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Screening of antibacterial and cytotoxicity of the copper (II) complexes of N-donor chelating ligand

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Copper (II) complexes, namely dichloromono(1-amidino-*O*-ethylurea)copper (II), (1) and bis(1-amidino-*O*-ethylurea)copper (II) bromide, (2) were synthesized and characterized. The antibacterial screening of complexes 1 and 2 was performed along with their corresponding metal salts ($CuCl_2.2H_2O$) and ($CuBr_2$), and ligand (dicyandiamide), against two human urinary pathogenic bacterial strains, *Escherichia coli* and *Proteus mirabilis*, by the disc diffusion method. All test compounds, except ligand have shown significant antibacterial activities on both bacterial strains. Their antibacterial effectiveness increased in a dose dependent manner, whereas their ability to inhibit the growth of both bacterial species decreased as their incubation time increased. The activity of complex 1 was found to be greater than its metal salt, while that of complex 2 was lower than the corresponding metal salt on both bacterial species. The *in-vitro* cytotoxic screening of complexes 1 and 2 was also assessed against human cervical adenocarcinoma cancer (HeLa) cell line using the MTT assay. Both complexes 1 and 2 have displayed cytotoxic effect in a dose-dependent manner with IC_{50} values of 59.64±6.68 µg/mL and 62.85±0.39 µg/mL, respectively. Overall, the highest antibacterial and cytotoxic activity of the present work is provided by complex 1.

Keywords: Antibacterial activity, Copper (II) complex, Cytotoxicity, Disc diffusion assay, MTT assay

Despite the extensive advances in scientific research and findings, infectious diseases remain one of the common health issues[1,2]. Considerable research effort has been directed globally in the search for novel drugs which can overcome the emergence of infectious and multidrug-resistance pathogenic microbes[3-6]. According to WHO, around 75% of the newly authorized drugs, which are under clinical trials, are derivatives of already approved compounds. Regardless of the fact that their multiple resistance mechanisms are well established, the clinical benefits are still limited[7].

Cancer is a major life-threatening noninfectious disease that humans encounter in addition to infectious diseases. Medicinal chemistry faces a difficult task in finding new/novel medications to combat this developing disease. The success of metal-based anticancer drug cisplatin has prompted young researchers to investigate more on the platinum-based complexes[8]. However, due to the various drawbacks of cisplatin and platinum-based agents, researchers have shifted their views towards other metal-based agents having higher cytotoxicity with minimum side effects[9-12]. In anticancer research, copper (II)-based compounds have emerged as promising candidates[13-15]. Copper metal ions have recently been employed to treat several disorders, including cancer, in a variety of inorganic pharmaceutical products[16]. Copper metal is also one of the bio-essential elements of the living system and it has been employed in a variety of medicinal therapies over the years[17].

In addition to metal ions, most ligands have biological properties of their own. They are the main constituents of metal complexes and play a major role in their stabilization. The chelation of ligands with metal ions has been shown to improve the biological activity of metal free ligands to some extent[18,19]. Metal complexes, on the other hand, have some limitations for usage as *in-vivo* medications due to their relatively high toxicity and limited water solubility[20,21]. To overcome these limitations, researchers have attempted to modify the ligand frameworks of metal complexes[22].

Given the above points, the present work aims to investigate the biological activity of two copper (II) complexes having N-donor chelating ligand. The selected complexes are highly soluble in water. Ligand also possesses several hydrogen bonding sites on its own. The biological importance of hydrogen bonding in regulating the growth of pathogenic microbes has been shown by a number of authors[23,24]. Biological activities

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of some copper (II) complexes containing N-donor ligands have already been reported by our research group[25]. In this paper, we report the antibacterial screening of two copper (II) complexes against human urinary pathogenic bacterial strains, *Escherichia coli and Proteus mirabilis.* The cytotoxicity of both the complexes on human adenocarcinoma cancer cells (HeLa) has also been reported in this paper.

Materials and Methods

The reagent and chemicals, used here, were of analytical grade. The copper (II) chloride dihydrate ($CuCl_2.2H_2O$), copper (II) bromide ($CuBr_2$), and ligand dicyandiamide ($C_2N_4H_4$) were purchased from Merck, India. The elemental analysis was determined using CHNS/O elemental analyzer, Perkin–Elmer-2400- Series ll. Magnetic susceptibility was determined using a Sherwood Magnetic Susceptibility balance at room temperature. Copper sulphate pentahydrate ($CuSO_4.5H_2O$) was used as a standard and Pascal's constants were used for diamagnetic corrections.

Synthesis of the Complex

dichloromono(1-amidino-O-ethylurea)copper (II), [Cu(AEtUH) Cl_2].H_2O (Complex 1)

The complex 1 was synthesized by refluxing the copper (II) chloride dihydrate ($CuCl_2.2H_2O$) and ligand dicyandiamide ($C_2N_4H_4$) in a 1:1 stoichiometric ratio on a water bath for 3 h in ethanol solvent[26]. The resulting bright blue crystalline products were collected and thoroughly washed with ethanol, finally by acetone.

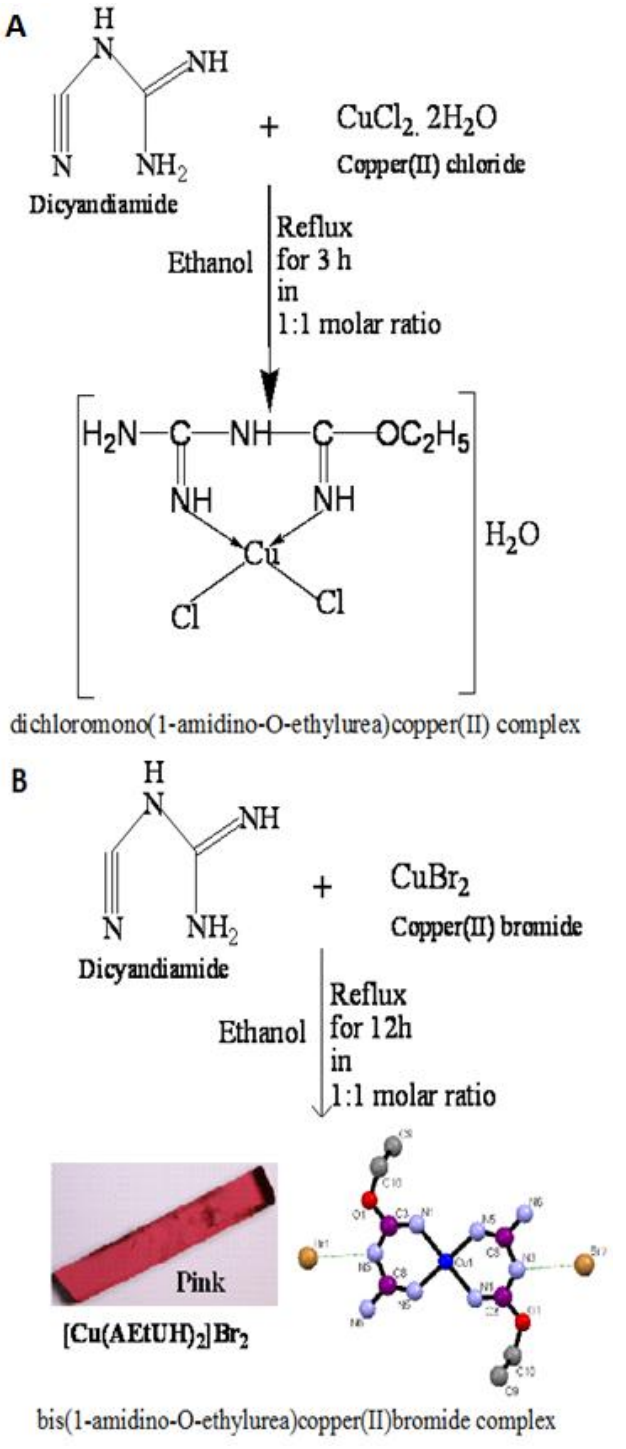
bis(1-amidino-O-ethylurea)copper (II) bromide, [Cu(AEtUH)$_2$]Br_2 (Complex 2)

The complex 2 was synthesized by refluxing the ethanolic solutions of metal salt copper (II) bromide ($CuBr_2$) and ligand dicyandiamide ($C_2N_4H_4$) together in 1:1 molar ratio at 45°C on the water bath for about 12 h[27]. Reaction mixture solution was filtered and the filtrate was kept for slow evaporation for few days. The resulting dark pink single crystals were collected and thoroughly washed with ethanol, finally by acetone.

The schematic diagrams for the synthesis of complex 1 and 2 are shown in (Scheme 1).

In vitro antibacterial screening

The *in vitro* antibacterial screening of complexes 1 and 2 was performed along with their corresponding metal salts ($CuCl_2.2H_2O$) and ($CuBr_2$), and ligand (dicyandiamide), against two human uropathogenic Gram-negative bacterial strains, *Escherichia coli* and *Proteus mirabilis.* Filter disc diffusion method[28] was used to screen the antibacterial activity using dimethylsulfoxide (DMSO) as a solvent. Experiments were done in triplicate and the resulting zone of inhibition corresponding to particular compounds were averaged and reported in the data.



Scheme 1 — Schematic diagrams for the synthesis of complex1 (A) and complex 2 (Hydrogen atoms are omitted for clarity) (B).

Antibacterial screening of the test compounds was carried out in two different ways:

(i) Test compounds of same weight dissolved in equal volume: The minimum inhibitory concentrations (MICs) of all the test compounds were determined by the serial dilution method, within the range of (0.01-6.0 mg/mL). The sensitivity of bacteria to the test compounds was observed by measuring the diameter of the zone of inhibition of bacterial growth after 24 h incubation. The antibiotic gentamycin (0.01 mg/mL) was used as a positive control.

(ii) Test compounds of same equimolar concentration: In this case, MICs were determined for the complexes 1 and 2 only, within the concentration range (1.0-40.0 × 10^{-3} M).The sensitivity of bacteria to the complexes was evaluated and compared by extending the incubation time to 24 h, 48 h, and 72 h.

In vitro cytotoxicity screening

Cytotoxicity screening of the complexes 1 and 2 was evaluated by MTT assay[29]. Human adenocarcinoma cancer (HeLa) cells were first grown in T-25 culture flask and they were harvested by the trypsinization process. Cells were further plated by taking approximate density 1 × 10^5 per well in the 96-well culture plates and then finally incubated for the confluency for 24 h. The medium from each well was removed and cells were washed with Dulbecco's Phosphate Buffer Saline and treated with an increasing amount of complexes. Each well containing 100 µL of different concentrations of complexes with serum-free DMEM was incubated for 24 h in 5% CO_2/95% air with relative humidity 90% at 37±0.2°C. After incubation, all the contents were replaced by equal amounts of 1.2 mM serum-free DMEM containing MTT and further incubated for another 3 h. The viable cells formed certain kinds of formazan crystals. To solubilize the formazan grains the contents were further replaced by taking an equal amount of DMSO solvent. The absorbance value was recorded at 570 nm with the help of a multi-well plate reader –Infinite M200, Tecan.

Statistical Analysis

All the data reported in this paper were expressed as standard deviation (mean ± SD) of triplicate readings. One-way ANOVA, supplemented with Tukey's HSD post hoc test, was used to perform the multiple comparisons between the groups which are more than two. To indicate the statistical significance, the values at $P < 0.05$ were considered.

Results and Discussion

Characterization of the complex 1 and 2

Both complexes 1 and 2 were stable at room temperature and also highly soluble in water. Magnetic moment values and elemental (CHN) analysis data of the complex 1 and 2 are shown in (Table 1).Other characterizations, such as spectroscopic and electrochemical data of the complex 1 and single crystals X-ray diffraction data of the complex 2 have already reported[27].

In vitro antibacterial screening

Test compounds of same weight dissolved in equal volume: The results of the antibacterial screening of all test compounds are displayed in (Fig. 1 & 2). Data obtained indicate that, all the test compounds, except ligand, exhibited significant antibacterial activity against both bacterial strains, though at a considerably lower level than the antibiotic gentamycin. The bacterial growth inhibition potential (diameter of zone of inhibition) of all tested compounds was also found to be increased in a dose-dependent manner. While comparing the antibacterial effectiveness of the complexes 1 and 2 with their respective metal salts ($CuCl_2.2H_2O$ and $CuBr_2$), complex 1 was more effective than its metal salt on both bacterial strains (Fig. 1A & B). The improved antibacterial effectiveness of complex1became more pronounced as their concentrations increased. MICs of the complex 1 and $CuCl_2.2H_2O$ were found to be (0.01 and 0.5 mg/mL), respectively, against *Escherichia coli*, while they showed a similar MIC value (0.5 mg/mL), against *Proteus mirabilis.* In contrast to

Table 1 — Elemental (CHN) analysis and magnetic moment data of the complex 1 and 2

Compound	Color	Yield	Analytical found (%) (calculated) (%)				μ_{eff} (B.M.)
			Cu	C	H	N	
$[Cu(AEtUH)Cl_2].H_2O$ Complex 1	Bright Blue	85	22.32 (22.48)	16.81 (16.98)	4.12 (4.24)	19.61 (19.82)	2.1
$[Cu(AEtUH)_2]Br_2$ Complex 2	Bright Pink	80	12.26 (13.10)	20.01 (19.86)	4.53 (4.10)	23.62 (23.17)	1.71

where AEtUH =1-amidino-*O*-ethylurea

complex 1, the antibacterial effectiveness of complex 2 was slightly lower than its metal salt on both bacterial strains (Fig. 2A & B). Their MICs were found to be (1.0 and 0.1 mg/mL), respectively, against *Escherichia coli* but they showed a similar MIC value (0.5 mg/mL), against *Proteus mirabilis.*

Test compounds of same equimolar concentrations: The antibacterial screening of all test compounds on both bacterial strains was investigated further by taking the same equimolar concentrations (40×10^{-3} M). Incubation times were also extended to 24 h, 48 h and 72 h (Table 2). When compared the antibacterial activity of all the test compounds to incubation time, their effectiveness decreased on average as the incubation time increased. The highest activity was shown by 24 h incubation. The ligand did not show any significant response during the entire incubation time. Complex 1 was found to be more effective than its metal salt, where as complex 2 was found to be less effective than its metal salt. MICs of complexes 1 and 2 were compared further for all incubation times. Complex 1 showed a similar MIC (5.0×10^{-3} M) on *Escherichia coli* during the entire incubation time, whereas somewhat different MICs (5.0×10^{-3} M) for 24 h and 48 h, and (10.0×10^{-3} M) for 72 h, respectively, (Table 3). Complex 2 showed a similar MIC (10.0×10^{-3} M) on both bacterial strains during the entire incubation time (Table 4). The variations in the antibacterial activity of all tested compounds recorded after 24 h incubation are demonstrated in (Fig. 3A-D).

Overall, the highest antibacterial activity among all of the test compounds is provided by complex 1. The exact mechanism of action of this complex is not certain. However, one of the main aspects that influences the

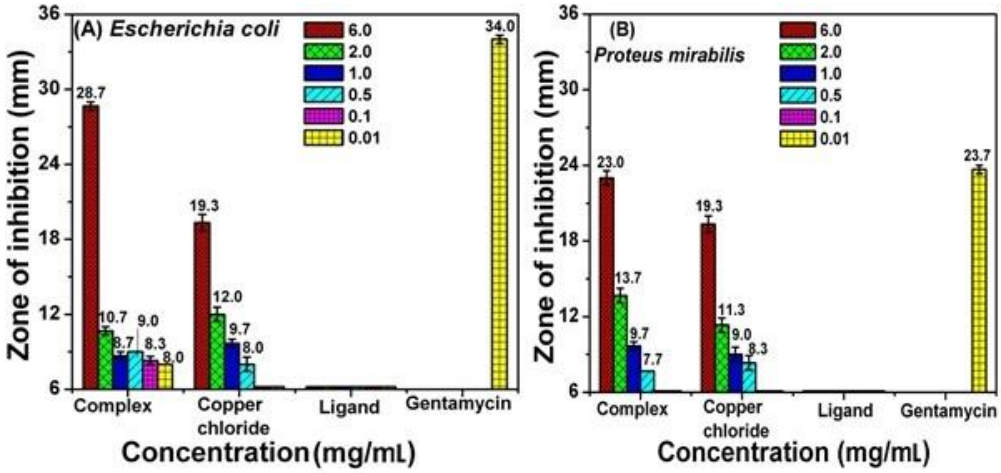


Fig. 1 (A & B) — Histograms showing MIC values of complex 1, $CuCl_2.2H_2O$ salt, ligand and positive control gentamycin at different concentrations against the bacterial strains (A) *Escherichia coli*; and (B) *Proteus mirabilis*, recorded at the end of 24 h

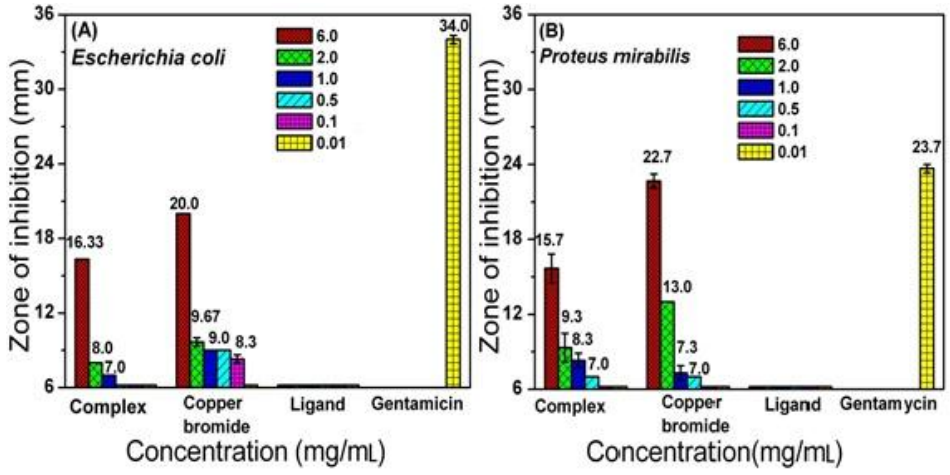


Fig. 2 (A & B) — Histograms showing MIC values of complex 2, $CuBr_2$ salt, ligand and positive control gentamycin at different concentrations against the bacterial strains (A) *Escherichia coli*; and (B) *Proteus mirabilis*, recorded at the end of 24 h

Table 2 — [a]Diameter of inhibition zone of the test compounds

Test compound (40×10^{-3} M)	*Escherichia coli*			*Proteus mirabilis*		
	24 h	48 h	72 h	24 h	48 h	72 h
Ligand	nil	nil	nil	nil	nil	nil
$CuCl_2.2H_2O$	19.0±0.0	17.3±7.0	15.0±0.0	21.0±0.0	17.7±0.0	17.0±0.0
Complex 1	21.5±0.7	18.7±3.0	16.3±7.0	22.0±0.5	19.3±0.0	18.7±1.0
$CuBr_2$	22.3±0.9	20.7±0.9	18.0±0.9	22.3±0.9	20.7±0.9	20.0±0.9
Complex 2	20.7±0.0	15.0±0.7	11.5±0.0	22.0±1.1	19.3±0.3	18.7±1.0

[a]Represents the diameter zone of inhibition of the test compounds after subtracting the inhibition zone value of DMSO solvent

Table 3 — [a]Diameter of inhibition zone of the complex 1 for different concentrations

Complex 1 (10^{-3} M)	*Escherichia coli*			*Proteus mirabilis*		
	24 h	48 h	72 h	24h	48 h	72 h
40	21.5±0.7	20.0±0.0	20.0±0.0	22.0±0.7	20.0±0.0	19.0±0.7
20	18.0±0.0	15.5±0.5	15.5±0.3	16.5±0.5	14.5±0.3	14.5±0.0
10	15.0±0.7	13.0±0.0	13.0±0.0	12.0±0.0	10.0±0.0	09.0±1.0
05	09.0±0.0	09.0±0.0	09.0±0.0	07.0±0.0	07.0±0.0	nil
01	nil	nil	nil	nil	nil	nil

[a]Represents the diameter zone of inhibition of the complex 1 after subtracting the inhibition zone value of DMSO solvent

Table 4 — [a]Diameter of inhibition zone of the complex 2 for different concentrations

Complex 2 (10^{-3} M)	*Escherichia coli*			*Proteus mirabilis*		
	24 h	48 h	72 h	24 h	48 h	72 h
40	20.7±0.0	19.5±0.5	18.0±0.0	21.0±0.0	19.5±0.5	18.0±0.0
20	18.0±0.7	13.5±0.5	10.0±0.0	15.0±0.7	13.5±0.5	10.0±0.5
10	11.5±0.5	09.0±0.0	07.0±0.0	11.5±0.5	09.0±0.0	07.0±0.0
05	nil	nil	nil	nil	nil	nil
01	nil	nil	nil	nil	nil	nil

[a]Represents the diameter zone of inhibition of the complex 2 after subtracting the inhibition zone value of DMSO solvent

antibacterial activity of test compounds is their lipophilic nature. According to ligand field theory (LFT), chelation of ligands with metal ions involves some overlap of ligand orbitals with metal ion orbitals. This orbital overlapping facilitates ligand electrons to delocalize to metal orbitals, lowering the positive charge (charge polarity) of the metal complexes. As a result, the lipophilic nature and cell penetration power of the metal complexes increase[6]. In complex 1, the presence of an N-donor chelating ligand as well as two monodentate chloride ligands might just have contributed to lowering its charge polarity, enhancing its lipophilicity. Furthermore, chloride ions, being labile in nature, can facilitate ligand substitution with bacterial biomolecules, enhancing the bactericidal effect of complex 1 in comparison to other test compounds. Schwartz *et al.*, (2001) have also reported the role played by the labile ligand in improving the antiviral activity of their cobalt complexes[30].

Slight variations in the antibacterial efficiency of a particular test compound, on different bacterial strains, might be induced by differences in bacterial cell wall

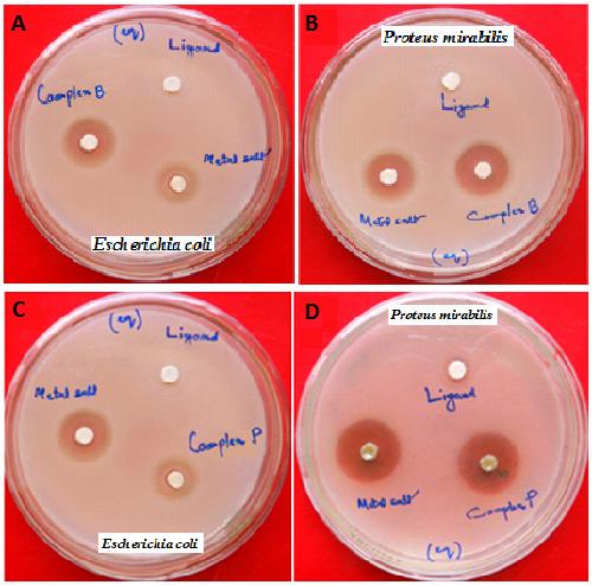


Fig. 3 (A-D) − Photographs showing the antibacterial activity (diameter of zone of inhibition) of tested compounds ($40 \times 1\ 0^{-3}$ M) against *Escherichia coli* and *Proteus mirabilis* recorded at the end of 24 h; (A & B) Complex 1, parent $CuCl_2.2H_2O$ salt and ligand dicyandiamide; (C & D) Complex 2, $CuBr_2$ salt and ligand dicyandiamide

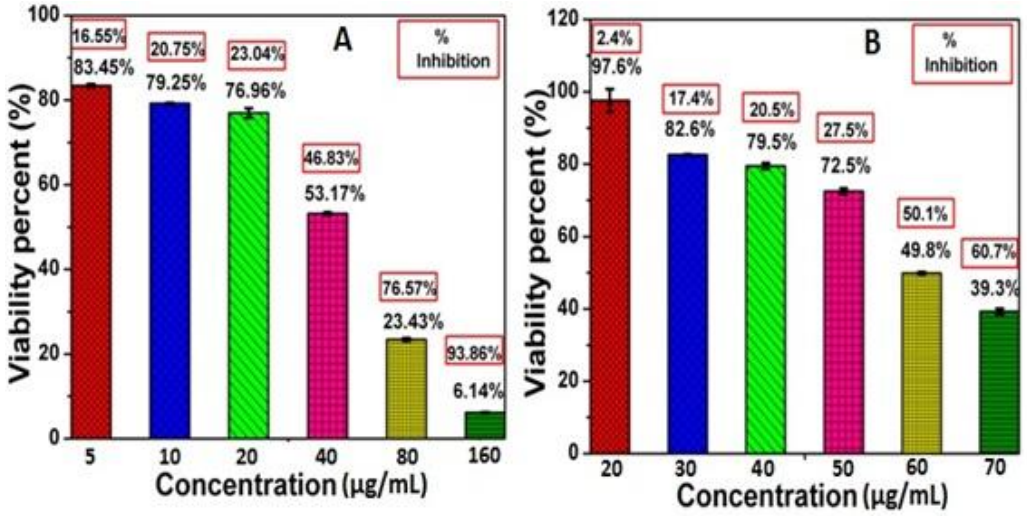


Fig. 4 (A & B) — Histograms showing the cytotoxicity effect of the (A) complex 1; and (B) complex 2 against HeLa cells. Each bar represents the mean ± SD

permeability or bacterial ribosomal content[31]. Other aspects that may have influenced the antibacterial properties of test compounds include chemical structure, the total charge of the complex, type of the donor ligand, and ion neutralizing the ionic complex[32-34].

***In vitro* cytotoxicity screening**

The results of *in vitro* cytotoxicity of the complex 1 and 2 against human cervical adenocarcinoma cancer (HeLa) cells were represented as the percentage (%) of viable cells. The relative number of surviving HeLa cells was decreased in a dose-dependent manner (Fig. 4A & B). The minimum concentration required to reduce the viable cancer cells by 50% (IC_{50}) was calculated from dose-response curves. The complex 1 was found to be more cytotoxic (IC_{50} = 59.64± 6.68 µg/mL) than complex 2 (IC_{50} = 62.85±0.39 µg/mL).

The variations in antiproliferative activity of the complexes 1 and 2 on HeLa cells can be attributed to differences in complex geometry and the nature of the metal-ligand bonds[35,36]. It has been reported that, anticancer property of a compound is significantly influenced by its strong DNA binding and breaking abilities[37-39]. Our research group has already reported the DNA binding capacities of some analogous copper (II) complexes[25] and investigation into complex 1 is currently underway.

Conclusion

The biological properties of complexes 1 and 2 were screened in terms of antibacterial and cytotoxic activity. The antibacterial screening of complexes 1 and 2 was performed along with their corresponding metal salts, and ligand, against bacterial strains, *Escherichia coli* and *Proteus mirabilis*. All the test compounds, except ligand, have shown significant antibacterial activity on both bacterial strains. Their antibacterial effectiveness also increased in a dose dependent manner, whereas their ability to inhibit the growth of both bacterial species decreased as their incubation time increased. The activity of complex 1 was found to be greater than its metal salt, while complex 2 showed lower activity than the corresponding metal salt, on both bacterial species. Antiproliferative activity of complexes 1 and 2 against HeLa cancer cells was found to increase dose-dependently. When comparing the cytotoxicity of the complexes, complex 1 was more cytotoxic than complex 2, with IC_{50} values of 59.64±6.68 µg/mL and 62.85±0.39 µg/mL, respectively. Observed changes in biological activity between complexes 1 and 2 can be attributed to differences in complex structures, nature of coordinating ligands, lipophilic nature, and DNA binding and breaking affinities. The most significant finding of this study is the remarkable antibacterial and cytotoxic activities of complex 1, which may provide a suitable candidate for further development of metal complex-based therapeutics.

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Conflict of Interest

All authors declare no conflict of interest.

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