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Study on synthesis, *in-vitro* anti-inflammatory and anticancer activity of L-Catechin Metal complexes.

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ABSTRACT : This article describes the isolation of L-catechin and a novel synthesis of L-catechin bioorganic complexes in water. The formation of the complex is deduced from the UV–visible spectra, which shows that the successive formation of complex occurs in a ratio of 1:1 (metal/ligand) stoichiometric. Five new complexes were synthesized by the reaction of ligand L-catechin with bioorganic metal ions such as Zinc (Zn^{2+}), Copper (Cu^{2+}), Ferrous (Fe^{2+}), Magnesium (Mg^{2+}), Calcium (Ca^{2+}). The composition of the complexes was characterized by elemental analysis, FTIR, mass spectrometry (MS) and ^{1}H NMR spectrometric techniques. The antioxidant activity of the complex was evaluated using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide radical scavenging method whereas; the anti-inflammatory activity was evaluated using the bovine serum albumin method. L-catechin bioorganic complexes were evaluated against three human cancer cell lines using breast, lungs, and leukemia cancer cell lines. In the following work, we have successfully demonstrated that the metal complexed flavonoids were much more effective free radical scavengers, than the free flavonoids.

Key words: catechin (-), bio-organic metal complex, antioxidant, anti-inflammatory, anticancer.

1. Introduction

The clinical success of cisplatin has triggered considerable interest in the search for the novel and improved metal-based drugs[1] . Several approaches have been applied over the last three decades with common goals, such as the design of new metal compounds that remain active against resistant cell lines, compounds with a wider spectrum of antitumor activity, and compounds with a lower toxicity than cisplatin. Since then, the numerous non-platinum metal complexes were studied, including ruthenium, iron, and cobalt complexes with different ligands. The vast amount of the literature [1-3], related to the synthetic metal complexes of flavonoids with strong anti-tumor activity has been reported in recent years. Flavonol (like quercetin), a kind of flavonoid, is mainly obtained from the natural sources such as some plant extracts and possess low toxicity and good potency. At the same time, catechin structure possesses many excellent properties, such as having a high degree of super-delocalized and complete π-bond conjugated system, containing strong coordination oxygen atoms with the appropriate spatial configuration, which makes it as a perfect metal ion chelating ligand [4]. The reported literature has suggested the synthesis of Naringin and copper complexes, also the anticancer activity study showed that the IC_{50} of these complex was far less than the ligand itself [5].Transition metal complexes with the 4′-methoxy-5, 7 dihydroxyisoflavone possess greater antitumor activities and selectivity than the parent isoflavone, even more, effective than the positive control cisplatin against the selected cell lines [6].All the above researchers showed that these complexes have a broad prospect to be chosen as anti-cancer

drugs. It is known that the metal complexes can bind to DNA via covalent and/or non-covalent interactions. The aim of this paper was to obtain ferrous (Fe^{2+}), zinc (Zn^{2+}), copper (Cu^{2+}), magnesium (Mg^{2+}), and calcium (Ca^{2+}) complexes with a ligand levo catechin as well as evaluation of their antioxidant potency, anti-inflammatory and anticancer activities.

2. Materials and Methods

All the reagents and substrates used were in their commercially available form and were used as received and purchased from Rarco Research Lab, Mumbai, India. All the solvents were used as received without further purification. All the reactions were monitored by thin-layer chromatography on silica gel plates (GF 254) and visualized with UV light. Melting points of the compounds were determined on DBK Prog. melting point apparatus and were uncorrected. Specific optical rotation was measured using Accumax Polarimeter. IR spectra were recorded in KBr pellets on Shimadzu IR Affinity-1 FTIR spectrophotometer. ^{1}H NMR spectra were recorded in $CDCl_3$, DMSO-d6, D_2O (if applicable) on a Joel FT/1H NMR 300 MHz spectrometer with tetramethylsilane (TMS) as an internal reference at IIT, Bombay, India. The chemical shifts are given in δ (ppm) referenced to the respective solvent peak and coupling constants are reported in Hz. Mass spectra were recorded on Agilent MSD at Institute of Science, Mumbai, India. The purity of complex is done by DSC. Almost all compounds were characterized by FTIR, ^{1}H NMR and GCMS.

This part describes the exact procedure for the complex synthesis and determination of the structure and purity of these analogs by various analytical methods. The yield of the product synthesized by a particular reaction is mentioned below the procedure followed.

Starting materials used for each reaction and the products obtained were assessed for purity by physical constant determination, and Thin Layer Chromatography (TLC). The final product obtained was found to be a solid powder, in all the reactions.

2.1 General Procedure for extraction of ligand (L-Catechin)

Ligand was isolated by standard procedure from Literature [7]. Recrystallization was done by using different solvent such as water, ethanol, acetic acid. The pure L-Catechin was obtained using water as solvent (13%).

2.3. General Procedure for synthesis of complex (2a-2e) (scheme 1)

L-Catechin (1mM) was dissolved in distilled water, at 60^{o}C followed by introduction of 1mM of bioorganic metal salt(M) slowly via a syringe. The reaction was carried out till completion at 60^{0}C, and then allowed stand at room temperature at for 2 hrs. Resulting solid products were washed with cold water, rinsed with diethyl ether and dried under vacuum. Solid obtained was extracted with ethyl acetate (3X 20 mL) and organic layer was evaporated under vacuum and crude product was then recrystallized with distilled water (60%-75%).

2.4 . Antioxidant studies

2.4.1. Nitric oxide radical scavenging activity[8]

8 ml of SNP solution was added to the test containing 2 ml of phosphate buffer and incubated for 30 min at room temperature, then at every 30 min interval 1 ml of sample was removed and mixed well with 1 ml of griess reagent and absorbance of the resultant pink coloured solution was taken at 540 nm. Effect of Test compound on NO radical scavenging activity was studied by solubilizing complexes **2a-2e** in distilled water. 2 ml of each solution was removed and added to the appropriate test tubes containing 2 ml of SNP solution and 1ml of phosphate buffer, mixed well and allowed to incubate at 30 min. This served as test solution. Blank solution will contain 2 ml of distilled water in appropriate test tubes along with the 2 ml of SNP and 1 ml of griess regent and absorbance of each solution was measured at 540 nm by using shimadzu UV-Vis spectrophotometer. This procedure was repeated after every 30 min interval for 120 min. Quercetin and L-Catechin was used as standard. The amount of NO release in 30, 60, 90, 120, min time interval was calculated from difference (D) between

absorbance of test and blank solutions. The % inhibition of NO release by drug was calculated by using formula {(O.D.Blank – D)/O.D.Blank}X 100.2a, 2b, 2c, 2d, 2e in the activity evaluation are referred as ZnC, MgC, CuC, CaC and FeC respectively.

2.4.2. 1,1-Diphenyl 2 picryl Hydrazyl (DPPH) free radical scavenging activity.

The reaction mixture consisted of 1 ml of o.1 mM DPPH in methanol, 1 ml of methanol and 0.05 ml of methanolic solution of drug /complex **2a-2e**, at various concentration 10, 20, 40, 60, 80, 100 ug/ml. L-Catechin was used as positive control. The absorbance of the mixture was measured at 517 nm exactly for 30 sec. After adding test material the percentage of scavenging activity was determined by comparing the result with Quercetin and L-Catechin. The radical scavenging activity was expressed as the inhibition percentage and monitored as per the equation Inhibition (%) =[(control –test) X 100/control. The result was expressed as IC_{50} value that is the concentration of extract required for 50% inhibition of DPPH radical.

2.5. Anti-inflammatory studies.

2.5.1 Bovine serum albumin *in-vitro* model.[9]

The test solution (0.5ml) consists of 0.45ml of Bovine serum albumin (5%W/V aqueous solution) and 0.05ml of test compounds at concentration of 10, 20, 40, 60, 80, 100 µg/ml. Test control solution (0.5ml) consist of 0.45ml of bovine serum albumin (5%W/V aqueous solution) and 0 .05ml of distilled water. Product control (0.5ml) consists of 0.45ml of distilled water and 0.05 ml of test solution (10, 20, 40, 60, 80, 100)ug/ml). Standard solution (0.5ml) consists of 0.45ml of Bovine serum albumin (5%w/v aqueous solution) and 0.05ml of Diclofenac sodium (10, 20, 40, 60, 80, 100 µg/ml). All the above solutions were adjusted to pH 6.3 using 1N HCl. The samples were incubated at 37°C for 20 min. and the temperature was increased to keep the samples at 57°C for 3 min. After cooling, 2.5 ml of phosphate buffer was added to the above solutions. The absorbance was measured using UV-Visible spectrophotometer at 416nm. [11-12]The percentage inhibition of protein denaturation can be calculated as, percentage inhibition = [100-(optical density of test solution – optical density of product control) ÷ (optical density of test control)] ×100. The control represents 100% protein denaturation.

2.6 Anticancer screening studies[10].

2.6.1. SRB Assay

Anticancer activity of extracted L-catechin and synthesized L-catechin bio-organic complex was determined by Sulphorhodamine B (SRB) assay at ACTREC, Mumbai, India. The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For screening experiment, cells were inoculated into 96 well microtiter plates in 100 µl. After inoculation, the microtiter plates were incubated at 37 °C, 5 % CO_2, 95 % air and 100 % relative humidity for 24 h prior to the addition of experimental drugs. After 24 h, one 96 well plate containing $5x10^3$ cells/well was fixed *in situ* with TCA to represent a measurement of the cell population (Tz) at the time of drug addition. Experimental drugs were initially solubilized in dimethyl sulfoxide at 100 mg/ml and diluted to 1 mg/ml using water and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to 10µg/ml, 20 µg/ml, 40 µg/ml and 80 µg/ml with complete medium. Aliquots of 10 µl of these different drug dilutions were added to the appropriate microtiter wells already containing 90 µl of medium, resulting in the required final drug concentrations i.e.10µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml. Adriamycin used as control.

2.6.2. End point measurement.

After addition of compounds, plates were incubated at standard conditions for 48 hours and assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 µl of cold 30 % (w/v) TCA (final concentration, 10 % TCA) and incubated for 60 minutes at 4°C. The supernatant was discarded; plates were washed and air dried. SRB

solution (50 µl) at 0.4 % (w/v) in 1 % acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing with 1 % acetic acid and air dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm reference wavelength. Percent growth has been calculated on a plate-by plate basis for test wells relative to control wells. Percent growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells X 100. Six absorbance measurements [at time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)] were used to calculate the percentage growth inhibition. Percentage growth inhibition at each of the drug concentration was calculated as:

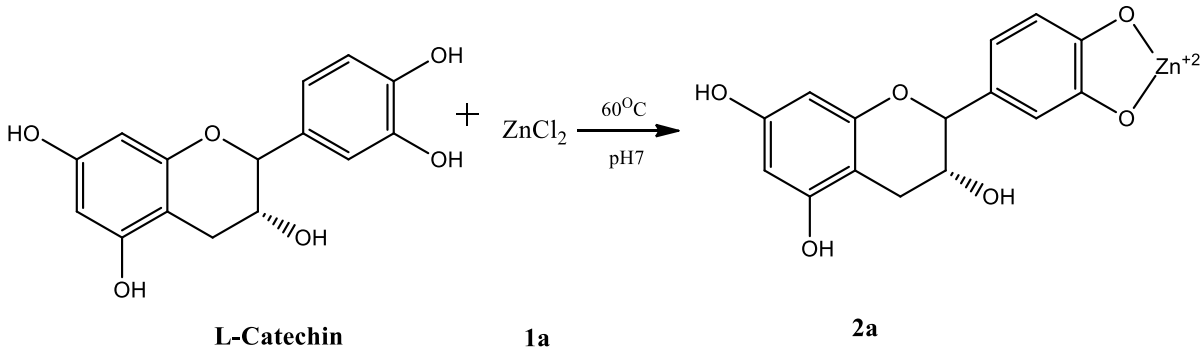
[(Ti–Tz)/(C–Tz)] x 100 for concentrations for which Ti ≥ Tz (Ti–Tz) positive or zero

[(Ti–Tz)/Tz] x 100 for concentrations for which Ti <Tz. (Ti–Tz) negative.

3. Results and discussion

3.1 Synthesis

The model reaction (Scheme 1) was carried out using equimolar mixture of L-Catechin and inorganic salt **1a,** in water as solvent at 60^{O}C. It was noticed that stoichiometric amount of bioorganic metal salt is required to afford **2a** in significant yield. (Table 1, entry 4). The use L-Catechin: Metal salt in 1:4 ratio afforded **2a** in 70% yield (Table 1 entry 4).



L-Catechin **1a** **2a**

Scheme 1. Synthesis of L-Catechin-Zn^{+2} complex **2a**.

Table 1. Effect of concentration of inorganic metal salt on synthesis of L-Catechin-Zn complex **2a**[a].

Entry	$ZnCl_2$ amount (mol)	Yield[c]
1	1	20
2	2	30
3	3	52
4	4	70
5	5	70

[a] Reaction conditions: 1 mmol of L-Catechin , 1-4 mmol of $ZnCl_2$ **(1a)**, in Water (10 ml) heated at 60^{o}C (oil bath temp.) for 2 hrs

To derive the optimal reaction parameters such as effect of temperature, pH, and influence of solvent for synthesis of L-Catechin-Metal complex formation; model reaction was performed using different variations of these parameters. Keeping all parameter constant and varying temperature from 60^{o}C to 110^{o}C effect on yield of complex formation was studied. The maximum yield was found at 70^{o}C (Table 2, entry 3), as the temperature was increased above 90^{0}C, the yield was decreased may be because of formation of polymer which is oxidized product of parent compound in which metal act as catalyst (Table 2, entry 7). The influence of solvent was also evaluated on model reaction. The reaction was performed using hydrocarbon, and polar solvents like water, methanol and ethanol. Water was found to be best solvent (74%, Table 2, entry 14). This may be attributed to good solubility of starting materials in water as a solvent. The effect of pH on reaction was also studied by carrying out the reaction at pH 1-12. It was found that formation of complex is less in strong acidic medium, whereas pH 7 was found to be best for reaction. (Table 2, entry 3). By utilizing the optimized procedure various L-Catechin metal complexes (2a-2e) were synthesized with significant yield (Fig. 1). The complexes were isolated as powdered solids in different yields, the purity of which was confirmed by thin-layer chromatography (TLC) and elemental analysis. In addition, all the complexes were slight soluble in water, soluble in methanol and soluble in DMSO or mixture of water/DMSO.

Table 2. Optimization of L-Catechin-Zn^{+2} complex synthesis (**2a**)[a]

Entry	pH	Solvent	Time (Hrs.)	Temp (^{0}C)	% Yield[b]
1	7	Water	2	60^{o}C	70
2	7	Water	2	65^{o}C	72
3	7	Water	2	70^{o}C	74
4	7	Water	2	75^{o}C	74
5	7	Water	2	80^{o}C	74
6	7	Water	2	85^{o}C	73
7	7	Water	2	90^{o}C	50
8	7	Water	2	110^{o}C	15
9	7	Water	2	30^{o}C (RT)	20
10	5	Water	2	70^{o}C	42
11	2	Water	2	70^{o}C	12
12	8	Water	2	70^{o}C	22
13	12	Water	2	70^{o}C	39
14	7	Water	2	70^{o}C	74

15	7	Ethanol	2	70^0C	79
16	7	Methanol	2	70^0C	80
17	7	Benzene	2	Reflux[c]	14
18	7	Neat	2	70^0C	23

[a] Reaction conditions: 1 mmol of L-catechin **(1a)**, 4 mmol of $ZnCl_2$ **(1a)** in presence of various solvents (10 ml) heated at different temperatures (oil bath temp.) for the indicated time period. [b] Isolated yield of **2a**. [c] Oil bath temp. 70ºC.

3.2. Spectroscopic data

The synthesized complexes were then subjected to UV, FTIR, ^{1}H NMR and DSC studies. The coordination sites and the binding properties of Ligand were determined by using FTIR spectroscopy. Important information can be obtained by comparing the FTIR spectra of Ligand with the bioorganic metal complex. The stretching mode of the free L-catechin occurs at 3000cm^{-1} to 3500cm^{-1}, by the formation of the complex the intensity of functional group decreases. This shift suggests the coordination of hydroxyl group with a metal ion. The decrease in the bond order of C-O and 3-OH, when connected with the metal in the complex, may give rise to a coupling of the vibrations of these two bonds. The new bands

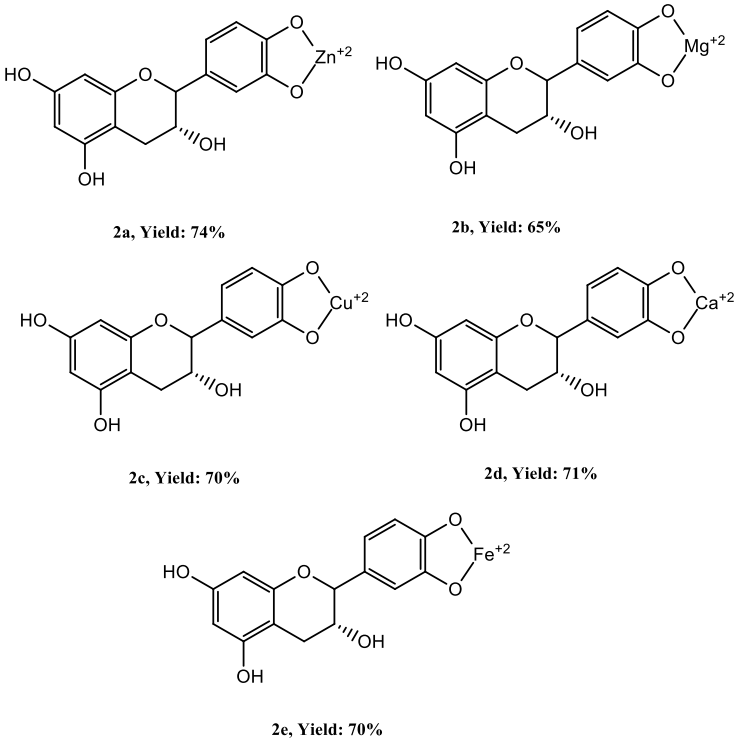


Figure 1: Variety of synthesized L-Catechin- metal complexes (2a-2e).

approximately appear with difference of $40cm^{-1}/50cm^{-1}$ can be considered as associated with the antisymmetric and symmetric stretching modes of the C–O group at the chelating site, respectively. The band situated at 1611 and 1261 cm^{-1} are related to m(C = C) and m(C–O–C) frequencies, which are slightly shifted by the complex. The m(C–O–H) deformation mode observed at 1026.13 cm^{-1} in the ligand, is shifted to 1143.79cm^{-1} in the complex, indicating an increase in bond order, which is normally observed when metal coordination involves with the ortho-phenolic m(O– H) group on the Ligand B ring. Moreover, the presence of stretching vibration/intensity vibration at indicates the formation of metal complex, while the ligand exhibits no such band, which is also coincident with the results of thermal analysis.[1]H NMR studies of the L catechin and its complex show that the L catechin is able to chelate metal ions via 3' or 4' phenol groups. Upon complexation, the metal ion removes two hydrogens from the ligand. In solution, the ligand is in a relatively rapid equilibrium. Both structures have an easily removable H atom, whereas the other H atoms are intramolecularly bonded. The [1]H NMR signals show that the structure of the complex is either (A) or (B) (Fig. 2). The [1]H NMR spectra of the bioorganic metal complex reveal the absence of hydrogen of 3'-OH and 4'-OH group. The other proton signals of the complex are slightly shifted as compared to the free flavonoid, and the signals appeared at downfield, as expected; this is probably due to the increase of the conjugation caused by the effect of coordination when the complex is formed. The complex is paramagnetic in nature due to the availability of the unpaired electron which is localized in the complex. This information clearly indicates that during complex formation, two protons of the free ligand are deprotonated and ligand behaves in a dibasic tetra dentate fashion. All the spectroscopic details are available in supplementary information of this article.

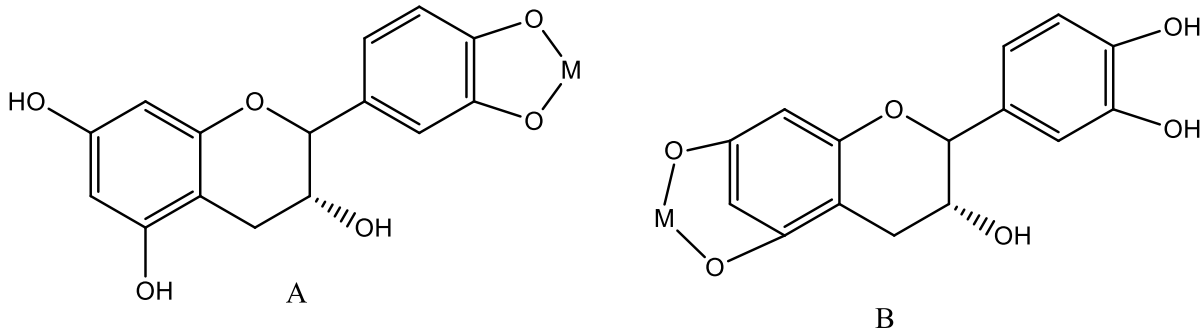


Figure 2: Proposed structure of L-Catechin-Metal complexes

All compounds have been investigated by positive/negative mass spectrometric measurements, giving valuable structural information. As expected, the molecular ions peak of complexes was indicating that each complex containing one metal ion and one molecule ligands was formed. The appearance of the characteristic cluster of isotopic peaks of the corresponding metal ion isotopes demonstrated that metal ions have been successfully coordinated to the ligand. In addition, the predominant peaks corresponding to ions containing Zn^{2+}, Mg^{2+}, Cu^{2+}, Ca^{2+} and Fe^{2+} in the complexes are those with metal bonded to one ligand molecule at m/z 354, 313, 355, 339 and 345 respectively. Complexes showed ions obtained by loss of hydroxyl, which was observed at m/z 337,296,338,322 and 328.
The data of elemental analysis, FTIR, MS and [1]H NMR spectrum confirmed the bonding of ligand a to metal ions and the metals are bridged by the position of 3',4'-deprotonated phenolic oxygen . The elemental analysis, and mass spectrum agreed well with the formation of 1:1 (metal/ligand) stoichiometry.

3.3. Antioxidant studies

All metal co-ordinate complexes showed excellent nitric oxide scavenging capacity in comparison to standard, quercetin and L-catechin. Calcium, Ferrous, Magnesium, and Zinc complexes showed better nitric oxide scavenging capacity which is in direct correlation with their antioxidant potential. Further, the antioxidant activity of ligand and its bioorganic metal complex was also measured in terms of their radical scavenging ability using the stable DPPH. The free radical scavenging activity of L catechin and its bioorganic metal complex was greater than the free L catechin. This suggests that the metal ion significantly changes the chemical properties of the L catechin. From IC_{50} values of the compounds it has been observed that compound Zn^{2+},Ca^{2+},Mg^{2+} have excellent anti-oxidant activity with IC_{50} value of 12µg/ml, 4 µg/ml, 5 µg/ml respectively when compared to Quercertin (IC_{50} value = 29 µg/ml) and L-catechin(IC_{50} value = 54 µg/ml) (Fig. 3a , 3b).

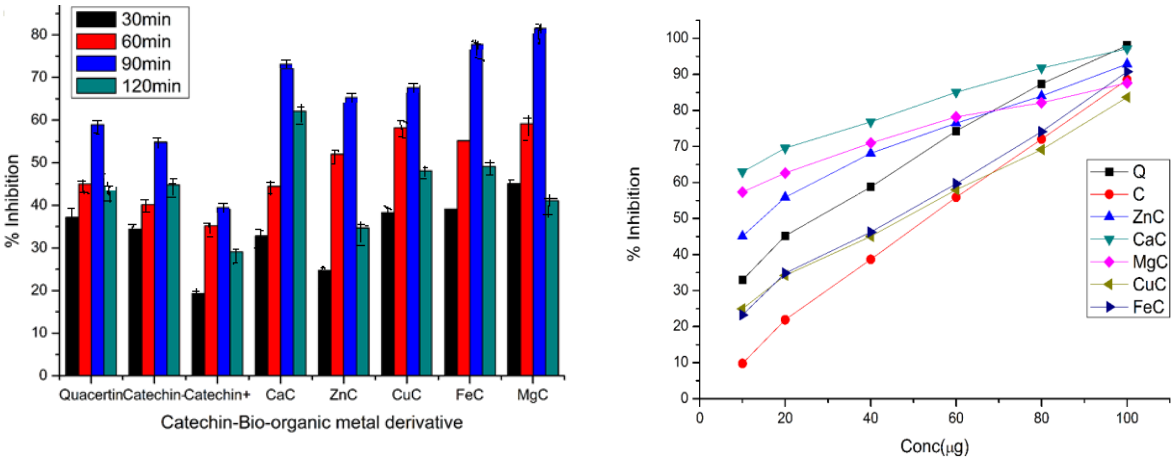


Figure 3. Results of antioxidant activity of L-catcehin metal complexes by a) NOs assay c) DPPH assay. Each point represents the mean ± SD of triplicate measurements.

3.4. Anti-inflammatory studies

Ligand is an excellent scavenger of both ROS and RNS. Consequently, the flavonoid might be used to reduce both oxidative stress, i.e. an imbalance between the production of and the protection against reactive species, and inflammation. The antioxidant defense network is composed of endogenous and dietary factors that act in a dynamic interrelationship, including complex sparing and recycling reactions that allow for quenching a variety of reactive species and also conserving elements of the network itself. Anti-inflammatory activity of ferrous complex with IC_{50} value of 38 µg/ml was found to better and potent than the standard compound diclofenac (IC_{50} value = 45 µg/ml), while other derivatives such as CaC, MgC, ZnC (IC_{50} = 45µg/ml, 45µg/ml, 47 µg/ml respectively) are equally active, in comparison with standard diclofenac and L-catechin (IC_{50} value = 48 µg/ml) alone. The Cu^{+2} complex of L-catechin (CuC) is comparatively less potent(IC_{50} = 50 µg/ml) than other (Fig 4.).

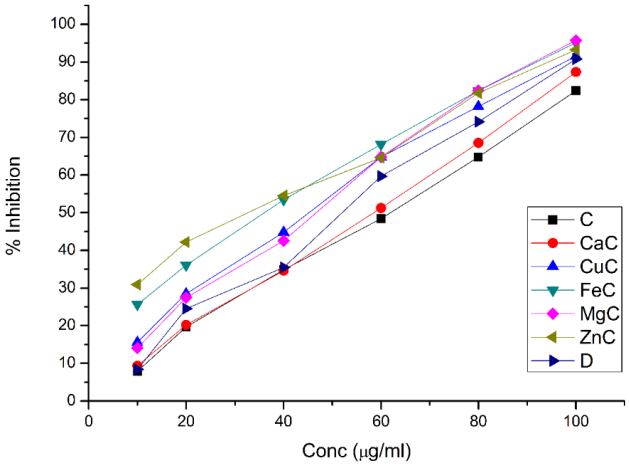


Fig.4.Results of *In-vitro* anti-inflammatory activity of synthesized L-catechin metal complexes.

3.5. Anticancer screening studies

In the present study, ligand and its complex of selected bioactive organic metal were evaluated for their anticancer potential. L-catechin metal complexes are found to be effective in controlling the growth of MCF-7 breast cancer cell lines when compared with Adriamycin as standard. The L-catechin- Fe^{+2} complex, L-catechin-Mg^{+2} complex, L-catechin Zn^{+2} complex have shown excellent activity compared to Adriamycin whereas L-catechin itself failed to control the growth of cancer cells. All the complexes of ligand and metals are very less effective in controlling the growth of human leukemia cell lines K562 but not as good as standard Adriamycin; although ability of all the complexes is much better than L-catechin itself. When evaluated using human hepatoma cell lines HepG2 all the complexes are better in comparison to L-catechin alone but less effective than standard Adriamycin. (Fig. 5, 6, 7).

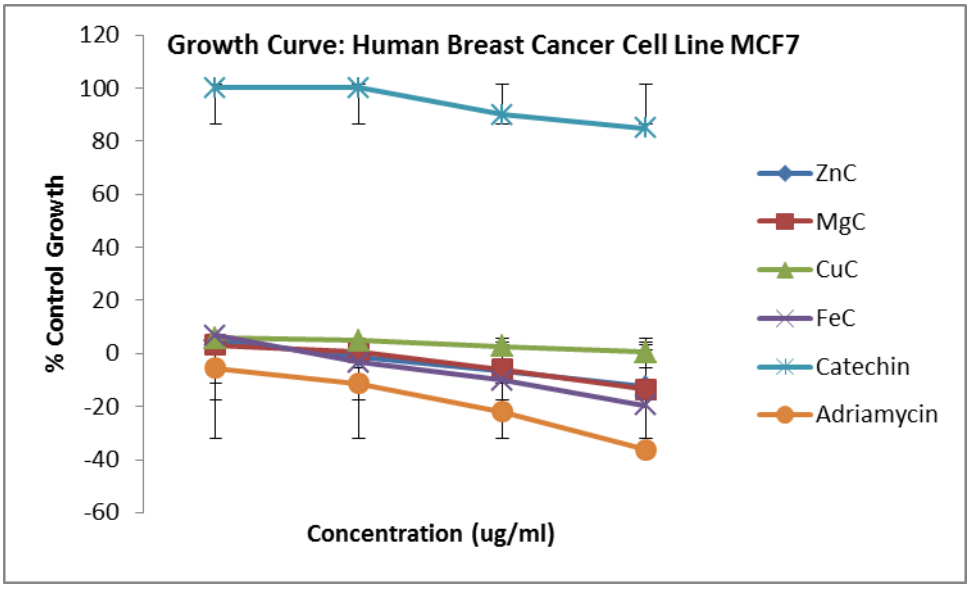


Fig.5. Results of *In-vitro* anticancer activity using Human Breast Cancer Cell Line MCF7. Each point represents the mean ± SD of triplicate measurements.

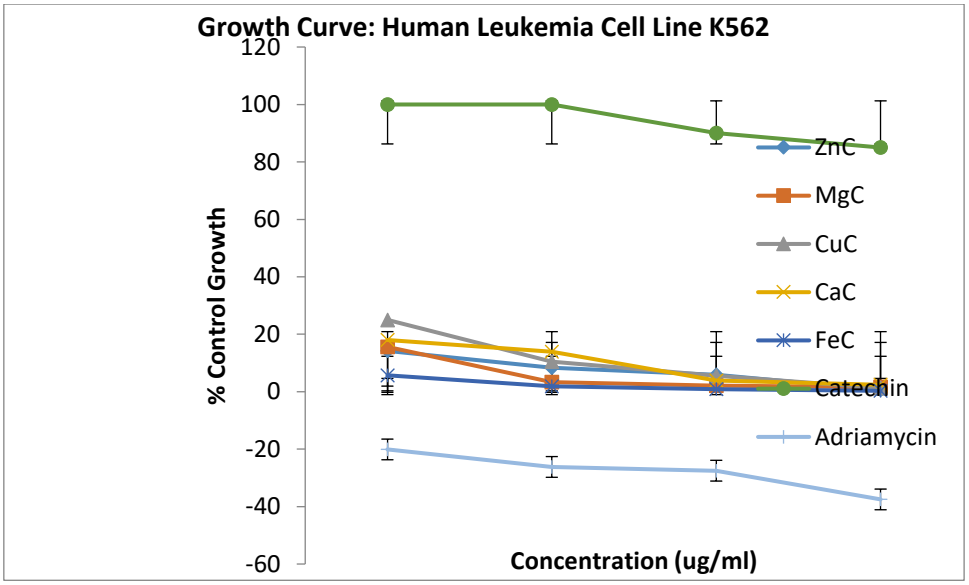


Fig.6. Results of *In-vitro* anticancer activity using Human Leukemia Cell Line K562. Each point represents the mean ± SD of triplicate measurements.

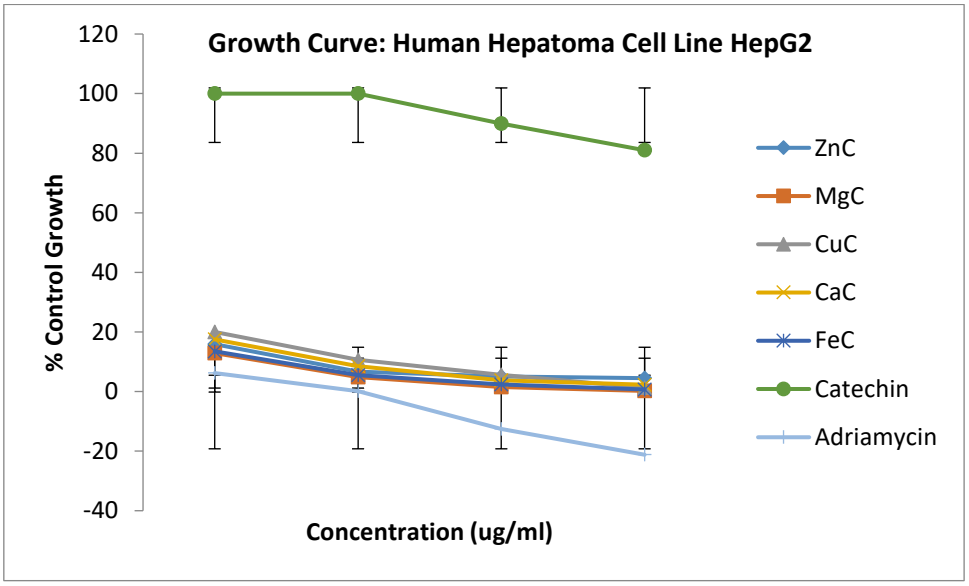


Fig.7. Results of *In-vitro* anticancer activity using Human Hepatoma Cell Line HEPG2. Each point represents the mean ± SD of triplicate measurements.

6. Conclusions

In, conclusion we have successfully synthesized L-catechin metal complexes from readily accessible reagents by simple, optimized, efficient protocol. All the complexes exhibited excellent antioxidant potential when evaluated by DPPH and Nitric oxide scavenging (NoS) method. Amongst all L-catechin, ferrous, zinc, magnesium, calcium complexes are better antioxidant with IC_{50} value 4-12 µg/ml, which is much better than L-catechin alone. The determination of *in-vitro* anti-inflammatory activity draws our attention to more potent L-catechin-Fe complex when compared with standard diclofenac; other complexes of ligand and magnesium, calcium and zinc are also active. Again the activity was found to be improved when compared with L-catechin alone. This helps us to establish the co-relation

between antioxidant activity of synthesized metal complexes and their *in-vitro* anti-inflammatory activity.

We demonstrated the anticancer activity of L catechin metal complexes on different cancer cells. The anticancer activity of metal complexes was enhanced several fold than L catechin alone and L-catechin-Fe^{+2} complex was found to be excellent in controlling the growth of MCF-7 cell lines when compared with standard adriamycin. This may explain the protective effect and suggest that the utilization of different bioorganic metal complexes may have enhanced anticancer activity. A further mechanistic study in depth is warranted to know the molecular interaction in the structure of the bioorganic metal complex and L catechin responsible for an enhanced anticancer activity.

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Conflict of interest

The authors declare no conflict of interest.

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