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Cytotoxic & Antitumour Activities of (4e,6e)-4,6-Bis (Aryl) Cyclohexane1,3 Dione and Their Copper Complexes

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ABSTRACT

Four curcuminoid equivalents and their copper (II) complexes with a metal ligand ratio 1:2 were prepared and characterized. Trypan blue exclusion method is adopted for the in vitro cytotoxic examination of the test compounds and their ML_2 stoichiometric complexes on the Daltons Lymphoma Ascites cells.It is found that (4E,6E)-4,6-bis(thiophen-2-ylmethylidene) cyclohexane-1,3-dione and its copper complexes shows a significant response in increasing the life period of tumour induced mice by decreasing the volume of the solid tumour.

KEYWORDS: Curcuminoids, IR, NMR, mass spectra, cytotoxicity, antitumour.

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INTRODUCTION

Curcumin which is an organic compound and accountable for the yellow pigment of turmeric.It also exhibit strong antiinflammatory[1-3], antifungal, ant proliferative, antioxidant [4] and anticarcinogenicproperties[5-11].Curcumin, demethoxycurcumin and bisdemethoxycurcumin are the three various types of curcuminoids present in turmeric. They possess linear 1, 7-diaryl-1,6-heptadiene-3,5-diones structure and continue in dynamic equilibrium with its enolic form. The curcuminoid analogues maintain α, β unsaturated 1,3-diketo component of the organiccurcumin except the aryl rings are altered. The inadequate bioavailability of the curcumin can be enhanced by adopting structural analogues of curcumin.

The metal chelates formation of curcuminoids is very similar to that of 1,3-diketones and it exhibit excellent biochemical properties[12]. The antioxidant and free radical scavenging properties of curcumin help them to possess anticarcinogenic character. Furthermore, they periphrastically enhance glutathione levels, by means of supporting the detoxification of mutagens and carcinogens. Curcuminoids are potent chelating operative and can be used in therapy. Chelation therapy is a remedial treatment that associates the application of chelating agents to eliminate heavy metals from the body[13,14]. These ligands tie heavy metals such as cadmium and lead, which may lead to the decreasing the toxicity of these heavy metals. The metal chelating abilities of curcuminoids is through the β diketo group [15,16]which in turn forms new structural entities with modified biochemical activities. The physical and chemical aspect like the planarity, aquaphobicity, dimensions and type of the ligand, as well as the coordination geometry of the metal chelates have taken a critical part in determining the bonding mode of copper complexes to DNA. In agreement with this information, much number of copper complexes has been tested as anticancer drugs.

In the current study, we have synthesized and characterized four curcuminoid analogues and their copper complexes from cyclic 1,3 diketones. EAC and DLA cells were selected for the checking the cytotoxic response of curcuminoid analogues

EXPERIMENTAL

Materials

The best reagent grade chemicals needed for the analysis and it were solicited from Sigma Aldrich India chemical company. Daltons Lymphoma Ascites cells and Erlich Ascites Carcinoma cells were obtained from Adayar Cancer Research Institute, Chennai, India. These biological samples were preserved as transplantable tumours in Swiss albino mice by introducing a suspension of cells intraperitoneally (ip). Swiss albino mice are required for the experiments and it were procured from Vetenary College, Thrissur, Kerala, India. These biological samples were preserved as

under standard circumstances of temperature and humidity in animal house of Amala Cancer Research Centre. The animals were nourished with normal mouse chow (Lipton India) and water *ad libitum.*

Synthesis of (4E,6E)-4,6-bis (aryl)cyclohexane1,3- dione

The compound was prepared by the reaction of aldehyde with cyclohexane-1,3 dione-boric oxide complexes. Ethyl acetate was used as solvent and the condensation is carried out in the presence of tributyl borate and n-butyl amine (Pabons Method)[17]. The synthesized compounds were purified by column chromatography by using a mixture of chloroform and acetone with a respective ratio 4:1 as eluent. For better purification it was recrystallized from hot benzene.

Synthesis of metal complexes of (4E,6E)-4, 6-bis (aryl)cyclohexane1,3- dione

To a retreated solution of diketone (0.002mol) in methanol (25ml), an aqueous solution of copper salt (0.001mol) was combined and the reaction blend was shrinkage for almost 4 hrs and lowered to room temperature. The precipitated complex was filtered, washed with a mixture of methanol and water with a ratio of 1:1 and recrystallized against hot methanol.

ANTITUMOR ACTIVITY

In vitro cytotoxicity studies

The test compounds were studied for short term in vitro cytoxicity using DLA cells. The tumour cells aspirated from the peritoneal cavity of tumour bearing mice were washed thrice with PBS or normal saline. Cell viability was determined by Trypan blue exclusion method[18]. Viable suspension (1×10^6 cells in 0.1 ml) was added to tubes containing various concentrations of the test compounds and the volume was made up to 1 ml using Phosphate Buffered Saline (PBS). Control tube contained only cell suspension. These assay mixture were incubated for 3 hour at 37^0C.Further cell suspension was mixed with 0.1ml of 1%Trypan blue and kept for 2-3 minutes and loaded on a haemocytometer. Dead cells take up the blue colour of Trypan blue while live cells do not take up the dye.

$$\%\, cytotoxicity = \left(\frac{No.of\ dead\ cells}{No.of\ dead\ cells + No.of\ live\ cells}\right) \times 100 \text{.....(1)}$$

Determination of tumour reducing activity

Albino mice (male,5-6 weeks old)weighing 18-25 kgwere used for the study purpose. Six animals form a study group and there were 14 such groups. Ascites tumour was made in these animals by introducing viable EAC cells in 0.1 ml of PBS into the peritoneal cavity. Out of 14 groups, one group was kept as control group which wasn't treated with any drug.Itwas labeled as

group1.Another group was treated with the standard drug,cyclophosphamide and it was labeled as group 2. The other groups were injected with different concentrations (20µg/ml, 10µg/ml and 5µg/ml) of the test compounds after the tumour formation and injections were continued regularly for ten days. The death rate of animals due to tumour problem was carefully examined and the percentage increase in life span (ILS) was evaluated by the definite formula.

$$\%ILS = \left(\frac{T-C}{C}\right) \times 100 \quad \ldots\ldots\ldots\ldots\ldots(2)$$

Where, T and C are mean survival time of treated and control mice respectively in days.

Determination of effects of compounds on solid tumour development

The solid tumour volume reductions of Swiss albino mice wereinvestigated by the administration of the synthesized test compounds. The synthesized test compounds and its copper metal chelates were effective for reducing solid tumour caused by DLA cell lines in mice because these analogues were in addition cytotoxic to DLA cell lines. The animals were classified under six groups. Viable DLA cells ($1x10^6$cells per animal) were transferred and introduced into the right back limb of the mice. Test compounds (50 and 100mg /kg of body weight) were inducedto the mice on alternative days for two weeks. The group that accepted/admitted only DLA cells kept as control group which wasn't treated with any drug. The generation of tumour on animals was examined by assessing the diameter of the tumour development in two perpendicular phases by vernier calipers, every third day for one month. The tumour volume was calculated by the formula

$$V = \frac{4}{3}\pi r_1 r_2^2 \quad \ldots\ldots\ldots\ldots(3)$$

Where, r_1 and r_2 are the minor and major radii respectively[19].

Analytical instruments

Microanalysis was done for determining Carbon and Hydrogen percentages (Heraeus Elemental analyzer) and metal percentage by Atomic Absorption Spectroscopy (AAS;Perkin Elmer 2380).The electronic spectra were noted on a Shimadzu UV-VIS-1601 Spectrophotometer. Infra-red (IR) spectra were taken on Perkin Elmer Fourier transform of IR(FTIR) spectrophotometer. The ^{1}H Nuclear Magnetic Resonance (NMR) spectra were recorded on a FT-NMR Spectrophotometer.The FAB mass spectra were recorded on a Joel SX–102 mass spectrophotometer from CDRI, Lucknow, India.

RESULTS AND DISCUSSION

Structural characterization of (4E,6E)-4,6-bis (aryl)cyclohexane1,3- dione

The prepared cyclic curcuminoid equivalents were evaluated by C, H & metal analysis. Further characterization was done using Ultra-violet (UV), IR, ^{1}H NMR and Mass spectral technique. (Table I).The synthesized dicarbonyl compounds showed two types of transitions in UV spectrum, one is n→π* and the other one is π→π*. The normal π-π* transition value lies in the range of 270-300nm and n-π* transition value lies in the range of 300-460nm. Here the value increases because of conjugation presence of α, β unsaturation shifts the wavelength for the carbonyl absorption to a greater value.

Table 1: Spectral data of prepared ligands

Compound	UV data λ_{max}(nm)	IR data cm^{-1} (Chelated C=O)	^{1}H NMR spectral data Chemical shift (ppm)			Mass spectral data (m/z)
			Methie	Phenyl	Alkenyl	
$C_{16}H_{12}O_2S_2$	264,360	1647	5.4	7.08-7.7	6.4-7.9	561,367,145,105,77
$C_{22}H_{20}O_2$	265,358	1634	5.44	7.47-7.49	6.933-7.812	413,264,131,105,77,69
$C_{28}H_{20}O_4$	293,334	1634	5.91	7.213-8.130	7.142-7.776	408,263,223,210,182
$C_{36}H_{23}O_2$	293,413	1639	5.12	7.614-7.760	6.6-8.99	489286,258,230,190

All the synthetic analogues have $\nu_{C=O}$ vibrations and these showed noticeable bands near the region 1600cm^{-1}to 2500cm $^{-1}$ as a result of the stretching of free carbonyl, intra-molecularly hydrogen bonded carbonyl and various alkenyl vibrations. Extensive analysis of the spectra it has been found that the compound exist in the H-bonded form. This is further supported by the occurrence of broad band near the region of 3500-2500cm^{-1}. The ν(C-C) alkenyl, ν_{as}(C-C-C) chelate ring, ν_s(C-C-C) chelate ring and β(C-H) chelate ring are responsible for the presence of further peaks. The ^{1}H NMR spectra of synthetic diketones also strengthness the enolic arrangement of the composite. Ligandsexhibited a one proton singlet at~16 ppm conveyable to strong intramolecularly hydrogen bonded enolic proton[20].

The several proton signals detected in the spectra. The peaks analogues to enolic, methane, alkenyl, methyl and phenyl group can be detected in the spectrum. The aromatic protons displayed signals in the region 7.08 – 7.76ppm and the alkenyl type protons exhibited signals in the region of 6.5 – 8.0ppm. The mass spectra give an idea about the various fragmentation modes of the compounds. The mass spectra can determine the molecular weight and molecular structure of the compounds. The peak with highest m/z value will be the molecular ion peak. The fragmentation

pattern gives the idea about the structure of the compound.The peaks for molecular ion, the loss of C_2H_2, CH2=C=O, CH2,-CH=C=O ion along with other important peaks are noticed in mass spectra of synthesized diketone analogue. Remaining parts like O, OH, CH_2 etc. are detached from the molecular ion and are displayed in the spectrum.

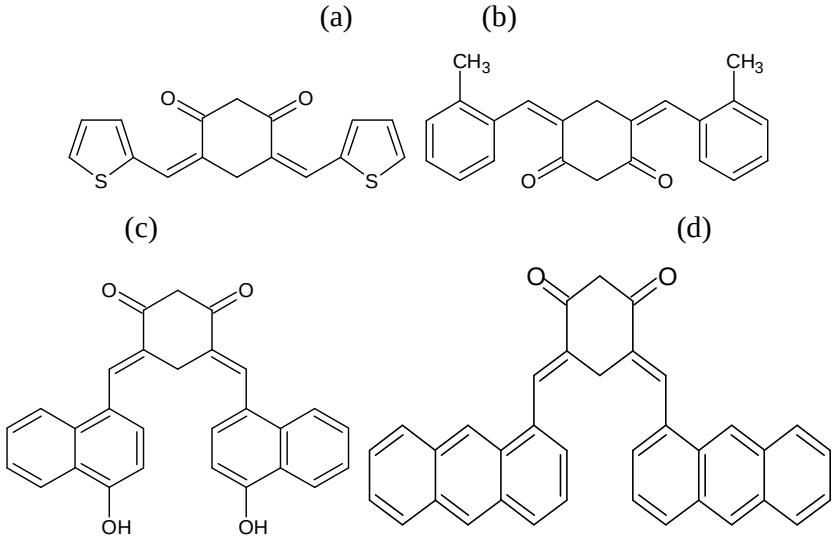

Figure 1. The structure of curcuminoid analogues

(a) (4E,6E)-4,6-bis(thiophen-2-ylmethylidene)cyclohexane-1,3-dione;($C_{16}H_{12}O_2S_2$)

(b)(4E,6E)-4,6-bis(2 methyl benzylidene)cyclohexane-1,3 dione;($C_{22}H_{20}O_2$)

(c) (4E,6E) - 4,6-bis (hydroxy naphthalene-ylmethylidene)cyclohexane -1,3dione;

($C_{28}H_{20}O_4$)

(d) (4E,6E)- 4,6- bis(anthraceneyl-methylidene)cyclohexane -1,3 dione($C_{36}H_{23}O_2$)

Structural characterization of copper complexes

After the complexation when the UV spectrum of the complex was compared with that of ligand there was not much difference in the absorption peaks. That is the complex formation did not alter the structure of the ligands. The association of carbonyl moiety in chelate formation can be detected from bathochromic shift of absorption maxima to longer wavelength. The lack of strong band in the region 1650-1800 is a clear indication of the metal complex formation. Newly appeared Metal coordinated carbonyl group having a peak at 1608cm^{-1} replaced the peak of intra-molecularly hydrogen bonded carbonyl group which had the peak of 1617. Nonexistence of broad band in the region of 2600-3500cm^{-1} is due to the replacement of enolic proton by a metal ion and there is a medium intensity bands of metal oxygen bond depicted in the region of 420-480cm^{-1}.

Table 2: Spectral data of the Cu (II) complexes

Compound	Elemental Analysis: found(calculated) %			IR data cm^{-1}		Mass spectral data (m/z)
	C	H	Cu	ν C=O	ν M-O	
$[M(a)_2]$	56.44(57.87)	3.29(3.61)	9.11(9.57)	1616	466,453	1182,834,740,274,198,169
$[M(b)_2]$	76.24(75.91)	5.01(5.75)	9.01(9.13)	1584	455,413	886,685,603,275,199,172
$[M(c)_2]$	74.39(74.41)	4.17(4.42)	6.86(7.03)	1588	459,407	903,617,331,483,307,187
$[M(d)_2]$	82.08(83.156)	4.18(4.61)	6.007(6.11)	1597	458,428	1035,681,565,489,327,211

Enolic proton in the ligand is supposed to be responsible for the peak at 16 ppm,but after the complex formation it is completely absent from the spectrum. The main reason for this is the replacement of enolic proton by a transition metal. Whereas, the other two protons namely phenyl and alkenyl do not take part in complex formation, so their peak doesn't show much changes in spectrum and also methine signals showed a downfield shift. Thus in effective both the ligand spectra and complex spectra were almost identical and the only difference is in the case of enolic proton. The configuration and stoichiometry of the synthetic analogues can be very well formulated by the mass spectroscopy. It can be inferred from the study of mass spectra that bit- by- bit elimination of aromatic part is a unique character. Electronic and steric effect of the groups associated to the diketo function greatly affects the existence of numerous fractions generated by the mass spectra. The recommended formulation and configuration of synthetic analogues of ligand are evidently in accordance with the detected spectra of chelates. From the examined peaks in the spectrum of complexes, it is implied that a few fragments reshuffle to form well-built cyclic species. Peaks due to [ML]+, and fragments of L+ are also identified in the spectrum.The spectrum provides solid proof for ML_2stochiometry for the complex. Smaller species such as O, OH, CH etc. are also eliminated.

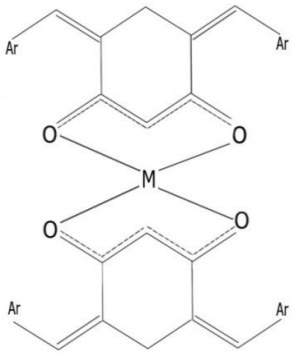

Figure 2 . The structure of metal complex of diketone- curcuminoid analogue

In vitro cytotoxicity

The test compounds were studied for short term in vitro cytoxicity using DLA cells. The entire compounds possessed extreme activity at higher concentations namely 200µg/ml. When the concentration of compounds increases the % of cell death also increases. That is the concentration play an important role in death rate. It could be concluded from this result that, metal chelation increases cytotoxic activity of any ligand. Metal complexes showed reasonable activity even at lower concentrations. Relating the ligands, $C_{16}H_{12}O_2S_2$withthiophene groups was found to be more cytotoxic than others. Among the metal complexes the activity follows the order $[M(a)_2]$> $[M(b)_2]$)> $[M(c)_2]$>$[M(d)_2]$. The copper complex of (4E,6E)-4,6-bis(thiophen-2-ylmethylidene)cyclohexane-1,3-dione was found to be very operative and created 98% cell death. It is because of heterocyclic thiphene ring in the system. The results of *in vitro* cytotoxicity of ligands and their copper complexes towards DLA cells are given in TableIII.

Table 3: Percentage cytotoxicity against DLA cells for diketone- curcuminoid analogues and Cu(II) chelates

Compounds	% cell death at various concentrations				
	10µg/ml	20µg/ml	50µg/ml	100µg/ml	200µg/ml
A	14	23	54	62	89
B	12	21	46	59	82
C	11	20	43	57	79
D	10	18	37	53	74
$[Cu(a)_2]$	23	35	61	73	97
$[Cu(b)_2]$	20	32	57	71	95
$[Cu(c)_2]$	18	29	52	68	88
$[Cu(d)_2]$	14	22	47	66	79

Effect of compounds on ascites tumour reduction (in vivo)

The survival span of control group implanted with Ehrlich ascites tumour cells observed to be 16.5 ± 1.29 days(Table IV) whereas it is 27.4.±1.2 days for the group which is implanted (4E,6E)-4,6–bis(thiophen-2-ylmethylidene)cyclohexane-1,3-dione having 20µg/ml concentration. A maximum extension in the life period (90.90%)of mice was observed under the implant of thiophene based copper complex with concentration fixed at 20µg/ml. Table IV represents the performance of test analogues (concentration 20µg/ml) on reducing the ascites tumour. Fig.III displays the performance of copper complexes on Ascites tumour reduction.

Table 4: Effect of synthesized compounds (of concentration 20µg/ml) on ascites tumour reduction

Sl. No.	Animal groups	No. of animals with tumour	No. of days survived	Percent ILS
1	control	5/5	16.5 ± 1.29	
2	a	5/5	27.4 ± 1.2	66.06
3	b	5/5	26.8 ± 2.01	62.42
4	c	5/5	24.9 ± 2.9	50.90
5	d	5/5	20.2 ± 1.5	22.42
6	[Cu(a)$_2$]	5/5	31.5 ± 1.4	90.90
7	[Cu(b)$_2$]	5/5	28.4 ± 2.36	72.12
8	[Cu(c)$_2$]	5/5	26.3 ± 1.13	59.39
9	[Cu(d)$_2$]	5/5	21.7 ± 2.85	31.51

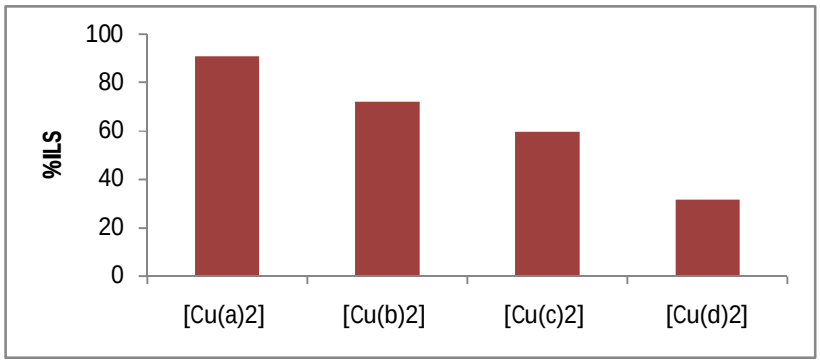

Figure 3. Effect of copper complexes of curcuminoid analogues on Ascites tumour reduction

Solid tumour reduction (in vivo)

The graphical representation of Suppressive reaction of test diketone-metal complexes on the advancement of tumour in the animals by the intraperitoneal induction of drugs is shown in Fig.IV. The graph evidently indicates that both the synthetic diketone analogue of curcumin and its metal chelates play pivotal rolein minimizing the volume of the tumour. It is found that the metal chelates are better in minimizing the volume of tumour when compared to its ligand.The 30th day measurement of tumour volume for a,b,c,d and control group are1.824cm^3, 2.146cm^3, 2.290cm^3, 2.459cm^3 and 3.731cm^3 respectively whereas corresponding metal chelates are 0.640cm^3, 1.043cm^3, 1.282cm^3 and 1.217cm^3 respectively. Metal chelates exhibit a broader spectrum of performance and is lesser toxic when compared to the platinum based medicine and are implied to have more ability in overcoming hereditary and/or acquired immunity than cisplatin.

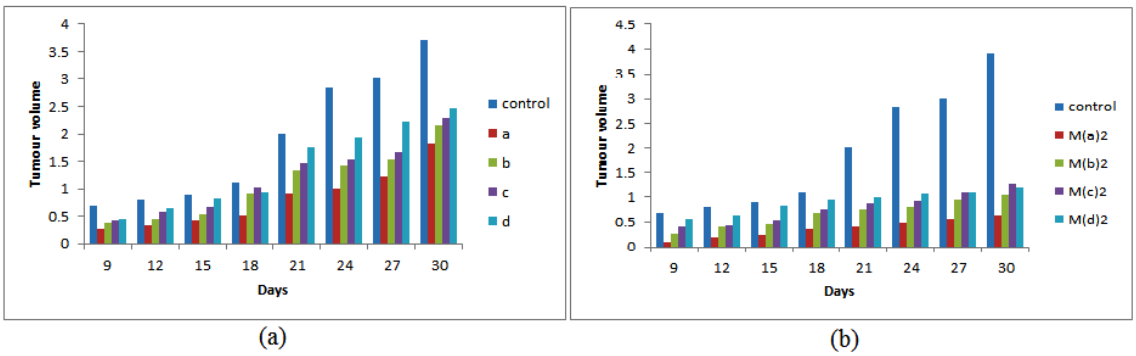

Figure 4. Effect of diketone-curcumin analogues on solid tumour growth (a), metal complexes on solid tumour growth (b).

CONCLUSIONS

Various spectral procedures were used to investigate the characteristic of recently manufactured cyclic diketonecurcuminoid analogues and their copper complexes in order to uncover their potential cytotoxic and anticancer responses. The current study indicates that chelates of metal displays a better positive response against cytotoxins and tumours when compared to its ligands and Cu(II) complex of (4E, 6E)-4, 6-bis(thiophen-2 ylmethylidene) cyclohexane-1,3-dione, has shown the maximum activity with cell death of 91% . The compound d ($C_{36}H_{23}O_2$)which is having anthracenyl ring is the infinitesimally active test diketone compound in comparison to the rest of the compounds. The antitumourexamination (in vivo) on mice reveals that the (4E, 6E)-4, 6-bis(2 methyl benzylidene)cyclohexane-1,3 dione, (4E,6E)-4,6-bis (hydroxy naphthalene-ylmethylidene)cyclohexane -1,3 dione and their chelates of metal have outstanding inhibitory influence on Erlich Ascites Carcinoma cells(EAC) and Daltons Lymphoma Ascites cells (DLA) cells. (4E,6E)-4,6-bis(thiophen-2-ylmethylidene)cyclohexane-1,3-dioneand the its copper complex were found to be better active to extend life period of tumour-induced mice and decrease the volume of tumour (EAC cells) in animals. The copper complexes have highest rate of activity which is equivalent to a reference anticancerous drug. The investigation discloses that the chelation has significantly improved the in vitro and in vivo antitumour activities.

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