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Investigation of Novel Tetrahalometallate Complexes of Cetrimonium Bromide Surfactant against *in vitro* Human Tumour Cell Lines of Lung, Colon and Liver

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Authors' contributions

This work was carried out in collaboration between all authors. Author HEAA prepared the synthesized compounds, managed the literature searches and designed the study. Authors MFZ and AMB managed the analyses of the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

The synthesis of the tetrahalo ***Cu***(II) and tetrahalo ***Zn***(II) metal complexes with two cetrimonium bromide (CTAB) ligands is reported. Potentiometric studies showed that these complexes in aqueous solution showed no metal release, thus accounting for their high *in vitro* toxicity against three human cancer cell lines: A-549 (non-small cell lung carcinoma), HCT-116 (colon carcinoma cells), and HepG-2 (Hepatocellular carcinoma cells). The tetrahalo ***Cu***(II) and ***Zn***(II) metal complexes were synthesized by solid state grinding. Metal complexes of chelating CTAB with metal ion were studied on the basis of FT-IR, ^{1}H-NMR and atomic absorption spectroscopic data. The tetrahalo ***Cu***(II) and ***Zn***(II) metal complexes induced cancer cell apoptosis. The tetrahalo complex of ***Cu***(II) or ***Zn***(II) inhibited *in vitro* the growth of three tumor cell lines at low concentrations. The tetrahalo copper(II) complex displayed against A-549 (non-small cell lung carcinoma) human cancer

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cell lines, IC_{50} values in mM range was similar to that of the antitumor drug cis-platin and they are considered for further stages of *in vitro* screening as potential antitumor activity.
Study Design: Antiproliferative activities were evaluated using a SRB and MTT assays. Effects on the cell cycle were assessed by flow cytometry and on apoptosis-related proteins (active caspase-3, -8 and -9, procaspase-3, -8, -9, PARP, Bid, Bcl-xL and Bcl-2) by Western blotting. Cu(II) and Zn (II) metal complexes displayed high inhibition potency against A-549 (non-small cell lung carcinoma), HCT-116 (colon carcinoma cells), and HepG-2 (Hepatocellular carcinoma cells).
Place and Duration of Study: Regional Center Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt, between March 2016 and June 2016.

Keywords: Solid state grinding; cetyltrimethylammonium bromide (CTAB); cytotoxicity, anticancer.

1. INTRODUCTION

Apoptosis (from Ancient Greek "falling off") is a process of programmed cell death that occurs in multicellular organisms (Fig. 1). Biochemical events lead to characteristic cell changes (morphology) and death. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and global mRNA decay. Between 50 and 70 billion cells die each day due to apoptosis in the average human adult. For an average child between the ages of 8 and 14, approximately 20 to 30 billion cells die per day. Research on apoptosis has increased substantially since the early 1990s. In addition to its importance as a biological phenomenon, defective apoptotic processes have been implicated in a wide variety of diseases. Excessive apoptosis causes atrophy, whereas an insufficient amount results in uncontrolled cell proliferation, such as cancer. Some factors like Fas receptors and caspases promote apoptosis, while some members of the BCL-2 family of proteins inhibit apoptosis.

Similar to oncogene addiction, in which tumor cells rely on a single dominant gene for survival, tumor cells may also become dependent on B-cell (BCL-2) in order to survive. In response to stress signals and malignant cells may express pro-apoptotic activators. Some cancer cells overexpress BCL-2, which can damage this pro-apoptotic response. The result is in many cases an abundance of pro-apoptotic activators bound and sequestered by BCL-2. In this scenario, cancer cells are thought to be “primed” for apoptosis, in that they may contain sufficient amounts of the pro-apoptotic activators, if displaced from BCL-2, to induce programmed cell death (Fig. 2). Cancers that depend on BCL-2 for survival in this way are likely to be sensitive to BCL-2 modulation. BCL-2 expression in solid tumors BCL-2 may also play a role in nonhematologic tumors, and inappropriate expression has been observed in solid tumors such as prostate, breast, and small cell and non-small cell lung cancers. In small cell lung cancer, high BCL-2 expression is >90% of patients has been reported. Ovarian, neuroblastoma, bladder, colorectal, and some head and neck cancers have all exhibited significant levels of BCL-2 expression. Cellular stresses endured by nearly all cancer cells—limited blood supply, reactive oxygen species, and exposure to chemotoxins—can lead to increases in pro-apoptotic proteins, much like they would be in normal cells. When BCL-2 is overexpressed in cancer cells, it may inhibit the pro-apoptotic signals, allowing the cancer cells to survive under stressful conditions. The high levels of pro-apoptotic proteins bound and sequestered by increased BCL-2 may result in what is called a primed state (Fig. 2). Primed cells are thought to have a high apoptotic potential: in other words, displacement of pro-apoptotic proteins from BCL-2 can result in a large enough increase in free pro-apoptotic proteins to initiate apoptosis. The primed state provides a strong relationship for conducting research related to the targeting and inhibition of BCL-2. Scavenger receptors which are a group of receptors that recognize modified low-density lipoprotein (LDL) by oxidation or acetylation. This naming is based on a function of cleaning (scavenging): Scavenger receptors (endocrinology) widely recognize and take up macromolecules that have negative charges, like modified LDL. Scavenger receptors are thought to participate in the removal of many foreign substances and waste materials in the living body by extensive ligand specificity and a variety of receptor molecules [1].

Takashi and et al. found inhibition of cancer cells growth by Cyclic diguanosine monophosphate (c-di-GMP), a ligand of the stimulator of interferon genes (STING) signal pathway, as a new biological activity [2]. Andrew and et al. have

prepared four new trinuclear $[Cu_3(RCOO)_4(H_2TEA)_2]$ copper(II) complexes as well as dimeric species $[Cu_2(H_2TEA)_2(RCOO)_2]\bullet_2H_2O$ by adding triethanolamine (H_3TEA) at ambient conditions to hydrated $Cu(RCOO)_2$ salts. To the best of our knowledge, this is the first evaluation of two scavengers against cancer. Apoptosis induced by antitumor agents is known to be related to alterations of cellular signalling pathways [3].

Solubilities of a given solute (drug) are typically expressed in terms of how many part of a given solvent is needed to dissolve 1 part of the solute. The relationship between the solvent and the solute are given as follow [4]:

Terms	Approximate volume of solvent in millilitres per gram of solute
Very soluble	< 1
Freely soluble	1-10
Soluble	10-30
Sparingly soluble	30-100
Slightly soluble	100-1000
Very slightly soluble	1000-10000
Practically insoluble or insoluble	>10000

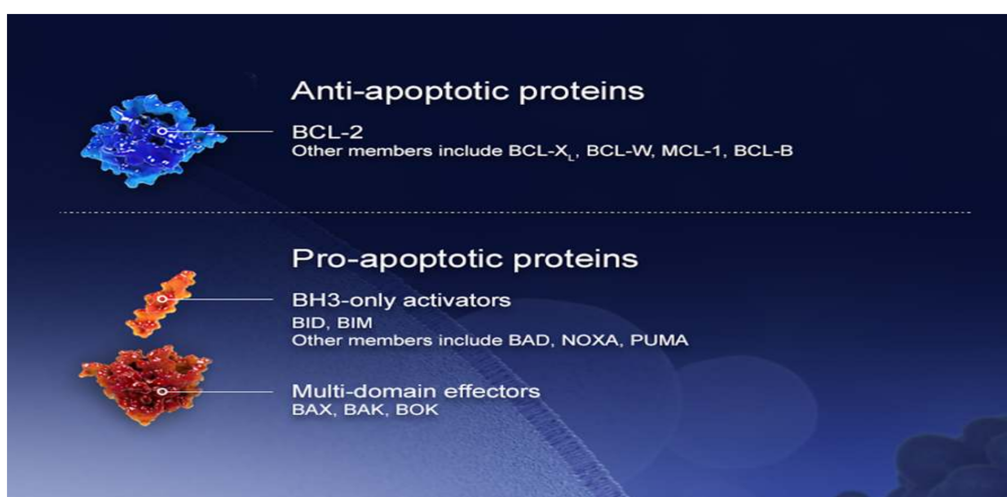


Fig. 1. Apoptosis process
Source: www.health.zone. Apoptosis Process Cell

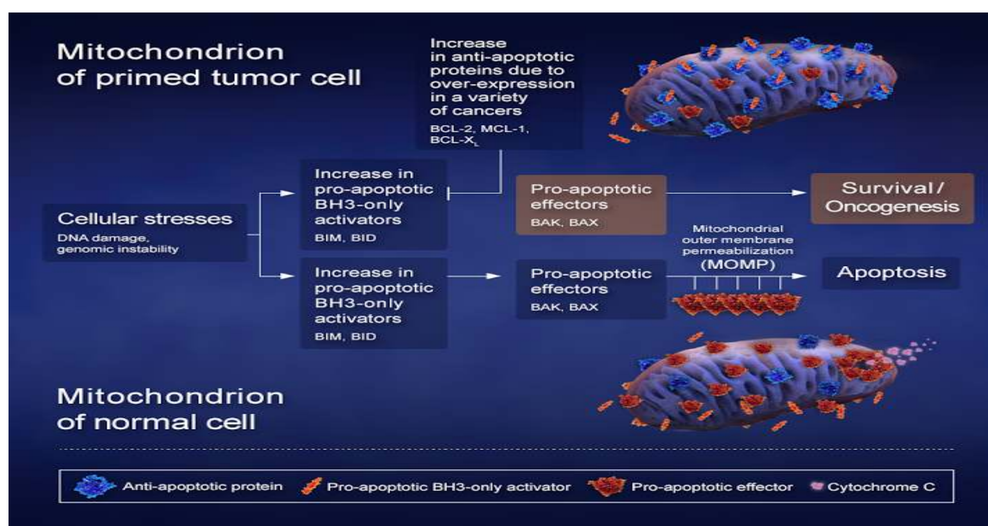


Fig. 2. Cell priming
Source: www.cell.com. Cell priming

2. EXPERIMENTAL

2.1 Materials

All other reagents and solvents were of high purity and used as purchased without any further purification. Cetrimonium bromide $((C_{16}H_{33})\ N^+ (CH_3)_3\ Br^-)$ or hexadecyltrimethylammonium bromide, CTAB, was purchased from Aldrich. ***Cu***(II) and ***Zn***(II) chlorides were purchased from El Nasr Chemical Co. Elemental analyses were performed using a Varian Elemental. FT-IR spectra were recorded on a Perkin Elmer-spectrum one spectrophotometer in the 4,000-400 cm range using KBr discs. ^{1}H-NMR spectra was recorded on Varian Gemini 200 MHz instrument in CDCl solution. The tested human carcinoma cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The established *in vitro* human cancer cell line A-549 (non-small cell lung carcinoma), HCT-116 (colon carcinoma cells), and HepG-2 (Hepatocellular carcinoma cells) were applied: The cell lines are maintained in the Regional Center Mycology and Biotechnology (Al-Azhar University).

2.2 Synthesis

2.2.1 Synthesis of bis-cetrimonium-dibromo-dichloro-metallate

By solid-state reaction: An agate mortar and pestle was used to grind in presence of CTAB and a metal chloride ($CuCl_2$ or $ZnCl_2$) in 2:1 molar ratio in few minutes with occasional mixing and crushing. The reaction product was white in color (Scheme 1) [5].

2.3 Characterization

Table 1 shows Elemental analysis for the scavengers. Formation of copper and zinc complex is confirmed by comparing the ^{1}H-NMR of ligand and its metal complex (chart 1).

^{1}H-NMR spectra for Cetrimonium bromide

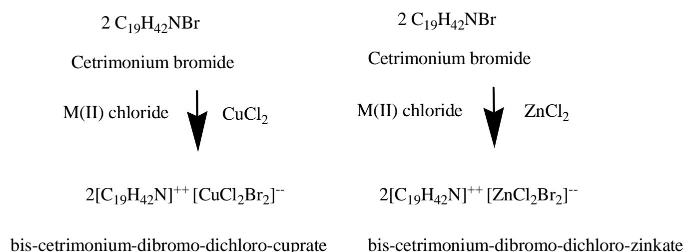
^{1}H-NMR: d = 3.3 ppm (t, 3H, CH_3-N^+), 3.24 ppm (m, 2H, CH_2-N^+), 1.73 ppm (m, 2H, CH_2-CH_2-N^+), 1.33 ppm (m, 2H, CH_3-CH_2), 1.29 ppm (m, 24H, $(CH_2)_n$), 0.96 ppm (m, 3H, CH_3-CH_2-).

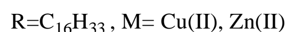
^{1}H-NMR spectra for Bis-cetyltrimethy-lammonium dichloro-dibromo-cuprate $\{2[C_{19}H_{42}N]^{++}[CuCl_2Br_2]^{--}\}$

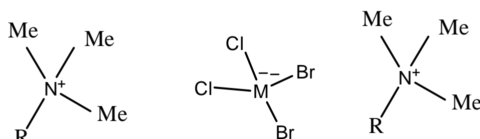
^{1}H-NMR: d = 3.36 ppm (t, 3H, CH_3-N^+), 3.32 ppm (m, 2H, CH_2-N^+), 1.79 ppm (m, 2H, CH_2-CH_2-N^+), 1.41 ppm (m, 2H, CH_3-CH_2), 1.35 ppm (m, 24H, $(CH_2)_n$), 1.06 ppm (m, 3H, CH_3-CH_2-).

^{1}H-NMR spectra for Bis-cetyltrimethy-lammonium dichloro-dibromo-zinkate $\{2[C_{19}H_{42}N]^{++}[ZnCl_2Br_2]^{--}\}$

^{1}H-NMR: d = 3.8 ppm (t, 3H, CH_3-N^+), 3.29 ppm (m, 2H, CH_2-N^+), 1.78 ppm (m, 2H, CH_2-CH_2-N^+), 1.35 ppm (m, 2H, CH_3-CH_2), 1.34 ppm (m, 24H, $(CH_2)_n$), 1.09 ppm (m, 3H, CH_3-CH_2-).







Scheme 1. Synthesis route for ligand and metal salts as well as the structure of synthesized complexes (bis-cetyltrimethylammonium dichloro-dibromo-metallate)

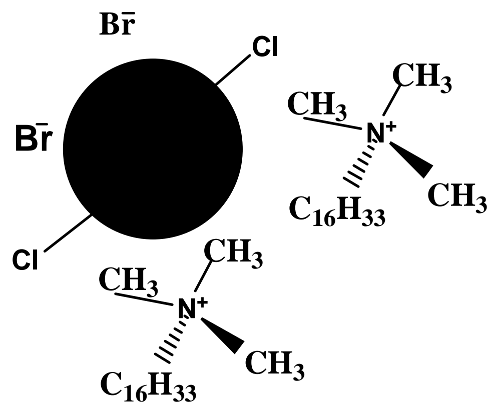


Chart 1. Schematic illustration of combined electrostatic and steric double layer around a diammonium tetrahalometallate

Table 1. Elemental microanalysis data for the scavengers

S	MF	MW	Analysis data calc / found%					
			C	H	N	Cl	Br	M
Cu	$C_{38}H_{84}N_2Cl_2Br_2$ Cu 2 CTAB	863	52.86/52.85	9.81/9. 80	3.24/3.23	8.21/8.20	18.51/18.5	7.36/7.35
Zn	$C_{38}H_{84}N_2Cl_2Br_2Zn$ 2 CTAB	865	52.75 / 52.74	9.79 /9.78	3.24/3.23	8.20 /8.19	18.47/18.5	7.56/7.55

Table 2. Selected IR frequencies (cm^{-1})

S	v (N^+- stret.)	v (CH_2-stret.)	v (CH_3- stret.)	v (CH-bend.)	v (N^+- bend.)	v (M-X)
A3	3600-3015	2919	2850	1450	1610	962-454
A4	3600-3015	2919	2850	1450	1610	962-454

2.4 Infrared Spectra Analysis

Table 2 shows FTIR spectra for the scavengers (scheme 1). There are several stretching bands observed in the spectra which is 3500 cm^{-1}, 3015 cm^{-1}, 2919 cm^{-1}, 2850 cm^{-1}, 1610 cm^{-1}, 1450 cm^{-1}, 962 cm^{-1} and 454 cm^{-1} that represent different functional group. The band at 3500 cm^{-1}, 3015 cm^{-1} and 1610 cm^{-1} respectively assigned for quaternary compound of ammonium salt which were formed by the reaction of CTAB with ***Cu*** or ***Zn*** dichlorides. The band at 2919 cm^{-1}, 2850 cm^{-1} and 1450 cm^{-1} respectively assigned for CH_2 and CH_3 stretch of alkane, besides CH bending occurs at 1450 cm^{-1} which were formed by solid state grinding. The band 454 cm^{-1} could be assigned to metal halide stretching mode from the chelating of CTAB with metallic ions, besides strong adsorption bands appear at 962 cm^{-1} and can be assigned to metal ions in the dried powder [6].

2.5 Antiproliferative Assay *In vitro* [7]

Compounds. Test solutions of the compounds tested (1 mg/mL) were prepared by dissolving the substance in 100 mL of DMSO completed with 900 mL of tissue culture medium. Afterwards, the tested compounds were diluted in culture medium to reach the final concentrations of 100, 10, 1 and 0.1 mg/mL. The solvent (DMSO) in the highest concentration used in test did not reveal any cytotoxic activity.

Cells. A-549 and HCT-116 cells were determined by the sulforhodamine B assay [8,9], and HepG-2 cells by the MTT assay [10,11].

SRB assay. The details of this technique were described by Skekhan et al. [12]. The cytotoxicity assay was performed after 72 h exposure of the cultured cells to varying concentrations (from 0.1 to 100 mg/mL) of the tested agents. The cells attached to the plastic were fixed by gently layering cold 50% trichloroacetic acid (TCA) on the top of the culture medium in each well. The plates were incubated at 4℃ for 1 h and then washed five times with tap water. The background optical density was measured in the wells filled with culture medium, without the cells. The cellular material fixed with TCA was stained for 30 min with 0.4% sulforhodamine B dissolved in 1% acetic acid. Unbound dye was removed by rinsing with 1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered Tris base for determination of optical density (at 540 nm) in a computer-interfaced, 96-well microtiter plate.

MTT assay. This technique was applied for cytotoxicity screening against HepG-2 cells growing in suspension culture. An assay was performed after 72 h exposure to varying concentrations (from 0.1 to 100 mg/mL) of the tested agents. For the last 3-4 h of incubation 20 mL of MTT solution was added to each well [MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma); stock solution: 5 mg/mL]. The mitochondria of viable cells reduce a pale yellow MTT to a navy blue formazan, so if more viable cells are present in the well, more MTT will be reduced to formazan. When the incubation time was completed (4 h), 80 mL of the lysing mixture was added to each well (lysing mixture: 225 mL dimethylformamide, 67.5 g sodium dodecylsulfate (both from Sigma) and 275 mL of distilled water). After 24 h, when formazan crystals had dissolved, the optical densities of the samples were read on an Elisa spectra max 190 photometer at 570nm wavelength. Each compound at a given concentration was tested in triplicate in each experiment, which was repeated three times.

3. RESULTS AND DISCUSSION

3.1 Scavenging Properties of the Metal Ion Compounds in Cell-Free System

As shown in Table 3, within the tested concentration range, the compounds under study showed a good dose-effect relationship due to scavenging effects. On the other hand, the spectrophotometric measurements already showed that the complexation of CTAB-Metal, Tables 1 & 2. With this notable exception, Cu(II) complex appeared to be more potent than Zn(II) complex [13].

3.2 Cytotoxic Activity

The *in vitro* growth inhibitory activity of the synthesized compounds was investigated in comparison with the well-known anticancer standard drug (cisplatin) under the same conditions using colorimetric MTT and SRB assays. Data generated were used to plot a dose response curve of which the concentration of test compounds required to kill 50% of cell population (IC_{50}) was determined. The results revealed that all the tested compounds showed inhibitory activity to the tumor cell lines in a concentration dependent manner. Cytotoxic activity was expressed as the mean IC_{50} of three independent experiments. Interestingly, the results are represented in Table 3 and Figs. 3 & 4 showed

Table 3. The antitumor activities of the tested scavengers expressed as IC_{50} values and compared with reference standard drugs evaluated on A-549 (non-small cell lung carcinoma), HCT-116 (colon carcinoma cells), and HepG-2 (Hepatocellular carcinoma cells) cancer cell lines

Cell viability %	Sample Conc. µg/ml	Cu-scavenger			Zn-scavenger		
		A-549	HCT-116	HepG-2	A-549	HCT-116	HepG-2
	500	6.52	3.45	3.51	9.84	5.37	6.78
	250	11.37	5.82	6.78	17.49	9.84	12.35
	125	18.94	9.74	9.82	21.83	15.22	17.84
	62.5	23.18	12.89	13.95	27.65	23.81	25.06
	31.25	28.21	21.93	23.84	35.48	29.74	31.43
	15.6	36.98	28.46	31.73	41.96	35.68	38.95
	7.8	45.73	36.74	40.95	53.47	43.29	47.52
	3.8	57.48	44.18	48.72	62.54	50.76	56.83
	2	68.92	57.29	61.43	75.31	59.13	67.42
	1	79.43	66.51	70.88	89.62	67.04	75.19
	0	100	100	100	100	100	100
IC_{50} µg/ml		6.38	3.11	3.8	10.2	4.3	6.76

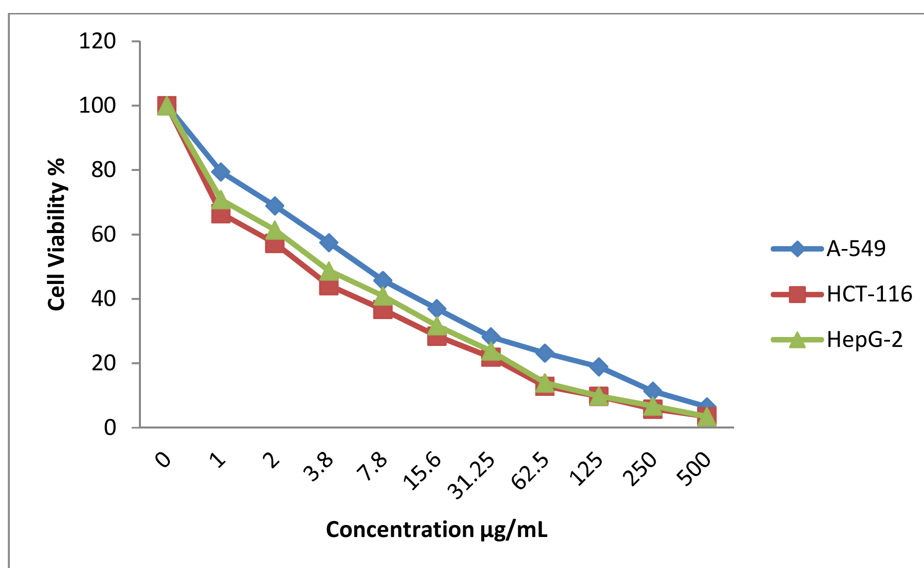


Fig. 3. Scavenging potency of the Cu(II) metal complex, added SRB/MTT medium expressed as percent inhibition of the accumulation

that ***Cu*** complex was the most active against the liver carcinoma cell line (HepG-2), showing more activity than the ***Zn*** complex with IC_{50} value of 3.8 µg/mL compared to ***Zn*** complex with IC_{50} value of 6.76 µg/mL. The tested compounds exhibited good antitumor activity against the colon carcinoma cell line (HCT-116) with IC_{50} value of 3.11 µg/mL for ***Cu*** complex as well as IC_{50} value of 4.3 µg/mL for ***Zn*** complex, respectively. ***Zn*** complex showed lower tendency to inhibit the non-small cell lung carcinoma cells (A-549) than those observed for ***Cu*** complex. The order of activity against cancer cell lines (A-549, HCT-116 and HepG-2) was ***Cu*** and ***Zn***, respectively. Moreover, metal ions were less active among their analogues against the A-549 cell lines [14].

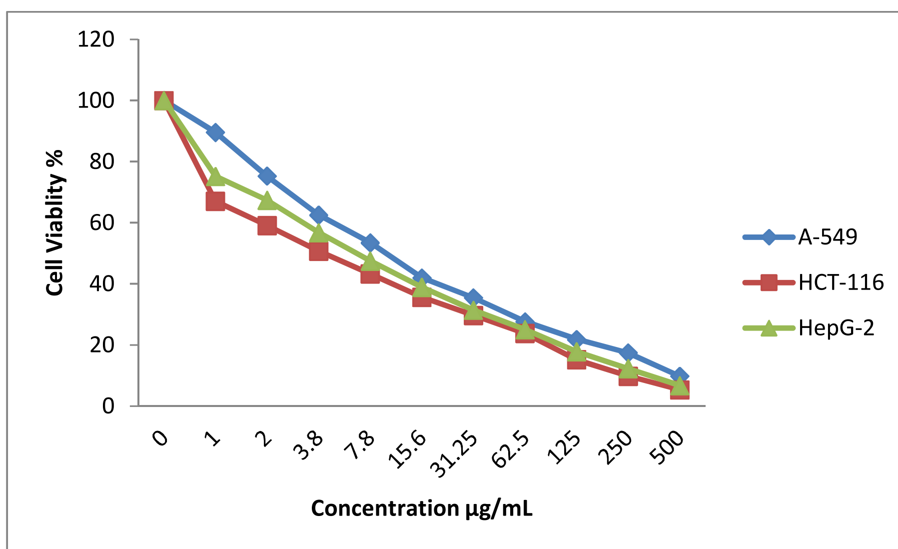


Fig. 4. Scavenging potency of the Zn(II) metal complex, added SRB/MTT medium expressed as percent inhibition of the accumulation

3.3 Antiproliferative Assay *in vitro*

The results of cytotoxic activity *in vitro* are expressed as IC_{50} – the concentration of compound (in mM) that inhibits a proliferation rate of the tumor cells by 50% as compared to control untreated cells, (Table 3). CTAB - Metal complexes were tested for their antiproliferative activity *in vitro* against the cells of three human cancer cell lines: A-549 (non-small cell lung carcinoma), HCT-116 (colon carcinoma cells), and HepG-2 (Hepatocellular carcinoma cells) and the results are compared with the known chemotherapeutic cisplatin. The IC_{50} value for the ***Cu*** complex against the A-549 cell line is 6.38 µg/mL. The IC_{50} values against HCT-116 and HepG-2 are 3.11 and 3.8 µg/mL respectively. The IC_{50} value for the ***Zn*** complex against the A-549 cell line is 10.2 µg/mL. The IC_{50} values against HCT-116 and HepG-2 are 4.3 and 6.76 µg/mL respectively [15].

4. CONCLUSION

The tetrahalometallate complexes as $\{2[C_{19}H_{42}N]^{++}[CuCl_2Br_2]^{--}\}$ and $\{2[C_{19}H_{42}N]^{++}[ZnCl_2Br_2]^{--}\}$ were synthesized and characterized. The complex $\{2[C_{19}H_{42}N]^{++}[CuCl_2Br_2]^{--}\}$ exhibited the highest inhibitory effect against A-549 (non-small cell lung carcinoma), HCT-116 (colon carcinoma cells), and HepG-2 (Hepatocellular carcinoma cells). The complex $\{2[C_{19}H_{42}N]^{++}[CuCl_2Br_2]^{--}\}$ exhibited a strong *in vitro* inhibitory effect at 500 µg/ml (IC_{50} are 10.2, 4.3 and 6.76), superior than the inhibition induced by $\{2[C_{19}H_{42}N]^{++}[ZnCl_2Br_2]^{--}\}$ at the same molar dose (IC_{50} are 6.38, 3.11 and 3.8). These compounds may prove useful for treating a variety of antitumor diseases and may lead to the development of new drugs. $\{2[C_{19}H_{42}N]^{++}[CuCl_2Br_2]^{--}\}$ and $\{2[C_{19}H_{42}N]^{++}[ZnCl_2Br_2]^{--}\}$ are considered as agents with potential antitumor activity, and can therefore be candidates for further stages of screening *in vitro*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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