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Research Article

Antitumour and antimicrobial activities of chloro derivatives of Synthetic Curcumin and their metal chelates

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Abstract

Curcumin and its analogues have been extensively studied as effective medicine for the treatment of cancer and related diseases. Recently several curcuminoid analogues were synthesized and their chemotherapeutic potential has been revealed. In the present study, the synthesis and characterization of three new chlorine containing curcuminoid analogues and their metal chelates Cu(II) and Al(III) are discussed here. The curcuminoid analogues namely 1,7-di(4-chloro phenyl)-1,6-heptadiene-3,5-dione(HL1), 1,7- di(2-chloro phenyl)-1,6-heptadiene-3,5-dione(HL2), and 1,7-bis(3,4 dichloro phenyl)-1,6-heptadiene-3,5-dione(HL3) and their Cu(II) and Al(III) chelates were synthesized and were characterized using UV, IR, 1H NMR and mass spectral data. *In vitro* cytotoxic studies were done with ligand and metal complexes (Cu & Al) against DLA and EAC cells using Tryptan blue exclusion method and antibacterial study of the compounds were done using agar well diffusion method. The *in vivo* antitumour activity of the ligand and complexes were determined by using DLA cells in mice and compared with standard anticancer drug cyclophosphamide. The life spans of the treated animals were increased upto 60-70%. The present investigation reveals that the Cu(II) complexes show enhanced cytotoxic activity where as the Al(III) complexes have greater activity towards *in vivo* antitumour studies and antibacterial studies.

Keywords: 1,7–diaryl heptanoids; NMR; mass spectra; antitumour; cytotoxicity

1. Introduction

Curcumin is the main component of turmeric (Curcumin Longa Linn) and has been used in ancient ayurvedic medicine. It is a yellow pigment and has been isolated from the ground rhizome part of the curcuma plant species. Curcumin has several biological effects exhibiting anti inflammatory[1-4] and antioxidant [5-8] activities. It has also been studied extensively as a chemo preventive agent in several cancer cells[9-16]. Structurally curcuminoids are linear diaryl heptanoids which exist in tautomeric forms as α,β unsaturated 1,3-diketo form and enol form. Curcuminoid analogues prepared by synthesis retain the α,β unsaturated 1,3-diketo moiety and their metal chelates possess remarkable biochemical activity[17-18].

In the present study, aldehydes namely p-chloro benzaldehyde, o-chloro benzaldehyde and dichloro benzaldehyde were condensed with acetylacetone in presence of B_2O_3 using tri-secondary butyl borate and n-butyl amine as the condensing agent [19]. The ligands prepared [1,7-di(4-chloro phenyl)-1,6-heptadiene-3,5-dione(HL1), 1,7- di(2-chloro phenyl)-1,6-heptadiene-3,5-dione(HL2), and 1,7-bis(3,4 dichloro phenyl)-1,6-heptadiene-3,5-dione(HL3)] (Fig.1.1) were complexed with Cu(II) and Al(III) to form metal chelates (Fig.1.2). The curcuminoid analogues and their metal chelates were subjected to *in vitro* cytotoxic studies using trypan blue exclusion method. *In vivo* antitumour studies were conducted in DLA induced mice. The chloro derivatives of curcuminoid analogues and their metal chelates were administered intraperitoneally (i.p.) as drug into the mice and the % increase in life span was calculated and compared with standard drug. The ligands and the metal complexes were also subjected to antibacterial activity against the test organisms *Escherichia coli, Klebsiella pneumoniae and Bacillus subtilis*.

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Fig.1.1: Chloro derivatives of 1,7- diaryl heptanoids

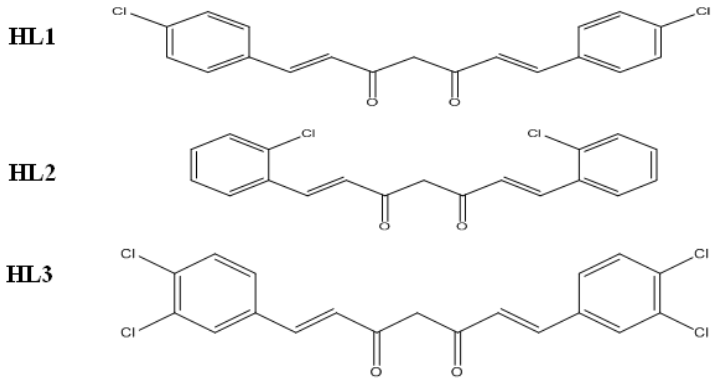
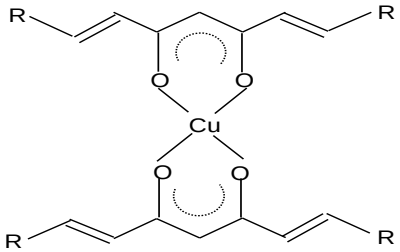


Fig.1.2: Metal chelates of 1,7-diaryl heptanoids



2. Materials and methods

The chemicals required were obtained from Sigma Aldrich chemical suppliers and are of analar grade. Daltons Lymphoma ascites (DLA) and Ehrilich Ascites Carcinoma (EAC) cells were obtained from the Adayar Cancer Research Institute, Chennai, India and propagated as transplantable tumours in Swiss albino mice by injecting a suspension of cells ($1X10^6$ cells/ml) intraperitoneally. Bacterial strains namely *Escherichia coli*, *Klebsiella pneumoniae and Bacillus subtilis* were obtained from the culture collection of Institute of Microbial Technology (IMTECH), Chandigarh, India.

Swiss albino mice were obtained from the Small Animal Breeding Station (SABS), Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur, Kerala. They were kept under standard conditions of temperature and humidity in animal house of Amala Cancer Research Centre. All animal experiments in this study were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC) and were conducted strictly according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (No.149/1999/CPCSEA).

2.1. Analytical instruments

UV spectra were recorded on a Schimadzu UV-VIS-1601 spectrophotometer. IR spectra (KBr pellets) were recorded on 8101 Schimadzu FTIR spectrophotometer. The ^{1}H NMR spectra were recorded on a Varian 300 NMR spectrophotometer. The FAB mass spectra were recorded on a Joel SX–102 mass spectrophotometer from CDRI, Lucknow, India.

2.2. Synthesis of chloro derivatives of 1,7-diaryl-1,6-heptadiene-3,5-diones

The curcuminoid analogues were prepared by the condensation of aldehydes, (p-chloro benzaldehyde, o-chloro benzaldehyde and dichloro benzaldehyde) with acetylacetone-boric oxide complex in ethyl acetate medium in presence of tributyl borate and n-butyl amine. The product was purified by column chromatography over silica gel (60–120 mesh) using 4:1 (v/v) chloroform:acetone mixture as the eluent and recrystallised twice from hot benzene to get pure crystalline material.

2.3. Synthesis of metal complexes.

The Al(III) complexes were prepared by adding a methanolic solution of aluminium nitrate $Al(NO_3)_3.9H_2O$ (25 ml, 0.001mol) to a solution of diketone (25 ml, 0.003 mol) in methanol and refluxed gently for 2 h. After reducing the volume to half, the solution was cooled to room temperature. The precipitated complex was filtered, washed with 1:1, methanol:water mixture and recrystallised from hot methanol. The Cu(II) complexes were

prepared by adding a methanolic solution of copper(II) acetate (25 ml, 0.001mol) to a solution of diketone (25 ml, 0.002 mol) in methanol and the above procedure is repeated.

2.4. *In vitro* cytotoxicity studies

In vitro cytotoxicity studies were carried out using the diketone, Cu(II) and Al(III) complexes dissolved in minimum quantity of DMSO. The tumour cells aspirated from the peritoneal cavity of tumour bearing mice were washed with PBS (Phosphate Buffered Saline) and centrifuged for 15min. at 1500 rpm. Cell viability was determined by trypan blue exclusion method. Viable cells (1×10^6 cells in 0.1 ml) were added to tubes containing various concentrations of the test compounds and the volume was made up to 1ml using PBS. Control tube contains only cell suspension. These mixtures were incubated for 3h at 37^{o}C. Further, cell suspension was mixed with 0.1mol of 1% trypan blue and kept for 2-3 min. and loaded on a haemocytometer. The number of stained (dead) and unstained (live) cells were counted and percentage cytotoxicity was evaluated by trypan blue exclusion method [20].

2.5. Antibacterial assay (Agar well diffusion method)

Agar plates were prepared using sterile Muller-Hinton (MH) agar medium. Bacterial strains of *Escherichia Coli, Klebsiella Pneumoniae* and *Bacillus Subtilis* of 24 h culture were evenly spread into the surface of the agar plates using sterile swab sticks. Wells were cut into agar plates with sterile gel puncture. The curcuminoid analogues and their metal chelates in the concentration 5 mg/ml in DMSO were added in the cells. The pure solvent DMSO act as negative control and streptomycin (5mg/ml) served as positive control. The plates were incubated at 37^{o}C for 24 h and observed for zones of inhibition. The antibacterial activity was measured in terms of mean diameter of the zone of inhibition in mm.

2.6. *In vivo* antitumour activity

Animals (male mice, 6-8 weeks old) weighing 28-30g were divided into 11 groups of 5 animals each. Viable DLA cells ($1X10^6$) in 0.1ml of phosphate buffered saline (PBS) were injected into the peritoneal cavity of mice. Group1, Control: Oral administration of 0.1 ml of distilled water/animal. Group 2, Standard: Cyclophosphamide 25mg/kg body weight. Group 3-5: Ligand, 1,7-di(4-chloro phenyl) -1,6-heptadiene -3,5-dione with concentrations 20µg/ml, 10µg/ml and 5µg/ml was given as drug. Group 6-8 & 9-11: Al(III) & Cu(II) metal chelates as drug with concentrations 20µg/ml, 10µg/ml & 5µg/ml respectively. Ligand, complexes and cyclophosphamide were given by i.p. injection from the 1stday of tumour induction upto 10 days. The death pattern of animals due to tumour burden was noted and the percentage increase in life span (ILS) was calculated.

[% ILS= {(T – C)/ C} X 100, where T and C are mean survival of treated and control mice respectively.]

3. Results

3.1. Structural characterization of chloro analogues of 1,7-diaryl-1,6-heptadiene-3,5-diones

The synthesized compounds, 1,7-di(4-chloro phenyl)-1,6-heptadiene-3,5-dione(HL1), 1,7- di(2-chloro phenyl)-1,6-heptadiene-3,5-dione(HL2), and 1,7-bis(3,4 dichloro phenyl)-1,6-heptadiene-3,5-dione(HL3) were characterized on the basis of UV, IR, ^{1}HNMR and Mass spectral data **(Table 3.1).**

Table 3.1: UV, IR, 1H NMR & Mass Spectral data of chloro substituted 1,7-diaryl heptanoids.

Compound	UV data Λ max (nm)	IR data cm-1 ν(C=O)	1H NMR spectral data Chemical shift (ppm)				Mass spectral data (m/z)
			Enol	Methine	Phenyl	Alkenyl	
HL1	269,376	1620	15.92	5.89	7.16-7.78	6.64-8.07	345,329 289,229
HL2	225,307	1639	16.025	5.92	7.4-7.8	6.4-8.05	344,230 169,115
HL3	246,332	1628	16.02	5.9	7.35-7.75	6.4-8.1	414,382 347,339

3.2. Structural characterization of metal complexes

Chloro derivatives of1,7-diaryl heptanoids form well defined crystalline complexes with Al(III) and Cu(II) ions. Analytical and mass spectral data are given in (**Table 3.2).** The approximate formulae of the metal complexes are CuL_2 and AlL_3.

Table 3.2: Spectral data of Cu(II) and Al(III) complexes of chloro derivatives of 1,7-diaryl heptanoids.

Complex	UV spectra Λ max (nm)	IR data (cm-1)		Mass spectral data (m/z)
		V C=O	V M-O	
$Cu(L1)_2$	272,378	1593	423, 462	755,528,345,302,226,113
$Cu(L2)_2$	228,304	1591	423,472	754,718,682,529,345,303,227
$Cu(L3)_2$	250,333	1598	430,460	894,747,600,415,290,145,115
$Al(L1)_3$	270,378	1598	410,465	1062,836,610,346,226,113
$Al(L2)_3$	230,305	1600	430,465	1064,835,609,346,226,115
$Al(L3)_3$	253,334	1608	441,475	1270,976,666,416,288,143

3.3. *In vitro* cytotoxicity

The results of *in vitro* cytotoxicity of 1,7-di(4-chloro phenyl)-1,6-heptadiene-3,5-dione(HL1), 1,7- di(2-chloro phenyl)-1,6-heptadiene-3,5-dione(HL2), and 1,7-bis(3,4 dichloro phenyl)-1,6-heptadiene-3,5-dione(HL3) and their complexes (Cu(II) and Al(III)) towards EAC and DLA are given in (**Fig. 3.1& Fig. 3.2**). The graph was plotted with % cell death on y axis. The diketones and their metal complexes are given as drug in concentrations 200µg/ml, 100µg/ml, 50µg/ml, 20µg/ml & 10µg/ml. The number of stained and unstained cancer cells were counted and evaluated as % cell death.

Fig. 3.1: *In vitro* cytotoxicity of chloro compounds and their metal chelates towards EAC

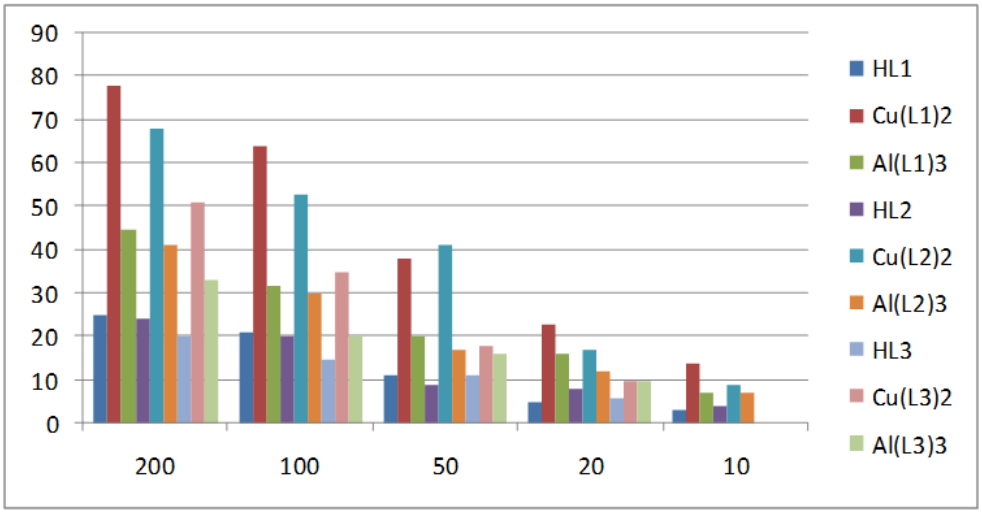
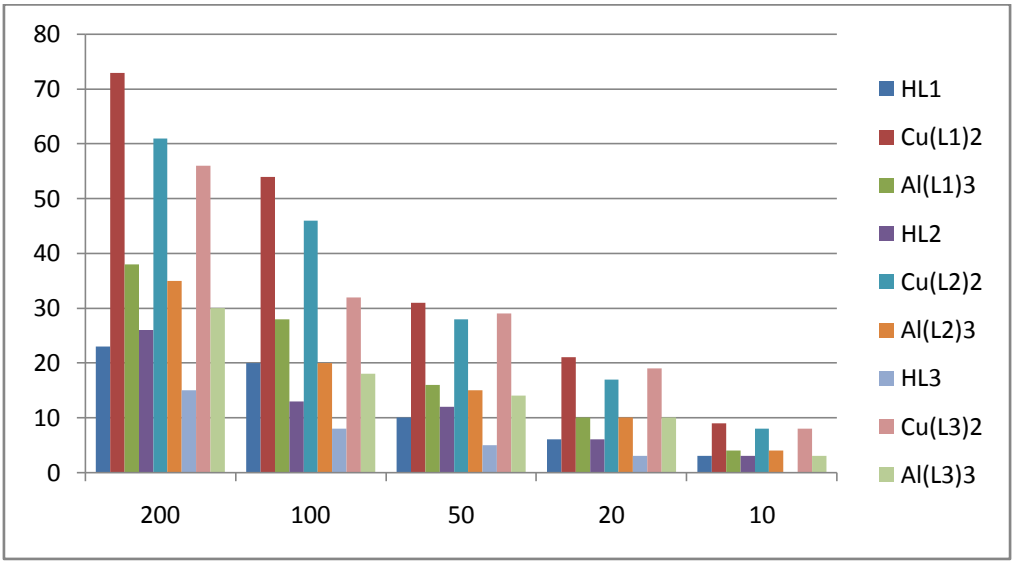


Fig. 3.2: *In vitro* cytotoxicity of chloro compounds and their metal chelates towards DLA



3.4. Antibacterial activity

The results of the antibacterial activity of 1,7-di(4-chloro phenyl)-1,6-heptadiene-3,5-dione(HL1), 1,7-di(2-chloro phenyl)-1,6-heptadiene-3,5-dione(HL2), and 1,7-bis(3,4- dichloro phenyl)-1,6-heptadiene-3,5-dione (HL3) and their metal complexes (Cu II & Al III) are given in **Table 3.3.**

Table 3.3: Antibacterial activity of chloro derivatives 1,7-diaryl heptanoids and their metal complexes

Bacteria	Diameter of zone of inhibition in mm.								
	HL1	$Cu(L1)_2$	$Al(L1)_3$	HL2	$Cu(L2)_2$	$Al(L2)_3$	HL3	$Cu(L3)_2$	$Al(L3)_3$
E Coli	13.5	16	17.5	13	17	19.5	11	15.5	18
Klebsiella	11	12.5	15	12	15	17	7.5	9	13.5
Bacillus	4.5	6	8.5	6	11	13.5	4	6	8.5
Streptomycin (std)	25	25	25	25	25	25	25	25	25

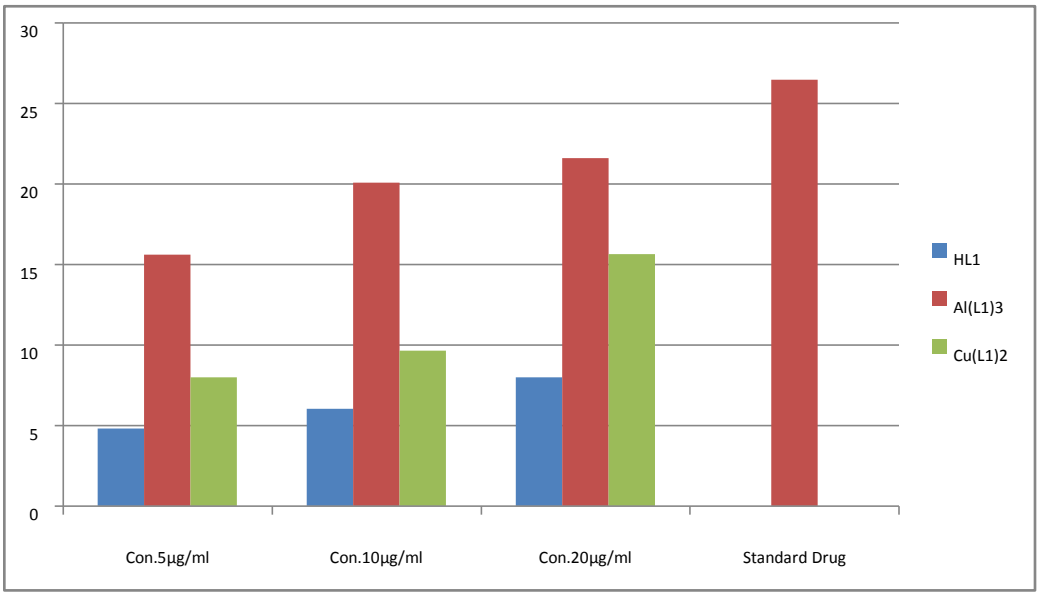
3.5. Effect of compounds on ascites tumour reduction

The ligand p-chloro 1,7 diaryl heptanoid (HL1) & its metal complexes were given as drug and the survival of animals are given in **Table 3.4**. The control and the group with std. drug cyclophosphamide are also given in the table. The values of No. of days survived are means of five determinations ±SD (standard deviation). The increase in life span corresponding to drugs HL1, $Al(L1)_3$ and $Cu(L1)_2$ with varying concentrations is also given in **Fig. 3.3.**

Table 3.4: Effect of compounds on ascites tumour reduction (*in vivo*)

Animal groups	Concentration µg/ml	No. of animals With tumour	No. of days Survived	% ILS
1. Control		5/5	16.6±1.49	
2.Standard drug		5/5	21.0±5.09	26.5
3.HL1	20	5/5	18.0±2.60	8.0
4.HL1	10	5/5	17.6±2.72	6.02
5.HL1	5	5/5	17.4±2.87	4.8
6.Al(L1)3	20	5/5	20.2±2.63	21.6
7.Al(L1)3	10	5/5	19.6±2.65	20.1
8.Al(L1)3	5	5/5	19.2±2.71	15.6
9.Cu(L1)2	20	5/5	19.2±2.92	15.6
10Cu(L1)2	10	5/5	18.4±2.15	9.64
11.Cu(L1)2	5	5/5	18.0±2.83	8.0

Fig.3.3: *In vivo* antitumour activity (% ILS)



4. Discussion

4.1 Characterization of chloro analogues of 1,7-diaryl-1,6-heptadiene-3,5-diones

The synthesized chloro derivatives of 1,7-diaryl heptanoids were characterized by various analytical techniques **(Table 3.1**). The UV spectra of the compound in methanol show two absorption maxima corresponding to $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions. The IR spectra of compounds show a strong band ~ 1620 cm^{-1} assignable to intra molecularly hydrogen bonded carbonyl function. The ^{1}HNMR spectra of the compounds show peaks due to enol, methine, phenyl and alkenyl group. Peaks corresponding to step wise elimination of aryl groups and small fragments are present in the mass spectra.

4.2 Characterization of metal complexes

Analytical and mass spectral data (**Table 3.2**) clearly suggest a ML_3 stoichiometry for Al(III) and ML_2 for Cu(II) complexes. In the IR spectra of metal chelates, the bond due to intra molecularly hydrogen bonded carbonyl function of the ligand at ~1620 cm^{-1} disappeared and instead a strong band assignable to stretching of the coordinated carbonyl moiety appeared at ~ 1600 cm^{-1}. Additional bands appear at ~ 475 cm^{-1} and ~ 420 cm^{-1} assignable to ν (M–O) vibration. The mass spectra of complexes showed relatively intense peak at m/z corresponding to AlL_3 and CuL_2, respectively.

4.3 *In vitro* cytotoxicity

The % cytotoxicity was plotted in the graph. Both in ligands as well as metal complexes, 200µg/ml concentration show maximum activity. It is also noted that metal chelation enhances cytotoxicity of compounds considerably. The copper complexes of 1,7-diaryl heptanoids show better results than that of ligands as well as aluminium complexes in almost all concentration.

4.4 Antibacterial activity

The results clearly reveal that the ligands and their complexes possess comparable antibacterial activity to that of standard drug streptomycin. In all the cases, metal complexes possess better antibacterial activity than that of ligands, which means that metal complexation enhance activity. Out of the two metals, aluminium complexes show maximum antibacterial activity. Also, it is found that, out of the chloro compounds, o-chloro derivative show better results than p-chloro and dichloro compounds.

4.5. Effect of compounds on ascites tumour reduction (*in vivo*).

The animals of the tumour control group inoculated with DLA survived for a period 16.6±1.49 days. The treatment with cyclophosphamide, survived for 21±5.09 days. The animals which were given the drug 1,7-di(4-chloro phenyl)-1,6-heptadiene 3,5-dione, survived for18±2.6 days with the concentration 20µg/ml. The values of ligand and complexes given are comparable to that of std. drug cyclophosphamide. The increase in life span for $Al(L1)_3$ was maximum (21.6%) with 20µg/ml con. This is also comparable to that of cyclophosphamide (26.5%).

The ongoing discussion reveals that the chloro derivatives of 1,7-diaryl heptanoids and their metal complexes possess enhanced antitumour (both *in vivo & in vitro*) activity. The metal chelation considerably enhances the cytotoxicity of these compounds. Also it is found that Cu(II) complex of p-chloro derivative, is the most active compound in *in-vitro* cytotoxicity studies both with EAC and DLA than Al(III) complexes. The antibacterial studies clearly show that both ligand and metal complexes enhanced the activity. The Al(III) complexes show better antibacterial activity than Cu(II) complexes and ligands. The *in vivo* antitumour studies of p-chloro derivative of Al(III) complexes show more activity than Cu(II) and ligands. The results are very much comparable to that of std. drug cyclophosphamide.

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