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Cytotoxic and Antifungal activities of Curcuminoid analogue with Methyl substituted phenyl ring and the Transition metal chelates

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Abstract: *Several plant species are known to exert wide range of physiological effects in addition to aroma and flavour . In recent years there has been an increased enthusiasm in treating the diseases by natural products.Curcumin,the bioactive pigment in Turmeric has found a wide application in this context.Curcumin , its analogues and metal complexes have been extensively studied for their biological activities including antimicrobial,anti-inflammatory,antioxidant,anticarcinogenic etc. Recently a number of compounds structurally related to curcuminoids were synthesized and their biological activities have been studied. In the present study, the synthesis and characterization of a curcuminoid analogue with dimethyl substituted phenyl ring and its metal chelates Cu(II),Zn(II) ,Oxovanadium(IV) & Ni(II) are discussed here. The curcuminoid analogue 1,7-bis(2,5-dimethyl phenyl)hepta-1,6-diene-3,5-dione(L1) and the metal 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 against DLA cells(Daltons Lymphoma Ascites)using Trypan blue exclusion method and antifungal study of the compounds were done using Kirby Bauer method. The present investigation reveals that the Cu(II) complex show enhanced cytotoxic activity and Vanadyl complex showed better antifungal activity than the curcuminoid analogue and other metal complexes.*

Keywords: *1,7–dimethylheptanoids;IR; NMR; mass spectra; antitumour; cytotoxicity;antifungal*

I. Introduction

Curcumin is an important phytochemical derived from the rhizomes of turmeric (Curcuma longa Linn) , a member of ginger family, Zingaberaceae. In Ayurveda, turmeric has been used as medicine for various indications.All curcuminoids are components of turmeric and often referred to simply as curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione].Turmeric contains a variety of different curcuminoids namely curcumin, demethoxycurcumin and bisdemethoxy curcumin.Curcuminoids has been isolated from the ground rhizome part of the Curcuma plant species. Curcumin has several biological effects exhibiting anti inflammatory[1-5], antioxidant activities[6-9], anticarcinogenic[10-15] and antimicrobial activities.[16,17]. It has also been studied extensively as a chemo preventive agent in several cancer cells[18,19]. 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 but the aryl ring in natural curcumin is modified and here in the present study it is replaced with a dimethyl substituted phenyl ring .

Curcuminoids are expected to form metal complexes similar to other 1,3-diketones.They are powerful chelating agents.[20,21] Scientific research spanning over more than four decades have confirmed the diverse pharmacological effects of curcumin and is still attracting the researchers from all over the world. Inorganic chemists have used its metal chelating abilities through the β diketo group to form new structural entities with modified biochemical activities. Complexation with transition metals has attracted much interest over the past years as one of the useful requirement to treat deadly diseases.Curcuminoids and their metal chelates possess remarkable biochemical activity[20,23].HereCu(II),Zn(II),Ni(II) and Oxovanadium(IV)complexes of curcuminoid analogue is synthesized and characterized.

In the present study, 2,5-dimethyl benzaldehyde was condensed with acetylacetone in presence of B2O3 using tri-secondary butyl borate and n-butylamine as the condensing agent[22]. The ligand prepared was complexed with Cu(II), Zn(II), Ni(II) and VO(IV) ions to form metal chelates . The curcuminoid analogue and the metal chelates were subjected to in vitro cytotoxic studies using trypan blue exclusion method[19]. The ligand and the metal complexes were also subjected to antifungal activity against the fungi species Aspergillus niger,Penicillium Chrysogenum and Alternaria alternate.

II. Materials And Methods

The chemicals required were obtained from Sigma Aldrich chemical suppliers and are of analar grade. Daltons Lymphoma Ascites (DLA) 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.Fungi species namely Aspergillus niger,Penicillium Chrysogenum and Alternaria alternata were obtained from the culture collection of Institute of Microbial Technology (IMTECH), Chandigarh, India.

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 1,7-bis(2,5-dimethyl phenyl)hepta-1,6-diene-3,5-dione(L1)

The curcuminoid analogue was prepared by the condensation of 2,5-dimethyl benzaldehyde with acetyl acetone-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.

Table 1. Structure of 1,7-bis(2,5-dimethyl phenyl)hepta-1,6-diene-3,5-dione(L1)

Compound	Aldehyde used for Synthesis	Structure of Ligand	Systematic name	Yield %
L1	**2,5-dimethyl benzaldehyde**		1,7-bis(2,5-dimethyl phenyl) hepta-1,6-diene-3,5-dione	**75**

2.3. Synthesis of metal complexes.

The Cu(II),Zn(II),Ni(II) &VO(IV) complexes were prepared respectively by adding a methanolic solution of copper(II) acetate, zinc(II)acetate, vanadyl sulphate, nickel acetate(25 ml, 0.001mol) to a solution of curcuminoid analogue (25 ml, 0.002 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 transition metal complexes were synthesized. The Fig. below represents the structure of Cu(II) complex of the ligand.

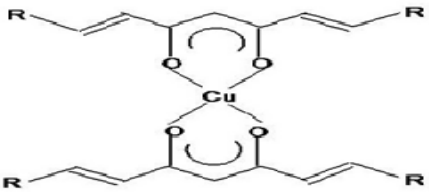

Figure 1.Structure of Cu(II) chelate of ligand.(R=2,5-dimethyl phenyl)

2.4.Characterisation of ligand and metal complexes.

The ligand prepared **1,7-bis(2,5-dimethyl phenyl)hepta-1,6-diene-3,5-dione(L1)** and the transition metal chelates with Cu(II) ,Zn(II) ,Ni(II) &VO(IV) are characterized by various spectral techniques like UV,IR, ^{1}H NMR and mass spectral data.

2.5. In vitro cytotoxicity studies against DLA cancer cells

In vitro cytotoxicity studies were carried out using the diketone and Cu(II) ,Zn(II),Ni(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 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 0.1ml 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 .

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

2.6. Antifungal activity (Kirby Bauer or Disc diffusion method)

Antifungal test was carried out by disc diffusion method. The fungal cultures were maintained in Sabouraud's Dextrose broth. Each culture was uniformly distributed on SDA plates using sterile swabs. Sterile filter paper discs of 3mm diameter were placed on the surface of SD agar plates at a distance of 2cm using sterile forceps. 2 % DMSO was used to dissolve the drug, which was found to have no adverse effect on the fungal cultures. Drugs of different concentrations [100,250,500µg/ml] were added on each disc with a micropipette. Disc with DMSO but, without drug was used as control. Then the plates were incubated at room temperature for 2-3days. After incubation, zone diameter in mm was measured.

III. Results

3.1. Structural characterization of synthesized ligand:

The synthesized curcuminoid analogue namely 1,7-bis(2,5-dimethyl phenyl)hepta-1,6-diene-3,5-dione(L1) was synthesized and characterized.Analytical and UV spectral data are given in **TABLE 2**.The IR spectral data of the compound is given in **TABLE 3.**The IR spectrum of the ligand is represented in **Fig.2**.The NMR spectral details are given in **TABLE 4** and the spectrum is given in **Fig.3.**The Mass spectral details are given in **TABLE 5** and the mass spectrum is given in **Fig.4.**The ligand has been characterized by various spectral techniques like UV,IR,^{1}H NMR& Mass spectrum.

Table 2:Analytical & UV Spectral data of 1,7-bis(2,5-dimethyl phenyl)hepta-1,6-diene-3,5-dione(L1)

Compound	MP.(^{o}C)	Elemental analysis(%)		Molecular weight	UV Λmax (nm)
		C	H		
		Found/(Calculated)			
L1	159	82.45(83.13)	6.95(7.22)	331(332)	281, 393

Table 3 . IR spectral data of methyl substituted 1,7-diaryl heptanoids

Compound L1	Probable IR assignments
1622	V(C=O) chelated
1564,1554	V(C=C) phenyl
1521	V(C-C) alkenyl
1444	V_{as}(C-C-C) chelate ring
1404	V_{s}(C-C-C) chelate ring
1157,1067	β(C-H) chelate ring
974	V(CH=CH) trans

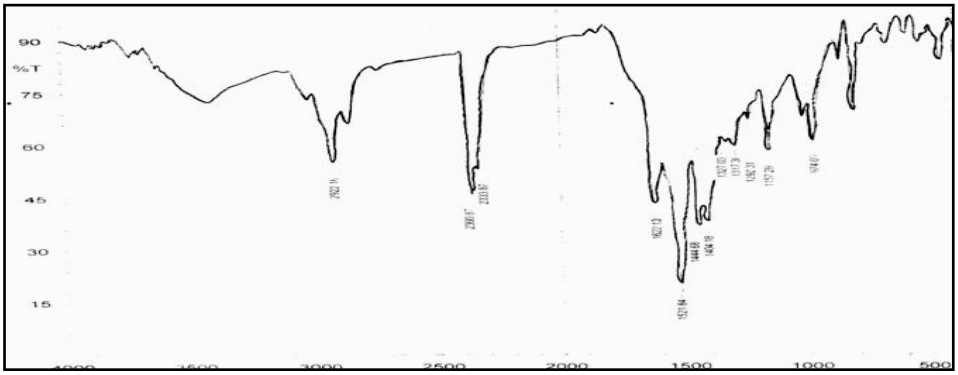

Fig.2 IR spectrum of 1,7-bis(2,5-dimethyl phenyl)hepta-1,6-diene-3,5-dione(L1)

Table 4 . ^{1}HNMR spectral data of methyl substituted 1,7-diaryl heptanoids

Compound	Chemical shifts (δ ppm)				
	Enolic	Methine	Alkenyl	Phenyl	Methyl
L1	15.941	5.835	6.536-7.977	7.199-7.269	2.463

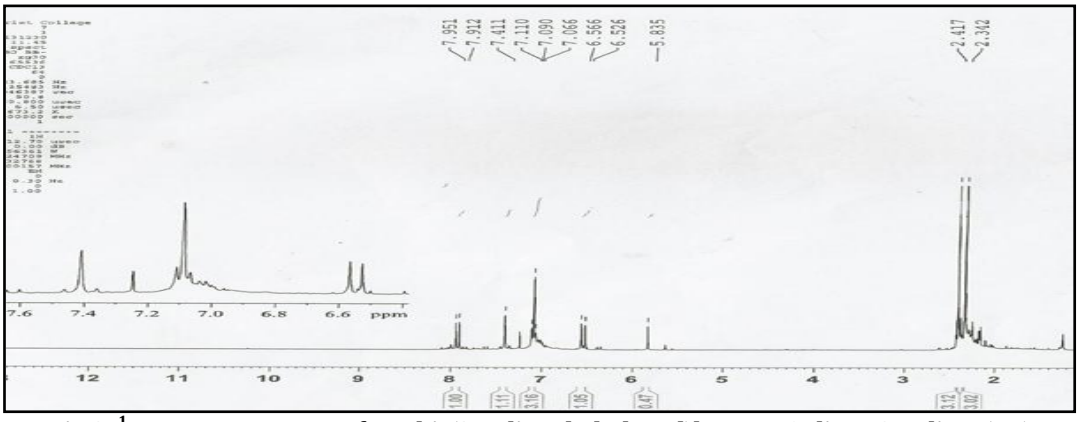

Fig.3 ^{1}H NMR spectrum of 1,7-bis(2,5-dimethyl phenyl)hepta-1,6-diene-3,5-dione(L1)

Table 5 Mass spectral data of of 1,7-bis(2,5-dimethyl phenyl)hepta-1,6-diene-3,5-dione(L1)

Fragments*	Ligand	M+/M+1 ion	Fragment ion peaks							
Mass pattern	L1	332	173	159	115	128	106	127	227	202

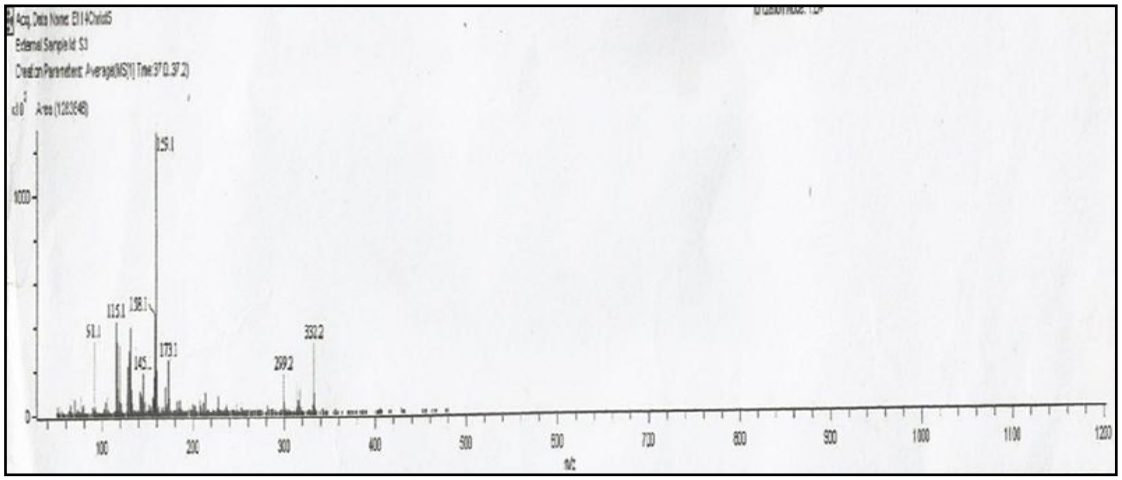

Fig.4 Mass spectrum of 1,7-bis(2,5-dimethyl phenyl)hepta-1,6-diene-3,5-dione(L1)

3.2. Structural characterization of metal complexes with Cu(II),Zn(II),Ni(II),&VO(IV)ions

The ligand L1 form well defined crystalline complexes with Cu(II),Zn(II),Ni(II) and VO(IV) ions. Analytical and mass spectral data of synthesized metal complexes are given in **Table 5.** The approximate formulae of the metal complexes has been found to be as expected ie two ligands are coordinated with the metal ion.(ML2).

Table 6: Spectral data of Cu(II) Zn(II),Ni(II)&Oxovanadium(IV)complexes of ligands

Complex	UV spectra Λ max (nm)	IR data (cm-1)		Mass spectral data (m/z)
		V (C=O)	V (M-O)	
Cu(L1)2	284,394	1608	460,430	726,516,395,306,185,121
Zn(L1)2	289,397	1610	470,420	728,518,397,308,187,
Ni(L1)2	286,395	1602	465,435	721,511,390,301,180,121
VO(L1)2	290,398	1615	479,438	729,519,398,309,188,121

3.3. In vitro cytotoxicity

The results of in vitro cytotoxicity of ligand namely 1,7-bis(2,5-dimethyl phenyl)hepta-1,6-diene-3,5-dione(L1) and the complexes Cu(II)),Zn(II),Ni(II)and VO(IV) towards DLA cells are given in **Table.7.** 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.The cytotoxicity is expressed in terms of % cell death.

Table 7 shows % cytotoxicity towards DLA cells for curcuminoid analogues and Cu(II),Zn(II),Ni(II),VO(IV) chelates

Concentration	L1	$Cu(L1)_2$	$Zn(L1)_2$	$VO(L1)_2$	$Ni(L1)_2$
200µg	20	80	34	43	38
100 µg	10	66	20	40	21
50µg	5	47	8	20	13
20µg	4	35	5	7	6
10µg	2	15	4	5	5

3.4. Antifungal activity

The results of the antifungal activity of **1,7-bis(2,5-dimethyl phenyl)hepta-1,6-diene-3,5-dione(L1)** and its complexes with Cu(II)), Zn(II) ,VO(IV)are given in **Table 8.**For comparison the diameter of zone of inhibition in mm given by the std.drug has also been included in the table.

Table 8. Antifungal studies of 1,7-bis(2,5-dimethyl phenyl) hepta-1,6-diene-3,5-dione (L_1) and its Zn(II) & Vanadyl complexes

Fungi	Diameter of zone of inhibition in mm								
	L_1			$Zn(L_1)_2$			$VO(L_1)_2$		
	100µg	250µg	500µg	100µg	250µg	500µg	100µg	250µg	500µg
Aspergillus	8	10.5	12	10	11	14	12	14	16
Penicillium	11	13	14.5	12	15	17	14	17	19
Alternaria	10.5	12.5	15	11	13	16	13	16	17
Flucanazole(std.drug)			20			20			20

IV. Discussion

4.1 Characterization of methyl substituted analogue of 1,7-diaryl-1,6-heptadiene-3,5-diones

The synthesized methyl substituted derivative of 1,7-diaryl heptanoids were characterized by various analytical techniques . The UV spectra of the compound in methanol show two absorption maxima corresponding to $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions The UV spectra of the compound L1 in methanol show two absorption maxima at 393nm &281nm respectively due to the $n \rightarrow \pi *$ and $\pi \rightarrow \pi *$ transitions. The value at 270-300nm are due to $\pi \rightarrow \pi *$ transition and at 360-460nm are due to $n \rightarrow \pi *$ transitions based on earlier reports.The presence of α,β unsaturation increases the wavelength of carbonyl absorption maxima.

IR spectra of L1 is characterized by the presence of a strong band at 1622 cm^{-1} due to the enolised conjugated C=O group. The stretching frequency of acetyl carbonyl group and carbonyl stretching frequency of aroyl group are at ~ 1710 cm^{-1} and at ~ 1660 cm^{-1} respectively. The C=O frequency decreases due to hydrogen bonding and increased conjugation. There is no other band in the region 1600-1800 cm^{-1} which is assignable due to free or bound C=O group.This shows that the compound exist in the intramolecularly hydrogen bonded enolic form.In the spectra, the intramolecular hydrogen bonded enolic group shows a broad band in the region 2550-3600 cm^{-1}. There are a number of medium intensity vibrations observed in the region 1570-1400 cm^{-1} due to various stretching vibrations of the phenyl group, alkenyl & chelate ring.The band in the region 974 cm^{-1} is assigned to the trans CH=CH vibration.

The ^1H NMR spectra of methyl substituted 1,7-diaryl heptanoids also supports the enolic structure of the compound.The peaks corresponding to enolic, methine, alkenyl, methyl and phenyl groups can be observed in the spectrum.**.** Ligand **L1** displayed a one proton singlet at ~ 16ppm assignable to strong intramolecularly hydrogen bonded enolic proton . Another one proton singlet at ~ 5.8ppm can be assigned to the strong intramolecularly hydrogen bonded methine proton. 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 two methyl groups on aryl ring of L1 are present at ~ 2.5ppm.

The most important application of mass spectra is in the determination of molecular weight of compounds.The molecular ion peaks as well as fragment ion peaks are observed in the spectra.The mass spectra also gives idea about the various fragmentation modes of the substance. The mass spectra of compound showed intense molecular ion peak. Elimination of important groups like CH2,C2H2,C2H2O,CH2=C=O from the molecule gives different fragments.Important fragment ion peaks that appeared in the spectra of compound can be conveniently accounted by the fragmentation pattern . The M+ ion of L1 is observed at 332.The remaining important peaks are that due to the fragment ions.

4.2 Characterization of metal complexes

Analytical and mass spectral data clearly suggests a stochiometry ML2 for complexes. The UV absorption bands of the ligand were almost unaffected by complexation with metal ions. The spectra of complexes closely resembles the spectra of respective ligand. So there is no much change in the structure due to complex formation. There is a bathochromic shift of absorption maxima to longer wavelength which indicate the involvement of the carbonyl moiety in chelate formation.

In the IR spectra of metal chelates,additional bands appear at ~ 475 cm^{-1} and ~ 420 cm^{-1}assignable to ν (M–O) vibrations. The absence of a strong band in the region 1650-1800 cm^{-1} is one characteristic feature of the metal complex.But the peak due to intramolecularly hydrogen bonded carbonyl group which is present at ~ 1622 cm^{-1} disappeared and a new band appeared at ~ 1595 cm^{-1}. The new band can be assigned to the metal coordinated carbonyl group .The replacement of enolic proton by a metal ion is also evident from the absence of the broad band in the region of 2600 -3500 cm^{-1} present in the ligand.

In their mass spectra,all the complexes showed relatively intense peaks at m/z corresponding to ML_2 stochiometry,where M is metal and L is ligand.Mass spectral fragments are another important tool in elucidating the structure of metal complexes. It was found that some fragments rearrange to form stable cyclic species . The mass spectral analysis shows that stepwise removal of aryl groups is a characteristic feature of all the complexes. In all the cases $[ML_2]+$ ion is the most intense peak. Smaller molecules like O, OH, CH etc. are also eliminated.Peaks due to $[ML_2]+$,L+ and fragments of L+ are also detected in the spectrum.

4.3 In vitro cytotoxicity

Invitrocytotoxicity studies towards DLA cells revealed that both ligands and complexes exhibited greater %cell death at higher concentrations ie 200µg/ml.As concentration of drug compound increases the % cell death increases.All the metal complexes showed significant increase in % cell death than the ligand.Among the metal complexes the activity followed the order Cu(II)>VO(II)>Ni(II) > Zn(II). It is also noted that metal chelation enhances cytotoxicity of compounds considerably. The copper complex of di methyl substituted 1,7-diarylheptanoids show better results than that of ligand in almost all concentrations. Cu(II)chelate of ligand 1,7-bis(2,5-dimethyl phenyl)hepta-1,6-diene-3,5-dione(L2)showed maximum cytotoxicity among the metal complexes.The complex was very effective in increasing %cell death and produced 80%cell death.The activity of Cu(II) complex was nearly four times that of the ligand.

4.4 Antifungal activity

For all the tested compounds, they show maximum antifungal activity at a higher concentration of 500µg/ml.It is observed that antifungal nature increases with the concentration of the compounds.The ligand L1 exhibited more antifungal activity against Alternaria with a zone of inhibition of 15mm.The compound gave a zone of inhibition of 14.5mm and 12mm for Penicillium and Aspergillus species respectively.Both Zn(II) and VO(IV) complexes exhibited greater antifungal activity than the ligand.

Comparing Zn(II) and VO(IV) complexes of the ligand, it was observed that vanadyl complexes exhibited more antifungal activity.The Zn(II) complex gave inhibitory activity against fungal cultures which was only slightly greater than the ligand.But the VO(IV) complex of ligand had appreciable antifungal activity against all fungal cultures.It produced a maximum zone of inhibition of 19mm against Penicillin which is comparable with the zone of inhibition produced by the std.drug.

V. Conclusion

The ligand 1,7-bis(2,5-dimethyl phenyl)hepta-1,6-diene-3,5-dione(L1) and the complexes Cu(II)),Zn(II),Ni(II)and VO(IV) were synthesized and characterized by various spectral techniques.The ongoing discussion reveals that the methyl substituted derivatives of 1,7-diaryl heptanoids and their metal complexes possess enhanced cytotoxic activity. The metal chelation considerably enhances the cytotoxicity of these compounds. Also it is found that Cu(II) complex of 1,7-bis(2,5-dimethyl phenyl)hepta-1,6-diene-3,5-dione(L1) is the most active compound in in-vitro cytotoxicity study with DLA cells . The antifungal studies clearly show that both ligand and metal complexes possess significant antifungal activity. The VO(IV)complex of 1,7-bis(2,5-dimethyl phenyl)hepta-1,6-diene-3,5-dione show better antifungal activity than Zn(II) complex and ligand. The ligand 1,7-bis(2,5-dimethyl phenyl)hepta-1,6-diene-3,5-dione(L2) was found to be not very effective in cytotoxic activity when compared with its Cu(II) complex.So metal chelation of the curcuminoid analogue has increased its cytotoxic and antifungal activity.

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