



SYNTHESIS AND EVALUATION OF ANTIMICROBIAL ACTIVITY OF SOME GUANIDINE DERIVATIVES

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ABSTRACT

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A series of substituted piperazine guanidine derivatives were synthesized and evaluated for in vitro antimicrobial activity. The cup plate agar diffusion method was used to evaluate the antibacterial activity of synthesized compounds. This classic method yields a quantitative result for the amount of antibacterial agent needed to inhibit the growth of specific bacterial strains. The minimum inhibitory concentration (MIC) determination was carried out to find out the minimum concentration of antimicrobial compound found to inhibit the growth of particular test microorganisms.

Guanidine derivatives, MIC

INTRODUCTION:

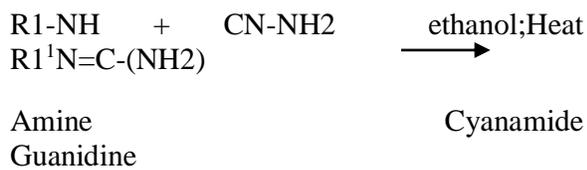
The guanidine and guanidine derivatives constitutes a very important class of therapeutic agent suitable for the treatment of wide spectrum of diseases. It can be found as a side chain of arginine, it is also present in many natural product. Chiral Guanidines have been widely used as a Bronsted base catalyst and phase transfer catalyst in enantioselective reactions [1]. Guanidine is a nitrogenous analogue of carbonic acid, replaced by a C=NH group and each OH is replaced by NH₂ group [2]. The central bond within the group is that of an imine which is structurally related to

amidines and ureas. It is colourless solid and dissolves in polar solvents. It is found in urine as a normal product of protein metabolism [3]. Guanidine is a functional group on the side chain of arginine with pKa 13.6 [4] meaning the guanidine is a very strong base in water [5]. Guanidines are categorized as organic superbases with pKa value of conjugated acids being ~ 13. The highly basic nature and remarkable stability is conferred to the guanidinium skeleton on protonation by the so called Y aromaticity that is resonance through three canonical forms [1].

Compounds containing guanidine as a functional group have attracted considerable synthetic interest [6] due to both the hydrogen bond mediated interactions of guanidinium ions and a wide variety of biological activities those substances display. Synthetic guanidines found uses in the engineering of advanced molecular recognition devices, sensors, organic material and phase transfer catalysts [7]. However, that guanidinium ion is one of the most hydrophilic functional group known [8]. Guanidine does not seem to be an ideal scaffolding template on which may be appended side chain specifically oriented to occupy the same relative regions of space as key side chains of the known ligands [9]. However, polyvalence of guanidine compounds and the variations of their chemical, biochemical and pharmacological activities make them of great importance to the design and development of novel therapeutic agents [10], [11]. The guanidinium cation can engage in a spatial interactions between ligand/receptor or enzyme/substrate. Therefore guanidine group is of great interest in medicinal chemistry and has become a key motif in many clinical drugs [12]

The compounds containing guanidine backbone possesses diverse chemical, biochemical and pharmacological properties which makes them important in the design and developments of novel drugs. A series of synthetic analogues and its computational models by a quantitative structure activity relationship (QSAR) program revealed that aminopropylguanidine as well as to piperazine-1-carboxamide (PZC) as potent agmatinase inhibitors [13].

General scheme of synthesis of guanidine:



Guanidine is synthesized by reacting, amines and cyanamide using suitable reagents at optimum conditions⁴. Guanidine compounds can be synthesized using alkyl amino chain or aryl (piperazine) molecule [14]. The side chains can also serve as modulators of lipid affinity and therefore, cellular bioavailability; so when the size or number of side chains is increased, this enhances the membrane permeability [15]. Guanidine derivatives have antibacterial, antifungal, antitumor activities. Guanidine have effect on CNS, anti-inflammatory agents, inhibitors of Na⁽⁺⁾/H⁽⁺⁾exchanger, inhibitors of NO synthase, anti-thrombotic, anti-diabetic and chemotherapeutic agents as well as guanidinium-based transporters and vectors [16].

Guanidine derivatives have wide variety of classes such as, guanidines acting at the CNS, anti-cancer guanidines, Anti- viral guanidines, anti-bacterial guanidines, fungicidal guanidine, etc [17].

Material and Method

Scheme: Synthesis of guanidine using piperazine molecule

2.1 Synthesis of 1-(diphenylmethyl)guanylpiperazine

In 500 ml of round bottomed flask, 5.0 g of 1-(diphenylmethyl)piperazine was taken along with 0.9 g cyanamide and 100 ml ethanol. Mixedwell and refluxed for 6 hrs. the condenser was removed and mixture was evaporated to 50 ml. the product was filtered, washed with diethyl ether and dried [17], [18].

2.2 Synthesis of guanylpiperazine

Step 1: The piperazine hydrochloride was prepared by treating 2.0 g of piperazine with an excess (about 3 ml) of hydrochloric acid in a small beaker, cool, filtered, washed with a small volume of ether and dried between filter paper [18].

Step 2: In a 500 ml of round bottomed flask, 5.0 g of piperazine hydrochloride was taken along with 3.4 g cyanamide and 100ml ethanol. Mixed well and refluxed for 6 hrs. The condenser was removed and the mixture was evaporated to 50ml. The product was filtered, washed with diethyl ether and dried [17], [18].

2.3 Synthesis of 1,4-diguanylpiperazine

In a 500ml of round bottomed flask, 5.0 g of piperazinedihydrochloride monohydrate was taken along with 1.3 g of cyanamide and 100ml ethanol. Mixed well and refluxed it for 6 hrs. the condenser was removed and the mixture was evaporated to 50 ml. the product was filtered, washed with diethyl ether and dried [17], [18].

3 Physicochemical tests

3.1 Solubility study

The solubility of synthesized compounds were determined using different solvents such as water, ethanol, 5% sodium hydroxide, 5% hydrochloric acid, conc. sulphuric acid, methanol, chloroform, acetone etc [19].

3.2 Test for Aliphaticity and Aromaticity

The Aliphaticity or Aromaticity of the synthesized compounds were determined using flame (Bunsen burner). Small amount of sample was taken in spatula and hold on flame to note the colour of smoke.

3.3 Element Detection: The synthesized compounds were checked for the presence of elements such as nitrogen, sulphur and halogens. The sodium fusion test was performed for the following.

Nitrogen: Pour 2-3 mL of the filtered fusion solution into a test tube, containing 0.1-0.2g ferrous sulphate crystals. Heat the mixture gently with shaking, and add drops of dil. Sulphuric acid. Prussian blue colour indicates presence of nitrogen.

Sulphur: Acidify 2mL of fusion solution with dilute acetic acid, and add a few drops of lead acetate solution. Black precipitate indicates presence of sulphur.

Halogen: Acidify portion of fusion solution with dilute nitric acid and add an excess of silver nitrate solution. A precipitate indicates the presence of halogen. The test was performed and results were recorded in [20].

3.4 pH determination

Dissolve 0.1g of the synthesized compounds in 10mL double distill water to make 1% solution. The resulting solutions were used for determination of pH [21].

3.5 Identification (Sakaguchi) Test

5mL of test solution was taken in test tube which is placed in ice bath. 1mL of NaOH solution (10%) and 1mL of α -naphthol solution (0.02%) was added and mixed well. Cool for 2-3min in ice bath, add 0.1-0.2mL of sodium hypobromite solution, shake the tube for 4-6sec, 1mL of urea solution (40%) was added and thoroughly mixed. The red coloration indicates the presence of guanidine moiety [7].

3.6 Ultraviolet absorption spectral studies (UV)

The solutions of the synthesized compound (10 μ g/mL) was prepared in methanol and scanned in the region from 210 to 400nm using Jasco v-63 Spectrophotometer [22].

3.7 Thin layer chromatography (TLC)

The purity and homogeneity of the synthesized compounds were ascertained by Thin Layer Chromatography (TLC) using glass plates coated with silica gel G or precoated silica plates and chloroform : methanol : ammonia : acetic acid (3:5:2:0.5) as solvent system. The solution of synthesized compounds were prepared in methanol and spotted on TLC plates by using glass capillaries. The spot were visualized in UV chamber at 254 nm and 366nm. All the

synthesized compounds gave a single spot without tailing, indicating the purity and homogeneity of the compounds. The Rf values of the compounds were calculated [23].

3.8 Infrared spectral studies (IR)

The IR spectra's of the synthesized compounds were recorded using KBr pellets on FTIR 8400s Spectrophotometer. The IR spectra and absorption bands occurring in various regions of compounds are given [24].

4. MICROBIOLOGICAL STUDY

4.1 Cup-plate agar diffusion method:

Preparation of solutions of compounds: Before testing of synthesized compounds for antibacterial activity, they were completely dried at room temperature. Each compound was then dissolved in sterile Distill water individually to obtained 1000 µg/mL concentration [25].

Preparation of inoculums: The bacterial cultures were grown on nutrient agar slants and nutrient broth for 24h at 37°C in incubator. They were stored at 4°C and sub culturing was done after one week. For evaluation of antibacterial activity, 24h fresh culture of bacteria was suspended in nutrient broth. The culture of bacteria was obtained from Hislopcollege, Nagpur, India.

Nutrient Agar: The media for antibacterial activities were prepared by dissolving 28.0g of ingredients in 1 L of distilled water and sterilized in an autoclave at 121°C at 15 lbs/inch pressure for 20min.

Potato Dextrose Agar: The media for antibacterial activities were prepared by dissolving 39.0g of ingredients in 1 L of distilled water and sterilized in an autoclave at 121°C at 15 lbs/inch pressure for 20min.

4.2 Antibacterial Activity: The synthesized compounds tested against bacterial strains; gramPositive *S.aureus* and gram-negative *E.coli*. Broth culture (0.75 mL) was added to

75 mL of nutrient agar medium at 45°C, mixed well, and then poured into a sterile Petri plate. The media was allowed to solidify and 8 mm wells were dug with a sterile metallic borer. Then a DMSO and Water solution of test sample (60 µL) at 1 mg/mL was added to the respective wells. DMSO and Water served as negative control, and the standard antibacterial drug (1 mg/mL) were used as positive control. Triplicate plates of each bacterial strain were prepared which was incubated aerobically at 37°C for 24 h. The activity was determined by measuring the diameter of zone showing complete inhibition (mm). The growth inhibition was calculated with reference to the positive control [19].

4.3 Antifungal Activity: The synthesized compounds tested against fungal strain *Candida albicans*. Broth culture (0.75 mL) was added to 75 mL of Potato dextrose agar medium at 45°C, mixed well, and then poured sterile petri plate. The media was allowed to solidify and 8 mm wells were dug with a sterile metallic borer. Then a DMSO and Water solution of test sample (60 µL) at 1 mg/mL was added to the respective wells. DMSO served as negative control, and the standard antibacterial drug (1 mg/mL) were used as positive control. Triplicate plates of each bacterial strain were prepared which was incubated aerobically at 27°C for 2-3 days. The activity was determined by measuring the diameter of zone showing complete inhibition (mm). The growth inhibition was calculated with reference to the positive control [19].

4.3 Minimum inhibitor concentration (MIC)

Minimum inhibitory concentration (MIC) is the minimum concentration of antimicrobial compound found to inhibit the growth of a particular test microorganism. It is applied to disinfectant, antiseptic, preservative and antibiotics. MIC values are usually expressed in terms of µg/mL or units/mL

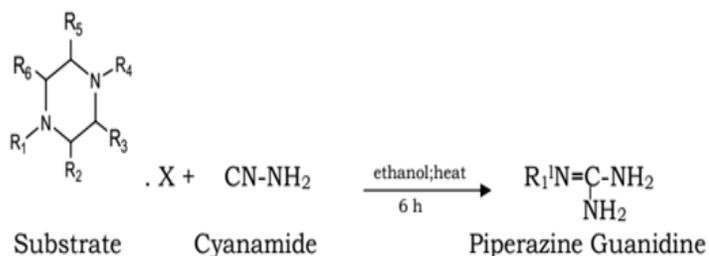


Fig.1: Scheme of synthesis showing mechanism

SR.NO	R1	R2	R3	R4	R5	R6	X
1.	H	H	H	H	H	H	HCL
2.	H	H	H	H	H	H	2HCL.2H ₂ O
3.		H	H	H	H	H	-

Table no. 1: Groups substituted in the particular position in synthesis

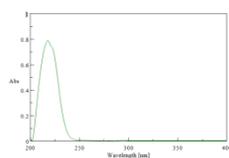


Fig.1

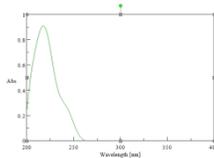


Fig.2

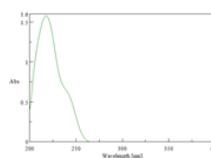
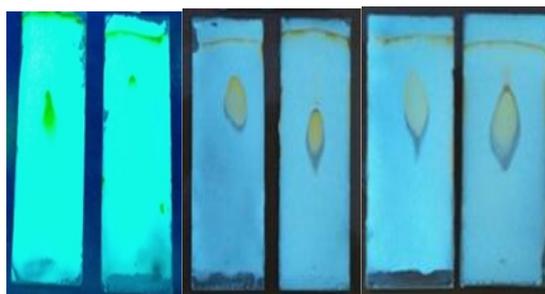


Fig.3

UV Spectra of Compound No. a) 2, b) 3 and c) 4

5.8 Thin Layer Chromatography Study

The R_f value of compound d-1, 2 & 3 was found to be 0.709, 0.815 & 0.830 respectively. The spots were visualized in UV chamber at 254nm, 366nm and in visible light. All compounds shows single spot without tailing, indicating purity and homogeneity of compounds.



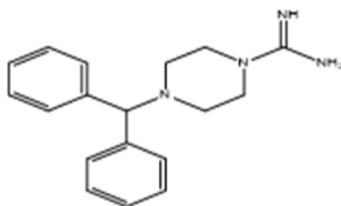
Compound1

Compound 2

Compound 3

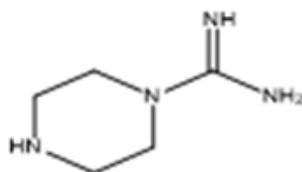
5.9 Infra Red (IR) Spectral Analysis

Compound 1



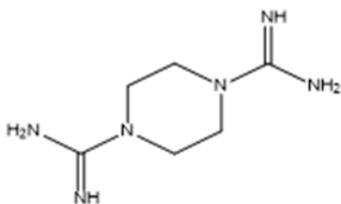
1-(Diphenylmethylguanyl)piperazine

Compound 2



Guanylpiperazine

Compound 3



1, 4-Diguanylpiperazine

I.R. Spectrum of compounds

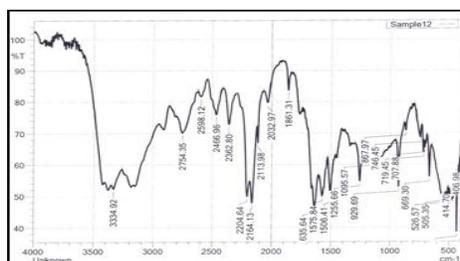


Fig. 5: I.R. Spectrum of compound-1

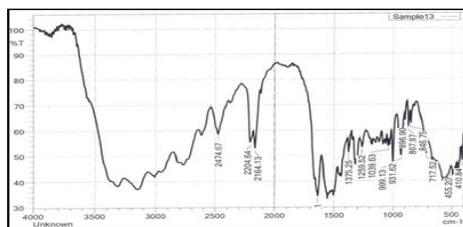


Fig.6: I.R. Spectrum of compound-2

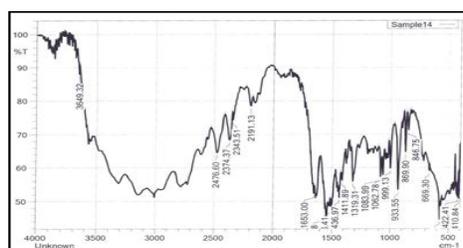


Fig.7: I.R. Spectrum of compound-3

I.R. Absorption peak of compounds

IR ABSORPTION PEAKS(cm^{-1})	FUNCTIONAL GROUP
3450.00	N-H stretching
1575.84	N-H bending
1255.66	C-N stretching
1861.00	C=N stretching
3390.00	Secondary amine stretching
3334.92	Tertiary amine stretching
2164.13	Piperazine stretching
1635.64	Guanidine stretching

Table no. 2: Absorption peaks of compound 1

IR ABSORPTION PEAKS(cm^{-1})	FUNCTIONAL GROUP
3390.00	N-H stretching
1570.00	N-H bending
1259.52	C-N stretching
1500.00	C=N stretching
3300.00	Secondary amine stretching
3150.00	Tertiary amine stretching
2164.13	Piperazine stretching
1633.71	Guanidine stretching

Table no. 3: Absorption peaks of compound 2
 IR ABSORPTION FUNCTIONAL GROUP
 PEAKS(cm^{-1})

3600.00	N-H stretching
1544.98	N-H bending
1319.31	C-N stretching
1506.41	C=N stretching
3270.00	Secondary amine stretching
3010.00	Tertiary amine stretching
2191.13	Piperazine stretching
1653.00	Guanidine stretching

Table no. 4: Absorption peaks of compound 3

5.10 Anti-microbial activity

a. Anti-bacterial activity of synthesized compounds by cup-plate method using DMSO as vehicle

Compound No.	Mean Zone of inhibition (mm)	
	E. coli (gram negative)	S. aureus (gram positive)
Compound-1	24.0 ± 6.1*	24.2 ± 2.4*
Compound-2	24.2 ± 1.0*	25.0 ± 4.3*
Compound-3	24.0 ± 3.0*	23.8 ± 4.5*
Streptomycin (S)	26.7 ± 5.1*	27.5 ± 2.0*
Vehicle Control (D)	No activity	No activity

Table no.5: Zone of inhibition of compounds by cup-plate method using DMSO as a vehicle

*Standard deviation of zone of inhibition

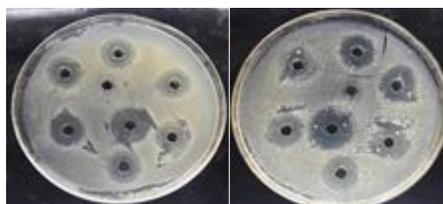


Fig. 5: Zone of inhibition using S. aureus Fig. 6: Zone of inhibition using E. coli

b. Anti-bacterial activity of synthesized compounds by cup-plate method using Water as vehicle:

Compound No.	Mean Zone of inhibition (mm)	
	E. coli (gram negative)	S. aureus (gram positive)
Compound-1	26.2 ± 1.3	28.5 ± 0.5
Compound-2	27.1 ± 1.0	28.5 ± 0.4
Compound-3	25.5 ± 0.5	26.4 ± 0.5
Streptomycin (S)	29.5 ± 0.5	29.9 ± 1.0
Vehicle Control (W)	No activity	No activity

Table no.6: Zone of inhibition of compounds by cup-plate method using Water as a vehicle.



Fig. 7: Zone inhibition using S. aureus



Fig. 8: Zone of inhibition using E.coli

All the compounds showed effective zone of inhibition for anti-bacterial activity using E.coli and S.aureus when compared with streptomycin as standard. Activity against S.aureus is more as compared to E.coli.

c. Anti-fungal activity of synthesized compounds by cup-plate method using Candida albicans in DMSO

Compound No.	Mean Zone of inhibition (mm)
Compound-1	2.37
Compound-2	1.80
Compound-3	2.05
Ofloxacin (S)	2.50
Vehicle Control	No activity

Table no.6: Zone of inhibition for Anti-fungal activity of synthesized compounds using Candida albicans in DMSO.

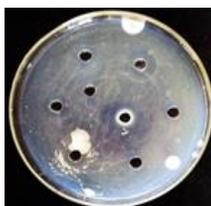


Fig.9: Zone of inhibition by cup plate method using C.albicans in DMSO

Procedure for MIC: 1

Broth dilution method: Prepare nutrient broth (double strength) test tubes and label them. In first test tube (UT), inoculum is not added which is used for checking the sterility of medium and as a negative control. Other all test tubes, inoculums (three to four drops) is added to reach the final concentration of microorganisms is 10⁶ cells/mL. In all test tubes, test antimicrobial compound is added ranging from 0.5 to 5 mL except uninoculated (negative control) and control (positive) tube. The positive control tube is used to check the

suitability of the medium for growth of the test microorganism and the viability of the inoculum. Adjust the final volume (10 mL) in all test tubes by using sterile water. All test tubes are properly shaken and then incubated at 37°C for two days [19].

Solid dilution method: In this method, first test chemical is mixed into molten agar and then poured into Petri plates. After solidification, inoculum is spread on the surface of agar medium. All plates are incubated at 37°C for two to three days. After incubation, all test tubes or petri plates are

examined for the growth in the form of turbidity and colonies, respectively. Record the result and calculate the minimum inhibitory concentration by comparing all results with positive and negative control [19].

5 Result and discussion

5.1 Physical characteristics: Compound 1 was white amorphous having melting point 90-92, compound 2 & 3 were white crystalline having melting point 171-173 & 251-254 respectively. The melting point of all compound were sharp, uncorrected and different from their starting chemical.

5.2 Solubility study: The compounds were soluble in polar solvent. As the polarity changes, the solubility also changed. The solubility was found to be less in non-polar solvent.

5.3 Test for Aliphaticity or Aromaticity: Except compound-1, which give sooty flame the remaining compounds shows non sooty flame corresponds to the proposed structure.

5.4 Element Detection: The compound 1, 2 and 3 showed positive test for nitrogen by indicating Prussian blue coloration and negative test for Sulphur and halogen atoms, indicates synthesized compounds contains nitrogen.

5.5 pH determination: The pH of synthesized compounds was found in the range of 7-11, suggested the synthesized compound found to be basic in nature.

5.6 Identification (Sakaguchi) test: The compound-2 showed prominent red colour whereas compound-3 is less prominent i.e. Pinkish red colour, which illustrated positive sakaguchi test and concluded the presence of guanidine moiety. Compound-1 showed orange colour, might present guanidine.

5.7 U.V. Spectrophotometric Analysis: λ max of synthesized compounds was found to be in between 217.0 and 218.0

The guanidine moiety of metformin shows the peak maxima at 232 nm. The synthesized compounds exhibited peak maxima between 217.0 to 218.0 nm i.e. near to reported value, which might be differ due to the substituents present on the guanidine moiety.

5.8 Thin Layer Chromatography Study:

The R_f value of compound d-1, 2 & 3 was found to be 0.709, 0.815 & 0.830 respectively. The spots were visualized in UV chamber at 254nm, 366nm and in visible light. All compounds shows single spot without tailing, indicating purity and homogeneity of compounds.

All the compounds showed effective zone of inhibition for anti-bacterial activity using E.coli and S.aureus when compared with streptomycin as standard. Activity against S.aureus is more as compared to E.coli.

All the compounds were tested for antifungal activity using candida albicans against oxconazole. All compound exhibited zone of inhibition for antifungal activity when compared with oxconazole as standard. Compound-2 shows more activity as compare to others.

5.11 Minimum inhibitory concentration (MIC) Study

MIC was appropriately calculated by broth dilution method. The solid dilution method was performed but, not given the proper interpretation of results. MIC against E.coli was found to be, 70 μ g/ml for compound-1, 70 μ g/ml for compound- 2 and 70 μ g/ml for compound-3. MIC against S.aureus was found to be, 60 μ g/ml for compound 1, 70 μ g/ml for compound-2 and 70 μ g/ml for compound-3.

6. CONCLUSION:

In present investigation, attempts have been made to synthesize some substituted piperazine guanidine derivative for possible unreported antibacterial and antifungal activity. Targeted compounds were

synthesized by using routine method of guanidine synthesis i.e. using amine and cyanamide. In all, three different compounds were synthesized by using different amines. All synthesized compound were crystallized and subjected to physicochemical studies like melting point, element detection, functional group test, solubility etc. beside this, TLC was performed to ascertain the purity and homogeneity of compounds. Spectral studies like UV and IR were also performed to ascertain the proposed structures. Typical UV band with λ_{max} around 216-218nm was present in all compounds, where as typical IR peaks at 3600-3000 (N-H stretch), 2050-1500 (C=N stretch), 1650-1500 (N-H bending), 1350-1150 (C-N stretch) along with specific peaks for a proposed structures were noted in IR spectra. Difference in melting point as compared to starting compound, UV studies and typical IR peaks give some sort of conformation to the proposed structures. All the synthesized compounds were screened for antibacterial and antifungal activity. For screening purpose, one gram positive (*Staphylococcus aureus*), one gram negative (*Escherichia coli*) bacterial strain and fungal strain (*Candida albicans*) were utilized. Compounds were tested for possible antibacterial and antifungal activity by cup plate method and minimum inhibitory concentration was determined by solid dilution and broth dilution method. Almost all synthesized compounds shows antibacterial activity when compared with standard streptomycin against selected gram positive and gram negative bacteria. Activity was found to be more against gram positive bacteria than gram negative bacteria. Compound-2 (guanylpiperazine) shows promising result in comparison with streptomycin. Similarly, Synthesized compounds, specifically compound-1[1Diphenylmethyl]guanylpiperazine] possesses antifungal activity when compared with Oxconazole. So, it can be clearly evident that synthesis of some more guanidine derivatives and their screening for antibacterial and antifungal activity will

provide more inside into their structural activity relationship. Studies on their possible mechanism of action and correlation with structure will open a new arena for most needed novel antibacterial drugs.

7. Conflict of interest: The authors declare no conflict of interest.

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