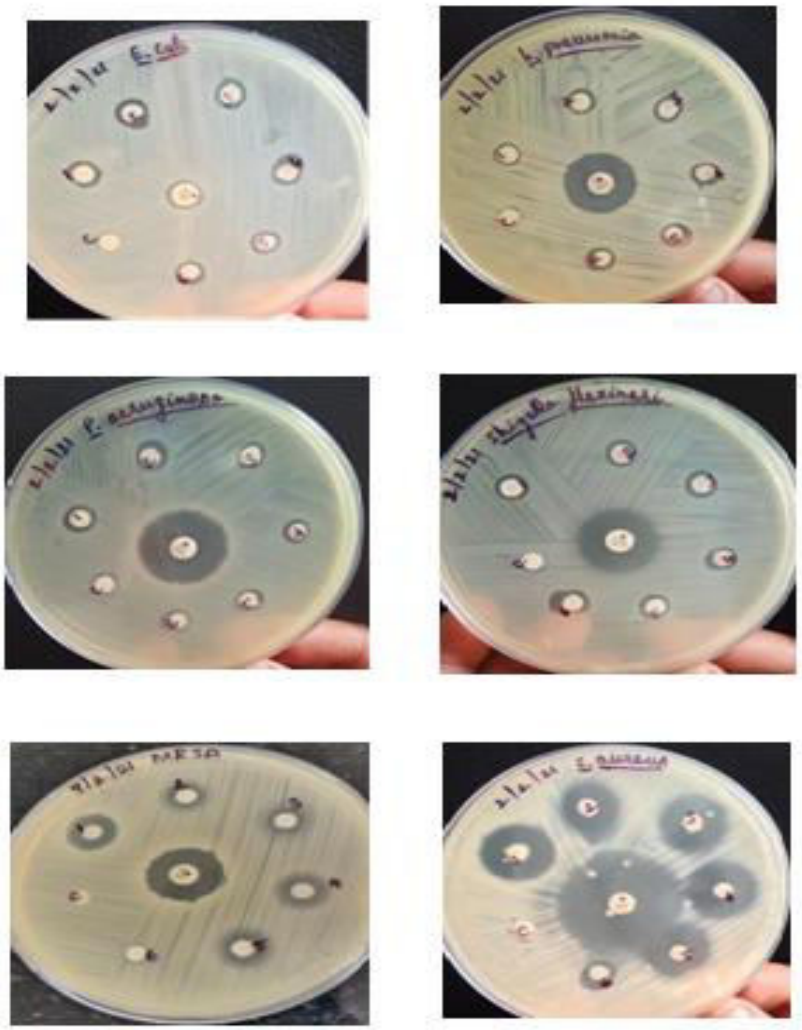


Figure.2 Methicillin Resistant *Staphylococcus aureus*

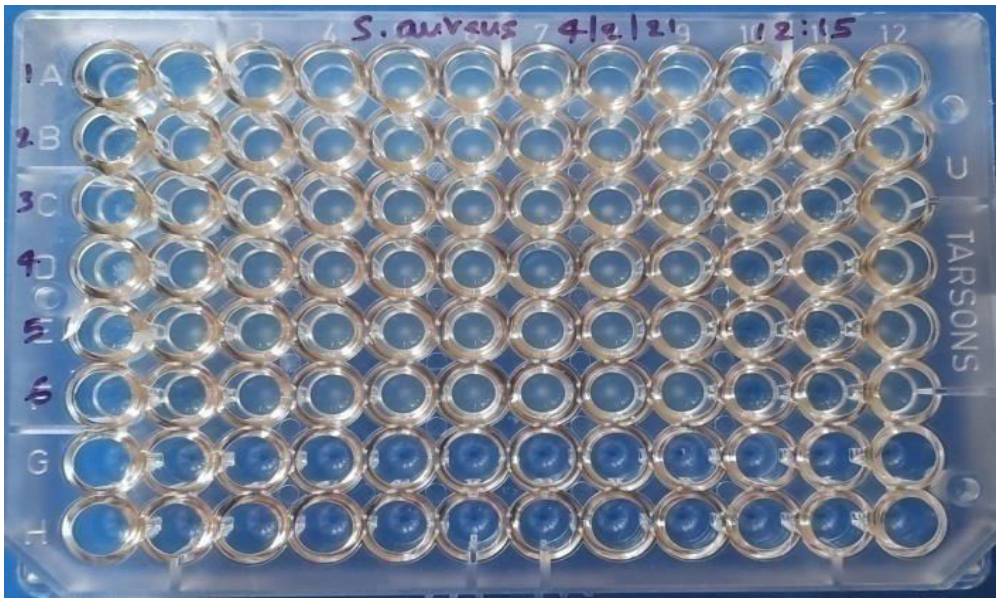


Figure.3 Methicillin Resistant *Staphylococcus aureus*

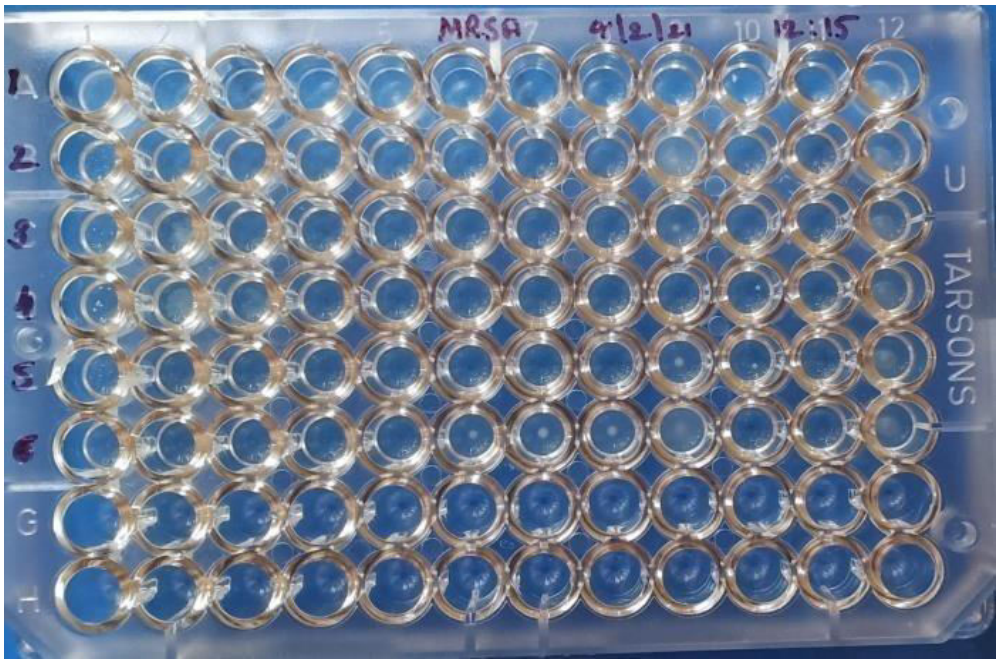


Figure.4 Methicillin Resistant *Staphylococcus aureus*

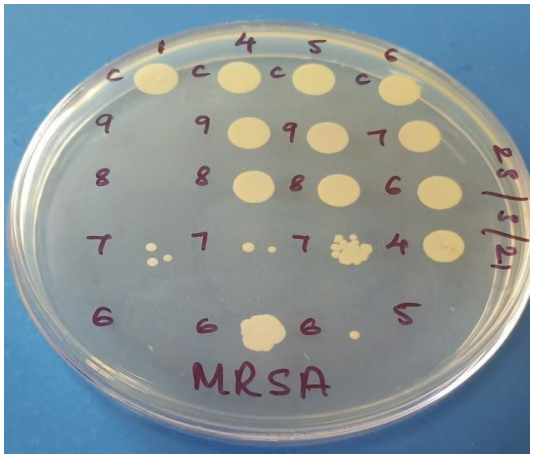
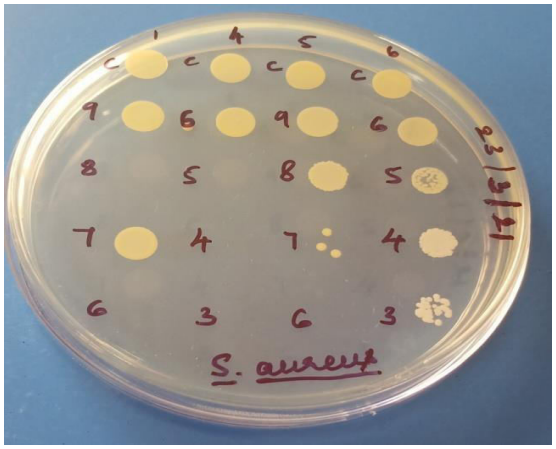


Figure.5 *Staphylococcus aureus*



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Author Contribution

S. S. Thammanna Gowda: Investigation, formal analysis, writing—original draft. A. N. Priyadharshini: Validation, methodology, writing—reviewing. Lokehs Ravilla:—Formal analysis, writing—review and editing. Parimala Hanumesh: Investigation, writing—reviewing. Shobith Rangappa: Resources, investigation writing—reviewing. T. A. Dhanalakshmi: Validation, formal analysis, writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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