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SYNTHESIS,CHARACTERIZATION, CYTOTOXIC& ANTITUMOUR ACTIVITIES OF SCHIFF BASES OF CURCUMINOID ANALOGUES AND THEIR COPPER COMPLEXES

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Abstract
Synthesizedand characterized Schiff bases of four curcuminoidanalogues and their copper(II) complexes of ML_2 stoichiometry. In vitro cytotoxic studies of the synthesized Schiff bases and their copper complexes were done against Daltons Lymphoma Ascites cells usingTrypan blue exclusion method. Compound I**a**and the corresponding copper complex were found to be more active towards increase in life span of tumour-bearing mice and reducing thesolid tumour(ErlichAscites Carcinoma cells) volume in mice.
Keywords: Curcuminoids;IR; NMR; mass spectra; cytotoxicity;antitumour.

Introduction

Curcuminis a naturalpolyphenolic compound which is responsible for the yellow colour of the turmeric, possess potent antiinflammatory[i-iii], antifungal, antiproliferative, antioxidant [iv] and anticancer activities[v-xi]. Turmericcontains three different kinds of curcuminoids namely curcumin,demethoxycurcumin and bisdemethoxycurcumin.Structurally they are linear 1,7-diaryl-1,6-heptadiene-3,5-diones which exist in rapid equilibrium with its tautomericenol form. The synthesized curcuminoid analogues retain the α,β unsaturated 1,3-diketo moiety of the natural curcuminbut the aryl ringsare modified.The poor bioavailability of the curcumin can be improved by the use of structural analogues of curcumin.
Curcuminoidsform metal complexes in the same manner as 1,3-diketones do.The curcuminoids and their metal complexes possess remarkable biochemical activities[xii]. The anticarcinogenic activity of curcumin is due to the direct antioxidant and free radical scavenging properties as well as their ability to indirectly increase glutathione levels, thereby assisting in detoxification of mutagens and carcinogens.Curcuminoids are powerful chelating agents and can be used in therapy. Chelation therapy is a medical procedure that involves the administration of chelating agents to remove heavy metals from the body[xiii,xiv]. These ligands bind to heavy metals such as cadmium and lead, thereby reducing the toxicity of these heavy metals.The metal chelating abilities of curcuminoids is through the β diketogroup[xv,xvi]which in turn forms new structural entities with modified biochemical activities. The physicochemical features such as the planarity, hydrophobicity and size and nature of the

ligand, as well as the coordinationgeometry of the metal complex all haveimportant roles in determining the binding/intercalating mode of copper complexes to DNA. According to these observations, a large number of copper complexes has been and is still being tested as anticancer drug.

In the present study,as continuation of our work we synthesized and characterized Schiff bases of four curcuminoid analogues and their copper complexes.We have focused on the cytotoxic effects of curcuminoids onErlichAscites Carcinoma cells(EAC) and Daltons Lymphoma Ascites(DLA)tumour cell lines.

Experimental

Materials

The chemicals required were procuredfrom Sigma Aldrich chemical suppliers and were of analytical reagent grade. Daltons Lymphoma Ascites cells and ErlichAscites Carcinoma cells were obtained from the Adayar Cancer Research Institute, Chennai, India and maintained as transplantable tumours in Swiss albino mice by injecting a suspension of cells intraperitoneally(ip). Swiss albino mice for the experiments were purchased from Vetenary College, Thrissur, Kerala, India.The animals were fed with normal mouse chow (Lipton India) and water *ad libitum*.

Synthesis of 1,7-di aryl-hepta-1,6diene-3,5-diones.

The curcuminoid analogues wereprepared by the condensation of aromatic aldehydeswithacetyl acetone-boric oxide complex in ethyl acetate medium in the presence of tributyl borate and n-butylamine as reported earlier (Pabons Method)[xvii].The product was purified by column chromatography over silica gel (60-120 mesh) using 2:1 (v/v) chloroform: acetone mixture as the eluent and recrystallized twice from hot benzene to get pure crystalline material

Synthesis of Schiff Bases

The Schiff bases were synthesized by the coupling of benzene diazonium salt with β-diketones[xviii]. Benzene diazonium salt was prepared and added drop by drop to a solution of the β-diketone kept below 0^0c with constant stirring. The precipitated compound was filtered, washed with water and recrystallized from ethanol to get chromatographically pure (TLC) material.

Synthesis of metal complexes

The Cu (II) complexes were prepared by adding a methanolic solution of copper (II) acetate (25 ml, 0.001mol) to a solution of curcuminoidanalogue (25 ml, 0.002mol) in methanol and refluxed gently for 3 hrs. 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 recrystallized from hot methanol.

Antitumor Experiments

In vitro cytotoxicity studies

In vitro cytotoxicity studies were carried out using the Schiff bases and their Cu(II) 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 15minutes at 1500 rpm. Cell viability was determined usingTrypan blue exclusion method. ViableDLA 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 contained only cell suspension. These mixtures were incubated for 3hrs at 37^oC.

Further, cell suspension was mixed with 0.1ml of 1% trypan blue and kept for 2-3 minutes 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[xix].

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

Determination of tumour reducing activity

The animals (male mice, 6-8 weeks old) weighing 20-25g were divided into groups of 5 animals each. EACcells ($1x10^6$cells per animal) were injected into the peritoneal cavity of mice. One group of animals was kept as control, without injecting any test compound. The other groups were injected with different concentrations (20µg/ml, 10µg/ml and 5µg/ml) of the test compounds afterthe tumour formation and injections were continued for ten days. The mortality rate of animals due to tumour burden was noted and the percentage increase in life span (ILS) was calculated using the given formula.

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

Where, T and C are mean survival time of treated and control mice respectively in days[19].

Determination of effects of compounds on solid tumourdevelopment

The Swiss albino mice were used to study the effect of the synthesized compounds on solid tumourvolume reduction.Since the synthesized Schiff bases and their copper complexes are found to be more cytotoxic to DLA celllines, they were used to study its potential to reduce solid tumour induced by DLA cell lines in mice. The animals were divided into groups of six.Viable DLA cells ($1x10^6$cells per animal) were transplanted subcutaneously into the right hind limb of the mice. Drugs (50 and 100mg /kg of body weight) were injected to the animals on alternate days for two weeks. The group that received only DLA cells served as the control. The tumour development on animals was determined by measuring the diameter of the tumour growth in two perpendicular phases using vernier calipers, every third day for one month.The tumour volume was calculated using the formula given,

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

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

Analytical instruments

Carbon and hydrogen percentages were determined by microanalysis (Heraeus Elemental analyzer) and metal content by AAS (Perkin Elmer 2380). The electronic spectra were recorded on a Shimadzu UV-VIS-1601 Spectrophotometer. IR spectra were recorded on Perkin Elmer FTIR spectrophotometer. The ^{1}H 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 1,7-di aryl-hepta-1,6diene-3,5-diones

The synthesized curcuminoid analogues were characterized by CHN analysis, UV, IR, ^{1}H NMR and Mass spectral data (Table I). The UV spectra of the compounds in methanol show two absorption maxima corresponding to n→ π* and π→ π* transitions. The values at 260-300nm are due to $\pi \rightarrow \pi *$ transition and at 300-460nm are due to n→ $\pi *$ transitions of fully conjugated systems based on earlier reports. The presence of α, β unsaturation shifts the wavelength for the carbonyl absorption to a higher value.

Table I : Spectral data of synthesized 1,7-di aryl-hepta-1,6diene-3,5-diones

Compound	UV data λ_{max}(nm)	IR data cm^{-1} (Chelated C=O)	^1H NMR spectral data Chemical shift (ppm)			Mass spectral data (m/z)
			NH	Phenyl	Alkenyl	
HL^1	278,354	1629	9.695	7.233-7.438	6.856-7.734	561,367,145,105,77
HL^2	280,356	1626	9.936	7.359-7.565	6.78-7.85	413,264,131,105,77,69
HL^3	273,349	1615	9.607	7.365-7.430	7.157-7.415	513,333,179,131,105,77
HL^4	266,353	1621	9.036	7.371-7.837	6.983-7.954	582,377,204,105,77

IR spectra of synthesized ligands are characterized by the presence of three strong bands in the region 1590 cm^{-1} to 1710 cm^{-1} respectively due to the stretching of free carbonyl, intramolecularly hydrogen bonded carbonyl and C=N stretching vibrations . This shows that the compound exist in the intra-molecularly hydrogen bonded hydrazone-keto form. In the spectra, the strong intra-molecular hydrogen bonding shows a broad band in the region 2550-3600 cm^{-1}. There are a number of medium intensity vibrations observed in the region 1550-1600 cm^{-1} due to various stretching vibrations of the phenyl group, alkenyl& chelate ring. The band in the region 979 cm^{-1} and 974 cm^{-1} is assigned to the trans CH=CH vibration. The ^1H NMR spectra of synthesized curcuminoids also support the hydrazone-keto structure of the compound. The peaks corresponding to NH proton, alkenyl, and phenyl groups can be observed in the spectrum. Ligands displayed a one proton singlet at $\delta \sim 9.4$ppm assignable to strong intra-molecularly hydrogen bonded NH proton[xxi]. The aryl protons show signals in the region 7.1 – 7.5ppm and the alkenyl protons show 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 of the synthesized Schiff bases showed intense molecular ion peaks. Important peaks appeared in the spectra of compounds can be accounted by the fragmentation pattern. The peaks for [M+1] ion, $[NH=N-C=C=O]^+$ion together with other significant peaks are observed in mass spectra of synthesized Schiff bases. The remaining important peaks are that due to the fragment ions.

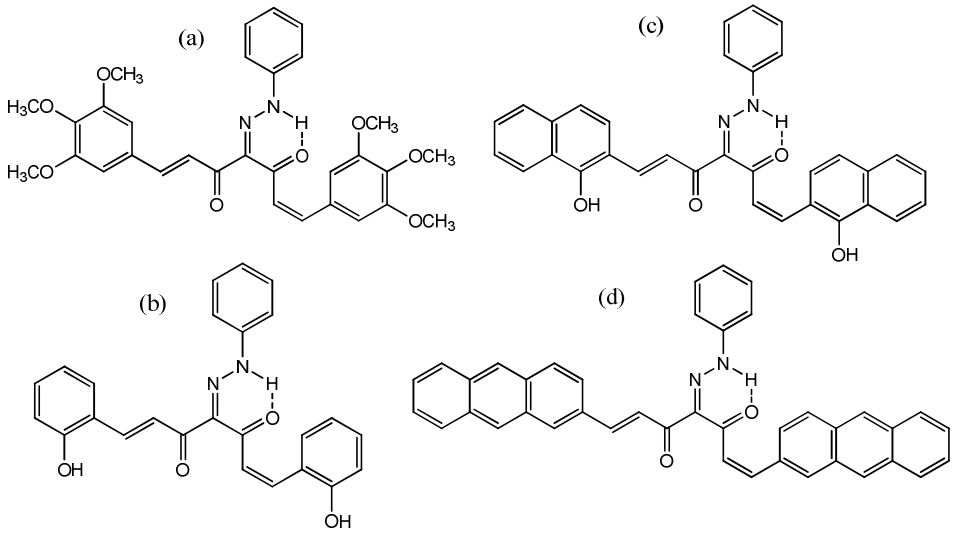

Fig.I. The structure of curcuminoid analogues
(a) 1,7-bis(3,4,5 tri methoxy phenyl)-4-(phenyl-hydrazono)-hepta-1,6diene-3,5-dione,(HL^1);
(b)1,7-bis(2 hydroxy phenyl)-4-(phenyl-hydrazono)-hepta-1,6diene-3,5-dione,(HL^2);
(c)1,7-bis(2hydroxynaphthyl)-4-(phenyl-hydrazono)-hepta-1,6diene-3,5-dione,(HL^3);
(d)1,7-bis(anthracenyl)-4-(phenyl-hydrazono)-hepta-1,6diene-3,5-dione,**(**HL^4**).**

Structural characterization of copper complexes

The complexes have absorption maxima relatively same as that of ligands indicating that the structure of the ligands remains almost unaltered in complexes. There is a slight bathochromic shift of absorption maxima of complexes which shows the involvement of the carbonyl moiety in chelate formation. In the IR spectra of metal chelates, the band at ~1626 cm^{-1} due to intra molecularly hydrogen bonded carbonyl function disappeared and instead a strong band assignable to stretching of the metal coordinated carbonyl moiety appeared at ~1554 cm^{-1}. Additional bands appeared at ~405cm^{-1} to ~420 cm^{-1} are assignable to ν (M–O) vibrations. The replacement of NH proton by a metal ion is also evident from the absence of the broad band in the region of 2600 -3500 cm^{-1}which was present in the ligand.

Table II: 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	
$[M(L^1)_2]$	62.82(62.96)	5.43(5.28)	5.31(5.37)	1559	417,407	1182,834,740,274,198,169
$[M(L^2)_2]$	67.78(67.70)	4.42(4.5)	7.24(7.16)	1554	422,405	886,685,603,275,199,172
$[M(L^3)_2]$	72.82(72.95)	4.36(4.26)	5.74(5.84)	1557	418,410	1086,786,692,212,181,105
$[M(L^4)_2]$	80.21(80.40)	4.58(4.60)	5.11(5.18)	1554	420,408	1224,856,762,274,197,170

The main feature of NMR spectra of metal complex is the absence of singlet signal at δ ~ 9.4ppm which was due to the NH proton in the ligands. This indicates the replacement of NH proton by metal atom in metal complexes. The phenyl and alkenyl protons are not altered much since they are not involved in metal complexation. Thus the spectra of ligand and complexes are much similar except those of NH proton. In the mass spectra of complexes, all the compounds showed relatively intense peaks at m/z corresponding to ML_2 stoichiometry, where M is metal and L is ligand. Mass spectral fragmentation pattern plays an important role

in elucidating the structure of metal complexes. It was found that some fragment ions undergo rearrangement to form stable species. The mass spectral data shows that stepwise removal of aryl groups is the characteristic feature of all complexes.Smaller species such as O, OH, CH etc. are also eliminated. In all the cases $[ML_2]^+$ ion is the most intense peak.. Peaks due to $[ML_2]^+$, $[ML]^+$,L^+ and fragments of L^+ are also detected in the spectrum.

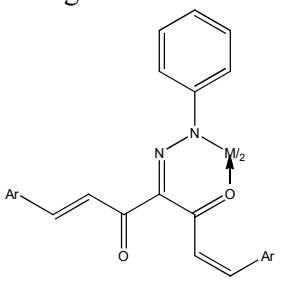

Fig.II.The structure of metal complex of curcuminoid analogue

In vitro cytotoxicity

*Invitro*cytotoxicity studies towards DLA cells revealed that both ligands and complexes exhibited greater %cell death at higher concentrations i.e., 200µg/ml.As concentration of drug compound increases from10µg/ml to 200µg/ml the %cell death increases.Comparing the ligands,$HL^1$with three methoxygroups was found to be more cytotoxic than other three ligands.All the metal complexes showed significant increase in %cell death than the ligands which shows that metal chelation enhances cytotoxicity of compounds considerably. Among the metal complexes the activity follows the order $[M(L^1)_2]$> $[M(L^2)_2]$)> $[M(L^3)_2]$>$[M(L^4)_2]$. The copper complex of 1,7-bis(3,4,5 tri methoxyphenyl)-4-(phenyl-hydrazono)-hepta-1,6diene-3,5-dionewas found to be very effective and produced 98%cell death.The results of *in vitro* cytotoxicity of ligands and their copper complexes towards DLA cells are given in Table**.** III.

Table III: Percentage cytotoxicity towards DLA cells for curcuminoid analogues and Cu(II) chelates

Compounds	% cell death at different concentrations				
	10µg/ml	20µg/ml	50µg/ml	100µg/ml	200µg/ml
HL^1	13	22	52	65	90
HL^2	11	22	43	65	80
HL^3	11	20	40	63	78
HL^4	9	17	35	59	76
$[M(L^1)_2]$	20	34	58	77	98
$[M(L^2)_2]$	18	31	53	76	95
$[M(L^3)_2]$	17	27	55	71	90
$[M(L^4)_2]$	15	25	49	65	80

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

The animals of the tumour control group inoculated with Ehrlich ascites tumour cells survived for a period 16.6±1.49 days (Table IV). The group of animals which were given the drug 1,7-bis(3,4,5 tri methoxy phenyl)-4-(phenyl-hydrazono)-hepta-1,6diene-3,5-dione

survived for 26.3±1.5 days with the concentration 20µg/ml. The increase in life span for corresponding copper complex was maximum (86.7%) with 20µg/ml concentration. Effect of synthesized compounds (concentration 20µg/ml) on ascites tumour reduction is presented in Table IV. The effect of copper complexes of curcuminoid analogues on Ascites tumour reduction are shown in Fig.III.

TableIV: 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	16.6±1.49	
2	HL^1	5/5	26.3±1.5	58.4
3	HL^2	5/5	25.2±2.7	51.8
4	HL^3	5/5	24.3 ± 1.9	46.4
5	HL^4	5/5	18.4 ± 1.9	10.8
6	$[M(L^1)_2]$	5/5	31.0±1.7	86.7
7	$[M(L^2)_2]$	5/5	27.5±1.3	65.6
8	$[M(L^3)_2]$	5/5	25.2±2.63	51.8
9	$[M(L^4)_2]$	5/5	22.5 ± 1.8	26.2

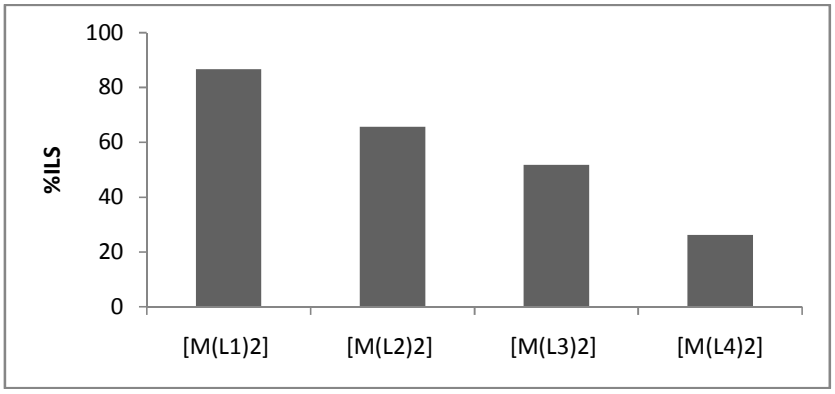

Fig.III. Effect of copper complexes of curcuminoid analogues on Ascites tumour reduction

Solid tumourreduction(*in vivo*)

Inhibitory effect of compounds on tumour growth in mice by the intraperitoneal administration of compounds is shown graphically in Fig.IV. The graphs show that the curcuminoids and their complexes reduce the tumour volume in a good manner. The copper complexes are found to be more efficient in reducing tumour volume than their corresponding ligands.Tumour volumes on the thirtieth day for HL^1, HL^2, HL^3,HL^4 and control are 1.675cm^3, 2.023cm^3, 2.210cm^3 2.329$cm^3$and 3.624cm^3 respectively and for their copper complexesare 0.940cm^3, 1.093cm^3, 1.182cm^3 and 1.203cm^3 respectively. Copper complexes have been shown to possess a broader spectrum of activity and a lower toxicity

than the platinum drugs and are suggested to be able to overcome inherited and/or acquired resistance to cisplatin.

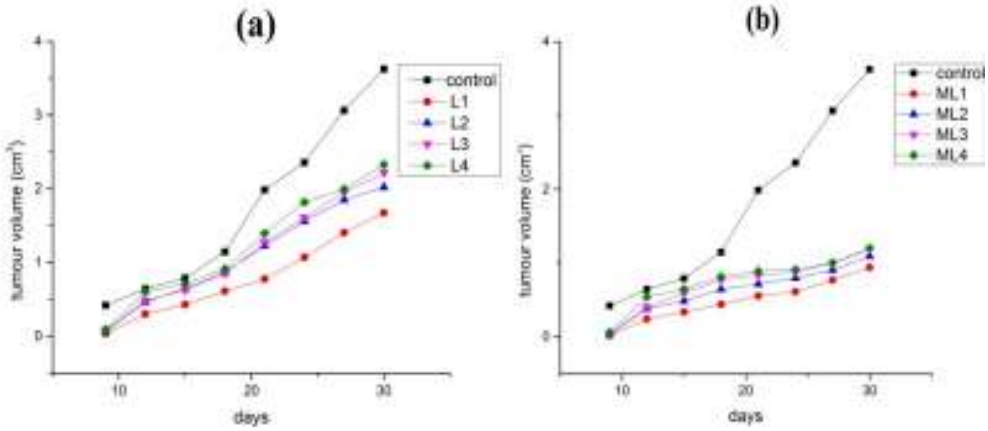

Fig.IV: Effect of curcuminoid analogues on solid tumour development(a), metal complexes on solid tumour development (b).

Conclusion

The newlysynthesized four Schiff bases of curcuminoid analogues and their copper complexes were characterized using different spectral techniques and investigated for their possible cytotoxic and anticancer activities. All the metal complexes exhibited enhanced cytotoxicand antitumouractivities than their ligands. Theinvitro cytotoxicity studiesreveal that metal chelation considerably increases the cytotoxicity of the Schiff bases towards DLA cells. The Cu(II) complex of1,7-bis(3,4,5 tri methoxy phenyl)-4-(phenyl-hydrazono)-hepta-1,6diene-3,5-dione was found to be the most active compound by producing 80% cell death. HL^4 with unsubstitutedanthracenyl ring is the least active compound compared to the other compounds.The antitumour studies (in vivo) on mice show that the hydroxyl and methoxy derivatives of diketones and their metal complexes havegreaterinhibitory effect on EAC and DLA cells. $HL^1$and the corresponding copper complex were found to be more active towards increase in life span of tumour-bearing mice and reducing the solid tumour(EAC cells) volume in mice. The copper complexes possess maximum activity which is comparable with a standard anticancerous drug. The studies reveal that chelation remarkably enhanced the in vitro and in vivoantitumour activities.

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