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SYNTHESIS, CHARACTERIZATION, AND BIOLOGICAL STUDIES OF 1,7-DIHETEROARYL-1,6-HEPTADIENE-3,5-DIONE AND THEIR METAL COMPLEXES

S.Sindhu, Seena Thomachan, John V.D.

Department of Chemistry, Christ College, Irinjalakuda, Kerala, India. 680125
Affiliated to University of Calicut
E mail: sindhujoshy1995@gmail.com

Abstract:
The present investigation mainly emphasis on the synthesis and structural characterization of two new curcuminoid analogues containing heterocyclic ring systems (thiophene and related compounds) and their Cu(II) and Al(III) chelates. The anti-tumour (*in vivo & in vitro*) activity and antibacterial activity of these ligands and their metal complexes were also investigated. The synthesis of the curcuminoid analogues were done by the condensation of acetyl acetone with a heterocyclic aldehyde in presence of boron trioxide, tri -secondary butyl borate using n-butyl amine as the condensing agent. The compounds synthesized were characterized by UV, IR, ^{1}HNMR, ^{13}C and Mass spectral techniques. Structurally curcuminoid analogues are 1,7 – diaryl heptanoids which due to the presence of α,β – unsaturated 1,3 - diketo moiety are expected to form a large number of coordination compounds with metals like Al(III), Cu(II) etc. The curcuminoid analogues and their metal complexes were studied for their antibacterial and antitumour (*in vitro)* activity using agar well diffusion method and trypan blue exclusion method respectively. 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 span of the treated animals were increased upto 77% than that of the standard drug with thiophene Al(III) complex. 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–diheteroarylheptanoids; curcuminoids; cytotoxicity; antimicrobial activity; antitumour studies.

Introduction

Curcuminoids are bioactive yellow orange pigments from turmeric, Curcuma Longa. *Linn*. Turmeric has been used for centuries in Ayurvedic medicine. Based on this traditional usage dietary supplements containing turmeric rhizome and turmeric extracts are also being used nowadays. The curcuminoids occurring naturally in turmeric are curcumin, demethoxy curcumin and bis demethoxy curcumin. Structurally curcuminoids are linear diaryl heptanoids which exist in tautomeric forms including a α,β – unsaturated 1,3 – diketo form

and an enol form. Curcuminoid analogues prepared by synthesis, retain the α,β – unsaturated 1,3 – diketo moiety in them. In the present study the aryl rings in natural curcuminoids are replaced with heterocyclic and substituted heterocyclic rings. Research has identified curcumin as the agent responsible for the most of the biological activity of turmeric exhibiting anti-inflammatory [i-v], antioxidant & anifungal activities [vi-xiii]. Chemopreventive activities of curcumin has also been well studied [xiv-xvi]. There were very few literatures available with the use of heterocyclic aldehydes for the synthesis of curcumin related compounds. Here introduction of heteroclic compounds was done by condensing thiophene - 2-carboxaldehyde and 3-methyl thiophene-2-carboxaldehyde with acetyl acetone in presence of B_2O_3 & tri – sec. butyl borate using n-butyl amine as the condensing agent [xvii]. The products formed are 1,7-di(thiophenyl)-1,6-heptadiene-3,5-dione(HL_1) and 1,7-di(3-methyl thiophenyl)-1,6-heptadiene-3,5-dione(HL_2) respectively. Curcumin has a highly conjugated β – diketo moiety and thus act as a powerful natural chelating agent. Complexation of curcumin with metals has attracted much interest over the past years. Curcuminoid analogues can also form stable chelate complexes with great range of metal ions. Metals like Cu(II) & Al(III) were used for complexation in the present study. The curcuminoid analogues with heterocyclic rings and their metal complexes were studied for their antibacterial and antitumour activity using agar well diffusion method and trypan blue exclusion 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

Materials and methods

The chemicals required were obtained from Sigma Aldrich chemical suppliers and are of analar grade. Two different heterocyclic aldehydes, thiophene-2-carboxaldehyde and 3-methyl thiophene-2-carboxaldehyde were used along with the 1,3 - diketone, acetyl acetone. Metal salts used for synthesis were Cu(II) acetate mono hydrate and Al(III) nitrate. Commercial solvents like methanol, acetone, chloroform, ethyl acetate were used in the synthesis of curcuminoid analogues and their metal complexes. Daltons Lymphoma Ascites (DLA) and Ehrilich Ascites Carcinoma (EAC) cells were obtained from the Cancer research institute, Mumbai, India. Bacterial stains namely Escherichia Coli, Klebsiella Pneumoniae & 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)

Analytical Instruments:

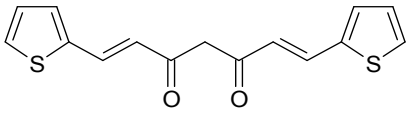
UV spectra were recorded on a Schimadzu UV – VIS – 1601 spectrophotometer. IR spectra (KBr pellets) were recorded on Schimadzu 81101 A FT IR spectrophotometer. The ^{1}H NMR spectra were recorded on a Varian 300 NMR spectrophotometer. The FAB mass spectra were recorded on a Joel SX – 102 mass spectrophotometer from CDRI, Lucknow, India.

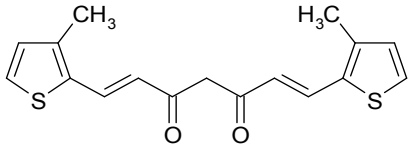
Synthesis of 1,7 – diheteroaryl – 1,6 – heptadiene – 3,5 – diones:

The curcuminoid analogues were prepared by the condensation of aldehydes (thiophene – 2-carboxaldehyde and 3– methyl thiophene –2carboxaldehyde) 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(Figure 1).

HL_1 (1,7-di(thiophenyl)-1,6-heptadiene-3,5-dione)





HL_2 (1,7-di(3-methyl thiophenyl)-1,6-heptadiene-3,5-dione)

Figure 1. 1,7-Diheteroaryl heptanoids

Preparation of metal complexes :
The Al(III) complexes were prepared by adding a methanolic solution of aluminium nitrate $Al(NO_3)_3.9H_2O$ (25ml, 0.001mol) to a solution of diketone (25ml, 0.003mol) in methanol and refluxed gently for 2 hours. 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 (Figure 2) were prepared by adding a methanolic solution of copper(II) acetate (25ml, 0.001mol) to a solution of diketone (25ml, 0.002mol) in methanol and the above procedure is repeated.

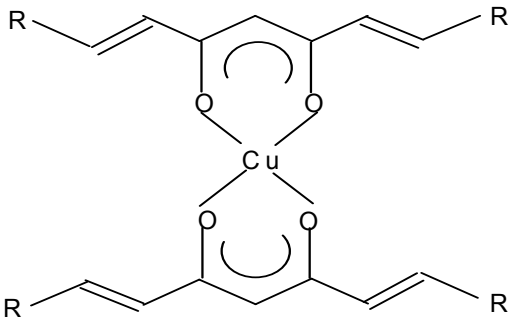


Figure 2. Metal chelates of 1,7-diheteroaryl heptanoids

In vitro cytotoxicity study:
In vitro cytotoxicity studies were carried out using the diketone, Cu(II) and Al(III) complexes. These compounds (as drugs) with concentrations 200, 100, 50, 20 & 10 μg/ml, were dissolved in minimum quantity of DMSO. The tumour cells (DLA & EAC), aspirated from the peritoneal cavity of tumour bearing mice were washed with PBS (Phosphate buffered saline) solution. Cell viability was determined by Trypan blue exclusion method. Viable cell separation (1×10^6 cells in 0.1ml) was added to tubes containing various concentrations of the test compounds and the volume was made up to 1ml using PBS. Control tube contained only cell suspension. These assay mixture were incubated for 3 hours at 37^oC. Further cell suspension was mixed with 0.1mol of 1% Trypan blue and kept for 2-3 minutes

and loaded on a haemocytometer. The number of stained (dead cells) and unstained (live) cells were counted and % cytotoxicity was evaluated by Trypan blue exclusion method [xviii].

Antibacterial assay (agar well diffusion method):

Agar plates were prepared using sterile Muller-Hinton (MH) agar medium. Bacterial strains of Escherichia Coli, Klebsiella Pneumoniae & Bacillus Subtilis of 24 hour culture were evenly spread onto 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 5mg/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 hours and observed for zones of inhibition. The antibacterial activity was measured in terms of mean diameter of the zone of inhibition in mm.

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 ofdistilled water/animal. Group 2, Standard: Cyclophosphamide 25mg/kg body weight.Group3-5: Ligand, 1,7-di(thiophenyl)-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 ip. 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.]

Results and Discussion

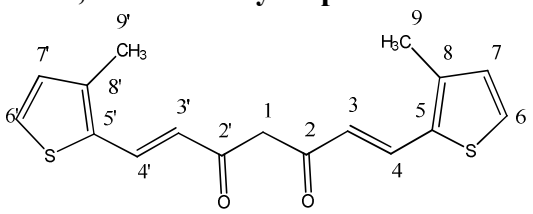
Structural characterization of 1,7-diheteroaryl-1,6-heptadiene-3,5-diones

1,7– Dithiophenyl –1,6 –heptadiene – 3,5 – dione and 1,7–di – (3 – methyl thiophenyl) – 1,6 heptadiene 3,5 – diones synthesized are crystalline in nature, black in colour, show sharp melting points and are freely soluble in organic solvents. The compounds prepared were characterized on the basis of UV, IR, ^{1}HNMR, ^{13}C and Mass spectral data (Table 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 ~ 1640 cm^{-1} assignable to intra molecularly hydrogen bonded carbonyl function. The ^{1}H NMR spectra of the compounds show peaks due to enol, methine, thiophenyl and alkenyl proton. Additionally compound HL_2 shows a peak at δ ~ 2.1 ppm due to methyl group. Peaks corresponding to step wise elimination of aryl groups and small fragments are present in the mass spectra. The ^{13}C spectra of the compounds are given in Table 3. A peak at δ~ 106 ppm is a clear evidence of methine which is flanked between two keto groups. Alkenyl peaks are also present at δ ~ 60 ppm. The elemental analysis data, molecular determination and mass spectral data of the compounds suggest that two equivalents of the heterocyclic aromatic aldehyde has condensed with one equivalent of acetyl acetone resulting in a bis – condensation product.

Table.1. UV, IR, ^{1}H NMR & Mass spectral data of 1,7- diheteroaryl heptanoids

Ligand	UV data λ max (nm)	IR data cm^{-1} ν(C=O)	1HNMR spectral data (δ ppm)					Mass spectral data (m/z)
			Enol	Meth-ine	Methyl	Thio-phenyl	Alkenyl	
HL_1	233, 331	1647	16.1	6.3	-----	7-7.15	6.7-7.9	290,246,137,109,65
HL_2	243, 338	1638	16.2	6.1	2.1	7.0-7.3	6.9-7.6	317,275,233,193, 151,123,111

Table.2. ^{13}C NMR spectra of 1,7-diheteroaryl heptanoids



Ligand	C1	C2,C2'	C3,C3'	C4,C4'	C5,C5'	C6,C6'	C7,C7'	C8,C8'	C9,C9'
HL1	106.8	190.99	60.96	56.28	153.67	143.7	143.7	131.73	___
HL2	107.43	189.5	59.45	57.63	150.13	146.25	140.22	141.3	21.6

Structural characterization of metal complexes

1,7-Dihetero aryl heptanoids form well defined crystalline complexes with Al(III) and Cu(II) ions. Analytical and mass spectral data of metal complexes are given in Table 3 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 ~1640 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. In the UV spectra, the peaks corresponding to $n\rightarrow \pi*$ and $\pi \rightarrow \pi*$ transitions in ligands are not altered much. The mass spectra of complexes showed relatively intense peak at m/z corresponding to AlL_3 and CuL_2, respectively. Thus the analytical and mass spectral data clearly suggest a ML_3 stoichiometry for Al(III) and ML_2 for Cu(II) complexes

Table.3. Spectral data of Cu (II) and Al (III) complexes of 1,7 – diheteroaryl heptanoids

Complex	UV spectra λ_{max}	IR data cm-1		Mass spectral data (m/z)
		$\nu(C=O)$	ν(M-O)	
$Cu(L_1)_2$	235,330	1614	479,423	639,475,289,166,83
$Cu(L_2)_2$	335,242	1606	492,435	697,682,500,307,119
$Al(L_1)_3$	229,337	1591	477,423	890,725,642,290,165
$Al(L_2)_3$	240,332	1610	485,440	977,890,665,636,587,389

In vitro cytotoxicity

The diketones (HL_1 & HL_2) and their Cu(II) and Al(III) complexes show much *in vitro* cytotoxicity towards EAC and DLA cells. The results indicate that metal chelation enhance cytotoxicity of compounds considerably. The compounds (as drugs) with concentration 200µg/ml show maximum activity. The thiophene Cu(II) complex show a value of about 92% cell death. The copper complexes of 1,7-diharyl heptanoids show better results than that of ligands as well as aluminium complexes (Figure 3 & Figure 4)

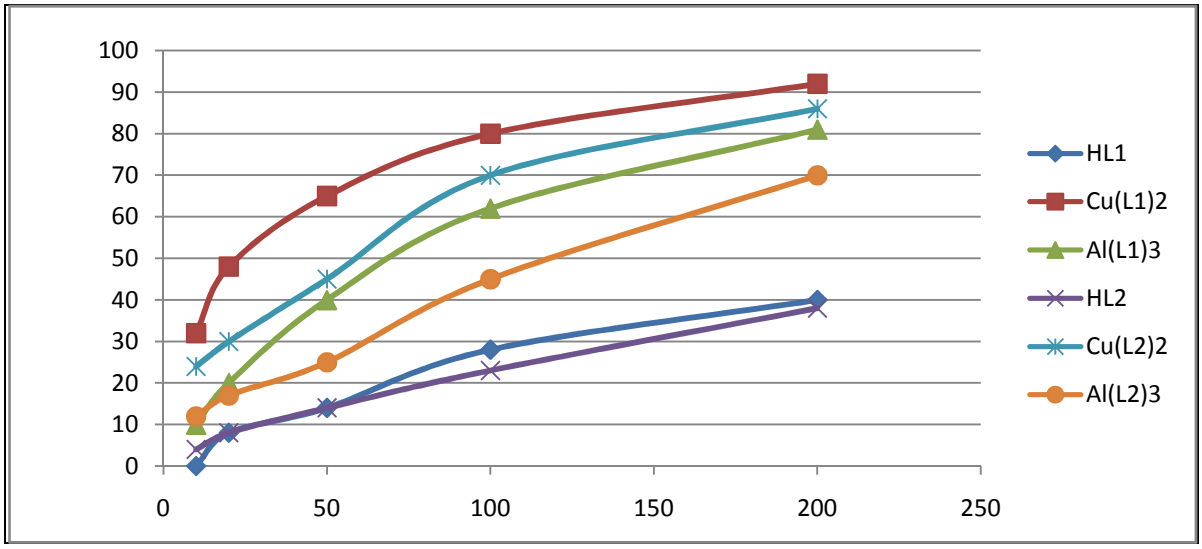


Figure 3. *In vitro* cytotoxicity of compounds towards EAC

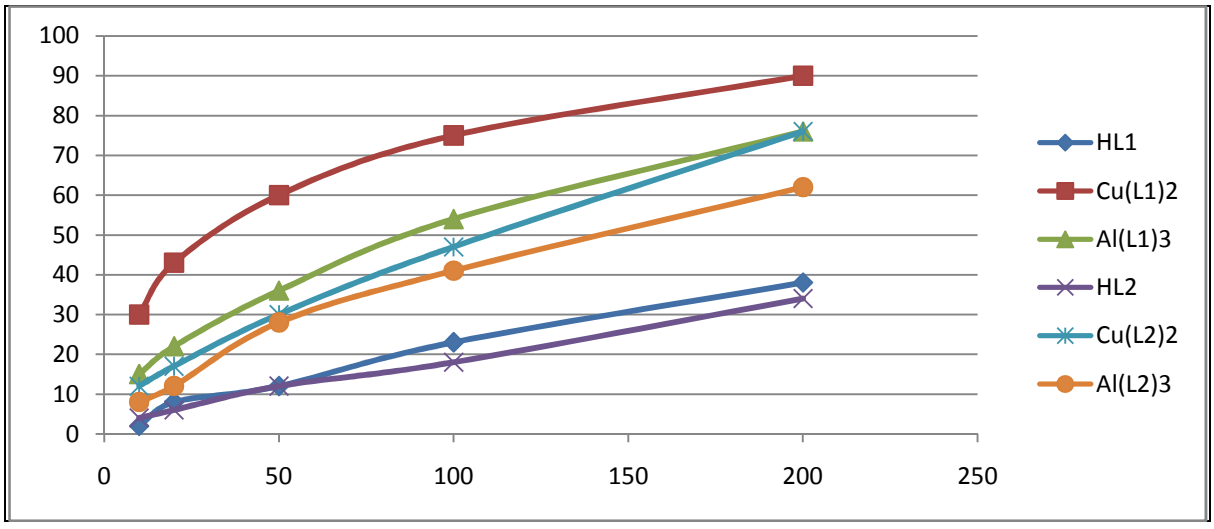


Figure 4. *In vitro* cytotoxicity of compounds towards DLA

Antibacterial activity

The results of the antibacterial activity of 1,7-diaryl heptanoids and their complexes revealed 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 (Figure 5 & Figure 6).

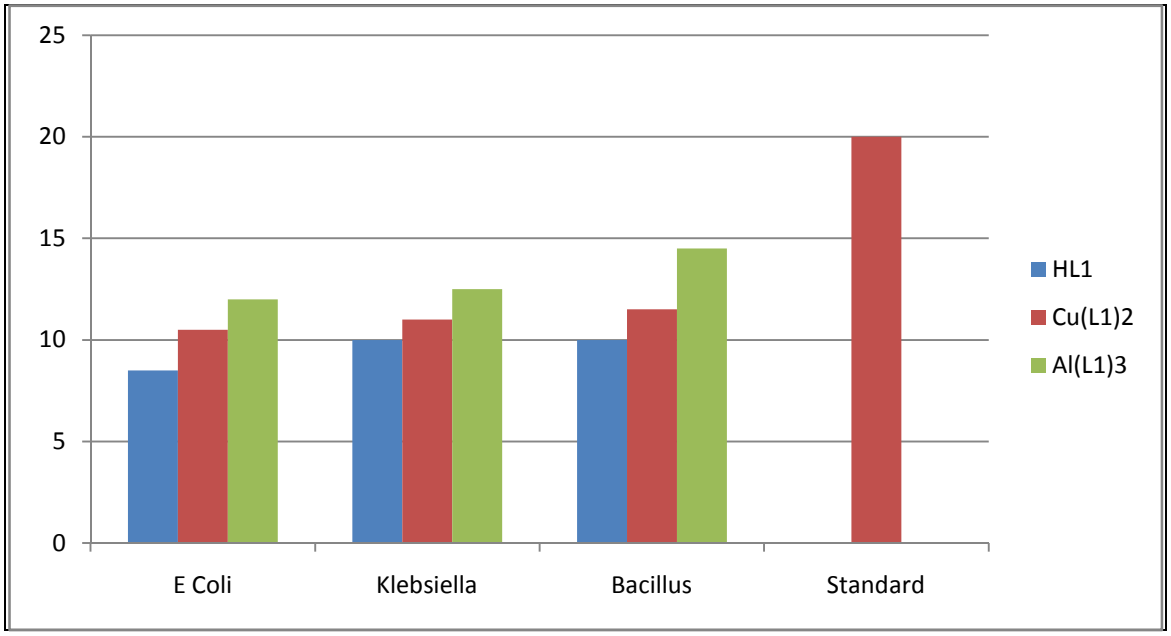


Figure 5. Antibacterial activity of 1,7-di(thiophenyl)-1,6-heptadiene-3,5-dione and their metal complexes.

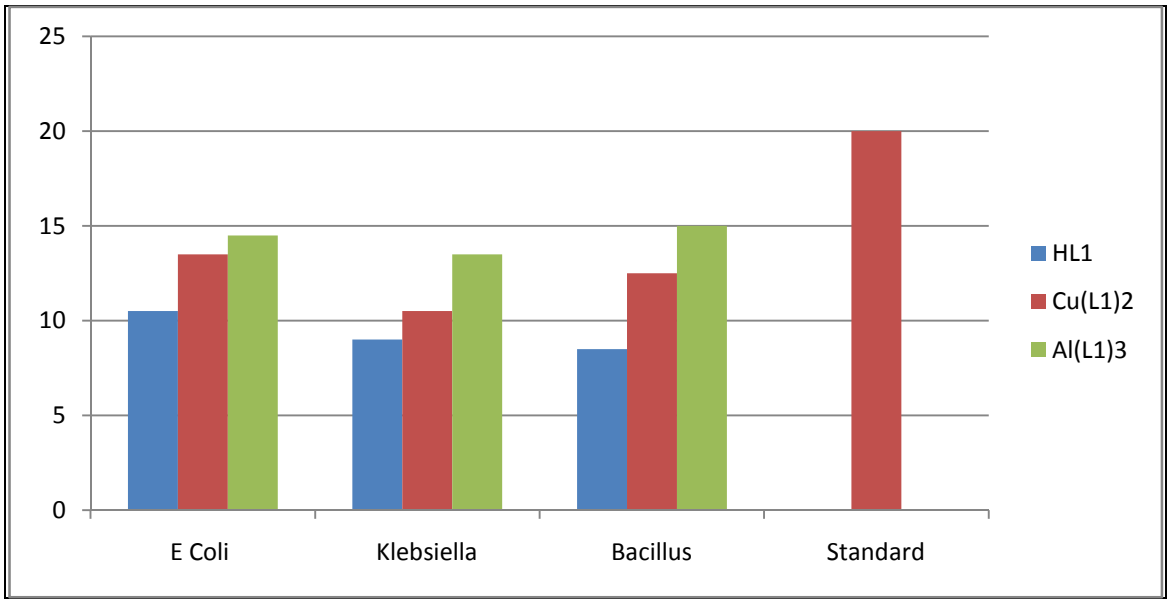


Figure 6. Antibacterial activity of 1,7-di(3-methyl thiophenyl)-1,6-heptadiene-3,5-dione and their metal complexes

Effect of compounds on ascites tumour reduction(in vivo)

The ligand 1,7-di(thiophenyl)-1,6-heptadiene-3,5-dione(HL_1) & its metal complexes Al(III) & Cu(II) were given as drug and the survival of animals are given in Table 4. The control group 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 $\pm SD$ (standard deviation). The increase in life span (% ILS) corresponding to drugs HL_1, $Al(L_1)_3$ and $Cu(L_1)_2$ with varying concentrations compared with std. drug cyclophosphamide is also given in Figure 7.

Table 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.HL_1	20	5/5	17.8±3.74	7.2
4.HL_1	10	5/5	17.±2.82	2.4
5.HL_1	5	5/5	17.2±2.15	3.66
6.$Al(L_1)_3$	20	5/5	24.4±3.26	46.9
7.$Al(L_1)_3$	10	5/5	20.4±3.26	19.4
8.$Al(L_1)_3$	5	5/5	19.0±3.22	14.5
9.$Cu(L_1)_2$	20	5/5	19.8±2.85	19.28
10$Cu(L_1)_2$	10	5/5	18.8±2.71	13.25
11.$Cu(L_1)_2$	5	5/5	17.4±2.65	4.82

The animals of the tumour control group inoculated with DLA survived for a period 16.6±1.49days. The treatment with cyclophosphamide, survived for 21±5.09 days. The values of ligand and complexes given are comparaed to std. drug cyclophosphamide. The animals which were given the drug, the aluminium complex of 1,7-di(thiophenyl)-1,6-heptadiene-3,5-dione, survived for 24.4±3.26 days with the concentration 20µg/ml. This value is astonishing since the results are higher than that of std. drug . The increase in life span for $Al(L_1)_3$ was maximum (46.9%) with 20µg/ml con. This is also more than that of cyclophosphamide (26.5%).

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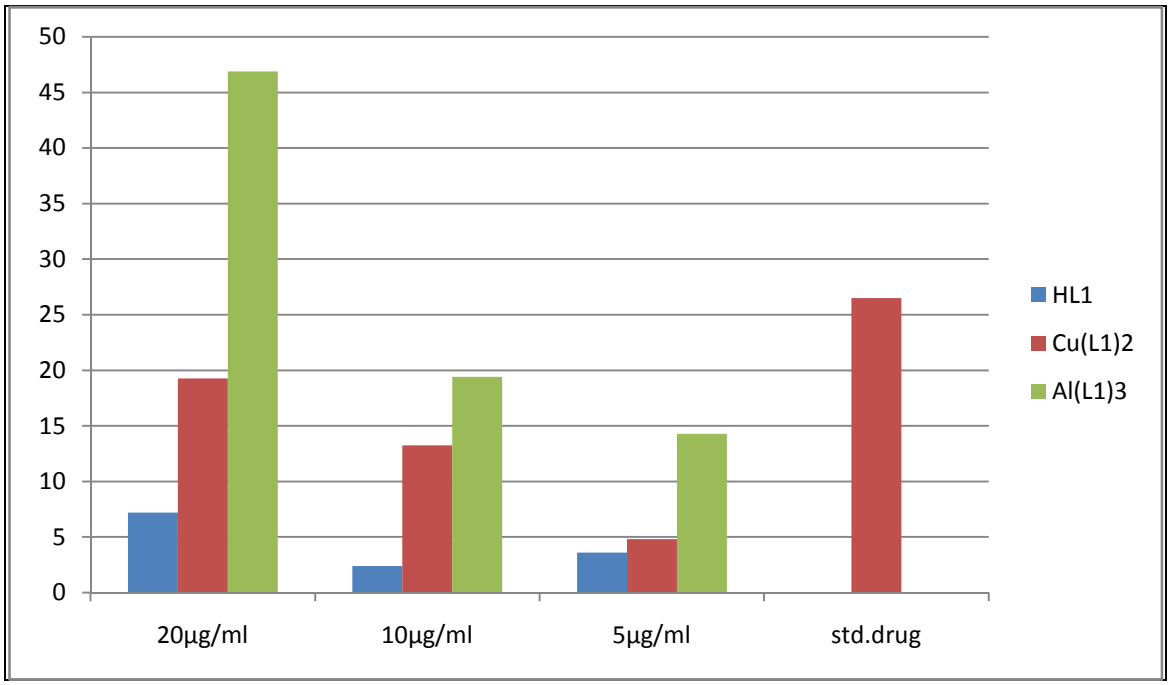

Figure 7. Percentage increase in life span in *in vivo* anti tumour studies

Conclusion

The ongoing discussion reveals that the derivatives of 1,7-diheteroaryl heptanoids and their metal complexes possess enhanced antitumour (both *in vivo & in vitro*) activity. The metal chelation considerably enhance the cytotoxicity of these compounds. Also it is found that Cu(II) complexes are the most active compounds 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 have enhanced activity. The Al(III) complexes show better antibacterial activity than Cu(II) complexes and ligands. The *in vivo* antitumour studies of 1,7-dithiophenyl heptanoids of Al(III) complexes show more activity than Cu(II) and ligands. The Al(III) complexes with con.200μg/ml show tremendous *in vivo* anti tumour activity which even more than that of std. drug cyclophosphamide.

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