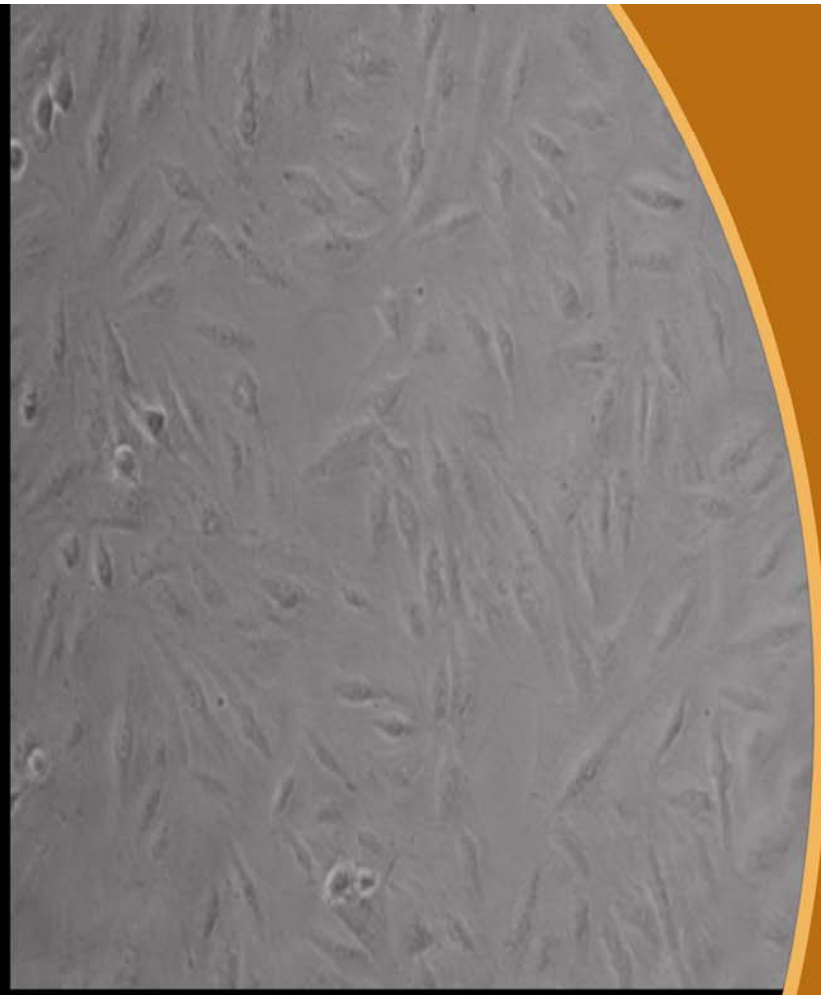

Department of Applied Chemistry
Cochin University of Science and Technology
Kochi – 682 022

Ph.D Thesis
May 2013

Curcumin and its derivatives as Antioxidants and DNA Intercalators

Priya Rajan. S

Ph.D Thesis

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By

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Department of Applied Chemistry
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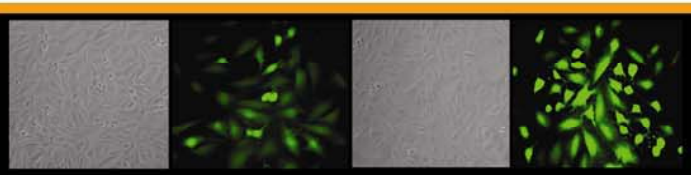

“Curcumin and its derivatives as Antioxidants and DNA Intercalators”

Thesis submitted to
Cochin University of Science and Technology
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Chemistry
under the Faculty of Science

By

Priya Rajan S

Superwising Guide
Dr. P.V. Mohanan

Department of Applied Chemistry
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May 2013

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Certificate

*This is to certify that the thesis entitled "**Curcumin and its derivatives as Antioxidants and DNA Intercalators**" is an authentic record of research work carried out by **Mrs. Priya Rajan. S** under my supervision in partial fulfillment of the requirements for the degree of Doctor of Philosophy of Cochin University of Science and Technology and further that no part thereof has been presented before for any other degree.*

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Declaration

I hereby declare that the thesis entitled **"Curcumin and its derivatives as Antioxidants and DNA Intercalators"** submitted for the award of Ph.D. Degree, is the result of investigations carried out by me at Department of Applied Chemistry, Cochin University of Science and Technology under the guidance of **Dr. P .V. Mohanan**, Asst Professor and further that it has not previously formed the basis for the award of any other degree.

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Priya Rajan. S

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Preface

Over the last few decades, there has been an increasing awareness in plant-derived polyphenols with respect to human health. Dietary polyphenols show evidence of many biologically significant functions such as, protection against oxidative stress and degenerative diseases as cardiovascular disease, atherosclerosis, type II diabetes and cancer. 50% of the anticancer therapeutic agents used in contemporary medicine are derived from plant and characterised by stereo specificity. Plant based chemoprevention agents consist of the vast array of polyphenols (bioflavonoids, stilbenoids, etc) and non polyphenols (isothiocyanates, terpenoids etc, as well as the more familiar vitamins A, C, D, and E). Experimental data specify that most of these biological actions can be ascribed to their intrinsic antioxidant capabilities. A dietary antioxidant is a substance in food that significantly decreases the undesirable effects of reactive oxygen and nitrogen species on physiological functions in humans. When the balance between reactive oxygen species production and antioxidant defences is lost, 'oxidative stress' results which through a series of events deregulates the cellular functions and leads to various pathological conditions, viz. AIDS, ageing, cataract, diabetes, neurodegenerative diseases etc.

Turmeric, derived from the rhizomes of plant *Curcuma longa,* is a golden-yellow coloured spice commonly used on the Indian subcontinent, for health care, preservation of food and as a yellow dye for textiles. Since the time of Ayurveda (1900 BC) numerous therapeutic activities have been assigned to turmeric for a wide variety of diseases and conditions, including those of the skin, pulmonary, gastro-intestinal systems, aches, pains, wounds and liver disorders. The significance of turmeric as medicine has gained acceptance since the discovery of its antioxidant properties, which is mainly due to the phenolic part in curcumin. The biological properties of curcumin were attributed to its antioxidant activity.

The active centre for antioxidant activity of curcumin is suggested as its two phenolic groups, enol form of the diketone moiety and extended conjugated structure, which is still a controversy.

Studies on metal based anticancer drugs and anti-arthritic agents are some currently active topics of investigation in bio-inorganic chemistry. The interest in preparation of new metal complexes gained the tendency of studying on the interaction of metal complexes with DNA for their applications in biotechnology and medicine. The metal complexes bind to DNA by non-covalent interactions such as electrostatic binding, groove binding and intercalative binding. The metal-chelation by curcumin can take place through either the diketone moiety or the o-methoxy phenol moiety, in most cases chelation is observed only through the diketo group. The o-methoxy group may influence the electron density on the diketo group, however, can affect their chelating ability.

Objective of the present study

The scope of the work was to synthesis few biologically active derivatives of curcumin. The derivatives were prepared by altering the keto-enol centre of curcumin by various reagents. This particular reaction centre for preparing derivative was selected keeping in mind the controversy regarding the major site responsible for antioxidant mechanism of curcumin. Most of the mechanistic study done earlier was by varying the constituents in one or both of the phenol ring present in the curcumin. The alterations at the keto-enol moiety may throw an insight into the role of the diketo moiety towards the antioxidant mechanism. Since recently curcumin has been suggested as a chemotherapeutic agent for various ailments, we also decided to check the DNA intercalating property of the derivatives synthesised. The main objectives of the present study can be summarised as follows.

Thesis is divided to seven chapters.

***Chapter 1*: Curcumin and its biological importance**

This chapter presents a general introduction of curcumin and its various application study done so far. The chapter discusses about the structure activity relationship for the antioxidant property and various mechanistic path way suggested for it. It also provides a brief review on the importance of metal complexes as DNA intercalators.

***Chapter 2*: Metal chelating property of curcumin and the possible role of metal complexes of curcumin as antioxidant**

This chapter discusses the procedure for separation of curcumin-I and II, its characterization using UV, IR and NMR. The chapter also deals with synthesis and characterization of metal complexes of curcumin-I along with the study of antioxidant activity using DPPH in methanol.

***Chapter 3*: Kinetic solvent effect on antioxidant property of curcumin-I & II and metal complexes of curcumin-I**

This chapter shows the effect of solvent medium on the antioxidant activity of curcumin-I and II as well as Manganese, Magnesium and Copper complex of curcumin-I. The solvent selected for the study were methanol, acetonitrile, acetone, ethyl acetate and 1,4-dioxane. The antioxidant activity showed a regular trend for all the substrate which was interpreted in terms of substrate-solvent interaction, polarity and dielectric constant of the medium selected for the study. By comparison of the result obtained a substrate–activity relation was established

and a mechanism was suggested with respect to the medium in which study has been carried out.

***Chapter 4*: Antioxidant activity of Knoevenagel condensates and its biological interaction in terms of its capacity to prevent/attenuate cataract formation-*in vitro* study.**

This chapter discusses the synthesis and characterization of Knoevenagel condensates of curcumin-I using salicylaldehyde and benzaldehyde. The antioxidant activity was compared with the parent curcumin to establish a structure activity relationship. The effectiveness of curcumin and Knoevenagel condensates of curcumin against selenite induced cataract in organ cultured rat lens was evaluated in terms of Reactive oxygen species (ROS), the activity of Ca^{2+}ATPase, the activity of Super oxide dismutase (SOD) and the amount of calcium.

***Chapter 5*: DNA binding study and cytotoxicity of metal complexes of curcumin-I**

This chapter deals with the study of metal complexes of curcumin-I as DNA intercalators. Techniques such as UV, Cyclic Voltammetry (CV) and Circular Dichroism (CD) were used to analyse the mode of binding of complexes to DNA. The cytotoxicity of the metal complexes was compared to that of curcumin-I.

***Chapter 6*: Synthesis of curcumin pyrazole and its metal complexes: Antioxidant activity and DNA binding study of curcumin pyrazole complexes**

This chapter discusses the synthesis and characterization of curcumin pyrazole and its metal complexes. The antioxidant activity of curcumin pyrazole

was studied in different solvent using DPPH. The mode of binding of complexes to DNA were analysed using UV, Cyclic Voltammetry (CV) and Circular Dichroism (CD).

***Chapter* 7: Conclusions**

This chapter presents the summary and important conclusions of the work done. Further scope of the work is also being discussed in this chapter.

References are given at the end of each chapter.

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Curcumin and its biological importance

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1.1 Curcumin its relevance and activity

Turmeric, derived from the rhizomes of plant *Curcuma longa,* is a golden-yellow coloured spice commonly used on the Indian subcontinent, for health care, preservation of food and as a yellow dye for textiles. Since the time of Ayurveda (1900 BC) numerous therapeutic activities have been assigned to turmeric for a wide variety of diseases and conditions including those of the skin, pulmonary, gastro-intestinal systems, aches, pains, wounds, sprains and liver disorders. The significance of turmeric in medicine has changed considerably since the discovery of its antioxidant properties, which is mainly due to the phenolic part in curcumin [1]. Turmeric contains protein (63%), fat (5.1%), minerals (3.5%), carbohydrates (6.94%) moisture (13.1%) and essential oil (0.8%). The steam distillate of turmeric i.e., essential oils contains α-phelladrene (1.00%), sabinene (0.6%), cineol (1.00%), borneol (0.5%), zingiberene (25%) and sesquiterpenes (50%) and curcumin (3.4%) which is responsible for yellow colour [2].

The most active component of turmeric is curcumin, which makes up 2 to 5% of the spice curcumin, giving the yellow colour to turmeric, was first isolated by Vogel in 1842. The structure of curcumin ($C_{21}H_{20}O_6$) was described in 1910 by Lampe and Milobedeska [3]. Extensive research within the last half a century has

proven that most of these activities, once associated with turmeric, are due to curcumin. Even though curcumin is the major component, it contains many powerful antioxidants and anti inflammatory compounds. Large number of applications of turmeric extracts [4] was observed as in **Table-1.1**.

Table 1.1–Biological Activity of Turmeric extracts and its components [4]

Compound(extract)	Biological activity
Turmeric powder	Wound healing
Ethanol extract	Anti inflammatory, hypolipemic, anti tumor, anti protozoan
Pet. Ether extract	Anti inflammatory, anti fertility.
Alcoholic extract	Anti bacterial.
Crude ether extract	Anti fungal.
Chloroform extract	Anti fungal.
Aqueous extract	Anti fertility.
Volatile oil	Anti inflammatory, anti bacterial, anti fungal.
Curcumin	Antibacterial, anti protozoan, anti viral, hypo lipemic, antioxidant, hypo glycemic, anti coagulant, anti tumor, anti carcinogenic.
Ar-Tumerone	Anti venom.
Sodium curcuminate	Anti inflammatory, anti bacterial.

1.1.1 Structure of natural curcuminoids

Curcumin isolated from turmeric, once believed to be a single component later found to have three closely related species [5] and are shown in **Fig 1.1**. They differ by the methoxy group attached to the phenolic rings, Curcumin-II is chemically demethoxy curcumin (DMC) and Curcumin-III is bis-demethoxy curcumin (BDMC). Curcumin and other related components are generally called by the name curcuminoids.

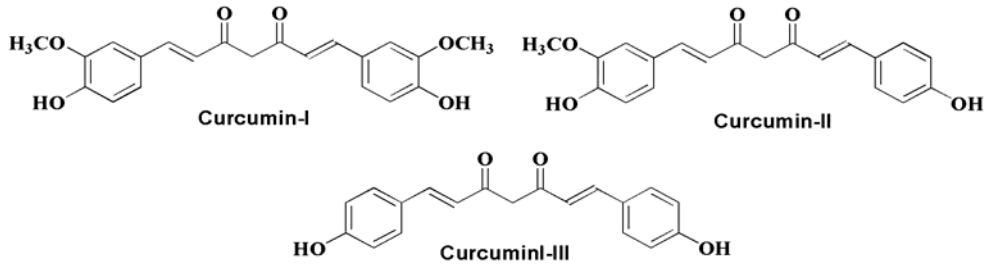


Figure-1.1 Structure of natural curcuminoids

Curcumin-I **1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione**
Curcumin-II **1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)hepta-1,6-diene-3,5-dione**
Curcumin-III **1,7-bis(4-hydroxyphenyl)hepta-1,6-diene-3,5-dione**

Natural curcumin isolated from turmeric contains, curcumin-I (77%), as the major component, melting point of 180–186^{o} C, forms a reddish brown salt with alkali and is soluble in ethanol, alkali, acetic acid and chloroform. It can exist in two tautomeric forms keto and enol as in **Fig 1.2**. The enol form is more energetically stable in the solid phase and in solution [6].

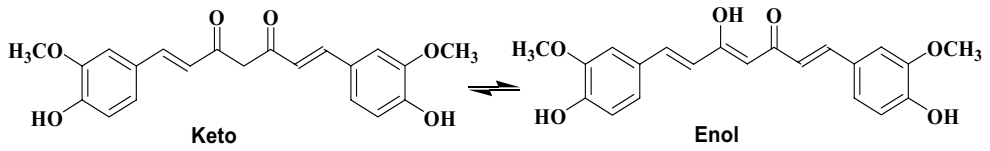


Figure-1.2 The keto-enol tautomerism exhibited by curcumin in solution

1.1.2. Curcuminoids and its activity

In the last few decades, efforts have been made to isolate curcuminoids from different sources, including Curcuma longa, Curcuma zedoaria, and Curcuma aromatica. Several research groups have investigated and compared their antioxidant, cardioprotective, neuroprotective, antidiabetic, antitumor and chemopreventive activities, employing them either individually or as mixtures. The curcuminoids have been shown to be scavengers of free radicals and reactive

oxygen species (ROS), such as hydroxyl radicals, superoxide radicals, singlet oxygen, peroxyl radicals and peroxynitrite, whose production is implicated in the induction of oxidative stress [7-11].

Curcumin, DMC and BDMC exhibit cardio protective, antidiabetic and nematocidal activities. The three compounds inhibited proliferation of bovine vascular smooth muscle cells stimulated by oxidized low-density lipoproteins (LDL) and delayed development of arteriosclerosis [12]. Again, curcumin was the most efficient cardio protective agent of the three. Turmeric extract containing the three curcuminoids could cause lowering of the blood glucose level in type 2 diabetic KK-Ay mice, its hypoglycaemic effect improved when administered in combination with sesquiterpenes [13]. It is the binding of curcuminoids to peroxisome proliferators activated receptor-γ (PPAR- γ) and their acting as PPAR- γ agonists that are responsible for their hypoglycaemic effect.

The neuroprotective effects of curcuminoids have been investigated by various groups. Curcumin, DMC and BDMC protected PC12 rat pheochromocytoma and normal human umbilical vein endothelial cells against β-amyloid-induced oxidative stress even better than α-tocopherol [14]. Curcuminoids have been found to be inhibitors of lead acetate Pb(II)-induced neurotoxicity in primary hippocampal neurons [15], the decreased lipid peroxidation, improved neuron viability and prevented decrease in glutathione levels in rat brain. Under similar treatment, curcumin was the most effective, DMC moderately effective and BDMC the least effective. Curcumin and DMC, but not BDMC reduced Pb(II)-induced memory deficits in rats. BDMC, on the other hand exhibited potent immuno stimulatory effects and was able to correct immune defects of Alzheimer's disease patients by enhancing phagocytosis of β -amyloid and regulation of the transcription of β-1,4-mannosyl-glycoprotein 4-β-N-acetyl gluosaminyl transferase and toll-like receptors [16].

1.1.3. Structure-activity relationship.

Although curcumin, DMC and BDMC differ in their chemical structures only with regard to methoxy substitution, they exhibit significantly different antioxidant, antitumor and anti-inflammatory activities. To date there has been no systematic study that clearly correlates the physicochemical and molecular properties of the three curcuminoids with their biological activities.

Since many reports suggest that curcumin has better radical scavenging and antioxidant ability than the other two and that DMC is superior to BDMC in this activity, the o-methoxy substitutions are certainly involved in this activity. The hydrogen bonding interaction between the phenolic -OH and the o-methoxy groups in curcumin markedly influences the O–H bond energy and H-atom abstraction by free radicals, thus making it a better free radical scavenger than BDMC [9]. The ability of curcuminoids to act as antioxidants or prooxidants in the presence of metals such as Cu(II), Fe(II) or Pb(II) arises mainly from their chelating power [7,15]. Although transition metal-chelation by curcumin can take place through either the diketone moiety or the o-methoxy phenol moiety, in most cases chelation is observed only through the diketo group. Since the three curcuminoids possess similar diketone moieties, their effects on metal-induced toxicity should be similar. The o-methoxy group may influence the electron density on the diketo group, however, which in turn can affect their chelating ability.

1.1.4 Natural analogues of curcumin.

The curcumin molecule is unique in its physiological effects, however having a greater number of molecular targets than any other molecule so far reported. A number of naturally occurring bioactive compounds have shown some structural similarity **Fig.1.3** to the curcumin molecule [17] and at least have a pharmacophore containing one aryl function with 3,4 substitution, that is, either a

methoxylated phenol or catechol. They include ferulic acid, cinnamic acid, caffeic acid, chlorogenic acid, capsaicin, gingerol, paradol, eugenol, dibenzoylmethane, dehydrozingerone, cassumuin and yakuchinone.

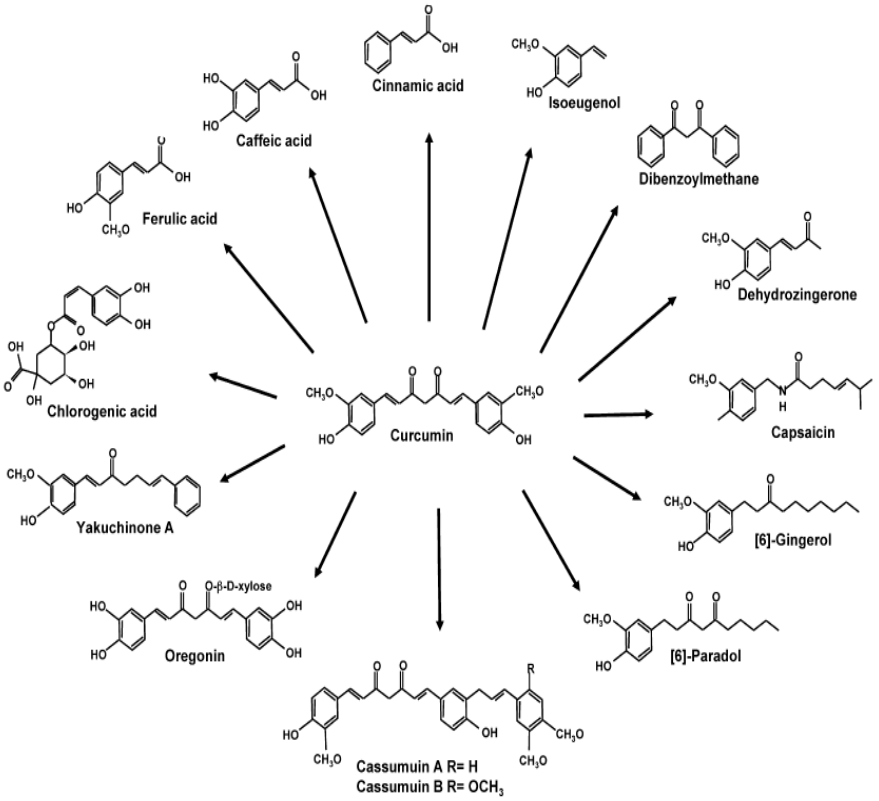


Figure 1.3 Natural analogues of curcumin [17].

Though comparative studies on the antioxidant potential of different naturally occurring analogues of curcumin are not reported, a look at **Table 1.2** [17] and **Fig 1.3** indicates that an ortho-methoxylated phenolic chromophore is desirable [18–20], which may be present in a single aromatic ring (e.g., ferulic acid, caffeic acid, chlorogenic acid, capsaicin, gingerols, zingerone, eugenols) or in two aromatic rings (e.g., oregonin, the potent nitric oxide synthase inhibitor (iNOS), dehydroguairetic acid, yakuchinones, cassumunins). The same

chromophore is responsible for both the antioxidant and prooxidant properties of curcumin and its analogues, which may be due to its radical-generating or hydrogen bond donor/acceptor properties.

Table 1.2–Relative potency of curcumin and its natural analogues [17]

- Caffeic acid and ferulic acid but not cinnamic acid are more potent than curcumin in inhibiting lipid peroxidation [21]
- Caffeic acid, ferulic acid and chlorogenic acid are less potent than curcumin in inhibiting TPA-induced inflammation and promotion of skin tumors [22]
- Dibenzoylmethane is several times more potent (10-fold) than curcumin in inducing phase II enzymes, in inhibiting DMBA-induced mammary tumors in rodents and in inhibiting TPA-induced skin inflammation and tumor promotion [22–24]
- 6-gingerol is more potent (107-fold) mutagen than curcumin whereas less potent in inhibiting TPA-induced inflammation, epidermal ornithine decarboxylase activity and skin tumor promotion in mice [25,26]
- Capsaicin is more potent than curcumin in lowering acidic glycoprotein and inflammation in arthritic rats [27]
- Capsaicin and curcumin are more potent (1000-fold) than eugenol in inhibiting superoxide radical generation [28]
- Capsaicin and curcumin are equally potent in inhibiting arachidonic acid metabolism [29]
- Dehydrozingerone is less active than curcumin in inhibiting formation of conjugated dienes and spontaneous lipid peroxidation [30]
- Dehydrozingerone is as active as curcumin but less active than isoeugenol in inhibiting Epstein–Barr virus antigen early antigen activation [31]
- Yakuchinone A and B are as potent as curcumin in inhibiting LPS-induced nitric oxide production, TPA-induced superoxide production and lipid peroxidation [32,33]
- Cassumunins A and B are more active than curcumin in protecting thymocytes from H_2O_2-induced toxicity [34]

Note: DMBA (7,12-dimethylbenz[a]anthracene); H_2O_2 (hydrogen peroxide); LPS (lipopolysachharide);TPA (12-O-tetradecanoylphorbol-13-acetate.).

1.2 Antioxidants and its relevance

An antioxidant is any substance that when present at low concentrations significantly delays or prevent oxidation of cell content like proteins, lipids, carbohydrates and DNA. Antioxidants can be classified into three main types: first, second and third line defence antioxidants. The first line antioxidants are those which are present within the human system (endogenous), second line of antioxidants are of plant origin (polyphenolic compounds) and third line consist of complex enzymes involved in repair of damaged DNA and proteins. SOD, CAT, glutathione reductase and some minerals like Se, Mn, Cu, and Zn come under first line defence antioxidants. SOD mainly acts as quencher of superoxide, catalase catalyses decomposition of hydrogen peroxide to water and oxygen. Glutathione peroxidise is a selenium containing enzyme which catalyses the reduction of hydrogen peroxide and lipid hydro peroxide to water using reduced glutathione as substrate. Cu exerts its antioxidant activity through the cytosolic superoxide dismutase. Zn is essential for normal growth and is a component of several enzymes like cytosolic superoxide. Glutathione (GSH), vitamin C, uric acid, albumin, vitamin E (α–tocopherol), carotenoids, flavanoids etc, comes under second line defence antioxidants. β-carotene is an excellent scavenger of singlet oxygen. Vitamin E scavenges peroxyl radical intermediates in lipid peroxidation. Flavanoids are phenolic compounds present in several plants which inhibit lipid peroxidation. The third line of antioxidants is complex group of enzymes like lipase, protease, reductase, etc for repair of damaged DNA, damaged protein and also to stop propagation of peroxyl lipid radical.

Free radicals are defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbital [35]. The unpaired electron(s) gives a considerable degree of reactivity to the species. The addition of one electron to dioxygen generates the superoxide anion radical ($O_2^{\cdot-}$) [36], mostly

within mitochondria. The superoxide anion, generated through metabolic processes or following oxygen "activation" by physical irradiation, is considered as the "primary" reactive oxygen species (ROS), and which can further interact with other molecules to generate "secondary" ROS, either directly or through metal-catalysed or enzyme processes [37]. Superoxide can disproportionate by the action of superoxide dismutase to yield hydrogen peroxide, which in the presence of partially reduced metal ions, particularly iron, is subsequently converted through Fenton and Haber-Weiss reactions to a hydroxyl radical as in equations (1-4). The hydroxyl radical, $OH^{\bullet}$, is a very dangerous radical and has a high reactivity with an exceedingly short *in vivo* half-life of approximately 10^{-9} s [38], that is near diffusion rate. Thus when produced *in vivo* $OH^{\bullet}$ reacts close to its site of formation and can interact with nucleic acids, lipids and proteins which significantly contributes to oxidative stress.

$$O_2 \xrightarrow{e^-} O_2^- \quad (1)$$

$$O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (2)$$

$$H_2O_2 + Fe^{2+} \longrightarrow 2OH^- + Fe^{3+} \quad \text{(Fenton Reaction)} \quad (3)$$

$$O_2^- + H_2O_2 \xrightarrow{Fe^{3+}} O_2 + 2OH^- \quad \text{(Haber-Weiss Reaction)} \quad (4)$$

Free radicals are produced from a number of sources, among which are enzymatic, mitochondrial and redox metal ion-derived sources [39]. Aging, leads to loss of free radical scavenging ability by endogenous mechanisms [39]. Hence, the normal balance between free radical generation and free radical scavenging is disrupted with aging and leads to oxidative stress conditions [40]. This condition in which endogenous mechanisms stop working as radical scavengers, could be substituted by polyphenols, natural substance universally present in fruits, vegetables and curcumin, as well as, beverages obtained from plants such as tea and red wine. The remarkable antioxidant activity of these compounds is conferred by the numerous

phenolic hydroxyl groups on the aromatic ring. The rapid donation of a hydrogen atom to lipid peroxyl radical results in the formation of the polyphenol phenoxyl radical ($PP^{\bullet}$), the reaction that can be stabilized by further donation of another hydrogen or by reacting with another radical. In addition, flavanoids present efficient iron chelating activity, for which the 3-OH is important [41]. Once ingested, these compounds are capable of elevating the redox and antioxidant level [42]. In red blood cells, polyphenols enhance cell resistance to oxidative insult [43], as well as inhibit LDL oxidation in plasma [43]. The importance of these molecules in protecting cells from oxidative stress goes beyond the simple radical oxygen species (ROS) scavenging properties.

1.2.1 Antioxidant-prooxidant equilibrium and its significance

When the balance between reactive oxygen species production and antioxidant defences is lost, "oxidative stress" results. Oxidative stress through a series of events deregulates the cellular functions and leads to various pathological conditions, viz AIDS, ageing, cataract, diabetes, neurodegenerative diseases etc. The delicate balance between beneficial and harmful effects of free radicals is important for living systems and is gained by mechanisms called "redox regulation". The process of "redox regulation" protects living organisms from oxidative stress and thereby maintains "redox homeostasis" by controlling the redox status *in vivo* [44].

Normal cellular metabolism gives rise to oxygen free radicals or reactive oxygen species (ROS), as well as reactive nitrogen species (RNS). ROS and RNS play a dual role as both deleterious and beneficial species, as they can be either harmful or beneficial to living systems [45]. Beneficial effects of ROS/RNS involve physiological roles in cellular responses to noxious stimuli as, for example, in defence against infectious agents and occur at low/moderate concentrations. The harmful effect of free radicals termed as oxidative stress causes potential biological

damage and is commonly known as nitrosative stress [46,47]. This occurs in biological systems when there is an overproduction of ROS/RNS on one side and a deficiency of non-enzymatic and enzymatic antioxidants on the other. Thus oxidative stress results from the metabolic reactions that use oxygen and due to the disturbance in the equilibrium status of prooxidant/antioxidant reactions in living organisms. In the last two decades, there has been an increasing interest in plant-derived polyphenols with respect to human health [48,49] and its competence to substitute the enzymatic antioxidants. Plants have found to generate novel compounds with extreme stereospecificity, biological importance and help in maintaining delicate balance of prooxidant/antioxidant. Once such compound derived from plant, found to have multipotent target is curcumin, also an excellent antioxidant.

Curcumin an antioxidant contains two electrophilic β̃unsaturated carbonyl groups, which can react with nucleophiles such as glutathione [50]. By virtue of its Michael reaction acceptor function and its electrophilic characteristics, curcumin and several other polyphenolic compounds have been recently demonstrated to induce the activities of Phase I and Phase II detox system [23,51], e.g., inhibition of COX-1 and COX-2 enzymes [52] and stimulation of glutathione-S-transferase [53]. In addition to its ability to scavenge carcinogenic free radicals [54,55], curcumin also interferes with cell growth through inhibition of protein kinases. Although the exact mechanisms by which curcumin promotes these effects remains to be elucidated, the antioxidant properties of this yellow pigment appear to be an essential component underlying its pleiotropic biological activities. Of particular interest is the ability of curcumin to inhibit lipid peroxidation and effectively to intercept and neutralize ROS (superoxide, peroxyl, hydroxyl radicals) [56] and NO-based free radicals (nitric oxide and peroxynitrite) [57]. In this regard, curcumin has been demonstrated to be several times more potent than vitamin E [58] in reducing oxidative stress.

1.3 Metal complexes and its relevance

Metal complexes play important role in biological systems and have been used as diagnostic and therapeutic agents. The use of inorganic substances in medicine has its origin from the time of Hippocrates and he recommended the medicinal use of metallic salts. However, the logical bases for understanding the role of inorganic species in medicine have been established only after the advances in the field of bio-inorganic chemistry. It is well known that metal ions present in complexes accelerate the drug action and the efficacy of the organic therapeutic agents [59].

Studies on metal based anticancer drugs and anti-arthritic agents are some currently active topics of investigation in bio-inorganic chemistry. The interest in preparation of new metal complexes gained the tendency of studying on the interaction of metal complexes with DNA for their applications in biotechnology and medicine. The metal complexes bind to DNA by non-covalent interactions such as electrostatic binding, groove binding and intercalative binding [60,61] and among all, intercalation is one of the most important DNA binding mode. The intercalating ability of the complex depends on the planarity of ligand, the coordination geometry, ligand donor atom type and the metal ion type [62]. The intercalation process induces modifications of the structure and properties of DNA [63] and is considered as a preliminary step to mutagenesis for several substances [64]. The π-stacking interactions between the aromatic heterocyclic groups of base pairs and the aromatic moieties of an intercalating agent along with hydrogen bonding, electrostatic and hydrophobic interactions contribute to stabilize the binding of these small flat molecules to the DNA helix. Metal complexes are well suited for the study of these interactions because they offer the possibility of evaluating the effects of electronic and sterric factors on the binding with the nucleic acids through systematic changes of the metal and the coordination sphere, e.g., square planar copper(II) complex has a remarkable ability to cleave DNA [65].

1.4 Structure of DNA

Deoxyribonucleic acid (DNA) is the hereditary material in humans and almost all other organisms. DNA is located in the cell nucleus (nuclear DNA), but a small amount is also found in the mitochondria (mitochondrial DNA or mtDNA). The total DNA is the information bank governing all life processes of the organism which is unique to an organism and is termed the '*Genome'*. Stretches of DNA called '*genes*' have the extremely important function of coding for proteins. The information for coding is stored by the order or sequence of four chemical bases: adenine (A), guanine (G), cytosine (C) and thymine (T). DNA bases pair up with each other, A with T and C with G, to form units called base pairs. The two types of base pairs form different numbers of hydrogen bonds, AT forming two hydrogen bonds, and GC forming three hydrogen bonds, **Fig 1.4**. Human DNA consists of about 3 billion bases, and more than 99% of these bases are the same in all people.

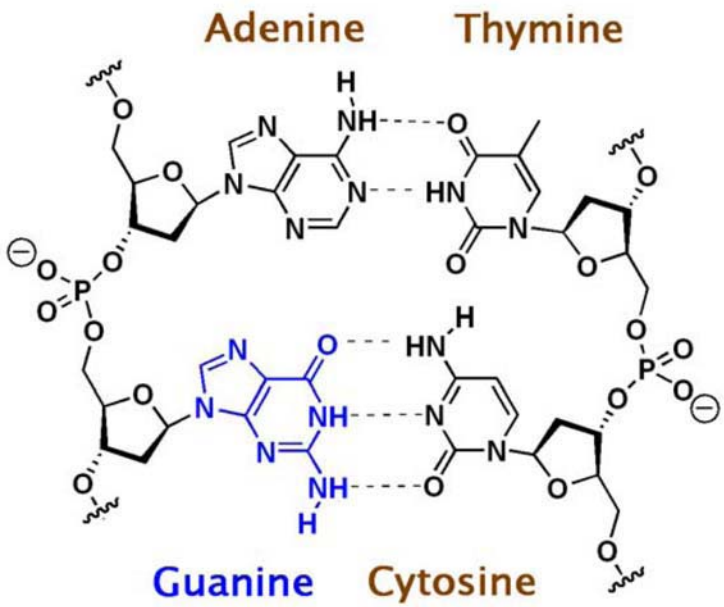


Fig.1.4 Hydrogen bonds in DNA

Each base is also attached to a sugar molecule and a phosphate molecule. Together, a base, sugar and phosphate are called a nucleotide. Nucleotides are arranged in two long strands that form a spiral called a double helix. The structure of the double helix **Fig 1.5** is somewhat like a ladder, with the base pairs forming the ladder's rungs and the sugar and phosphate molecules forming the vertical sidepieces of the ladder.

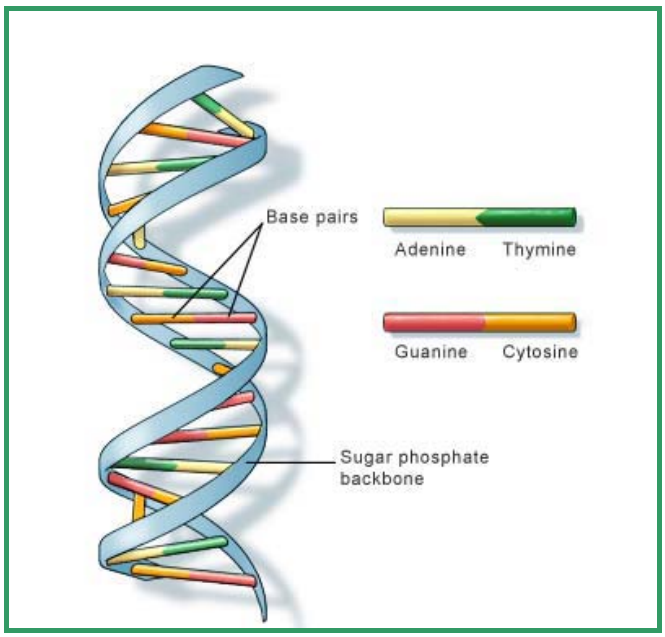


Fig.1.5 Structure of DNA

1.4.1 DNA-Drug interaction

DNA starts transcribing or replicating only when it receives a signal, which is often in the form of a regulatory protein binding to a particular region of the DNA. Thus, if the binding specificity and strength of this regulatory protein can be mimicked by a small molecule, then DNA function can be artificially modulated, inhibited or activated by binding this molecule instead of the protein. Thus, this synthetic/natural small molecule can act as a drug when activation or inhibition of

DNA function is required to cure or control disease. DNA activation would produce more quantities of the required protein or could induce DNA replication, depending on which site the drug is targeted. DNA inhibition would restrict protein synthesis or replication and could induce cell death. Though both these actions are possible, mostly DNA is targeted in an inhibitory mode, to destroy cells for anti-tumour and antibiotic action. The anticancer agents interact through characteristic binding modes with the DNA of cancer infected cell in such a way that the cell cannot replicate further. This inhibition of replication finally leads to the death of the infected cell [66]. Especially the coordination complexes are known to perturb the 3D-structure of DNA with the interaction of N-7 nitrogen of nucleotide and hence inhibiting the replication [66].

Drugs bind to DNA both covalently as well as non-covalently. Covalent binding in DNA are irreversible and invariably leads to complete inhibition of DNA processes and subsequent cell death. Cis-platin (cis-diamminedichloroplatinum) is a known covalent binder used as an anticancer drug and makes an intra/interstrand cross-link via the chloro groups with the nitrogens on the DNA bases [66].

1.4.2 DNA binding modes

Small molecules can interact with DNA through different binding modes as depicted **Fig 1.6**. Intercalation involves the insertion of a planar molecule between DNA base pairs, which results in a decrease in the DNA helical twist and lengthening of the DNA [67]. There is a significant free energy cost for the establishment of the intercalation cavity (approximately 4 kcal mol^{-1}), which is provided by favourable contributions like hydrophobic, ionic, hydrogen bonding and van der Waals interactions which result in association constants of 10^5 to 10^{11} M^{-1} [68]. Intercalation is traditionally associated with molecules containing fused

bi/tricyclic ring structures, typical intercalations with non-fused rings systems is reported [69].

Groove binding is considered similar to standard lock-and-key models for ligand–macromolecular binding [68] and does not induce large conformational changes in DNA. Groove binders are usually crescent-shaped molecules. The binding is stabilized by intermolecular interactions and the cost in free energy required for binding is negligible [68]. Like intercalators, groove binders also have proven clinical utility as anticancer and antibacterial agents, as exemplified by mitomycin (which is also a DNA cross linker) [66]. Notably, the anthracyclines, a class of clinically important compounds with antineoplastic and antibacterial properties, take advantage of both modes of binding as they possess an intercalative unit as well as a groove-binding side chain. The base pair stack at the core of the double helix and insertion into the minor groove are the other binding modes observed.

Intercalation is typically observed for cationic molecules having planar aromatic rings. The positive charge need not be part of the ring system, but rather could be on a substituent. This binding mode requires two adjacent base pairs to separate from one another to create a binding pocket for the ligand [67]. Minor groove binders, on the other hand usually have at least limited flexibility since this allows the molecule to adjust its structure to follow the groove as it twists around the central axis of the helix [70,71]. Binding in the minor groove requires substantially less distortion of the DNA compared with intercalative binding.

Electrostatic interaction happens in the case of positively charged molecules. They electro statically interact with the negatively charged phosphates backbone of DNA chain. Electrostatic attraction is generally weak under physiological conditions. Cations such as Mg^{2+} usually interact in this way [70].

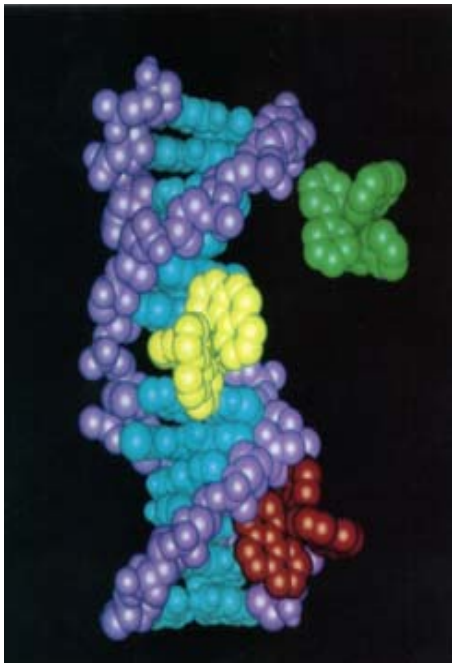
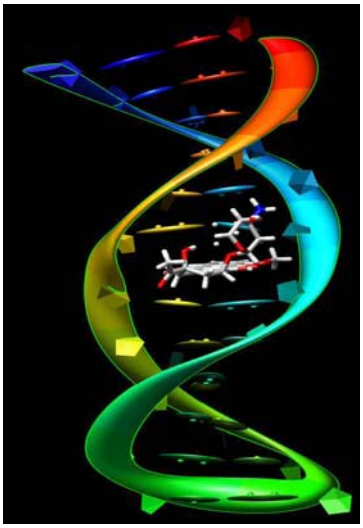
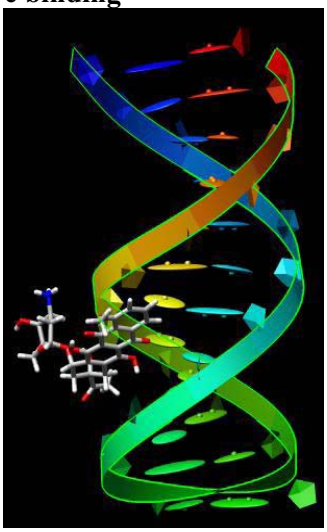


Fig 1.6 Three Modes of Binding in DNA
Green : Surface binding
Yellow : Intercalation
Red : Groove binding



Intercalator



Minor groove binding

Fig 1.7 Non covalently bound drugs

Few examples are listed in the **Table 1.3** shown below [66].

Table.1 3 Drug, action and mode of binding for some DNA binding drugs [73]

NO	DRUG	ACTION	MODE OF BINDING
1	Netropsin	Antitumor, Antiviral	Minor groove binding
2	Distamycin	Antitumor, Antiviral	Minor groove binding
3	Nogalamycin	Antitumor	Intercalation
4	Menogaril	Antitumor-topoisomerase II poison	Intercalation
5	Cis-Platin	Anticancer antibiotic	Covalent cross linking

1.4.3 Methods for the determination of binding modes

The commonly used methods to provide insight into the binding modes of small molecules are UV-visible spectroscopy, fluorescence spectroscopy, cyclic Voltammetry (CV), Circular dichroism (CD) and linear dichroism (LD). Electronic absorption spectroscopy is one of the powerful techniques for probing metal ion-DNA interactions. Electrochemical method is complementary to UV-visible spectroscopy method of investigation. Circular Dichroism (CD) is a useful technique to probe non-covalent drug-DNA interactions, where spectrum of DNA will be modified with increasing concentration of small drugs or metal complexes and characteristic conformation changes would be observed in DNA.

Binding to DNA will often cause a change in the absorption maximum or peak extinction coefficient interactions. “Hyperchromic effect” and “Hypochromic effect” are the spectral features of DNA concerning its double helix structure. Hypochromism results from the contraction of DNA in the helix axis, as well as from the change in conformation of DNA, while hyperchromism results from the damage of the DNA double helix structure [72].

Titration with UV absorption spectroscopy is an effective method to examine the binding mode of DNA with compounds. After compounds intercalating into the DNA base pairs, the π* orbital of the intercalated compounds can couple with the π orbital of the DNA base pairs, thus decreasing the π→π* transition energy and resulting in bathochromism. On the other hand, the coupling π orbital is partially filled by electrons, decreasing the transition probabilities and concomitantly resulting in hypochromism. Generally, the binding of an intercalative molecule to DNA is always accompanied by hypochromism and/or significant bathochromism in the absorption spectra due to the strong stacking interactions between the aromatic chromophore of the compounds and DNA base pairs [73]. The metal complexes which bind non-intercalatively or electrostatically with DNA may result in hyperchromism or hypochromism [74].

1.5 Aim and Objective of the study

The major objective of the study was to synthesis few biologically active derivatives of curcumin by the condensation at the active methylene site of curcumin which is considered as one of the active centre responsible for the antioxidant activity. This particular reaction centre for preparing derivative was selected keeping in mind the controversy regarding the major antioxidant mechanism site of curcumin. Most of the mechanistic study done earlier was by varying the constituents in one or both phenol rings present in the curcumin. The new reaction centre selected for the present study would throw an insight into the role of the diketo moiety towards the antioxidant mechanism. We also decided to study the DNA intercalating properties of the metal complexes synthesised since recently curcumin has been suggested as a chemotherapeutic agent. The main objectives of the work can be summarised as follows

1. Separation of three components of curcumin from the natural curcumin and to study its antioxidant mechanism with respect to solvents selected such as methanol, acetone, ethyl acetate, 1,4-dioxane, acetonitrile and also in water–methanol system.
2. Prepare Knoevenagel condensates of curcumin so as to prevent the keto-enol tautomerism observed in curcumin.
3. Study the changes in antioxidant behaviour of Knoevenagel condensates and its biological interaction using its capacity to prevent/attenuate cataract formation-*in vitro* study.
4. Synthesizing metal complexes of curcumin-I and establish their structural features.
5. Evaluate the antioxidant activity of metal complexes with respect to the solvents such as methanol, acetone, ethyl acetate, 1,4-dioxane and acetonitrile. The scope of the work is to evaluate the contribution of 1,3-diketo system towards the antioxidant mechanism.
6. Evaluating the DNA intercalating property of metal complexes of curcumin and its cytotoxicity
7. Synthesizing pyrazole derivative of curcumin-I and its metal complexes. Evaluating the DNA intercalating property of pyrazole metal complex and also its antioxidant activity.

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Chapter-2
Metal chelating property of curcumin and the possible role of metal complexes of curcumin as antioxidant

Contents

2.1 Introduction
2.2 Materials
2.3. Physico-Chemical Characterization.
2.4 Experimental
2.5 Characterisation of curcumin-I, II and metal complexes of curcumin-I
2.6 Results and Discussion
2.7. Conclusions
2.8 References

2.1 Introduction.

The involvement of metal in neurodegenerative disorder such as Alzheimer disease (AD), mitochondrial disorder, Wilson's disease and Parkinson's disease were recently reported [1]. Several clinical trials were performed to test the ability of natural antioxidant including curcumin to slow down the progression of AD. The key process believed to be involved in the pathogenesis of AD by curcumin is its anti-oxidant, anti-inflammatory [2] and cholesterol-lowering [3] property, along with the metal chelating ability. Epidemiological studies revealed that India has the lowest prevalence rates of AD, where consumption of turmeric via dietary ingredient is widespread [4]. The curcumin taken by the traditional method of dissolving turmeric in fat during cooking was found to be effective to improve absorption, which could play a role in low prevalence of AD in India [5]. The AD

was found to be caused by amyloid Aβ protein loaded senile plaques in the brain cell combined with oxidative stress and inflammation. Human brain is a known concentrator of metal, where potentially toxic level of copper, iron, zinc and manganese could accumulate. In animal study's, curcumin was found to remove heavy metal accumulation in rat brain thus preventing lipid peroxidation induced by metal [6]. Curcumin exhibit unique charge and bonding characteristic [7] that facilitate penetration into the blood brain barrier superior to other known nonsteroidal anti-inflammatory drug (NSAID) [8] and Congo red [9]. The latter is known to be toxic and its charged character is not well suited for penetration into the blood-brain barrier. The polar enolic and phenolic groups of curcumin separated by a nearly neutral hydrophobic conjugated hydrocarbon bridge would facilitate the penetration into the blood-brain barrier, thereby promoting the binding to amyloid β oligomer in the brain cell. The polar group provide a channel of deprotonation and subsequent binding of the resulting anionic species to the protein binding site through hydrogen bonding [7]. The effectiveness of curcumin against oxidative stress is well established and is directly related to the decrease in plaque formation in brain cell [9]. The diketo group can chelate to the metal ion forming a ring structure, analogue to the enol form of curcumin [7]. All these observation have lead to the conclusion about the preventive / curative role of curcumin in AD. The complex formation of curcumin with metal prevents the accumulation of metal in brain cell which in turn reduces the oxidative stress. In the present study the antioxidant property of the ligand curcumin and its complexes were studied to establish the possible role of complexes as antioxidants.

The details of the general experimental techniques adopted, analytical procedures, materials employed and solvent purification are described in this chapter. The chapter also discuss the synthesis and evaluation of antioxidant properties of curcumin-I and its metal complexes.

2.2 Materials

Curcumin	E.MERCK
Piperidine	E.MERCK
Cupric chloride	s. d. fiNE CHEM LTd. Mumbai
Magnesium sulphate	s. d. fiNE CHEM LTd. Mumbai
Cobalt chloride hexahydrate	s. d. fiNE CHEM LTd. Mumbai
Manganous chloride	s. d. fiNE CHEM LTd. Mumbai
Nickel chloride	s. d. fiNE CHEM LTd. Mumbai
2, 2-Diphenyl-1-pikryl-hydrazyl	ALDRICH

Solvents

Common solvents like acetone, ethanol, methanol, chloroform, dimethylsulphoxide, acetonitrile, petroleum ether and ethyl acetate used at various stages of this work were purified according to the standard procedures described either in Weissberger series [10] or purification of Laboratory chemical Perrin [11].

2.3. Physico-Chemical characterization.

2.3.1 Electronic spectra

Electronic spectra of the ligands and their complexes were recorded in DMSO on a Thermoelectron Nicolet evolution 300 UV-vis spectrophotometer.

2.3.2 FT-IR spectroscopy

Fourier Transform Infrared Spectroscopy (FT-IR) is a popular tool for identifying and characterizing materials. FT-IR spectra of the ligands and simple complexes were recorded as KBr pellets with a JASCO-8000 FT-IR spectrophotometer in the 400-4000 cm^{-1} range.

2.3.3 Elemental analyses

Elemental analyses of all the synthesised compounds were done using an Elementar Vario EL III CHN analyzer at Sophisticated Test and Instrumentation Centre (SAIF), Cochin University of Science and Technology, Kochi, India.

2.3.4 Estimation of metal ions

The estimation of metals was carried out on a Thermo Electron Corporation; M series Atomic Absorption Spectrophotometer. For the estimation of metal, organic part of the complexes were completely eliminated with the following procedure. Accurately weighed sample of the complexes (0.05 g) was treated with concentrated sulphuric acid (5 mL) followed by concentrated nitric acid (20 mL). As the reaction subsides, perchloric acid (5 mL, 60%) was added. The mixture was refluxed until the colour of the solution changes to that of the corresponding metal salt. The clear solution thus obtained was evaporated to dryness. After cooling, concentrated nitric acid was added and evaporated to dryness on a water bath. The residue was dissolved in water and this neutral solution was used for the estimation of metals.

2.3.5 Conductivity measurements

The molar conductivities of the complexes in dimethylsulphoxide (DMSO) solutions (10^{-3} M) at room temperature were measured using direct reading conductivity meter (Systronics conductivity bridge type 305).

2.3.6 Estimation of Chloride

Chloride present in the complex was converted to soluble sodium chloride by the peroxide fusion. A mixture of the complex (0.2 g), sodium carbonate (3 g) and sodium peroxide (2 g) was fused in a nickel crucible for nearly 2 h. It was then treated with concentrated nitric acid. Chloride was then volumetrically estimated by Volhard's method [12]. Chloride ion was precipitates as silver chloride by

addition of a known volume of standard silver nitrate solution. The excess of silver nitrate was then titrated against standard ammonium thiocyanate solution using ferric alum as indicator.

2.3.7 TG/DTG

TG-DTG analysis of the complexes were performed on Perkin Elmer Pyris Diamond 6 Thermogravimetric Analyzer in nitrogen atmosphere in the temperature range of 40-800° C and heating rate of l0° C per min. Powdered samples (3 mg) were sealed in standard platinum pans. The instrument was calibrated using indium and tin as standards. Sample residual weight (TG curves) and its derivative (DTG curves) versus temperature were automatically generated by Pyris software.

2.3.8 NMR spectra

^{1}H NMR spectra were recorded in DMSO on a Burker Advance DRX 300 FT-NMR spectrometer with TMS as the internal standard.

2.4 Experimental

2.4.1 Preparation of DPPH solution

The free radical scavenging ability of curcumin and the metal complexes were studied using DPPH assay [13]. Curcumin-I (1 mg mL^{-1}) and metal complex (0.5 mg mL^{-1}) solutions in DMSO were prepared separately and added to a methanol solution of DPPH (0.01 mmol) within the range of 10-150 μL and made up to a final volume of 3 mL using methanol as solvent. The scavenging ability of curcumin and its metal complexes were monitored spectrophotometrically in terms of decrease in absorbance at 517 nm after 20 min. Percentage inhibition was calculated using equation (1).

$$\% \text{ Inhibition} = \left\{ \frac{\text{ABS control} - \text{ABS sample}}{\text{ABS control}} \right\} * 100 \quad (1)$$

From concentration (μM) against absorbance graph, 50% fall in absorbance of DPPH solution was determined. The above concentration values were used for the determination of IC_{50} values in μM.

2.4.2 Separation of curcumin-I and II.

Commercial curcumin was subjected to silica gel (60-120 mesh) column chromatography [14] initially run by $CHCl_3$ till the oily layer started eluting. Curcumin is a mixture of three components **Fig 2.1,** curcumin-I (77%), curcumin-II (18%) and curcumin-III (3%). When the three colour bands, yellow, dark yellowish orange and brown bands starts to separate out, the polarity of eluting solvent was increased by using chloroform-methanol mixture in the ratio 9:1. The fractions were collected separately and components identified using thin layer chromatography (TLC) and MS. The first fraction eluted was named as curcumin-I, 78% yield, (m.p. 186° C). The second fraction eluted was named as curcumin-II, 58% yield, (m.p. 176–177° C). The third fraction which was only 3% of the mixture was not attempted to separate.

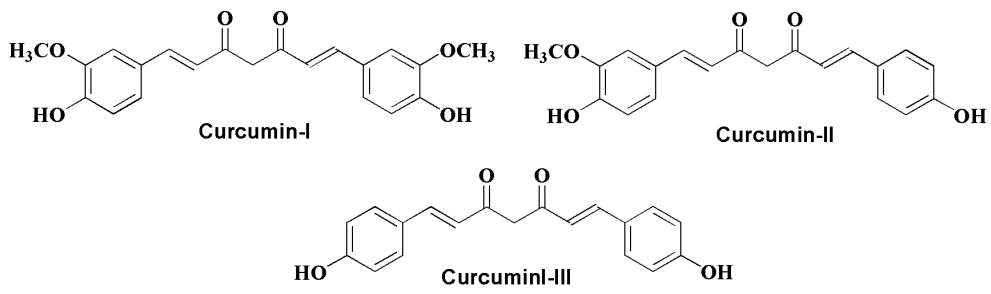
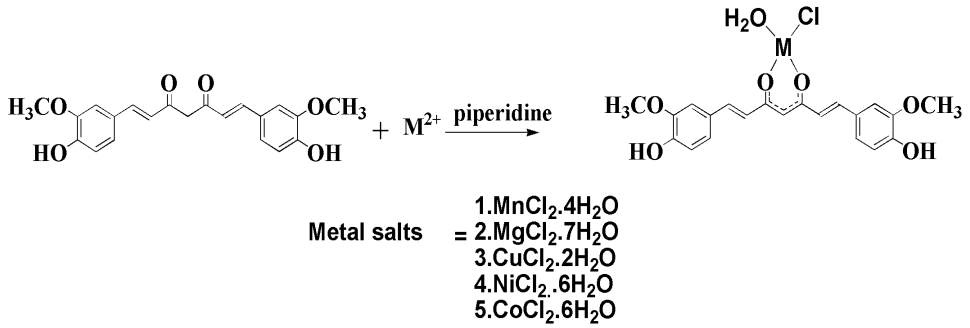


Figure 2.1 Structure of curcumin-I, II and III

2.4.3 Preparation of metal complexes of curcumin-I (1-5).

Curcumin-I (0.27 mmol) and the metal salts (0.27 mmol) solution were prepared separately in methanol. To the curcumin solution containing catalytic amount of piperidine, the metal salt solution was added with continuous stirring, **Scheme-1** [15]. The resultant solution was kept for stirring for 4 h and the product separated was filtered, washed with cold methanol several times to remove the residual reactant and dried in vacuum.



Scheme-1 Synthesis of metal complexes of curcumin-I

2.4.4 Cardiomyocyte model in H9c2 cells

H9c2 cells derived from rat embryonic cardiomyocytes were obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were cultured in DMEM supplemented with FBS, 100 U penicillin mL^{-1} and 100 µg streptomycin mL^{-1} and cultured in 5% CO_2 at 37^0 C. Cells were passaged regularly and subcultured to 80% confluence before the experiments. Experimental group consist of (a) control cells; (b) cells for H_2O_2 treatment (positive control); (c) cells pre-treated with curcumin (5 µM); (d) cells pre-treated with Cu-curcumin (5 µM); (e) cells pre-treated with Mn- curcumin (5 µM); (f) cells pre-treated with Ni-curcumin (5 µM) and (g) cells treated with Co-curcumin (5 µM) for a period of 24 h. Experimental groups (b-h) were treated with H_2O_2 for 15 min, prior to staining.

2.4.5 Generation of reactive oxygen species (ROS)

Intracellular ROS content was determined by oxidative conversion of cell-permeable DCFH-DA to fluorescent 2′,7′ dichlorofluorescein (DCF). H9c2 cells were seeded in 96-well plate at a density of 5000 cells per well. DCFH-DA solution in serum free medium was added at a concentration of 10 μM and co-incubated with H9c2 cells at 37^0 C for 20 min. After three washes, DCF fluorescence was measured by fluorimetry (570 nm) in multiwell plate reader (Biotek Synergy 4, US) and fluorescent imaging was done (BD Pathway™ Bioimager system, USA) to detect the difference in the intensity of fluorescence emitted.

2.4.6 Statistical analysis

All experiments were performed in triplicates (n=3). Data are reported as mean ± SD of control and treated cells. The data were subjected to one way analysis of variance (ANOVA) and the significance of differences between means were calculated by Duncan's multiple range test using SPSS for windows, standard version 7.5.1 and the significance accepted at $P \leq 0.05$.

2.5 Characterisation of curcumin-I, II and metal complexes of curcumin-I

2.5.1 Curcumin–I

2.5.1.1 Electronic spectra

UV spectrum were recorded in the range of 300-600 nm in DMSO. Maximum absorption band of curcumin was obtained at 434 nm indicating the n-π^* transition.

2.5.1.2 Infrared spectra

The FTIR spectrum obtained is as shown **Fig 2.2.** The broad absorption band centred at 3440 cm^{-1} was interpreted as the result of ν(O-H) stretching. The peak at 2930 cm^{-1} is characteristic to ν(C-H). The sharp band at 1623 cm^{-1}, characteristic to ν(C=O). The intense band at 1500 cm^{-1} can be attributed to ν(C=C). The sharp peak at 1271 cm^{-1} corresponds to ν(C-O) of phenol. A medium intense band at 1029 cm^{-1} can be ascribed to ν(O-CH_3).

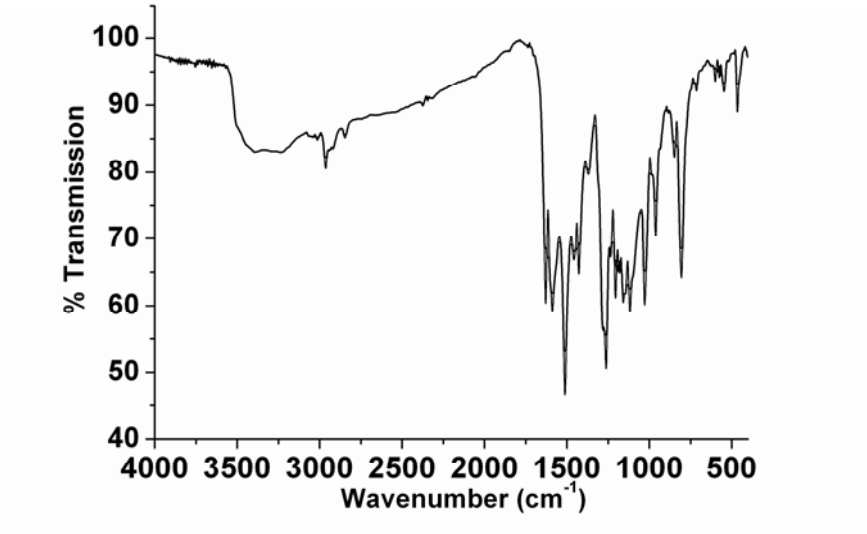


Figure 2.2 FTIR spectrum of curcumin-I

2.5.1.3 NMR spectra

^{1}H N.M.R. (300MHz, DMSO-D_6) δ 3.9 (s,6H,-OCH_3), 6.065 (s,1H), 6.73 (d,2H,J=16Hz), 6.81-7.32 (6H,aromatic), 7.5 (d,2H,J=16Hz), 9.66 (s,2H,-OH) is represented in **Fig 2.3**

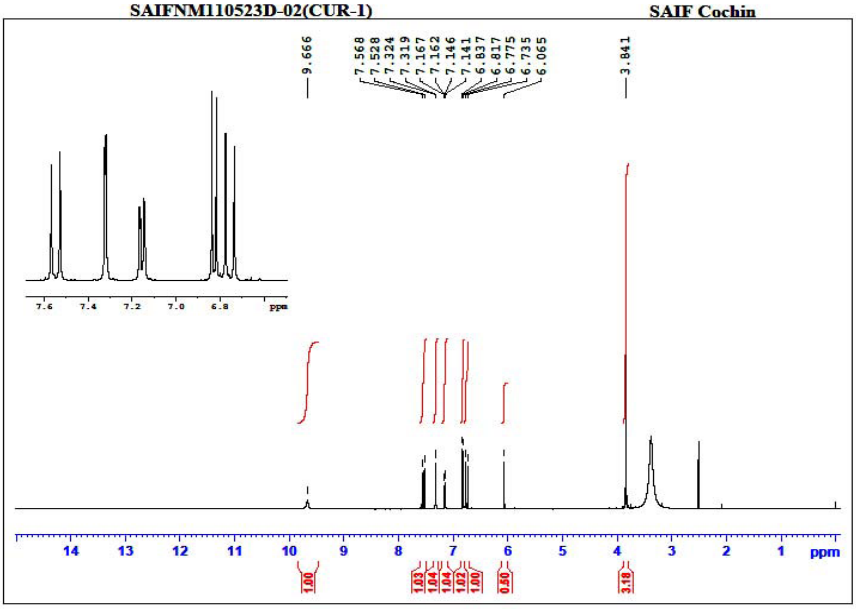


Figure 2.3 ^{1}H NMR spectra of curcumin-I

2.5.2 Curcumin–II

2.5.2.1 Electronic spectra

UV spectrum were recorded in the range of 300-600 nm in DMSO. Maximum absorption band of curcumin was obtained at 438 nm indicating the n-π* transition.

2.5.2.2 Infrared spectra

The strong broad absorption band centred at 3440 cm^{-1} was interpreted as the result of ν(O-H) stretching. The peak at 2930 cm^{-1} is characteristic to ν(C-H). The sharp band at 1628 cm^{-1} can be assigned to ν(C=O). The intense band at 1500 cm^{-1} may be due to ν(C=C).The sharp peak at 1271 cm^{-1} corresponds to ν(C-O) of phenol. A medium intense band at 1029 cm^{-1} can be ascribed to ν(O-CH_3). The spectrum obtained was as in **Fig 2.4.**

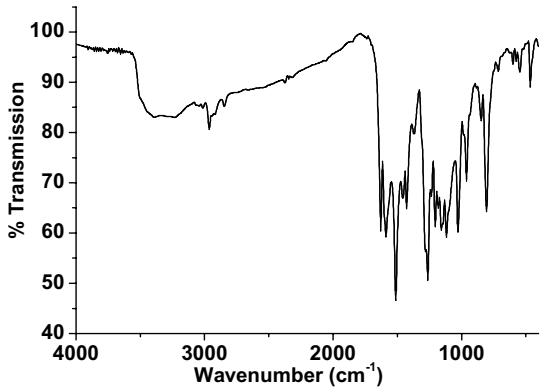


Figure 2.4 FTIR spectrum of curcumin-II

2.5.2.3 NMR spectra

^{1}H N.M.R. (300MHz, DMSO-D_6) δ 3.9 (s,3H,-OCH_3), 6.065 (s,1H), 6.73 (d,2H,J=16Hz), 6.81-7.32 (7H,aromatic), 7.5 (d,2H,J=16Hz), 9.66 (s,1H,-OH). 10.1 (s,1H,-OH).is represented in **Fig 2.5.**

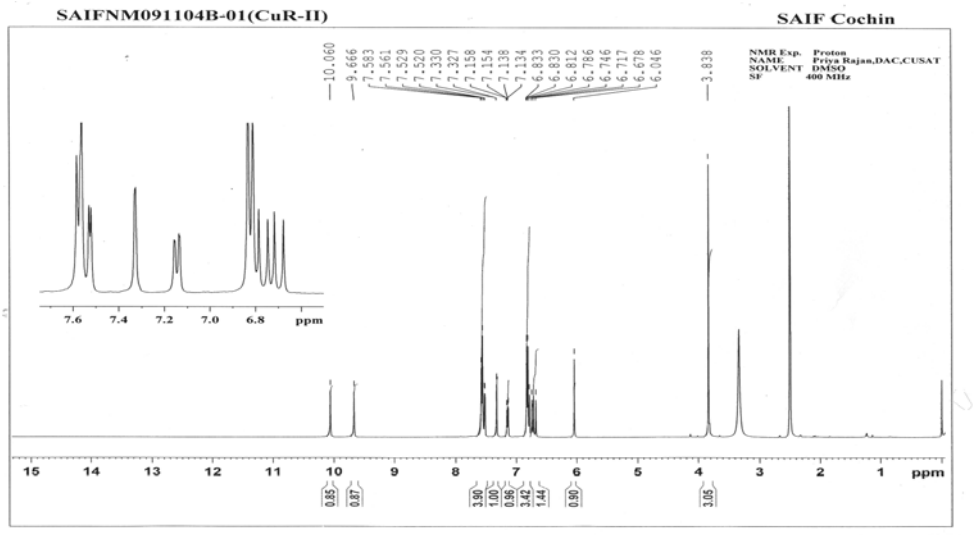


Figure 2.5 ^{1}H NMR of curcumin-II

2.5.3 Characterisation of metal complexes of curcumin–I (1-5)

2.5.3.1 Elemental analysis.

50 Analytical data suggest that all the complexes are mononuclear and have a metal to curcumin ratio of 1:1 and are as show in the **Table 2.1**

Table -2.1 Analytical and conductance data of metal complexes of curcumin-I

Sl. No	Compound	C (%) obtain (Cal)	H (%) obtain (Cal)	Metal obtain (Cal)	Cl%	Molar conductance (Ohm^{-1} cm^2 mol^{-1})
1	Mn-Cur	53.88 (53.01)	4.70 (4.45)	11.30 (11.55)	7.85 (7.45)	6
2	Mg-Cur	56.76 (56.66)	4.56 (4.76)	5.9 (5.46)	8.21 (7.96)	17
3	Cu-Cur	52.17 (52.07)	4.25 (4.37)	13.18 (13.12)	7.66 (7.32)	20
4	Ni-Cur	52.34 (52.60)	4.50 (4.41)	12.50 (12.24)	7.98 (7.39)	22
5	Co-Cur	52.14 (52.57)	4.73 (4.51)	12.50 (12.28)	7.89 (7.39)	20

Based on the analytical and molar conductance data complex have been assigned the molecular formula $[Mn(Cur)H_2O.Cl_2]$, $[Mg(Cur)H_2O.Cl_2]$, $[Cu(Cur)H_2O.Cl_2]$, $[Ni(Cur)H_2O.Cl_2]$ and $[Co(Cur)H_2O.Cl_2]$.

2.5.3.2 Electronic spectra

Electronic spectra of the complexes in DMSO are given in the **Table-2.2**

Table-2.2 UV-visible spectral data of curcumin-I and its metal complexes in DMSO 10^{-5} $molL^{-1}$

Sl .No	Compound	λ max, nm (cm^{-1})
	Cur	434
1	Mn-Cur	442
2	Mg-Cur	437
3	Cu-Cur	439
4	Ni-Cur	443
5	Co-Cur	438

2.5.3.3 Infrared spectra

The various IR bands of curcumin-I and its metal complexes and their assignments are given in the **Table 2.3**. The strong C=O stretching frequency of curcumin at 1628 cm^{-1} is being shifted to lower wave length region in all its metal complexes. The C=O stretching frequencies of complex of curcumin with Mn(II), Mg(II), Cu(II), Ni(II) and Co(II) were 1579 cm^{-1}, 1598 cm^{-1}, 1606 cm^{-1}, 1619 cm^{-1} and 1579 cm^{-1} respectively. This being indicative that it is the ionic enol form of curcumin that chelate with metals [16]. In the IR spectra of curcumin and its metal complexes **Fig 2.6-2.10** the O-H band of phenol do not show considerable shift from 3440 cm^{-1}. Hence it is suggestive that the phenolic O-H group are not involved in the complex formation. All these confirm the association of the ionic enol form in complex formation.

Table-2.3 FTIR band's of curcumin-I and its metal complexes and their assignments

Sl.No	Compound	ν(O-H) (cm^{-1})	ν(C=O) (cm^{-1})	ν (C=C) (cm^{-1})	ν(C-O phenol) (cm^{-1})	ν(-OCH_3) (cm^{-1})
	Curcumin-I	3440	1628	1500	1271	1029
1	Mn-Cur(1)	3433	1579	1500	1287	1015
2	Mg-Cur(2)	3428	1598	1500	1277	1023
3	Cu-Cur(3)	3429	1606	1500	1276	1019
4	Ni-Cur(4)	3422	1619	1500	1270	1031
5	Co-Cur(5)	3434	1579	1500	1279	1027

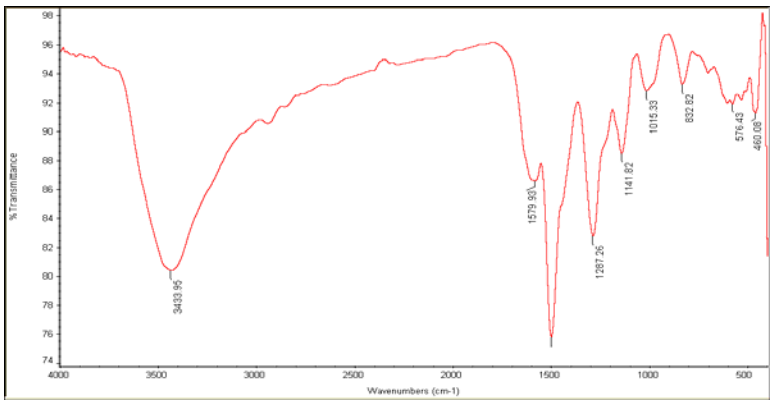


Figure 2.6 FTIR spectrum of Mn-curcumin complex

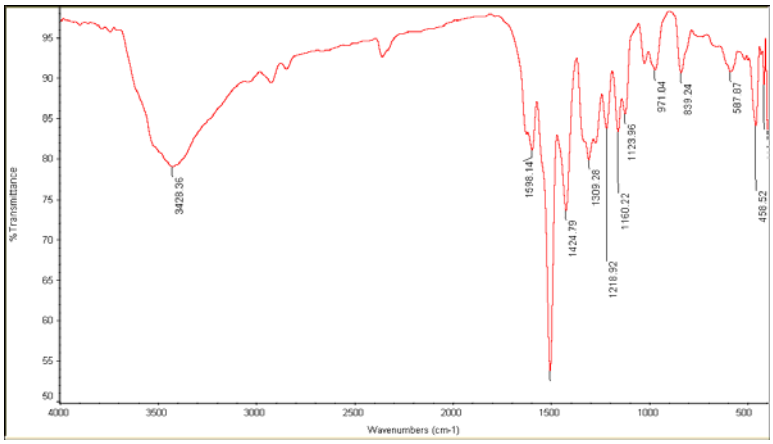


Figure 2.7 FTIR spectrum of Mg-curcumin complex

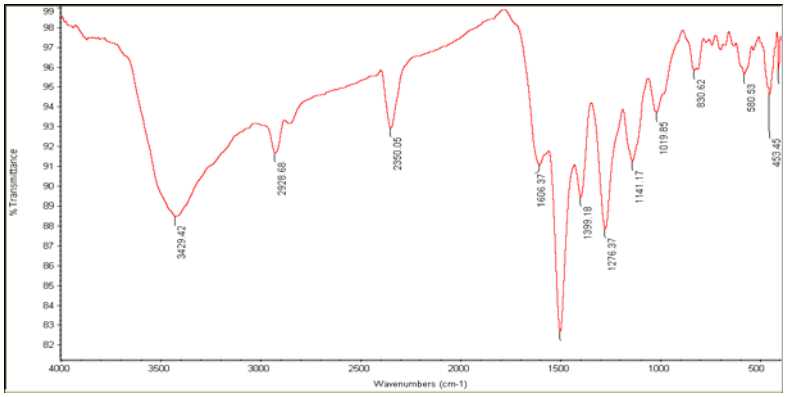


Figure 2.8 FTIR spectrum of Cu-curcumin complex

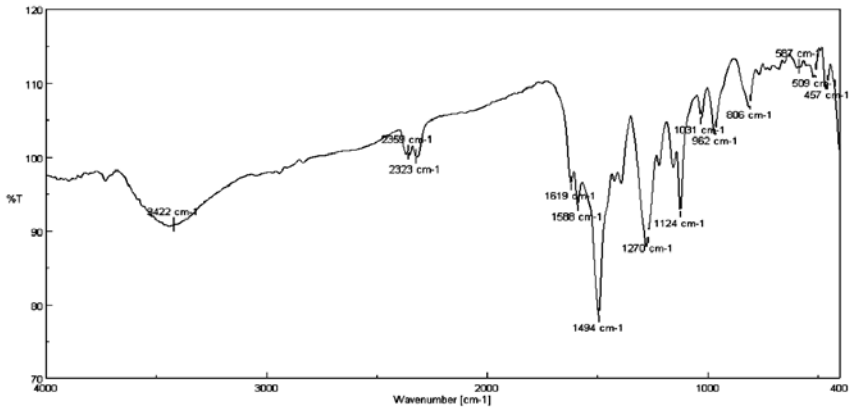


Figure 2.9 FTIR spectra of Ni-curcumin complex

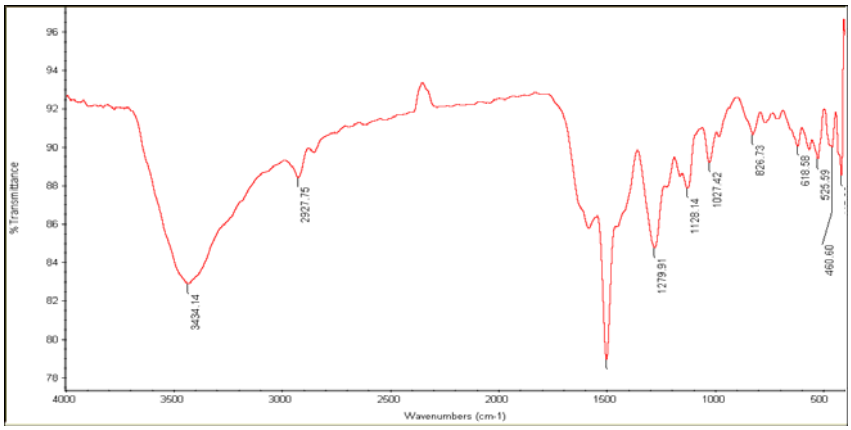
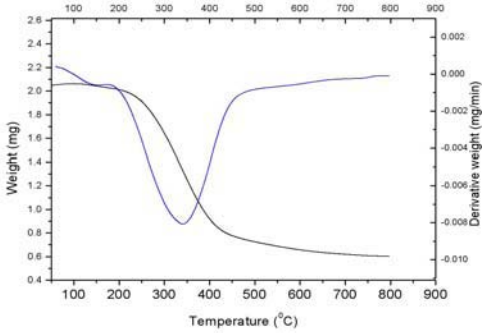


Figure 2.10 FTIR spectra of Co-curcumin complex

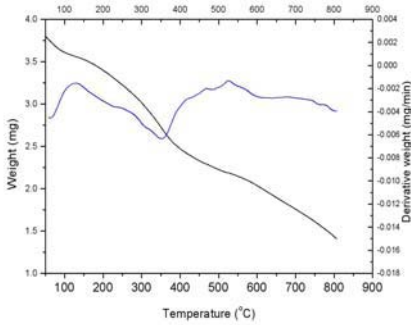
2.5.3.4 Thermal Analysis

TGA was used to determine degradation temperatures and absorbed moisture content. Curcumin was stable up to 150^{o} C and the DTG peak observed at 160^{o} C can be attributed to the dehydroxylation of two -OH groups and after 400^{o} C there is complete decomposition [17]. The Mn(II) complex showed a weight loss around 175^{o} C corresponding to loss of coordinated water (weight loss found: 3.7%, calcd 3.8%). The Mg(II) complex showed a weight loss at 165^{o} C due to loss

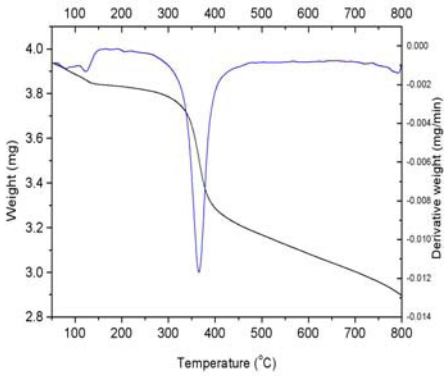
of coordinated water (weight loss found: 3.8%, calcd 4.0%). Similarly Copper, Nickel and Cobalt complexes showed a weight loss in the region 166^{o} C, 162^{o} C and 196^{o} C due to loss of coordinated water, ie, weight loss of 4.0%, 3.5% and 3.6% against the calculated values of 3.7%, 3.6% and 3.8% respectively. All the complexes showed a peak in the region of 300^{o} C corresponding to the loss of halogen. TG curves **Fig 2.11** suggest that there is coordinated water in the complexes. Decomposition of the complexes occurs at a higher temperature when compared to the ligand curcumin-I.



(i)



(ii)



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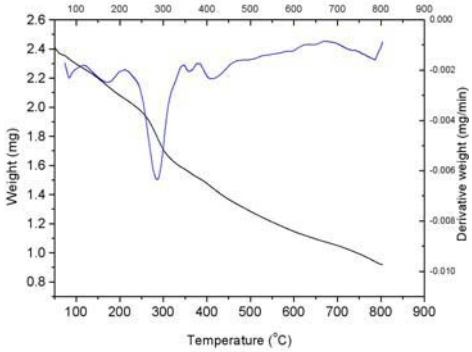

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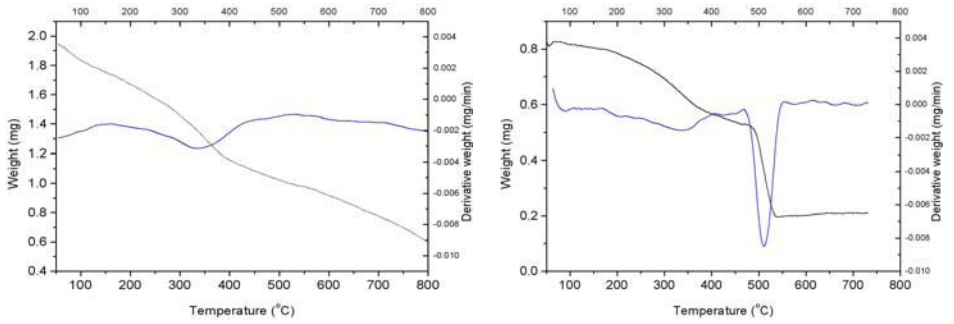


(v) **(vi)**

Figure 2.11 TG-DTG curves of (i) Curcumin-I, (ii) Mn-curcumin, (iii) Mg-curcumin, (iv) Cu-curcumin, (v) Ni-curcumin, (vi) Co-curcumin

2.6 Results and Discussion

2.6.1 Physico-chemical study

The synthesized metal complexes of curcumin have 1:1 ligand to metal ratio as shown by the CHN and AAS used to estimate the metal content. The metal to ligand ratio of 1:1 was in agreement with previously reported copper and manganese complex of curcumin [18,19]. In order to establish the relative importance of phenolic and enolic centre to the antioxidant activity of curcumin, the synthesis of only 1:1 complexes were attempted even though 1:2 complexes of curcumin were also reported [18]. This was to retain the number of phenolic group as two, similar to the parent curcumin-I. The UV spectra of curcumin-I exhibited a peak at 434 nm and in metal complex (1-5) the absorption maxima was shifted to higher region of 437-442 nm. The complex (1-5) exhibited a shoulder peak in the range of (450-463 nm) indicating Curcumin→Metal (M^{2+}) charge transfer transition. The absorption data obtained was in agreement with the data for 1:1 complexes as suggested by Barik et al [18]. For 1:2 complex Barik et al showed an absorption in the range of 370 nm. Curcumin exhibit keto-enol tautomerism, the

enol form that predominate in basic condition easily gets deprotonated to give enolate ion, which is capable of forming very stable complex with a vast range of metal ion as in **Fig. 2.12**. In acidic condition the diketone form predominate which can also undergo metal chelation. The various possible mode of metal chelation in this region are depicted **Fig. 2.13.** The strong C=O stretching peak observed for curcumin-I at 1623 cm^{-1} showed a blue shift in metal complex and the value assigned are 1579 cm^{-1},1598 cm^{-1}, 1606 cm^{-1}, 1619 cm^{-1} and 1579 cm^{-1} for Mn(II), Mg(II), Cu(II), Ni(II) and Co(II) complex respectively. The IR data of all the synthesized complexes suggest typical **Type A** chelation mode as shown in **Fig. 2.13,** where the ionic enol form is chelated with metal. Type A chelation was reported for Cd(II) and Pb(II) complexes of curcumin by Daniels [6]. In the IR spectrum of curcumin and its metal complexes the O-H band of phenol do not show considerable shift from 3433 cm^{-1} hence concluded that the phenolic O-H is not involved in complex formation.

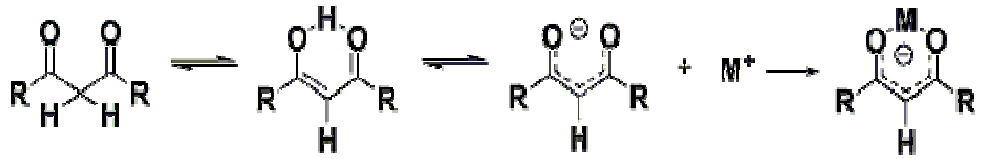


Figure 2.12 Enolate ion formation of 1, 3 diketones in the basic medium

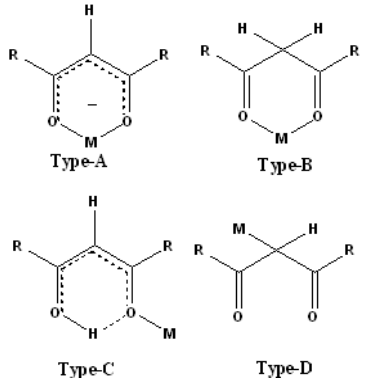


Figure 2.13 The various possible modes of chelation of the 1,3-diketone system with metal

In 1:1 metal:enolate curcumin complex of Type A as depicted in **Fig. 2.13**, one of positive charge of metal is satisfied by the negative charge of the enolate ion. The conductance value of the Mn(II), Mg(II), Cu(II), Ni(II) and Co(II) complexes of curcumin-I are found to be 6, 17, 20, 22, and 20 ohm^{-1} cm^2 mol^{-1} respectively. The molar conductance values of complexes in DMSO (10^{-3} mol) indicates non-electrolytic nature of the complexes. Hence, it can be concluded that the chloride ions are being coordinated to the metal ion in the complexes.

The thermal analysis plot of the complexes and ligand, are as in **Fig 2.11** which shows the presence of coordinated water in the complexes. The TG/DTG and conductance measurement suggest a neutral coordination sphere. The structure of the complexes can be represented as in **Fig 2.14.**

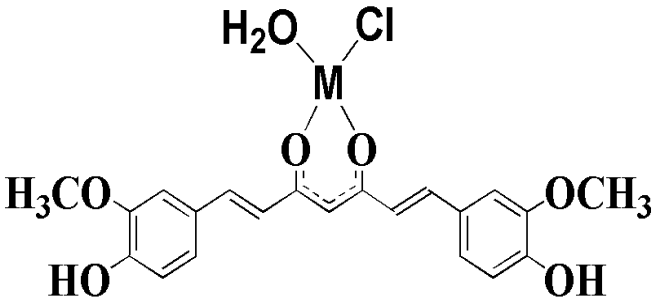


Fig 2.14. The proposed structure of 1:1 complexes of curcumin

The DPPH scavenging activity of metal complexes were less than that of curcumin-I **Fig. 2.15**. The antioxidant activity of the complexes decreases in the order of Mn(II) > Mg(II) > Cu(II) > Ni(II) > Co(II) **Table 2.4**. The difference in activity of curcumin-I and its complexes can be inferred in terms of involvement of different reaction centre of curcumin in free radical quenching.

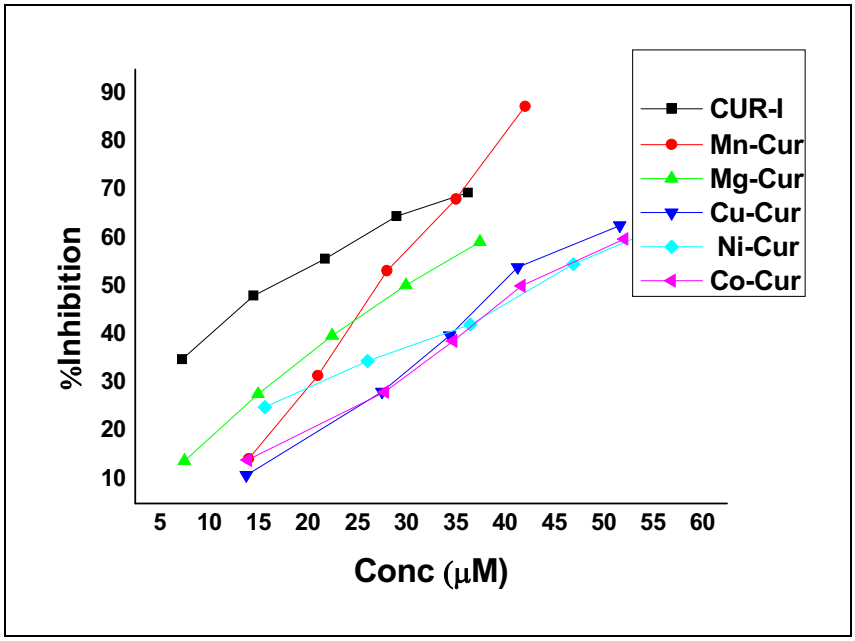


Fig. 2.15 The DPPH scavenging activity of curcumin-I and its metal complexes

Table-2.4 IC_{50} value in μM of curcumin-I and its metal complexes

Sl. No	Compound	IC_{50} (μM)
	Cur	17.88
1	Mn-Cur	27.68
2	Mg-Cur	30.28
3	Cu-Cur	41.32
4	Ni-Cur	42.92
5	Co-Cur	43.56

Different radical intermediate **Fig 2.16** was suggested by various group [20-25] for explaining the antioxidant mechanism of curcumin. Accordingly hydrogen atom transfer (HAT) can take place from (i) phenolic-OH, (ii) enolic-OH, (iii) active methylene group [$-CH_2$] of 1,3 diketo form and the other intermediates by the resonance of the phenoxide ion.

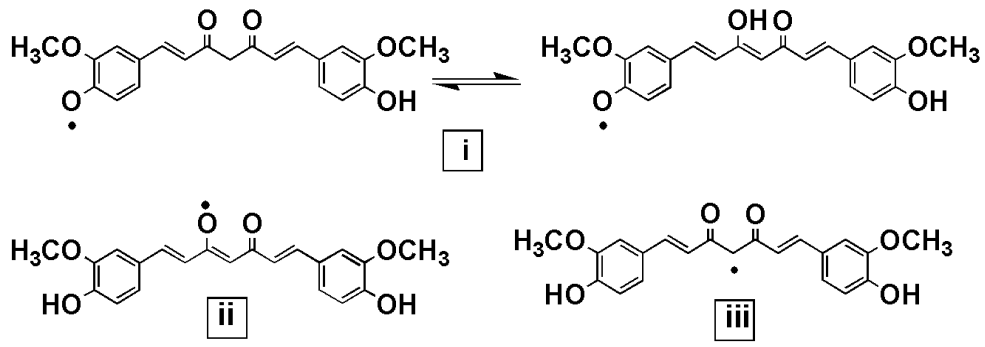


Fig 2.16 Free radical intermediate generated by curcumin

In the metal complexes enol proton is unavailable, still appreciable free radical quenching is shown. This rejects the possible involvement of keto-enol moiety in antioxidant activity. Consequently contribution of reactive intermediates (ii and iii) in antioxidant mechanism can be neglected. Of all the reactive intermediate suggested, the only possible one that could be generated in complexes is from phenolic-OH which is expected to release hydrogen in polar protic solvent like methanol by SPLET mechanism [20]. ln SPLET, a single electron transfer to DPPH takes place from ArO^-, the details would be discussed in Chapter 3. The changes in electronic effect at the diketo part of curcumin can influence the electron availability at ArO^- group there by the antioxidant activity. An electron donating group enhances the electron density at the ArO^- group increasing the antioxidant activity. In curcumin, the enolate centre act as good electron donor, where as in the metal complexes the negative charge is transferred to metal **Fig 2.12** thereby decreasing the antioxidant activity. The present observation was in agreement with the Priyadarsini et al [23], suggestion that the phenolic hydrogen is responsible for antioxidant activity and free radical kinetics of curcumin.

2.6.2 Biological study

The fluorescence imaging data **Fig 2.17** shows that curcumin and its metal complexes of Mn(II), Cu(II), Ni(II) and Co(II) were found to be reduce the ROS generation in cardiac myocytes significantly at 5 μM concentration. In **Fig 2.17, (a)** shows slight fluorescence due to intracellular ROS generation in untreated cells and **(b)** shows very high DCF fluorescence in H_2O_2 stress induced positive control cells. The intensity of fluorescence was decreased in the H_2O_2 stress induced groups that were subjected to pre-treatment with curcumin/its metal complexes (**c-g)** for 24 h. This support the fact that even in the cell lines the metal complexes in which the enol centre was blocked shows activity comparable to curcumin, further emphasizing the findings of the DPPH study that phenolic centre is the major centre for antioxidant activity.

The fluorescence imaging data was used to quantify the presence of ROS in H9c2 cells. The stain used for the purpose was cell-permeable DCFH-DA (non-fluorescent) which in presence of ROS like H_2O_2 was converted to DCF (fluorescent). The normal cells **(a)** showed very low ROS as compared to cell line induced with H_2O_2 **(b)**. Cell lines from **(c-g)** were pretreated with 5 μM of curcumin, Cu-curcumin, Mn-curcumin, Ni-curcumin and Co-curcumin for a period of 24 h. Experimental groups **(b-g)** were treated with H_2O_2 for 15 min, prior to staining with DCFH-DA. In the presence of ROS, DCFH-DA was converted to DCF by oxidative conversion. This was done to compare the ability of curcumin and its metal complex to quench the reactive oxygen species in terms of its fluorescence. The normal cell line [control cell, **(a)**] due to low metabolic activity shows a relatively low amount of ROS, while the cell line induced with H_2O_2 for 15 min, prior to staining taken as positive control **(b)** shows high fluorescence**.**

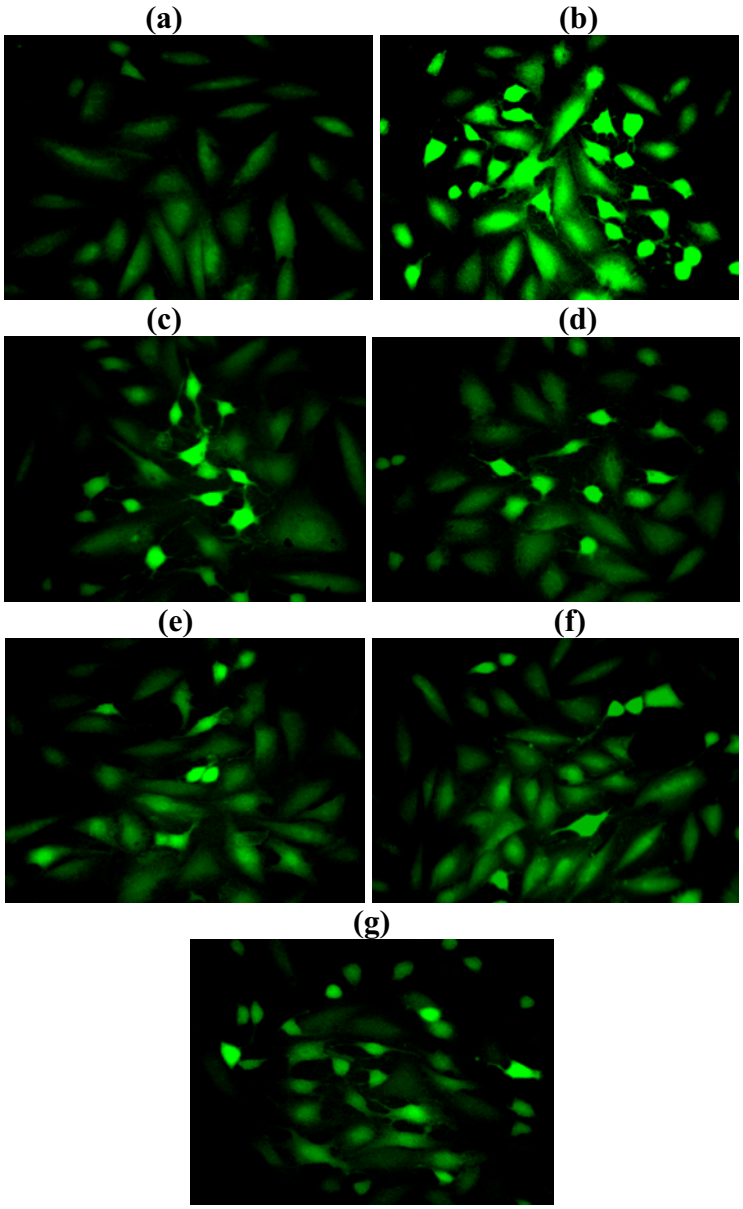


Fig 2.17 Generation of reactive oxygen species

(a) control cells; (b) cells for H_2O_2 treatment (positive control); (c) cells pre-treated with curcumin (5 µM); (d) cells pre-treated with Cu-curcumin (5 µM); (e) cells pre-treated with Mn- curcumin (5 µM); (f) cells pre-treated with Ni-curcumin (5 µM) and (g) cells treated with Co-curcumin (5 µM) for a period of 24 h. Experimental groups (b-g) were treated with H_2O_2 for 15 min, prior to staining.

The quantitative comparison by spectro-fluorimetric analysis **Fig 2.18** also supported the data obtained by imaging. The 5 μM curcumin was able to bring the level of ROS in H_2O_2 treated cell lines to the level of control. The activity of curcumin and complexes in cardiac myocytes were comparable. Curcumin showed the highest activity of all the samples taken and among the complexes Co(II) showed the highest reducing power and Cu(II) the least. The biological activity of curcumin like anti-inflammatory, cholesterol-lowering, anti-Alzheimer was ascribed to its antioxidant property. It has been suggested that antioxidant property of curcumin plays a role against AD via metal chelation of curcumin. The mechanism suggested for the action was the removal of metal related plaque deposited in brain. There hasn't been any mention about the possible role of curcumin complexed with metal as antioxidants. In the present study, it was shown that the metal complexes of curcumin have comparable antioxidant activity to curcumin and thus even after metal chelation in brain tissues by curcumin, it can act as antioxidant and retain its other biological activity. Curcumin can act simultaneously as a metal chelator and antioxidant, thus an efficient brain protector (**Scheme 2).** This property of binding of curcumin to metals and its utility as a multipotent agent for combating to oxidative stress and AD treatment have potential applications in its medication.

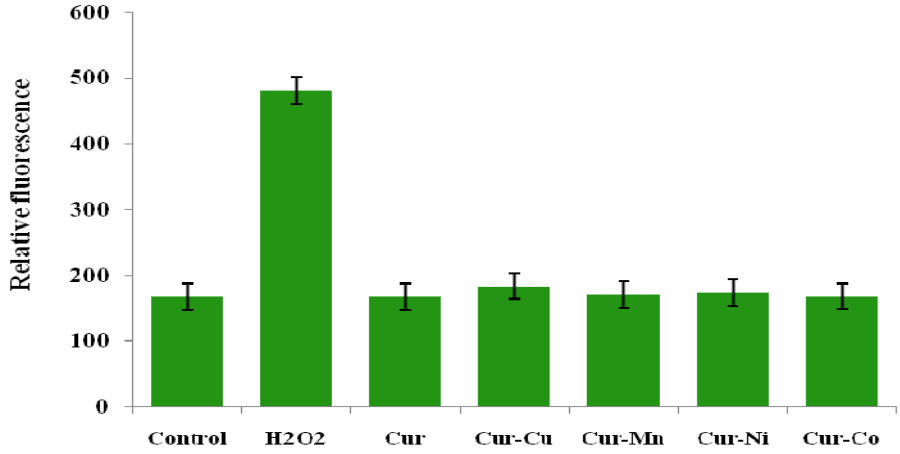
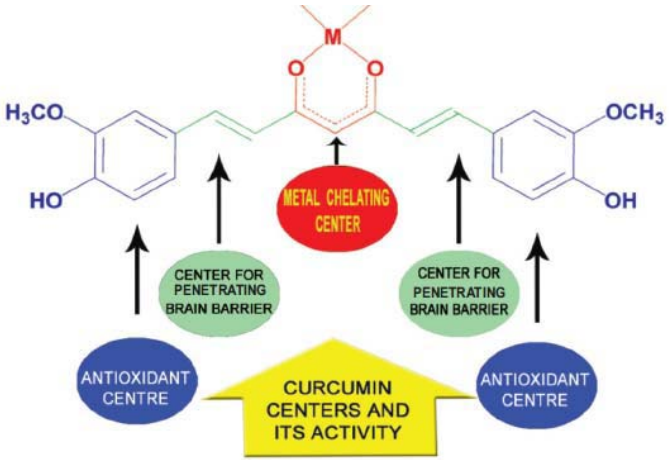


Fig 2.18 Relative fluorescence quenching property relative to reactive oxygen species of curcumin-I and its Copper, Manganese, Nickel and Cobalt complex



Scheme 2: Curcumin its reactive centres and activity

2.7. Conclusions

In the complex of curcumin with Mn(II), Mg(II), Cu(II), Ni(II) and Co(II) the enolate form of curcumin ligands to the metal. The complexes have comparable antioxidant activity to parent curcumin-I, establishing the minimal involvement of keto-enol moiety of curcumin as the antioxidant centre and hold up the phenolic -OH as the prime centre for the antioxidant activity. Thus it can concluded that different parts of curcumin help in different way to accelerate its biological property. The C=O group as metal chelator reducing oxidative stress, phenol as antioxidant centre and neutral hydrophobic conjugated hydrocarbon bridge facilitate the penetration into the blood-brain barrier **(Scheme 2).**

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Chapter-3
Kinetic solvent effect on antioxidant property of curcumin–I &II and Metal complexes of curcumin-I

Contents

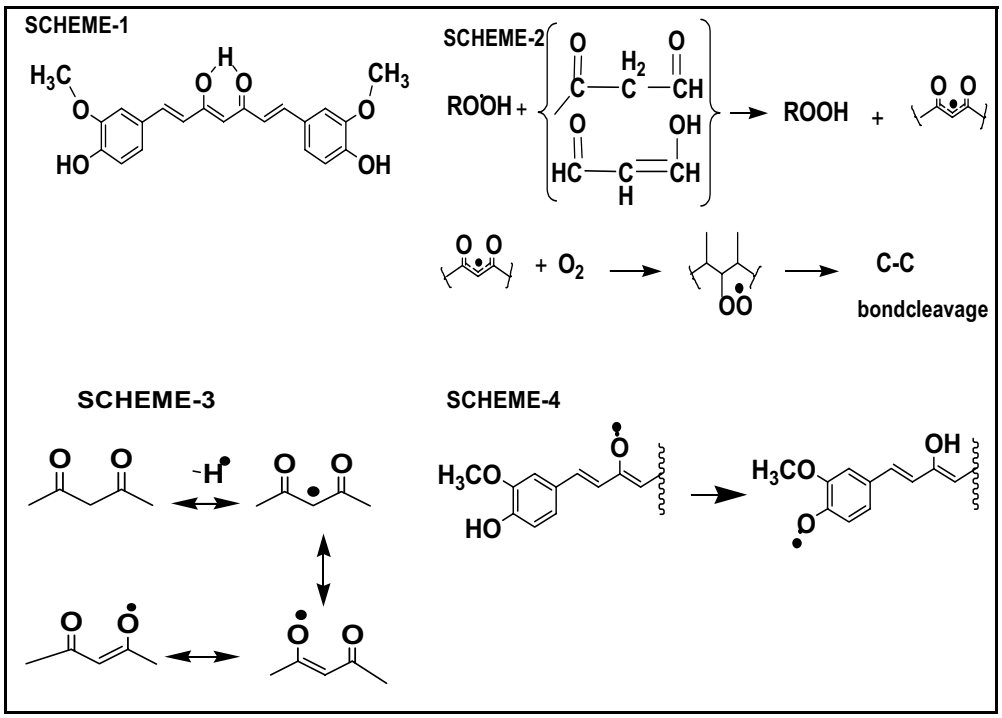
3.1. Introduction
3.2 Curcumin-antioxidant mechanism and its structural co-relation
3.3 Experimental
3.4 Results and Discussion.
3.5 Conclusions
3.6 References

3.1 Introduction

Curcumin is well known for its antioxidant, anti-inflammatory, antimicrobial and anticarcinogenic activities. Most of the biological properties of curcumin are attributed to its antioxidant activity. The free radical reaction centers of curcumin has been suggested as its two phenolic groups, enol form, active methylene of the 1,3-diketone moiety and extended conjugated structure. Different mechanistic paths were suggested for the electron transfer from the phenolic group for the antioxidant activity, among them the HAT and SPLET mechanism are the most accepted. To distinguish these mechanisms Litwinienko [1] suggested solvent effect study using flash photolysis for radical generating reaction for antioxidants such as curcumin. Later Priyadarsini et al [2] concluded that the DPPH method used for antioxidant studies corresponds to the free radical kinetics.

3.2 Curcumin-antioxidant mechanism and its structural co-relation.

Curcumin is a phenolic antioxidant and ability to scavenge radical differ in three curcumin in the order, curcumin > DMC > BDMC in methanol [3]. The methoxy group substitution at ortho position increases the electron availability at the carbon attached to the phenolic OH and thus retards the O-H bond breaking. Contrary to the electro donating effect ortho methoxy group can also involve in H-bonding with the phenolic OH which probably increases the bond length of O-H bond and facilitates the O-H bond breaking thereby increasing the antioxidant property. The hydrogen bonding interaction between the phenolic OH and the o-methoxy groups in curcumin markedly influences the O–H bond energy and H-atom abstraction by free radicals, thus making it a better free radical scavenger than BDMC [3].



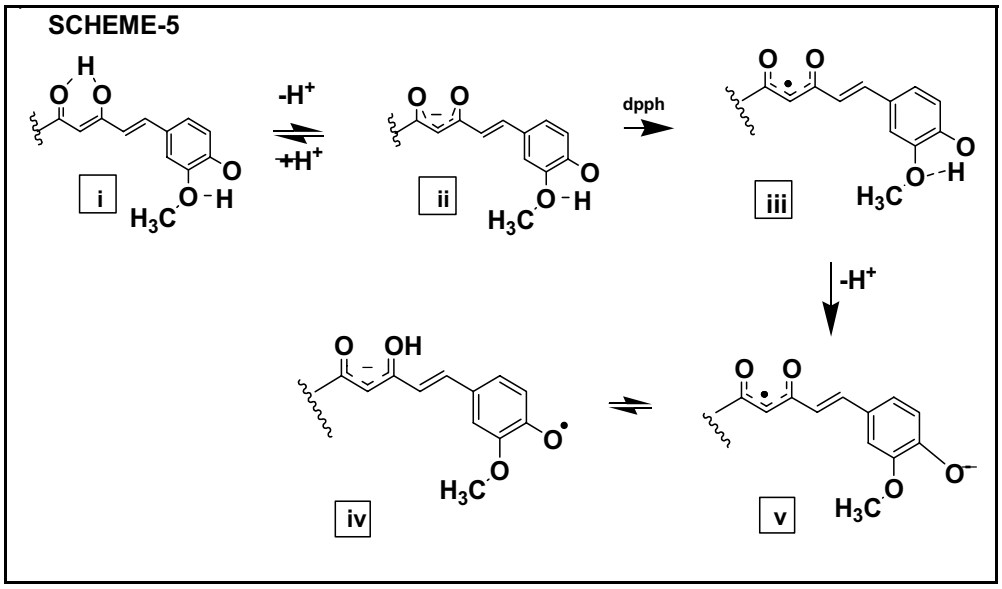


Fig 3.1 Review on antioxidant mechanism suggested for curcumin

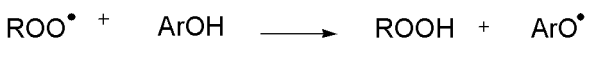
The different mechanistic path suggested for the antioxidant activity of curcumin is depicted in **Fig 3.1**. Tonnesen et al [4] has shown cis-enol form in solution is involved in scavenging of oxygen radicals **Fig 3.1, Scheme 1**; while Sugiyama et al [5], showed that radical of 1,3-dicarbonyl moieties do react with O_2 which is the main criteria for the antioxidant activity of curcumin **Fig 3.1, Scheme 2**. As acetylated curcumin failed to react with DPPH radical Sreejayan & Rao [6] concluded that the phenolic group is responsible for the antioxidant property shown by curcumin. In 1999 Jovanovic et al [7], on other hand voiced the importance of diketo moiety and claimed that "an extraordinarily potent H-atom donor-curcumin is due to delocalization of the unpaired electron on the adjacent oxygen's **Fig 3.1, Scheme 3**. Barclay et al [8] supported Rao's view and showed that synthetic non-phenolic curcuminoids exhibited no antioxidant activity. In 2001 Jovanovic et al [9] however ignored Barclay's conclusion that curcumin is a *phenolic* chain breaking antioxidant and suggested an alternative site that decide the antioxidant character of curcumin, the diketo moiety **Fig 3.1, Scheme 4**.

The controversy on the antioxidant sites of curcumin type compounds still continue. However the importance of phenolic moiety is well accepted, though the contribution of the diketo moiety and the conjugated alkene connected to curcumin are not yet well defined. Ingold [1] suggested the importance of solvent effect studies in understanding the mechanism of antioxidants **Fig 3.1, Scheme 5**. In all the **Schemes (1-5)** the free radical reaction initiation centre is assigned to be the α,γ-keto/enol moiety, even though the final product is the formation of stable phenoxide ion through the migration of the free radical along the conjugated structure of curcumin as shown in the **Scheme 4** and **Scheme 5** of **Fig 3.1.** Even though previous result suggest that the α,γ-keto-enol moiety cannot be spared in terms of its contribution to the antioxidant activity, our study with metal complexes in Chapter 2 have eliminated the possible importance of enol centre.

3.2.1 Mechanism's suggested for hydrogen abstraction from phenolic antioxidants

Free radicals ($ROO^{\bullet}$) react with phenols (ArOH) via the following prominent mechanisms (a) The direct abstraction of the hydrogen atom of ArOH by free radicals called hydrogen atom transfer (HAT) where the bond dissociation energy of O….H bond determine the rate of reaction. The HAT are predominant in apolar solvents and nonprotic polar solvents with low dielectric constant (ε_r) [9]. These solvents do not support ionisation of ArOH, hence the release of hydrogen as free radical from ArOH is slow thereby reducing the rate of ArOH-free radical reactions (b) The electron-transfer process (ET mechanism) from ArOH or its phenolate anion (ArO-) to free radicals [10]. (c) The sequential proton loss electro transfer mechanism (SPLET) which is supported predominantly by polar protic solvents like methanol and ethanol. The study of these reaction mechanisms has shown that medium of reaction influence the existence of HAT, ET and SPLET mechanism [1].

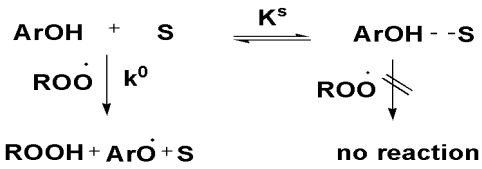
3.2.1.1 HAT



(1)

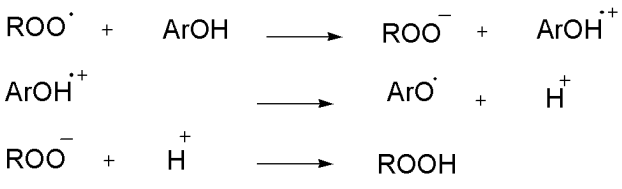
Ease of hydrogen atom transfer is related to low-bond dissociation enthalpy (BDE) of O-H bond which can be rationalised by the simple chemistry as in **Scheme 6 [1]**. The other factor that decides rate of hydrogen transfer (HAT) from ArOH to ROO$^{\bullet}$ is kinetic solvent effects (KSEs). The HAT does not occur when ArOH is a hydrogen bond donor (HBD) and the reaction is carried out in a hydrogen bond accepting solvent (HBA) solvent (S) due to sterric reasons. In such cases rate constant k^0 will be less compared to k^{HAT} of solvent free interaction from **Scheme 7.** This is because phenol molecule that is involved as HBD to solvent is unreactive towards radicals [1]. Thus a solvent–independent rate constant for proton transfer, k^0 occur via HAT involving only that fraction of ArOH that is not H-bonded as in **Scheme 7** [1].

Scheme- 6 [1] HAT in absence of kinetic solvent effects



Scheme-7 [1] Cause of kinetic solvent effects in a HAT reaction

3.2.1.2 SET-PT



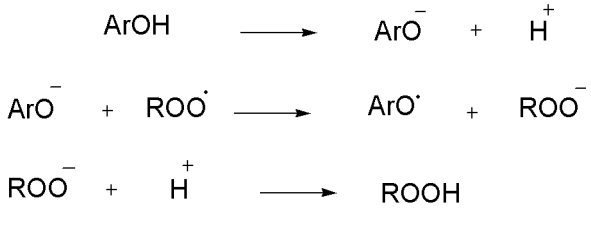
(2a)

(2b)

(2c)

Ionization potential (IP) & proton dissociation enthalpy (PDE) are the important factors determining this mechanism. This mechanism occurs in HBA solvents where phenols are largely H-bonded to a solvent molecule. Electron transfer to $ROO^{\bullet}$ generates an H-bonded radical cation ($ArOH^{\bullet+}$) very much acidic than its neutral parent which facilitate proton loss at extremely faster rate. This mechanism is expected to dominate at low pH and the concerted process at high pH [11].

3.2.1.3 SPLET



$$ArOH \longrightarrow ArO^{-} + H^{+} \quad (3a)$$

$$ArO^{-} + ROO^{\bullet} \longrightarrow ArO^{\bullet} + ROO^{-} \quad (3b)$$

$$ROO^{-} + H^{+} \longrightarrow ROOH \quad (3c)$$

Proton affinity (PA) & electron transfer enthalpy (ETE) are the important factors which determine this process. This mechanism was discovered by Foti et al [12]. Key observation was esters of cinnamic acid were more reactive than the cinnamic acid itself. This phenomenon when applied to phenolic antioxidants showed suppression in ionisation with addition of acetic acid [1] in step (3a) and hence residual reaction occurred by HAT with a slower rate.

3.2.1.4 Comparison of HAT, SET-PT and SPLET in terms of rate of free radical reaction of phenolic antioxidant in different solvent

HAT is expected to take place at slower rate since it is independent of solvent influence and depends only on O-H bond dissociation energy as in **Scheme 6** [1]**,** thus is prevalent in non-ionising solvent with low dielectric constant. The SPLET mechanism is prevalent in solvent that support ionisation and with high dielectric constant, namely methanol and ethanol. In such solvent the rate of proton loss will

be very fast as they support ionisation and thereby the overall reaction rate will be several folds higher when compared to the HAT [11]. For phenolic antioxidant so far there has been no concrete evidence of SET-PT mechanism [13]. The KSEs on rate of the reaction were measured using pulse radiolysis with respect to DPPH quenched by the antioxidants. Recently there has been a report in which the IC_{50} value of the phenolic antioxidant with DPPH has been found to be in consistent with the rate value [2].

3.3 Experimental

3.3.1 Separation of curcumin-I, II and preparation of complexes of curcumin-I

Separation of curcumin fractions and preparation and characterization of metal complex are discussed in Chapter 2 along with solvent purification techniques adopted.

3.3.2 Solvent effect on antioxidant property of curcumin-I, II and metal complexes of curcumin-I using DPPH

To study the solvent effect on antioxidant activity of curcumin-I, II and the metal complexes of curcumin-I, five solvents with varying polarity were selected namely, methanol, a polar protic solvent; acetonitrile, acetone and ethyl acetate which are polar aprotic solvents and 1,4-dioxane, non polar solvent. To study the effect of solvents on the antioxidant activity, the solution of DPPH were prepared using that respective solvent in an appropriate concentration of 1 mmol. Since complexes where only soluble in DMSO the solution of metal complexes of curcumin (0.5-1 mg mL^{-1}) were prepared in DMSO and added to the DPPH solution with respective solvent in the range of 0.01-1.3 mL and made up to final volume of 3 mL using the same solvent. The scavenging ability of the curcumin and its complexes were studied spectrophotometrically in terms of decrease in absorbance at 517 nm after 20 min. Percentage inhibition was calculated. Four

trials were done for each concentration and the average values are used (SD ± 4%) for plotting the graph concentration (μM) against the % inhibition. From the graph IC_{50} value (the concentrations at which 50% fall in absorbance of DPPH solution occur) can be calculated. The magnitude of IC_{50} value was interpreted in terms of rate of reacting antioxidants with DDPH in that particular solvent. Smaller the IC_{50} value, higher the rate of quenching of DPPH by antioxidants.

3.4 Results and Discussion

3.4.1 Solvent effect on antioxidant property of curcumin-I & II

The comparison of IC_{50} value and % inhibition curve of curcumin-1 & II using DPPH assay method were as shown in **Fig 3.2** and **Fig 3.3**. The curcumin-I was found to be more active than curcumin-II in all the solvents used for the study, exhibiting maximum activity in methanol and minimum in 1,4-dioxane. The trend in antioxidant activity of curcumin-I and II were the same in all the solvent used for the study. The magnitude of antioxidant activity in the five solvents in terms of IC_{50} value in μM is as given in **Table 3.1.**

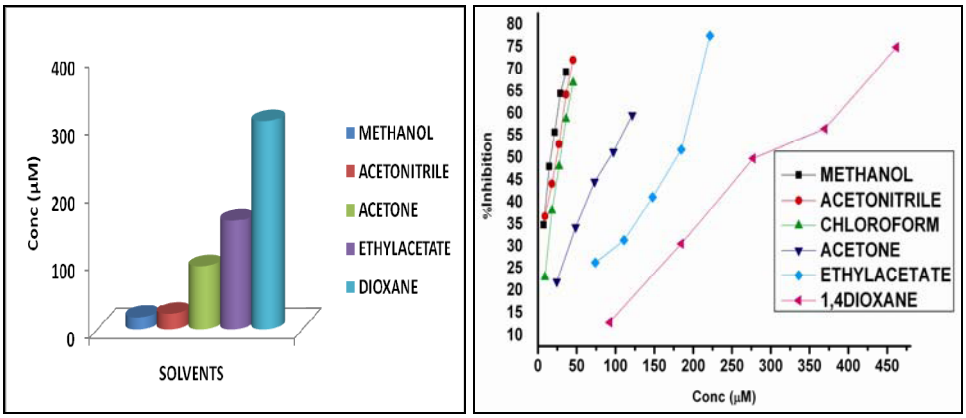


Fig 3.2 Kinetic solvent effect on antioxidant activity of curcumin-I

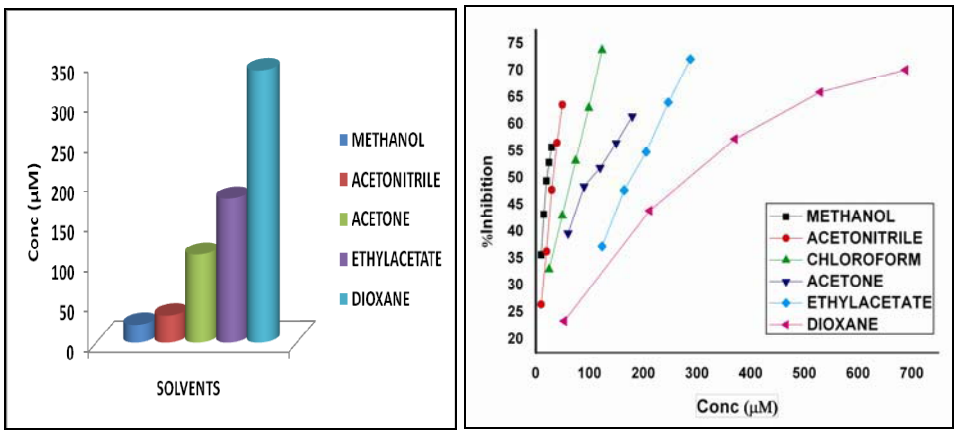


Fig 3.3 Kinetic solvent effect on antioxidant activity of curcumin-II

Table 3.1 IC_{50} values of curcumin-I & II in various solvents

SOLVENTS	Curcumin-I IC_{50} (µM)	Curcumin-II IC_{50} (µM)
Methanol	17	22
Acetonitrile	23	33
Acetone	110	145
Ethyl acetate	180	222
1,4 Dioxane	307	341

The antioxidant activity in the five solvents follow the order of 1,4-dioxane < ethyl acetate < acetone < acetonitrile < methanol for curcumin-I and II. From the IC_{50} values in **Table 3.1** for curcumin-I & II a correlation between activity and medium of study could be established.

3.4.1.1 Effect of water on antioxidant property of curcumin-I & II

The antioxidant activity of curcumin-I and II in 20%, 40% and 60% of water in methanol system was studied using DPPH. The IC_{50} value and % inhibition curve of curcumin-I & II using DPPH assay is as shown in **Fig 3.4** and **Fig 3.5**. The antioxidant activity follow the order of 60% H_2O < 40% H_2O < 20%

H_2O < methanol for curcumin-I and II. From the IC_{50} values in **Table 3.2** for curcumin-I & II it was evident that the antioxidant activity decreases with increase in percentage of water.

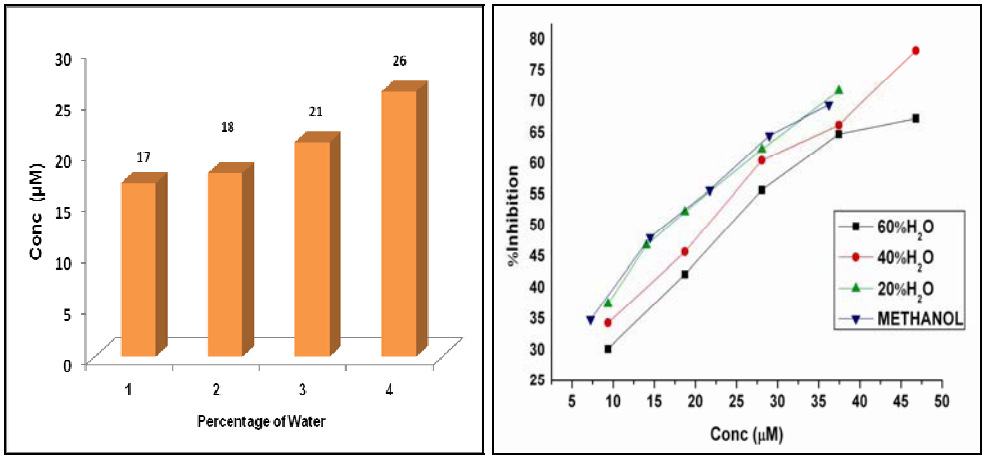


Fig 3.4 Effect of water on antioxidant activity of curcumin-I

Activity in (1) Methanol system (2) 20%H_2O (3) 40%H_2O and (4) 60%H_2O

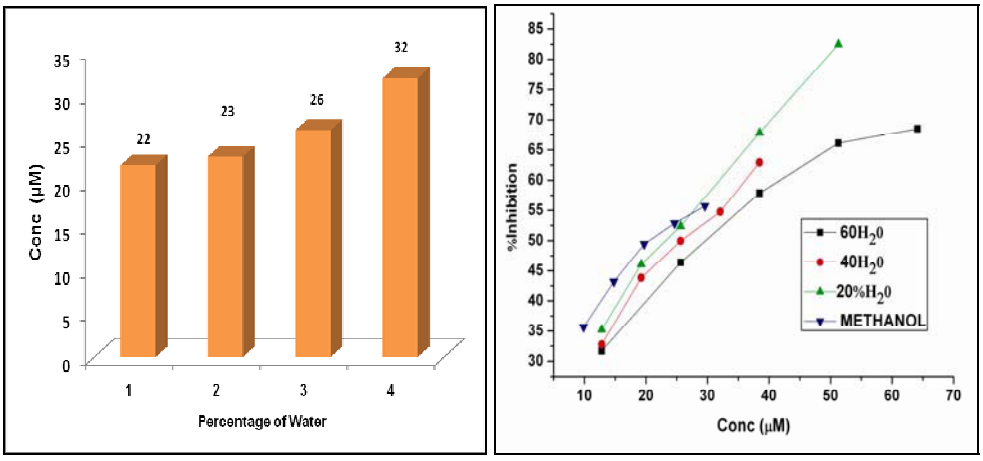


Fig 3.5 Effect of water on antioxidant activity of curcumin-II

Activity in (1) Methanol system (2) 20%H_20 (3) 40%H_20 and (4) 60%H_20

Table 3.2 IC_{50} values of curcumin-I & II in methanol-water system

SOLVENTS	Curcumin-I IC_{50}(μM)	Curcumin-II IC_{50}(μM)
Methanol	17	22
20% H_2O	18	23
40% H_2O	21	26
60% H_2O	26	32

3.4.2 Solvent effect on antioxidant property of metal complexes of curcumin-I

The antioxidant activity metal complexes [Mg(II), Mn(II) and Cu(II)] of curcumin-I using DPPH assay using different solvent system and its trend in terms of IC_{50} value and % inhibition curve were as shown in **Fig 3.6, 3.7** and **3.8**. Comparing the antioxidant activity, Mn-Curcumin was found to be more active followed by Mg-Curcumin and Cu-Curcumin.

3.4.2.1 Solvent effect on antioxidant property of Manganese–curcumin complex

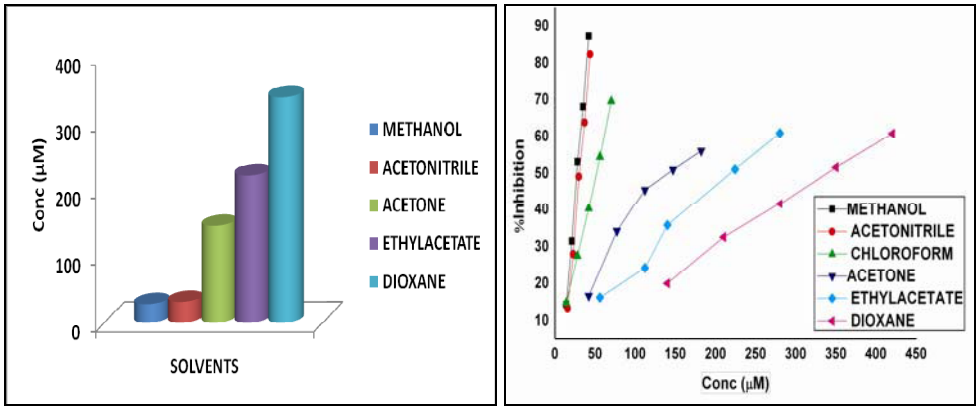


Fig 3.6 Kinetic solvent effect on antioxidant activity of Manganese–curcumin complex

3.4.2.2 Solvent effect on antioxidant property of Magnesium–curcumin complex

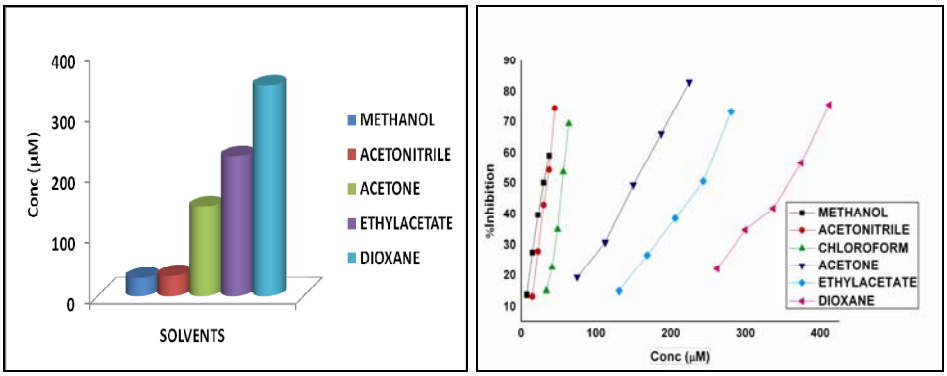


Fig 3.7 Kinetic solvent effect on antioxidant activity of Magnesium–curcumin

3.4.2.3 Solvent effect on antioxidant property of Copper–curcumin complex.

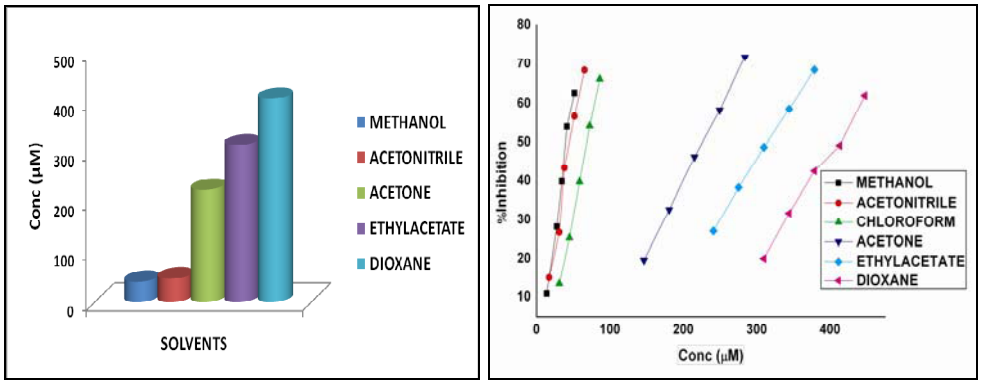


Fig 3.8 Kinetic solvent effect on antioxidant activity of Copper–curcumin

Table 3.3 IC_{50} values of Mn-curcumin, Mg-curcumin and Cu-curcumin in various solvents

SOLVENTS	Mn-Curcumin IC_{50} (µM)	Mg-Curcumin IC_{50} (µM)	Cu-Curcumin IC_{50} (µM)
Methanol	27	30	41
Acetonitrile	30	33	47
Acetone	145	147	226
Ethyl acetate	222	230	315
1,4 Dioxane	340	347	408

Table 3.4 Properties of solvents used for the study

Solvent	*k^s (M^{-1} s^{-1}) [1]	Dielectric constant	Dipole moment	HBA activities (β_2^H)[14]	Type
Methanol	16000	33	1.7	0.41	Polar protic
Acetonitirle	-	37.5	3.92	0.42	Polar aprotic
Acetone	-	21	2.88	0.50	Polar aprotic
Ethylacetate	9.0	6.02	1.78	0.48	Polar aprotic
1,4 Dioxane	1.4	2.3	0.45	0.47	Non polar

***k^s bimolecular rate constant**

The order of antioxidant activity in the five solvents are the same for metal complexes of curcumin-I, that is 1,4-dioxane < ethyl acetate < acetone < acetonitrile < methanol **Table 3.3**. The order of IC_{50} values in solvent namely in methanol, ethyl acetate and 1, 4-dioxane are closely related to the bimolecular rate constant k^s ($M^{-1}s^{-1}$) **Table 3.4,** reported by G. Litwinienko and K.U. Ingold [1] for the reaction of DPPH with curcumin**.** Priyadarsini et al showed that there is close relation in the rate constant values and IC_{50} values [2] of simple phenols like p-aminophenol and p-hydroxy acetophenone. Even though the rate constant values are more appropriate in predicting the mechanistic path of the reactions, the close relation as shown by Priyadarsini et al suggested that the IC_{50} value study are sufficient and their trends are suggestive to the mechanistic path way as in rate constant k^s values.

3.4.3 Kinetic solvent effects and assumptions

The order of antioxidant activity in different solvents can be explained as follows,

(i) In methanol ArOH….S (solvent) interactions [14] is the primary factor contributing to the H-abstraction from a phenol to a free radical

(ii) Hydroxylic solvent which have higher dielectric constant have greater ability to support SPLET reaction mechanism [1]. This is because such solvents support ionisation of phenols by abstraction of H^+ generating ArO^-

(iii) The importance of SPLET relative to HAT is observed in solvents having higher dielectric constant [15] **Table 3.4**. The reactions in such solvents will have higher reaction rate as ionisation is faster and thereby showing higher activity

(iv) HAT mechanism which is slower when compared to SPLET is predominant apolar and nonprotic polar solvents [16] as these solvent do not support ionisation and reaction take place in solvent independent mode where rate is determined only by O-H bond dissociation energy

(v) An increase in the dielectric constant of the solvent would increase the ease of separation of the ion pair formed in the initial step the ET-PT [13]

(vi) The decreasing order of antioxidant activity in different solvent was as follows, methanol > acetonitrile > acetone > ethyl acetate > 1,4-dioxane. The order of reactivity could be better explained with respect to the **Table 3.4** giving the bimolecular rate constant, dielectric constant, dipole moment and HBA of the solvent used

The kinetic solvent effect exhibited by curcumin and its metal complex could be explained from the above criteria's.

3.4.3 1 Antioxidant activity in polar protic solvents

Among the solvent used to study the reaction with DPPH and antioxidant, it was in the methanol mediated reactions that the antioxidants showed the maximum quenching ability. This anomalous behavior was due to protic nature of methanol forming intermolecular hydrogen bonding among solvent thereby inhibiting ArOH…S interaction as in **Scheme 8**. Hence, ArOH (substrate) to S (solvent) equilibrium do not exist in methanol, facilitating the ionisation of ArOH depending on the bulk property of the solvent, its relative permittivity (dielectric constant, ε_r), molecular property, its relative ability to solvate stabilising anions (ArO^-), as

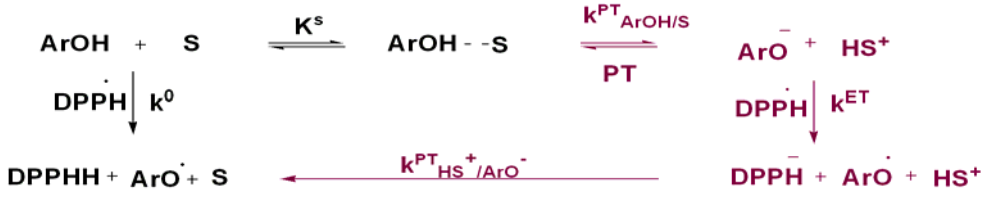
quantified by Swain et al's A value [17]. Methanol having high ε_r and A values (33, 0.75) supports ionisation, ArOH looses proton to form stable phenoxide ion at very fast rate. Consequently quenching of DPPH occurs at low concentration of ArOH, giving a very low IC_{50} value [11]. Hence concluded that the ionisation tendency of solvent medium support SPLET or release of proton as equation (**3a**).

Scheme 8 Anomalous behavior of phenolic antioxidants in methanol

3.4.3.2 Antioxidant activity in polar aprotic solvents

Comparing the antioxidant activity in polar aprotic solvents the order is acetonitrile > acetone > ethyl acetate. The IC_{50} values were as in **Table 3.1** for curcumin-I, II and **Table 3.3** for the metal complexes of curcumin-I. The solvents belonging to the class of polar aprotic solvent with high dielectric constant (37, 21) was expected to give high activity in terms of dielectric constant [1], which was true for acetonitrile but not for acetone. The high activity in acetonitrile as when compared to the very low activity in acetone can be interpreted in terms of solvent-ArOH interaction as represented in **Fig 3.9.** The hydrogen bonding interactions between highly electronegative oxygen of acetone and hydrogen of phenol give rise to strong ArOH….S (solvent) interactions [15], the primary factor contributing to the low rate of H-abstraction from a phenol to a free radical. Hence phenol deprotonation is difficult and only those molecules which are free for the deprotonation as in Scheme 7 [1] will be available to interact with DPPH to form DPPHH via HAT. HAT being slow will subsequently result in higher IC_{50} value for all the substrates in acetone as when compared to very low value in acetonitrile. As acetone supports ionization the measured rate would be the sum of the rates for the HAT (black) and SPLET (red) **Scheme 9** [14]. Whether the SPLET mechanism will be significant compared with the HAT mechanism will depend on the phenol's

acidity, the radical's electron affinity, the ability of the solvent to support phenol ionization. In acetone contribution from HAT will be more when compared to SPLET as evident from higher IC_{50} value. Acetonitrile in which solvent interaction is very less the major reaction is expected to proceed via SPLET as evident from its low IC_{50} value, comparable to the activity in methanol. This similarity of antioxidant activity in methanol and acetonitrile is due to Hydrogen Bonding Ability (HBA) being similar (0.41 and 0.42) as in **Table 3.4**. The relatively low activity in acetone can be due to its high HBA value (0.50) as compared to methanol and acetonitrile.



Scheme-9 [14] Origin of kinetic solvent effects in a mixed HAT and SPLET reaction

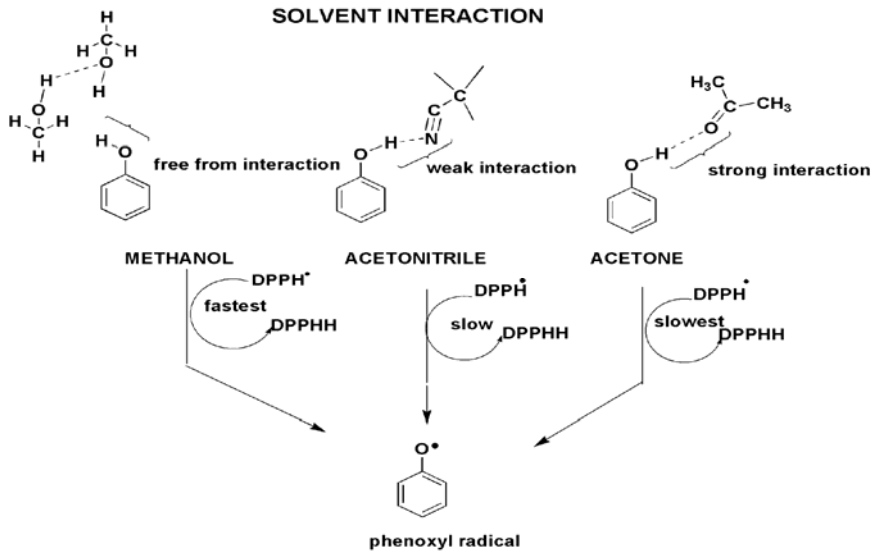


Figure 3.9 Solvent interactions with phenolic antioxidants-methanol, acetonitrile and acetone

The comparatively high IC_{50} value and hence low activity in ethyl acetate, a polar aprotic solvent may be due to the prevalent HAT mechanism that was expected from a medium that do not support ionisation of ArOH. HAT will generally dominate ArOH/DPPH reactions in solvents having low ε_r and *A* values [11], notably ethyl acetate (6.0, 0.21) and the reaction follows **Scheme 6**. The relatively low activity in ethyl acetate can also be owed to its high HBA value (0.48) and low dielectric constant **Table 3.4**.

3.4.3.3 Antioxidant activity in non-polar solvents

In 1,4-dioxane the non polar solvent used to study the reaction kinetics, as expected the activity was very low as compared to methanol due to the low ε_r and *A* values (2.2, 0.19) [11]. In solvent, S that supports ionization the measured rate is the sum of the rates for the HAT (**Scheme 8, black**) and SPLET (**Scheme 9**, **red**). Whether the SPLET mechanism will be significant compared with the HAT mechanism will depend on the phenol's acidity, the radical's electron affinity, the ability of the solvent to support phenol ionization. Dioxane being a non polar solvent that dose not support ionisation it is assumed that reaction will take a path of slower HAT as shown in **Scheme 10** [13]. HAT being very slow will require higher concentration of the antioxidant to bring 50% inhibition of DPPH, which in turn would result in higher IC_{50} value.

Scheme 10 HAT mechanism in non polar, non ionising solvent

3.4.3.4 Antioxidant activity in methanol-water system

Water being polar solvent which is slightly acidic will tend to prevent the ionisation of ArOH. The decrease in activity with increases in water percentage could be interpreted in terms of decreasing tendency of water to support ionization of phenols [11].

3.5 Conclusions

The study of antioxidant property of curcumin-I, II and metal complexes of curcumin-I using DPPH in five solvent showed an activity in the order of methanol > acetonitrile > acetone > ethyl acetate > 1,4-dioxane. The very low IC_{50} value for all the substrates in protic solvent methanol suggested a SPLET mechanism[14]. Among polar non protic solvent used, for acetonitrile which have a highest dielectric constants and comparatively low ArOH....S interaction, the IC_{50} value obtained was comparable to that of methanol indicating that the reaction proceed predominantly by SPLET. In medium like acetone with high dielectric constant and strong ArOH....S solvent interaction result's in a higher IC_{50} value, where it is assumed that reaction proceeds through the contribution of SPLET and HAT [14] that is prevalent in ionising solvents, **Scheme 9**. From a very high IC_{50} value for all the substrates in ethyl acetate and 1,4 dioxane, it is suggestive that the reaction proceeds through HAT, **Scheme 10**. HAT is generally prevalent in non ionising solvents with low dielectric constant.

The complex in which enol form was blocked also showed the same trend as curcumin-I and II but slightly less active when compared to the parent compound. This point's to the fact that even though the enol form is not available for the radical formation as in **Scheme 5,** sufficient scavenging of DPPH occurs and can be concluded as the consequence of phenoxide ion formation. Since the electron transfer takes place from ArO^- species, an electron donating group can enhance antioxidant activity or rate of reaction and an electron withdrawing group decreases the rate. Curcumin-I is more reactive than curcumin-II in all the solvent selected for the study, curcumin-I has two methoxy groups which is electron donating compared to one methoxy in curcumin-II. In the complexes formed it was the enolate ion that chelate to metals. Metals being electropositive decrease the electron density at -OH group thereby declining the possible electron donation to

DPPH and hence the antioxidant activity. This is consistent with the observation that metal complexes are slightly less active than the parent compound in all the solvent. Since the activity of curcumin-I and its complexes do not show a marked difference, it is in agreement with the Priyadarsini et al [18] conclusion that phenolic hydrogen's play a major role in the antioxidant activity of curcumin. As curcumin-I, II and metal complexes show the same trend in activity in solvents selected for the study, it is suggestive that mechanistic path way followed by the substrates are the same. To resolve the controversy in antioxidant activity of curcumin, Litwinienko [1] suggested the contribution of solvent on hydrogen atom abstractions, **Scheme 5**. The mechanistic path suggested in the **Scheme 5,** with enol as reaction centre for hydrogen atom abstractions for DPPH reaction points to the fact that the controversy doesn't end with solvent effect study of curcumin. The effect of solvent on antioxidant activity of metal complexes of curcumin was done to establish the structure activity relationship, in terms of involvement of the enol centre towards the activity. In the metal complexes the probability of radical center (ii and iii) of **Fig 2.16** being generated could be neglected. The activity of complexes selected for the study was comparable to curcumin-I in all the solvents. Hence it is concluded that antioxidant activity was merely due to the contribution of radical centre (i) **Fig 2.16 Chapter 2** and hence the role of radical centre (ii and iii) in deciding the activity could be avoided. The change in activity in different medium is entirely ascribed to the role of medium in deciding the hydrogen atom abstraction tendency from the phenolic part for quenching of DPPH.

3.6 References

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Chapter-4
Antioxidant activity of Knoevenagel condensates and its biological interaction in terms of its capacity to prevent/attenuate cataract formation-*in vitro* study

Contents

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4.2 Methods and Materials
4.3 Results and Discussion
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4.5 References

4.1. Introduction

Blindness is a common condition globally, affecting approximately 45 million people and more than a two third of blindness is caused by cataract [1]. Approximately 25% of the population with age over 65 and about 50% over 80 has serious loss of vision because of cataract [2]. Cataract is a protein aggregation disease affecting vision in humans. At present, the most effective treatment of cataract is surgical extirpation of the opaque lens, but it is expensive and not free from risk factors [3]. It is widely accepted that oxidative stress is a significant factor in the genesis of cataract [4]. Oxidative stress may result from an imbalance between the production of reactive oxygen species (ROS) and the cellular antioxidant defence mechanisms. Oxidative metabolism is clearly important in maintaining the lens in a transparent state. The lens is metabolically active and requires nourishment in order to maintain its growth and transparency. Like other organs, lens has a well designed defence system against oxidation. It uses both enzymatic and non-enzymatic defence system to neutralize free radicals which are clearly involved in the pathophysiological changes in the eye [5]. Selenite-

overdose cataract is an extremely rapid and convenient model to study experimental cataract. Major events of selenite cataract are loss of calcium homeostasis, reactive oxygen species generation and decreased water soluble proteins [6].

Medicinal plants often contain highly active pharmacological compounds. Many of these compounds, isolated from a part or whole of the medicinal herbs, act as pharmaceutical drugs having therapeutic potential against cataract [7-11]. Curcumin is well known for its flavouring and medical properties and shown to exhibit antioxidant, anti-inflammatory, anti-microbial and anti-carcinogenic activities. The medicinal properties of curcumin is attributed to its radical scavenger activity and is shown to scavenge free radicals such as hydroxyl radicals, superoxide radicals, singlet oxygen, peroxyl radicals and peroxynitrite [12]. Thus, curcumin can reduce the oxidative stress in tissues and hence a potential candidate as an anticataract agent. Inspite of its efficacy and safety, curcumin has not yet been approved as a therapeutic agent because of its poor aqueous solubility and relatively low bioavailability [13] and consequently search for better curcumin derivatives without these problems and with efficacy equal to or better than that of curcumin is ongoing.

In the present study, two derivatives of curcumin, salicylidenecurcumin (CD1) and benzalidenecurcumin (CD2) were prepared by the Knoevenagel condensation of curcumin-I with the aldehydes-salicylaldehyde and benzaldehyde along the active methylene group and their efficacy evaluated against selenite cataract model. The study was designed to compare the biological activity of curcumin and two of its Knoevenagel condensates, salicylidenecurcumin (CD1) and bezaledenecurcumin (CD2) on *in vitro* selenite induced cataract models. The Knoevenagel condensates prepared the enol formation was blocked and condensates has got a 1,3-diketo structure.

4.2 Materials and methods

4.2.1 General

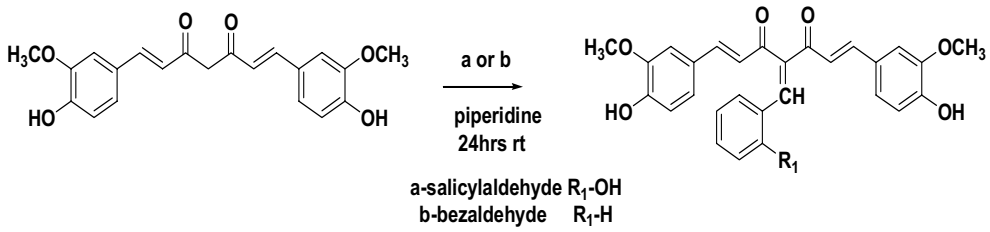
The commercial sample of curcumin was purchased from Kancor Ingradients Limited, Angamaly, Kerala. The curcumin-I separated from commercial sample is used for the preparation of CD1 and CD2 which were separated by column chromatography [14,15]. Sodium selenite, DMEM culture medium, FBS and DPPH were purchased from Sigma-Aldrich Co, St. Louis, USA. All other reagents and solvents used were of analytical grade and were purchased from Spectrochem, India.

4.2.2 Separation of curcumin–I

Commercial curcumin was subjected to silica gel (60-120 mesh) column chromatography, to separate curcumin I & II are discussed in detail in Chapter 2.

4.2.3 Preparation of Knoevenagel condensate.

Curcumin-I obtained as the first fraction from column separation of commercial curcumin was subjected to Knoevenagel condensation **Scheme 1** by dissolving curcumin (1 mmol) in minimum amount of methanol along with (1.5 mmol) piperidine as catalyst followed by slow addition of salicylaldehyde/ benzaldehyde (1 mmol) each. The resultant solution was stirred for 48 h with cooling and the reaction was monitored using TLC. After the product separation, the solution was evaporated under vacuum in dark. Solid salicylidenecurcumin (CD1)/ benzalidenecurcumin (CD2) were washed with petroleum-ether and then subjected to silica gel column chromatography using 9:1 chloroform-methanol as eluent similar to commercial curcumin. The third fraction collected is of Knoevenagel condensate.



Scheme 1 Preparation of Knoevenagel condensates

Salicylidenecurcumin (CD1)-Yield: 56%, (m.p. 98^{o} C). MS m/z: 471.2 $(M-H)^{+}$, UV (λ_{max}) = 357 nm (CH_3OH), IR (KBr cm^{-1}) ν_{OH} = 3429, $\nu_{C=O}$ = 1699, $\nu_{C=C}$ = 1586, ν_{C-O} (phenol) = 1263, νc-o (-OCH_3) = 1020, ^{1}H.NMR. (300MHz, DMSO) δ 3.88 (s,6H,OCH_3), 8.46 (s,1H,-CH=C-), 6.57-6.7 (6H,aromatic), 6.75-7.28 (10H,aromatic), 7.06 (d,2H), 7.66 (d,2H), 9.68 (s,1H,-OH).

Benzalidenecurcumin (CD2)-Yield: 58%, (m.p. 120^{o} C) MS m/z: 457 $(M+H)^{+}$, UV(λ_{max}) = 360 nm (CH_3OH), IR (KBr cm^{-1}) ν_{OH} = 3430,$\nu_{C=O}$ = 1679, $\nu_{C=C}$ = 1580, ν_{C-O} (phenol) = 1260, ν_{C-O} (-OCH_3) = 1025, ^{1}H NMR. (300MHz, $CDCl_3$) δ 3.89 (s,6H,OCH_3), 6.68-7.15 (m,6H,aromatic), 7.18 (d,2H), 7.35-7.48 (m,5H aromatic), 7.8 (d,2H), 8.16 (s,1H, -CH=C-).

4.2.4 In vitro antioxidant activities-DPPH free radical scavenging activity

The DPPH radical scavenging activity was measured by the method of Blois [16]. 0.1 mM solution of DPPH in ethanol was prepared and 0.1 mL of this solution was added to test solution (curcumin, CD1 and CD2 in minimum quantity of Dimethyl Sulphoxide) at different concentrations (20 M - 220 M) and made up to 3 mL with appropriate solution of ethanol-water to get 90% ethanol water system. After incubation for 20 min, the absorbance was measured at 517 nm. The IC_{50} value was used to compare DPPH scavenging activity. Lower the IC_{50} value, higher the free radical scavenging activity.

4.2.5 Biological studies

Lenses were extracted through posterior approach from the eyes of two month old female Sprague-Dawley strain rats under deep anesthesia. A total of 30 rats were used for the experiment with two lenses obtained from each rat. All ethical guidelines were followed for the conduct of animal experiments in strict compliance with the Institutional Animal Ethical Committee and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India [Sanction No: IAEC-KU-6/10-11-BC-AA], [17]. Lenses were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS, 100 U mL^{-1} penicillin, 0.1 µg mL^{-1} streptomycin and 0.25 µg mL^{-1} amphotericin under 5% CO_2 at 37° C in a CO_2 incubator. Lenses were immediately transferred to culture medium maintained at 37° C. Organ culture of lens was carried out in a 24 well culture plate with 2 mL of medium/well with change of medium every 24 h. Lenses developing opacification in the first 24 h were discarded. Lenses were grouped as five with six lenses in each group.

GI - Control; lenses incubated in DMEM

G II- Selenite induced (100 µM Sodium selenite)

G III- Selenite induced (100 µM Sodium selenite) + Curcumin (2.5 µg mL^{-1})

G IV- Selenite induced (100 µM Sodium selenite) + Salicylidenecurcumin (CD1) (2.5 µg mL^{-1})

G V - Selenite induced (100 µM Sodium selenite) + Benzalidenecurcumin (CD2) (2.5 µg mL^{-1})

The lenses were incubated for 72 h at 37° C in 24 well cell culture plates maintained in an incubator with an atmosphere of 5% CO_2. After incubation, quantitative analyses of enzymatic and non- enzymatic antioxidants were carried out.

4.2.6 Analytical procedures

4.2.6.1 Superoxide dismutase (SOD)

Activity of Superoxide Dismutase in the lens samples was measured by the method of Kakkar et al., [17]. The assay mixture contained 1.2 mL sodium pyrophosphate buffer (0.052 M, pH 8.3), 0.1mL of 186 µM PMS, 0.3 mL of 300 µM NBT, 0.2 mL of 780 µM NADH, appropriately diluted enzyme preparation and water added to make up the final volume to 3 mL. Reaction was started by the addition of NADH and incubated at 30° C for 1 min. The reaction was stopped by the addition of 1.0 mL glacial acetic acid and the mixture stirred vigorously. 4.0 mL n-butanol was added to the mixture and shaken well. The mixture was allowed to stand for 10 min, centrifuged at 1000 rpm for 10 min, the butanol layer taken out and measured at 560 nm against a butanol blank. A system devoid of enzyme served as the control.

4.2.6.2 Determination of reactive oxygen species (ROS)

The level of ROS was determined by the method of Davidson et al., [18] which is expressed as fluorescence intensity (FI) mg^{-1} lens protein. A 10% (w v^{-1}) lens homogenate was prepared in phosphate buffer (100 mM, pH 7.4), from this aliquots were taken for ROS estimation and protein estimation. 150- 200 µL (5 mg protein) lens homogenate was taken, made up to 990 µl with homogenate buffer and 10.0 µL of dichlorofluoroscin diacetate (1 mM) was added to each tube including the buffer blank and allowed to incubate at 37° C for 30 min and centrifuged at 10,000 rpm for 15 min and the fluorescence of supernatant was recorded at an absorbance of 502 nm and an absorbance of 523 nm using a spectrofluorimeter.

4.2.6.3 Estimation of the activity of Ca^{2+}ATPase

The activity of Ca^{2+} ATPase in the lens samples was measured by the

method of Rorive and Kleinzeller [19]. 0.25 ml of Tris–HCl buffer (0.4 M, pH 7.4) and 0.25 mL of 40 mM ATP (Tris salt) are taken in three test tubes kept in ice. To tubes 1 and 3, 0.25 mL of 40 mM $CaCl_2$ was added. At time zero, the reaction was started by the addition of 0.25 mL of enzyme extract to tubes 1 and 2. The volume in all the tubes was adjusted to 2 mL with distilled water. The tubes were incubated at 37° C with gentle shaking for 30 min. (Under these conditions, the release of phosphate will be linear for up to 60 min). The reaction was stopped by placing the tubes in ice and 0.4 mL of ice-cold 35% TCA added to this. The tubes were then centrifuged for 10 min at 10,000 rpm in a refrigerated centrifuge. The phosphate content was estimated by the method of Friske and Subbarow [20]. Because of the instability of ATP in acid solutions containing molybdate, tube 3 was used as a control to determine the phosphate liberated in the absence of enzyme. Results are expressed as micromoles of phosphate liberated mg^{-1} $protein^{-1}$ h^{-1}.

4.2.6.4 Determination of calcium

The lenses were weighed, cleaned and washed with phosphate buffered saline. Then the tissue was digested with necessary volume of concentrated nitric acid: perchloric acid mixture (5:1). After complete digestion, the lenses were dried and diluted with 1% nitric acid. Calcium concentrations in the lens samples were analyzed flame photometrically with standard prepared from $CaCO_3$ and deionized water. The results were expressed as µmoles g^{-1} wet tissue [21].

4.2.6.5 Estimation of protein content

The protein content of the samples was determined by the method of Lowry et al., [22] using bovine serum albumin as the standard. To 0.04 mL of sample, 1.0 mL of distilled water and 5.0 mL alkaline copper sulphate reagent were added and incubated for 10 min at room temperature. To this, 0.5 mL of Folin's phenol reagent was added, incubated for 20 min at room temperature and absorbance

measured at 670 nm. Protein concentration was calculated from a standard curve taking 20-100 µg BSA and measured against a reagent blank.

4.2.6.6 Statistical analysis

All statistical calculations were carried out with statistical package for Social Sciences (SPSS) Software Program. The values were expressed as the mean ± SD. The data were statistically analyzed using one-way analysis of variance (ANOVA) and significant difference of means was determined using Duncan's multiple range tests at the level of $p < 0.05$ [23].

4.3 Results and Discussion

Curcumin and its Knoevenagel condensates were synthesized and tested for their antioxidant activities using DPPH radical scavenging assay. Activities of SOD and Ca^{2+}ATPase, concentrations of calcium and ROS were compared with control, selenite induced and treated groups. The IC_{50} (50% inhibition of radical) of curcumin, CD1 and CD2 were indicating appreciable antioxidant activity for the compounds. The IC_{50} values of curcumin, CD1 and CD2 are 70, 42 and 60 µmol mL^{-1}. The antioxidant property thus increases in the order curcumin < CD2 < CD1. The results are shown in **Fig 4.2**.

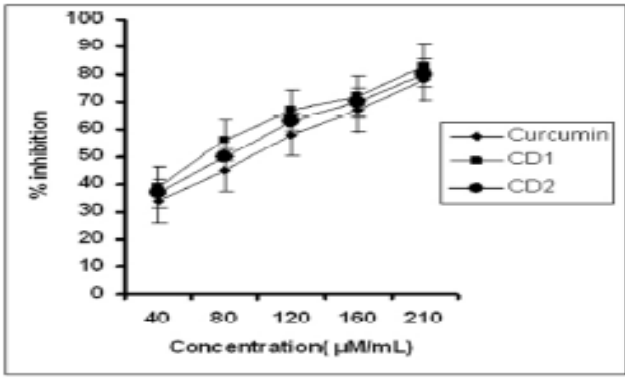


Figure 4.2 DPPH scavenging ability of Salicylidenecurcumin(CD1) Benzalidenecurcumin (CD2)

The activities of SOD, Ca^{2+}ATPase in the eye lens were decreased following selenite administration. The mean activity of SOD and Ca^{2+} ATPase in G II lenses were significantly reduced ($P < 0.05$) when compared to G I. Cultured lenses belonging to G III, G IV and G V in presence of curcumin, CD1 and CD2 produced no appreciable change in SOD and Ca^{2+} ATPase activity when compared to G II lenses and the levels were near to that observed with control lenses **Fig 4.3 and Table 4.1**. CD1 treated group show activities of these enzymes more similar to control group and hence, better protective activity.

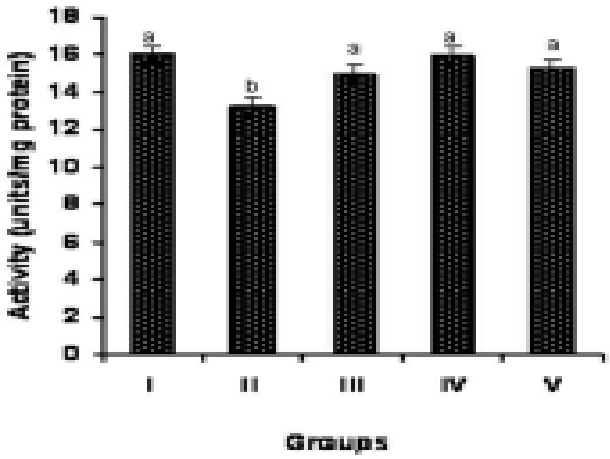


Figure 4.3 SOD activity

G-I: Control, G-II: Selenite-induced (100 µM), G-III: Selenite-induced + curcumin (2.5 µg mL^{-1}) treated, G-IV: Selenite-induced + CD1 (2.5 µg mL^{-1}) treated, G-V: Selenite-induced + CD2 (2.5 µg mL^{-1}) treated. Activity of SOD: U mg^{-1} protein. Comparison between groups, different alphabets indicate significant difference at $p < 0.05$. Each value represents mean ± SD of six values.

Table 4.1 Activity of Ca^{2+} ATPase

Enzyme analysed (unit of activity)	GI	GII	GIII	GIV	GV
Ca^{2+} ATPase (μmole phosphate liberated /mg protein/h)	4.26 ± 0.026[a]	2.46 ± 0.016[b]	4.08 ± 0.021[c]	3.69 ± 0.37[d]	4.09 ± 0.12[e]

Table 4.1 Activity of Ca^{2+} ATPase G-I: Control, G-II: Selenite-induced (100 µM Sodium selenite), G-III: Selenite-induced (100 µM Sodium selenite) + curcumin (2.5 µg mL^{-1}), G-IV: Selenite-induced (100 µM Sodium selenite) + CD1 (2.5 µg mL^{-1}) treated, G-V: Selenite-induced (100 µM Sodium selenite) + CD2 (2.5 µg mL^{-1}) treated. Ca^{2+}ATPase (µmoles phosphate liberated mg^{-1} $protein^{-1}$ h^{-1})

Selenite induction caused an increase in lenticular ROS and calcium concentration in G II compared to control. However, there was significant difference in the level of ROS and calcium in curcumin, CD1 and CD2 administered group. The level of ROS and calcium in CD1 treatment showed appreciable normalization compared to others **Table 4.2**.

Table 4.2 Concentrartion of ROS and calcium component in the cultured lens

Component (Unit of activity)	GI	GII	GIII	GIV	GV
ROS(fluorescence intensity/mg protein)	7.26 ± 0.26[a]	11.46 ± 0.16[b]	6.08 ± 0.21[c]	6.69 ± 0.37[d]	6.09 ± 0.12[e]
Calcium(µmole/g dry tissue)	0.470 ± 0.004[a]	0.989 ± 0.006[b]	0.789 ± 0.001[c]	0.510 ± 0.007[d]	0.634 ± 0.003[e]

Table 4.2 Concentrartion of ROS and Calcium content. G-I: Control, G-II: Selenite-induced (100 µM Sodium selenite), G-III: Selenite-induced (100 µM Sodium selenite) + curcumin (2.5 µg mL^{-1}), G-IV: Selenite-induced (100 µM Sodium selenite) + CD1 (2.5 µg/ml) treated, G-V: Selenite-induced (100 µM Sodium selenite) + CD2 (2.5 µg mL^{-1}). Concentration of ROS values expressed as fluorescence intensity mg^{-1} protein. Comparison between groups, different alphabets indicate significant difference at p <0.05.Each value represents mean ± SD o six values.

Although there is no way to cure or reverse the effects of cataract, prevention strategies can slow the rate at which a cataract forms or even prevent one from developing in the first phase. Curcumin is a known commercial product extracted from Curcuma longa with antioxidant property [24]. Curcumin was reported as effective against selenite induced cataractogenesis in Wistar rat pups [25]. In this study, we have synthesized Knoevenagel condensates of curcumin-CD1 and CD2, evaluated their antioxidant property in comparison to curcumin and their effectiveness against selenite induced cataract in organ cultured rat lens.

DPPH free radical scavenging is an excellent method used to evaluate antioxidant property based on their ability to donate a hydrogen ion [26]. Curcumin and its derivatives CD1 and CD2 exhibited higher antioxidant property and the order of increase in antioxidant property was curcumin < CD2 < CD1 **Fig 4.2**. The salicylidenecurcumin (CD1) have an additional OH in the salicylidene when compared to bezaledenecurcumin and curcumin, resulting in an appreciable increase in its antioxidant property, indicating that this OH is functioning as a radical quenching active centre. Thus the mere increase of phenolic OH group will enhance the antioxidant property. Knoevenagel condensates keto-enol tautomerism is being blocked by condensation of aldehyde through active methylene group. Hence the involvement of free radical intermediates (ii and iii) **Fig 2.16 Chapter 2** can be avoided. The only reactive intermediate possible for free radical quenching reaction is the (i) with phenolic OH as the hydrogen donating centre. Accordingly the salicylidenecurcumin (CD1) with three phenolic OH shows the maximum activity, following is benzaledenecurcumin (CD2). The higher activity of benzaledene-curcumin (CD2) when compared to curcumin with the same number of phenolic OH may be due to the extended conjugated system.

The activities of SOD and Ca^{2+}ATPase in the eye lens were decreased following selenite administration. SOD is a specific scavenger of superoxide anion

and selenite is shown to induce O^{2-} generation in eye lens. Superoxide and its derivatives have been implicated in many pathological conditions including cataract and ageing [27,28]. Both SOD and Ca^{2+}ATPase activities in cultured lenses of Group II were significantly reduced. In lenses from Groups III, IV and V, which were pretreated with curcumin, CD1 and CD2 respectively along with selenite, the SOD and Ca^{2+}ATPase activities were similar to control groups **Fig 4.3 and Table 4.1**, indicating a complete reversal of selenite induced cataract. Our results are in agreement with the earlier reports [29-35].

Reactive oxygen species (ROS) are known mediators of intracellular signaling cascades. Excessive production of ROS may, however, lead to oxidative stress, loss of cell function and ultimately apoptosis or necrosis [36-38]. A balance between oxidant and antioxidant intracellular systems is hence vital for cell function, regulation and adaptation to diverse growth conditions. Selenite induced group show high elevation of ROS when compared to that of control. Decreased level of ROS was found in treated groups (curcumin, CD1 and CD2). Among these, CD1 reduced the level of ROS when compared to that of others. The same results were obtained in the case of calcium content. Previously published reports from our laboratory [39,40] also agree with the obtained results.

4.4 Conclusions

While several experimental reports show that curcumin has antioxidant property, our present study has developed derivatives of curcumin and compared their antioxidant and anti-cataractogenic property. Selenite cataract is an extremely rapid and convenient model of nuclear cataracts. Reactive oxygen species (ROS) such as superoxide O^{-}, H_2O_2 and hydroxyl radicals (OH^{-}) are by products of normal metabolism and attack certain biological molecules, leading to destabilization and disintegration of cell membranes in cataractous lenses, such as in selenite induced animal models. In the human body, the toxic effects of ROS are

combated regularly by a number of endogenous defense and protective mechanisms which include various enzymes and non-enzymatic antioxidants.

The derivatives of curcumin, CD1 and CD2 were found to be effective in overcoming biochemical changes occurring in cataractous lenses. In comparison, the derivatives of curcumin, CD1 was superior to CD2 in the attenuation of selenite induced cataract. The presence of an additional -OH group in the salicyledene is proposed as the cause of an appreciable increase in antioxidant property. Our findings suggest that the curcumin derivative, salicyledenecurcumin (CD1) is endowed with enhanced antioxidant property and keto-enol structure is not a necessary requirement for deciding antioxidant activity.

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Chapter-5
DNA binding study and cytotoxicity of metal complexes of curcumin-I

Contents

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5.2 Experimental
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5.1 Introduction

Over the past 20 years, there has been an increasing interest in plant-derived polyphenols with respect to human health. All the compounds in plants have been selected by evolution to have biological importance and that plants also generate novel compounds with extreme stereospecificity. Consequently, 50% of the anticancer therapeutic agents used in modern medicine are derived from plants. One such polyphenolic compound with a wide range of pharmacological property is curcumin, vibrant yellow spice derived from the rhizome of the plant, *Curcuma longa*. Curcumin has been used for long in traditional medicines of China and India [1]. It is one of the most potent and thoroughly researched dietary phytochemical in cancer management. The clinical trials using curcumin suggests it to be viable alternative to non-steroidal anti-inflammatory drugs or COX-2 inhibitors as a potential colorectal cancer chemopreventive agent [2]. Various mechanisms of action like G1/S arrest and apoptosis induction other than the mitotic block in different tumour cell lines are responsible for anti cancer activity of curcumin [3,4]. A multitude of molecular targets for curcumin like cell cycle

proteins, cell surface adhesion protein, transcription factors, enzymes and cytokines are been revealed from experimental fact [5,6]. Further curcumin is the first polyphenolic compound found to bind in the minor groove of nucleic acids [7].

Curcumin exhibits strong chelating ability with a great number of metal ions to form complexes of the type 1:1 and 1:2 with copper, iron and other transition metals [8-10]. This property of binding of curcumin to metals like iron and copper have made it viable as a multipotent agent for combating oxidative stress and Alzheimer disease (AD) treatment [11]. In animal studies, curcumin was found to remove heavy metal accumulation in rat brain thus preventing lipid peroxidation induced by metal [12]. Several metal complexes of curcumin have been synthesized, characterized and evaluated for various biological activities. Copper complex of curcumin as SOD mimick [8], Manganese complex with neuroprotective activity [10] and Vanadyl, Gallium and Indium complexes of curcumin with medicinal applications and reduced cytotoxic potential [13] are been reported.

Although curcumin has an evident anti-cancer activity, relative poor stability has been highlighted as one of the major problems in therapeutic applications. Consequently attempts are being furnished by researchers to synthesis curcumin analogue showing efficacy and stability superior to that of curcumin. Nevertheless to ensure the safety of such congeners, extensive and elaborate long-term toxicity studies would be necessary. The subject matter would be worth exploration as antioncogenic signalling properties of curcumin may render its possibility as a suitable “cheap alternative” to molecular-targeted anti-cancer drugs. Intuitively, minor alteration of the parent curcumin stand a better chance of retaining the safety features as compared to more complex alterations. As the metal complex of curcumin has only minor alteration as compared to the parent curcumin, it has been selected for cytotoxicity and DNA binding studies.

5.2 Experimentals

5.2.1 Materials

Deoxyribonucleic Acid	SRL
Tris (Hydroxymethyl)Aminomethane	SRL
Acetic acid glacial	S. D. FINE CHEM LTd. Mumbai

5.2.2 Methods

5.2.2.1 Separation of curcumin-I and preparation of metal complexes of curcumin-I

Details of separation and characterization of curcumin-I along with preparation and characterization of metal complex are discussed in Chapter 2.

5.2.2.2 Preparation of stock solution of DNA

The stock solution of Herring sperm DNA (HS-DNA) was prepared in Tris-HCl buffer and the concentration was determined by measuring the absorption intensity at 260 nm with a known ε value of 6600 M^{-1} cm^{-1}[14]. The stock solutions were stored at 4^0 C and used within 4 days.

5.2.2.3 Cardiomyocyte model in H9c2 cells

Details are provided in Chapter 2

5.2.2.3.1 *In vitro* cytotoxicity assay

5.2.2.3.1.1 Morphological analysis

H9c2 cells in the exponential growth phase were plated at 5×10^4 cells in 24-well plate. After 48 h, cells from all experimental groups were checked for morphological alterations under the phase-contrast microscope (Nikon Eclipse TS100, Japan) at 40 x magnification.

5.2.2.3.1.2 MTT assay

Cells in the exponential growth phase were suspended in the medium. A total of 5×10^4 cells per well were seeded in 24-well plate. After 48 h of incubation at 37^0 C, cells were subjected to various treatments. 350 µL of MTT solution (5 mg MTT mL^{-1} DMEM) was added to each well and incubated for 3-4 h at 37^0 C. The formazan crystals thus formed were dissolved in DMSO. Then the plates were read after 45 min in a microplate reader (Biotek Synergy 4, US) at 570 nm.

5.2.2.4 Electronic absorption titration

Spectrophotometric titration has been employed to ascertain the binding of curcumin complexes of Mn(II), Co(II), Mg(II), and Cu(II) with DNA. The DNA binding experiments were performed in Tris–HCl/NaCl buffer (50 mM Tris–HCl/1 mM NaCl buffer, pH 7.1) using dimethylsulphoxide (DMSO) solution of the complexes of curcumin. The absorption spectral titration was done with fixed concentrations of the complex and varying concentrations of DNA stock solution while maintaining the total volume constant (3 mL). The change in UV–vis absorption of the curcumin complexes with increasing amounts of HS-DNA could be monitored as a function of added DNA. The magnitude of spectral perturbation is an evidence for extent of binding [15].

5.2.2.5 Cyclic voltammetric studies

Cyclic voltammetric measurements of the all the synthesised complexes were carried out on a Bio-Analytical System (BAS) model CV-50 W electrochemical analyser. The three-electrode cell comprised a reference Ag/AgCl, a counter electrode as platinum wire and a working platinum electrode with surface area of 0.7 $cm^{2.}$ The electrode was polished with 0.3 and 0.005 nm alumina before each experiment and when necessary the electrode was sonicated in distilled water for 10 min. Dissolved oxygen was removed by purging the solution with pure

nitrogen for about 15 min before each experiment. Scanning the cyclic voltammogram for a blank solution was done to check the purity of the supporting electrolyte and the solvent.

Cyclic voltametric studies of the complex (1×10^{-5} M) were carried out in DMSO with Tetrabutylammonium perchlorate as the supporting electrolyte, in a BAS CV-27 Electrochemical Analyser using platinum working electrode. A Pt wire and Ag/AgCl were used as counter and reference electrodes, respectively.

5.3 Results and Discussion

5.3.1 Effects on morphological features of treated H9c2 cells

The morphological observation showed that cells had normal spindle shaped morphology at all (1, 5, 10, 15 and 20 μM) concentrations of various samples **Fig.5.1**.

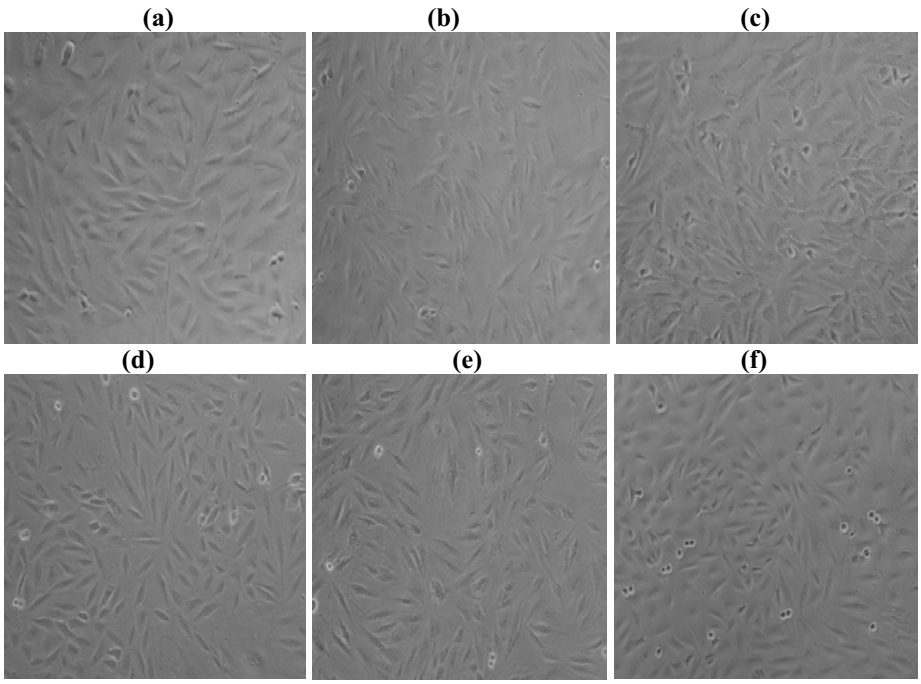

Fig 5.1 Morphological features of treated H9c2 cells

(a) Control (b) Curcumin (c) Cu-Cur (d) Mn-Cur (e) Ni-Cur (f) Co-Cur

5.3.2 Effect on cytotoxicity in H9c2 cells

The MTT results **Fig.5.2** showed that curcumin and its various metal complexes [Cu(II), Mn(II), Ni(II) and Co(II)] showed a reduction in cell viability in a dose-dependent manner when compared to untreated cells. The cell deaths observed were statistically insignificant when compared to untreated control.

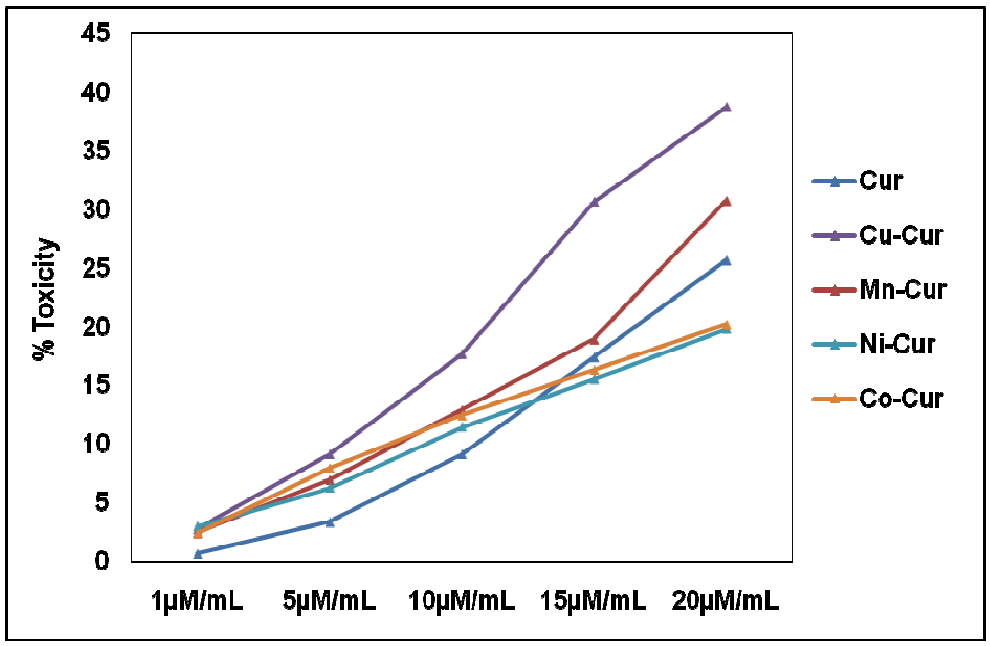

Fig.5.2 Cell viability assay with MTT

5.3.3 DNA-binding studies

To demonstrate the existence of non-covalent bonding interactions between different biomolecules (DNA, enzymes, proteins) and drugs or potentially mutagenic agents several sensitive analytical techniques are required. Small molecule have found to react with specific site along a DNA strand, which has been used as a model for protein nucleic acid interaction and has provided a new

route to drug design and development. Interaction of metal complexes with DNA is used as probe for DNA structure in solution and as chemotherapeutic agent [16,17]. The interaction of metal complexes of curcumin with DNA has been studied using spectral, electrochemical and CD techniques.

5.3.3.1 Electronic absorption titration

The two effect characteristics of interaction of metal complexes with DNA are hypochromism and hyperchromism. Hypochromic effect is attributed to interaction between electronic states of complex with DNA bases [18]. Hyperchromism can be ascribed to external contact or partial uncoiling of helical structure exposing more bases of the DNA [19].

Spectrum of curcumin complexes in Tris-HCl buffer was characterized by an intense ligand centred transition in the visible region at 360 nm, 424 nm, 428 nm and 404 nm for Mn(II), Co(II), Mg(II) and Cu(II) complex of curcumin. In the electronic absorption spectral data obtained **Fig 5.3(a), 5.4(a), 5.5(a) and 5.6(a)** using fixed amount of each metal complex of curcumin with increasing concentration of HS-DNA, all the complexes exhibited hypochromism, with no change in band position.

To further illustrate the binding strength of the curcumin complexes with DNA, the intrinsic binding constant K_b was determined from the spectral titration data. By monitoring the changes in absorption at corresponding λ_{max} with increasing HS-DNA concentration and K_b can be calculated using the Eq (1) [20].

$$\frac{[DNA]}{(\varepsilon_a-\varepsilon_f)} = \frac{[DNA]}{(\varepsilon_b-\varepsilon_f)} + \frac{1}{K_b\,(\varepsilon_b-\varepsilon_f)} \qquad (1)$$

Where [DNA] is the concentration of DNA in base pair, ε_a is the apparent extinction coefficient for each addition of DNA to the complex (A_{abs}/[Complex]), $\varepsilon_{f,}$ extinction coefficient for free metal complex and $\varepsilon_{b,}$ extinction coefficient for metal complex in the fully bound form.

A plot of [DNA]/(ε_a-ε_f) versus [DNA] gives binding constant K_b as the ratio of slope by intercept. The magnitude of K_b value gives the extent of binding. The absorption spectra of curcumin complexes (1.5×10^{-5} M) in Tris-HCl buffer pH 7.1 in the absence (R=0) and presence of increasing amount of DNA are represented in **Fig 5.3(a), 5.4(a), 5.5(a) and 5.6(a)**. A plot of [DNA]/(ε_a-ε_f) versus [DNA] for the titration of DNA with complex for binding constant are represented in **5.3(b), 5.4(b), 5.5(b) and 5.6(b)**. The binding constant values obtained for the curcumin complexes can be represented as in **Table 5.1**. From the binding constant values the order obtained is Mn(II) < Co(II) < Mg(II) < Cu(II). The values and order obtained where comparable to that of complexes of macrocyclic tetraaza diacetyl curcumin ligand [21] with Cu(II), Co(II) and Mn(II) as coordinated metals. The comparisons of binding constant values are represented in **Table 5.1**.

Table 5.1 The binding constant, K_b (M^{-1}) for curcumin complexes and tetraaza diacetyl curcumin complexes

SL No	Complexes	Binding Constant K_b (M^{-1})	
		Curcumin -complex	Tetraaza diacetyl curcumin [21]
1	Mn(II)	1.9×10^4	3.5×10^4
2	Co(II)	2.2×10^5	5.2×10^4
3	Mg(II)	2.3×10^5	-
4	Cu(II)	3.5×10^5	1.4×10^5

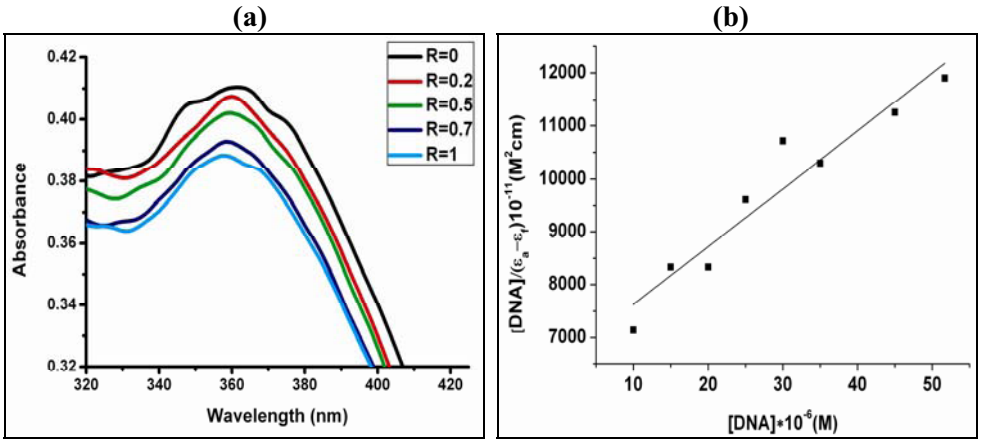

Fig 5.3 (a) Absorption spectra of Mn-curcumin complex (1.5×10^{-5} M) in Tris-HCl buffer of pH 7.1 in the absence (R=0) and presence (R=0.2, 0.5, 0.7 & 1) of increasing amount of DNA, R=[DNA]/[Complex]

Fig 5.3 (b) A plot of $[DNA]/(\varepsilon_a-\varepsilon_f)$ versus [DNA] for the titration of DNA with complex

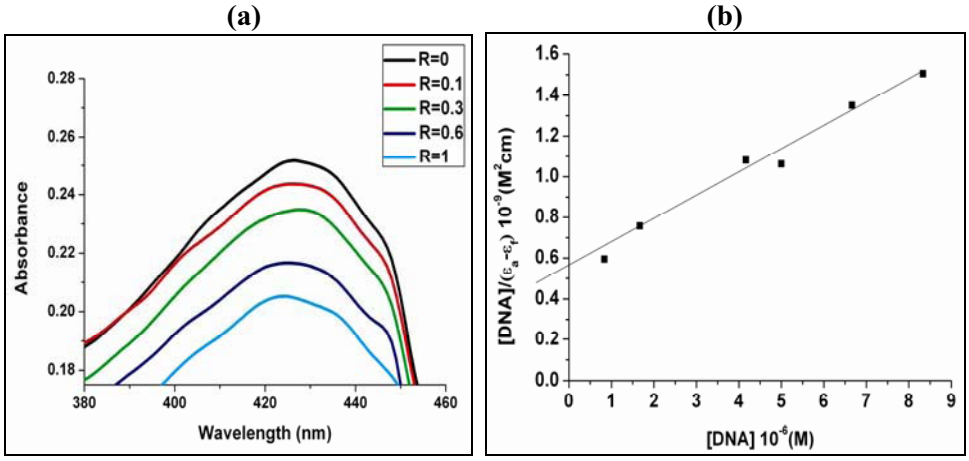

Fig 5.4 (a) Absorption spectra of Co-curcumin complex (1.5×10^{-5} M)) in Tris-HCl buffer of pH 7.1 in the absence (R=0) and presence (R= 0.1, 0.3, 0.6 & 1) of increasing amount of DNA, R=[DNA]/[Complex]

Fig 5.4 (b) A plot of $[DNA]/(\varepsilon_a-\varepsilon_f)$ versus [DNA] for the titration of DNA with complex

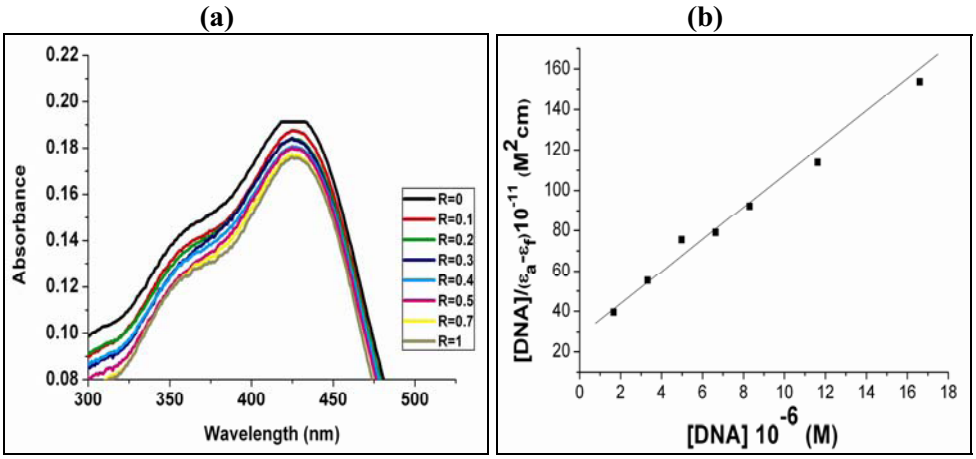

Fig 5.5 (a) Absorption spectra of Mg-curcumin complex (1.5×10^{-5} M)) in Tris-HCl buffer of pH 7.1 in the absence (R=0) and presence (R=0.1, 0.2, 0.3, 0.4, 0.5, 0.7 & 1) of increasing amount of DNA, R=[DNA]/[Complex]

Fig 5.5 (b) A plot of $[DNA]/(\varepsilon_a-\varepsilon_f)$ versus [DNA] for the titration of DNA with complex

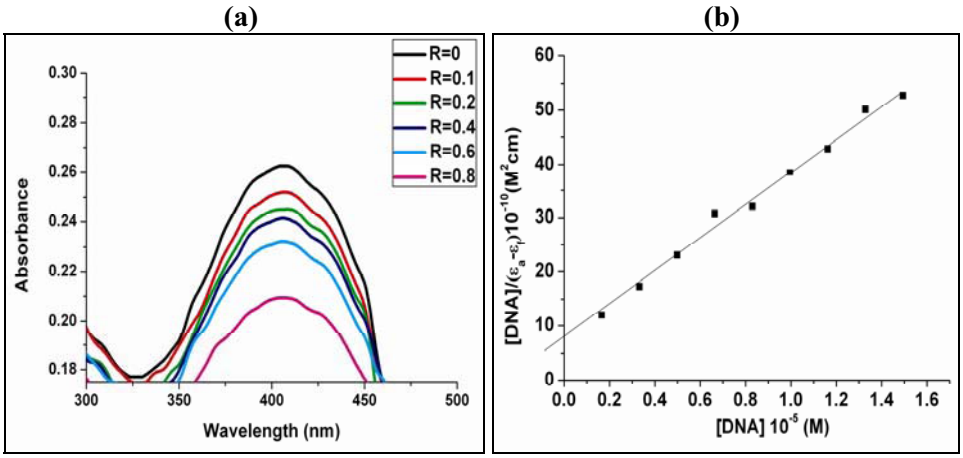

Fig 5.6 (a) Absorption spectra of Cu-curcumin complex (1.5×10^{-5} M) in Tris-HCl buffer of pH 7.1 in the absence (R=0) and presence (R=0.1, 0.2, 0.4, 0.6 & 0.8) of increasing amount of DNA, R=[DNA]/[Complex]

Fig 5.6 (b) A plot of $[DNA]/(\varepsilon_a-\varepsilon_f)$ versus [DNA] for the titration of DNA with complex

5.3.3.2 Cyclic Voltammetric studies

The cyclic voltammogram of curcumin complexes in DMSO are given in the **Fig 5.7-5.11** and **Table 5.2.** Studies were done with complexes of (1×10^{-5} M) in DMSO. The I_{pc}/I_{pa} shows that a one electron transport takes place in all the complexes.

Table 5.2 Cyclic voltammetric data of curcumin complexes

Compound	E_{pc}(mV)	E_{pa}(mV)	$E_{1/2}$(mV)	ΔE_p(mV)	I_{pc}/I_{pa}
Mn-Cur	-809	-724	-767	85	0.95
Mg-Cur	-829	-525	-677	304	1.3
Cu-Cur	130	467	-299	337	0.97
Ni-Cur	-963	-912	-938	51	1.2
Co-Cur	-912	-832	-872	80	1.3

E_{pc} = cathodic potential; E_{pa} = anodic potential; I_{pc} = cathodic peak current; I_{pa} = anodic peak current; $E_{1/2} = (E_{pa} + E_{pc})/2$; $\Delta E_p = E_{pa}-E_{pc}$; scan rate 100mVs^{-1}

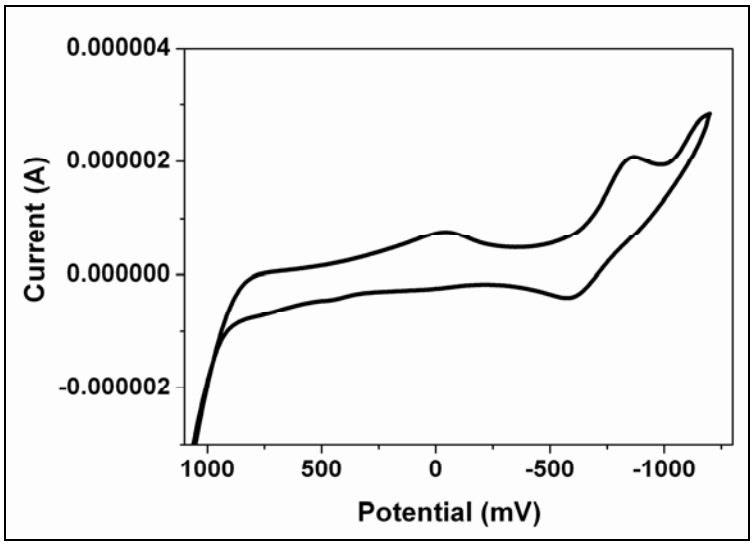

Fig.5.7 Cyclic voltammogram of Mn-curcumin

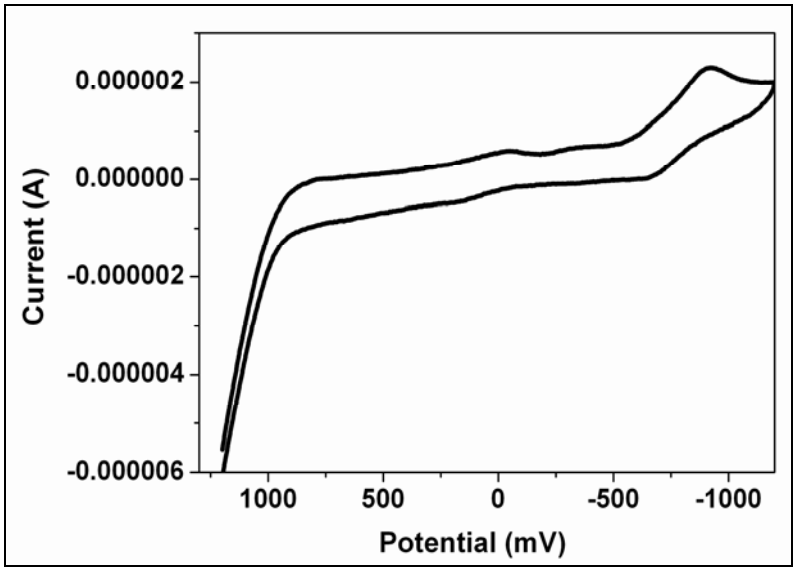

Fig.5.8 Cyclic voltammogram of Mg-curcumin

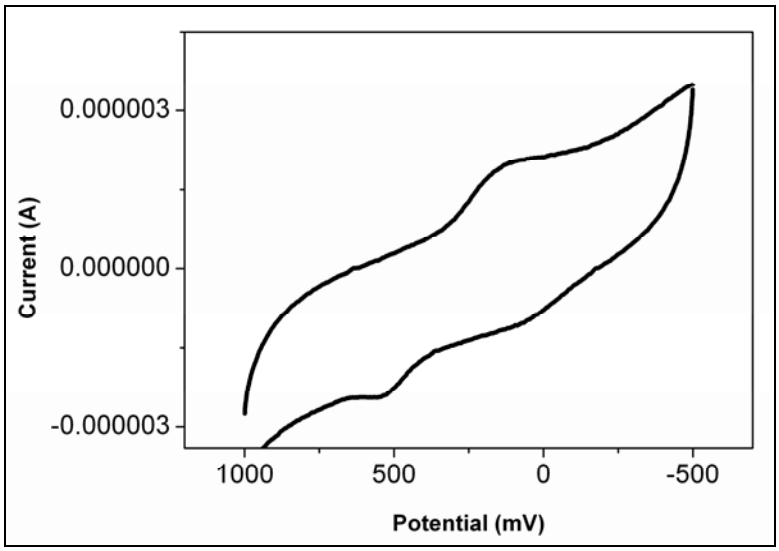

Fig.5.9 Cyclic voltammogram of Cu-curcumin

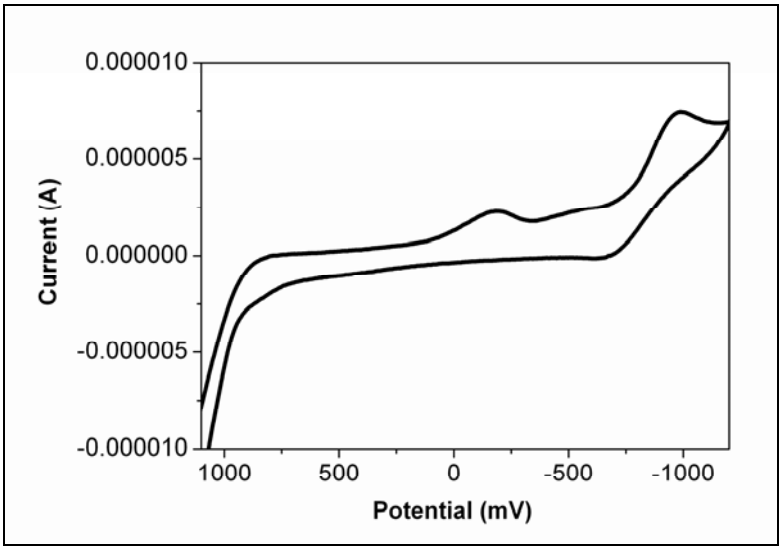

Fig.5.10 Cyclic voltammogram of Ni-curcumin

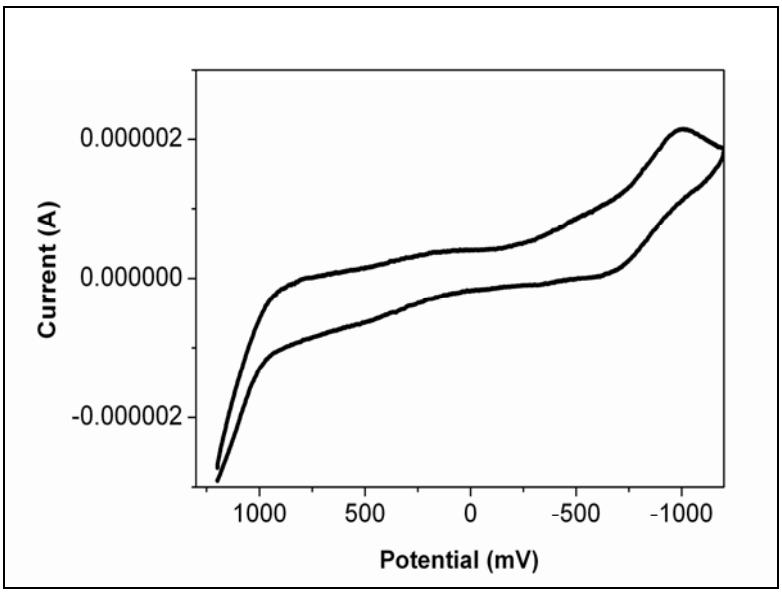

Fig.5.11 Cyclic voltammogram of Co-curcumin

To provide further evidence about interacting effect complexes of curcumin with DNA, electrochemical method which complements the UV–Vis spectroscopy were used. The application of electrochemical method as a marker line between the metallointrecalation and coordination of the metal ions with the DNA is well established. The advantages of this technique are that multiple oxidation states of the same species as well as mixtures of several interacting species can be observed simultaneously [22]. The Cu(II) and Mg(II) complex of curcumin were selected for voltammetric studies**.** The redox behaviour of curcumin complex was studied in the absence and presence of DNA at room temperature within the potential range of 1000 to -400 mV at the scan rate of 100 mVs^{-1}.

The cyclic voltammogram **Fig 5.12(a) & 5.13(a)** recorded for the Cu(II) and Mg(II) complex of curcumin indicated the mode of binding of complexes to DNA. A decrease in peak currents (both the Ipc and Ipa) and the Epc and Epa **Table 5.3** with increasing concentrations of DNA was observed in both the complex. The decrease of peak current implied forming a new association complex. The decrease in voltammetric current in these CV experiments may be attributed to the slow diffusion of complexes bound to the large, slowly diffusing DNA molecule [22]. Among the three kinds of binding modes for small molecules to DNA, the shift of Epc to a lower value is indicative of interaction mode of electrostatic or groove binding as reported by Bard [22]. The Differential pulse voltammogram (DPV) **Fig 5.12(b) & 5.13(b)** also certify the changes found in CV experiments.

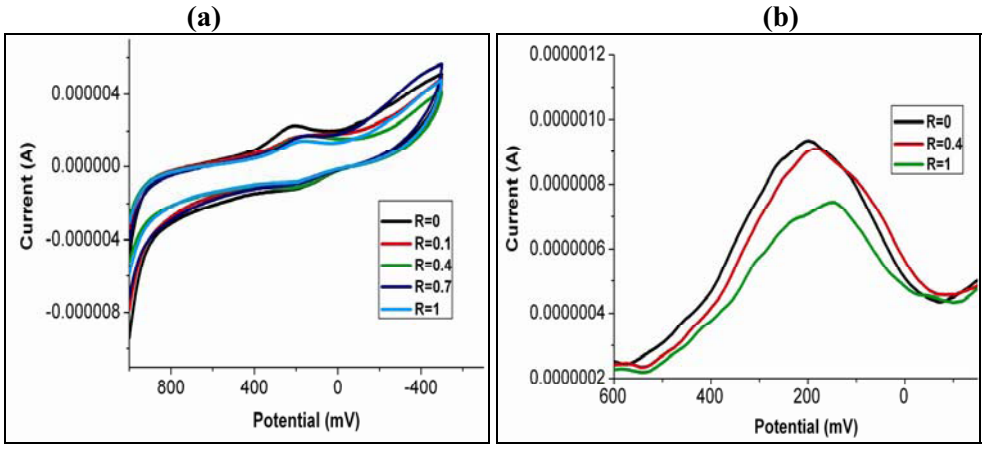

Fig 5.12 (a) Cyclic voltammogram (R= 0, 0.1, 0.4, 0.7 & 1) Cu-curcumin complex (1.5×10^{-5} M) in Tris-HCl buffer of pH 7.1

Fig 5.12 (b) Differential pulse voltammogram of Cu-curcumin complex (1.5×10^{-5} M) in Tris-HCl buffer of pH 7.1 in the absence (R=0) and presence (R=0.4 & 1) of increasing amount of DNA

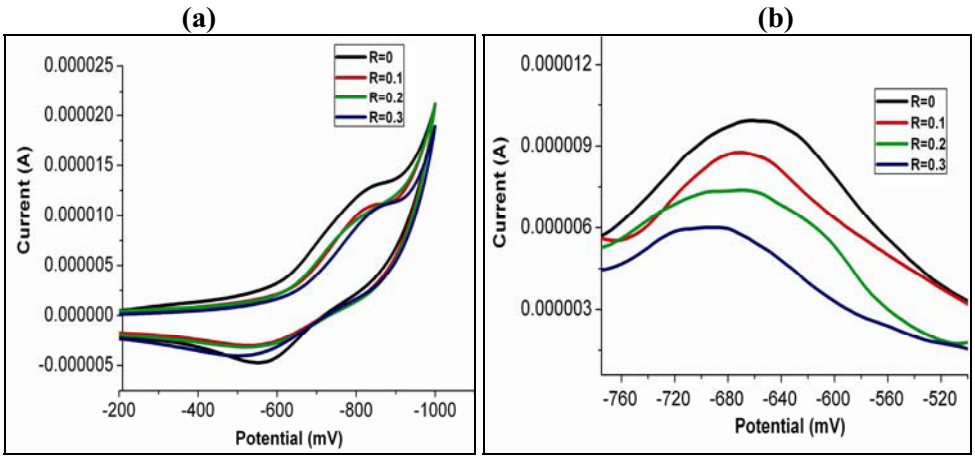

Fig 5.13 (a) Cyclic voltammogram (R= 0, 0.1, 0.2 & 0.3) Mg-curcumin complex (1.5×10^{-5} M)) in Tris-HCl buffer of pH 7.1

Fig 5.13 (b) Differential pulse voltammogram of Mg-curcumin complex (1.5×10^{-5} M)) in Tris-HCl buffer of pH 7.1 in the absence (R=0) and presence (R= 0.1, 0.2 &0.3) of increasing amount of DNA

Table-5.3 Voltammetric behaviour of curcumin complexes in Tris HCl buffer pH 7.1 in the absence and presence of DNA

Compound	R	E_{pc}(mV)	E_{pa}(mV)	ΔE_p(mV)	$E_{1/2}$(mV)
Cu-Curcumin	0	189	287	98	238
	0.4	154	264	110	209
	0.6	148	247	99	198
	1.0	138	245	107	192
Mg-Curcumin	0	-649	-554	-95	-602
	0.4	-658	-557	-101	-608
	0.6	-662	-559	-103	-611
	1.0	-678	-562	-116	-620

5.3.3.3 Circular dichroism studies

Circular dichroic (CD) spectral technique is useful in diagnosing changes in DNA morphology during complex–DNA interactions. The interaction of complexes with DNA may often change the CD signals of DNA which can be assigned as due to the corresponding changes in DNA structure [23]. A CD spectra of DNA solution shows a positive band (275 nm) from base stacking interaction and a negative band at (245 nm) from right-handed helicity of DNA, characteristic of DNA in the right-handed B form [24]. Depending on the binding modes, spectrum of DNA will be modified with increasing concentration of small drugs or metal complexes and characteristic conformation changes would be observed in DNA. A simple groove binding and electrostatic interaction of small molecules show less or no perturbation on the base-stacking and helicity bands, while intercalation enhances the intensities of both the bands stabilizing the right-handed B conformation of HS-DNA as observed for the classical intercalator methylene blue [25].

CD spectral variations of Herring sperm DNA (HS-DNA) were recorded by the respective addition of the curcumin complexes of copper and magnesium to the DNA. Each sample solution was scanned in the range of 220–320 nm. A CD spectrum was generated **Fig 5.14 (a) and (b)** represented the average of three scans

from which the buffer background had been subtracted. The concentration of DNA was 10^{-5} M. The change in intensity of DNA band **Table 5.4** suggests a conformational change in DNA which is relative to the mode of binding of complex to DNA. The CD spectra shows slight perturbation of the bands with decrease in intensity in both positive and negative bands and zero cross over at 258 nm suggesting the stacking mode and disturbance in the orientation of base pairs of DNA. With increasing concentration of curcumin complex of Mg(II) and Cu(II), the DNA peaks at 275 and 245 nm shift to 1–3 nm without any change in the zero-cross over at 258 nm is indicative of groove binding mode [23]. This suggests that binding of the complexes to DNA induces certain conformational changes in DNA of B-like to a more A-like structure [26]. The simple groove binding and electrostatic interaction of small molecules show less or no perturbation on the base-stacking and helicity bands, while for the classical intercalator like methylene blue enhances the intensities of both the bands stabilizing the right-handed B conformation of DNA [25].

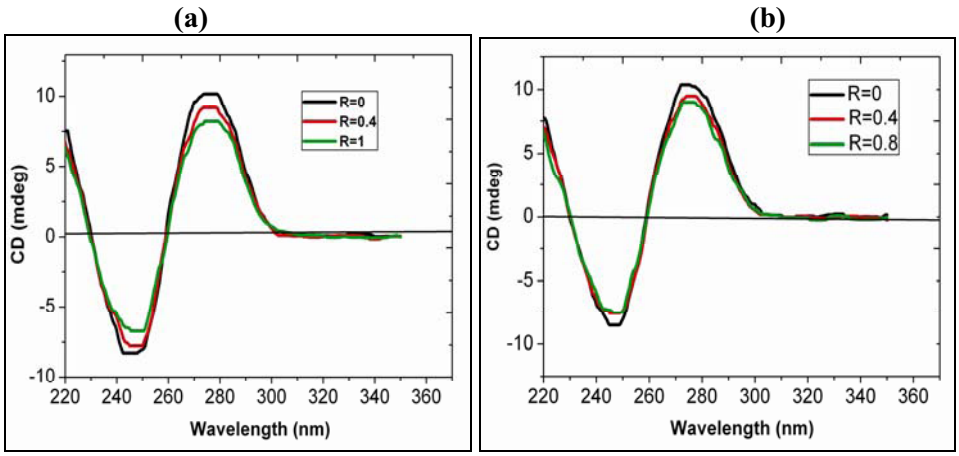

Fig 5.14 (a) CD spectra of HS-DNA (1×10^{-5} M)) in Tris-HCl buffer of Ph 7.1 in the absence (R=0) and presence (R=0.4 &1) of Cu-curcumin

Fig 5.14 (b) CD spectra of HS-DNA (1×10^{-5} M)) in Tris-HCl buffer of pH 7.1 in the absence (R=0) and presence (R=0.4 & 0.8) of Mg-curcumin

Table 5.4 CD parameter for DNA-complex interaction of curcumin complex of Copper and Magnesium

Complex	R	Positive band		Negative band	
		λmax (nm)	CD (mdeg)	λmax (nm)	CD (mdeg)
Cu-Cur	0.00	275	10.2888	245	-8.5786
	0.40	275	9.4205	247	-8.4927
	1.00	277	8.6200	248	-7.4920
Mg-Cur	0.00	275	10.4141	245	-8.5218
	0.40	275	9.6608	247	-7.8223
	0.80	278	9.2118	248	-7.8134

5.4 Conclusions

Morphological alterations of H9c2 cells after 48 h of treatment with curcumin and its metal complexes of [Cu(II), Mn(II), Ni(II) and Co(II)] showed that cells had normal spindle shaped morphology at all (1, 5, 10, 15 and 20 μM) concentrations of various samples. MTT assay showed that curcumin and its metal complexes showed a reduction in cell viability in a dose-dependent manner when compared to untreated cells.

Curcumin complexes have been subjected to DNA binding studies using various techniques like UV-Vis, CV and CD. The results showed that with increasing concentration of DNA, the complexes interact with DNA. From the plot of [DNA]/(ε_a-ε_f) versus [DNA] obtained from the titration of DNA with complex using UV, the binding constant (K_b) value was calculated. The order obtained for binding constant was Mn(II) < Co(II) < Mg(II) < Cu(II).

Cyclic voltammetric studies were done with curcumin complex of Cu(II) and Mg(II). On increasing the concentrations of DNA, the peak currents and Epc decreased. The variation in current and formal potential suggested an association of

the complex with DNA. From the electrochemical data the mode of binding was suggested as electrostatic or groove binding.

Circular dichroism studies were also carried out to ascertain the mode of binding of complex to DNA. In CD fixed concentration of DNA was titrated against increasing concentration of curcumin complex of Cu(II) and Mg(II). The spectra showed slight perturbation on the bands with decrease in intensity in both positive and negative bands with increasing concentration of curcumin complex of Mg(II) and Cu(II). The peaks at 275 and 245 nm of DNA with addition of Mgt(II) and Cu(II) complex shift to 2–3 nm without any change in the zero-cross over at 258 nm which was indicative of groove binding mode.

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Chapter-6

Synthesis of curcumin pyrazole and its metal complexes: Antioxidant activity and DNA binding study of curcumin pyrazole complexes

Contents

6.1 Introduction
6.2 Experimental
6.3 Results and Discussion
6.4 Conclusions
6.5 References

6.1 Introduction

Cancer has been a cause of concern for human throughout. With the second half of the 20th century molecular and cellular basis of cancer was understood. Based on these findings effective treatment have been developed. Regardless of all the developments made so far cancer is still a major cause of concern due to a great number of limitations [1]. Cisplatin synthesised by Peyrone in 1844 has been used as effective anticancer drug owing to its ability to bind to DNA via a covalent mode [2]. However the need for new metal based complexes has risen due to the limitation of cisplatin in terms of its resistance to tumour cells. The mechanism of cisplatin has been extensively monitored and was found to bind to DNA preferentially through covalent intrastrand binding. Lerman [3] suggested non-covalent mode such as intercalation for binding of small molecules with nucleic acid. This mode of binding was found to induce structural and property modification of DNA [4], which is the primary step to mutagenesis [5]. Various

interactive forces like hydrogen bonding, hydrophobic and electrostatic interaction stabilizes the binding of small molecules to DNA. The antibiotic actinomycin D or daunomycin [6] have shown intercalative mode of binding. Two additional non-covalent mode of binding has been reported: groove binding and external electrostatic effect [7,8].

Metal complexes are suited to study the mode of binding to DNA as they offer the possibility of evaluating the effect of sterric and electronic factor through systematic changes of the metal and coordination sphere. The non-covalent interactions between the ancillary ligands of the metal complexes and DNA are employed to induce selectivity of binding [9,10] towards particular DNA sequence depending on the steric complementarity between the bound complex and the DNA back bone. Among the other factors that affects binding affinity [11,12] are size, shape and hydrophobicity of the intercalating ligand.

Curcumin a spice used to give flavour and yellow colour to curry has found to exhibit various biological applications including anti-tumour property that include apoptosis induction and growth inhibition in vitro, also the ability to inhibit tumorigenesis in vivo [13-16]. Large quantity of curcumin can be consumed without toxicity; as a result it remains as a valuable compound for drug development. The advantage of bringing about changes in curcumin scaffold is the lack of toxicity. One such analogue of curcumin is curcumin pyrazole which has been evaluated for anti-malarial activity [17], enhancement of memory [18] and restoration of membrane homeostasis disrupted after brain trauma [19]. Pyrazole, characterized by a 5-membered ring structure consist of three carbon atoms and two nitrogen atoms in adjacent position. Pyrazole are aromatic compound of the heterocyclic diazole. They belong to a class of alkaloids due to its pharmacological effect on human. Pyrazole derivatives are subjected to many research studies as they have widespread biological activity such as anti-inflammatory [20], antiviral [21], antihistaminic [22], fungicides [23] and anti-cancer [24]. The pyrazole

derivate of curcumin and its complexes has been synthesised with the aim evaluating its antioxidant and DNA binding property.

6.2 Experimentals

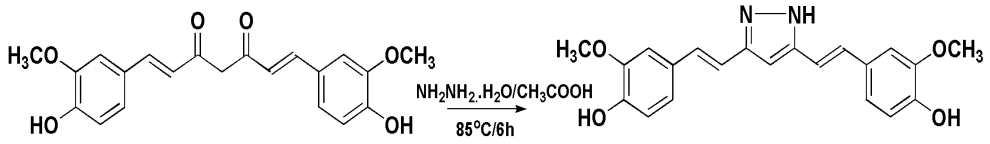
6.2.1 Materials

Curcumin	E.MERCK
Hydrazine hydrate	E.MERCK
Deoxyribonucleic Acid	SRL
Tris (Hydroxymethyl) Aminomethane	SRL
Acetic acid glacial	S. D. FINE CHEM LTd. Mumbai

6.2.2 Methods

6.2.2.1. Preparation of curcumin pyrazole

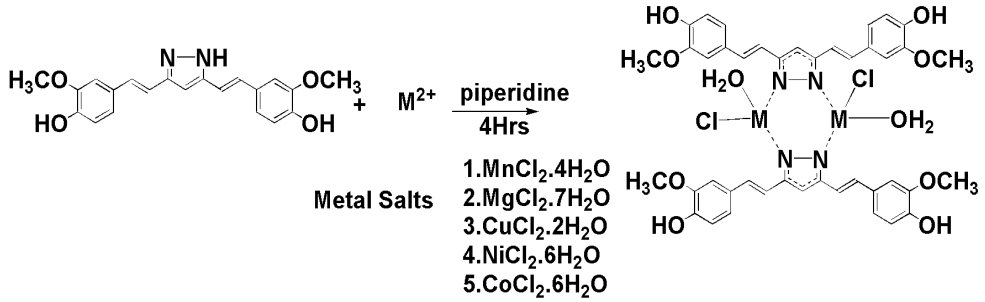
Curcumin-I (1.2 mmol) was dissolved in glacial acetic acid (5 mL) and Hydrazine hydrate (1.5 mL) was added to the solution as described in the **Scheme 1**. The solution was refluxed for 8 h and solvent was removed by vacuum. The obtained solid residue was dissolved in ethyl acetate and washed with water. Organic portion was collected, dried over sodium sulphate and concentrated in vacuum. Crude product was purified by column chromatography using silica gel with hexane/ ethyl acetate 60:40 as eluent [17].



Scheme-1 Preparation of curcumin pyrazole

6.2.2.2 Preparation of metal complexes of curcumin pyrazole

Curcumin pyrazole (0.27 mmol) and the metal salts (0.27 mmol) solution were prepared separately in methanol. To the curcumin pyrazole solution containing catalytic amount of piperidine, the metal salt solution was added with continuous stirring, **Scheme 2**. The resultant solution stirred for 4 h and the product separated was filtered, washed with cold methanol several times to remove the residual reactant and dried in vacuum.



Scheme-2 Preparation of metal complexes of curcumin pyrazole

6.2.2.3 Cyclic voltammetric studies

The cyclic voltammogram of curcumin pyrazole complexes were done with complexes of (1×10^{-5} M) in DMSO with Tetrabutylammonium perchlorate as the supporting electrolyte.

6.2.2.4 Preparation of stock solution of DNA

The stock solution of Herring sperm DNA was prepared in Tris-HCl buffer and the concentration was determined by measuring the absorption intensity at 260 nm with a known ε value of 6600 M^{-1} cm^{-1}[25]. The stock solutions were stored at 4^0 C and used within 4 days.

6.3 Result and Discussion

6.3.1 Characterisation of curcumin Pyrazole

6.3.1.1 Electronic absorption spectra

Maximum absorption band of Curcumin pyrazole was obtained at 328 nm indicating the π-π* transition in DMSO.

6.3.1.2 Infrared spectra

Infrared spectroscopy was used to determine the chemical bonds in a molecule. The broad absorption band centred at 3487 cm^{-1} can be interpreted as ν(O-H) stretching. A peak at 3148 cm^{-1} corresponds to ν(N-H) of pyrazole. The sharp band at 1623 cm^{-1} which is characteristic to ν(-C=O) of curcumin, disappeared and a new band around 1549-1600 cm^{-1} appeared was assigned to pyrazole ring ν(-C=N). The intense band at 1513 cm^{-1} can be attributed to ν(C=C).The sharp peak at 1271 cm^{-1} corresponds to ν(–C-O) of phenol. A medium intense band at 1029 cm^{-1} can be ascribed to ν(-OCH_3). The obtained IR spectra was as in **Fig 6.1**.

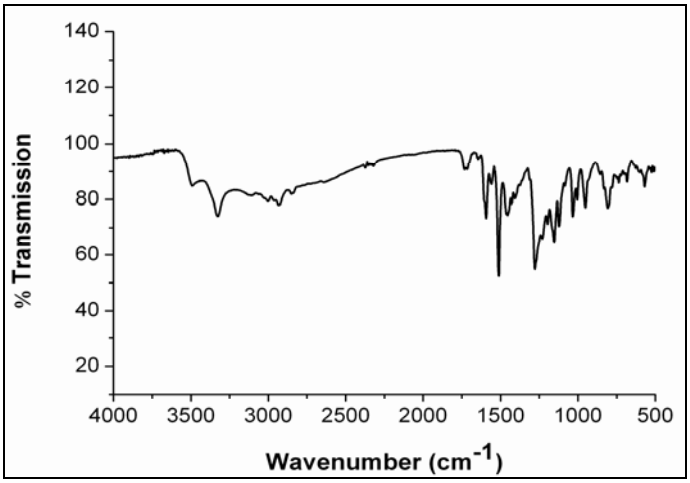


Fig 6.1 IR spectra of curcumin pyrazole

6.3.1.3 NMR spectra

^{1}H NMR (300MHz, DMSO-D_6) δ 3.9 (s,6H,-OCH_3), 6.73 (d,2H,J= 16Hz), 6.81-7.32 (6H,aromatic), 7.5 (d,2H,J=16Hz), 9.66 (s,2H,-OH) is represented in **Fig 6.2**.

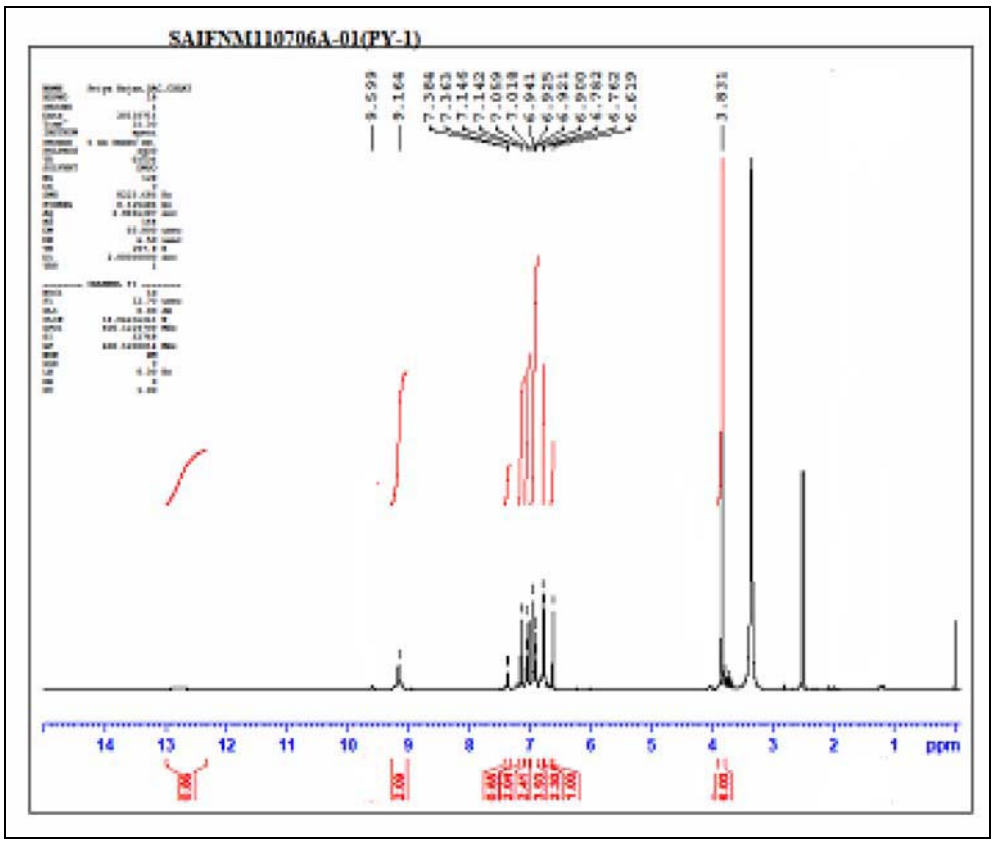


Figure 6.2 NMR of curcumin pyrazole

6.3.2 Characterisation of metal complexes curcumin pyrazole (1-5)

6.3.2. 1 Elemental analysis

Analytical data obtained are as shown in the **Table 6.1**.

Table -6.1 Elementary data of metal complexes of curcumin pyrazole

Sl. No	Compound	C (%) obtain (Cal)	H (%) obtain (Cal)	N % Obtain (Cal)	Metal obtain (Cal)	Cl%	Molar conductance (Ohm^{-1} cm^2 mol^{-1})
1	Mn-CurPy	53.68 (53.46)	4.90 (4.49)	5.67 (5.94)	11.70 (11.64)	7.67 (7.51)	8
2	Mg-CurPy	57.65 (57.17)	4.98 (4.80)	6.77 (6.35)	5.78 (5.51)	8.06 (8.04)	19
3	Cu-CurPy	52.87 (52.50)	4.55 (4.41)	5.77 (5.83)	13.76 (13.23)	7.78 (7.38)	16
4	Ni-CurPy	53.24 (53.04)	4.88 (4.45)	5.97 (5.89)	12.45 (12.34)	7.67 (7.46)	17
5	Co-CurPy	53.76 ((53.92)	4.99 (4.52)	5.57 (5.99)	12.98 (12.60)	7.79 (7.58)	12

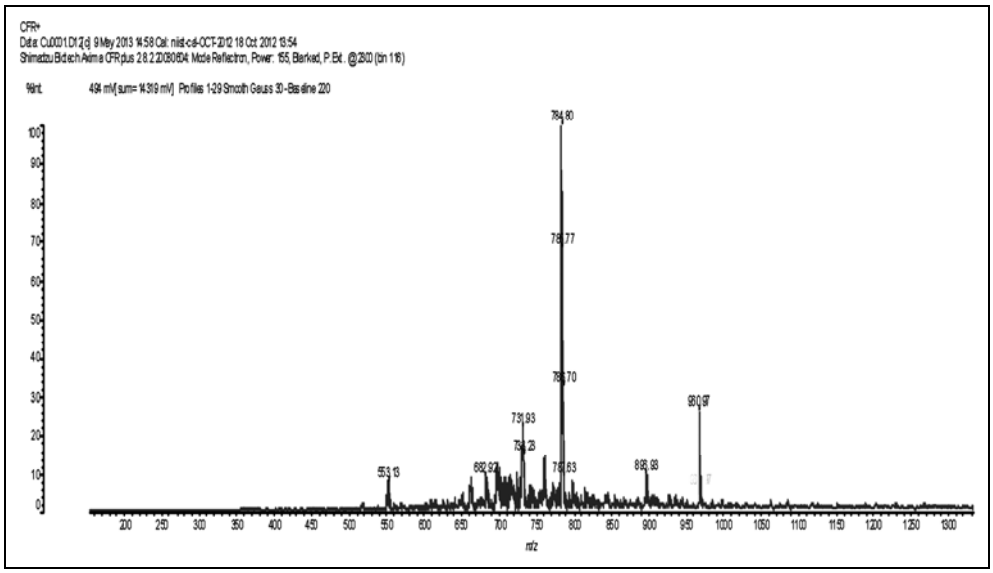


Fig 6.3 MALDI of Cu-curcumin pyrazole

The detection of mass using MALDI (Shimadsu Biotech Axima in +mode) suggests the formation of 2:2 complexes for curcumin pyrazole. **Fig 6.3** represents the mass spectrum of Cu-curcumin pyrazole where the highest peak at 960 corresponds to the 2:2 complex formed, other being the molecular cation of various fragments of the complex [26]. The molar conductance measurement indicates non-electrlytic nature of all the complexes.

Based on the analytical, molar conductance and mass data complexes have been assigned the molecular formula $[M_2(CurPy)_2(H_2O)_2.Cl_2]$.

6.3.2.2 Electronic spectra

Electronic spectra of the complexes in DMSO are given in the **Table-6.2.**

Table-6.2 UV-visible spectral data of curcumin pyrazole metal complexes in DMSO 10^{-5} mol L^{-1}

Sl .No	Compound	λmax (nm)
1	Mn-CurPy	342
2	Mg-CurPy	337
3	Cu- CurPy	339
4	Ni- CurPy	340
5	Co- CurPy	338

6.3.2.3 Infrared spectra

The various IR bands and their assignments of curcumin pyrazole and its metal complexes are given in the **Table 6.3**. The strong C=N stretching frequency of curcumin pyrazole at 1549cm^{-1} is being shifted to higher wave length region in its metal complexes. The C=N stretching frequencies of curcumin pyrazole in its complex with Mn(II), Mg(II), Cu(II), Ni(II) and Co(II) were 1589 cm^{-1}, 1588 cm^{-1},

1592 cm^{-1}, 1597 cm^{-1} and 1591c m^{-1} respectively. The high frequency shift of the ring ν(-C=N) suggest pyrazole ring nitrogen are involved in ligation. In the IR spectra of curcumin pyrazole complexes **Fig 6.4-6.8**, the -OH band do not show considerable shift from 3447 cm^{-1}. Hence indicating that the phenolic -OH groups are not involved in the complexation.

Table-6.3 IR bands and their assignments for curcumin pyrazole and its metal complexes

Compound	ν(O-H) (cm^{-1})	ν(N-H) pyrazole (cm^{-1})	ν(-C=N) pyrazole (cm^{-1})	ν (C=C) (cm^{-1})	ν(C-O phenol) (cm^{-1})	ν(-OCH_3) (cm^{-1})
Curcumin pyrazole	3447	3148	1549	1513	1271	1029
Mn-CurPy(**1**)	3443	-	1589	1512	1272	1020
Mg-CurPy(**2**)	3428	-	1594	1510	1275	1023
Cu-CurPy (**3**)	3428	-	1592	1512	1270	1018
Ni-CurPy (**4**)	3426	-	1597	1514	1265	1019
Co-CurPy (**5**)	3440	-	1591	1512	1269	1020

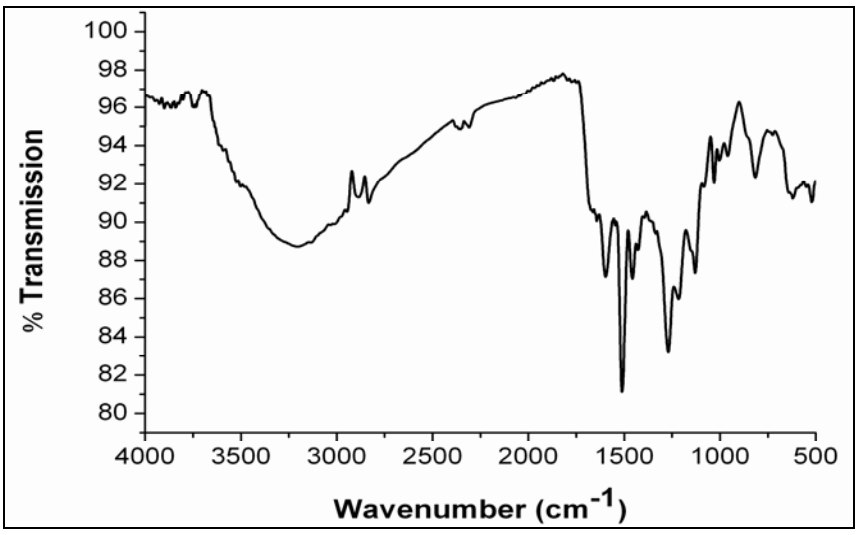


Figure 6.4 IR spectra of Mn-curcumin pyrazole complex

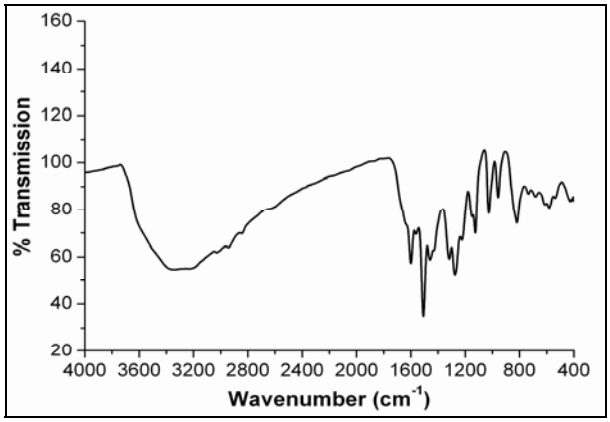


Figure 6.5 IR spectra of Mg-curcumin pyrazole complex

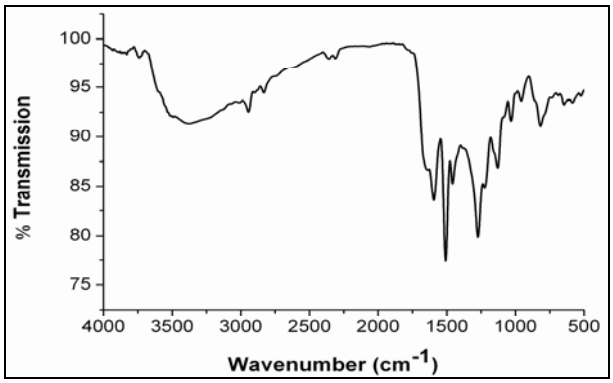


Figure 6.6 IR spectra of Cu-curcumin pyrazole complex

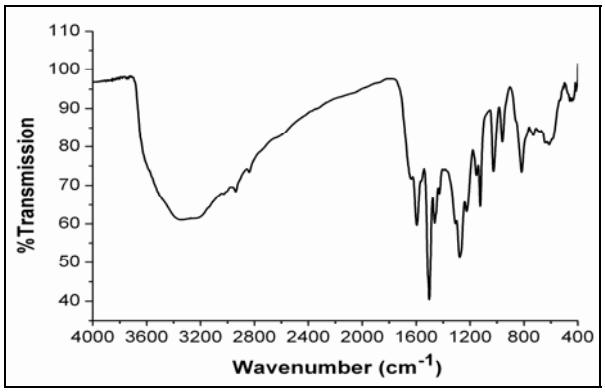


Figure 6.7 IR spectra of Ni-curcumin pyrazole complex

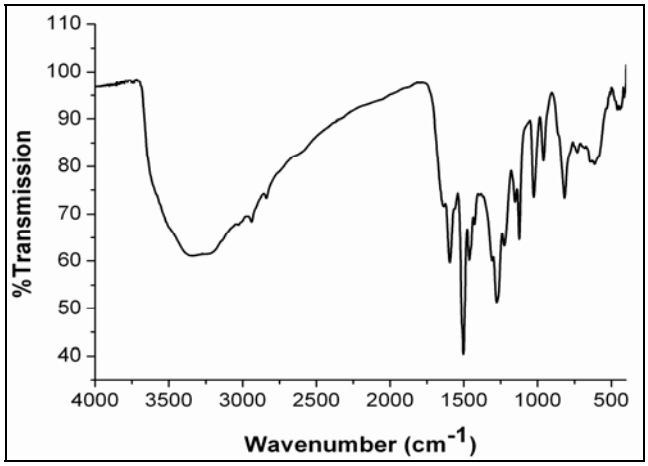
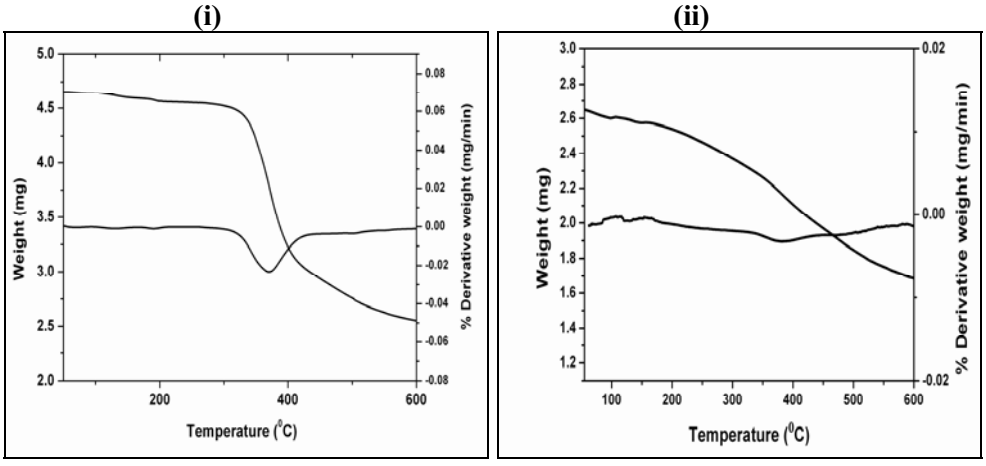


Figure 6.8 IR spectra of Co-curcumin pyrazole complex

6.3.2.4 Thermal Analysis

TGA was used to determine degradation temperatures and absorbed moisture content. TG curves **Fig 6.9**, suggest that there is coordinated water in the complexes. Decomposition of the complexes occurs at a higher temperature when compared to the ligand curcumin pyrazole.



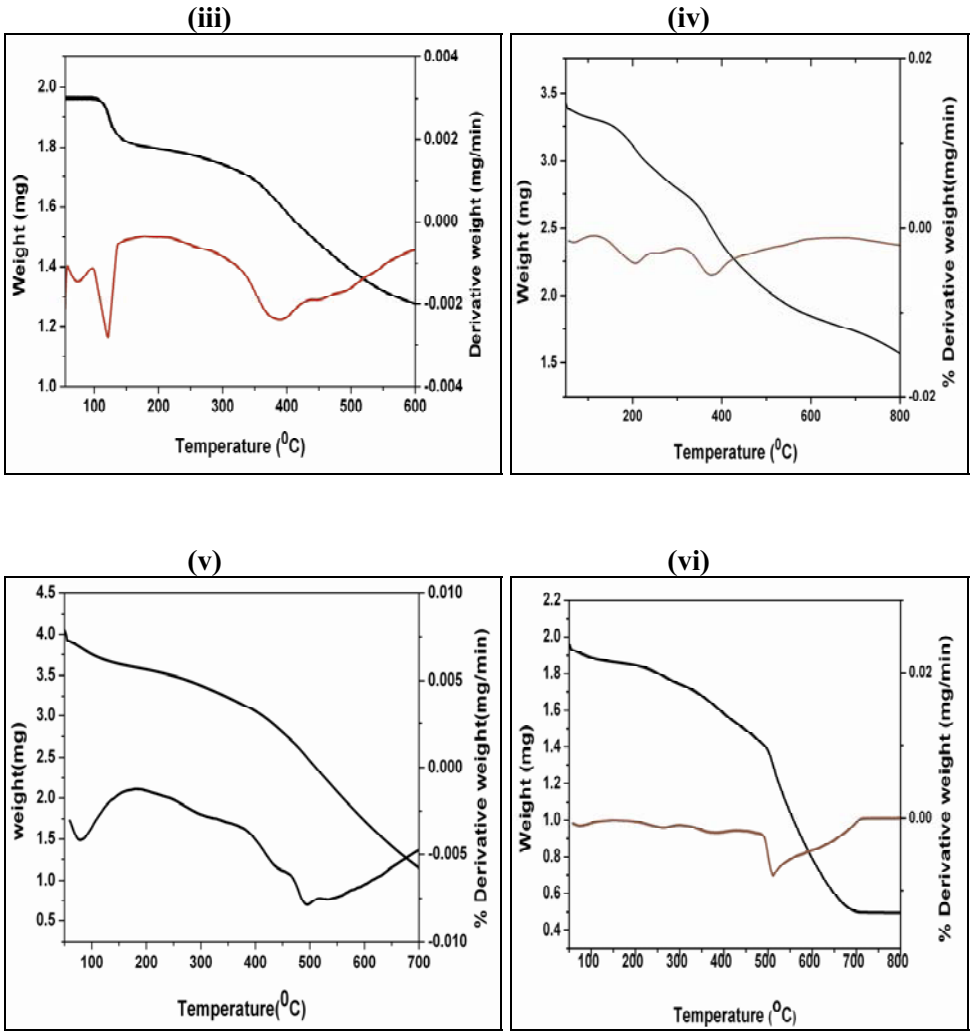


Figure 6.9 TG-DTG curves (i) Curcumin pyrazole (ii) Mn-curcumin pyrazole (iii) Mg-curcumin pyrazole, (iv) Cu-curcumin pyrazole (v) Ni-curcumin pyrazole (vi) Co-curcumin pyrazole

Curcumin pyrazole was stable up to 200°C and after 400°C it underwent complete decomposition. All complexes showed the presence of coordinated water.

6.3.2.5 Cyclic voltammetric studies

The cyclic voltammogram of curcumin pyrazole complexes in DMSO are given in the **Fig 6.10-6.14** and **Table 6.4**. Study were done with complexes of concentration (1×10^{-5} M) in DMSO with Tetrabutylammonium perchlorate as the supporting electrolyte. The I_{pc}/I_{pa} shows a one electron transport take place in all the complexes.

Table 6.4 Cyclic voltammetric data of curcumin pyrazole complexes

Complexes	E_{pc}(mV)	E_{pa}(mV)	$E_{1/2}$(mV)	ΔE_p(mV)	I_{pc}/I_{pa}
Mn-CurPy	-491	-245	-368	246	.895
Mg-CurPy	-861	-707	-784	154	.913
Cu-CurPy	-884	-690	-787	194	1.1
Ni-CurPy	-992	-747	-670	245	1.1
Co-CurPy	-901	-712	-807	189	1.0

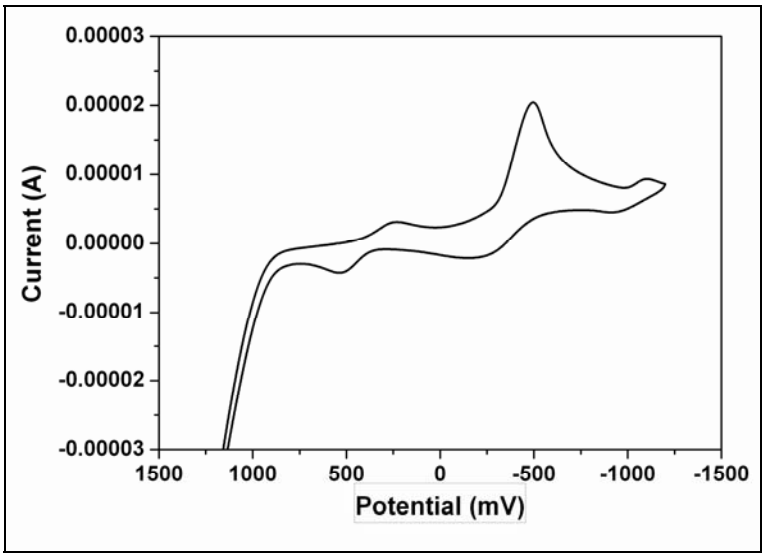


Fig.6.10 Cyclic voltammogram of Mn-curcumin pyrazole

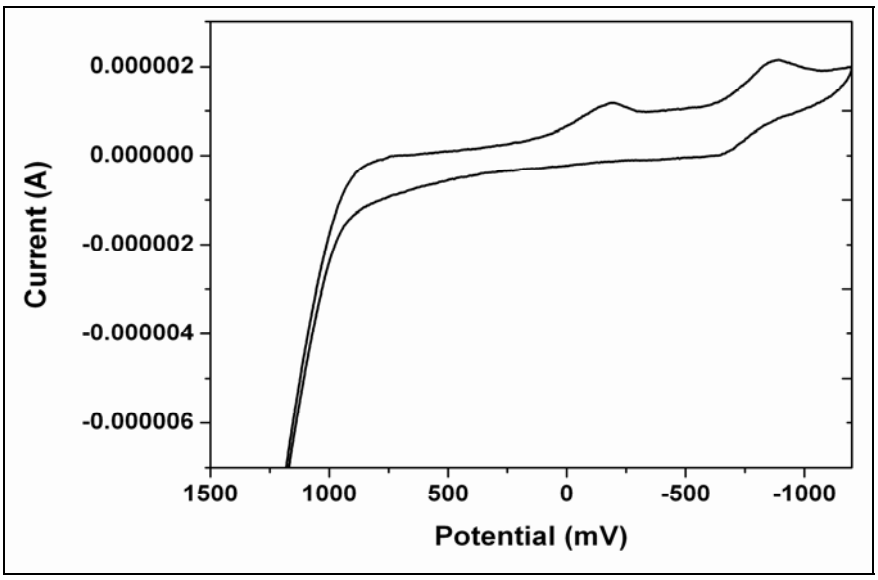


Fig.6.11 Cyclic voltammogram of Mg-curcumin pyrazole

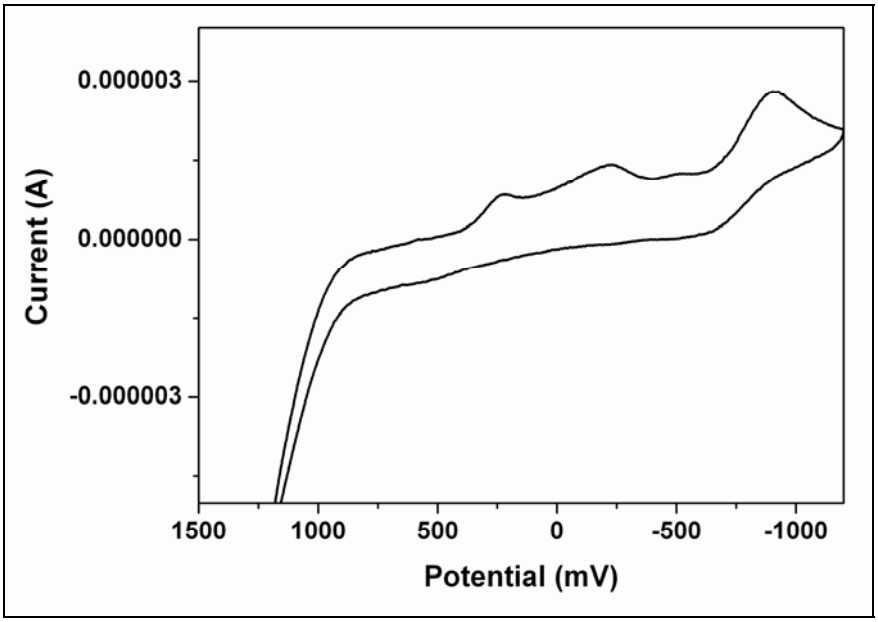


Fig.6.12 Cyclic voltammogram of Cu-curcumin pyrazole

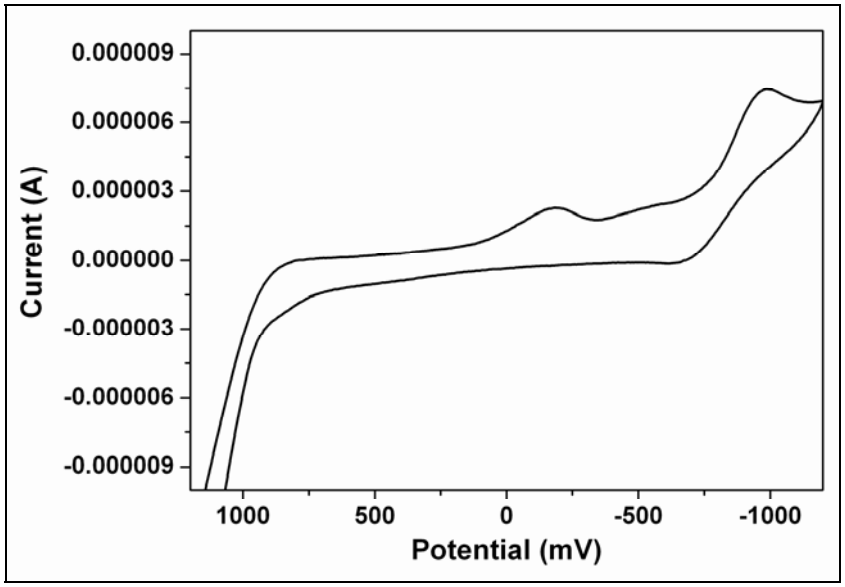


Fig.6.13 Cyclic voltammogram of Ni-curcumin pyrazole

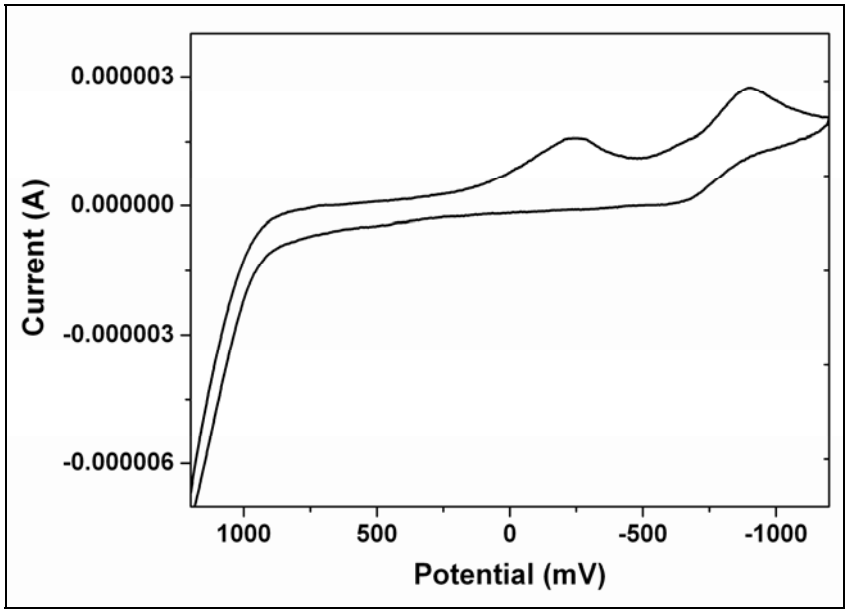


Fig.6.14 Cyclic voltammogram of Ni-curcumin pyrazole

6.3.3 Antioxidant activity study

The details of the DPPH solution prepared are discussed in Chapter 2. The concentration of curcumin pyrazole used for the DPPH assay varied form 13-30 mmol. The solvents used for the study were methanol, acetonitrile, acetone, ethyl acetate and 1,4-dioxane.

6.3.3.1 Solvent effect on antioxidant property of curcumin pyrazole

The result of antioxidant activity of curcumin pyrazole using DPPH assay method is as shown in **Fig 6.15**. The solvents used for the study were methanol, acetonitrile, acetone, ethyl acetate and 1,4-dioxane.The antioxidant activity was found to be far high in methanol and least in 1,4-dioxane. The magnitude of antioxidant activity in the five solvents follow the order 1,4-dioxane < ethyl acetate < acetone < acetonitrile < methanol. IC_{50} values of curcumin pyrazole are as given in **Table 6.5** from which it were evident that the antioxidant activity is dependent on medium in which study has been done.

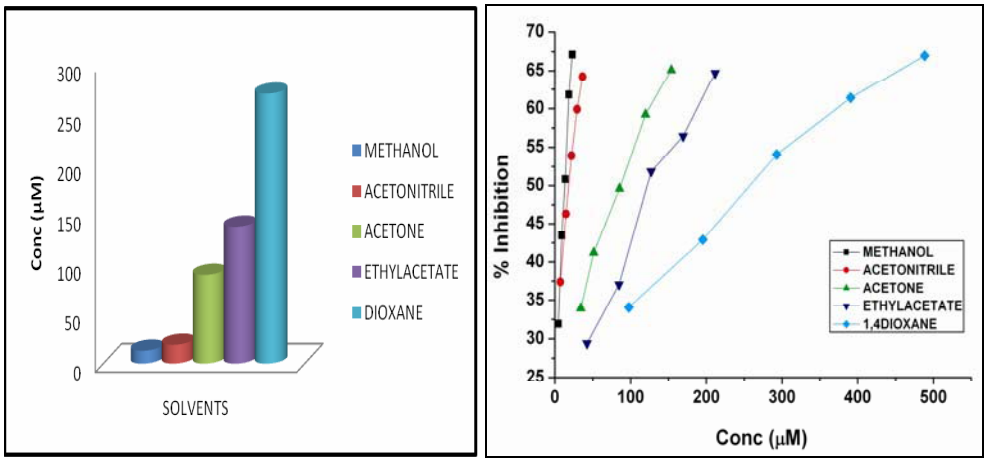


Fig 6.15 Kinetic solvent effect on antioxidant activity of curcumin pyrazole

Table 6.5 IC_{50} values of curcumin- pyrazole and curcumin-I in various solvents

SOLVENTS	Curcumin-I IC_{50}(μM)	Curcumin-pyrazole IC_{50}(μM)
Methanol	17	13
Acetonitrile	23	19
Acetone	110	89
Ethyl acetate	180	137
1,4 Dioxane	341	271

The antioxidant activity of curcumin pyrazole was found to be more in comparison to curcumin-I. This might be due to resonance stabilization of the free radical centre of pyrazole ring similarly as of phenols and are as depicted in **Fig 6.16**.

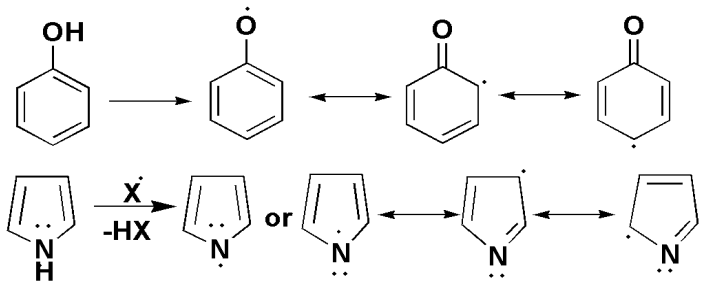


Fig 6.16 Resonance stabilisation of phenoxide and pyrrole radical

6.3.3.1.1 Antioxidant activity in polar protic solvents

Among the solvents used to study the antioxidant property of curcumin pyrazole by DPPH assay, the maximum activity was shown in methanol mediated reactions. This anomalous behaviour was due to protic nature of methanol forming intermolecular hydrogen bonding among solvent thereby preventing ArOH…S

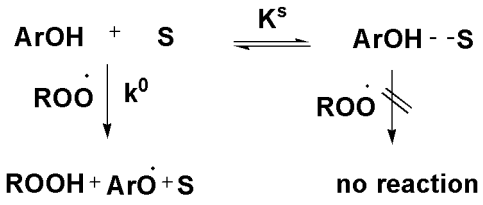
interaction as in **Scheme 3**. Hence, ArOH (substrate) to S (solvent) equilibrium do not exist in methanol, facilitating the ionisation of ArOH depending on the bulk property of the solvent, its relative permittivity (dielectric constant, ε_r), molecular property, its relative ability to solvate stabilising anions (ArO^-), as quantified by Swain et al's A value [27]. Methanol having high ε_r and A values (33, 0.75) supports ionisation and hence ArOH looses proton to form stable phenoxide ion at very fast rate. Consequently quenching of DPPH occurs at low concentration of ArOH, giving a very low IC_{50} value [28]. All these fact support the existence of SPLET in methanol.

Scheme 3 Anomalous behaviour of ArOH in protic solvent- methanol

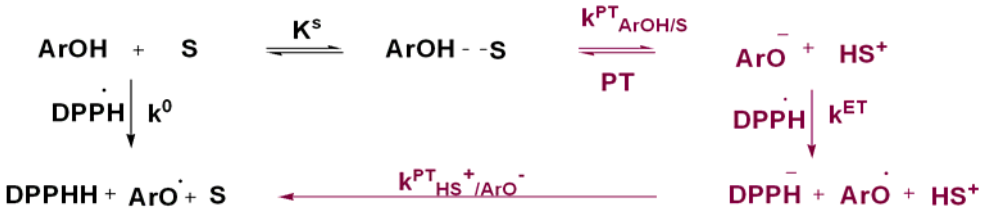
6.3.3.1.2 Antioxidant activity in polar aprotic solvents

In polar aprotic solvents the order of antioxidant activity was acetonitrile > acetone > ethyl acetate. The IC_{50} values were as in **Table 6.5**. The polar aprotic solvent with high dielectric constant (37, 21) was expected to give high activity in terms of dielectric constant [29], which was true for acetonitrile but not for acetone. The high activity in acetonitrile as when compared to the very low activity in acetone can be interpreted in terms of the solvent interaction of these medium as represented in **Fig 3.9** in Chapter 3**.** The hydrogen bonding interactions between highly electronegative oxygen of acetone and hydrogen of phenol give rise to strong ArOH….S (solvent) interactions [30], the primary factor contributing to the low rate of H-abstraction from a phenol to a free radical. Hence phenol deprotonation is difficult and only those molecules which are free for the deprotonation as in **Scheme 4** [29] will be available to interact with DPPH to form DPPHH via HAT. HAT being slow will subsequently lead to higher IC_{50} value for all the substrates in acetone as when compared to very low value in acetonitrile. As

acetone supports ionization, the measured rate would be the sum of the rates for the HAT (black) and SPLET (red) **Scheme 5** [31]. Whether the SPLET mechanism will be significant compared with the HAT mechanism will depend on the phenol's acidity, the radical's electron affinity, the ability of the solvent to support phenol ionization. In acetone, contribution from HAT will be more when compared to SPLET as evident from higher IC_{50} value. Acetonitrile in which solvent interaction is very less and the major reaction is expected to proceed via SPLET, as evident from its low IC_{50} value and antioxidant activity comparable to that in methanol.



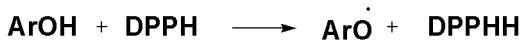
Scheme-4 [11] Origin of kinetic solvent effects in a HAT reaction in polar aprotic solvents –that do not support ionisation



Scheme-5 [26] Origin of kinetic solvent effects in a mixed HAT and SPLET reaction in polar aprotic solvents –that support ionisation

6.3.3.1.3 Antioxidant activity in non- polar solvents

In the non polar solvent 1,4-dioxane used to study the reaction kinetics the activity was very low when compared to methanol due to the low ε_r and *A* values (2.2, 0.19) [28]. Dioxane being a non polar solvent that may not support ionisation, it is assumed that reaction will take a path of slower HAT as shown in **Scheme 6** [32]. HAT being very slow will require higher concentration of the antioxidant to bring 50% inhibition of DPPH. This in turn is expected to give higher IC_{50} value in solvent like 1,4-dioxane.



Scheme 6 [31] HAT mechanism in non polar, non ionising solvent

6.3.3.2 Antioxidant property of metal complexes of curcumin pyrazole

Curcumin pyrazole (0.5 mg mL^{-1})/metal complex (0.5 mg mL^{-1}) solutions in DMSO were prepared separately and added to a methanol solution of DPPH (0.01 mmol) within the range of 10-120 μL and made up to a final volume of 3 mL using methanol as solvent. The scavenging ability of curcumin pyrazole and its metal complexes were monitored spectrophotometrically in terms of decrease in absorbance at 517 nm after 20 min.

The DPPH scavenging activity of metal complexes were less than that of curcumin pyrazole and are depicted in **Fig. 6.17**. The IC_{50} value of the complexes decreases in the order of Mn(II) > Mg(II) > Cu(II) > Ni(II) > Co(II) as shown in **Table 6.6**.

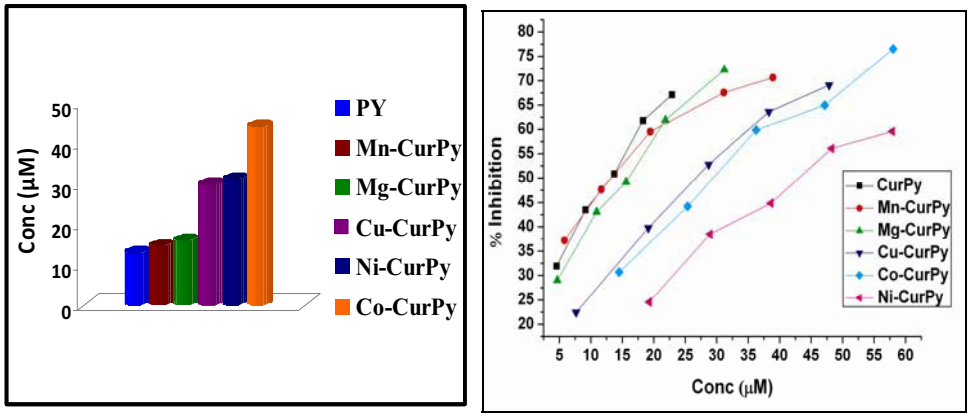


Fig 6.17 Antioxidant activity of metal complexes of curcumin pyrazole

Table-6.6 IC_{50} value in µM of Curcumin pyrazole and its metal complexes

Materials	IC_{50} (µM)
Curcumin-I	13
Mn-CurPy	14
Mg-CurPy	16
Cu-CurPy	29
Ni-CurPy	31
Co-CurPy	44

6.3.4 DNA- binding studies

Interaction of metal complexes with DNA has lead to investigation for new chemotherapeutic agent in biotechnology and medicine [33]. Small molecule have found to react with specific site along a DNA strand, which has been used as a model for protein nucleic acid interaction and has provided a new route to drug design and development. Interaction of metal complexes with DNA is used as probe for DNA structure in solution and as chemotherapeutic agents [33,34]. In our

work, interaction of metal complexes of curcumin pyrazole with DNA has been studied using spectral, electrochemical and CD techniques.

6.3.4.1 Electronic absorption titration

Electronic absorption spectroscopy is one of the most common technique used in DNA-binding studies of metal complexes. The magnitude of spectral perturbation is an evidence for extent of binding [35]. The two effect characteristics of interaction of metal complexes with DNA are hypochromism and hyperchromism. Hypochromic effect is attributed to interaction between electronic states of complex with DNA bases [36]. Hyperchromism can be ascribed to external contact or partial uncoiling of helical structure exposing more bases of the DNA [37].

The electronic absorption titrations were carried out using fixed amount of each metal complex of curcumin pyrazole (30 μM) with increasing concentration of DNA as in **Fig 6.18(a)-6.21(a)**. The Mn(II), Mg(II), Cu(II) and Co(II) complexes (**1,2,3 & 5**) of curcumin pyrazole gave absorption band in the region of 320-328 nm in DMSO- buffer mixture were assigned due to π-π* transition. All the complexes exhibited hypochromism in their absorption maximum with no change in band position.

To further illustrate the binding strength of the curcumin pyrazole complexes with DNA, the intrinsic binding constant K_b was determined from the spectral titration data. By monitoring the changes in absorption at corresponding λ_{max} with increasing HS-DNA concentration and K_b can be calculated using the Eq (1) [38].

$$\frac{[DNA]}{(\varepsilon_a-\varepsilon_f)} = \frac{[DNA]}{(\varepsilon_b-\varepsilon_f)} + \frac{1}{K_b\,(\varepsilon_b-\varepsilon_f)} \qquad (1)$$

Where [DNA] is the concentration of DNA in base pair, ε_a is the apparent extinction coefficient for each addition of DNA to the complex (A_{abs}/[Complex]), $\varepsilon_{f,}$ extinction coefficient for free metal complex and $\varepsilon_{b,}$ extinction coefficient for metal complex in the fully bound form.

A plot of [DNA]/(ε_a-ε_f) versus [DNA] gives binding constant K_b as the ratio of slope by intercept. The magnitude of K_b value gives the extent of binding. The absorption spectra of pyrazole complexes of Mn(II), Mg(II), Cu(II) and Co(II) of (3×10^{-5}M) in Tris-HCl buffer pH 7.1 in the absence (R=0) and presence of increasing amount of DNA are represented in **Fig 6.18(a)-6.21(a)**. A plot of [DNA]/(ε_a-ε_f) versus [DNA] for the titration of DNA with complex for binding constant are represented in **Fig 6.18(b)-6.21(b)**. The binding constant values obtained for the present curcumin pyrazole complexes can be represented as in **Table 6.7**. From the binding constant values the obtained order was Mn(II) < Co(II) < Mg(II) < Cu(II). These values and order obtained where comparable to that of complexes of macrocyclic tetraaza diacetyl curcumin ligand [39] with Cu(II), Co(II) and Mn(II) as metals. The comparison of binding constant values can be represented as in **Table 6.6**.

Table 6.7 The binding constant, K_b (M^{-1}) for curcumin pyrazole complexes and tetraaza diacetyl curcumin complexes

SL No	Complexed metal	Binding Constant K_b (M^{-1})	
		Curcumin -pyrazole	Tetraaza diacetyl curcumin [39]
1	Mn (II)	2.9×10^4	3.5×10^4
2	Co (II)	5.4×10^4	5.2×10^4
3	Mg (II)	1.6×10^5	-
4	Cu (II)	2.4×10^5	1.4×10^5

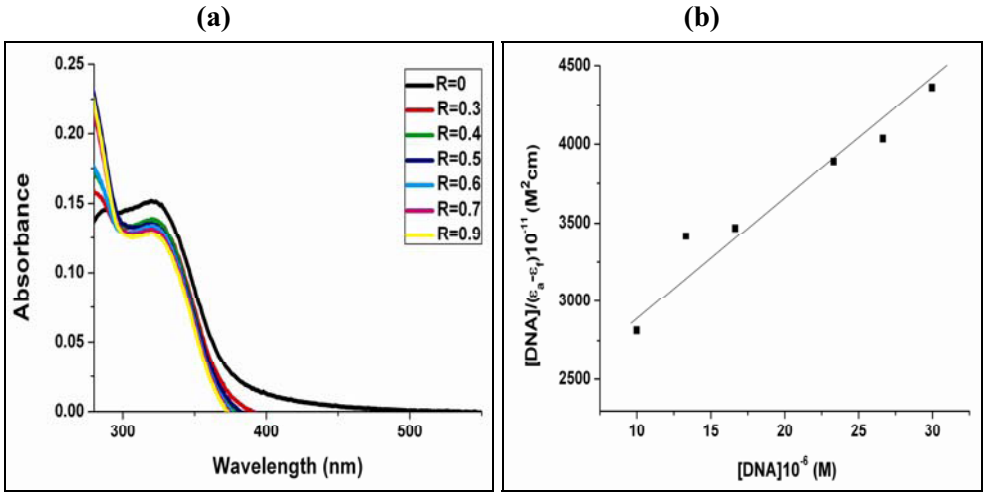


Fig 6.18 (a) Absorption spectra of Mn-CurPy complex (3×10^{-5}) in Tris-HCl buffer of pH 7.1 in the absence (R=0) and presence (R=0.3, 0.4, 0.5, 0.6, 0.7& 0.9) of increasing amount of DNA. R=[DNA]/[Complex]

Fig 6.18 (b) A plot of $[DNA]/(\varepsilon_a-\varepsilon_f)$ versus [DNA] for the titration of DNA with complex

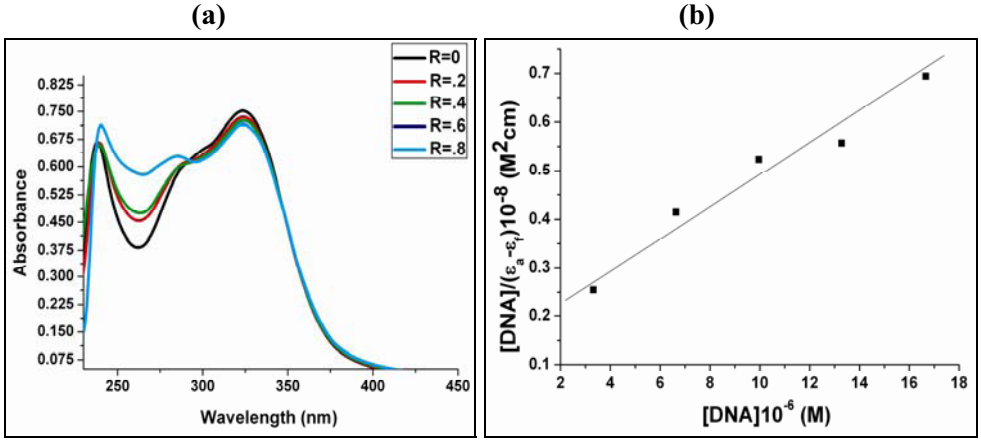


Fig 6.19 (a) Absorption spectra of Co-CurPy complex (3×10^{-5}) in Tris-HCl buffer of pH 7.1 in the absence (R=0) and presence (R= 0.2, 0.4, 0.6, & 0.8) of increasing amount of DNA. R=[DNA]/[Complex]

Fig 6.19 (b) A plot of $[DNA]/(\varepsilon_a-\varepsilon_f)$ versus [DNA] for the titration of DNA with complex

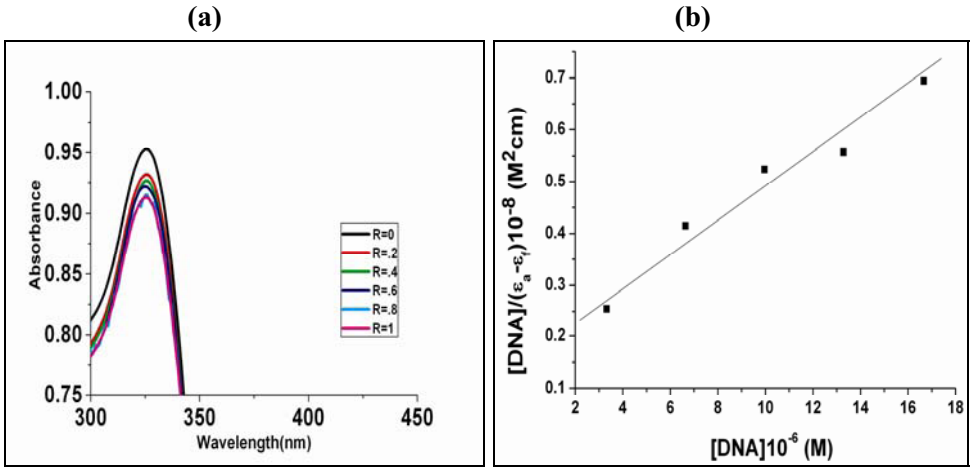


Fig 6.20 (a) Absorption spectra of Mg-CurPy complex (3×10^{-5}) in Tris-HCl buffer of pH 7.1 in the absence (R=0) and presence (R=0.2, 0.4, 0.6, 0.8&1) of increasing amount of DNA. R=[DNA]/[Complex]

Fig 6.20 (b) A plot of $[DNA]/(\varepsilon_a-\varepsilon_f)$ versus [DNA] for the titration of DNA with complex

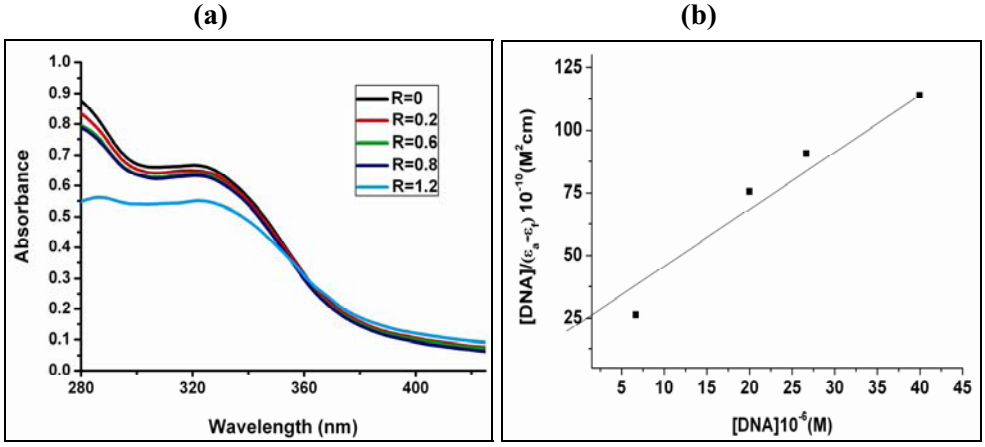


Fig 6.21 (a) Absorption spectra of Cu-CurPy complex (3×10^{-5}) in Tris-HCl buffer of pH 7.1 in the absence (R=0) and presence (R=0.2, 0.4, 0.6, 0.8 & 1.2) of increasing amount of DNA. R=[DNA]/[Complex]

Fig 6.21 (b) A plot of $[DNA]/(\varepsilon_a-\varepsilon_f)$ versus [DNA] for the titration of DNA with complex

6.3.4.2 Cyclic Voltammetric studies

Electrochemical method is complementary to UV method of investigating metallointercalation and coordination of metal ions to DNA. Voltammetric techniques can be used as a tool to study interactions of small molecules with either weak absorption bands or those having electronic transitions overlapping with the DNA molecule. The advantage of this technique is that multiple oxidation states of the same species as well as mixtures of several interacting species can be observed simultaneously [40]. The Cu(II) and Mn(II) complex of curcumin pyrazole were selected for voltammetric studies **Fig 6.22 & 6.23** and their redox behaviour were studied in the absence and presence of DNA at room temperature at the scan rate of 100 mVs^{-1}. On increasing the concentrations of DNA, the peak currents and Epc decreased, **Table 6.8**.

Electrochemical studies can be used to quantify the binding of the metal complex to DNA, in terms of change in current and formal potential upon addition of DNA, due to the difference in diffusion rate of an equilibrium mixture of free and DNA-bound metal complex to the electrode surface [40]. The effect of varying concentration of DNA with fixed concentration of complex, was a decrease in anodic and cathodic peak currents. The decrease in current could be attributed to slow diffusion of the metal complex bound to the large, slowly diffusing DNA [40]. The variation in current and formal potential suggests an association of the complex with DNA. According to Brads report [40], the decrease in potential (E_{pc}) and current (I_{pc}), imply that the interaction mode is electrostatic or groove binding. The DPV of copper complex of curcuminpyrazole ascertain the changes found in CV.

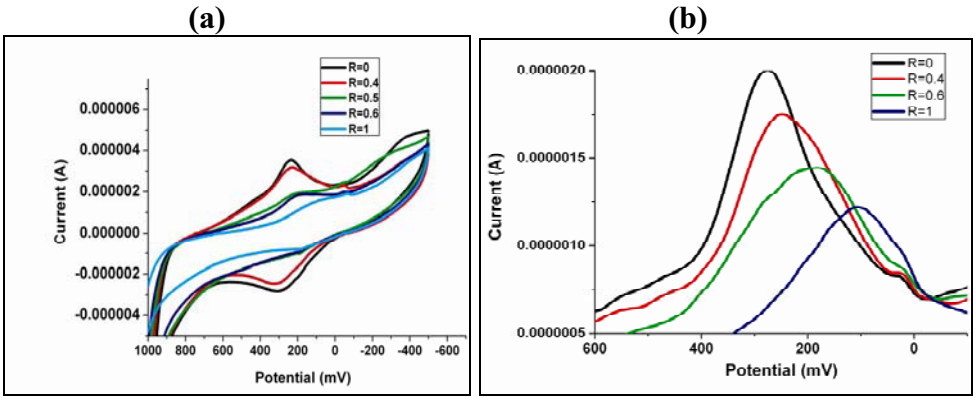


Fig 6.22 (a) Cyclic voltammogramand (b) Differential pulse voltammogram of Cu-pyrazole complex (3×10^{-5}) in Tris-HCl buffer of pH 7.1 in the absence (R=0) and presence (R= 0.4, 0.6 & 1) of increasing amount of DNA

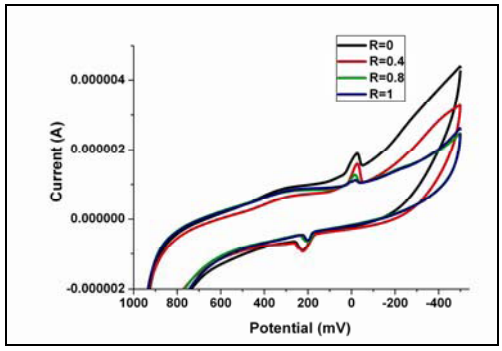


Fig 6.23 Cyclic voltammogramof Mn-pyrazole complex (3×10^{-5}) in Tris-HCl buffer of pH 7.1 in the absence (R=0) and presence (R= 0.4, 0.6 & 1) of increasing amount of DNA

Table-6.8 Voltammetric behaviour of pyrazole complexes in Tris HCl buffer pH 7.1 in the absence and presence of DNA

Compound	R	E_{pc}(mV)	E_{pa}(mV)	ΔE_p(mV)	$E_{1/2}$(mV)	$\Delta E_{1/2}$ (V)
Cu-CurPy	0	276	310	34	294	0.015
	0.4	240	317	77	279	0.089
	0.6	180	230	50	205	0.140
	1.0	96	211	115	154	
Mn-CurPy	0	224	-19	-243	102	0.008
	0.4	216	-27	-243	94	0.015
	0.6	202	-28	-230	87	0.019
	1.0	198	-32	-230	83	

6.3.4.3 Circular dichroism studies

Circular Dichroism (CD) is a useful technique to probe non-covalent drug-DNA interactions, which affect the electronic structure of the molecules and also alter their electronic spectroscopic behaviour [41,42]. CD spectroscopy allows for quick characterization of the drug-DNA interaction using a small amount of sample, as low as (20 μg mL^{-1}) [43]. When electromagnetic radiation reaches DNA, the macromolecules present a certain degree of alineation in the direction of the electric field vector and this molecular alignment is measured by the light polarised absorbance. Depending on the binding modes, spectrum of DNA will be modified with increasing concentration of small drugs or metal complexes and characteristic conformation changes would be observed in DNA.

The theoretical (i.e.quantum chemical) description of CD spectra of molecules as large as DNA is very complex and hence do not provide structural information on molecules at atomic level. CD spectroscopy is therefore used primarily for empirical studies of DNA and it has many advantages over other methods of conformational analysis. It is extremely sensitive, permitting work with very low amount of DNA (20 μg mL^{-1}), the samples can easily be titrated with various agents (like salts, alcohols or acids) that induce conformational isomerizations in DNA and CD measurements are fast and relatively inexpensive that allows comparative studies of related molecules of DNA under many conditions [43].

A solution of HS-DNA shows a positive band (275 nm) from base stacking interaction and a negative band at (245 nm) from right-handed helicity of DNA characteristic of DNA in the right-handed B form [44]. When HS-DNA was titrated with increasing concentration of curcumin pyrazole complex of Mn(II) and

Cu(II) the bands were modified **Fig.6.24 (a)** and **(b).** The change in intensity of DNA band **Table 6.9**, suggests a conformational change in DNA which is relative to the mode of binding of complex to DNA. The CD spectra shows slight perturbation of the bands with decrease in intensity in both positive and negative bands and zero cross over at 258 nm suggesting the stacking mode and disturbance in the orientation of base pairs of DNA. With increasing concentration of curcumin pyrazole complex of Mn(II) and Cu(II), the peaks at 275 and 245 nm of DNA shift to 1–3 nm without any change in the zero-cross over at 258 nm is indicative of groove binding mode [45]. This suggests that binding of the complexes to DNA induces certain conformational changes in DNA of B-like to a more A-like structure [46]. The simple groove binding and electrostatic interaction of small molecules show less or no perturbation on the base-stacking and helicity bands, while for the classical intercalator like methylene blue enhances the intensities of both the bands stabilizing the right-handed B conformation of DNA [47].

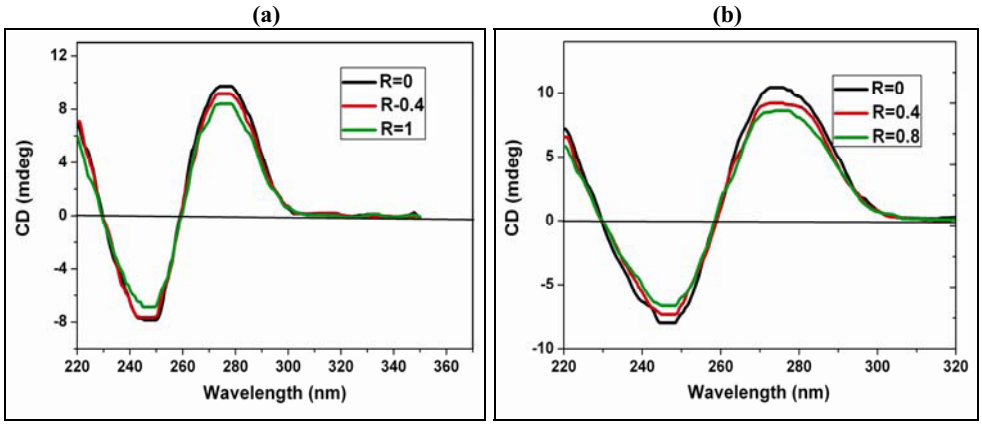


Fig 6.24 (a) CD spectra of HS-DNA (3×10^{-5}) in Tris-HCl buffer of Ph 7.1 in the absence (R=0) and presence (R=0.4 & 1) of Cu- CurPyrazole

Fig 6.24 (b) CD spectra of HS-DNA (3×10^{-5}) in Tris-HCl buffer of pH 7.1 in the absence (R=0) and presence (R=0.4 & 0.8) of Mn-CurPyrazole

Table 6.9 CD parameter for DNA-complex interaction of pyrazole complex of Copper and Manganese

Compound	R	Positive band λmax (nm)	Positive band CD (mdeg)	Negative band λmax (nm)	Negative band CD (mdeg)
Cu-CurPy	0.00	275	9.9892	245	-7.7100
	0.40	275	9.5114	246	-7.5944
	1.00	278	8.5809	247	-6.5000
Mn-CurPy	0.00	275	10.6248	245	-8.1075
	0.40	275	9.2534	246	-7.6403
	0.80	278	8.9438	247	-6.9819

6.4 Conclusions

Curcumin pyrazole has been synthesised and are structurally characterised. The antioxidant property of the compound has been studied using DPPH. Pyrazole derivative of curcumin was found to be more active than curcumin. Solvent effect on antioxidant property of pyrazole was checked in methanol, acetonitrile, acetone, ethyl acetate and 1,4-dioxane. The observed antioxidant activity was found to be high in methanol and least in 1,4-dioxane. The magnitude of antioxidant activity in the five solvents follow the order 1,4-dioxane < ethyl acetate < acetone < acetonitrile < methanol. The variation in antioxidant activity with solvent could be explained in terms of type of solvent used depending on its dielectric constant and interaction of the pyrazole with solvent medium.

Pyrazole derivative were used as ligand for the synthesis of metal complexes which has been subjected to DNA binding studies using various techniques like UV-Vis, CV and CD. The observed results showed that the curcuminpyrazole complexes interact with DNA with increasing concentration of DNA. From the plot of [DNA]/(ε_a-ε_f) versus [DNA] of the titration of DNA with

complex, the binding constant (K_b) value was calculated and the order obtained is Mn(II) < Co(II) < Mg(II) < Cu(II).

Cyclic voltammetric measurement studies were done with curcumin pyrazole complex of Mn(II) and Cu(II). On increasing the concentrations of DNA, the peak currents and Epc decreased. The variation in current and formal potential suggested an association of the complex with DNA. From the electrochemical data the mode of binding was suggested as electrostatic or groove binding.

Circular dichroism studies were also carried out to ascertain the mode of binding of complex to DNA. In CD fixed concentration of DNA was titrated against increasing concentration of curcumin pyrazole complex of Cu(II) and Mn(II). The spectra showed slight perturbation on the bands with decrease in intensity in both positive and negative bands with increasing concentration of curcumin pyrazole complex of Mn(II) and Cu(II). The peaks at 275 and 245 nm of Mn(II) and Cu(II) complex shift to 1–3 nm without any change in the zero-cross over at 258 nm which was indicative of groove binding mode.

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Chapter-7

Conclusions

Over the past few decades there has increasing demands on plant derived compounds as antioxidant. The unanswered question of the present scenario, whether medicines discovered today are safer, more effective and more affordable than the traditional age-old medicines that are in use for centuries for the welfare of mankind. Since the above thought advises us to revisit and revive the use of traditional remedies which far more superior than the modern medicine in terms of efficiency and having fewer side effects. Curcumin is one such medicine that has been used by the people of the Indian subcontinent for centuries with no known side effects, not only as a component of food, but also to treat a wide variety of ailments. Its history goes back over 5000 years, to the heyday of Ayurveda (which means the science of long life). Although its capability to preserve food is been utilised, the ability to give colour to food and to add taste to the food is also well acknowledged, its health-promoting effects are less well documented or appreciated. It was once considered a cure for jaundice, an appetite suppressant and digestive. In Indian and Chinese medicines, turmeric was used as anti-inflammatory agents to treat gas, colic, toothaches, chest pains and menstrual difficulties. This spice was also used to help with stomach and liver problems, heal wounds and scars and as a cosmetic.

In the thesis we represent our findings related to curcumin and its derivatives in terms of structure activity relationship. Efforts have been furnished to synthesis few derivatives modifying the 1,3-diketomoeity and keeping the phenolic part intact. This mode of study was selected keeping in mind the controversy regarding the active part of curcumin in deciding its antioxidant

activity. From the study result we were able to furnish conclusions regarding the relation between structure and activity.

Thesis is divided into six chapters. The first chapter provides an insight into the different biological activity of curcumin. The studies that have attempted to explain the activity in terms of its structure is also discussed in the chapter; subsequently the relevance and scope of the present work.

Chapter 2: The metal [Mn(II), Mg(II), Cu(II), Ni(II) and Co(II)] complexes of curcumin with 1:1 ligand to metal ratio has been synthesized and characterized. The DPPH scavenging activity of metal complexes were less than that of curcumin-I. The antioxidant activity of the complexes decreases in the order of Mn(II) > Mg(II) > Cu(II) > Ni(II) > Co(II). The difference in activity of curcumin-I and its complexes can be inferred in terms of involvement of different reaction centre of curcumin in free radical quenching. Intracellular ROS content was determined by oxidative conversion of cell-permeable DCFH-DA to fluorescent 2',7' dichlorofluorescein (DCF) in H9c2 cells. The fluorescence imaging data was used to quantify the presence of ROS in H9c2 cells. The intensity of fluorescence was decreased in the H_2O_2 stress induced groups that were subjected to pre-treatment with curcumin/its metal complexes for 24 h. This hold up the fact that even in the cell lines the metal complexes in which the enol centre was blocked shows activity comparable to curcumin, further emphasizing the findings of the DPPH study that phenolic centre is the major centre for antioxidant activity. The complexes have comparable antioxidant activity to parent curcumin-I, establishing the minimal involvement of keto-enol moiety of curcumin as the antioxidant centre and hold up the phenolic -OH as the prime centre for the antioxidant activity.

Chapter 3: To establish mechanistic paths for the electron transfer from the phenolic group of curcumin to free radical (DPPH) solvent effect study has been done. The study of antioxidant property of curcumin-I, II and metal complexes of curcumin-I using DPPH in five solvent showed an activity in the order of methanol > acetonitrile > acetone > ethyl acetate > 1,4-dioxane. The complex in which enol form was blocked also showed the same trend as curcumin-I and II but slightly less active when compared to the parent compound. This point's to the fact that even though the enol form is not available for the radical formation, sufficient scavenging of DPPH occurs and can be concluded as the consequence of phenoxide ion formation. Curcumin-I is more reactive than curcumin-II in all the solvent selected for the study, curcumin-I has two methoxy groups which is electron donating compared to one methoxy in curcumin-II. In the complexes formed it was the enolate ion that chelate to metals. Metals being electropositive decrease the electron density at -OH group thereby declining the possible electron donation to DPPH and hence the antioxidant activity. This is consistent with the observation that metal complexes are slightly less active than the parent compound in all the solvent. As curcumin-I, II and metal complexes show the same trend in antioxidant activity in solvents selected for the study, it is suggestive that mechanistic path way followed by the substrates are the same.

Chapter 4: Two derivatives of curcumin, salicylidenecurcumin (CD1) and benzalidenecurcumin (CD2) were prepared by the Knoevenagel condensation of curcumin-I with the aldehydes-salicylaldehyde and benzaldehyde along the active methylene group and their efficacy evaluated against selenite cataract model. The study was designed to compare the biological activity of curcumin and two of its Knoevenagel

condensates, salicylidenecurcumin (CD1) and bezaledenecurcumin (CD2) on *in vitro* selenite induced cataract models. The study establishes the contribution of diketo group (in the absence of keto/enol tautomerism) in deciding its activity. The derivatives of curcumin, CD1 and CD2 were found to be effective in overcoming biochemical changes occurring in cataractous lenses. In comparison, the derivatives of curcumin, CD1 was superior to CD2 in the attenuation of selenite induced cataract. The presence of an additional -OH group in the salicyledene is proposed as the cause of an appreciable increase in antioxidant property. Our findings suggest that the curcumin derivative, salicyledenecurcumin (CD1) is endowed with enhanced antioxidant property and keto-enol structure is not a necessary requirement for deciding antioxidant activity. The order of increase in antioxidant property was curcumin < CD2 < CD1. The higher activity of benzaledene-curcumin (CD2) when compared to curcumin with the same number of phenolic OH may be due to the extended conjugated system.

Chapter 5: Curcumin complexes have been subjected to DNA binding studies using various techniques like UV-Vis, CV and CD. The results showed that with increasing concentration of DNA, the complexes interact with DNA in groove binding mode. Morphological alterations of H9c2 cells after 48h of treatment with curcumin and its metal complexes of [Cu(II), Mn(II), Ni(II) and Co(II)] showed that cells had normal spindle shaped morphology at all (1, 5, 10, 15 and 20 μM) concentrations of various samples. MTT assay showed that curcumin and its metal complexes showed a reduction in cell viability in a dose-dependent manner when compared to untreated cells.

Chapter 6: Curcumin pyrazole has been synthesised and structurally characterised. The antioxidant property of the compound has been studied using DPPH. Pyrazole derivative of curcumin was found to be more active than curcumin. The magnitude of antioxidant activity in the five solvents follow the order 1,4-dioxane < ethyl acetate < acetone < acetonitrile < methanol. Pyrazole derivative were used as ligand for the synthesis and characterisation of metal complexes which has been subjected to DNA binding studies using various techniques like UV-Vis, CV and CD. The binding constant (K_b) value was calculated and the order obtained was Mn(II) < Co(II) < Mg(II) < Cu(II) and the mode of binding was suggested as electrostatic or groove binding.

Scope: Since curcumin pyrazole was found to be more active than parent curcumin, the *in vitro* study in terms of cytotoxicity and cell availability could be checked to establish its advantage over the parent material. ROS quenching ability of curcumin pyrazole and its metal complexes in cell lines could also be monitored so as to confirm its activity.

ABBREVIATIONS

DMC	Demethoxy curcumin
BDMC	Bis-demethoxy curcumin
PPAR- γ	Peroxisome proliferators ativated receptor-γ
Pb	Lead
Cu	Copper
Fe	Iron
Mn	Manganese
Se	Selenium
Zn	Zinc
Mg	Magnesium
Ni	Nickel
Co	Cobalt
iNOS	Nitric oxide synthase inhibitor
DMBA	7,12-dimethylbenz[a]anthracene
H_2O_2	Hydrogen peroxide
TPA	12-O-tetradecanoylphorbol-13-acetate
LPS	Lipopolysachharide
DNA	Deoxyribonucleic acid
CAT	Catalase
SOD	Superoxide dismutase
GSH	Glutathione
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
A	Adenine
T	Thymine
G	Guanine
C	Cytosine
N	Nitrogen
UV	Ultra-violet
CV	Cyclic Voltammetry
CD	Circular Dichroism
LD	Linear Dichroism
AD	Alzheimer disease

NSAID	Nonsteroidal anti-inflammatory drug
DMSO	Dimethylsulphoxide
DPPH	2, 2-Diphenyl-1-pikryl-hydrazyl
FT-IR	Fourier Transform Infrared Spectroscopy
DMEM	Dulbecco's Modified Eagle Medium
DCFH-DA	Dichlorofluoroscin diacetate
DCF	2′,7′ dichlorofluorescein
HAT	Hydrogen atom transfer
SPLET	Sequential Proton Loss Electron Transfer
SET-PT	Sequential Electron Transfer- Proton Transfer
ET	Electron-transfer
ETE	Electron-transfer enthalpy
PA	Proton affinity
BDE	Bond dissociation enthalpy
KSEs	Kinetic solvent effects
HBA	Hydrogen bond accepting
HBD	Hydrogen bond donor
S	Solvent
PA	Proton affinity
ETE	Electron transfer enthalpy
IP	Ionization potential
PDE	Proton dissociation enthalpy
FBS	Fetal bovine Serum
NBT	Nitro blue Tetrazolium chloride
PMS	Phenazine methosulphate
FI	Fluorescence intensity
TCA	Trichloroacetic acid
BSA	Bovine Serum Albumin
SPSS	Social Sciences Software Program
HS	Herring sperm
MTT	3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide
BAS	Bio-Analytical System
DPV	Differential pulse voltammogram

Curriculum Vitae

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Publications

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