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Curcuminoid analogues with substituted naphthyl ring and their transition metal chelates- Antibacterial and Antitumor studies

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Abstract
Curcumin and its analogues have been extensively studied for their biological activities including antimicrobial, anti-inflammatory, antioxidant, anticarcinogenic etc. Recently compounds structurally related to curcuminoids were synthesized and their chemotherapeutic potential has been revealed. In the present study, the synthesis and characterization of the curcuminoid analogues namely 1, 7-dinaphthyl-1, 6-heptadiene-3, 5-dione(L1);1, 7-Bis(2-hydroxynaphthyl)-1, 6-heptadiene-3, 5-dione(L2) and 1, 7-Bis(2-methoxy naphthyl)-1, 6-heptadiene-3, 5-dione (L3) and their metal chelates with Cu(II), Zn(II) and Oxovanadium (IV) using UV, IR, 1H NMR and mass spectral data. *In vitro* cytotoxic studies were done with ligands and metal complexes against EAC cells using Trypan 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. The present investigation revealed that the Cu (II) complexes show enhanced cytotoxic activity and antibacterial activity than the curcuminoid analogues, Zn (II) and Oxovanadium (IV) complex.

Keywords: 1, 7–dinaphthylheptanoids, antibacterial, cytotoxicity, curcuminiods, mass spectra, NMR, turmeric

1. Introduction
Curcuminoids are bioactive yellow orange pigments present in the rhizomes of the perennial herb turmeric (*Curcuma longa* Linn.) and has been used in ancient ayurvedic medicine. Curcumin has several biological effects exhibiting anti-inflammatory [1-4], antifungal, anticarcinogenic and antioxidant activities [5-8]. It has also been studied extensively as a chemo preventive agent in several cancer cells [9-16]. Structurally curcuminoids are linear 1, 7-diaryl-1, 6-heptadiene-3, 5-diones 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. Curcuminoids and their metal chelates possess remarkable biochemical activity [17-18].

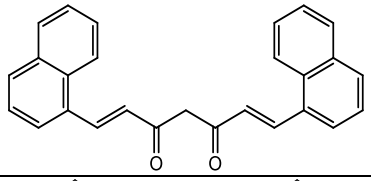
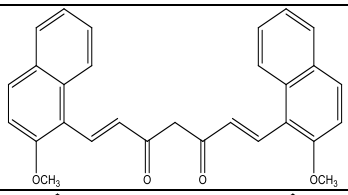
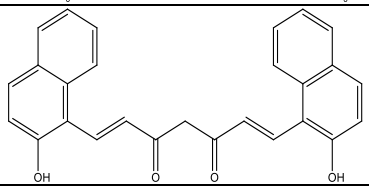
Curcuminoids are expected to form metal complexes similar to other 1,3-diketones. They are powerful chelating agents. Here Cu(II), Zn(II),Oxovanadium (IV)complexes of curcuminoid analogues are synthesized and characterized. In the present study, aldehydes namely 1-Naphthaldehyde, 2-hydroxynaphthaldehyde and 2-methoxynaphthaldehyde were condensed with acetylacetone in presence of B2O3 using tri-secondary butyl borate and n-butylamine as the condensing agent [19]. The ligands prepared were complexed with Cu(II), Zn(II), oxovanadium (IV) to form metal chelates. The curcuminoid analogues and their metal chelates were subjected to *in vitro* cytotoxic studies using trypan blue exclusion method [20]. *In vivo* antitumour studies were conducted in DLA induced mice. The naphthyl 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*.

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

(1X106 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. 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 1H 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 substituted derivatives of 1, 7-dinaphthyl-1, 6-heptadiene-3, 5-diones
The curcuminoid analogues were prepared by the condensation of aldehydes (1-naphthaldehyde, 2-hydroxynaphthaldehyde and 2-methoxynaphthalehyde) with acetyl acetone-boric oxide complex in ethyl acetate medium in presence of tributyl borate and n-butyl amine [19]. 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.

Compound	Aldehyde used for Synthesis	Structure of Ligands	Systematic name
L1	1-Naphthaldehye		1, 7-dinaphthyl hepta-1, 6-diene-3, 5-dione
L2	2–methoxynaphth-aldehyde		1, 7-bis (2-methoxy naphthyl) hepta-1, 6-diene-3, 5-dione
L3	2–hydroxynaphth-aldehyde		1, 7-bis (2-hydroxy naphthyl) hepta-1, 6-diene-3, 5-dione

2.3. Synthesis of metal complexes
The Cu(II) complexes were prepared by adding a methanolic solution of copper (II) acetate (25 ml, 0.001 mol) to a solution of curcuminoid analogue (25 ml, 0.002 mol) in methanol and refluxed gently for 2 h. The precipitated complex was filtered, washed with 1:1, methanol: water mixture and recrystallized from hot methanol [16]. The Zn(II) and Oxovanadium (IV) complexes were prepared by adding a methanolic solution of zinc(II)acetate (25 ml,. 001mol), vanadyl sulphate respectively to a solution of curcuminoid analogue (25 ml,. 002 mol) in methanol and refluxed gently for 2h. The precipitated complex was filtered, washed with 1:1, methanol: water.

2.4. *In vitro* cytotoxicity studies
Studies were carried out using the diketone and Cu(II), Zn(II) and oxovanadium (IV) 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 15 min. 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 ºC. Further, cell suspension was mixed with 0.1mol of 1% trypan blue and kept for 2-3 min. and loaded on a haemocyto meter. The number of stained (dead) and unstained (live) cells were counted and percentage cytotoxicity was evaluated by trypan blue exclusion method %

Cytotoxicity=(No:of dead cells/No:of dead cells+No:of live cells)x100

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 hr 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 ºC 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* anti-tumour activity
Animals (male mice, 6-8 weeks old) weighing 28-30g were divided into 8 groups of 5 animals each. Viable DLA cells (1X106) 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-no test compound is injected. Group 2, Standard: standard anticancer drug Cyclophosphamide 25 mg/kg body weight. Group 3-5: Ligand, 1, 7-Bis(2-hydroxynaphthyl) -1, 6-heptadiene -3, 5-dione with concentrations 20 µg/ml, 10 µg/ml and 5 µg/ml was given as drug. Group 6-8 Cu(II) metal chelates as drug with same concentrations. Ligand, complexes and cyclophosphamide were given by i.p. injection from the 1stday of tumour induction up to 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 substituted compounds of 1, 7-dinaphthyl-1, 6-heptadiene-3, 5-diones

The synthesized analogues namely 1, 7-dinaphthyl-1, 6-heptadiene-3, 5-dione(L1), 1, 7-Bis (2-hydroxynaphthyl)-1, 6-heptadiene-3, 5-dione(L2), and 1, 7-Bis(2-methoxynaphthyl)-1, 6-heptadiene-3, 5-dione(L3) were characterized by UV, IR, 1HNMR and Mass spectral data (**Table 1**).

Table 1: UV, IR, ^{1}H NMR and mass spectral data of substituted 1, 7 dinaphthyl heptanoids

Compound	UV data λ^{max} (nm)	IR data cm-1 ν (C=O)	^{1}H NMR spectral data Chemical shift (ppm)					Mass spectral data (m/z)
			Enol	Methine	Functional group	Phenyl	Alkenyl	
L1	260,386	1620	15.989	5.95		7.84-8.28	6.78-8.56	377, 250, 223, 181
L2	259,385	1637	16.041	5.909	10.789 (hydroxy)	7.413-8.335	7.142-7.97	408, 237, 198, 165, 154
L3	263,391	1640	16.10	5.96	4.058 (methoxy)	7.86-9.28	7.290-7.765	436, 279, 253, 225, 212, 184, 171

3.2. Structural characterization of metal complexes

All the three ligands L1, L2, L3 form well defined crystalline complexes with Cu(II), Zn(II) and vanadyl ions. Analytical and mass spectral data are given in (Table 2). The approximate formulae of the metal complexes are ML2.

Table 2: Spectral data of Cu(II), Zn(II), Oxovanadium (IV) complexes of methoxy and hydroxyderivatives of 1, 7-dinaphthyl heptanoids

Complex	UV spectra Λ max (nm)	IR data (cm-1)		Mass spectral data (m/z)
		V C=O	V M-O	
Cu $(L_1)_2$	262, 385	1581	484, 428	814, 559, 439, 307, 181, 120
Cu $(L_2)_2$	263, 388	1595	467, 409	878, 641, 527, 460, 307
Cu $(L_3)_2$	265, 393	1590	460, 411	934, 620, 499, 306, 185, 121
Zn $(L_1)_2$	261, 385	1600	472, 419	816, 561, 441, 309, 183, 122
Zn $(L_2)_2$	264, 390	1580	455, 419	880, 643, 529, 462, 309
Zn $(L_3)_2$	270, 395	1585	465, 421	936, 622, 501, 308, 187
VO $(L_1)_2$	258, 390	1561	479, 422	817, 563, 442, 308, 254, 188
VO $(L_2)_2$	259, 392	1590	468, 419	937, 623, 502, 314, 309, 157
VO $(L_3)_2$	260, 398	1601	465, 409	881, 595, 474, 309, 286, 188, 143

3.3. *In vitro* cytotoxicity

The results of *in vitro* cytotoxicity of ligands namely 1, 7-dinaphthyl-1, 6-heptadiene-3, 5-dione(L1), 1, 7-Bis(2-hydroxynaphthyl)-1, 6-heptadiene-3, 5-dione(L2), and 1, 7-Bis (2-methoxynaphthyl)-1, 6-heptadiene-3, 5-dione (L3) and their complexes (Cu(II)), Zn(II) and oxovanadium (IV) towards EAC are given in (Table 3). The diketones and their metal complexes are given as drug in concentrations 200 µg/ml, 100 µg/ml, 50 µg/ml, 20 µg/ml & 10 µg/ml. The number of stained and unstained cancer cells were counted and evaluated as per cent cell death.

Table 3: Per cent cytotoxicity towards EAC cells for curcuminoid analogues and Cu(II),Zn(II),VO(IV) chelates

Concentration (µg)	Per cent cytotoxicity											
	L_1	Cu $(L_1)_2$	Zn $(L_1)_2$	VO $(L_1)_2$	L_2	Cu $(L_2)_2$	Zn $(L_2)_2$	VO $(L_2)_2$	L_3	Cu $(L_3)_2$	Zn $(L_3)_2$	VO $(L_3)_2$
200	34	72	45	40	40	84	55	50	36	80	40	38
100	20	45	35	32	30	55	42	40	22	50	32	30
50	10	30	20	18	20	37	30	28	11	32	28	20
20	5	15	10	8	12	22	20	18	6	18	10	8
10	2	8	4	3	8	12	10	8	4	9	6	5

3.4 Antibacterial activity

The results of the antibacterial activity of 1, 7-dinaphthyl-1, 6-heptadiene-3, 5-dione(L1), 1, 7-Bis (2-hydroxynaphthyl)-1, 6-heptadiene-3, 5-dione(L2), and 1, 7-Bis (2-methoxynaphthyl)-1, 6-heptadiene-3, 5-dione (L3) and their complexes (Cu(II)) and Zn(II) are given in Table 4.

Table 4: Antibacterial activity of ligands and their metal complexes

Bacteria			Diameter of zone of inhibition in mm						
	L_1	Cu $(L_1)_2$	Zn $(L_1)_2$	L_2	Cu $(L_2)_2$	Zn $(L_2)_2$	L_3	Cu $(L_3)_2$	Zn $(L_3)_2$
E. Coli	15	19	17	20	21	18	18	19	16
Klebsiella	12	15	14	15	17	12	14	16	10
Bacillus	8	10	9	11	12	10	10	11	8
Streptomycin (standard)	25	25	25	25	25	25	25	25	25

3.5 *In vivo* antitumor activity

The ligand (L2) and its metal complexes were given as drug and the survival of animals are given in Table 5. The control and the group with std. drug cyclophosphamide are also given in the table. The values of number of days survived are means of five determinations ±SD (standard deviation). The increase in life span corresponding to drugs L2 and Cu(L2)2 with varying concentrations is also given.

Table 5: Effect of compounds on ascites tumour reduction (*in vivo*)

Sl. No.	Animal groups	Concentration (µg/ml)	No. of animals with tumour	No. of days survived	Per cent ILS
1.	Control		5/5	16.6±1.49	
2.	Standard drug		5/5	21.0±5.09	26.5
3.	L2	20	5/5	19.6±2.65	20.1
4.	L2	10	5/5	18.0±2.60	8
5.	L2	5	5/5	17.6±2.72	6.02
6.	$Cu(L2)_2$	20	5/5	20.2±2.63	21.6
7.	$Cu(L2)_2$	10	5/5	19.6±2.65	20.1
8.	$Cu(L2)_2$	5	5/5	19.2±2.71	15.6

4. Discussion

4.1 Characterization of hydroxy and methoxy substituted analogues of 1, 7-dinaphthyl-1, 6-heptadiene-3, 5-diones

The synthesized derivatives of 1,7-dinaphthyl heptanoids were characterized by various analytical techniques (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 strongband ~1620 cm^{-1} assignable to intra molecularly hydrogen bonded carbonyl function. The 1HNMR spectra of the compounds show peaks due to enol, methine, phenyl, alkenyl, methoxy and hydroxyl group [21]. Peaks corresponding to step wise elimination of naphthyl groups and small fragments are present in the mass spectra.

4.2 Characterization of metal complexes

Analytical and mass spectral data (Table 2) clearly suggests a ML2 for 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 CuL2 [14, 15, 22].

4.3 *In vitro* cytotoxicity

Both in ligands as well as metal complexes, 200µg/ml concentration show maximum activity (Table 3). It is also noted that metal chelation enhances cytotoxicity of compounds considerably. The copper complexes of 1, 7-dinaphthylheptanoids show better results than that of ligands in almost all concentration. Among the ligands 1, 7-Bis(2-hydroxynaphthyl)-1, 6-heptadiene-3, 5-dione(L2) shows maximum cytotoxicity and its Cu(II) chelate [23, 24] showed maximum cytotoxicity among the metal complexes. The Zn(II)complex [25] and Oxovanadium (IV) complexes were found to be more cytotoxic than the corresponding ligands.

4.4 Antibacterial activity

The results (Table 4) 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. Among the ligands 1, 7-Bis(2-hydroxynaphthyl)-1, 6-heptadiene-3, 5-dione (L2) showed maximum antibacterial activity and its Cu(II) chelate showed maximum activity among the metal complexes [26].

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 (Table 5). The animals which were given the drug 1, 7-Bis(2-hydroxynaphthyl)-1, 6-heptadiene-3, 5-dione (L2), survived for19.6±2.65 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 $Cu(L2)_2$ was maximum (21.6%) with 20 µg/ml con [14, 15].

The ongoing discussion reveals that the hydroxyl and methoxy derivatives of 1, 7-dinaphthyl 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) omplex of 1, 7-Bis(2-hydroxynaphthyl)-1, 6-heptadiene-3, 5-dione(L2) is the most active compound in *in-vitro* cytotoxicity studies both with EAC and DLA. The antibacterial studies clearly show that both ligand and metal complexes possess enhanced antibacterial activity. The Cu(II)complexes show better antibacterial activity than Zn(II) complexes and ligands. The Cu(II) complex of ligand 1,7-Bis(2-hydroxynaphthyl)-1, 6-heptadiene-3, 5-dione(L3) showed maximum antibacterial activity. The ligand. 1,7-Bis (2-hydroxynaphthyl)-1, 6-heptadiene-3, 5-dione(L2) was found to be quite effective in reducing ascites tumour in mice and also its Cu(II) complex possessed maximum activity which is comparable with a standard anticancerous drug.

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6. Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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