

In-vitro antifungal study of Cu (II) complexes with a heterocyclic N donor ligand

Madhu Kumari Gupta* & Mrs. Sudipti**

*Assistant Professor, Department of Chemistry, Magadh Mahila College, Patna University, Patna,

**Research Scholar, Department of Chemistry, Patna University, Patna

Email : madhugupta8415@gmail.com; drmadhuchemmmcpu@gmail.com

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Abstract : Biguanide, $C_2H_7N_5$ is a nitrogen donor bidentate ligand and its derivative, piperazinedibiguanide $C_8H_{18}N_{10}$ is a nitrogen donor tetradentate ligand. The tetradentate ligand, piperazinedibiguanide was prepared and complexed with Cu (II) by reaction of the ligand with Cu (II) salts. The characterization of the complexes – elemental analysis and magnetic property was reported in laboratory along with spectral characteristics. All prepared Cu (II) complexes were paramagnetic which confirm its square planar structure. Further their antifungal activity against two common ascomycetes was studied by using disc dilution test method in Potato Dextrose Agar and Sabourand Dextrose Agar media. During the tests, the concentration of the complexes of copper with piperazinedibiguanide ligand were $400\mu\text{g/ml}$, $200\mu\text{g/ml}$ and $100\mu\text{g/ml}$. The tests reported a positive response against the growth of *Aspergillus niger* and *Aspergillus versicolor* in both the media. All complexes have shown more than 80% inhibition in fungal growth at concentration $400\mu\text{g/ml}$. One of the complexes, Copper piperazinedibiguanide hydroxide reported reduction in the fungal growth of *Aspergillus versicolor* by 100% and 99.10% in Potato Dextrose Agar and Sabourand Dextrose Agar media respectively. Thus, *in vitro* Minimum Inhibitory Concentration results during the study suggest further study of antifungal activity of transition metal complexes of said ligand with other microbes as well.

(Keywords : Biguanide; *Aspergillus versicolor*; *Aspergillus niger*; ascomycetes; piperazine-dibiguanide)

Introduction

Biguanide and its derivatives have been

used from a long time as drugs in medical science.¹ For example, a biguanide compound, metformin is used as an oral hypoglycemic drug for type 2 diabetic patients. Similarly, proguanil used as antimalarial drugs, phenyl biguanide as 5-HT₃ receptor agonist, phenylaminopropyl biguanide as disinfectant, antimalarial drug etc.^{2,3,4} Biguanide is actually a condensation product of two guanidine molecules with elimination of one ammonia molecule. This can be shown as below in figure 1:

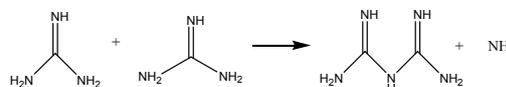


Fig 1. Condensation reaction of guanidine molecules to form biguanide with elimination of ammonia

Actually, biguanide is prepared by the fusion of guanidine hydrochloride at $180-185^{\circ}\text{C}$. Hence, biguanide is also called as guanylguanidine. Piperazinedibiguanidine is a tetradentate ligand of this class similar to ethylenedibiguanide ligand. The structure of both the compounds are shown in figure 2.

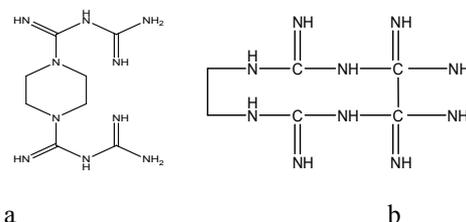


Fig 2 Structure of (a) piperazinedibiguanide and (b) ethylene dibiguanide

To explore the potential of metal-ligand complexes in antifungal applications, it is essential to consider earlier studies, that highlight the biological relevance of similar compounds. Coordination complexes of some thiocarbamoyl dihydropyrazoles with copper and nickel metal ions shows a good antifungal activity against several *Candida* strains.²⁹ The analysis shown more activity of copper complexes in comparison to its nickel complexes. Piperazine is used as medicine under category antihelminthics against common round worms (ascariasis) and pinworms (enterobiasis). This compound was reacted with dicyandiamide to form its biguanide derivative. Biguanide and its derivatives complexed with transition metal have been studied for a long time but the study of mentioned ligand is still lacking both in physiochemical characteristics as well as biochemical properties²⁴. In this present work, the authors have dragged the attention towards magnetic characteristic and antifungal activity of the complexes of piperazinedibiguanide. In order to achieve the goal, firstly the ligand was prepared by reported method. The ligand so obtained was then complexed with Cu (II) ion by reaction with its salts. After complexation, elemental elucidation, UV-visible spectroscopy & magnetic study were done. After that antifungal activity of the prepared complexes was studied and discussed in this research report. The two fungi used for the study are *Aspergillus niger* and *Aspergillus versicolor*. Biguanide iridium (III) complexes exhibit high antifungal potency towards *C. albicans* and *C. neoformans* with minimum inhibitory concentrations (MICs) in nanomolar range as low as 0.25 µg/ml.¹³ Cr(III), Ni(II) and Cu(II) ions complexed with p-phenylenedibiguanide ligand shown more than 80% inhibition in fungal growth of *A. niger* and *A. versicolor* at 400 µg/ml when compared with growth on respective ligand in PDA as well as SDA media.^{15,23}

Cobalt (III) complexes with mixed ligand having one biguanide moiety as ligand shown more than 70% inhibition at 400 µg/ml against *A. niger* in PDA and SDA media. Beside this

inhibition of more than 80% against *A. versicolor* in PDA and SDA medium at same concentration.^{12,16,23} Bis (phenylbiguanidium) nickel (II) chloride & α Nickelphenylbiguanidine (II) containing biguanide as ligand also exhibit antifungal activity against *A. niger* and *A. versicolor* in PDA and SDA media. The inhibition was more than 80% at 400 µg/ml concentration of the complexes in both media.^{14,23}

Antifungal activity of copper piperazinedibiguanide chloride and nickel piperazinedibiguanide chloride complexes shown 100% growth of *A. niger* and *P. digitatum* at 1000 µg/ml in both PDA and SDA media.¹⁷ The review study shows that there is a great opportunity in the research study of biochemical activity of biguanide derivatives as ligand complexed with metal ions. The two fungi which were used by the authors in the study are discussed below.

Aspergillus niger is a common fungus of genus *Aspergillus*.⁵ It causes black mold disease on fruits and vegetables. Mostly found in soil and commonly present indoor environment. *Aspergillus versicolor* is widely distributed in nature, mostly on substrates exposed to humidity or slow-decaying.^{6,7} Mainly found in cold regions and soil used for cultivation but very rare to be found in forests. *A. versicolor* is a toxic and pathogenic fungus for both man and animals. The aflatoxins it synthesizes are responsible for several cancers.

The main objective of the study is to find out whether the complexes have any antifungal activity. If the activity is satisfactory then it could be used as antifungal agent in different products of day-to-day life. These can also be used as alternate in future against the microbes which are becoming resistant to common antifungal chemicals.

Experimental Materials and methods

The ligand and its complexes were prepared by the reported method as described below:^{8,9,10,11} Piperazinedibiguanide sulphate $C_8H_{18}N_{10} \cdot 2H_2SO_4 \cdot 1.5H_2O$ For the preparation of above-mentioned compound, 4.3g of piperazine was heated with 8.4g of dicyandiamide with 75ml of water in a conical flask on water bath for 3 hours. While heating 5ml of aqueous solution of $CuSO_4$ was added in small intervals of 20 minutes. The mixture so obtained was then treated with aqueous solution of 2.8g sodium hydroxide. As a result, the mixture turns reddish (purple). The content was cooled and filtered so that the purple residue gets separated from the filtrate. The purple residue was then decomposed with dil. sulphuric acid (1:3). After cooling, white residue of piperazinedibiguanide sulphate starts depositing in the vessel. It was filtered and the white residue was washed with cold water until free from any copper ion. The white residue was dissolved further with ammonia containing little amount of sodium hydroxide. The basic solution was acidified with dil. sulphuric acid (1:3). On cooling, white shining crystals of piperazinedibiguanide sulphate starts getting deposited in the beaker. Shining crystals were filtered, washed with cold water and dried in air.

Copper piperazinedibiguanide hydroxide
[Cu Pip(BigH)₂](OH)₂·3H₂O

It was prepared by adding ammoniacal solution of 4.7g of ligand piperazinedibiguanide sulphate to ammoniacal solution of 1.6g of cupric sulphate with constant stirring. As a result, reddish violet precipitate of above said complex was obtained immediately. It was filtered and washed with cold water till it gets free from sulphate ion. It was dried in desiccator till it acquire constant weight.

Copper piperazine dibiguanide chloride
[Cu Pip(BigH)₂]Cl₂·4H₂O

To prepare this complex compound, copper piperazinedibiguanide hydroxide base was heated with aqueous ammonium chloride on water bath. The heating was stopped when the

ammonia gas evolution ceases. As a result, reddish violet precipitate was obtained which was washed with cold water and dried in air. It was found sparingly soluble in hot water and insoluble in alcohol.

Copper piperazinedibiguanide sulfate
[Cu Pip(BigH)₂]SO₄·5.5H₂O

It was obtained by the same way as chloride by treating complex base with aqueous solution of ammonium sulphate. The complex was violet in colour and insoluble in both water and alcohol.

Copper piperazinedibiguanide nitrate
[Cu Pip(BigH)₂](NO₃)₂·H₂O

It was prepared by treating complex base with a solution of ammonium nitrate. The complex nitrate so obtained was red powder insoluble in water and alcohol.

Antifungal activity

Antifungal activity was done by inoculating the above-mentioned fungus to PDA (Potato Dextrose Agar) and SDA (Sabourand and Dextrose Agar) medium. The medium was autoclaved before use and the ratio used for complex solution and medium was 1:10.^{12,14,18,19,20,21,22,27,28} The tests were done in triplicates for each Petri dish of mentioned concentrations and media. Further inhibition of mycelium was calculated by disc dilution test method. The media PDA and SDA were prepared as follows:

Preparation of PDA (Potato Dextrose Agar)

Potato tubers were taken peeled off and weighed 200g. It was chopped into small pieces and transferred to a beaker containing about 100ml of distilled water and boiled for 20 minutes and filtered with muslin cloth. 20g Dextrose, 15g agar and 2g peptone were added into the extract and gently heated. The filtrate so obtained was made to 1 litre. The pH of the solution was maintained at 5.6 by using 1N HCl or NaOH and kept in Erlenmeyer flask. This solution so

obtained was PDA medium and autoclaved at 121°C for 20 minutes before using.^{25,26}

Preparation of SDA (Sabourand and Dextrose Agar Medium)

It was prepared by combining the ingredients water, dextrose, agar, peptone & antibiotics separately, in many different variations. In the case of using premix, the proper amount (around 70gms) was mixed with one litre of water and heated to dissolve the agar. pH of the medium was adjusted with one molar solution of hydrochloric acid to lower pH. The pH was maintained at 5.5. The medium was then autoclaved and stored at room temperature. The medium can be used to inoculate with fungal spores and mycelium inhibition growth was counted by usual method.^{25,26} After preparation of the media, pure culture of the fungal spores was prepared and isolated by Warcup method.²⁶ On successive inoculation in new petri dish with the medium, pure culture of a particular fungal spore had been obtained. After getting separated, the fungus was identified under microscope and testing was done in UV chamber of the Chemistry Dept. of Magadh Mahila College, Patna University.

Results and Discussion

Piperazinedibiguanide sulphate is a quadridentate chelating ligand and its complexes

with bivalent metal ions are known.⁸ The ligand has been found to be co-ordinate with N^a & N^b of both the biguanide substituents forming usual six membered rings with Cu (II) metal ion (figure 3). UV-visible spectral study shows formation of bond between metal ion and nitrogen of the ligand. The elemental analysis is shown in table 1. When magnetic property was checked it was found that all copper (II) complexes with piperzinedibiguanide ligand were paramagnetic (table 2). The UV-visible spectroscopy reported peaks at peaks at 370nm, 240nm and 460 nm. This result reported complexation of the metal ion with the ligand and suggested the structure of the complexes of copper to be planar. The complexes prepared in laboratory were dissolved in suitable solvent at different concentrations and was then treated with the fungus isolated in UV chamber. These solutions showed antifungal property against two ascomycetes, *A. niger* and *A. versicolor*. Both these fungi were cultured over PDA & SDA medium and showed 100% control of the fungal growth when the concentration of the complexes had been increased to 500-800 ¼g/ml.¹⁷ It was interesting to note that one of the complexes of piperazinedibiguanide with Cu (II) inhibited the growth to 100% at 400¼g/ml also. The micelle inhibition was then counted, the data of which are as under (table 3 and 4).

Elemental Analysis in %

Compound		N	S	Cu	Cl	Water
Piperazinedibiguanide	Found	29	13.97			7.6
C ₈ H ₁₈ N ₁₀ ·2H ₂ SO ₄ ·1.5H ₂ O	Calculated	29.35	13.41			7.82
Copper piperazinedibiguanide hydroxide	Found	34.96		15.4		21.95
[Cu Pip(BigH ₂) ₂](OH) ₂ ·3H ₂ O	Calculated	34.5		15.6		22.10
Copper piperazinedibiguanide chloride	Found			14.09	15.78	15.30
[Cu Pip(BigH ₂) ₂]Cl ₂ ·4H ₂ O	Calculated			13.7	15.04	15.60
Copper piperazinedibiguanide sulphate	Found		6.47	12.98		19.12
[Cu Pip(BigH ₂) ₂]SO ₄ ·5.5H ₂ O	Calculated		6.2	12.4		19.31
Copper piperazinedibiguanide nitrate	Found			13.2		4.01
[Cu Pip (BigH ₂) ₂](NO ₃) ₂ ·H ₂ O	Calculated			13.8		3.9

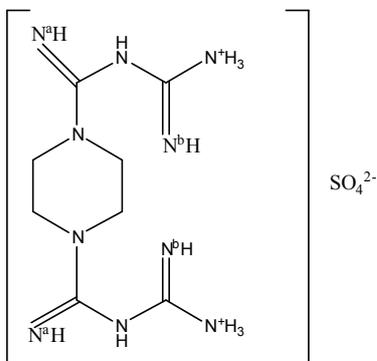


Figure 3. showing atoms of Piperazinedibiguanide sulphate coordinating to metal ions to form complexes

Table - 1
Elemental analysis of different element in ligand and metal complexes
Magnetic behaviour of complexes

Compound	Magnetic behaviour
Copper piperazinedibiguanide hydroxide	paramagnetic
Copper piperazine dibiguanide chloride	paramagnetic
Copper piperazinedibiguanide sulphate	paramagnetic
Copper piperazinedibiguanide nitrate	paramagnetic

Table 2. Magnetic behavior of complexes
Percentage inhibition of growth of fungus *Aspergillus niger* at indicated dose

Metal complex	Concentration	% Inhibition of fungal growth	
		PDA	SDA
Copper piperazinedibiguanide hydroxide	400 µg/ml	96.97%	94.30%
	200 µg/ml	78.49%	72.14%
	100 µg/ml	71.57%	69.91%
Copper piperazine dibiguanide chloride	400 µg/ml	95.80%	93.11%
	200 µg/ml	87.25%	79.59%
	100 µg/ml	78.52%	74.45%
Copper piperazinedibiguanide sulphate	400 µg/ml	97.15%	94.63%
	200 µg/ml	87.21%	84.08%
	100 µg/ml	79.70%	77.07%
Copper piperazinedibiguanide nitrate	400 µg/ml	93.01%	89.09%
	200 µg/ml	85.72%	81.37%
	100 µg/ml	75.36%	72.25%

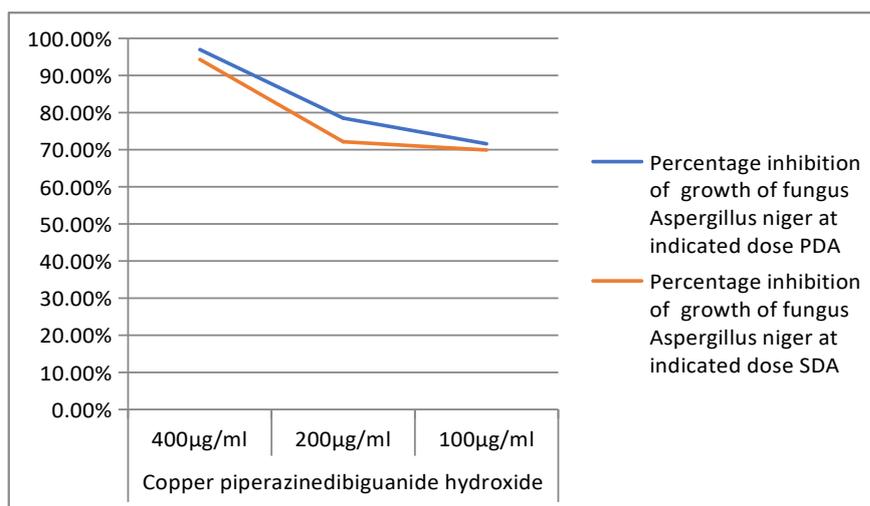
Table - 3
Percentage inhibition of mycelial growth of *A. niger* in PDA and SDA

Percentage inhibition of growth of fungus *Aspergillus versicolor* at indicated dose

Metal complex	Concentration	% Inhibition of fungal growth	
		PDA	SDA
Copper piperazinedibiguanide hydroxide	400 µg/ml	100%	99.10%
	200 µg/ml	88.89%	86.78%
	100 µg/ml	79.40%	77.40%
Copper piperazine dibiguanide chloride	400 µg/ml	98.50%	95.20%
	200 µg/ml	87.28%	79.27%
	100 µg/ml	69.51%	63.31%
Copper piperazinedibiguanide sulphate	400 µg/ml	95.00%	90.82%
	200 µg/ml	82.50%	79.75%
	100 µg/ml	69.57%	60.91%
Copper piperazinedibiguanide nitrate	400 ¼g/ml	92.31%	87.50%
	200 ¼g/ml	78.73%	70.58%
	100 ¼g/ml	62.72%	53.41%

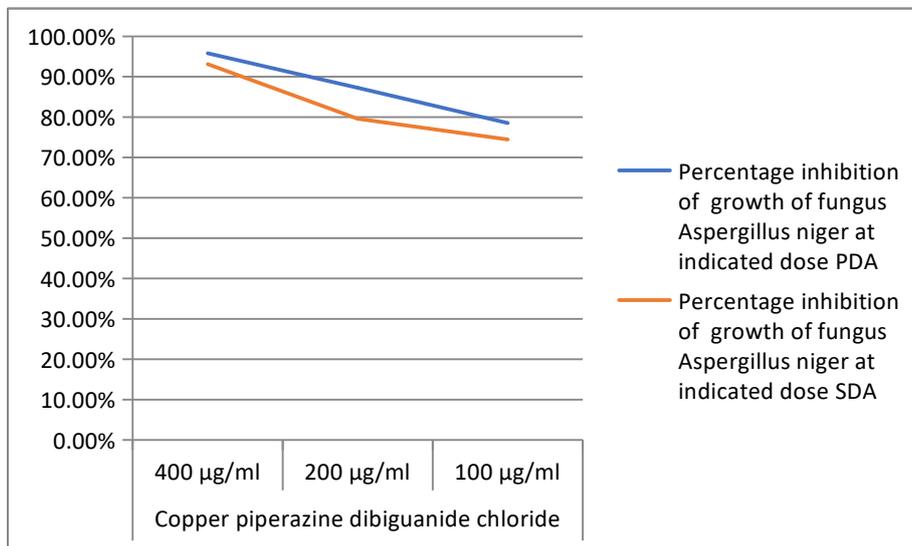
Statistical analysis : From table 3 following statistical graph have been obtained which give detailed view of comparative activity of

complexes against *A. niger* under different concentrations.



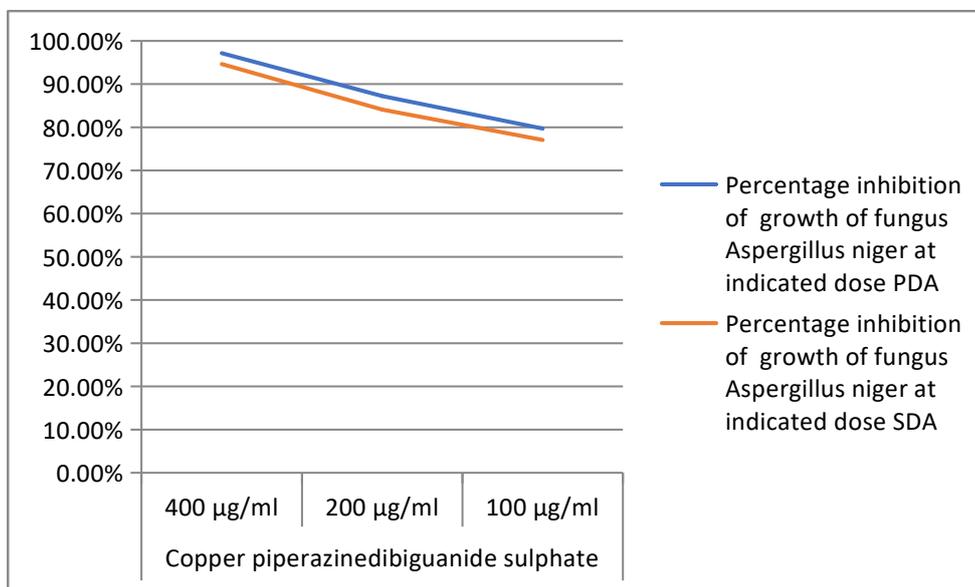
Graph 1 a

Comparative activity of complex Copper piperazinedibiguanide hydroxide against *A. niger*



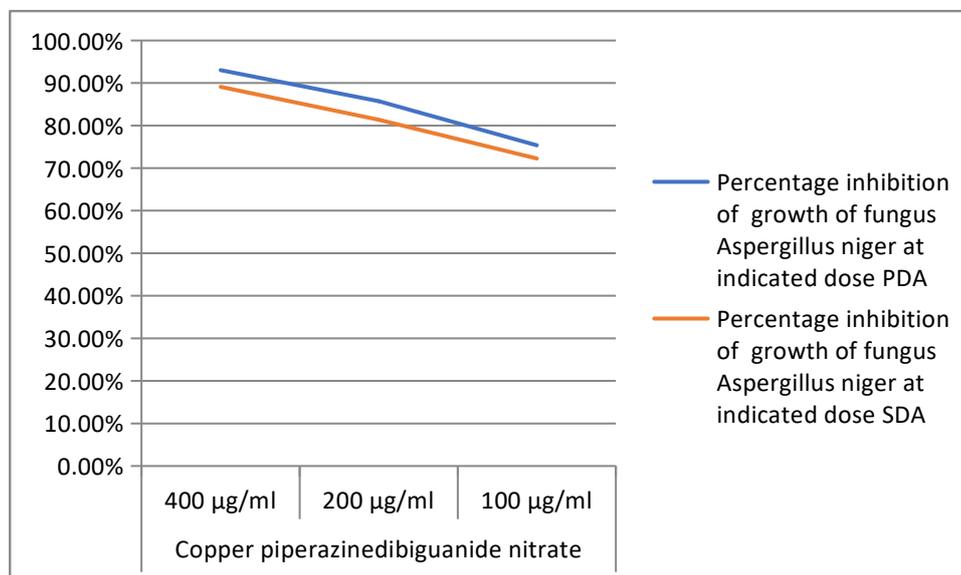
Graph 1 b

Comparative activity of complex Copper piperazinedibiguanide chloride against *A. niger*



Graph 1 c

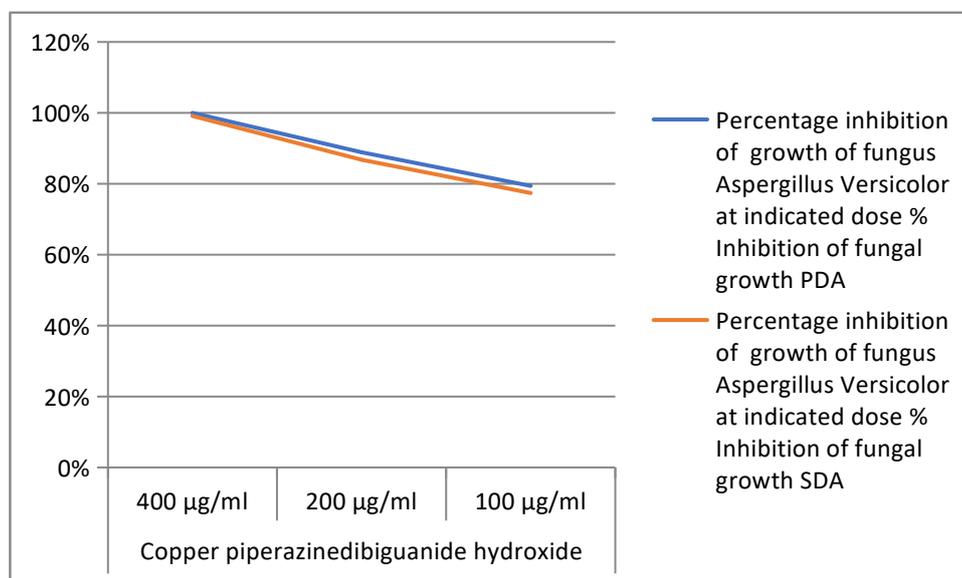
Comparative activity of complex Copper piperazinedibiguanide sulphate against *A. niger*



Graph 1 d

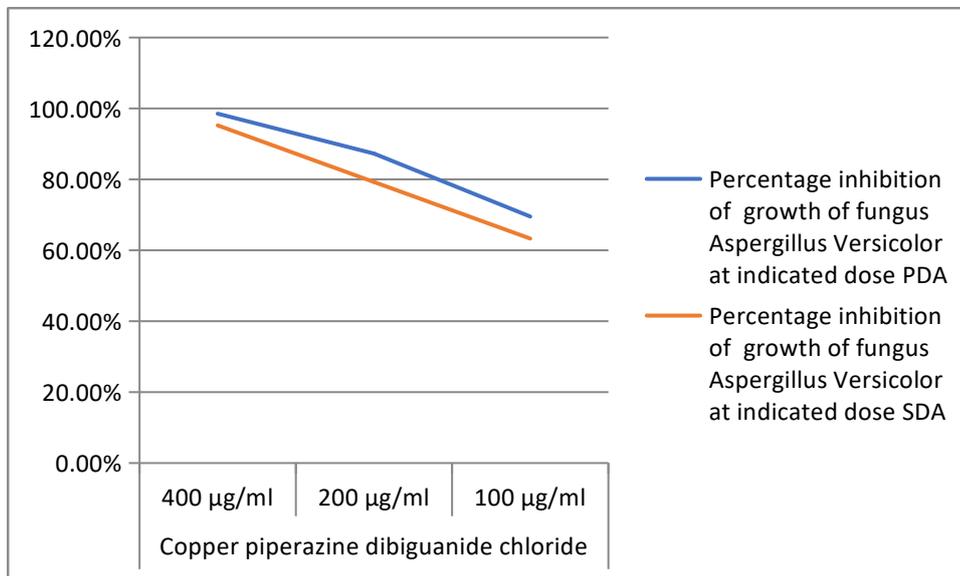
Comparative activity of complex Copper piperazinedibiguanide nitrate against *A. niger*

From table IV following comparison data have been obtained shown in graph against *A. versicolor* under different concentrations.



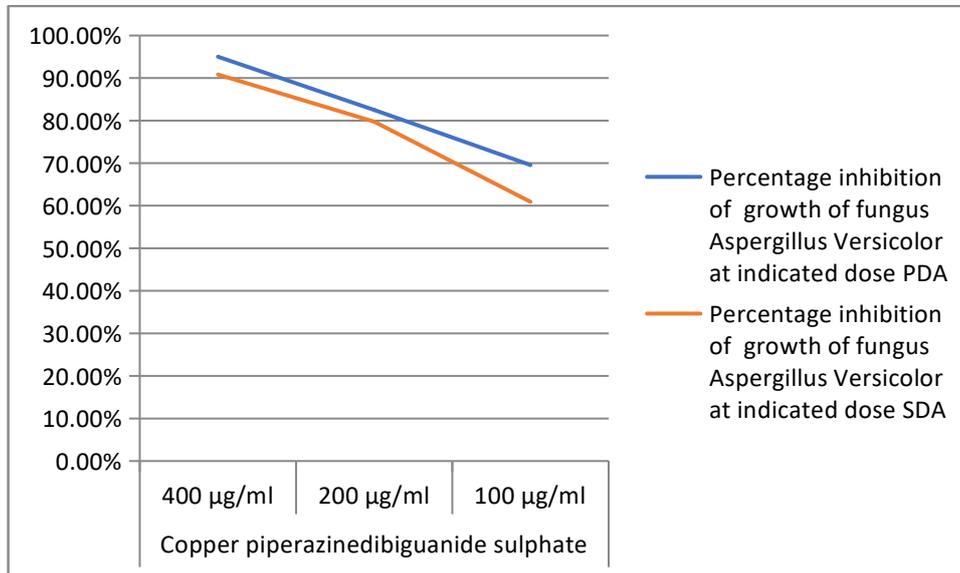
Graph 2 a

Comparative activity of complex Copper piperazine dibiguanide hydroxide against *A. versicolor*



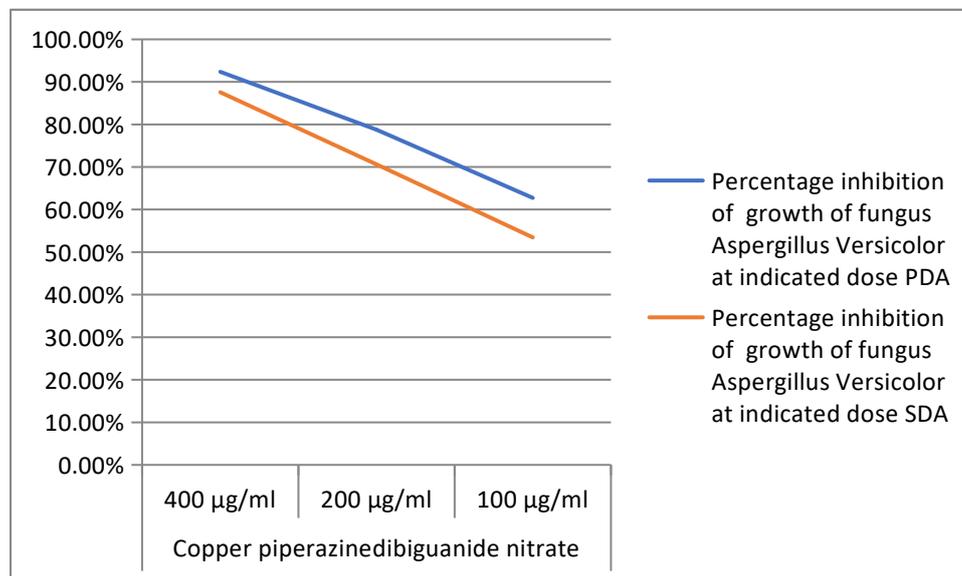
Graph 2 b

Comparative activity of complex Copper piperazine dibiguanide chloride against A. versicolor



Graph 2 C

Comparative activity of complex Copper piperazine dibiguanide sulphate against A. versicolor



Graph 2 d

Comparative activity of complex Copper piperazine dibiguanide nitrate against *A. versicolor*

Discussion and interpretation

Elemental analysis of ligands and complexes has been shown in table I. The data obtained matches with the theoretically calculated data. The UV-Visible spectral analysis shown peaks at 370nm, 240nm and 460 nm. This shows following transitions and confirms formation of metal ion bonding with nitrogen of the ligand.

370 nm → CT

240 nm → CT

460nm $^2B_{1g} \rightarrow ^2B_{2g}$

Table III describes that the compounds are paramagnetic in character. The data provided in table III and IV are further interpreted in graphs to show the biochemical activity statistically as reported in the eight graph plots. From the statistical graph from 1a to 1d represent that the complexes were more active against *A. niger* in PDA medium and controlled the growth more in it as compared to SDA medium. Similarly, when

we compare the growth inhibition of *A. versicolor* (graph 2 a to 2 d), it was found that it was more in PDA medium. In case of copper piperazinedibiguanide hydroxide the antifungal activity in both the media coincides, which mean the compound is equally efficient in both the media.

Conclusion

In the study a series of copper (II) complexes with tetradentate piperazinedibiguanide were synthesized which confirms the chelating properties of the ligand. The findings confirmed that the ligand effectively binds with Cu (II) ion, as verified by elemental analysis and UV-visible spectroscopic peaks by indicating ligand to metal charge transfer transitions along with d-d transitions. The paramagnetic character confirms the square planar structure of the complexes.

In the antifungal assays, these complexes exhibited significant activity against *Aspergillus niger* and *Aspergillus versicolor*. The data so obtained revealed that the higher concentrations particularly around 400 to 800 μ g/ml, resulted in near- total inhibition of fungal growth, with some complexes demonstrated complete suppression of growth in PDA medium. In the research study, the copper piperazinedibiguanide hydroxide complex showed the most effective antifungal performance across both media types PDA and SDA, suggesting a robust potential for practical application.

At last, the study concludes the application of copper complexes with the nitrogen donor tetradentate ligand piperazinedibiguanide as an effective alternative

for antifungal agent. As one knows, many fungi have become resistant to conventional drugs which trigger the researchers for further investigation of some alternatives. As a result, there is a need of more exploration and optimization of these complexes for the development of new, effective treatments in medical and environmental applications.

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