Studies on the Interaction of a Curcumin Analogue Dimethoxycurcumin with DNA and its Biochemical Consequences in Cells.

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CERTIFICATE

Certified that this Ph.D. dissertation entitled **"Studies on the Interaction of a Curcumin Analogue Dimethoxycurcumin with DNA and its Biochemical Consequences in Cells"** is a bonafide record of research work done by Mr. **Emmanuel Simon.**, in the Department of Zoology, University of Calicut, under my supervision and guidance. I further certify that no part of this dissertation has been presented elsewhere, for the award of any other degree or diploma.

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DECLARATION

I, **Emmanuel Simon**., do hereby declare that the thesis entitled **"Studies on the Interaction of a Curcumin Analogue Dimethoxycurcumin with DNA and its Biochemical Consequences in Cells"** is an authentic record of the research work carried out by me in the Department of Zoology, University of Calicut, under the guidance of **Dr. M. Gokuldas.** I further declare that no part of this thesis has been submitted previously for any other Degree/Diploma of this or any other university.

Calicut University Campus **Emmanuel Simon**
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Emmanuel Simon

CONTENTS

ABBREVIATIONS

AGE	:	Agarose gel electrophoresis
ADP	:	Adenosine diphosphate
ATP	:	Adenosine triphosphate
BC	:	Before Christ
BDMC	:	Bisdemethoxycurcumin
BLC	:	Bovine liver catalase
BSA	:	Bovine Serum Albumin
CAT	:	Catalase
CD	:	Circular dichroism spectroscopy
CHO	:	Chinese Hamster Ovary
CHOP	:	CCAAT-enhancer-binding protein homologous protein
CON	:	Control
Con A	:	Concanavalin A
CVD	:	Cardiovascular diseases
Ct-DNA	:	Calf thymus deoxyribonucleic acid
Cyt c	:	Cytochrome c
DAPI	:	4′,6-diamidino-2-phenylindole
DAC	:	Diacetylcurcumin
DIMC	:	Dimethoxycurcumin
DMEM	:	Dulbecco's Modified Eagle Medium
DMSO	:	Dimethyl sulfoxide
DMC	:	Demethoxycurcumin
DNA	:	Deoxy ribonucleic acid
DS-DNA	:	Double stranded Deoxy ribonucleic acid
DSBs	:	Double-strand breaks
DTNB	:	5,5-dithiobis2-nitro benzoic acid
EDTA	:	Ethylenediamine tetra acetic acid
ER	:	Endoplasmic reticulum
FBS	:	Fetal Bovine Serum

FRET	:	Fluorescence resonance energy transfer
FTIR	:	Fourier transform infrared
GR	:	Glutathione reductase
GSH	:	Reduced glutathione
GSSG	:	Oxidized glutathione
HEK 293	:	Human embryonic kidney 293 cell line
HPLC	:	High Performance Liquid Chromatography
HRP	:	Horseradish peroxidase
IL	:	Interleukin
IFN-γ	:	Interferon gamma
IOC	:	Isoxazolcurcumin
LSM	:	Lymphocyte separation medium
MCF-7	:	Michigan Cancer Foundation-7 cell line
MDA	:	Malondialdehyde
MS	:	Mass spectroscopy
NFκB	:	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMR	:	Nuclear magnetic resonance
NK	:	Natural killer cells
NMR	:	Nuclear magnetic resonance
PBMC	:	Peripheral blood mononuclear cells
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PMT	:	Photomultiplier tube
PVDF	:	Polyvinylidene fluoride
RHD	:	Rel-homology domain
RONS	:	Reactive oxygen and nitrogen species
ROS	:	Reactive Oxygen Species
RNA	:	Ribonucleic acid
RPMI	:	Roswell Park Memorial Institute
RT-PCR	:	Real time-polymerase chain reaction
SDS	:	Sodium dodecyl sulphate
SGOT	:	Serum glutamic-oxaloacetic transaminase

SGPT	:	Serum glutamic pyruvic transaminase
SSA	:	Sulphosalicylic acid
SEM	:	Scanning electron microscope
TAD	:	Transcription activation domain
TBARS	:	Thiobarbituric acid reactive substances
TBE	:	Tris Borate EDTA
TEMED	:	N, N, N′, N′-Tetra methyl ethylene diamine
TBST	:	Tris-buffered saline tween 20
TNFα	:	Tumor necrosis factor alpha
TRI	:	TRIzol
TSCC	:	Tongue squamous cell carcinoma
UV	:	Ultraviolet

CHAPTER 1
INTRODUCTION

The dietary spice turmeric had wide use in ancient Asian medicine. Susruta's Ayurvedic Compendium, dating back to 250 BC, recommends an ointment containing turmeric to relieve the effects of poisoned food, (Suśruta, Ācārya and Acharya 1998) but of course the active principles involved in it were not known at that time. It is now known that curcumin is the main active principle probably responsible for most of the biological activities of turmeric. In 1815, Vogel and Pelletier reported the isolation of "yellow coloring-matter" from the rhizomes of *Curcuma longa* (turmeric) and named it curcumin. In 1842, Vogel Jr. obtained a pure preparation of curcumin but did not report its chemical formula. In 1910, Milobedzka and Lampe identified the chemical structure of curcumin as diferuloyl-methane, or 1, 6-heptadiene-3, 5 dione-1, 7-bis (4-hydroxy-3-methoxyphenyl) -(1E, 6E) and subsequently in 1913 succeeded in synthesizing the compound. Curcumin is a yellow-orange compound obtained from turmeric, the powdered root of the herb *Curcuma longa*. It is one of three curcuminoids present in turmeric along with two others namely, dimethoxycurcumin and bis-demethoxycurcumin (Ruby *et al.* 1995). It is sold as herbal supplement, cosmetics ingredient, food flavoring and food

coloring agent. It is a tautomeric compound existing in enolic form in organic solvents, and as keto form in water. Turmeric has approximately 1 to 6 % curcuminoids, among which curcumin has been known to exhibit a range of biological activities, the molecular mechanism of which has been shown to have multiple targets inside the cell including various macromolecules in the cell (Zhou *et al*. 2011).

Research and clinical trials carried out over the past couple of years have confirmed that curcumin possesses diverse pharmacological activities such as anti-carcinogenic, anti-diabetic, anti-inflammatory, anti-oxidant and anti-microbial activities. These activities are mediated by the modulation of several important molecular targets, including transcription factors, various enzymes, proteins involved in cell cycle, various cytokines, receptors and cell surface adhesion molecules (Chattopadhyay *et al.* 2004; Strimpakos *et al.*2008). However, one of the major problems with developing curcumin as a drug is its poor bioavailability and poor metabolic stability, which appears to be primarily due to poor absorption, rapid metabolism, and rapid systemic elimination in human body. In order to circumvent these issues a number of methods have been tested in various studies (Hatcher *et al*. 2008).

Recently there has been significant interest in the development of curcumin analogues/congeners that have better

activity than curcumin (Anand *et al*. 2008). Curcumin congeners have been reported to induce apoptosis, sensitize and overcome resistance to chemotherapeutic agents in diverse human cancer cells (Aggarwal *et al*.2006). Dimethoxycurcumin (DIMC), a synthetic analogue of curcumin, has been found to be about three-fold more metabolically stable and expressed better anti-tumor activity against HCT 116 colon cancer cells when compared to curcumin (Tamvakopoulos *et al*. 2007; Pae *et al*. 2008). Recent literature has confirmed that DIMC is a more effective anti-inflammatory and anti-tumour agent than curcumin or its analogues viz., bis-demethoxycurcumin and tetrahydro-curcumin (Aggarwal *et al.* 2009; Aggarwal *et al.* 2007; Sandur *et al.* 2007; Jeong *et al.* 2009; Simon *et al.* 1998). Most of the anticancer chemotherapeutic drugs that are broadly and successfully used today are DNA-binding agents. However, the clinical potential of DNA-damaging agents is limited by the adverse side effects caused by them and increased risk of secondary cancers (Gurova 2009).

DNA binding agents bind to DNA differently based on the site of binding, intercalative mode of binding is where ligand intercalates between base pairs of DNA whereas in groove binding mode ligands bind to the major groove or minor groove of DNA. Groove binders usually bind to the minor groove of DNA. Curcumin is one such important natural polyphenol, which

has been shown to be a minor groove binder of DNA/RNA, monitored by spectroscopic methods based on absorption, circular dichroism and Fourier transform infrared spectroscopy (Zsila *et al*. 2004; Nafisi *et al.* 2009). However, other binding modes have also been proposed for curcumin. In one study it was found that curcumin binds ds-DNA with electrostatic interaction at lower ionic strength and intercalate at high ionic strength (Senthil *et al.* 2009). However, many investigators have reported the minor groove mode of binding for curcumin. Binding of curcumin derivatives like diacetyl curcumin and isoxazole curcumin to DNA has been reported and such interactions are being explored to understand the molecular mechanisms responsible for their overall biological activity (Sahoo *et al*. 2008; Sharma 1976; Bera *et al*. 2008).

Curcumin is a robust antioxidant *in vitro* and *in vivo* as evidenced by many reports that have been published over the years. The antioxidant activity of curcumin has been mainly attributed to its phenolic -OH group (Wright 2002; Sun *et al*. 2002; Priyadarsini *et al*. 2003; Chen *et al*. 2006). If this is true then DIMC would be a poor antioxidant since its lacks the phenolic -OH groups. On the other hand, prooxidant activity of curcumin and DIMC has also been reported (Banerjee *et al*. 2008; Kunwar *et al*. 2008; 2012). A possible role for the beta-diketone moiety was also suggested. However, the phenolic -OH

group is supposed to be of paramount importance according to many reports. (Sugiyama *et al.*1996; Jovanovic *et al.* 1999).

Small molecules that bind to DNA duplexes hold promise for diagnostic and therapeutic applications, but the precise details of how they interact with DNA need to be better understood. Several studies on DNA binding activity of curcumin and some of its analogs had already been reported (Zsila *et al.* 2004; Zaitsev *et al.* 1998; Bera *et al.* 2008; Sahoo *et al.* 2008; Nafisi *et al*. 2009). However, DIMC has not been studied in terms of its interaction with DNA.

In the present work, we had analyzed the binding of DIMC, a more bioactive derivative of curcumin with DNA in different systems employing various spectroscopic techniques like UV-Visible spectroscopy, Fluorescence spectroscopy and Circular dichroism spectroscopy. We employed FRET studies to observe the effect of DIMC on DMSO induced destabilization of double stranded DNA. Nuclear localization of small molecules is a key factor when considering their biological role as a DNA minor groove binder because interaction with DNA could be considered to be of any biological significance only if such molecules can penetrate the nuclear envelope and access DNA. In an earlier work it was shown that curcumin could indeed be localized in nucleus when treated to MCF-7 breast cancer cells

(Kunwar *et al.* 2008). Confocal microscopy was used to monitor the nuclear localization of DIMC in MCF-7 cells.

Oxygen is very essential for survival of living organisms but at the same time it is a highly reactive molecule that damages living organisms by producing reactive oxygen species (Davies 1995). However, reactive oxygen species also have useful cellular functions, such as redox signaling. Antioxidants are compounds that inhibit oxidation. Oxidation is a chemical reaction that can produce free radicals, thereby leading to chain reactions that may damage macromolecules like DNA or proteins in cells of organisms. To balance the oxidative state, plants and animals maintain complex systems of overlapping antioxidants, such as glutathione and various enzymes (e.g., catalase and superoxide dismutase) or the dietary antioxidants vitamin A, vitamin C and vitamin E. Thus, the function of antioxidant systems is not to remove oxidants entirely, but instead to keep them at an optimum level (Rhee 2006).

The reactive oxygen species produced in cells include hydrogen peroxide (H_2O_2), hypochlorous acid (HClO) and free radicals such as the hydroxyl radical (·OH) and the superoxide anion (O^{2-}) (Valko *et al*. 2007). The hydroxyl radical is particularly unstable and very reactive it will react rapidly and non-specifically with most biological molecules. This species is produced from hydrogen peroxide in metal-catalyzed redox

reactions such as the Fenton reaction (Stohs *et al*. 1995). These oxidants can damage cells by starting chemical chain reactions such as lipid peroxidation or by oxidizing DNA, RNA or proteins (Sies 1997). Damage to DNA can cause mutations and possibly cancer, if not reversed by DNA repair mechanisms, while damage to proteins causes enzyme inhibition, denaturation and protein degradation.

The antioxidant activities of curcumin and related compounds have been investigated by a variety of assay systems, by many investigators in both *in vitro* and *in vivo* conditions. The disparity in assay conditions makes exact comparisons rather difficult. However, in one of the early reports on the antioxidant activity of curcumin and its derivatives, Sharma (1976) observed that the phenolic hydroxyl groups are needed for antioxidant activity and that the presence of more than one of these groups, as in the curcumin derivative bis (3,4-dihydroxycinnamoyl)-methane, confers better activity than that of curcumin itself. The mechanistic aspects of antioxidant activity of curcumin have been more recently investigated at length. Studies by Wright (2002), Sun *et al.* (2002), Priyadarsini *et al.* (2003) and Chen *et al.* (2006) seem to suggest that the phenolic OH groups are important in the antioxidant activity. We compared curcumin with its synthetic analogue DIMC in terms of its ability to modulate antioxidant enzymes in peripheral blood mononuclear

cells (PBMC). For this, we compared the effect of curcumin and DIMC on activity of catalase as well as glutathione reductase (GR). We also studied mRNA level expression of these two enzymes. Further, their effect on lipid peroxidation and reduced glutathione (GSH) levels in normal PBMC were analyzed. Apart from this, we also compared DIMC with curcumin in terms of their anti-tumor activity.

Nuclear factor kappa B (NFκB), is a transcription factor that plays key role in the expression of genes involved in immune response, inflammation, metastasis, cell survival and many other important cellular functions. It also regulate the expression of hundreds of genes that are involved in regulating cell growth, cell differentiation, development and apoptosis (Liu *et al*. 2017; Wong *et al.* 2009; Oeckinghaus *et al.* 2009). Reactive Oxygen Species (ROS) are produced in the cell as a result of various cellular reactions. While certain NFκB-regulated genes play a major role in regulating the level of ROS in the cell, ROS have various inhibitory or stimulatory roles in NFκB signaling. The mammalian NFκB proteins consist of five different related family members that bind as homodimers or heterodimers to 10-base pair κB sites. All of these family members have a Rel-homology domain (RHD) that is essential for DNA binding and dimerization (Morgan *et al.* 2011; Brasier 2006). The three Rel members of the family, RelA (also known as p65), RelB, and

cRel, have a C-terminal transcription activation domain (TAD) that serves to positively regulate gene expression. A wide variety of agents can activate NFκB through canonical and noncanonical pathways (Gupta *et al.* 2010; Hoesel *et al.* 2013). Canonical pathway involves various steps including the phosphorylation, ubiquitination, and degradation of the inhibitor of NFκB (IκBα), which leads to the nuclear translocation of the p50-p65 subunits of NFκB followed by p65 phosphorylation, acetylation and methylation, DNA binding and gene transcription.

It has been reported that curcumin inhibits TNFα-activated NFκB signaling (Gonzales *et al.* 2008; Hayden *et al.* 2006; Tak *et al.* 2001). In the present work we compared curcumin and DIMC in terms of their ability to modulate NFκB activation, for this we quantified nuclear as well as cytosolic fractions of NFκB by western blot after treatment with curcumin or DIMC, in addition to this we also performed NFκB reporter assay to supplement the western blot assay.

Binding of any ligand with DNA can be analyzed with computational tools like molecular docking. Molecular docking is one of the most frequently used computational tool in structure-based drug design, due to its ability to predict the binding-conformation of small molecule ligands to the relevant target binding site (Lengauer *et al.*1996; Kitchen *et al.* 2004; Wei *et al.* 2004; Sarma *et al.* 2018; Yang *et al.* 2018; Kalyanaraman 2018).

In the field of molecular modeling, docking is one of the methods that predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex (Mohamad *et al*. 2018; Wang *et al.* 2017; Heitz and Rupp 2018). Knowledge of the preferred orientation in turn may be used to predict the strength of association or binding affinity between two molecules. One can think of molecular docking as a problem of "lock-and-key", in which one wants to find the correct relative orientation of the "key" which will open up the "lock" (where on the surface of the lock is the key hole, which direction to turn the key after it is inserted, etc.) (Ali *et al*. 2017; Damayanti *et al.* 2016; Ghosh *et al.* 2017). Molecular docking is a key tool in structural molecular biology and computer-assisted drug design (Kitchen *et al*. 2004). The goal of ligand-protein docking is to predict the predominant binding mode(s) of a ligand with a protein of known three-dimensional structure. Successful docking methods search high-dimensional spaces effectively and use a scoring function that correctly ranks candidate dockings (Wei et al. 2004). In the present work, AutoDock 4.2.6 developed by the Scripps research institute has been used for the molecular docking studies. This computational tool can be downloaded from The Scripps Research Institute website and is used in the present work for non-commercial research purpose only.

CHAPTER 2
REVIEW OF LITERATURE

2.1. FREE RADICALS

A free radical is any species, which is capable of independent existence and contain one or more unpaired electrons. Unpaired electrons alter the chemical activity of an atom or molecule, usually making it more reactive than the corresponding non-radical. These highly reactive atoms or molecules are produced in the body during normal metabolic functions or introduced from environment. They are also formed when oxygen interact with certain molecules. They are inherently unstable since they contain extra energy (Martínez-Cayuela 1995; Zwart *et al*. 1999; Viña 2019; Imlay *et al.* 2019).

Free radicals such as superoxide, nitric oxide and their adducts peroxy nitrite, hydrogen peroxide, hydroxyl radical as well as alkyl peroxy radicals are involved in several disease conditions. Reactive oxygen species have been implicated in the pathophysiology of several disorders, including ischemia, myocardial infarction, rheumatoid arthritis, neurodegenerative disorders, atherosclerosis, acute hypertension, hemorrhagic shock, diabetes mellitus etc. (Fang *et al.* 2002; Tangvarasittichai *et al.* 2019; Shu *et al*. 2019).

2.2. REACTIVE OXYGEN SPECIES (ROS)

Reactive oxygen species (ROS) is a collective term that includes both oxygen radicals and certain oxidizing agents that are easily converted into radicals (Buonocore *et al.* 2010). ROS are normal byproducts of aerobic metabolism. Several sources of ROS in the cells are proposed, from mitochondrial electron transport chain, microsomal cytochrome P-450 and their electron donating enzyme systems. (Adam-Vizi 2005; Goncalves *et al.* 2014) Generation of ROS through normal cellular metabolism and due to exogenous factors is a constant problem against which cells have developed multiple defense mechanisms. An imbalance between free radical generation and sequestration leads to oxidative stress (Matés 2000; Matés *et al.* 1999). Reactive oxygen species present a paradox in their biological function. On one hand, they prevent disease by assisting the immune system, mediating cell signaling and playing an essential role in apoptosis. On the other hand, they can damage important macromolecules in cells (Dröge 2002). DNA appears to be one of the most important target of ROS in cells (Bhutia *et al.* 2018). It can cause DNA base changes, strand breaks, damage to tumor suppressor genes and enhanced expression of proto-oncogenes and has been shown to induce malignant transformation of cells (Liou *et al.* 2010). Protein damage is a major consequence of excess ROS generation *in vivo*, in addition damage to DNA

polymerase could alter their fidelity. Oxidative protein damage could also affect the activity of DNA repair enzymes (Seifried *et al.* 2007). Another possible mutagenic effect of ROS involves their attack on lipid to initiate lipid peroxidation. Peroxidation can decompose lipids to a range of mutagenic carbonyl products, For example, 4-hydroxynonenal is genotoxic to lymphocytes and hepatocytes (Wells *et al*. 2005).

Reactive oxygen species can also damage mitochondrial DNA. Mitochondrial DNA damage has been suggested to be important in several human diseases and in the aging process. ROS generated from mitochondrial electron transport chain are responsible for such DNA damage (Matés 2000). *In vitro* oxidative DNA damage caused by ROS is influenced by a number of factors, especially the concentration and the specific type of ROS produced (Buonocore *et al.* 2010).

2.3. ANTIOXIDANTS

Antioxidants have gone through a gradual transition from "Miracle Molecules" to "Marvelous Molecules" to "Physiological Molecules". No doubt, they are vital cogs in numerous metabolic reactions and are co-players in redox homeostasis. Further, many antioxidants perform equally or even more importantly, non-antioxidant duties (Kowaltowski *et al.* 2009). Many types of antioxidants with different functions play

their role in the defense network *in vivo*. The free radical scavenging antioxidants are one of the important classes of antioxidants (Niki 2010).

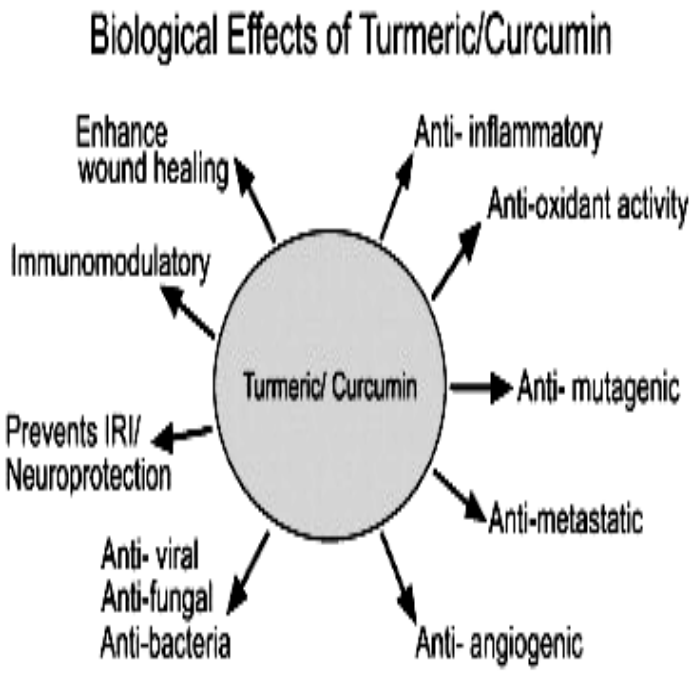
The role played by antioxidants against various disorders and diseases induced by oxidative stress have received much attention over the years. In a healthy cell, ROS are detoxified by antioxidant defenses. Cellular antioxidants may be enzymatic (catalase, superoxide dismutase, glutathione peroxidase) or nonenzymatic (glutathione, thiols, some vitamins and metals, or phytochemicals such as isoflavones, polyphenols, and flavonoids) (Seifried *et al.* 2007). In the normal metabolic state of a cell, a balance exists between the generation of ROS and their quenching by antioxidants (Manju *et al.* 2008). Sometimes the cellular antioxidant defense system is overwhelmed by reactive oxygen and nitrogen species (RONS), this results in oxidative stress and damage to cells. The oxidative stress theory of aging hypothesizes that age-associated functional losses are due to the accumulation of RONS-induced damages (Pérez *et al.* 2009; Buffenstein *et al*. 2008). Oxidative stress is also involved in several age-related conditions (i.e., cardiovascular diseases [CVDs], chronic obstructive pulmonary disease, chronic kidney disease, neurodegenerative diseases, and cancer) (Liguori *et al.* 2018).

2.4. Curcumin

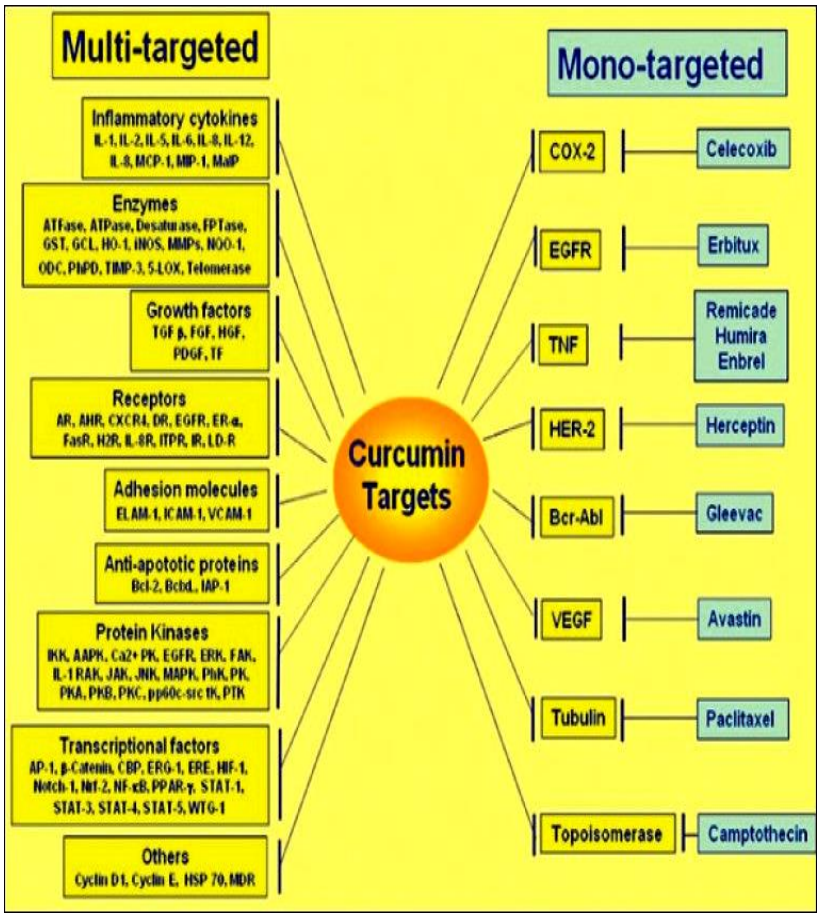
Turmeric has been widely used in Asian culinary culture since time immemorial (Cavaleri 2018). It has approximately 1-6 % curcuminoids, (Ruby *et al*. 1995) among which curcumin has been known to exhibit a range of biological activities as shown in scheme 1, the molecular mechanism of which has been shown to have multiple targets including various critical macromolecules in the cell (Zhou *et al.* 2011) as depicted in scheme 2, which outlines some of the molecular targets of curcumin. Chemically curcumin is (1E,6E)-1,7-Bis(4-hydroxy-3-methoxyphenyl) hepta-1,6-diene-3,5-dione, it is also known as (1E,6E)-1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione or simply as, diferuloylmethane (Priyadarsini 2013).

2.5. Chemistry of Curcumin

Curcumin is the yellow pigmented fraction of curcuma longa, first isolated in 1815, obtained in crystalline form in 1870 and identified as (1,7-bis (4-hydroxy 3-methoxy phenyl)-1,6-heptadiene-3,5-dione) or diferuloylmethane. Curcumin, which exists as enolic and beta-diketone forms, is stable at acidic pH but is unstable at neutral and basic pH and is degraded to ferulic acid and feruloylmethane. Curcumin is less soluble in water but quite soluble in organic solvents such as dimethyl sulfoxide, ethanol, methanol or acetone and has a melting point of 183^{o}C, molecular



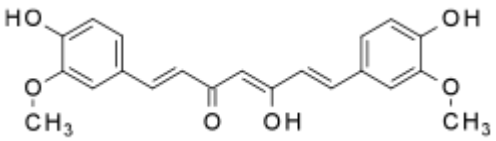
Scheme 1. Biological effects of curcumin (Maheshwari et al. 2006).



Scheme 2. Molecular targets of curcumin

(http://flipper.diff.org/app/items/4490)

formula of $C_{21}H_{20}O_6$ and molecular weight of 363.87 g/mol (Anand *et al*. 2008; Aggarwal *et al.* 2009; Aggarwal *et al*. 2007).



Curcumin

2.6. Biological activities of Curcumin

Various cell culture, animal and clinical studies suggests that curcumin has extensive and diverse biological activities, some of the published work on the biological activities of curcumin has been reviewed here.

Srimal *et al.* (1973) carried out a study on some pharmacological actions of curcumin on rats, mice and cats and found that the compound possesses significant anti-inflammatory activity in acute as well as in chronic models of inflammation. They also found that it was as potent as phenylbutazone in the carrageenan oedema test but only half as potent in chronic tests and it prevents the inflammation induced increase in SGOT and SGPT level.

Kuttan *et al*. (1985) evaluated the anticancer activity of the rhizomes of turmeric in Chinese Hamster Ovary (CHO) cells and *in vivo* in mice using Dalton's lymphoma cells grown as ascites.

They found that turmeric extract inhibited the cell growth in CHO cells at a concentration of 0.4 mg/ml and was cytotoxic to lymphocytes and also inhibited Dalton's lymphoma cells at the same concentration. Cytotoxic effect was found within 30 min at room temperature (30ºC). The active constituent was identified as curcumin which showed cytotoxicity to lymphocytes and Dalton's lymphoma cells at a concentration of 4 µg/ml.

Mahady *et al*. (2002) studied the effect of a methanol extract of turmeric as well as curcumin on 19 strains of *H. pylori*, including 5 cagA+ strains. *H. pylori* is a Group 1 carcinogen and is associated with the development of gastric and colon cancer. They found that both the methanol extract and curcumin inhibited the growth of all strains of *H. pylori in vitro* with a minimum inhibitory concentration range of 6.25-50.0 µg/ml. They proposed that this may be one of the mechanisms by which curcumin exerts its chemo preventative effects against gastric and colon cancer.

Gallardo *et al.* (2016) tried to determine the potential effect of curcumin on EMT, migration and invasion. They evaluated the effect of curcumin (30 µM for 48 h) on expression of EMT-related genes by RT-qPCR. Results showed that curcumin decreased E-cadherin, N-cadherin, β-catenin, Slug, AXL, Twist1, Vimentin and Fibronectin protein expression.

Curcumin also decreased migration and invasive capabilities in comparison to their own controls.

Yen *et al*. (2018) created back wounds in 72 mice and treated them with or without topical curcumin (0.2 mg/ml) in Pluronic F127 gel (20%) daily for 3, 5, 7, 9, and 12 days. They found that topical application of curcumin resulted in fast wound closure with well-formed granulation tissue dominated by collagen deposition and regenerating epithelium. They also found that levels of collagen were significantly higher in curcumin-treated wounds.

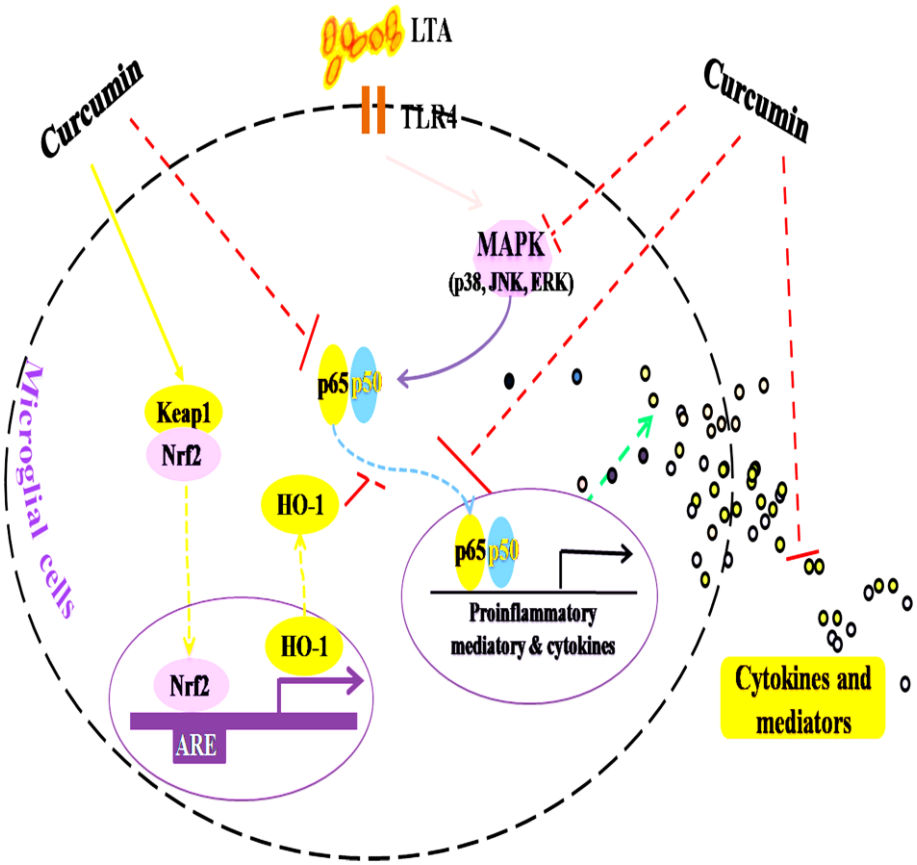
Ardito *et al*. (2018) conducted an *in vitro* study on the effects of curcumin on squamous cell carcinoma of tongue. They found that IC*50* value of curcumin is about 10 μM and there have been inhibitory effects even for treatments at low concentrations. They also found that curcumin reduces migration and progression of TSCC cells and it promotes apoptosis and inhibits tumorigenesis.

Jafarpour *et al*. (2018) conducted a study to investigate the effects of curcumin and trehalose on the level of DNA double-strand breaks (DSBs) caused by ^{131}I in human lymphocytes. They concluded that the use of curcumin and trehalose as antioxidant can reduce the numbers of DSBs caused by ^{131}I and that the radioprotective effect of curcumin was more than trehalose.

Lee *et al.* (2018) studied the effect of curcumin on breast cancer cells by increasing the activity of natural killer (NK) cells. They found that curcumin had an immune stimulatory effect on NK-92 by increasing the surface expression of the CD16+ and CD56dim. They also confirmed that the cytotoxic effect of NK-92 on MDA-MB231 was significantly enhanced in the presence of curcumin, which was associated with the activation of Stat4 and Stat5 proteins in NK-92. In addition to this, they also found that this improved anti-cancer effect of curcumin was correlated with decreased expressions of pErk and PI3K in MDA-MB231.

Yu *et al*. (2018) investigated anti-neuroinflammatory properties of curcumin in LTA-stimulated BV-2 microglial cells. They found that Inflammatory cytokine tumor necrosis factor-α [TNF-α, prostaglandin E2 (PGE2), and Nitric Oxide (NO] secretion in LTA-induced microglial cells were inhibited by curcumin. Their studies also revealed that curcumin inhibited LTA-induced phosphorylation of mitogen-activated protein kinase (MAPK) including ERK, p38, Akt and translocation of NF-κB in microglial cells. The summary of their study is represented in scheme 3.

Machado *et al.* (2019) analyzed the action of curcumin-nanoemulsion, as a photosensitizing agent in photodynamic therapy in an in vitro breast cancer model, MCF-7 cells. They observed high phototoxic effect after activation at 440 nm, decreasing viable tumor cells to <10% after two irradiations and



Scheme 3. Anti-inflammatory Effects of Curcumin in Microglial Cells. (Yu et al. 2018b)

increasing the reactive oxygen species (ROS) production. They also found an increase in the levels of caspase 3/7 activity, indicating that this therapy triggers apoptosis. They concluded that this formulation had great potential in treating breast cancer.

2.7. Curcumin analogs

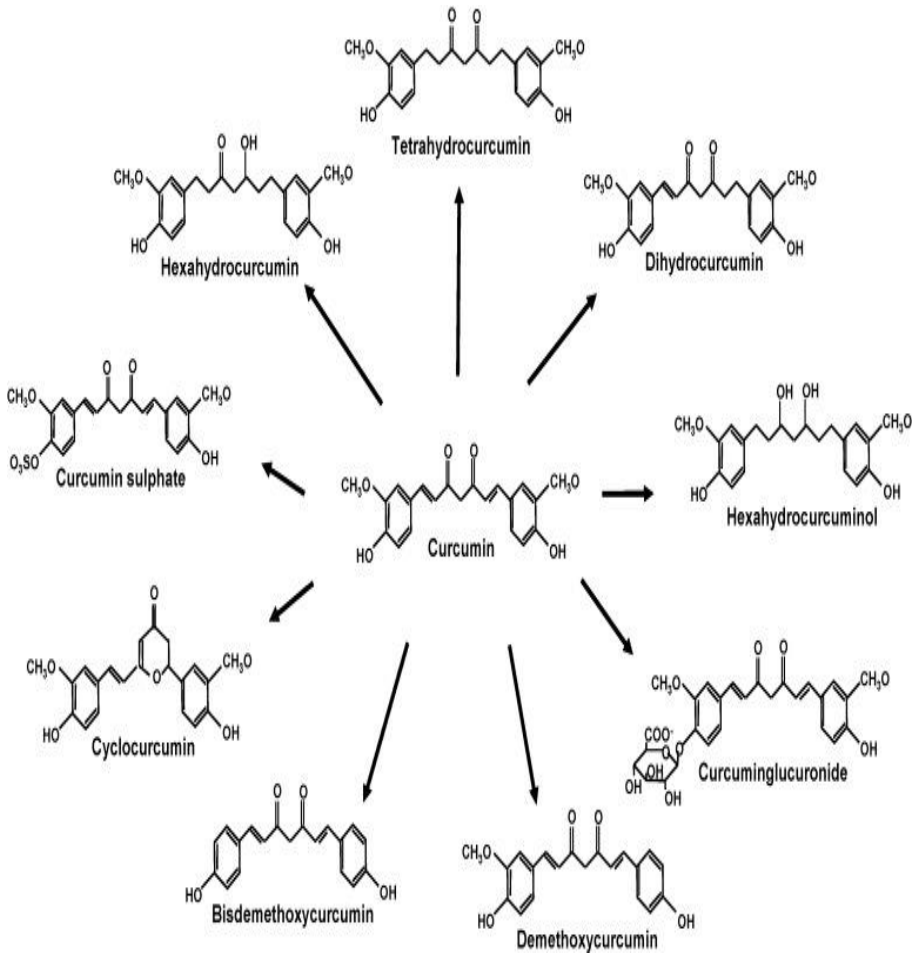
Structurally, there are three sections in the molecular structure of curcumin that have been modified in the course of attempting to produce analogs with better activity. These include the aromatic rings, the beta-diketone moiety, and the two flanking double bonds conjugated to the latter. Successful synthesis of several such analogues has resulted in the development of potential anticancer candidates that target various stages and/or processes in cancer cell growth. Successful anticancer compounds based on curcuminoids structures ordinarily retain the conjugated α,β-unsaturated ketone moieties other than a few exceptions (Vyas *et al*. 2013; Jordan *et al*. 2016; Nagaraju *et al.* 2018). Turmeric contains three main analogues, curcumin, demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC). Collectively called curcuminoids, the three compounds differ in methoxy substitution on the aromatic ring. While curcumin has two symmetric o-methoxy phenols linked through the, β-unsaturated β-diketone moiety, BDMC, also symmetric, is deficient in two o-methoxy substitutions, and DMC has an asymmetric structure

with one of the phenyl rings having o-methoxy substitution (Anand *et al.* 2008).

Curcumin is the most abundant in turmeric, followed by DMC and BDMC. A lesser known curcuminoid from turmeric is cyclocurcumin, first isolated and characterized by Kiuchi *et al*. (1993). Structurally, cyclocurcumin differs from curcumin in the β-diketone moiety. There have been few biological studies on cyclocurcumin. Simon *et al*. (1998) reported that this analogue was ineffective in inhibiting MCF-7 tumor cell proliferation and arrest of cell cycle progression. Curcumin derivatives are generally synthesized by derivatization, starting from curcumin. For example, the phenolic hydroxy group may be acylated, alkylated, glycosylated, or amino acylated. The methoxy groups may be demethylated to hydroxy groups. The reactive methylene group of the linker may be acylated, alkylated or substituted by an arylidene group (ArCH) thereby introducing substituents on the C7 chain (Anand *et al.* 2008). Some of the analogs of curcumin has been represented in scheme 4.

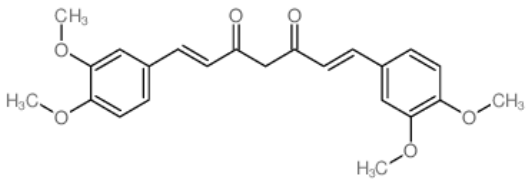
2.8. Dimethoxycurcumin

Dimethoxycurcumin (DIMC) is a synthetic analogue of curcumin obtained by methylation of both free phenolic groups in the parent compound. Studies have shown that DIMC is more potent that curcumin in terms of its cytotoxic activity towards cancer cells.



Scheme 4 Some analogs of curcumin (Anand *et al*. 2008)**.**

The metabolic stability of DIMC is also higher when compared to curcumin (Tamvakopoulos *et al*. 2007; Pae *et al*. 2008).



Dimethoxycurcumin

There have been very few studies on the actions of DIMC over the years. Tamvakopoulos *et al*. (2007) compared the anticancer activity as well as metabolic stability of DIMC over curcumin. They found that DIMC is significantly more potent than curcumin in inhibiting proliferation and inducing apoptosis in HCT116 cells treated for 48 h. Nearly 100% of curcumin was degraded but <30% of DIMC was degraded in cells treated for 48 h, and incubation with liver microsomes confirmed the limited metabolism of dimethoxycurcumin. They also found that though both compounds were rapidly degraded *in vivo* however, dimethoxycurcumin was more stable.

Pae *et al*. (2008) investigated whether DIMC could inhibit NO production and iNOS expression in activated macrophages. Their results suggested that DIMC inhibits NO production, iNOS expression and NFκB activation in LPS-activated macrophages. They concluded that this may be due not only to the conjugated double bonds but also to the increased number of methoxy groups.

Jeong *et al*. (2009) investigated whether DIMC could induce HO-1 expression to the same extent as curcumin in RAW264.7 macrophages. It was found that DIMC and curcumin, induced HO-1 expression and Nrf2 nuclear translocation. They also found that blockage of Nrf2 synthesis by small interfering RNA abolished HO-1 expression by dimethoxycurcumin, indicating that dimethoxycurcumin may induce HO-1 expression via Nrf2 activation. They concluded that dimethoxycurcumin and curcumin had about the same effect on HO-1 expression, suggesting that dimethoxycurcumin retains the HO-1-inducing activity of its parent compound curcumin in RAW264.7 macrophages.

Lee *et al*. (2010) examined whether DIMC, like curcumin, induces apoptosis in Caki cells and also compared the apoptosis-inducing activity of DIMC with that of curcumin. They concluded that DIMC, like curcumin, may induce apoptosis in human renal carcinoma Caki cells through the production of ROS, the release of mitochondrial Cyt c, and the subsequent activation of caspase-3. They also found that DIMC is more potent than curcumin in its ability to induce apoptosis.

Patwardhan *et al.* (2011) investigated whether DIMC could exhibit anti-inflammatory activity in murine and human lymphocytes. They found that both curcumin and DIMC suppressed mitogen as well as antigen driven proliferation of

murine splenic lymphocytes. Further, mitogen and antigen-stimulated cytokine (IL-2, IL-4, IL-6 and IFN-γ) secretion by T cells was also abrogated by curcumin and DIMC. It was also established that curcumin and DIMC suppressed B cell proliferation induced by lipopolysaccharide and that they also inhibited Con A-induced activation of early and late T cell activation markers.

Lu *et al*. (2012) studied the protective effect of DIMC in Mitochondrial dysfunction in human TDP-43 transfected NSC34 cell lines. In this work they showed that dimethoxy curcumin (DIMC) could ameliorate mitochondrial dysfunction in mutated TDP-43 stably transfected cell lines.

Kunwar *et al*. (2012) evaluated the factors responsible for the induction of cell death by DIMC in human breast carcinoma MCF7 cells. They found that DIMC (5-50 μM) caused generation of reactive oxygen species, reduction in glutathione level and induction of DNA damage. Further, the mitochondrial dysfunction induced by DIMC was evidenced by the reduction in mitochondrial membrane potential and decrease in cellular energy status (ATP/ADP) monitored by HPLC analysis. They found that S-phase arrest and apoptosis could be correlated with the changes in the expressions of cell cycle proteins like p53, p21, CDK4, and cyclin-D1 and apoptotic markers like Bax and

Bcl-2. It was thus concluded that DIMC induced cell death in MCF7 cells through S-phase arrest and apoptosis.

Yoon *et al*. (2014) showed that dimethoxycurcumin is significantly more potent than curcumin in inducing cell death and reducing the clonogenicity of malignant breast cancer cells. They found that DIMC induced paraptosis accompanied by excessive dilation of mitochondria and the endoplasmic reticulum (ER); this was similar to curcumin, but a much lower concentration of DIMC was required to induce this process. Their results suggest that DIMC triggers a stronger proteasome inhibition and higher induction of CCAAT-enhancer-binding protein homologous protein (CHOP) compared with curcumin, giving it more potent anticancer effects on malignant breast cancer cells.

Hassan *et al.* (2015) compared the cytotoxicity, metabolism and the epigenetic changes induced by curcumin and DIMC in leukemia cells. They found that curcumin and DIMC were not cytotoxic to leukemia cells and that they induced G2/M cell cycle arrest. DIMC was more metabolically stable than curcumin and can induce epigenetic changes unlike curcumin.

Chen *et al.* (2016) studied the effect of DIMC on two colon cancer cells (HT-29 and SW480) and one normal human colon mucosal epithelial cell (NCM460). They observed that

DIMC significantly inhibited the growth of colon cancer cells by inducing apoptosis in dose-dependent manner both *in vivo* as well as *in vitro* also the expression of survivin was reduced and E-cadherin was enhanced in both cells *in vitro* and *in vivo*.

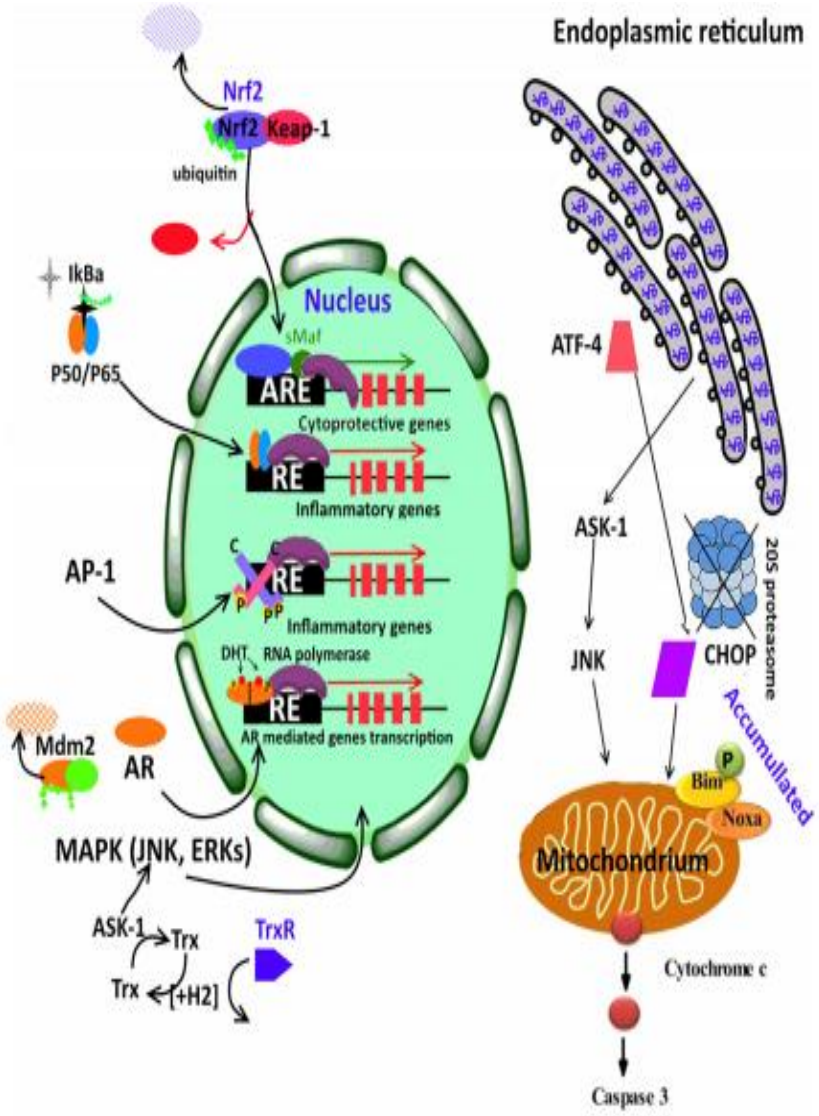
Jayakumar *et al*. (2016) investigated the radiosensitizing effect of DIMC in A549 lung cancer cells. They found that as compared to curcumin, DIMC showed a very potent radiosensitizing effect as seen by clonogenic survival assay. It was also shown that DIMC in combination with radiation significantly increased the apoptosis and mitotic death in A549 cells. Overall, they demonstrated that DIMC can synergistically enhance the cancer cell killing when combined with radiation by targeting thioredoxin system.

Adeyeni *et al*. (2016) studied, effects of curcumin and two analogs (bisdemethoxycurcumin and dimethoxycurcumin) on modulation of B-cell-specific Moloney murine leukemia virus integration site 1 (BMl1) expression in DLD-1 colorectal cancer cells. They found that curcumin significantly reduced BMl1levels more than bisdemethoxycurcumin and dimethoxycurcumin. They also found that while curcumin and bisdemethoxycurcumin inhibited survival of DLD-1 colorectal cancer cells by inducing apoptosis, dimethoxycurcumin did not induce apoptosis though it inhibited survival of DLD-1 colorectal cancer cells.

Hassan *et al.* (2016) looked into cytotoxic effects and epigenetic changes associated with the combination of DIMC and DNMT inhibitor decitabine (DAC) in primary leukemia samples and cell lines. They found that the combination demonstrated antagonistic cytotoxic effects and effect of DAC was not significantly altered when combined with DIMC. However, increased histone acetylation was noted when DAC was co treated with DIMC.

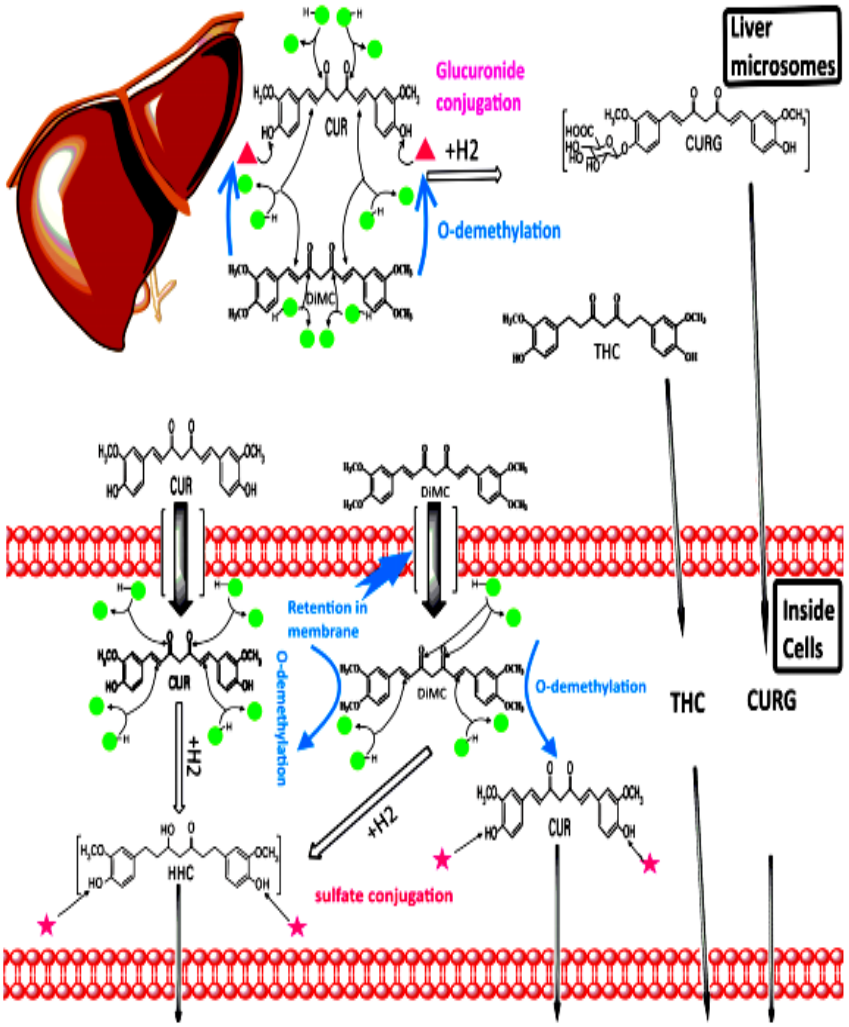
Teymouri *et al.* (2018) reviewed various biological activities of curcumin and DIMC. The cellular and molecular targets of DIMC that has been mentioned in the review is illustrated in scheme 5 and differential hepatic and cellular metabolism of curcumin (Cur) and dimethoxycurcumin (DIMC) leading to different cellular availability in scheme 6. DIMC induces the activity of the transcriptional factors of NF-kB and Nrf2 through inducing degradation of their inhibitory proteins, IkBα and Keap-1, respectively. On the other hand, DIMC hinders the activity of transcriptional factor of AP-1 and induces AR degradation mediated by Mdm2. ASK-1, MAPKs pathway modulation, TrxR inhibition also occurs following DIMC treatment**.**

Zhao *et al.* (2017) studied the synergistic effects of DIMC with 5-fluorouracil in SW480 and SW620 colon cancer cells. They found that DIMC- and/or 5-Fu-induced apoptosis,



Scheme 5. Modulation of various cellular proteins by DIMC:

(Teymouri *et al.* 2018)



Scheme 6. Differential hepatic and cellular metabolism of curcumin (Cur) and dimethoxycurcumin (DIMC): Differential hepatic and cellular metabolism of curcumin (Cur) and dimethoxycurcumin (DIMC) leading to different cellular availability and bearing. (Teymouri *et al*. 2018)**.**

stimulated G0/G1 phase arrest, increased ROS levels, decreased mitochondrial membrane potential, and enhanced endoplasmic reticulum expansion in colon cancer cells.

2.9. Nucleic acid binding activity of curcumin and its analogs

A few studies have been conducted on nucleic acid binding of curcumin and some of its analogs. Most of these studies have been reviewed in this section.

Zsila *et al.* (2004) studied interaction between curcumin and both natural and synthetic DNA duplexes using circular dichroism (CD) and absorption spectroscopy techniques. Upon addition of curcumin to calf thymus DNA and various polynucleotide solutions, they observed an intense positive induced CD band centered around 460-470 nm depending on the actual pH and Na^+ concentration of the medium; there was no CD signal, however, with single stranded poly(dC). They evaluated spectral data and molecular modeling calculations which suggested that curcumin, binds in the minor groove of the double helix.

Sahoo *et al.* (2008) studied the interaction of diacetylcurcumin (DAC) with calf thymus-DNA (ct-DNA) by spectroscopic and viscometric techniques. They found that fluorescence intensity of DAC was quenched by ct-DNA. The

mean binding constant obtained from the spectroscopic techniques was 3.97 ± 0.31 × 105 M^{-1}. The binding mode was analyzed by competitive binding between ethidium bromide (EB) and DAC for ct-DNA and also by viscometric studies. DAC was found to be a minor groove binder with a preference for the AT region compared to the GC region. They substantiated this by displacement studies with Hoechst 33258, a known minor groove binder. Docking studies corroborated with experimental results.

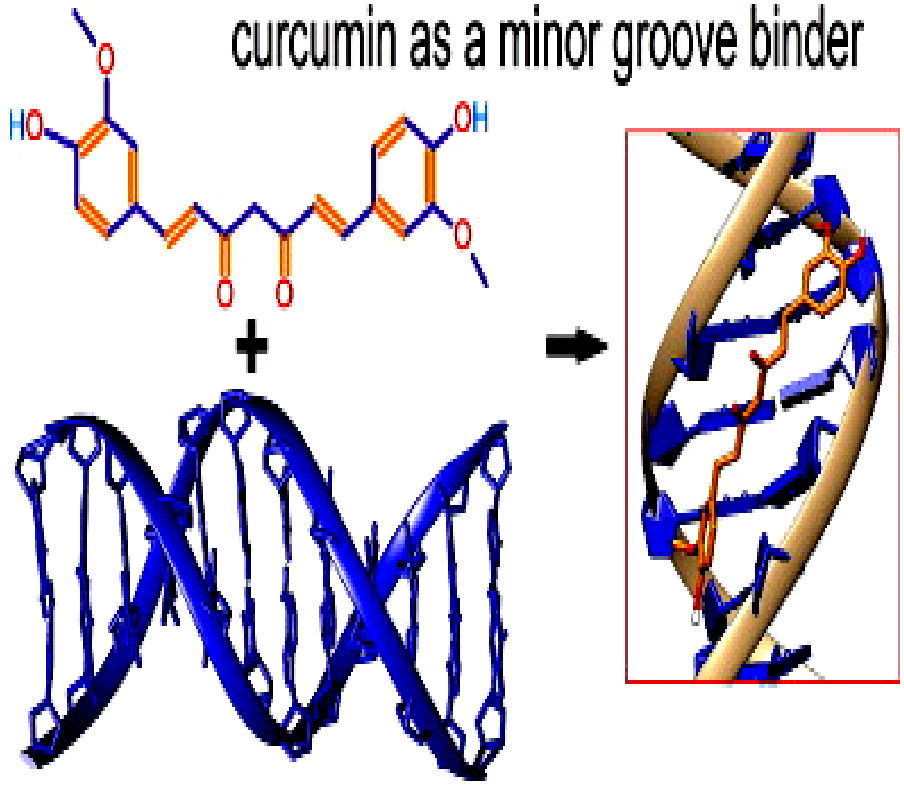
Bera *et al*. (2008) studied the interaction of isoxazolcurcumin (IOC), a synthetic derivative of curcumin, with calf thymus-DNA (ct-DNA) by UV–Vis, fluorescence and circular dichroism spectroscopy, viscosity measurements and docking studies. From the analyses, the binding constant, number of binding sites and mode of binding of IOC to ct-DNA had been determined by them. The binding constant of IOC to DNA was found to be in the 10^4 M^{-1} range. Analyses of fluorescence spectra, viscosity measurements and molecular modeling of IOC–DNA interactions by them indicated that IOC is a minor groove binder and preferentially binds to AT rich regions. Ethidium bromide displacement studies done by them revealed that IOC did not have any effect on ethidium bromide bound DNA which suggests that IOC does not intercalate between base pairs of DNA.

Nafisi *et al*. (2009) examined the interactions of curcumin with calf thymus DNA and yeast RNA in aqueous solution at physiological conditions, employing constant DNA and RNA concentration (6.25 mM) and varying curcumin/polynucleotide (phosphate) ratios of 1/120, 1/80, 1/40, 1/20, and 1/10. Fourier transform infrared (FTIR) and UV-visible spectroscopic methods were used to determine the ligand binding modes, the binding constants and the stability of curcumin-DNA and curcumin-RNA complexes in aqueous solution. They showed that curcumin binds to the major and minor grooves of DNA duplex and to RNA bases as well as to the backbone phosphate group with overall binding constants of K(curcumin-DNA) = 4.255 x 10^4 M^{-1} and K(curcumin-RNA) = 1.262 x 10^4 M^{-1}. Major DNA and RNA aggregation occurred at high pigment concentration. No conformational changes were observed upon curcumin interaction with DNA or RNA.

Wang *et al*. (2010) studied the interaction of curcumin with immobilized double-stranded-DNA (ds-DNA). They used 5 mol L^{-1}Tris-HCl (pH 7.0) solution with different ionic strength. The current and formal potential of curcumin at ds-DNA modified GCE were investigated using electrochemical method. They found that curcumin binds ds-DNA with electrostatic interaction at lower ionic strength and intercalate at high ionic strength.

Li *et al*. (2013) employed fluorescence spectroscopy in combination with UV-Vis absorbance spectroscopy to investigate the binding of curcumin to herring sperm DNA (hs-DNA). The mode of binding of curcumin to DNA duplex was reported to be principally intercalative. They reported binding affinity for curcumin with hs-DNA of the order 10^4 L mol $^{-1}$.

Haris *et al*. (2017) claims contrasting findings on the interaction of curcumin with DNA. They therefore conducted a study to understand the mechanism of recognition of curcumin dissolved in DMSO by DNA. Their investigation confirmed that curcumin solubilized in DMSO binds in the minor groove of the ct-DNA without causing significant structural alteration to the DNA. The mode of binding of curcumin with DNA according to Harris *et al*. has been illustrated in scheme 7.



Scheme 7. Minor groove binding by curcumin (Haris et al. 2017).

2.10. Objectives of the present project

2.10.1. To investigate the binding of DIMC with DNA and derive the binding constant.

2.10.2. To establish the site of binding and the nature of interaction.

2.10.3. To study the modulation of antioxidant enzymes by DIMC in comparison with curcumin in normal cells.

2.10.4. To investigate modulation of NF-κB pathway by DIMC in comparison with curcumin in normal cells.

2.10.5. To investigate whether DIMC localizes in the nucleus of cancer cells.

2.10.6. To study the anti-tumor activity of DIMC in comparison with curcumin in cancer cell lines.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials:

3.1.1. Chemicals & reagents:

1. Acrylamide
2. Agarose
3. Ammonium persulphate
4. Anti-Mouse IgG
5. Anti-NFκB antibody
6. Anti-rabbit IgG
7. Anti β- Actin antibody
8. Antibiotic/Antimycotic solution
9. ATP sodium salt
10. BSA (Bovine Serum Albumin)
11. Calcium chloride
12. Calf thymus-DNA (ct-DNA)

13. cDNA synthesis kit
14. Cell Proliferation Kit II (XTT)
15. Curcumin
16. D luciferin
17. 4′,6-diamidino-2-phenylindole
18. Dimethyl sulfoxide
19. Dimethoxy curcumin
20. Dithiothreitol EGTA
21. Ethidium bromide
22. Fetal Bovine Serum
23. Glycylglycine
24. Heparin sodium salt
25. Histopaque HiSep LSM 1077
26. Octylphenoxypolyethoxyethanol (IGEPAL)
27. Potassium chloride
28. Dipotassium hydrogen phosphate (K_2HPO_4)
29. Lipofectamine LTX reagent

30. Magnesium sulfate

31. N′ methylene bis-acrylamide

32. PBR 322 plasmid DNA

33. Proteinase inhibitor cocktail

34. RPMI-1640 media

35. Sodium dodecyl sulphate (SDS)

36. Sodium chloride

37. Syber Green Real time PCR master mix

38. N, N, N′, N′-Tetra methyl ethylene diamine (TEMED)

39. TRI Reagent

40. Triton X-100

41. Trizma

42. Trypan Blue

43. Tween-20

Calf thymus-DNA (ct-DNA) and all other reagents (analytical grade) were procured from SRL, India. Curcumin was purchased from Sigma Chemical Co. (St. Louis, MO, USA) through local suppliers. Oligonucleotides (55mers) for FRET

studies were Purchased from Metabion, Germany. Histopaque HiSep LSM 1077, RPMI-1640 media, FBS (Fetal Bovine Serum), BSA (Bovine Serum Albumin), Antibiotic Antimycotic solution and Molecular biology grade water were obtained from Himedia, Mumbai, India. Proteinase inhibitor cocktail, IGEPAL, Anti β- Actin antibody, Anti Mouse IgG, Anti rabbit IgG, Anti NFκB antibody, Tween-20, Acrylamide, Trypan Blue, SDS (Sodium dodecyl sulphate), N′ methylene bis-acrylamide, ammonium persulphate, Trizma, TEMED (N, N, N′, N′-Tetra methyl ethylene diamine) and DTT were procured from Sigma–Aldrich Co, (St. Loius, MS). TRI Reagent, cDNA synthesis kit and Syber Green Real time PCR master mix were obtained from Origin Diagnostics, Kerala, India. Triton X-100, Heparin sodium salt, Sodium chloride, Calcium chloride, Glycylglycine, MgSO4, EGTA, K2HPO4 and ATP sodium salt were obtained from SRL Laboratories, Mumbai, India. Lipofectamine LTX reagent and D luciferin were obtained from Invitrogen, Carlsbad, CA, USA. The Cell Proliferation Kit II (XTT) was purchased from Sigma–Aldrich Co. (St. Loius, MS).

All other chemicals were purchased from local suppliers. All the chemicals and reagents were of analytical grade or Molecular biology grade. Nanopure water from a Milli Q system (Millipore, France) was used throughout.

3.2. Methods:

3.2.1. Preparation of buffers, reagents and test solutions

3.2.1.1. Phosphate buffer saline

A 10 L stock of 10x PBS was prepared by dissolving

800 g NaCl, 20 g KCl, 144 g Na2HPO4 · 2H2O and 24 g KH2PO4 in 8 L of distilled water. After complete mixing, made up final solution to 10 L. Whenever required stock was diluted to 1x and used. The pH of 1x PBS was 7.4.

3.2.1.2. TBE buffer

A concentrated 1liter (5x) stock solution of TBE was prepared by weighing 54 g Tris base (FW = 121.14) and 27.5 g boric acid (FW = 61.83) and dissolving both in approximately 900 milliliters of deionized water then added 20 milliliters of 0.5 M EDTA (pH 8.0) and adjusted the solution to a final volume of 1 L. This buffer solution was diluted as per requirement before use.

3.2.1.3. Preparation of curcumin stock solution

Curcumin stock solution (100 mM) was prepared by dissolving 37.4 mg curcumin in 1 ml DMSO. Solution was diluted as per requirement with PBS (10mM) before each

experiment. A concentration of 5 µM was fixed for curcumin for assays involving cells since at higher concentration it may induce loss of cell viability (Banerjee et al. 2008)

3.2.1.4. Preparation of DIMC stock solution

DIMC stock solution was prepared by dissolving 49.6 mg DIMC in 1 ml DMSO. Solution was diluted as per requirement with PBS (10mM) before each experiment. A concentration of 5 µM was fixed for DIMC for assays involving cells since same concentration was used for curcumin.

3.2.2. Synthesis and evaluation of purity of samples

3.2.2.1. Evaluation of purity of ct-DNA

The purity of ct- DNA was checked by measuring the ratio of UV absorbance of DNA solution in PBS at 260 and 280 nm, which was found to be 1.9 indicating that it was sufficiently free from protein. The concentration of DNA was measured directly using a nanodrop spectrophotometer before each experiment.

3.2.2.2. Synthesis and evaluation of purity of DIMC

DIMC was synthesized according to previously reported method (Priyadarsini et al. 2003) and characterized by melting point (mp) analysis, ^{1}H NMR, ^{13}C NMR spectroscopy and MS,

after which the results were compared with literature values. The sample was found to be sufficiently pure of impurities.

3.2.3. Molecular docking of curcumin and DIMC with DNA:

Principle:

Docking is a method which predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex. The associations between biologically relevant molecules such as proteins, peptides, nucleic acids, carbohydrates and lipids play a central role in signal transduction. Furthermore, the relative orientation of the two interacting partners may affect the type of signal produced (e.g., agonism vs antagonism). Therefore, docking is useful for predicting both the strength and type of signal produced.

Method:

The coordinates for B-DNA used for docking were extracted from the Protein Data Bank with identifier 453D. The structure of DIMC generated by HyperChem Professional Release 8 (Hypercube Inc., FL, USA) was energy minimized with MMFF94 force field using MMFF94 charges. The macromolecule file was prepared for docking by adding polar hydrogen atoms and Gasteiger charges. No rotatable bonds were assigned and docking carried out with the AutoDock 4.2.6

Lamarckian Genetic Algorithm (GA). DNA was enclosed in a grid having 0.375 Å spacing in all directions. Other miscellaneous parameters were assigned the default values given by AutoDock. The output from AutoDock was rendered with PyMol and processed with UCSF chimera.

3.2.4. UV-Vis spectrophotometry:

Reagents:

Calf thymus DNA

DIMC (Stock and standard)

DMSO

PBS

Principle:

Spectrophotometric analysis of nucleic acids is based on the principles that nucleic acids absorb ultraviolet light at particular wavelength. In the case of DNA, changes in absorption maxima around 260 nm when in contact with small molecules indicates some sort of interaction between the two.

Procedure:

Ct-DNA solution was prepared in PBS and mixed with DIMC solution so that the concentration of ct-DNA was 40 µM

and that of DIMC varied from 5 to 30 µM in different Eppendorf tubes. The total reaction mixture was fixed at 1.0 ml. After homogenization in a sonicator absorption spectra were recorded on a Jasco Spectrophotometer V-650 UV–Vis spectrophotometer at room temperature, with a slit width of 2 nm and scan speed of 400 nm min^{-1}.

Derivation of binding constant from UV-Vis spectroscopy:

Assuming that DIMC does bind to DNA, the whole dynamic process can be represented as follows:

$$n\,(DIMC) + \text{ct-DNA} \rightleftharpoons \text{Complex} \qquad \text{Equation (1)}$$

The equilibrium constant for the above equilibrium is given by the equation:

$$K = \frac{[complex]}{[DNA][DIMC]^n} \qquad \text{Equation (2)}$$

For the above equilibrium, assuming 1:1 complex formation, the double reciprocal plot, also expressed as the Benesi-Hildebrand equation is written as follows:

$$\frac{1}{\Delta A} = \frac{1}{K\Delta\varepsilon_{260}[ct-DNA]}\left(\frac{1}{[Dimc]}\right) + \frac{1}{\Delta\varepsilon_{260}[ct-DNA} \qquad \text{Equation (3)}$$

Here, **Δ** A is the difference in the absorbance at 260 nm of a solution containing a mixture of ct-DNA and varying

concentrations of DIMC and the sum of the individual absorbance of ct-DNA and DIMC. The **Δ**ε260 is the difference in the molar extinction coefficient at 260 nm. For these studies, the concentration of ct-DNA was fixed at 40 µM and that of DIMC varied from 5–30 µM.

3.2.5. Circular dichroism studies:

Reagents:

Calf thymus DNA

DIMC (Stock and standard)

DMSO

PBS

Principle:

The phenomenon of circular dichroism is very sensitive to the secondary structure of polypeptides and proteins. Circular dichroism (CD) spectroscopy is a form of light absorption spectroscopy that measures the difference in absorbance of right- and left-circularly polarized light (rather than the commonly used absorbance of isotropic light) by a substance.

Procedure:

Circular dichroism spectra were recorded on a Jasco-810 automatic recording spectropolarimeter at 25 °C. DIMC (3.33 μM-25 μM) were added onto ct-DNA solution (32 μM) in 10 mM phosphate buffer saline pH 7.4 and spectra were recorded from 200–350 nm in a 1 cm cell at scan rate of 100 nm/min with a response time of 1 sec. Each spectrum was accumulated twice and the obtained results expressed as millidegrees (mdeg). A nitrogen atmosphere was maintained throughout the experiment.

3.2.6. Fluorescence spectrophotometry:

Reagents:

Calf thymus DNA

DIMC (Stock and standard)

DMSO

PBS

Principle:

The emission maxima of DIMC is around 550 nm when excited at 420 nm, on interaction with macromolecules like proteins or DNA there may be changes in the emission properties.

Procedure:

All fluorescence measurements were carried out at room temperature in a Hitachi F-4500 fluorescence spectrophotometer. The bandwidth of both excitation and emission slits were set at 5 nm. Fluorescence emission was recorded keeping the concentration of DIMC (5 μM) constant and varying the concentration of ct-DNA in PBS (2 μM-20 μM) taken in different Eppendorf tubes and the total reaction volume was 1 ml. Excitation wavelength was set at 420 nm.

3.2.7. Ethidium bromide (Etbr) competitive binding assay:

Reagents:

Calf thymus DNA

DIMC (Stock and standard)

DMSO

PBS

Ethidium bromide

Principle:

Ethidium bromide intercalates between base pair of DNA and its fluorescence emission is increased several fold. When a compound that competes with ethidium bromide for DNA

binding site is added to a solution containing DNA and Ethidium bromide, the enhanced fluorescence intensity of ethidium bromide decreases.

Procedure:

Ethidium bromide (Etbr) competitive binding assay was performed by incubating ct-DNA (9.3 μM) with Etbr (9.8 μM) followed by addition of increasing concentrations of DIMC (0.5 μM - 10 μM) in phosphate buffer (pH 7.4) at room temperature, after excitation at 480 nm fluorescence spectra was recorded in a Hitachi F-4500 fluorescence spectrophotometer.

3.2.8. DAPI displacement assay:

Reagents:

Calf thymus DNA

DIMC (Stock and standard)

DMSO

4′,6-diamidino-2-phenylindole (DAPI)

Principle:

DAPI is a well-known DNA double helix minor groove binder. Fluorescence of DAPI increases several fold on binding to the minor groove of DNA. When a compound that binds to

minor groove of DNA is added to a mixture containing DNA and DAPI, enhanced fluorescence of DAPI will decrease.

Procedure:

DAPI displacement assay was done by incubating DNA (20 μM) in phosphate buffer (pH 7.4) with Dapi (15 μM) followed by addition of increasing concentrations of DIMC (5 μM-20 μM) after which the fluorescence spectra were recorded in a Hitachi F-4500 fluorescence spectrophotometer at room temperature after excitation at 338 nm.

3.2.9. Fluorescence resonance energy transfer (FRET) assay

Reagents:

Calf thymus DNA

DIMC (Stock and standard)

DMSO

PBS

Phi-C

Phi-W

Principle:

Fluorescence resonance energy transfer (FRET), the nonradiative transfer of excited state energy from one fluorophore to another. Fluorescence resonance energy transfer is a distance-dependent physical process, by which energy is transferred nonradiatively from an excited molecular fluorophore (the donor) to another fluorophore (the acceptor) by means of intermolecular long-range dipole–dipole coupling. FRET can be an accurate measurement of molecular proximity at angstrom distances (10–100 Å) and highly efficient if the donor and acceptor are positioned within the Forster radius (the distance at which half the excitation energy of the donor is transferred to the acceptor, typically 3–6 nm).

Procedure:

FRET studies were done with the complementary oligonucleotides (55 mers) synthesized by Metabion, Germany with the following sequences.

Phi-C:
5’CGATACGCTCAAAGTCAAAATAATCAGCGTGACATTC AGAAGG GTAATAAGAACG-3’

Phi-W:

5’-CGTTCTTATTACCCTTCTGAATGTCACGCTGATTAT TTTGACTTTGAGCGTATCG-3’

Phi-C was labelled with rhodamine B at 3’ end, and Phi-W was labelled with fluorescein at 5’ end. The respective absorption and fluorescence maxima of fluorescein are at 490 and 522 nm, while those of rhodamine B are at 540 and 590 nm. Double-helical DNA was prepared from renaturation of Phi-W (27.5 μM) and Phi-C (27.5 μM) by mixing single stranded oligonucleotides in phosphate buffer (pH 7.4). FRET studies in this double stranded DNA were performed after incubating with different concentrations of DMSO (2.8–7 μM) or with DMSO (2.8–7 μM) containing DIMC (20–50 μM) at room temperature for 5 min in a total reaction volume of 100 μl in phosphate buffer (pH 7.4). The change in the emission intensity due to fluorescein as a result of FRET was measured at 522 nm after excitation at 490 nm. Since the absorption of DIMC at 490 nm is very low, the interference from its emission to FRET was negligible. The assays were carried out in real time, where reproducibly similar trends were observed in duplicate sets performed together as a set on a given day.

3.2.10. Agarose gel electrophoresis (AGE):

Reagents:

PBR322 Plasmid DNA

DIMC (Stock and standard)

DMSO

TBE buffer

Agarose

Principle:

Agarose gel electrophoresis is a method of gel electrophoresis used to separate a mixed population of macromolecules such as DNA or proteins in a matrix of agarose, one of the two main components of agar. The proteins may be separated by charge and/or size and the DNA and RNA fragments by length. Biomolecules are separated by applying an electric field to move the charged molecules through an agarose matrix, and the biomolecules are separated by size in the agarose gel matrix

Procedure:

AGE was carried out in a Tarson midi submarine gel electrophoresis apparatus. PBR322 Plasmid DNA was treated with different concentrations of DIMC (5 µM-75 µM) incubated for 15 min and resolved on 0.8% agarose gel. Gel was run at 80V for 2 h. 1X TBE buffer was used for this experiment. The gel was then visualized in a Bio-Rad gel documentation system.

3.2.11. Blood collection:

Blood was obtained from young, healthy, non-smoking volunteers and was collected aseptically in the presence of EDTA. Blood collection was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and written consent were obtained from all the donors.

3.2.12. PBMC isolation:

Reagents:

HiSep™ LSM 1084

RPMI-1640 cell culture medium

Fetal bovine serum

L-Glutamine

Penicillin

Streptomycin

Trypan blue solution

Principle:

Isolation of PBMC is based on the adapted method of isolating mononuclear cells using centrifugation techniques by Bøyum in which defibrinated blood is layered on a solution of

sodium diatrizoate and polysucrose and centrifuged at low speeds for 30 min. Differential migration following centrifugation results in the formation of several cell layers. Mononuclear cells (lymphocytes and monocytes) are contained in the banded plasma-LSM interphase due to their density, and the pellet that is formed contains mostly erythrocytes and granulocytes, which have migrated through the gradient to the bottom of the tube.

Procedure:

Isolation of PBMC from blood was done as per the instructions given in the manual provided with HiSep™ LSM 1084. Isolated PBMC were suspended in RPMI-1640 medium with 10% sterile fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin-streptomycin solution, and checked for cell viability by trypan blue exclusion. PBMC were adjusted to 1×10^6 cells/ml and used for further experiments.

3.2.12.1. Treatment of PBMC with test compounds

After isolation PBMC were treated with curcumin or DIMC (5 µM), and incubated for 6 h in a CO2 incubator (New Brunswick Galaxy 170 S, Eppendorf) after which cells were homogenized and experiments carried out as mentioned in each assay unless otherwise specified.

3.2.13. PBMC viability by Trypan blue exclusion:

Reagents:

Trypan blue (0.4% solution)

Principle:

Trypan blue exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue.

Procedure:

To the isolated PBMCs, equal volume of trypan blue suspension (0.4% solution) was added and kept at 37 °C for 7 min in a CO_2 incubator supplied with 5% CO_2. The cells were then counted in a hemocytometer, observed under light microscope. Dead cells take up the dye and appear blue whereas the live cells exclude the dye and appear colorless.

3.2.14. Cell culture:

Reagents:

HEK293 cells

MCF7 cells

DMEM medium

FCS 10%

Penicillin 100 IU/ml

Streptomycin 100 µg/ml

DIMC (Stock and standard)

Curcumin (Stock and standard)

DMSO

Procedure:

MCF7 cell line and HEK293 cells (obtained from NCCS, Pune, India) were grown in DMEM medium supplemented with 10% FCS, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Monolayer cultures in tissue culture flasks were incubated in 95% air, 5% CO_2 atmosphere at 37°C. The culture medium was changed every 48 h. Stock solution of DIMC or curcumin was prepared in DMSO and added to the culture medium at the desired concentrations. DMSO concentration did not exceed 0.1% of the total reaction volume during each experiment.

3.2.15. Estimation of protein:

Reagents

A. Na_2CO_3 2% in 0.1 N NaOH

B. NaK Tartrate 1% in H_2O

C. $CuSO_4.5\ H_2O$ 0.5% in H_2O

D. Reagent I: 48 ml of A, 1 ml of B, 1 ml C

E. Reagent II- 1 part Folin-Phenol [2 N]: 1 part water

BSA Standard - 1 mg/ ml

DIMC (Stock and standard)

Curcumin (Stock and standard)

DMSO

Principle:

The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the FolinCiocalteau phosphomolybdic phosphotungstic acid to heteropoly molybdenum blue by the copper-catalyzed oxidation of aromatic acids.

Procedure:

After treatment with curcumin (5 µM) or DIMC (5 µM) and 6 h incubation, PBMC were sonicated and 20 µl of the sample, BSA standard (10-200 µg) and buffer (blank) were taken in respective tubes and final volume made up to 0.5 ml with

distilled water. Added 4.5 ml of Reagent I and incubated for 10 min at room temperature. After incubation added 0.5 ml of Reagent II and mixed thoroughly and incubated for 30 min at room temperature. Measured the absorbance at 660 nm and a calibration curve was plotted. Protein content of the sample was calculated from the calibration curve and expressed as mg protein/ml (Lowry *et al*. 1951).

3.2.16. Assessment of lipid peroxidation:

Reagents

Trichloro acetic acid (TCA)

Thiobarbituric acid (TBA)

DIMC (Stock and standard)

Curcumin (Stock and standard)

DMSO

Principle:

Since malondialdehyde is a degradation product of peroxidised lipids, the development of pink colour with the absorption characteristics (Absorption maximum at 532 nm) as a TBA-MDA chromophore has been taken as an index of lipid peroxidation.

Procedure:

Thiobarbituric acid reactive substances (TBARS) in PBMC was measured according to the method described previously (Ohkawa *et al.* 1979) with slight modifications. After treatment with curcumin (5 µM) or DIMC (5 µM) and 6 h incubation, compounds and incubation, PBMC were sonicated and mixed with 10% ice cold TCA. Samples were centrifuged at 3000 rpm for 15 min, collected the supernatant, added 1% thiobarbituric acid (TBA) then boiled at 100 °C for 15 min. Samples were cooled and the absorbance were measured at 532 nm. The amount of malondialdehyde (MDA) formed was calculated on the basis of molar extinction coefficient of MDA-TBA chromophore (1.56×10^5 $m^{-1}cm^{-1}$) and the results were expressed as n moles of TBARS/mg of protein.

3.2.17. Assay of catalase (CAT):

Reagents

Hydrogen peroxide

PBS

DIMC (Stock and standard)

Curcumin (Stock and standard)

DMSO

Principle:

The enzyme catalyzes the decomposition of hydrogen peroxide, which is measured by decrease in absorbance at 240 nm.

$$2H_2O_2 \longrightarrow H_2O_2 + O_2$$

Procedure:

Catalase activity was measured according to the method described previously (Aebi 1984) after treatment with curcumin (5 µM) or DIMC (5 µM) and incubation for 6 h. Cells were sonicated and 40 µl each of PBMC cytosol from the different groups were added to 1 ml of H_2O_2 (30 mM) and 1.960 ml of 50 mM phosphate buffer pH 7.4. The rapid decomposition of H_2O_2 was determined for every 30 sec from the decrease in absorbance at 240 nm for 3 min. The results are expressed as Units/mg of protein (1 Unit=mM H_2O_2 decomposed/min/mg protein).

3.2.18. Assay of glutathione reductase (GR):

Reagents

Glutathione disulfide

PBS

NADPH

DIMC (Stock and standard)

Curcumin (Stock and standard)

DMSO

Principle:

Glutathione reductase is a flavoprotein that catalyzes the NADPH dependent reduction of glutathione disulfide to GSH.

$$NADPH + H^{+} + GSSG \longrightarrow NADP^{+} + 2GSH$$

Procedure:

Activity of the GR was determined as described by (Bellomo *et al*. 1987). The reaction mixture consisted of 0.1 M phosphate buffer (pH 7.0), 5 mM glutathione disulfide (GSSG), and 40 µl of PBMC cytosol obtained after sonication of cells treated with curcumin (5 µM) or DIMC (5 µM) and incubated for 6 h, in a final volume of 1.0 ml. The reaction was started by adding 0.1 mM NADPH and the consumption of NADPH was monitored spectrophotometrically at 340 nm. The results were expressed as Units/mg of protein (1 Unit=m moles of NAPDH oxidized /min/mg of protein).

3.2.19. Analysis of reduced glutathione (GSH):

Reagents

GSH

5-Sulphosalicylic acid

5,5-Dithiobis2-nitro benzoic acid (DTNB)

Phosphate buffer

NADPH

DIMC (Stock and standard)

Curcumin (Stock and standard)

DMSO

Principle:

In this method, 5,5-dithiobis2-nitro benzoic acid (DTNB) is reduced by -SH groups to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH.

The nitromercaptobenzoic acid anion released has an intense yellow colour and can be used to measure -SH groups at 405 nm.

Procedure:

GSH assay was performed according to the method described previously (Baker *et al.* 1990) with a minor modification. PBMC were incubated for 6 h after treatment with curcumin (5 µM) or DIMC (5 µM), various concentrations of GSH were prepared by serially diluting a 200 µM standard with 0.5 % 5-sulphosalicylic acid (SSA) to get 0, 0.09, 0.19, 0.375, 0.75, 1.5, 3, and 6 µM solutions. Samples or standards (50 µl) plus 100 µl reaction mixture made by mixing 5 ml of 5-5'-dithiobis [2-nitrobenzoic acid] (DTNB) (1 mM), 5 ml of NADPH (1 mM), 5.75 ml of $NaPO_4$ buffer (100 mM) and 0.1 ml of GSH reductase (200 U/ml in buffer) were added to each well of a 96 well flat microplate. The plate was kept at room temperature in the dark for 10 min. DTNB reduction by GSH was monitored by optical density measurements at 405 nm. Standard curves were constructed and GSH contents were determined (expressed in $\mu M/10^6$ cells).

3.2.20. Real-time PCR (qPCR) analysis:

Reagents

TRI reagent

RT buffer

DTT

RNase inhibitor

Reverse transcriptase

SYBR Green Real-Time PCR Master Mix.

DIMC (Stock and standard)

Curcumin (Stock and standard)

DMSO

Principle:

Quantitative reverse transcription PCR (RT-qPCR) is used when the starting material is RNA. In this method, RNA is first transcribed into complementary DNA (cDNA) by reverse transcriptase from total RNA or messenger RNA (mRNA). The cDNA is then used as the template for the qPCR reaction. RT-qPCR is used in a variety of applications including gene expression analysis.

Procedure:

Isolated PBMCs were treated with curcumin (5 µM) or DIMC (5 µM) at a concentration of 5 µM and total RNA was isolated using TRI reagent and quantified by Nanodrop spectrophotometer (Thermo scientific). cDNA was prepared from 1 µg of total RNA using cDNA synthesis kit containing random primers, 5 X RT buffer, DTT, RNase Inhibitor and Reverse

transcriptase in a 20 µl reaction system. The obtained cDNA was diluted to a ratio of 1:10 and subjected to real-time PCR in Roche light cycler 480 using SYBR Green chemistry. The nucleotide sequence of primer pairs used to determine the levels of expressions of catalase, glutathione reductase and GAPDH are as follows:

- Catalase sense primer 5'-CCATCGCAGTTCGGTTCT-3'and antisense primer 5'-GGGTCCCGAACTGTGTC-3'.
- Glutathione reductase sense primer 5'-ATGATCAGCACCAACTGCAC-3' and antisense primer 5'-CGACAAAGTCTTTTTAACCTCCTT-3'
- GAPDH sense primer 5'- AGCCACATCGCTCAGACA C-3' and antisense primer 5'- GCCCAATACGACCAA ATCC-3'

The internal reference used was GAPDH and proper non-target negative controls were maintained for each gene. The relative expression of all the genes were analyzed and quantified. Melt curve analysis was carried out to analyze the specificity of the primers used.

3.2.21. Anti-cancer activity of DIMC and curcumin:

Reagents:

MCF7 cells

DMEM medium

FCS 10%

Penicillin 100 IU/ml

Streptomycin 100 µg/ml

XTT reagent

DIMC (Stock and standard)

Curcumin (Stock and standard)

DMSO

Principle:

XTT is a colorless or slightly yellow compound that when reduced becomes brightly orange. XTT is reduced to a soluble, brightly colored orange derivative by a mix of cellular effectors.

Procedure:

Cell viability assay was done on MCF7 cells using the Cell Proliferation Kit II (XTT). MCF7 cells were seeded in

microplates (tissue culture grade, 96 wells, flat bottom) 4000 cells in a final volume of 100 µl culture medium per well, in a humidified atmosphere (37 °C, 5% CO_2). DIMC or curcumin were added to the appropriate wells at concentrations varying from 5 – 25 µM followed by incubation for 24 h. After the incubation period, added to each well, 50 µl of the XTT labeling mixture, prepared as described in the manual for the kit (final XTT concentration 0.3 mg/ml). Incubated the microplate for 4 h in a humidified atmosphere (37 °C, 5% CO_2). Readings were taken in a multiwell plate reader at 450 as well as 690 nm.

3.2.22. Western blot analysis for NFκB:

Reagents

Nuclear-cytoplasmic fractionation buffer

NaCl 150 mM

NP-40 1.0%

Tris-HCl 50 mM, pH 8.0

Sodium deoxycholate 0.5%

SDS (sodium dodecyl sulphate) 0.1%

Protease inhibitors

Tris-HCl 20 mM

SDS 4%

2-mercaptoethanol 10%

Glycerol 20%

Bromophenol blue 0.004%

Tris-HCl 0.125 M

Tris base 25 mM

Glycine190 mM

Tris base 25 mM

Methanol 20%

Tris 48 mM

Glycine 39 mM

SDS 0.04%

Milk or BSA (bovine serum albumin) 3–5%

DIMC (Stock and standard)

Curcumin (Stock and standard)

DMSO

Principle:

Western blotting uses specific antibodies to identify proteins that have been separated based on size by gel electrophoresis. The immunoassay uses a membrane made of nitrocellulose or PVDF (polyvinylidene fluoride). The gel is placed next to the membrane and application of an electrical current induces the proteins to migrate from the gel to the membrane. The membrane can then be further processed with antibodies specific for the target of interest, and visualized using secondary antibodies and detection reagents.

Procedure:

After treatment with curcumin (5 µM) or DIMC (5 µM) and incubation for 24 hours, PBMCs were collected and lysed in nuclear-cytoplasmic fractionation buffer containing 0.1% NP40. Cytoplasmic fraction was collected after centrifugation and the pellet was subjected to further lysis by sonication and collected as nuclear fraction after centrifugation at 10,0000 x g for 10 min. The collected fractions were mixed with 6x SDS PAGE loading dye and heated at 90°C for 10 min. The denatured proteins were separated on 10% SDS PAGE and transferred on to PVDF membrane by wet transfer method. The membrane was then blocked with 5% BSA in TBST and incubated with primary antibody overnight. After overnight incubation, the membranes

were washed with TBST and incubated in HRP conjugated secondary antibody for 1 h. The blots were then developed in Biorad VersaDoc system using chemiluminescent reagent. The probes for cross contamination of the nuclear or cytoplasmic fractions were also carried out using anti-histone H3 as nuclear marker and hexokinase as the cytoplasmic marker.

3.2.23. NFκB reporter assay:

Reagents:

HEK293 cells

DMEM medium

FCS 10%

Penicillin 100 IU/ml

Streptomycin 100 µg/ml

Lipofectamine LTX reagent

Triton X100

Glycyl–glycine

$MgSO_4$

EGTA

DTT

K_2HPO_4

1X luciferin

DIMC (Stock and standard)

Curcumin (Stock and standard)

DMSO

Principle:

The NFκB Reporter kit is designed for monitoring the activity of the NFκB signaling pathway in the cultured cells. The kit contains transfection-ready NFκB luciferase reporter vector. This reporter contains a firefly luciferase gene under the control of multimerized NFκB responsive element located upstream of a minimal promoter. The NFκB reporter is premixed with constitutively-expressing *Renilla* (sea pansy) luciferase vector, which serves as an internal control for transfection efficiency. The kit also includes a non-inducible firefly luciferase vector premixed with constitutively-expressing *Renilla* luciferase vector as a negative control. The non-inducible luciferase vector contains a firefly luciferase gene under the control of a minimal promoter, without any additional response elements. The negative control is critical to determining pathway specific effects and background luciferase activity.

Procedure:

Human Embryonic Kidney 293 cell cultures (70 % confluent) were transfected with NFκB Reporter plasmid using Lipofectamine LTX reagent. After transfection, the cells were treated with curcumin or DIMC at a concentration of 5 µM. 24 h post treatment, the cells were lysed in 200 µl lysis buffer containing triton X100, glycyl–glycine, $MgSO_4$, EGTA and DTT. The cell lysates were then mixed with 150 µl of assay buffer containing glycyl–glycine, K2HPO4, MgSO4, EGTA, DTT and ATP. To this mix, 100 µl of 1X luciferin was added and the luminescence was measured in a Perkin Elmer multimode plate reader. The luminescence units were normalized to the total cell protein.

3.2.24. Confocal microscopy:

Reagents:

MCF7 cells

DMEM medium

FCS 10%

Penicillin 100 IU/ml

Streptomycin 100 µg/ml

DIMC (Stock and standard)

Curcumin (Stock and standard)

DMSO

DAPI

Principle:

Confocal microscope uses point illumination and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus signal – the name "confocal" stems from this configuration. As only light produced by fluorescence very close to the focal plane can be detected, the image's optical resolution, particularly in the sample depth direction, is much better than that of wide-field microscopes. However, as much of the light from sample fluorescence is blocked at the pinhole, this increased resolution is at the cost of decreased signal intensity – so long exposures are often required. To offset this drop in signal after the pinhole, the light intensity is detected by a sensitive detector, usually a photomultiplier tube (PMT) or avalanche photodiode, transforming the light signal into an electrical one that is recorded by a computer.

Procedure:

Confocal microscopy was performed to study the localization of DIMC in the nucleus of MCF7 cells. Cells were incubated with or without DIMC (20 µM) in DMEM medium

containing 10% FBS. At the end of 4 h, the cells were centrifuged onto cover slips, fixed with paraformaldehyde, stained with DAPI (5 µg/ml) for nuclear staining and mounted onto glass slides. Slides were examined using a LSM510 scanning module (Carl Zeiss Microscopy, Jena GmbH, Germany) with a Krypton–Argon laser, coupled to an Orthoplan Zeiss photomicroscope using a 488 nm laser line and a 510 nm band pass filter. Overlay images were recorded by superimposing simultaneous images from each channel.

3.3. Instruments and Equipment:

1. Biorad VersaDoc system.
2. Carbon dioxide incubator (New Brunswick Galaxy 170 S, Eppendorf).
3. Deep freezer, -20 ^{o}C (Labline, India).
4. Desktop centrifuge (Remi Equipments).
5. Desktop computer with core i3-6006U processor and 8gb ram.
6. Hitachi F-4500 fluorescence spectrophotometer.
7. Jasco Spectrophotometer V-650.
8. Jasco-810 automatic recording spectropolarimeter.

9. LSM510 scanning module (Carl Zeiss Microscopy, Jena GmbH, Germany) with a Krypton–Argon laser, coupled to an Orthoplan Zeiss photomicroscope.

10. Nanodrop 8000 spectrophotometer (Thermo Scientific).

11. Perkin Elmer multimode plate reader.

12. Roche light cycler 480.

13. Tarson midi submarine gel electrophoresis apparatus.

3.4. Software:

1. AutoDock 4.2.6.

2. Discovery Studio 3.5 client (Accelrys).

3. Origin 8 software (Origin Lab, Northampton, Massachusetts, USA).

4. Pymol-1.1 (for educational use).

5. Python 2.5.2, MGL-Tools 1.5.4 (The Scripps Research Institute).

6. UCSF Chimera 1.11.2.

7. HyperChem Professional Release 8.

3.5. Statistical analysis:

Results were expressed as mean ± standard deviation. The intergroup variation was measured by one-way analysis of variance (ANOVA) and Turkey's HSD post hoc test and Duncan's post hoc analysis. The intergroup variation was accepted as statistically significant at $p<0.05$. Turkey's HSD post hoc test and Duncan's post hoc analysis revealed that the different groups (control group, curcumin treated group and DIMC treated group) differed significantly when compared to each other. The statistical analysis was done using Origin 8 software (Origin Lab, Northampton, Massachusetts, USA).

CHAPTER 4

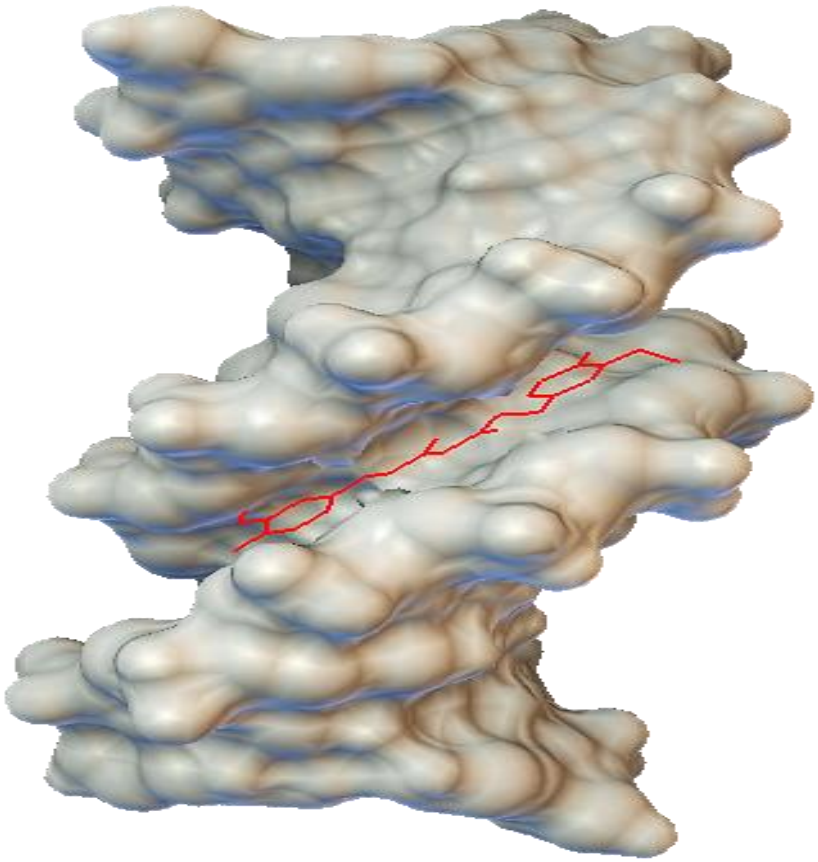
RESULT

Our first objective was to investigate the binding of DIMC with DNA and to derive the binding constant if indeed DIMC bound to DNA. Before commencement of wet lab experiments, we decided to investigate the interaction of DIMC with DNA using *in silico* methods, for this we used molecular docking method using AutoDock 4.2.6.

4.1. Molecular docking of curcumin and DIMC with DNA:

The coordinates for B-DNA used for docking were extracted from the Protein Data Bank with identifier 453D. The structure of DIMC generated by HyperChem Professional Release 8 (Hypercube Inc., FL, USA) was energy minimized with MMFF94 force field using MMFF94 charges. Docking was carried out as mentioned in section 3.2.3. Figures 1 and 5 are the docked structures of curcumin and DIMC with DNA respectively. The space filling model of DNA is shown here and red colored ring structure of curcumin in figure 1 and green colored ring structure of DIMC in figure 5 are distinctly visible.

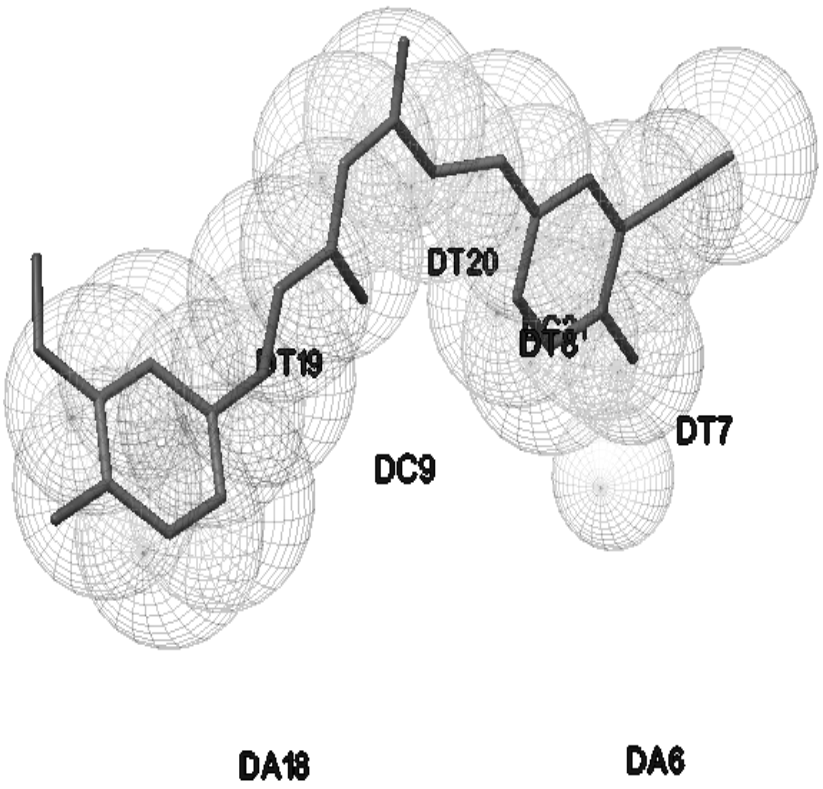
Figure 1: Docked structure of curcumin with DNA



- The macromolecule file was prepared for docking by adding polar hydrogen atoms and Gasteiger charges. No rotatable bonds were assigned and docking carried out with the AutoDock 4.2.6 Lamarckian Genetic Algorithm (GA). DNA was enclosed in a grid having 0.375 Å spacing in all directions. Other miscellaneous parameters were assigned the default values given by AutoDock.

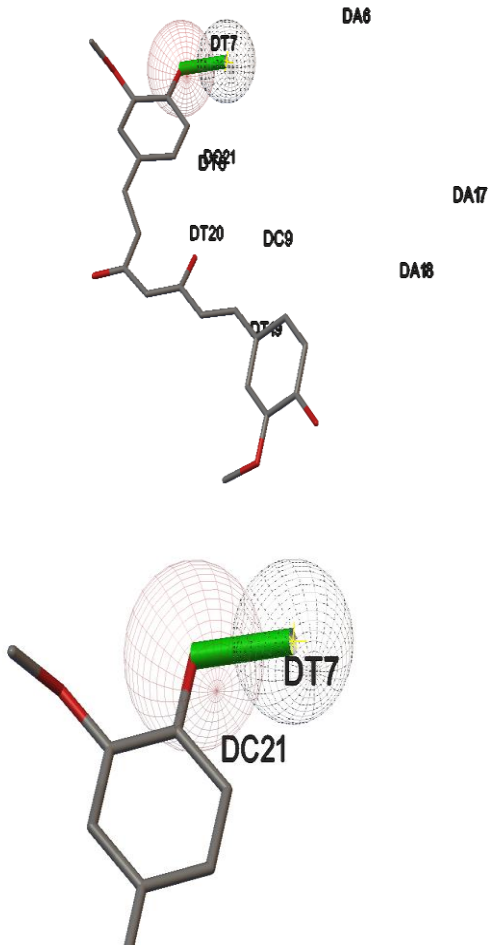
Figure 2 represents the interaction of curcumin with DNA, here DA, DG, DC and DT represents deoxy adenine, deoxy guanine, deoxy cytosine and deoxy thymine respectively. These are the nucleotides that were seen to directly interact with curcumin molecule. Interactions of curcumin atoms with nearby DNA atoms are shown as spheres. Figure 3 represents hydrogen bonding between deoxy thymine (DT7) of DNA with one of the phenolic hydroxyl group of curcumin. Interactions are shown as spheres and the length of hydrogen bond was depicted as green cylinder. Figure 4 represents the binding energy distribution of the different docked conformations. Figure 6 represents the interaction of DIMC with nearby atoms of DNA, interactions are represented as spheres. However, no hydrogen bonding was observed in the case of DIMC as with curcumin. The notations used are same as that described in the case of curcumin in figure 2. Figure 7 represents binding energy distribution of ranked conformations of binding of DIMC with DNA. It could be seen that the distribution of binding energies was different from that observed in curcumin. Ten ranked docked conformations were obtained in all after docking, rank one was expected to be the most preferred conformation. Docking studies revealed that both curcumin and DIMC binds to the minor groove of DNA with different affinities

Figure 2: Interaction of curcumin with DNA



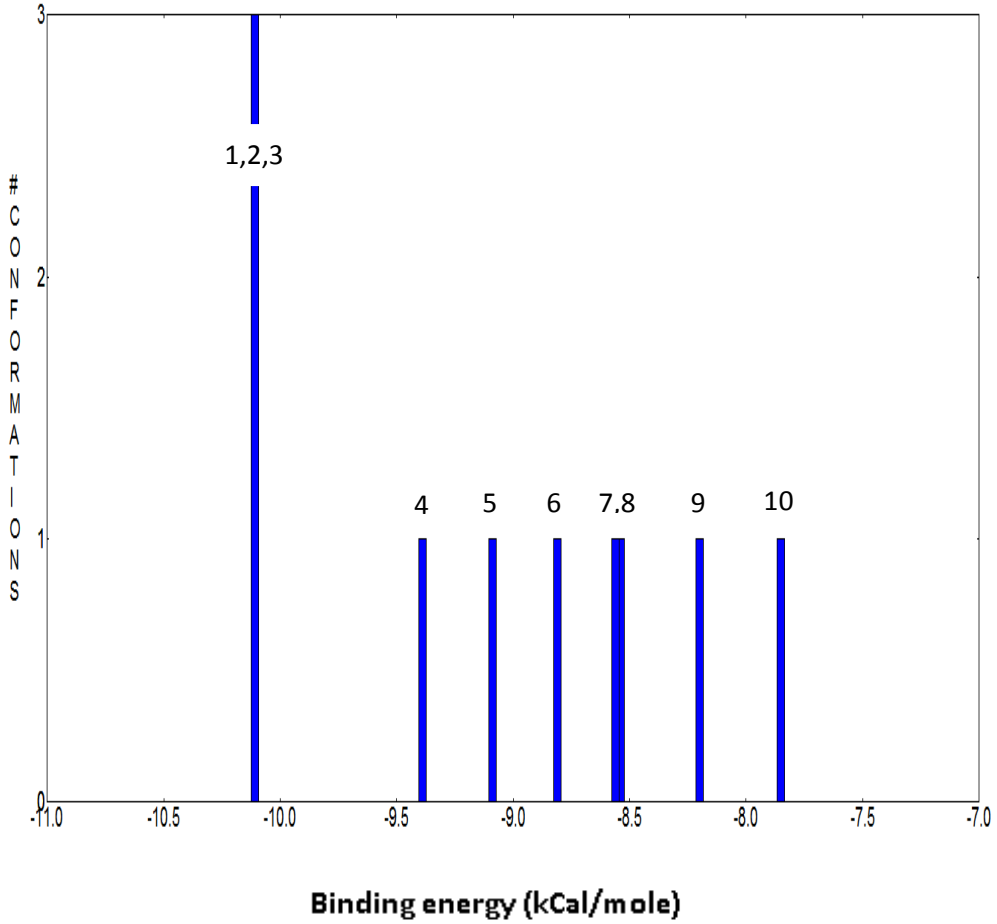
- DA, DG, DC and DT represents deoxy adenine, deoxy guanine, deoxy cytosine and deoxy thymine respectively, the numbers specify position of each nucleotide in the DNA molecule that was used for docking. The nucleotides shown here were seen to directly interact with curcumin molecule. Interactions of curcumin atoms with nearby DNA atoms are shown as spheres.

Figure 3: Hydrogen bonding involved in the interaction of curcumin with DNA



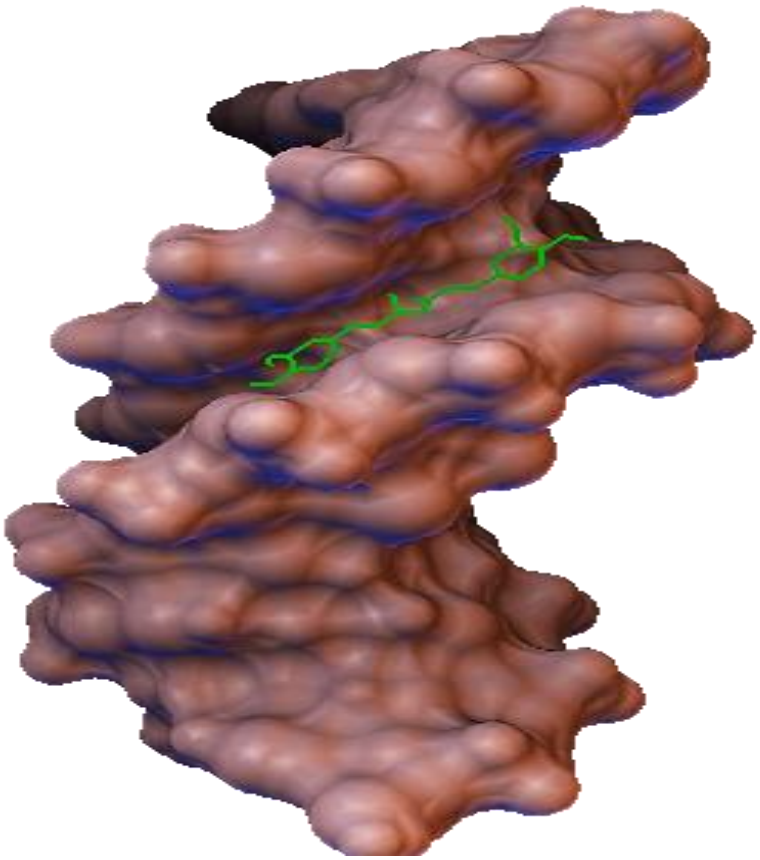
- Hydrogen bonding between deoxy thymine (DT7) of DNA with one of the phenolic hydroxyl group of curcumin is shown here. Interactions are shown as spheres and the length of hydrogen bond is depicted as green cylinder.

Figure 4: Binding energy distribution of Ranked conformations of binding of curcumin with DNA



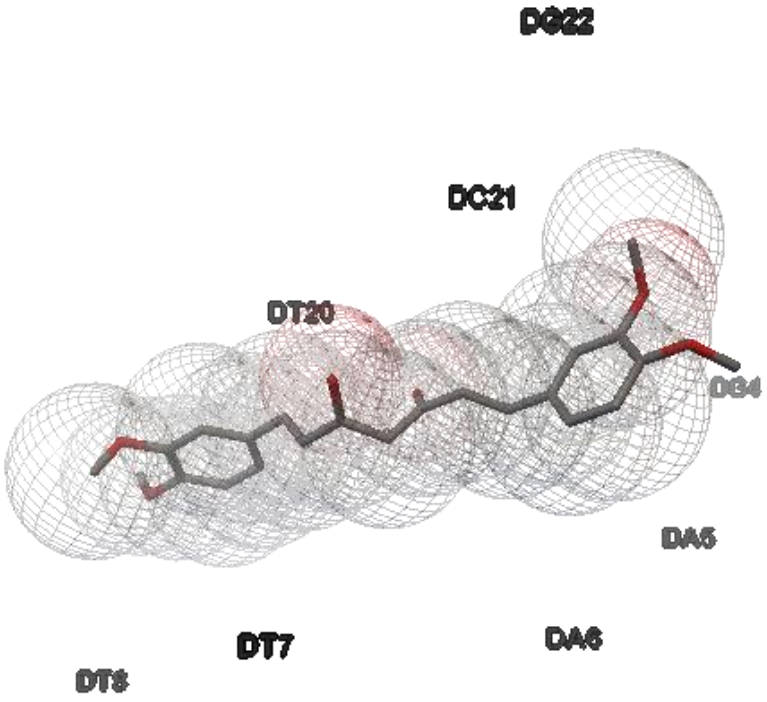
- It can be noted that out of the ten conformations obtained, three confirmations showed binding energy close to -10 kCal/mole and two conformations showed binding energy near -8.5 kCal/mole.

Figure 5: Docked structure of DIMC with DNA



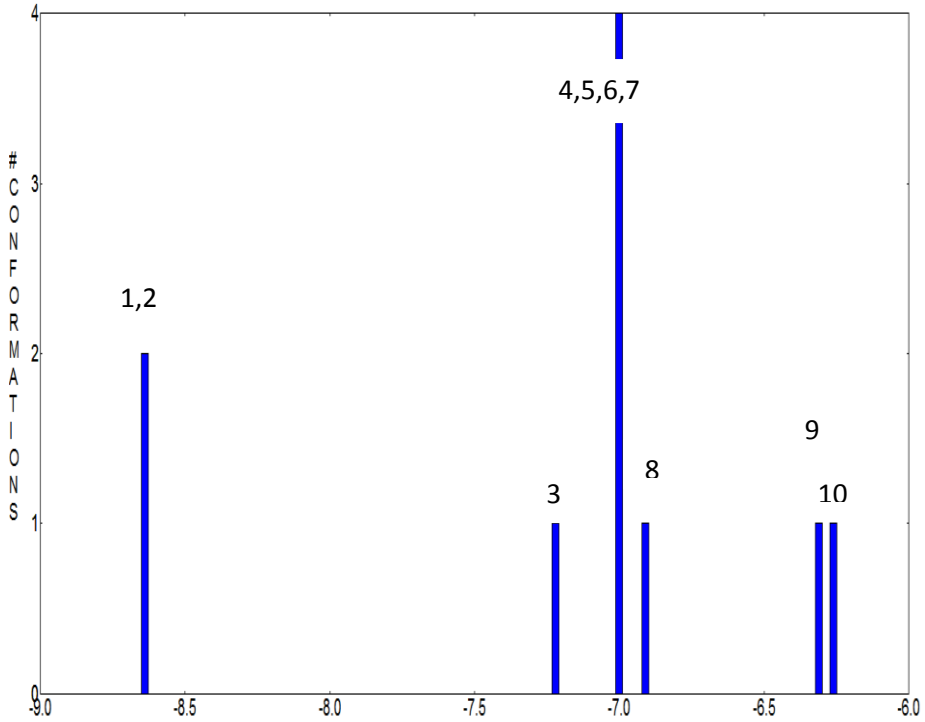
- The macromolecule file was prepared for docking by adding polar hydrogen atoms and Gasteiger charges. No rotatable bonds were assigned and docking carried out with the AutoDock 4.2.6 Lamarckian Genetic Algorithm (GA). DNA was enclosed in a grid having 0.375 Å spacing in all directions. Other miscellaneous parameters were assigned the default values given by AutoDock.

Figure 6: Interaction of DIMC with DNA



- DA, DG, DC and DT represents deoxy adenine, deoxy guanine, deoxy cytosine and deoxy thymine respectively, the numbers specify position of each nucleotide in the DNA molecule that was used for docking. The nucleotides shown here were seen to directly interact with DIMC molecule. Interactions of DIMC atoms with nearby DNA atoms are shown as spheres.

Figure 7: Binding energy distribution of Ranked conformations of binding of DIMC with DNA



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- It can be noted that out of the ten conformations obtained, four conformations showed binding energy close to -7 kCal/mole and two conformations showed binding energy near -8.4 kCal/mole.

and while curcumin forms hydrogen bond with DNA, DIMC does not form any such hydrogen bond.

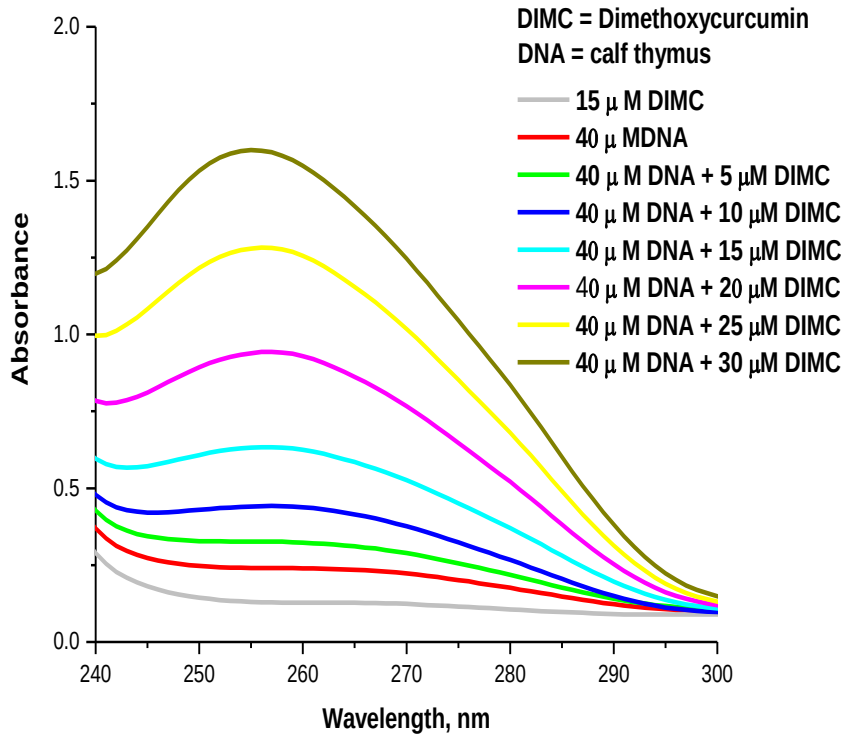
We needed to establish binding of DIMC to DNA employing wet lab experiments. To accomplish this, we performed three different experiments namely UV-Vis spectroscopy, CD spectroscopy and Fluorescence spectroscopy. Each of these spectroscopic techniques had the potential to independently establish the interaction of DIMC with DNA. Binding of curcumin to DNA had been studied exhaustively over the years, which has been reviewed in section 2.9. therefore, we concentrated on studying binding of DIMC with DNA.

4.2. Determination of binding of DIMC to ct-DNA and estimation of binding constant by UV-Visible spectroscopy:

Dimethoxycurcumin has absorption maxima around 418 nm. As ct-DNA does not have any absorbance above 300 nm it is convenient to monitor the absorbance of DIMC at its absorption maxima with addition of ct-DNA. Similarly, DIMC has negligible absorption at 280 nm therefore absorption of ct-DNA can be measured at 280 nm with addition of DIMC. Figures 8 shows the absorption spectra of 40 µM ct-DNA, in the absence and presence of DIMC (5 - 30 µM) in aqueous- DMSO (9:1) solutions at pH 7.4. The changes at 260 nm were prominent, an

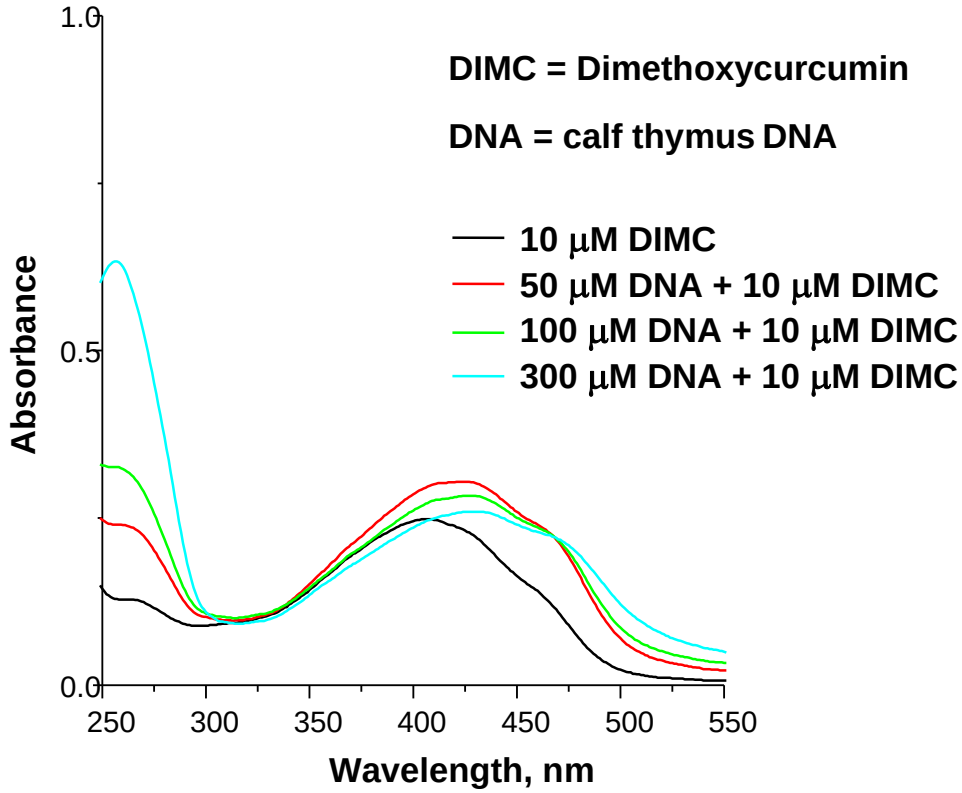
enhancement in absorption (hyperchromicity) at 260 nm and a slight blue shit of ~ 5 to 10 nm was observed. Figure 9 shows the absorption spectra of 10 µM DIMC, in the absence and presence of ct-DNA (50 - 300 µM) in aqueous- DMSO (9:1) solutions at pH 7.4. The binding of small molecules to DNA produces hypochromism, a broadening of band, and a red shift of the complex absorption band. Hypochomism followed by a red shift was observed when increasing amounts of DNA (50 µM-300 µM) were added to DIMC solution (10 µM) as shown in figure 9. In the case of groove binders, a large wavelength shift is usually correlated with a conformational change on binding or complex formation. Wavelength shift of ~20 nm was observed as shown in figure 9. Absorption maxima shifted from ~410 nm to ~430 nm. These results suggest that DIMC is a minor groove binder further, the isobestic point observed at ~475 nm confirms complex formation while reflecting homogeneity of the system. The difference in the absorbance at 260 nm (DA) obtained by subtracting the sum of the individual absorbance at 260 nm due to DIMC and ct-DNA from that of the mixture of ct-DNA and DIMC was used for quantitative estimation of the binding constant, K, for the equilibrium given in equation (1) in section 3.2.4. The ratio of the intercept and the slope of the plot of $1/\Delta A$ versus $1/[DIMC]$ according to equation (3) gave a K value of 5.5 $\pm$ 0.8 x 10^4 M^{-1}.

Figure 8: UV-VIS Absorption spectra of ct-DNA with DIMC



Steady state absorption spectra of different concentrations of Dimethoxycurcumin (5-30 μM) with calf thymus DNA (40 μM) in aqueous- DMSO (9:1) solutions at pH 7.4. DNA (40 μM) solution was prepared in PBS in Eppendorf tubes and varying amounts of DIMC (5-30 μM) working solution was added. Final volume was made up to 1 ml with PBS.

Figure 9: UV-VIS Absorption spectra of DIMC with ct-DNA



- Steady state absorption spectra of different concentrations of calf thymus DNA (50 μM–300 μM) with Dimethoxycurcumin (10 μM) in aqueous- DMSO (9:1) solutions at pH 7.4. Varying concentrations of DNA (50 μM–300 μM) solution was prepared in PBS in different Eppendorf tubes and added DIMC (10 μM) to all the tubes. Final volume was made up to 1 ml with PBS.

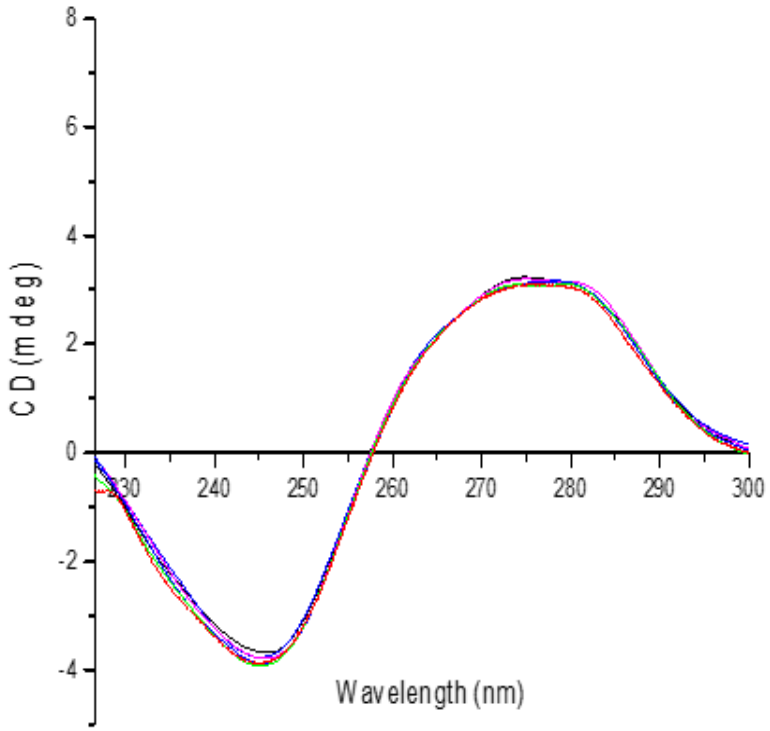
4.3. Effect of DIMC on ct-DNA conformation and base pairing by Circular dichroism spectroscopy:

CD spectra of ct-DNA (32 µM) in the absence and in presence of increasing concentrations of DIMC (3.33 µM -25 µM) (Figure 10) showed two conservative CD bands in the UV region; a positive band at around 273 nm (due to base stacking) and a negative band at around 245 nm (due to polynucleotide helicity). Upon addition of DIMC, only a slight decrease in the positive band and a slight enhancement in the negative band were observed with no change in band shape or induction of a new band. An increase in the molar ellipticity of positive bands suggests an intercalative mode of binding. It was observed that binding of DIMC with ct-DNA did not induce any conformational change and ⁄ or significant unwinding of DNA base pairs. These results point to a groove-binding mode for DIMC binding to ct-DNA.

4.4. Binding of DIMC to ct-DNA by fluorescence spectroscopy:

The change in fluorescence spectral intensities is often employed to assess the binding of drugs to DNA. Therefore, the fluorescence spectra of DIMC (5 µM) in the presence of ct-DNA (2–20 µM) in aqueous-DMSO (9:1) solutions at pH 7.4 were monitored after excitation at 420 nm. In the absence of ct-DNA,

Figure 10: Circular dichroism spectra of DIMC with ct-DNA



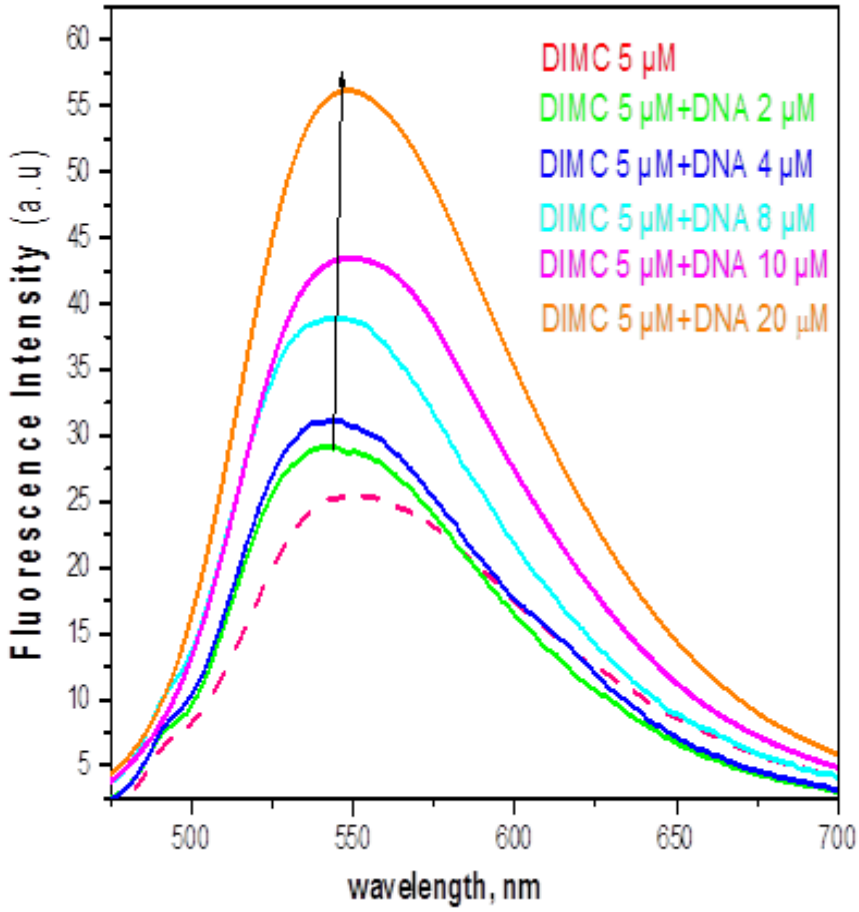
- DIMC (3.33 μM-25 μM) were added onto ct-DNA solution (32 μM) in 10 mM phosphate buffer saline pH 7.4 and spectra were recorded using Jasco-810 automatic recording spectropolarimeter at 25 °C from 200–350 nm in a 1 cm cell at scan rate of 100 nm/min with a response time of 1 sec.

DIMC showed weak fluorescence, but in presence of ct-DNA, its intensity increased several folds and the spectra showed small blue shift at lower concentrations of DNA (Figure 11).

Minor groove binders like DAPI and DNA intercalators like ethidium bromide show enhanced fluorescence upon entry into the minor groove or intercalation between base pair of DNA. Fluorescence emission of DIMC was found to increase steadily upon addition of increasing amount of ct-DNA, this indicates that DIMC interacts with ct-DNA. However, the exact nature of interaction cannot be ascertained by difference in fluorescence emission alone.

From docking studies, we could see that DIMC bound to the minor groove of DNA. Spectroscopic investigations revealed that DIMC did interact with DNA. However, the mode of binding could not be ascertained. In order to determine the mode of binding, i.e., groove binding mode or intercalative mode, we performed two assays namely Ethidium bromide (Etbr) competitive binding assay and DAPI displacement assay.

Figure 11: Fluorescence emission spectra of DIMC with ct-DNA

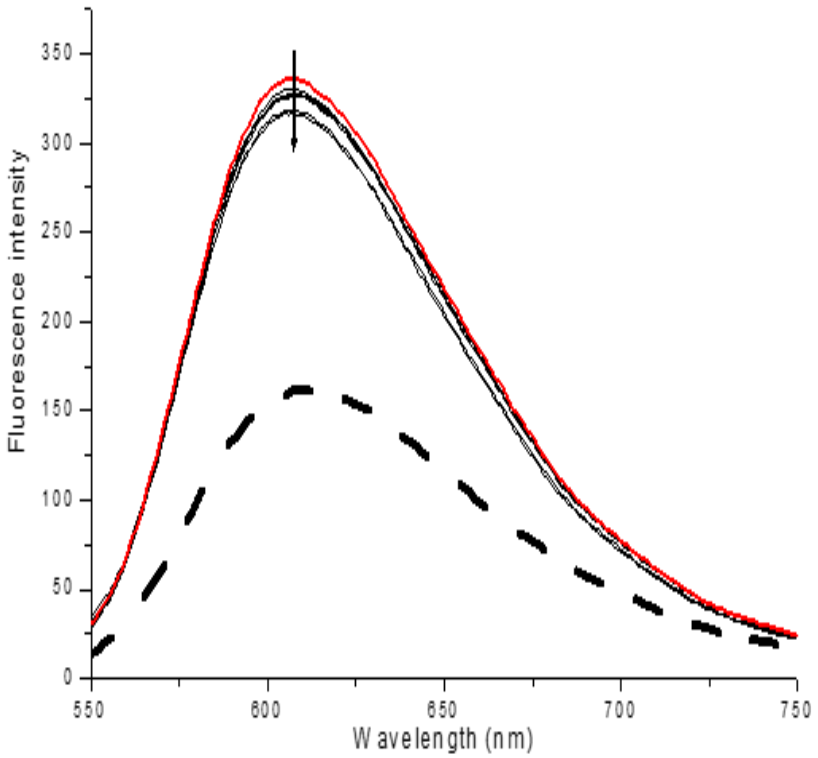


Fluorescence emission spectra of DIMC (5µM) in the presence and absence of varying concentrations of DNA (2-20 µM) in aqueous-DMSO (9:1) solutions at pH 7.4 were monitored using Hitachi F-4500 fluorescence spectrophotometer after excitation at 420 nm.

4.5. Binding mode of DIMC to ct-DNA determined by Ethidium bromide (Etbr) competitive binding assay:

EtBr is a well-known DNA intercalator, and its fluorescence intensity increases considerably after binding with DNA due to intercalation. In the presence of another molecule that intercalate between base pairs of DNA, there would be competition between the new probe and EtBr, for intercalation between base pairs of DNA, resulting in a decrease in the enhanced fluorescence of EtBr-DNA complex. On addition of increasing concentration of DIMC (5 μM - 20 μM) to solution containing EtBr (10 μM) and ct-DNA (20 μM) no changes in the fluorescence emission spectra of DNA-EtBr complex were noticed (Figure 12). From this result we confirmed that DIMC does not intercalate between base pairs of DNA like EtBr. Ethidium bromide (Etbr) competitive binding assay revealed that DIMC is not a DNA intercalator. In order to confirm that DIMC is indeed a minor grove binder as predicted by docking studies we performed DAPI displacement assay.

Figure 12: Fluorescence emission spectra of EtBr with ct-DNA



- Fluorescence emission spectra of EtBr (10 μM) (dashed line) and EtBr (10 μM) - ct-DNA (20 μM) mixture in the presence of increasing concentration of DIMC (5-20 μM) in 10 mM phosphate buffer (pH 7.4). After excitation at 480 nm fluorescence spectra was recorded using Hitachi F-4500 fluorescence spectrophotometer.

4.6. Binding mode of DIMC to ct-DNA determined by DAPI displacement assay:

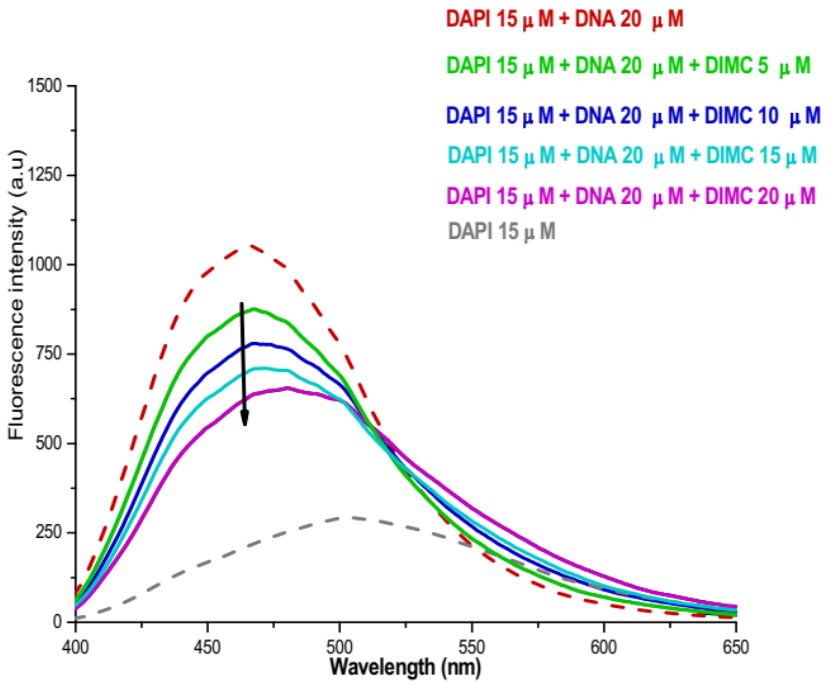
DAPI is a fluorogenic probe whose fluorescence intensity increase substantially upon interaction with DNA through minor groove binding. If a probe that bind to minor groove of DNA is added to a mixture of DAPI and DNA, the newly added probe would compete with DAPI for binding sites in the minor groove, thereby displacing some amount of DAPI molecules from minor groove subsequently decreasing the enhanced fluorescence of DAPI. A significant decrease in the fluorescence intensity of DAPI (15μM) bound to ct-DNA (20 μM) was observed in the presence of increasing concentration of DIMC (5-20 μM) (Figure 13). These results confirmed that DIMC competes with DAPI for binding space in the minor groove of ct-DNA and hence DIMC is indeed a minor groove binder.

With DAPI displacement assay we had established that DIMC is a minor groove binder. However, we wanted to study the effect of DIMC on denaturation of complementary oligonucleotides in the presence of DMSO. In order to study the effect of minor groove binding of DIMC on the stability of double stranded nucleotides we employed FRET assay.

4.7. Effect of DIMC on DMSO induced denaturation of complimentary oligonucleotides by fluorescence resonance energy transfer:

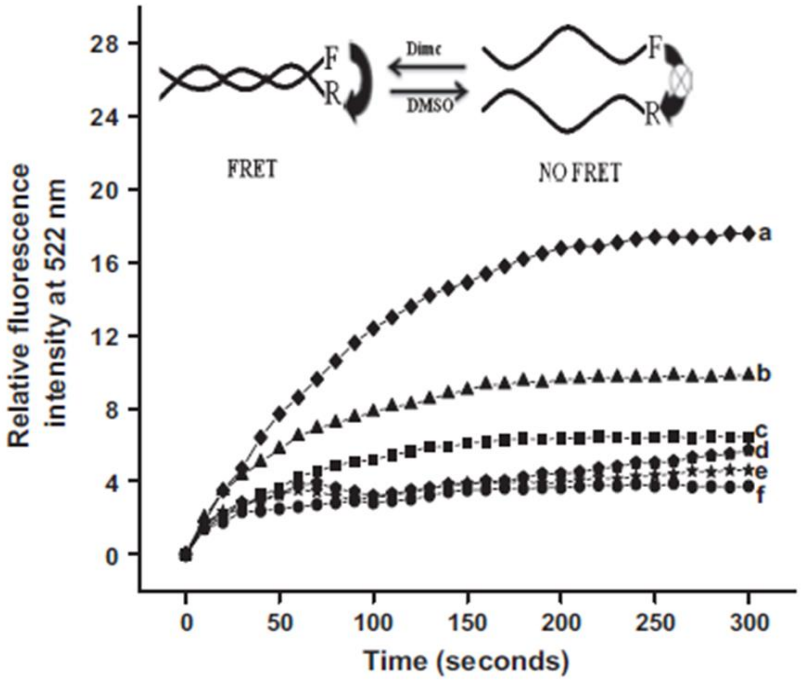
The assay is based on non-radiative resonance energy transfer between a donor (fluorescein) and an acceptor (Rhodamine B). FRET, being distance dependent between the donor and the acceptor moieties, has been used in many studies to understand the location of binding of the drugs to macromolecules. The design of the experiment is outlined in the inset of Figure 14. In this pair, initially the donor molecule fluorescein is excited at 490 nm, which emits fluorescence at 522 nm. Due to energy transfer to an acceptor like rhodamine B, loss of its emission intensity at 522 nm is observed. During renaturation of the two strands, Phi-W and Phi-C, the donor and acceptor molecules come closer and the energy transfer causes decrease in the emission intensity at 522 nm. During denaturation, the separation of these duplex strands causes loss of FRET and thereby results in increase in the emission intensity. The denaturation process occurs in a few seconds, therefore by monitoring the time-dependent emission changes at 522 nm, it is possible to monitor the kinetics of denaturation or stabilization.

Figure 13: Florescence emission spectra of DAPI with ct-DNA



- Florescence emission spectra of DAPI (15 μM) and DAPI (15 μM) – ct-DNA (20 μM) mixture in the presence of increasing concentration of DIMC (5-20 μM) in 10 mM phosphate buffer (pH 7.4). After excitation at 338 nm fluorescence spectra were recorded using Hitachi F-4500 fluorescence spectrophotometer.

Figure 14: FRET assay to demonstrate effect of DIMC on ds-DNA



- Denaturation reaction mediated by DMSO in the presence and absence of DIMC as monitored by fluorescence resonance energy transfer (FRET). Fluorescence intensity of fluorescein was plotted against time (a) DMSO (7 M); (b) DMSO (4.2 M); (c) DMSO (2.8 M); (d) DMSO (7.0 M) + DIMC (50 µM); (e) DMSO (4.2 M) + DIMC (30 µM); (f) DMSO (2.8 M) + DIMC (20 µM). Ex (□) -490 nm, and EM (□) -522 nm. Inset shows schematic representation of the FRET assay.

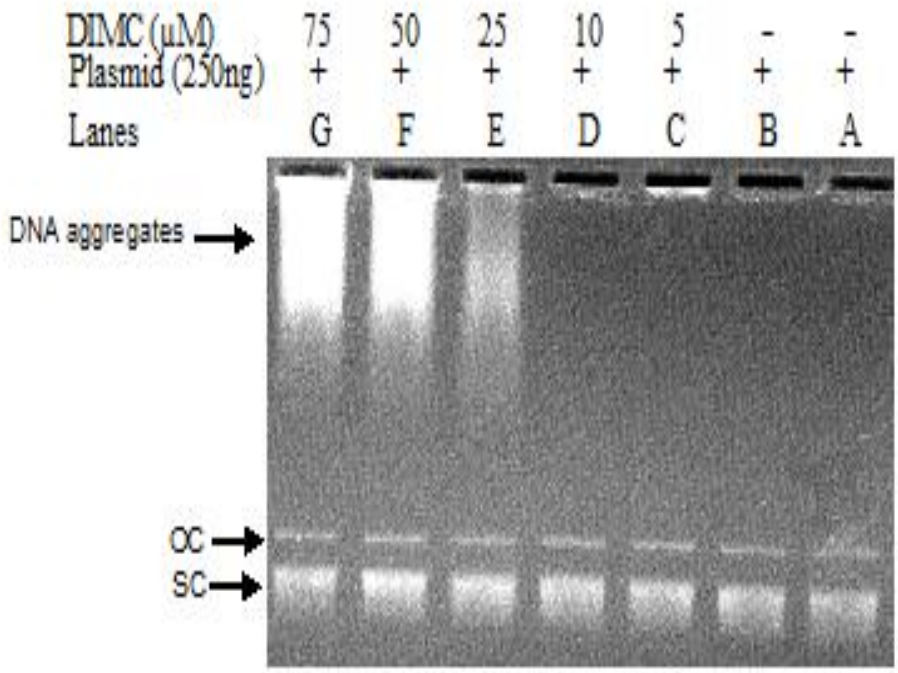
DMSO is a known denaturant of double-stranded DNA. Accordingly, with the addition of increasing concentration of DMSO from 2.8 to 7.0 M, the denaturing effect gradually increased and a clear increase in the rate and intensity of the fluorescence emission at 522 nm was observed (Figure 14). When DIMC (20 to 50 μM) was added along with DMSO to the same system, a clear inhibition of denaturation was seen and the emission intensity decreased (Figure 14). At low concentration of DMSO, no change in fluorescence was observed even in the presence of high concentration of DIMC. These results confirmed that DIMC inhibits the DMSO-mediated denaturation process of DNA, most likely through binding to the minor groove of DNA and thereby preventing the separation of the two strands.

We had established that DIMC bind to the minor groove of DNA and it stabilized double stranded oligonucleotides. We hypothesized that DNA bound to DIMC would cause gel mobility shift in DNA molecules, to ascertain this we conducted agarose gel mobility shift assay.

4.8. Effect of DIMC on gel mobility of plasmid DNA by Agarose gel electrophoresis

Agarose gel mobility of DIMC bound to DNA was checked by incubating DIMC (5 µM-75 µM) with pBR322 plasmid DNA and running the samples in an 0.87 % agarose gel. Controls for the experiment were pBR322 plasmid DNA alone and pBR322 plasmid DNA mixed with DMSO. Agarose gel electrophoresis revealed some unexpected bands or rather streaks in gel near the wells. Normally supercoiled pBR322 plasmid DNA resolved in agarose gel should show up as two distinct bands one for open circular and the other for supercoiled form. As expected, bands were obtained confirming the presence of supercoiled and very small amount of open circular form but streaks were seen much above these bands near the wells which glowed with bright fluorescence (Figure 15). Since all the bands were visible either in the well or just below the well, these may be very bulky complexes involving DNA and DIMC or aggregated DIMC alone. Since we had established that DIMC bind to minor groove of DNA like curcumin we were interested to study the biological consequences of minor groove binding.

Figure 15: Gel mobility shift assay of DIMC with Plasmid DNA



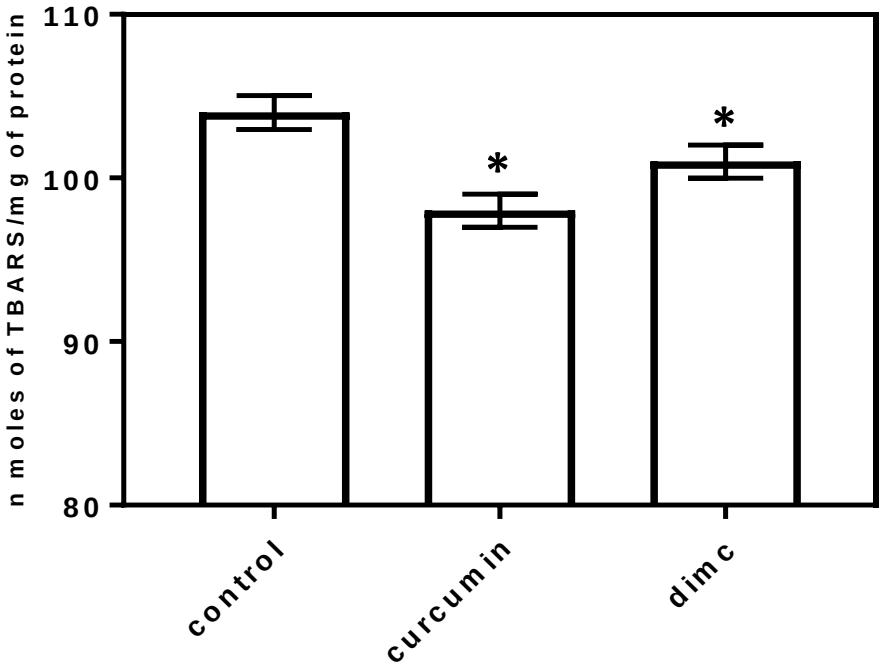
- Lanes C – G –PBR 322 plasmid DNA treated with different concentrations of DIMC (5 µM-75 µM) and resolved on 0.87 % agarose gel. Lanes A and B are control and DMSO control respectively. OC – open circular; SC – supercoiled.

We also wanted to study how DIMC interact with or modulates various components and systems in cells. We also wanted to compare DIMC with curcumin in terms of its antioxidant and anti-cancer activity as well as various other biological activities. First, we compared antioxidant status of PBMC after treatment with DIMC and curcumin by measuring parameters like lipid peroxidation, catalase activity, glutathione reductase activity and reduced glutathione levels.

4.9. Effect of curcumin and DIMC on lipid peroxidation in PBMC:

Lipid peroxidation is one of the main markers of oxidative damage to cells. We assayed lipid peroxidation after treatment with DIMC or curcumin as mentioned in 3.2.16. Treatment with curcumin and DIMC resulted in a slight decrease in thiobarbituric acid reactive substances (TBARS) levels in PBMC. However, curcumin had better activity in this regard (Figure 16 and table 1). Curcumin lowered TBARS levels from 104 ± 1.04-nmol/mg of protein (control) to 98 ± 1.01- nmol/mg of protein, whereas DIMC could lower it to 101 ± 1.02-nmol/mg protein only. Significant differences were noted in lipid peroxidation when treated with curcumin or DIMC when compared to control ($P<0.05$). These results confirm that curcumin is a better antioxidant compared to DIMC in terms of quenching lipid peroxides.

Figure 16: Lipid peroxidation assay with curcumin and DIMC in PBMC



- PBMC were treated with curcumin (5 µM) or dimethoxycurcumin (5 µM). After incubation, PBMC were washed with PBS, sonicated and treated with 10% TCA and centrifuged. Supernatant was used to determine the peroxidation product (MDA) using different concentrations of MDA standards. Data are expressed as mean ± S.D. Statistical analysis was performed using one-way Anova and Turkey's post hoc analysis., *$p<0.05$ when compared to control; n = 6.

Table 1: Effect of curcumin and DIMC on lipid peroxidation

Treated groups	Lipid peroxidation nmol of TBARS / mg of protein
control	104 ± 1.04
curcumin	98 ± 1.01
DIMC	101 ± 1.02

- PBMC were treated with curcumin (5 µM) or dimethoxycurcumin (5 µM). After incubation, PBMC were washed with PBS, sonicated and treated with 10% TCA and centrifuged. Supernatant was used to determine the peroxidation product (MDA) using different concentrations of MDA standards. Data are expressed as mean ± S.D. Statistical analysis was performed using one-way Anova and Turkey's post hoc analysis., *$p<0.05$ when compared to control; n = 6.

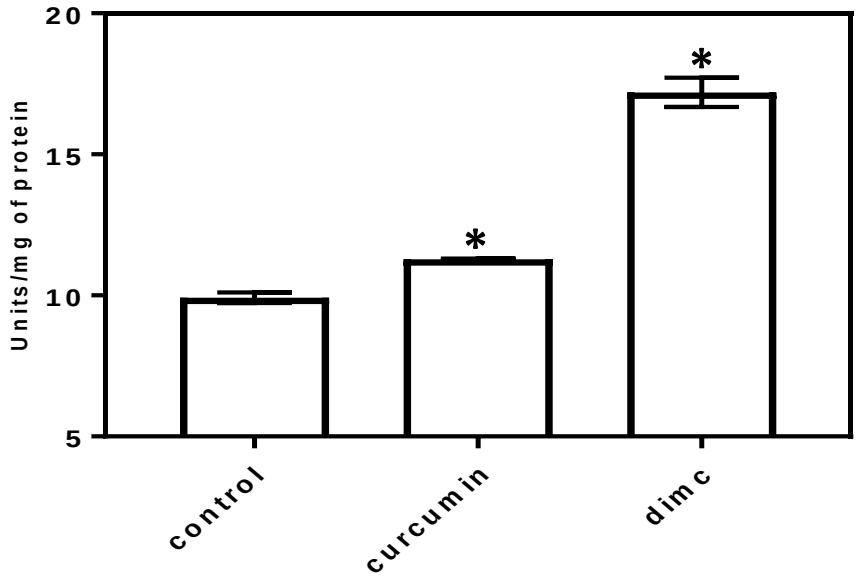
4.10. Effect of curcumin and DIMC on catalase activity in PBMC:

Catalase is an important enzyme present in almost all living systems. It catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is a key enzyme in protecting cells against oxidative damage. The assay of catalase activity was performed by incubating PBMC with curcumin or DIMC and measuring catalase activity afterwards as mentioned in 3.2.17. We found that catalase activity increased in the case of curcumin treated PBMC, to 11.28 ± 0.22 U/mg protein ($P<0.05$) from a control value of 9.92 ± 0.19 U/mg protein, whereas DIMC increased catalase activity to 17.2 ± 0.53 U/mg protein which is almost double the control value (Figure 17 and table 2). These results suggest that DIMC is able to increase catalase activity substantially.

4.11. Effect of curcumin and DIMC on glutathione reductase activity in PBMC:

Glutathione reductase plays a key role in the overall maintenance of antioxidant status of cells. It reduces glutathione disulfide to reduced glutathione which is critical for the functioning of glutathione peroxidase which in turn reduces hydrogen peroxide.

Figure 17: Effect of curcumin and DIMC on catalase activity



- PBMC were treated with curcumin (5 µM) or dimethoxycurcumin (5 µM). Catalase was estimated by the decomposition of hydrogen peroxide to release oxygen and water. The rapid decomposition of H_2O_2 was determined for every 30 sec from the decrease in absorbance at 240 nm for 3 min. The results were expressed as Units / mg of protein. Data expressed as mean ± S.D. Statistical analysis was performed using one-way Anova and Turkey's post hoc analysis. *$p<0.05$ when compared to control; n = 6.

Table 2: Effect of curcumin and DIMC on catalase activity

Treated groups	Catalase activity units / mg of protein
control	9.92 ± 0.19
curcumin	11.28 ± 0.022
DIMC	17.2 ± 0.52

- PBMC were treated with curcumin (5 µM) or dimethoxycurcumin (5 µM). Catalase was estimated by the decomposition of hydrogen peroxide to release oxygen and water. The rapid decomposition of H2O2 was determined for every 30 sc from the decrease in absorbance at 240 nm for 3 min. The results are expressed as Units / mg of protein. Data are expressed as mean ± S.D. Statistical analysis was performed using one-way Anova and Turkey's post hoc analysis. *$p<0.05$ when compared to control; n = 6.

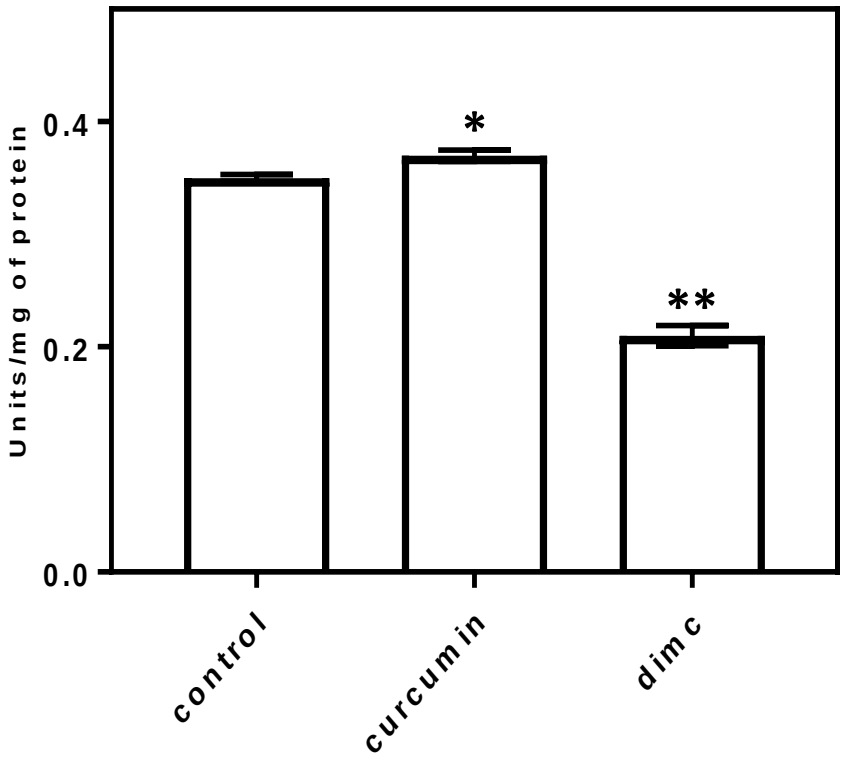
Activity of glutathione reductase was measured after treatment with DIMC or curcumin as mentioned in 3.2.18. There was an increase in the activity of glutathione reductase from a control value of 0.35 ± 0.003 U/mg protein to 0.37 ± 0.005 U/mg protein in the case of curcumin treated samples. However, the trend was reversed when treated with DIMC, here glutathione reductase activity was significantly reduced ($P<0.01$) to 0.21 ± 0.009-U/mg protein (Figures 18 and table 3).

DIMC treatment decrease glutathione reductase activity and hence the level of reduced glutathione would be expected to decrease, similarly curcumin increased the activity of glutathione reductase hence reduced glutathione levels will also be expected to increase with curcumin treatment. In order to ascertain this, we measured reduced glutathione levels.

4.12. Effect of curcumin and DIMC on reduced glutathione levels in PBMC:

Reduced glutathione levels were measured in PBMC after treatment with DIMC or curcumin according to the method mentioned in 3.2.19. There was an increase in reduced glutathione levels (1.58 ± 0.023 μM/10^6 cells) in the case of PBMC treated with curcumin. The control value for glutathione level was 1.47 ± 0.019 μM/10^6 cells.

Figure 18: Glutathione reductase assay on PBMC with curcumin and DIMC



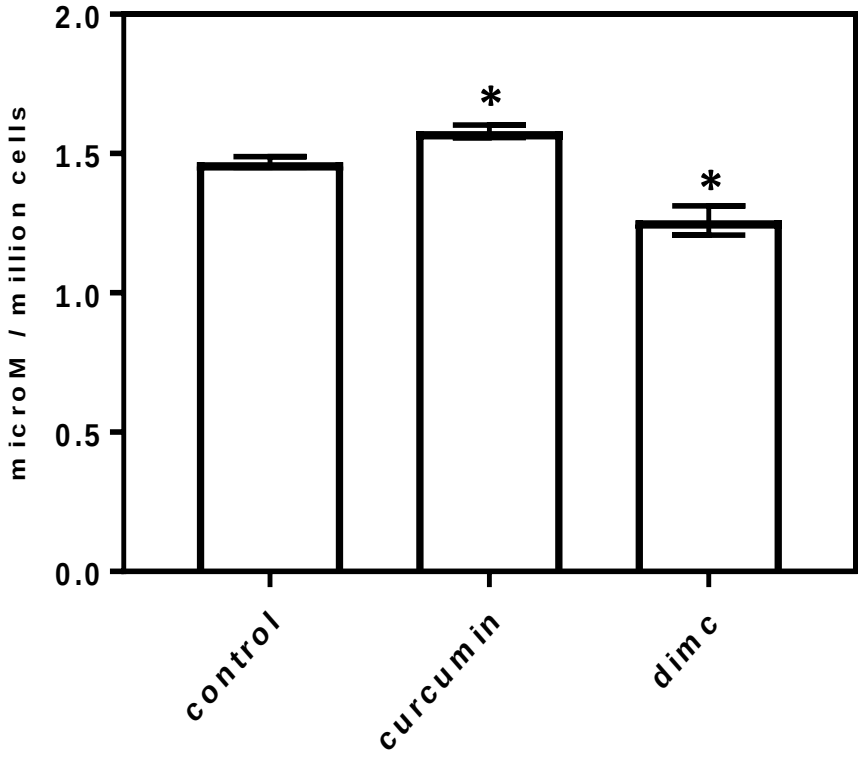
- PBMC were treated with curcumin (5 µM) or dimethoxycurcumin (5 µM) and activity of GR was measured by oxidation of NADPH to NADP during reduction of oxidized glutathione (GSSG). Data are expressed as mean ± S.D. Statistical analysis was performed using one-way Anova and Turkey's post hoc analysis. *$p<0.05$, **$p<0.01$ when compared to control; n = 6.

Table 3: Effect of curcumin and DIMC on glutathione reductase activity

Treated groups	Glutathione reductase activity units / mg of protein
control	0.35 ± 0.003
curcumin	0.37 ± 0.005
DIMC	0.21 ± 0.009

PBMC were treated with curcumin (5 µM) or dimethoxycurcumin (5 µM) and activity of GR was measured by oxidation of NADPH to NADP during reduction of oxidized glutathione (GSSG). Data are expressed as mean ± S.D. Statistical analysis was performed using one-way Anova and Turkey's post hoc analysis. *$p<0.01$ when compared to control; $n = 6$.

Figure.19: Effect of curcumin and DIMC on reduced glutathione levels



- PBMC treated with curcumin (5 µM) or dimethoxycurcumin (5 µM). The intracellular GSH concentration was determined by a colorimetric micro GSH assay and expressed as µM /106 cells. Data are expressed as mean ± S.D. Statistical analysis was performed using one-way Anova and Turkey's post hoc analysis. *$p<0.05$ when compared to control; n = 6.

Table 4: Effect of curcumin and DIMC on reduced glutathione levels

Treated groups	Glutathione reductase activity units / mg of protein
control	1.47 ± 0.019
curcumin	1.58 ± 0.023
DIMC	1.26 ± 0.052

- PBMC treated with curcumin (5 μM) or dimethoxycurcumin (5 μM). The intracellular GSH concentration was determined by a colorimetric micro GSH assay and expressed as μM /106 cells. Data are expressed as mean ± S.D. Statistical analysis was performed using one-way Anova and Turkey's post hoc analysis. *$p<0.05$ when compared to control; n = 6.

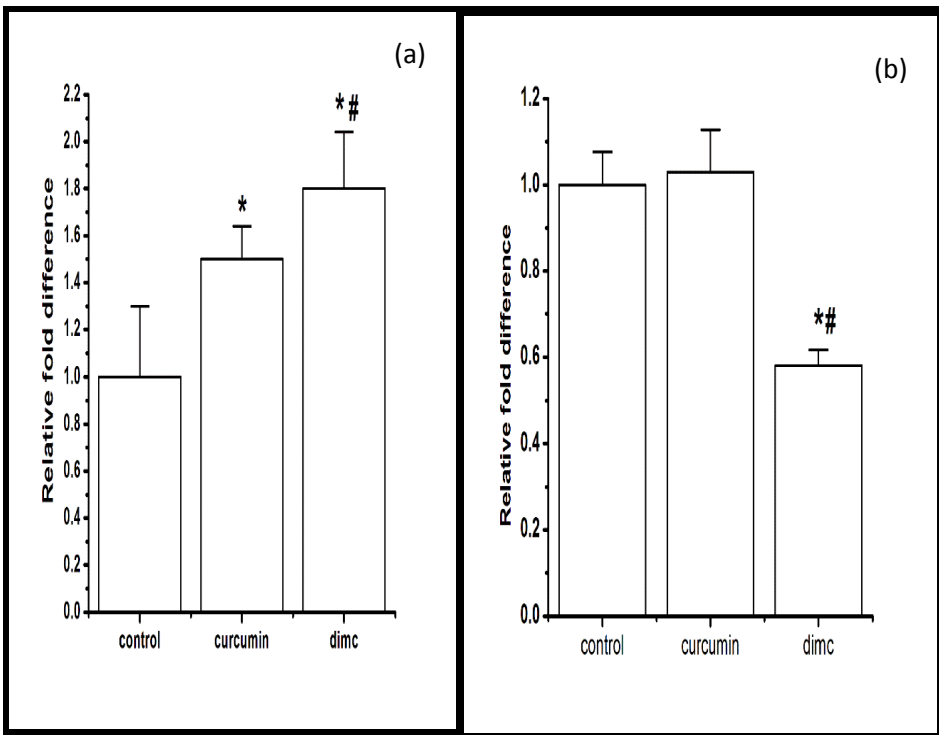
On treatment with DIMC reduced glutathione level was significantly reduced to 1.26 ± 0.052 mM/10^6 cells ($p<0.05$). Results are shown in figure 19 and table 4. These results were similar to that obtained in the case of glutathione reductase.

Results obtained from enzyme assays suggest that curcumin and DIMC differentially modulates enzymes involved in antioxidant defense system in PBMC. However, in order to determine if the enzyme activity is modulated by enhancing or repressing transcription of these enzymes at mRNA level, we performed Real-time PCR (qPCR) analysis to determine catalase and glutathione reductase mRNA levels in PBMC after treatment with curcumin or DIMC.

4.13. Effect of curcumin and DIMC on catalase and glutathione reductase mRNA levels by Real-time PCR (qPCR) analysis:

Isolated PBMCs were treated with curcumin and DIMC separately at a concentration of 5 µM and total RNA was isolated, Real time-polymerase chain reaction was performed using appropriate primers and internal reference as mentioned in 3.2.20. Real time-polymerase chain reaction (RT-PCR) analysis revealed significant difference ($P<0.05$) in the levels of catalase and glutathione reductase at mRNA level on treatment with curcumin as well as DIMC.

Figure 20: mRNA expression levels of catalase and glutathione reductase in PBMC



- PBMCs were treated with curcumin (5µM) or DIMC (5µM) and the expression of catalase mRNA (a) and glutathione reductase mRNA (b) were analyzed by qRT PCR. Results presented are average of three experiments ± SEM each done at least in triplicates, *$p<0.05$ when compared to 'control', #$p<0.05$ when compared to 'curcumin'. Statistical analysis was performed using one-way Anova and Duncan's post hoc analysis.

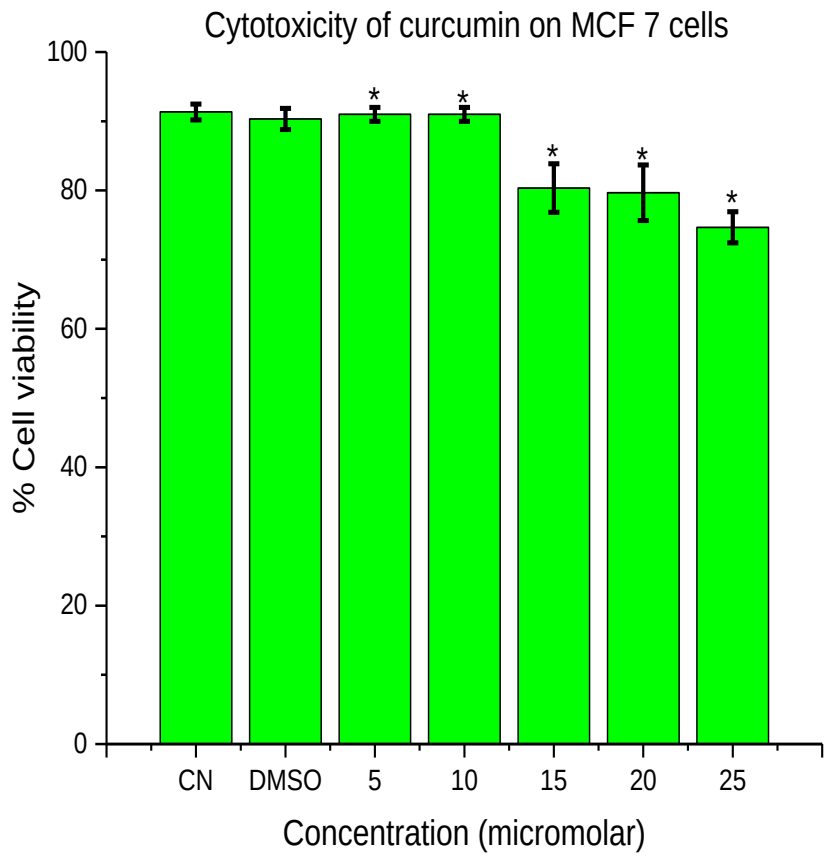
While curcumin increased catalase mRNA levels and did not have any significant effect on glutathione reductase mRNA levels, DIMC significantly increased both catalase mRNA levels and significantly decreased glutathione reductase mRNA levels, (Figure 20) these results agreed with PBMC enzyme assay results. This experiment confirmed that the enzyme activity is modulated by enhancing transcription of catalase while repressing transcription of glutathione reductase.

We also studied the anti-cancer activity of DIMC in comparison with curcumin. Cell viability assay was done on MCF 7 cell line after treatment with curcumin or DIMC.

4.14. Determination of anti-cancer activity of DIMC and curcumin in MCF 7 breast cancer cell line:

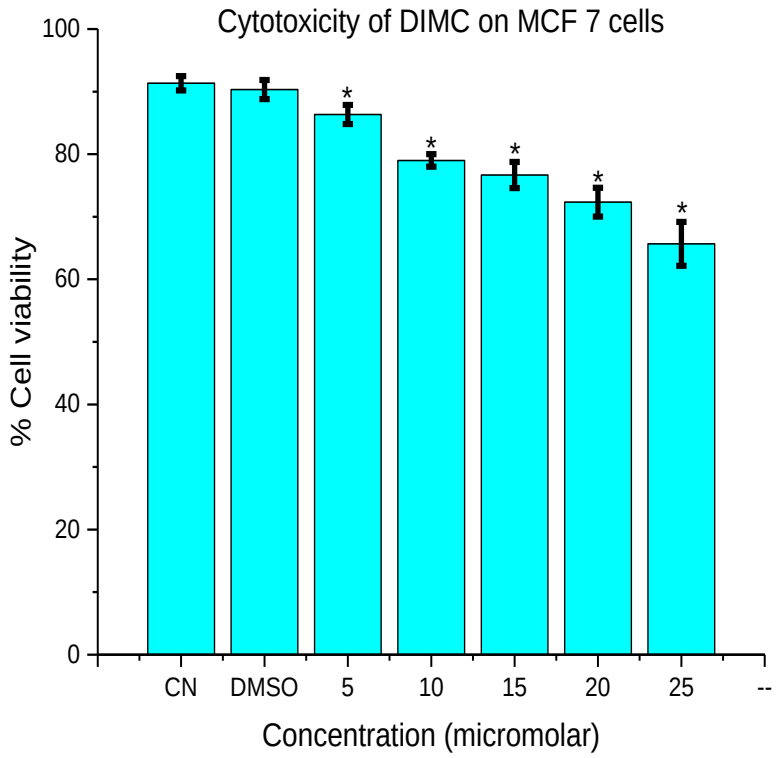
Dimethoxycurcumin or curcumin were added to the wells containing MCF 7 cells at concentrations varying from 5 – 25 µM as described in 3.2.21, followed by incubation for 24 h. After the incubation period, Cell viability was measured with proliferation kit II (Sigma – Aldrich). Figures 21 represents cytotoxic activity of curcumin against MCF 7 breast cancer cells. It can be seen that curcumin does not have any activity until 10 µM concentration and 25 µM concentration results in about 25 % cytotoxicity. Figure 22 shows the cytotoxic activity of DIMC against MCF 7 cells.

Figure 21: Anti-cancer activity of curcumin against MCF 7 breast cancer cell line



- Curcumin was added to the appropriate wells at concentrations varying from 5 – 25 µM followed by incubation for 24 h. After the incubation period, Cell viability was measured with proliferation kit II (Sigma – Aldrich). Data are expressed as mean ± S.D. n=3, *$p<0.05$.

Figure 22: Anti-cancer activity of DIMC against MCF 7 breast cancer cell line



- DIMC was added to the appropriate wells at concentrations varying from 5 – 25 µM followed by incubation for 24 h. After the incubation period, Cell viability was measured with proliferation kit II (Sigma – Aldrich). Data are expressed as mean ± S.D. n=3, *$p<0.05$.

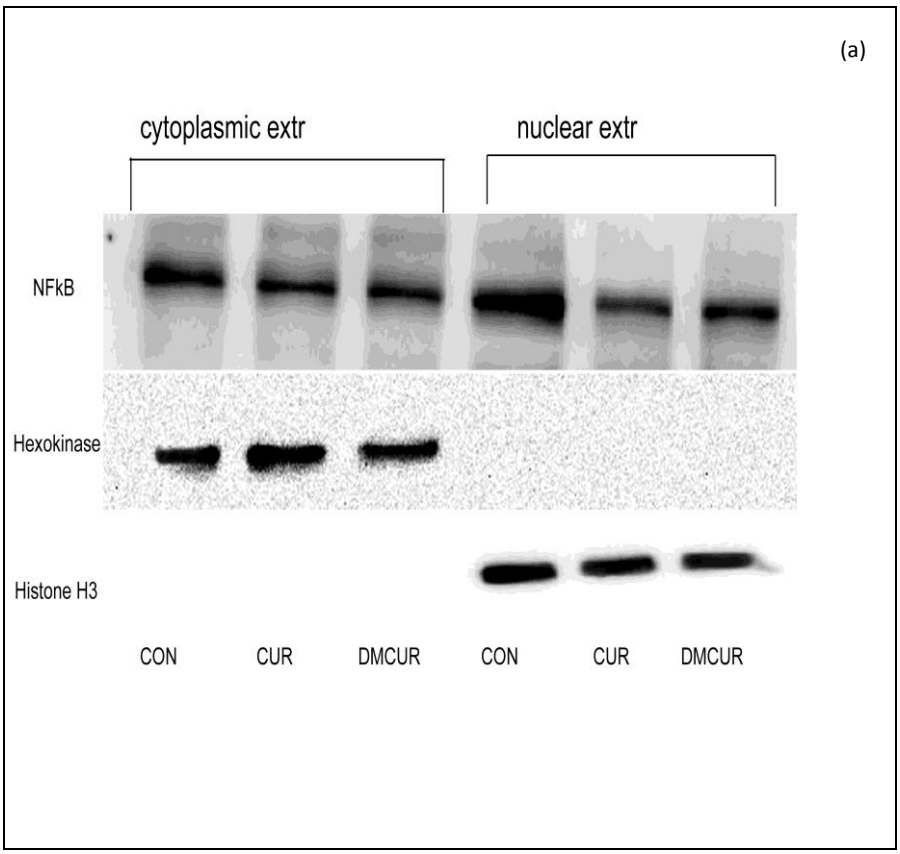
It can be noticed that DIMC has cytotoxic activity against MCF 7 cells right from 5 µM concentration and the activity increase steadily until 25 µM concentration. At 10 µM concentration DIMC shows about 20% cytotoxicity, while at 25 µM concentration it shows about 35% cytotoxicity.

Cell viability assay was done with cell proliferation kit II (Sigma-Aldrich) as described in 3.2.21. Percentage cytotoxity was calculated from the cell viability assay results. From our comparative studies of curcumin and DIMC on anti-oxidant status of normal cells and anti-tumor activity in cancer cell line, we found that these compounds had differential activity which we hypothesized could be due to subtle difference in their structures. We also knew that NFκB pathway played a major role in oxidative stress as well as cancer, therefore we studied the effect of curcumin and DIMC on activation of NFκB in PBMC by Western blot analysis as well as by NFκB reporter assay.

4.15. Effect of curcumin and DIMC on activation of NFκB in PBMC by Western blot analysis:

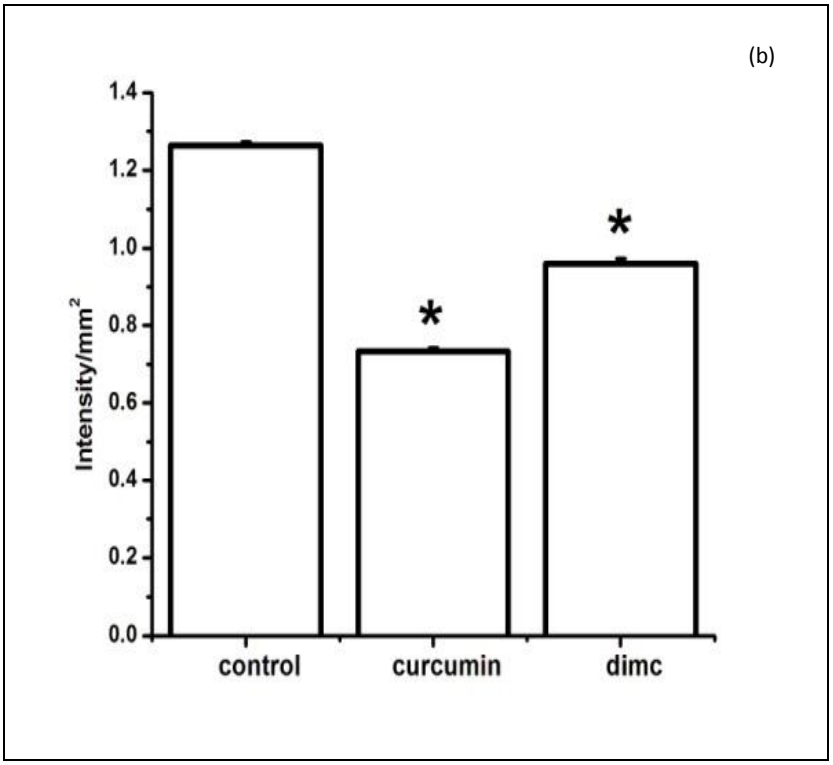
After treatment with curcumin (5 µM) or DIMC (5 µM) and incubation for 24 h, PBMCs were collected and lysed in nuclear-cytoplasmic fractionation buffer. Cytoplasmic and nuclear fractions were collected and western blot analysis was performed as described in 3.2.22.

Figure 23: Western blot analysis for NFκB in PBMC after curcumin and DIMC treatment



- Peripheral blood mononuclear cells were treated with curcumin (5 µM) or DIMC (5 µM). Protein was extracted from nucleus and cytoplasm separately. Western blot analyses were performed for NFκB, Hexokinase and Histone H3 proteins both in the nuclear and cytoplasmic extracts, CON- control, CUR – curcumin and DMCUR – DIMC.

Figure 24: Densitometric analysis of Western blot analysis for NFκB in PBMC shown in figure 23



- PBMCs were treated with curcumin (5 µM) or DIMC (5 µM). Protein was extracted from nucleus and cytoplasm separately. Western blot analysis was performed for NFκB. The ratio of intensity of protein bands in the nuclear to cytoplasmic extract was quantified by densitometric analysis. Data expressed as mean ± SEM. Statistical analysis was performed using one-way Anova and Duncan's post hoc analysis. *$p<0.05$ - statistically significant when compared to control; n = 6.

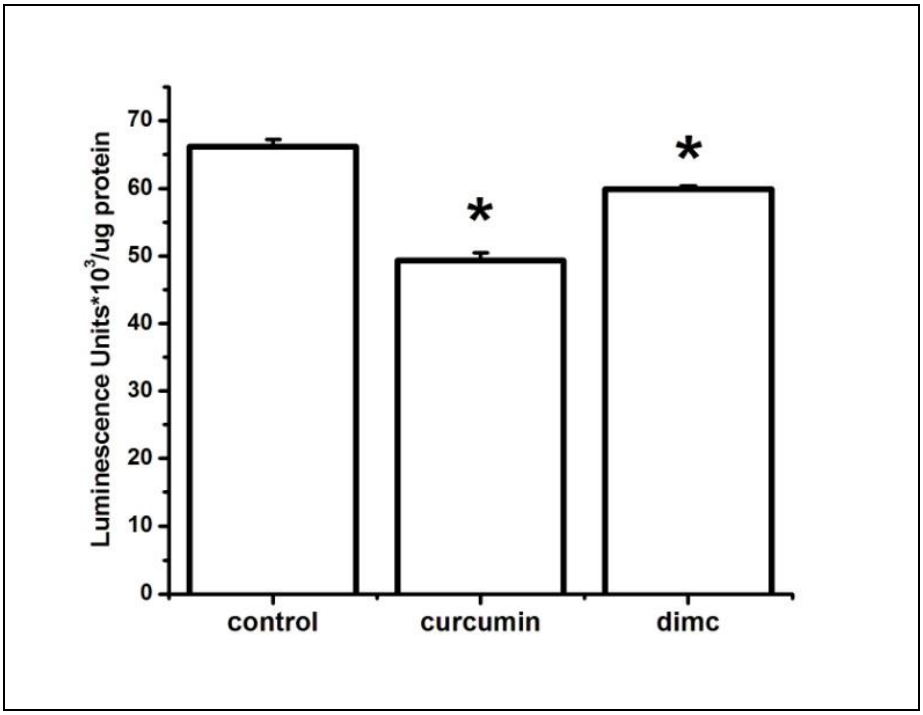
On treatment with curcumin, NFκB activation was inhibited significantly to almost half the control levels however, inhibition of NFκB activation by DIMC was significantly less ($p<0.05$) as seen in figure 23 and 24. Since NFκB pathway has a major role in cellular response to oxidative stress and carcinogenesis, it can be inferred that the differential activation of NFκB by curcumin and DIMC could be one of the reasons behind the differential modulation of antioxidant enzymes by these compounds as well as the difference in their anti-cancer activity.

4.16. Effect of curcumin and DIMC on expression of NFκB by NFκB reporter assay:

Human Embryonic Kidney 293 cell cultures were transfected with NFκB Reporter plasmid. After transfection, cells were treated with curcumin or DIMC at a concentration of 5 µM. After 24 h, cells were lysed and NFκB reporter assay performed as described in 3.2.23. This assay was performed to ascertain if similar results could be obtained as that of NFκB western blot assay in PBMC in a different system namely, HEK293 cell line.

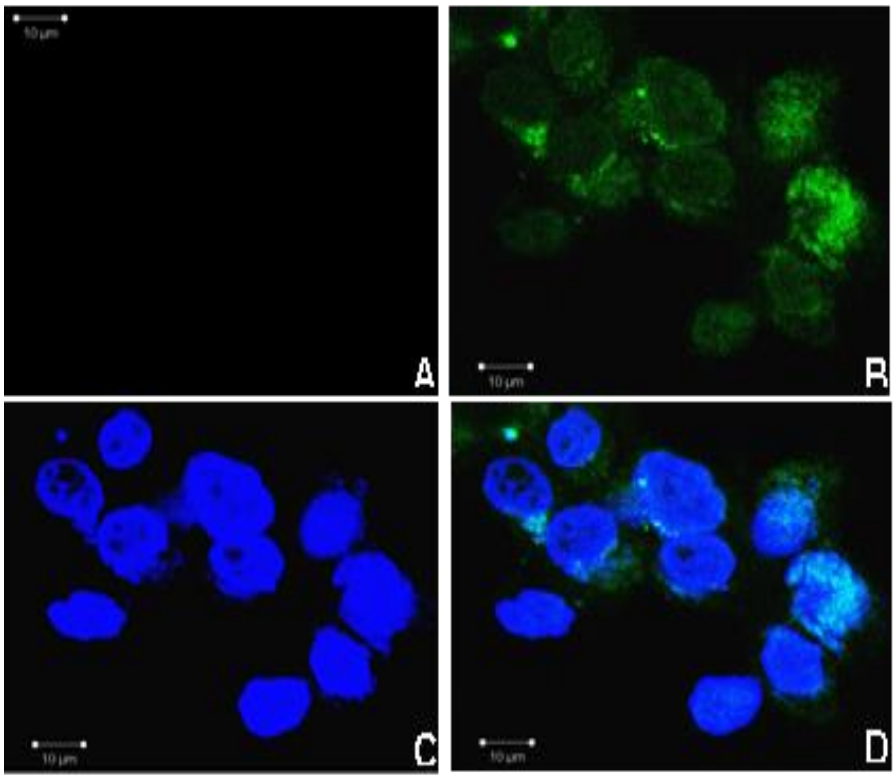
HEK293 cell line is a normal human embryonic kidney cell line routinely used in cell biology research because of their reliable growth and propensity for transfection.

Figure 25: NFκB reporter assay in PBMC.



- Human embryonic kidney 293 cells were transiently transfected with NFκB reporter vector containing luciferase gene. 24hrs post transfection the cells were treated with curcumin (5µM) or dimethoxy curcumin (5µM). 6 hrs post treatment cells were lysed and luciferase assay was performed with the cell lysate. Data expressed as mean ± SEM. Statistical analysis was performed using one-way Anova and Duncan's post hoc analysis. *$p<0.05$ - statistically significant when compared to control; n =6.

Figure 26: Confocal images of MCF 7 cells treated with DIMC and DAPI



- Confocal micrographs (100 x objective) of MCF7 cells treated with DIMC (20 µM) and DAPI (5 µlg ⁄ mL). (A) Image of control cells without DIMC and DAPI staining, (B) Image of cells stained with DIMC, (C) Image of cells stained with DAPI and (D) Image B and C merged together. λ_{ex} for DIMC is 458 nm and for DAPI is 720 nm.

Curcumin was observed to inhibit NFκB expression significantly whereas DIMC did not inhibit NFκB expression to the extent of curcumin, in fact the inhibition by DIMC was significantly less when compared to curcumin (Figures 25). These results confirm that effect of curcumin and DIMC on NFκB activation in HEK293 cells is similar to that in PBMC.

4.17. Determination of nuclear localization of DIMC by Confocal microscopy in MCF 7 cells:

Cells were incubated with or without DIMC (20 µM) in DMEM medium containing 10% FBS. At the end of 4 h, the cells were centrifuged onto cover slips, fixed with paraformaldehyde, stained with DAPI (5 µg/ml) for nuclear staining and mounted onto glass slides. Slides were examined using a confocal microscope as described in 3.2.24. 4′,6-diamidino-2-phenylindole (DAPI) is a fluorescent stain that binds strongly to DNA. It is used extensively in fluorescence microscopy as a nuclear stain as it can pass through an intact cell membrane. Figure 26 shows the confocal images of MCF7 cells under different treatment conditions. Figure 26A represents control, and Figure 26B–D represent fluorescence images of cells treated with DIMC and DAPI. The fluorescence from DIMC was visualized as green (Image 26B) and that from DAPI as blue (Image 26C). The two images when superimposed as shown in image 26D clearly showed green and blue areas overlapping, confirming the co-

localization of curcumin and DAPI in the nucleus. These results thus confirm that curcumin localizes in the nucleus, like DAPI.

CHAPTER V

DISCUSSION

Natural products derived from plants, animals or micro-organisms have been used for medicinal purposes by human beings since time immemorial. Paleoanthropological studies at the cave site of Shanidar, located in the Zagros Mountains of Kurdistan in Iraq, suggested that, Neanderthals might have been aware of the medicinal properties of various plants, more than 60,000 years ago as evidenced by pollen deposits in one of the graves at the site (Solecki 1975). Over the years humans have uncovered and made use of mind-boggling range of natural compounds. The latest version of the *Dictionary of Natural Products* has just over 214,000 entries (Ji *et. al.*2009).

During the past several decades natural products have taken a secondary role in drug discovery and drug development, especially after the studies at molecular level took off, which made possible the rational design of chemical compounds to target specific molecules in cells. However, natural products and their derivatives represent more than 50% of all the drugs in clinical use in the world (Gurib-Fakim 2006). The past few years, however, have seen a renewed interest in natural compounds, the massive volume of research done on compounds like curcumin,

genistein, catechins, resveratrol, quercetin etc. is testimony to this fact. It is an undeniable fact that most of the modern drug molecules have side effects or adverse reactions that counteracts the beneficial effects of such molecules. Natural products used as medicine on the other hand have seldom been reported to induce side effects. Probably this could be one of the reasons behind the renewed interest in natural products. Another reason for this interest could be the possibility of synergism or antagonism between drugs and natural products, Hema Iswarya *et. al* (2006) have listed out a number of drug herb interaction and their mechanism of action (Hema Iswarya *et. al.*2006).

In our studies we had evaluated some of the biological activity of dimethoxycurcumin (DIMC), which is a synthetic analog of natural compound curcumin. We had also studied the biological activities of curcumin in order to compare it with DIMC, if such studies where not reported for curcumin earlier.

Molecular docking using AutoDock

Molecular docking studies are usually used to determine molecular interactions between proteins and ligands (Lengauer *et. al.* 1996; Kitchen *et al*. 2004; Y. Wang *et al.* 2017; Ali *et al.* 2017). Studies involving Nucleic acids have been comparatively rare, (Holt *et. al*.2008). Our studies using AutoDock revealed that both curcumin and DIMC bind to the minor groove of DNA.

However, they differ in terms of binding energy. Curcumin forms hydrogen bond with DNA involving a thymine residue of DNA and phenolic -OH group of curcumin while no such hydrogen bonding was noticed in the case of DIMC. This could be because of the lack of phenolic -OH group in DIMC. In a previous work interaction of a curcumin analog namely, diacetylcurcumin (DAC) with DNA was studied. They found extensive van der Waals contacts between the docked DAC and the floor and walls of the minor groove suggesting that this type of interaction contributes significantly to the stabilization of the complex. The docking energy was -10.69 kcal mol^{-1} (Sahoo *et al*. 2008) which was similar to that of curcumin obtained in this study. However, DIMC showed a maximum binding energy of -8.6 kcal mol^{-1} in our study. Further, we found extensive non-covalent interaction between wall and floor of minor groove of DNA and both curcumin and DIMC. It has been reported that minor groove-binding molecules generally have aromatic rings connected by single bonds that allow for torsional rotation in order to fit into the helical curvature of the minor groove with displacement of water molecules. Both curcumin and DIMC has aromatic rings connected by single bonds. In a previous study involving docking of isoxazolcurcumin with DNA, results similar to that obtained by us were reported (Bera *et al*. 2008). The binding energy clustering of both compounds were quite different as can be seen in figures 19 and 22. Overall it can be concluded

from the molecular docking studies that although both compounds bind to minor groove of DNA, they are quite different in terms of the nature of interactions and binding energies.

DNA binding activity of DIMC

DNA is one of the most important targets of chemotherapeutic agents that are used in the treatment of human cancers. Therefore, agents that can bind to DNA are most sought after (Simon *et al*. 1998; Zsila *et al.* 2004; Nafisi *et al*. 2009; Senthil et al. 2009). Although it is well established that curcumin interacts with several cellular proteins, there are very few studies on the DNA-binding activity of curcumin or its analogues (Ruby *et al*. 1995; Zhou *et al.* 2011; Chattopadhyay *et al.* 2004). This prompted us to investigate the DNA-binding activity of a more potent derivative of curcumin, DIMC, using different spectroscopic methods. For these studies, ct-DNA, which is well characterized for spectroscopic studies, was used as a model system. The absorption and fluorescence spectral changes suggested binding of DIMC to DNA with similar binding constant as reported with curcumin (Zsila *et al.* 2004). Both intercalation and minor groove binding can cause such changes in absorption and fluorescence properties.

To resolve this, competitive binding assay with a known intercalator, EtBr, and with a known groove binder, DAPI, was performed. Accordingly, the addition of DIMC decreased the fluorescence of DAPI bound to DNA but not of EtBr bound to DNA. This further indicated that the preferential mode of interaction between DIMC and DNA is through minor groove region. FRET studies confirmed that DIMC prevents denaturation of complimentary strands of DNA possibly by direct interaction. Further, it was examined whether DIMC had any effect on DNA during gel electrophoresis. The agarose gel electrophoresis assay results suggested that probably DIMC formed some sort of bulky complex which was responsible for the bright fluorescence visible when the gel was visualized in a gel documentation system (Figure 6). It has to be noted that the streaking effect was seen only beyond 25 μM concentration, below this concentration no such effect was observed. This effect could probably be due to some sort of aggregation of DIMC at higher concentrations. DNA does not seem to be involved in these complex because intensity of plasmid bands did not seem to decrease as fluorescence near the wells increased. It is not known whether groove binding can manifest in such an effect.

These studies confirmed that DIMC, like curcumin, binds to DNA at the minor groove with similar efficiency, probably due to similar size. This also confirms that the phenolic OH group in

curcumin is not important for DNA interactions. The binding of curcumin and its analogues to the minor groove gain significance in addressing the biological actions of curcumin analogues.

Antioxidant and anticancer activity of DIMC

Anti-tumour and antioxidant activity of curcumin as well as its poor bioavailability and poor metabolic stability has been well documented (Anand *et al*. 2008; Tamvakopoulos *et al*. 2007; Pae *et al*. 2008). Developing curcumin congeners with better activity and stability has been one of the approaches adapted and worked upon for many years now by many researchers (Anand *et al*. 2008). DIMC is one such compound which is claimed to have better anti-tumour activity, better metabolic stability as well as better bioavailability compared to curcumin (Tamvakopoulos *et al*. 2007; Pae *et al*. 2008; Lee *et al*. 2010).

Oxidative stress associated with the production of reactive oxygen species (ROS) is believed to result in various tissue damages (Grattagliano *et al*. 2009). The toxicity to various organs is associated with the metabolic activation of foreign compounds to form free radicals or with the production of ROS such as superoxide anion, hydroxyl radicals, hydrogen peroxide radicals etc. These are responsible for tissue damaging effects such as lipid peroxidation and DNA damage (Davies 1995) . Curcumin has been shown to inhibit lipid peroxidation *in vitro*

and *in vivo* in several studies (Banerjee *et al*. 2008; Masuda *et al*. 2001; Sreejayan *et al.* 1994; Kalpana *et al.* 2004). Our results also showed that curcumin inhibits lipid peroxidation. However, DIMC seems to be less effective in inhibiting lipid peroxidation, possibly due to the absence of phenolic hydroxyl groups needed for antioxidant activity as described previously (Sharma 1976).

Most remarkably catalase activity was significantly enhanced when treated with DIMC whereas treatment with curcumin resulted only in a modest increase. In a recent work a possible mechanism by which curcumin activates bovine liver catalase (BLC) was suggested. This study reinforced the hypothesis that curcumin can increase enzyme activity by affecting re-arrangements of amino acid residues in structural pockets of the enzyme. Increase of distance between the residues of the bottleneck of narrow channel, which determines the amount of substrate entering the active site, facilitated the substrate access to the enzyme active site (Mofidi Najjar *et al.* 2017). Another study involving dose effect of curcumin on catalase messenger RNA and protein levels in ARPE-19 cells showed a dose dependent increase in catalase mRNA levels (Howell *et al.* 2013). We observed significant increase in catalase mRNA levels post curcumin or DIMC treatment. However, catalase mRNA levels increased more when treated with DIMC than when treated with same concentration of curcumin. Our

result suggests that overall antioxidant activity of DIMC is probably mediated through enhancement of catalase activity and this enhancement is possibly conferred through enhancement of mRNA levels thereby increasing protein levels as evidenced by our RT-PCR results.

Moreover, in DIMC both phenolic hydroxyl groups have been substituted with two methoxy groups and since, phenolic hydroxyl groups are essential for direct antioxidant activity (Anand *et al*. 2008; Chen *et al*. 2006) DIMC would be expected to perform as a poor antioxidant, therefore antioxidant activity expressed by DIMC would probably be exclusively through modulation of antioxidant enzymes.

Another aspect we focused was on GR enzyme activity and reduced GSH levels. GSH plays a key role in maintaining proper function and preventing oxidative stress in human cells. It can act as a scavenger for hydroxyl radicals, singlet oxygen, and various electrophiles. The ratio of GSSG/GSH present in the cell is a key factor in properly maintaining the oxidative balance of the cell. It is critical that cells maintain high levels of reduced glutathione and a low levels of oxidized glutathione disulphide (GSSG). This narrow balance is maintained by glutathione reductase, which catalyzes the reduction of GSSG to GSH (Deponte 2013; Pompella *et al.* 2003).

Our GR assay results as well as RT-PCR results show that curcumin enhances glutathione reductase activity and GSH levels in PBMC while DIMC significantly reduces GR activity as well as GSH levels. The decrease in GSH is justifiable because it is GR that reduces GSSG to GSH hence, reduction in GR activity would result in reduction in GSH level. GSH reduces the oxidized form of the enzyme glutathione peroxidase, which in turn reduces hydrogen peroxide (H_2O_2), a dangerously reactive species within the cell. Low GSH levels will result in reduced activity of glutathione peroxidase (GPx). Since GSH is needed for GPx activity, low GSH level would result in the accumulation of H_2O_2. Here, increase in catalase activity could compensate for the reduced GSH level and thereby GPx activity by scavenging the excess peroxide radicals. It has to be noted here that the overall level of H_2O_2 would therefore be a result of the cumulative action of both catalase as well as GPx in a cell.

Several studies suggest that DIMC exerts its cytotoxic activity through the production of ROS or rather build-up of ROS levels in cells (Kunwar *et al.* 2008; 2012; Banerjee *et al*. 2008). Our result suggests that probably the differential antioxidant activity of curcumin and DIMC could be due to the differential modulation of antioxidant enzymes by these compounds at mRNA level.

Anti-tumor activity of curcumin as well as DIMC has been studied earlier in many cell lines (Ruby *et al*. 1995; Tamvakopoulos *et al*. 2007; Kunwar *et al.* 2008; Lee *et al.* 2010; Kunwar *et al.* 2012). We studied the antitumor activity of DIMC as well as curcumin in MCF 7 breast cancer cell line. We found that DIMC had better cytotoxic activity against MCF 7 cell line when compared to curcumin. At 25 μM concentration, DIMC induced about 35% cell death while curcumin induced only 25% cell death. In an earlier study DIMC was compared with curcumin in terms of cytotoxic activity against MCF 7 cells and murine spleen lymphocytes. It was found that while DIMC had comparable cytotoxicity with curcumin against MCF 7 cells it was much less toxic to normal murine spleen lymphocytes (Kunwar *et al*. 2012). This study as well as our results kindle hope in DIMC being developed into an anticancer drug in future.

Nuclear localization of DIMC and involvement of NFκB pathway

To justify the biological significance of DIMC–DNA interaction, it is essential to know whether DIMC localizes in the nucleus, when added to cells. Earlier, using confocal microscopy, it was shown that the fluorescent properties of curcumin could be used to trace its location in cancer cells (Kunwar *et al*. 2008; Priyadarsini 2009). It was examined whether DIMC entered in to the nucleus, when added to cells. We found that DIMC localizes

in the nucleus in addition to other cellular compartments. The observed nuclear localization of DIMC may be due to its transport to nucleus via binding to nuclear proteins, which needs to be validated experimentally. DIMC has been shown to be taken up by tumour cells in a concentration-dependent manner (Kunwar *et al.* 2012), since DIMC interacts with DNA in the minor groove, nuclear localization of DIMC could be of significance in terms of its biological action

Key cellular processes such as cell survival, proliferation, and immunity are regulated through NFκB dependent transcription, other than this it is a central mediator in inflammatory response and stress response including oxidative stress. Dysfunction of NFκB pathway has been implicated in many diseases including cancer (Gilmore 2006). NFκB assay indicated a significant difference in activation post treatment with curcumin and DIMC. Curcumin inhibited NFκB activation significantly; DIMC activity differed significantly from that of curcumin in this regard. Curcumin is a well-known inhibitor of NFκB (Gupta *et al*. 2010; Leclercq *et al.* 2004; Singh *et al*. 1995). It is a well-established fact that ROS, particularly H_2O_2 induces NFκB as well as antioxidant enzymes such as catalase and GPx (Sies 1997; Morgan *et al*. 2011). It has been reported that expression of NFκB inhibitors reduces H_2O_2 induced increase in GPx and catalase expression in skeletal muscle cells (

Zhou *et al*. 2001). This report suggests that repression of NFκB also results in the repression of catalase and GPx. Our results agree with this, we found that curcumin represses NFκB more than DIMC and subsequently also repress the activation of catalase possibly by H_2O_2 more than DIMC. These results suggest that NFκB pathway could be involved in modulating the antioxidant enzymes directly or indirectly via other proteins it modulates. However, the increase in catalase levels post DIMC treatment suggest that there could be additional mechanism involved in the enhancement of catalase at mRNA level.

Overall, we have found that a subtle structural change in the structure of DIMC as compared to curcumin manifests in a significant difference in terms of biological activities of both these compounds. Our results suggest that modification in structure of curcumin not only results in enhanced metabolic stability and bioavailability but also result in altered biological activity.

Our studies show that merely replacing the two phenolic hydroxyl groups with two methoxy groups results in altered antioxidant and anti-cancer activity. However, DNA binding and nuclear localization potential are more or less retained by DIMC. In future while developing curcumin analogs in order to achieve better stability and bioavailability, measures must be taken to retain the desired biological activity. Other than concentrating on

structural modifications alone, studies can also be done on suitable delivery vehicles for curcumin and DIMC.

Curcumin and DIMC are both lipophilic compounds, hence suitable delivery vehicles would be required in order to study the effects of these compounds *in vivo* in murine and other models. Several studies have already been done in this regard, where liposomal formulations of curcumin and some of its analogs including DIMC have been made. *In vivo* as well as *in vitro* studies have been carried out on Liposomal curcumin as well as nano curcumin formulations (Feng *et al*. 2017; Wei *et al*. 2020; Hadjidemetriou *et al*. 2013; Nasery *et al*. 2020)

More studies are needed on curcumin analogs and various formulations of curcumin and its analogs including nano formulations, in order to completely understand the structure activity relationship of curcumin and its analogs. Studies in this direction could ultimately lead to promising molecules having diverse pharmaceutical potential.

CHAPTER VI

CONCLUSION

Docking studies revealed that curcumin and DIMC binds to minor groove with different binding energies and the interactions holding them in the minor groove are different. In the case of curcumin, it is Van der Waals forces as well as hydrogen bond whereas in DIMC it is Van der Waals forces and other non-covalent forces, there was no hydrogen bond involved.

From the biophysical studies that we conducted using various spectroscopic techniques like UV/visible spectroscopy, fluorescence spectroscopy, circular dichroism spectroscopy and FRET studies we conclude that DIMC bind to double stranded DNA in the minor groove probably through weak interactions like hydrogen bonding and Van der Waals force.

We had also conducted various enzyme assays, NFκB assays as well as real-time PCR analysis to determine if DIMC differs from curcumin in terms of modulation of various antioxidant enzymes and to determine the signaling pathway through which this is made possible. We conclude from these studies that DIMC differentially modulates antioxidant enzymes like catalase and glutathione reductase and that this modulation is at mRNA level as evident from our rt-PCR results. Further we

conclude that the mechanism behind this differential modulation of antioxidant enzymes could in fact be the difference in activation of NFκB by these two compounds. DIMC was found to the more effective in terms of cytotoxicity towards MCF-7 breast cancer cell line, when compared to curcumin. Overall, it can be said that the minor difference in structure between these two molecules perhaps have huge biochemical consequences in cells.

From the nuclear localization assay and computational biology approaches we conclude that DIMC indeed localizes in nucleus of MCF-7 cells, this could be one of the reasons for its diverse effects observed by as.

REFERENCES

Adam-Vizi, Vera. 2005. "Production of Reactive Oxygen Species in Brain Mitochondria: Contribution by Electron Transport Chain and Non–Electron Transport Chain Sources." *Antioxidants & Redox Signaling* 7 (9–10): 1140–49. https://doi.org/10.1089/ars.2005.7.1140.

Adeyeni, Temitope A., Natasha Khatwani, KayKay San, and Uthayashanker R. Ezekiel. 2016. "BMI1 Is Downregulated by the Natural Compound Curcumin, but Not by Bisdemethoxycurcumin and Dimethoxycurcumin." *Physiological Reports* 4 (16). https://doi.org/10.14814/phy2.12906.

Aebi, H. 1984. "Catalase in Vitro." *Methods in Enzymology* 105: 121–26.

Aggarwal, Bharat B., and Kuzhuvelil B. Harikumar. 2009. "Potential Therapeutic Effects of Curcumin, the Anti-Inflammatory Agent, against Neurodegenerative, Cardiovascular, Pulmonary, Metabolic, Autoimmune and Neoplastic Diseases." *The International Journal of Biochemistry & Cell Biology* 41 (1): 40–59. https://doi.org/10.1016/j.biocel.2008.06.010.

Aggarwal, Bharat B., and Shishir Shishodia. 2006. "Molecular Targets of Dietary Agents for Prevention and Therapy of

Cancer." *Biochemical Pharmacology* 71 (10): 1397–1421. https://doi.org/10.1016/j.bcp.2006.02.009.

Aggarwal, Bharat B., Chitra Sundaram, Nikita Malani, and Haruyo Ichikawa. 2007. "Curcumin: The Indian Solid Gold." *Advances in Experimental Medicine and Biology* 595: 1–75. https://doi.org/10.1007/978-0-387-46401-5_1.

Aggarwal, Bharat B., Young-Joon Surh, and Shishir Shishodia, eds. 2007. *The Molecular Targets and Therapeutic Uses of Curcumin in Health and Disease*. Advances in Experimental Medicine and Biology 595. New York, NY: Springer.

Ali, M. Rejwan, Mostafa Sadoqi, Simon G. Møller, Allal Boutajangout, and Mihaly Mezei. 2017. "Assessing the Binding of Cholinesterase Inhibitors by Docking and Molecular Dynamics Studies." *Journal of Molecular Graphics & Modelling* 76: 36–42. https://doi.org/10.1016/j.jmgm.2017.06.027.

Anand, Preetha, Sherin G. Thomas, Ajaikumar B. Kunnumakkara, Chitra Sundaram, Kuzhuvelil B. Harikumar, Bokyung Sung, Sheeja T. Tharakan, et al. 2008. "Biological Activities of Curcumin and Its Analogues (Congeners) Made by Man and Mother Nature." *Biochemical Pharmacology* 76 (11): 1590–1611. https://doi.org/10.1016/j.bcp.2008.08.008.

Ardito, F., D. Perrone, M. Giuliani, N.F. Testa, and L. Lo Muzio. 2018. "Effects of Curcumin on Squamous Cell Carcinoma

of Tongue: An In Vitro Study." *Current Topics in Medicinal Chemistry* 18 (3): 233–43. https://doi.org/10.2174/1568026618666180412153824.

Baker, M. A., G. J. Cerniglia, and A. Zaman. 1990. "Microtiter Plate Assay for the Measurement of Glutathione and Glutathione Disulfide in Large Numbers of Biological Samples." *Analytical Biochemistry* 190 (2): 360–65.

Banerjee, Arnab, A. Kunwar, B. Mishra, and K. I. Priyadarsini. 2008. "Concentration Dependent Antioxidant/pro-Oxidant Activity of Curcumin Studies from AAPH Induced Hemolysis of RBCs." *Chemico-Biological Interactions* 174 (2): 134–39. https://doi.org/10.1016/j.cbi.2008.05.009.

Bellomo, G., F. Mirabelli, D. DiMonte, P. Richelmi, H. Thor, C. Orrenius, and S. Orrenius. 1987. "Formation and Reduction of Glutathione-Protein Mixed Disulfides during Oxidative Stress. A Study with Isolated Hepatocytes and Menadione (2-Methyl-1,4-Naphthoquinone)." *Biochemical Pharmacology* 36 (8): 1313–20.

Bera, Rabindranath, Bijaya K. Sahoo, Kalyan S. Ghosh, and Swagata Dasgupta. 2008. "Studies on the Interaction of Isoxazolcurcumin with Calf Thymus DNA." *International Journal of Biological Macromolecules* 42 (1): 14–21. https://doi.org/10.1016/j.ijbiomac.2007.08.010.

Bhutia, RinchenDoma, MingmaLhamu Sherpa, Ta Singh, and Bidita Khandelwal. 2018. "Oxidative Stress in Metabolic

Syndrome & Its Association with DNA-Strand Break." *Indian Journal of Medical Research* 148 (4): 435. https://doi.org/10.4103/ijmr.IJMR_620_17.

Brasier, Allan R. 2006. "The NF-KappaB Regulatory Network." *Cardiovascular Toxicology* 6 (2): 111–30.

Buffenstein, Rochelle, Yael H. Edrey, Ting Yang, and James Mele. 2008. "The Oxidative Stress Theory of Aging: Embattled or Invincible? Insights from Non-Traditional Model Organisms." *AGE* 30 (2–3): 99–109. https://doi.org/10.1007/s11357-008-9058-z.

Buonocore, Giuseppe, Serafina Perrone, and Maria Luisa Tataranno. 2010. "Oxygen Toxicity: Chemistry and Biology of Reactive Oxygen Species." *Seminars in Fetal & Neonatal Medicine* 15 (4): 186–90. https://doi.org/10.1016/j.siny.2010.04.003.

Cavaleri, Franco. 2018. "Presenting a New Standard Drug Model for Turmeric and Its Prized Extract, Curcumin." *International Journal of Inflammation* 2018: 1–18. https://doi.org/10.1155/2018/5023429.

Chattopadhyay, Ishita, Kaushik Biswas, Uday Bandyopadhyay, and Ranajit K. Banerjee. 2004. "Turmeric and Curcumin: Biological Actions and Medicinal Applications." *Current Science* 87 (1): 44–53.

Chen, Dong, Fang Dai, Zhehang Chen, Saisai Wang, Xiaobin Cheng, Qinsong Sheng, Jianjiang Lin, and Wenbin Chen.

2016. “Dimethoxy Curcumin Induces Apoptosis by Suppressing Survivin and Inhibits Invasion by Enhancing E-Cadherin in Colon Cancer Cells.” *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research* 22 (September): 3215–22. https://doi.org/10.12659/msm.900802.

Chen, Wei-Feng, Shui-Ling Deng, Bo Zhou, Li Yang, and Zhong-Li Liu. 2006. “Curcumin and Its Analogues as Potent Inhibitors of Low Density Lipoprotein Oxidation: H-Atom Abstraction from the Phenolic Groups and Possible Involvement of the 4-Hydroxy-3-Methoxyphenyl Groups.” *Free Radical Biology & Medicine* 40 (3): 526–35. https://doi.org/10.1016/j.freeradbiomed.2005.09.008.

Damayanti, Dini Sri, Didik Huswo Utomo, and Chandra Kusuma. 2016. “Revealing the Potency of Annona Muricata Leaves Extract as FOXO1 Inhibitor for Diabetes Mellitus Treatment through Computational Study.” *In Silico Pharmacology* 5 (1): 3. https://doi.org/10.1007/s40203-017-0023-3.

Davies, K. J. 1995. “Oxidative Stress: The Paradox of Aerobic Life.” *Biochemical Society Symposium* 61: 1–31.

Deponte, Marcel. 2013. “Glutathione Catalysis and the Reaction Mechanisms of Glutathione-Dependent Enzymes.” *Biochimica Et Biophysica Acta* 1830 (5): 3217–66. https://doi.org/10.1016/j.bbagen.2012.09.018.

Dröge, Wulf. 2002. “Free Radicals in the Physiological Control of Cell Function.” *Physiological Reviews* 82 (1): 47–95. https://doi.org/10.1152/physrev.00018.2001.

Fang, Yun-Zhong, Sheng Yang, and Guoyao Wu. 2002. “Free Radicals, Antioxidants, and Nutrition.” *Nutrition (Burbank, Los Angeles County, Calif.)* 18 (10): 872–79.

Feng, Ting, Yumeng Wei, Robert Lee, and Ling Zhao. 2017. “Liposomal Curcumin and Its Application in Cancer.” *International Journal of Nanomedicine* Volume 12 (August): 6027–44. https://doi.org/10.2147/IJN.S132434.

Gallardo, Marcela, and Gloria M. Calaf. 2016. “Curcumin Inhibits Invasive Capabilities through Epithelial Mesenchymal Transition in Breast Cancer Cell Lines.” *International Journal of Oncology* 49 (3): 1019–27. https://doi.org/10.3892/ijo.2016.3598.

Ghosh, Shibaji, Nellore Bhanu Chandar, Kalyanashis Jana, and Bishwajit Ganguly. 2017. “Revealing the Importance of Linkers in K-Series Oxime Reactivators for Tabun-Inhibited AChE Using Quantum Chemical, Docking and SMD Studies.” *Journal of Computer-Aided Molecular Design* 31 (8): 729–42. https://doi.org/10.1007/s10822-017-0036-3.

Gilmore, T. D. 2006. “Introduction to NF-KappaB: Players, Pathways, Perspectives.” *Oncogene* 25 (51): 6680–84. https://doi.org/10.1038/sj.onc.1209954.

Goncalves, Renata L.S., Daniel E. Rothschild, Casey L. Quinlan, Gary K. Scott, Christopher C. Benz, and Martin D. Brand. 2014. “Sources of Superoxide/H2O2 during Mitochondrial Proline Oxidation.” *Redox Biology* 2: 901–9. https://doi.org/10.1016/j.redox.2014.07.003.

Gonzales, Amanda M., and Robert A. Orlando. 2008. “Curcumin and Resveratrol Inhibit Nuclear Factor-KappaB-Mediated Cytokine Expression in Adipocytes.” *Nutrition & Metabolism* 5 (June): 17. https://doi.org/10.1186/1743-7075-5-17.

Grattagliano, Ignazio, Leonilde Bonfrate, Catia V. Diogo, Helen H. Wang, David Q. H. Wang, and Piero Portincasa. 2009. “Biochemical Mechanisms in Drug-Induced Liver Injury: Certainties and Doubts.” *World Journal of Gastroenterology* 15 (39): 4865–76.

Gupta, Subash C., Chitra Sundaram, Simone Reuter, and Bharat B. Aggarwal. 2010. “Inhibiting NF-KB Activation by Small Molecules as a Therapeutic Strategy.” *Biochimica Et Biophysica Acta* 1799 (10–12): 775–87. https://doi.org/10.1016/j.bbagrm.2010.05.004.

Gurib-Fakim, Ameenah. 2006. “Medicinal Plants: Traditions of Yesterday and Drugs of Tomorrow.” *Molecular Aspects of Medicine* 27 (1): 1–93. https://doi.org/10.1016/j.mam.2005.07.008.

Gurova, Katerina. 2009. “New Hopes from Old Drugs: Revisiting DNA-Binding Small Molecules as Anticancer Agents.”

Future Oncology 5 (10): 1685–1704. https://doi.org/10.2217/fon.09.127.

Hadjidemetriou, Marilena, Natassa Pippa, Stergios Pispas, and Costas Demetzos. 2013. "Incorporation of Dimethoxycurcumin into Charged Liposomes and the Formation Kinetics of Fractal Aggregates of Uncharged Vectors." *Journal of Liposome Research* 23 (2): 94–100. https://doi.org/10.3109/08982104.2012.747534.

Haris, P., Varughese Mary, P. Aparna, K. V. Dileep, and C. Sudarsanakumar. 2017. "A Comprehensive Approach to Ascertain the Binding Mode of Curcumin with DNA." *Spectrochimica Acta. Part A, Molecular and Biomolecular Spectroscopy* 175 (March): 155–63. https://doi.org/10.1016/j.saa.2016.11.049.

Hassan, Hazem E., Samuel Carlson, Inas Abdallah, Thomm Buttolph, Karen C. Glass, and Tamer E. Fandy. 2015. "Curcumin and Dimethoxycurcumin Induced Epigenetic Changes in Leukemia Cells." *Pharmaceutical Research* 32 (3): 863–75. https://doi.org/10.1007/s11095-014-1502-4.

Hassan, Hazem E., Jean-Arnaud Keita, Lawrence Narayan, Sean M. Brady, Richard Frederick, Samuel Carlson, Karen C Glass, Senthil Natesan, Thomm Buttolph, and Tamer E. Fandy. 2016. "The Combination of Dimethoxycurcumin with DNA Methylation Inhibitor Enhances Gene Re-Expression of Promoter-Methylated Genes and

Antagonizes Their Cytotoxic Effect." *Epigenetics* 11 (10): 740–49. https://doi.org/10.1080/15592294.2016.1226452.

Hatcher, H., R. Planalp, J. Cho, F. M. Torti, and S. V. Torti. 2008. "Curcumin: From Ancient Medicine to Current Clinical Trials." *Cellular and Molecular Life Sciences: CMLS* 65 (11): 1631–52. https://doi.org/10.1007/s00018-008-7452-4.

Hayden, M S, A P West, and S Ghosh. 2006. "NF-KB and the Immune Response." *Oncogene* 25 (51): 6758–80. https://doi.org/10.1038/sj.onc.1209943.

Heitz, Mark P., and Jason W. Rupp. 2018. "Determining Mushroom Tyrosinase Inhibition by Imidazolium Ionic Liquids: A Spectroscopic and Molecular Docking Study." *International Journal of Biological Macromolecules* 107 (Pt B): 1971–81. https://doi.org/10.1016/j.ijbiomac.2017.10.066.

HemaIswarya, S., and Mukesh Doble. 2006. "Potential Synergism of Natural Products in the Treatment of Cancer." *Phytotherapy Research* 20 (4): 239–49. https://doi.org/10.1002/ptr.1841.

Hoesel, Bastian, and Johannes A Schmid. 2013. "The Complexity of NF-KB Signaling in Inflammation and Cancer." *Molecular Cancer* 12 (1): 86. https://doi.org/10.1186/1476-4598-12-86.

Holt, Patrick A., Jonathan B. Chaires, and John O. Trent. 2008. "Molecular Docking of Intercalators and Groove-Binders to Nucleic Acids Using Autodock and Surflex." *Journal of Chemical Information and Modeling* 48 (8): 1602–15. https://doi.org/10.1021/ci800063v.

Howell, Jennifer C., Eugene Chun, Annie N. Farrell, Elizabeth Y. Hur, Courtney M. Caroti, P. Michael Iuvone, and Rashidul Haque. 2013. "Global MicroRNA Expression Profiling: Curcumin (Diferuloylmethane) Alters Oxidative Stress-Responsive MicroRNAs in Human ARPE-19 Cells." *Molecular Vision* 19: 544–60.

Imlay, James A., Ramakrishnan Sethu, and Sanjay Kumar Rohaun. 2019. "Evolutionary Adaptations That Enable Enzymes to Tolerate Oxidative Stress." *Free Radical Biology and Medicine*, February. https://doi.org/10.1016/j.freeradbiomed.2019.01.048.

Jafarpour, SeyedMasoud, Mehdi Safaei, Mehran Mohseni, Morteza Salimian, Akbar Aliasgharzadeh, and Bagher Fahood. 2018. "The Radioprotective Effects of Curcumin and Trehalose Against Genetic Damage Caused By I-131." *Indian Journal of Nuclear Medicine* 33 (2): 99. https://doi.org/10.4103/ijnm.IJNM_158_17.

Jayakumar, Sundarraj, R. S. Patwardhan, Debojyoti Pal, Deepak Sharma, and Santosh K. Sandur. 2016. "Dimethoxycurcumin, a Metabolically Stable Analogue of Curcumin Enhances the Radiosensitivity of Cancer Cells:

Possible Involvement of ROS and Thioredoxin Reductase." *Biochemical and Biophysical Research Communications* 478 (1): 446–54. https://doi.org/10.1016/j.bbrc.2016.06.144.

Jeong, Sun-Oh, Gi-Su Oh, Hun-Yong Ha, Bon Soon Koo, Hak Sung Kim, Youn-Chul Kim, Eun-Cheol Kim, Kang-Min Lee, Hun-Taeg Chung, and Hyun-Ock Pae. 2009. "Dimethoxycurcumin, a Synthetic Curcumin Analogue, Induces Heme Oxygenase-1 Expression through Nrf2 Activation in RAW264.7 Macrophages." *Journal of Clinical Biochemistry and Nutrition* 44 (1): 79–84. https://doi.org/10.3164/jcbn.08-194.

Ji, Hong-Fang, Xue-Juan Li, and Hong-Yu Zhang. 2009. "Natural Products and Drug Discovery. Can Thousands of Years of Ancient Medical Knowledge Lead Us to New and Powerful Drug Combinations in the Fight against Cancer and Dementia?" *EMBO Reports* 10 (3): 194–200. https://doi.org/10.1038/embor.2009.12.

Jordan, Brian C., Charlotta D. Mock, Ramasamy Thilagavathi, and Chelliah Selvam. 2016. "Molecular Mechanisms of Curcumin and Its Semisynthetic Analogues in Prostate Cancer Prevention and Treatment." *Life Sciences* 152 (May): 135–44. https://doi.org/10.1016/j.lfs.2016.03.036.

Jovanovic, Slobodan V., Steen Steenken, Charles W. Boone, and Michael G. Simic. 1999. "H-Atom Transfer Is A Preferred Antioxidant Mechanism of Curcumin." *Journal of the*

American Chemical Society 121 (41): 9677–81. https://doi.org/10.1021/ja991446m.

Kalpana, C., and V. P. Menon. 2004. "Modulatory Effects of Curcumin on Lipid Peroxidation and Antioxidant Status during Nicotine-Induced Toxicity." *Polish Journal of Pharmacology* 56 (5): 581–86.

Kalyanaraman, Narayanan. 2018. "In Silico Prediction of Potential Vaccine Candidates on Capsid Protein of Human Bocavirus 1." *Molecular Immunology* 93: 193–205. https://doi.org/10.1016/j.molimm.2017.11.024.

Kitchen, Douglas B., Hélène Decornez, John R. Furr, and Jürgen Bajorath. 2004. "Docking and Scoring in Virtual Screening for Drug Discovery: Methods and Applications." *Nature Reviews. Drug Discovery* 3 (11): 935–49. https://doi.org/10.1038/nrd1549.

Kiuchi, F., Y. Goto, N. Sugimoto, N. Akao, K. Kondo, and Y. Tsuda. 1993. "Nematocidal Activity of Turmeric: Synergistic Action of Curcuminoids." *Chemical & Pharmaceutical Bulletin* 41 (9): 1640–43.

Kowaltowski, Alicia J., Nadja C. de Souza-Pinto, Roger F. Castilho, and Anibal E. Vercesi. 2009. "Mitochondria and Reactive Oxygen Species." *Free Radical Biology & Medicine* 47 (4): 333–43. https://doi.org/10.1016/j.freeradbiomed.2009.05.004.

Kunwar, A., A. Barik, B. Mishra, K. Rathinasamy, R. Pandey, and K.I. Priyadarsini. 2008. “Quantitative Cellular Uptake, Localization and Cytotoxicity of Curcumin in Normal and Tumor Cells.” *Biochimica et Biophysica Acta (BBA) - General Subjects* 1780 (4): 673–79. https://doi.org/10.1016/j.bbagen.2007.11.016.

Kunwar, A., S. Jayakumar, A. K. Srivastava, and K. I. Priyadarsini. 2012. “Dimethoxycurcumin-Induced Cell Death in Human Breast Carcinoma MCF7 Cells: Evidence for pro-Oxidant Activity, Mitochondrial Dysfunction, and Apoptosis.” *Archives of Toxicology* 86 (4): 603–14. https://doi.org/10.1007/s00204-011-0786-y.

Kuttan, R., P. Bhanumathy, K. Nirmala, and M. C. George. 1985. “Potential Anticancer Activity of Turmeric (Curcuma Longa).” *Cancer Letters* 29 (2): 197–202.

Leclercq, Isabelle A., Geoffrey C. Farrell, Christine Sempoux, Aileen dela Peña, and Yves Horsmans. 2004. “Curcumin Inhibits NF-KappaB Activation and Reduces the Severity of Experimental Steatohepatitis in Mice.” *Journal of Hepatology* 41 (6): 926–34. https://doi.org/10.1016/j.jhep.2004.08.010.

Lee, Hwan Hee, and Hyosun Cho. 2018. “Improved Anti-Cancer Effect of Curcumin on Breast Cancer Cells by Increasing the Activity of Natural Killer Cells.” *Journal of Microbiology and Biotechnology* 28 (6): 874–82. https://doi.org/10.4014/jmb.1801.01074.

Lee, Jea Whan, Hye Min Hong, Dong Deuk Kwon, Hyun-Ock Pae, and Hee Jong Jeong. 2010. “Dimethoxycurcumin, a Structural Analogue of Curcumin, Induces Apoptosis in Human Renal Carcinoma Caki Cells through the Production of Reactive Oxygen Species, the Release of Cytochrome C, and the Activation of Caspase-3.” *Korean Journal of Urology* 51 (12): 870–78. https://doi.org/10.4111/kju.2010.51.12.870.

Lengauer, T., and M. Rarey. 1996. “Computational Methods for Biomolecular Docking.” *Current Opinion in Structural Biology* 6 (3): 402–6.

Li, Xiao-Ling, Yan-Jun Hu, Ran Mi, Xiao-Yun Li, Pei-Qi Li, and Yu Ouyang. 2013. “Spectroscopic Exploring the Affinities, Characteristics, and Mode of Binding Interaction of Curcumin with DNA.” *Molecular Biology Reports* 40 (7): 4405–13. https://doi.org/10.1007/s11033-013-2530-6.

Liguori, Ilaria, Gennaro Russo, Francesco Curcio, Giulia Bulli, Luisa Aran, David Della-Morte, Gaetano Gargiulo, et al. 2018. “Oxidative Stress, Aging, and Diseases.” *Clinical Interventions in Aging* Volume 13 (April): 757–72. https://doi.org/10.2147/CIA.S158513.

Liou, Geou-Yarh, and Peter Storz. 2010. “Reactive Oxygen Species in Cancer.” *Free Radical Research* 44 (5): 479–96. https://doi.org/10.3109/10715761003667554.

Liu, Ting, Lingyun Zhang, Donghyun Joo, and Shao-Cong Sun. 2017. "NF-KB Signaling in Inflammation." *Signal Transduction and Targeted Therapy* 2 (July): 17023. https://doi.org/10.1038/sigtrans.2017.23.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. "Protein Measurement with the Folin Phenol Reagent." *The Journal of Biological Chemistry* 193 (1): 265–75.

Lu, Jinsheng, Weisong Duan, Yansu Guo, Hong Jiang, Zhongyao Li, Jing Huang, Kun Hong, and Chunyan Li. 2012. "Mitochondrial Dysfunction in Human TDP-43 Transfected NSC34 Cell Lines and the Protective Effect of Dimethoxy Curcumin." *Brain Research Bulletin* 89 (5–6): 185–90. https://doi.org/10.1016/j.brainresbull.2012.09.005.

Machado, Francielly Cristina, Renata Prandini Adum de Matos, Fernando Lucas Primo, Antonio Claudio Tedesco, Paula Rahal, and Marília Freitas Calmon. 2019. "Effect of Curcumin-Nanoemulsion Associated with Photodynamic Therapy in Breast Adenocarcinoma Cell Line." *Bioorganic & Medicinal Chemistry* 27 (9): 1882–90. https://doi.org/10.1016/j.bmc.2019.03.044.

Mahady, G. B., S. L. Pendland, G. Yun, and Z. Z. Lu. 2002. "Turmeric (Curcuma Longa) and Curcumin Inhibit the Growth of Helicobacter Pylori, a Group 1 Carcinogen." *Anticancer Research* 22 (6C): 4179–81.

Maheshwari, Radha K., Anoop K. Singh, Jaya Gaddipati, and Rikhab C. Srimal. 2006. “Multiple Biological Activities of Curcumin: A Short Review.” *Life Sciences* 78 (18): 2081–87. https://doi.org/10.1016/j.lfs.2005.12.007.

Manju, M., T. G. Sherin, K. N. Rajeesha, P. Sreejith, K. N. Rajasekharan, and O. V. Oommen. 2008. “Curcumin and Its Derivatives Prevent Hepatocyte Lipid Peroxidation in *Anabas Testudineus*.” *Journal of Fish Biology* 73 (7): 1701–13. https://doi.org/10.1111/j.1095-8649.2008.02044.x.

Martínez-Cayuela, M. 1995. “Oxygen Free Radicals and Human Disease.” *Biochimie* 77 (3): 147–61.

Matés, J. M. 2000. “Effects of Antioxidant Enzymes in the Molecular Control of Reactive Oxygen Species Toxicology.” *Toxicology* 153 (1–3): 83–104.

Matés, J. M., C. Pérez-Gómez, and I. Núñez de Castro. 1999. “Antioxidant Enzymes and Human Diseases.” *Clinical Biochemistry* 32 (8): 595–603.

Moballegh Nasery, Mahshid, Banafshe Abadi, Delaram Poormoghadam, Ali Zarrabi, Peyman Keyhanvar, Hashem Khanbabaei, Milad Ashrafizadeh, Reza Mohammadinejad, Shima Tavakol, and Gautam Sethi. 2020. “Curcumin Delivery Mediated by Bio-Based Nanoparticles: A Review.” *Molecules* 25 (3): 689. https://doi.org/10.3390/molecules25030689.

Mofidi Najjar, Fayezeh, Rahim Ghadari, Reza Yousefi, Naser Safari, Vahid Sheikhhasani, Nader Sheibani, and Ali Akbar Moosavi-Movahedi. 2017. "Studies to Reveal the Nature of Interactions between Catalase and Curcumin Using Computational Methods and Optical Techniques." *International Journal of Biological Macromolecules* 95 (February): 550–56. https://doi.org/10.1016/j.ijbiomac.2016.11.050.

Mohamad Rosdi, Mohamad Norisham, Shahkila Mohd Arif, Mohamad Hafizi Abu Bakar, Siti Aisyah Razali, Razauden Mohamed Zulkifli, and Harisun Ya'akob. 2018. "Molecular Docking Studies of Bioactive Compounds from Annona Muricata Linn as Potential Inhibitors for Bcl-2, Bcl-w and Mcl-1 Antiapoptotic Proteins." *Apoptosis: An International Journal on Programmed Cell Death* 23 (1): 27–40. https://doi.org/10.1007/s10495-017-1434-7.

Morgan, Michael J., and Zheng-gang Liu. 2011. "Crosstalk of Reactive Oxygen Species and NF-KB Signaling." *Cell Research* 21 (1): 103–15. https://doi.org/10.1038/cr.2010.178.

Nafisi, Shohreh, Maryam Adelzadeh, Zeinab Norouzi, and Mohammad Nabi Sarbolouki. 2009. "Curcumin Binding to DNA and RNA." *DNA and Cell Biology* 28 (4): 201–8. https://doi.org/10.1089/dna.2008.0840.

Nagaraju, Ganji Purnachandra, Leah Benton, Shipra Reddy Bethi, Mamoru Shoji, and Bassel F. El-Rayes. 2018. “Curcumin Analogs: Their Roles in Pancreatic Cancer Growth and Metastasis.” *International Journal of Cancer*, December. https://doi.org/10.1002/ijc.31867.

Niki, Etsuo. 2010. “Assessment of Antioxidant Capacity in Vitro and in Vivo.” *Free Radical Biology & Medicine* 49 (4): 503–15. https://doi.org/10.1016/j.freeradbiomed.2010.04.016.

Oeckinghaus, A., and S. Ghosh. 2009. “The NF- B Family of Transcription Factors and Its Regulation.” *Cold Spring Harbor Perspectives in Biology* 1 (4): a000034–a000034. https://doi.org/10.1101/cshperspect.a000034.

Ohkawa, H., N. Ohishi, and K. Yagi. 1979. “Assay for Lipid Peroxides in Animal Tissues by Thiobarbituric Acid Reaction.” *Analytical Biochemistry* 95 (2): 351–58.

Pae, Hyun-Ock, Sun-Oh Jeong, Hak Sung Kim, Seong Heak Kim, Yung Sun Song, Sung-kwun Kim, Kyu-Yun Chai, and Hun-Taeg Chung. 2008. “Dimethoxycurcumin, a Synthetic Curcumin Analogue with Higher Metabolic Stability, Inhibits NO Production, Inducible NO Synthase Expression and NF-KappaB Activation in RAW264.7 Macrophages Activated with LPS.” *Molecular Nutrition & Food Research* 52 (9): 1082–91. https://doi.org/10.1002/mnfr.200700333.

Patwardhan, Raghavendra S., Rahul Checker, Deepak Sharma, Vineet Kohli, K. I. Priyadarsini, and Santosh K. Sandur. 2011. “Dimethoxycurcumin, a Metabolically Stable Analogue of Curcumin, Exhibits Anti-Inflammatory Activities in Murine and Human Lymphocytes.” *Biochemical Pharmacology* 82 (6): 642–57. https://doi.org/10.1016/j.bcp.2011.06.024.

Pérez, Viviana I., Alex Bokov, Holly Van Remmen, James Mele, Qitao Ran, Yuji Ikeno, and Arlan Richardson. 2009. “Is the Oxidative Stress Theory of Aging Dead?” *Biochimica et Biophysica Acta (BBA) - General Subjects* 1790 (10): 1005–14. https://doi.org/10.1016/j.bbagen.2009.06.003.

Pompella, Alfonso, Athanase Visvikis, Aldo Paolicchi, Vincenzo De Tata, and Alessandro F. Casini. 2003. “The Changing Faces of Glutathione, a Cellular Protagonist.” *Biochemical Pharmacology* 66 (8): 1499–1503.

Priyadarsini, K. Indira. 2009. “Photophysics, Photochemistry and Photobiology of Curcumin: Studies from Organic Solutions, Bio-Mimetics and Living Cells.” *Journal of Photochemistry and Photobiology C: Photochemistry Reviews* 10 (2): 81–95. https://doi.org/10.1016/j.jphotochemrev.2009.05.001.

———. 2013. “Chemical and Structural Features Influencing the Biological Activity of Curcumin.” *Current Pharmaceutical Design* 19 (11): 2093–2100.

Priyadarsini, K. Indira, Dilip K. Maity, G. H. Naik, M. Sudheer Kumar, M. K. Unnikrishnan, J. G. Satav, and Hari Mohan. 2003. “Role of Phenolic O-H and Methylene Hydrogen on the Free Radical Reactions and Antioxidant Activity of Curcumin.” *Free Radical Biology & Medicine* 35 (5): 475–84.

Rhee, Sue Goo. 2006. “Cell Signaling. H2O2, a Necessary Evil for Cell Signaling.” *Science (New York, N.Y.)* 312 (5782): 1882–83. https://doi.org/10.1126/science.1130481.

Ruby, A. J., G. Kuttan, K. D. Babu, K. N. Rajasekharan, and R. Kuttan. 1995. “Anti-Tumour and Antioxidant Activity of Natural Curcuminoids.” *Cancer Letters* 94 (1): 79–83.

Sahoo, Bijaya Ketan, Kalyan Sundar Ghosh, Rabindranath Bera, and Swagata Dasgupta. 2008. “Studies on the Interaction of Diacetylcurcumin with Calf Thymus-DNA.” *Chemical Physics* 351 (1–3): 163–69. https://doi.org/10.1016/j.chemphys.2008.05.008.

Sandur, Santosh K., Manoj K. Pandey, Bokyung Sung, Kwang Seok Ahn, Akira Murakami, Gautam Sethi, Pornngarm Limtrakul, Vladimir Badmaev, and Bharat B. Aggarwal. 2007. “Curcumin, Demethoxycurcumin, Bisdemethoxycurcumin, Tetrahydrocurcumin and Turmerones Differentially Regulate Anti-Inflammatory and Anti-Proliferative Responses through a ROS-Independent Mechanism.” *Carcinogenesis* 28 (8): 1765–73. https://doi.org/10.1093/carcin/bgm123.

Sarma, Himakshi, and Venkata Satish Kumar Mattaparthi. 2018. "Structure-Based Virtual Screening of High-Affinity ATP-Competitive Inhibitors Against Human Lemur Tyrosine Kinase-3 (LMTK3) Domain: A Novel Therapeutic Target for Breast Cancer." *Interdisciplinary Sciences: Computational Life Sciences*, July. https://doi.org/10.1007/s12539-018-0302-7.

Seifried, Harold E., Darrell E. Anderson, Evan I. Fisher, and John A. Milner. 2007. "A Review of the Interaction among Dietary Antioxidants and Reactive Oxygen Species." *The Journal of Nutritional Biochemistry* 18 (9): 567–79. https://doi.org/10.1016/j.jnutbio.2006.10.007.

Senthil K, and Sarojini R. 2009. "Curcumin-DNA Interaction Studied by Fourier Transform Infrared Spectroscopy." *International Journal of Biotechnology & Biochemistry* 5: 251–59.

Sharma, O. P. 1976. "Antioxidant Activity of Curcumin and Related Compounds." *Biochemical Pharmacology* 25 (15): 1811–12.

Shu, Zunpeng, Yanni Yang, Liu Yang, Hai Jiang, Xiaojin Yu, and Yi Wang. 2019. "Cardioprotective Effects of Dihydroquercetin against Ischemia Reperfusion Injury by Inhibiting Oxidative Stress and Endoplasmic Reticulum Stress-Induced Apoptosis *via* the PI3K/Akt Pathway." *Food & Function* 10 (1): 203–15. https://doi.org/10.1039/C8FO01256C.

Sies, H. 1997. “Oxidative Stress: Oxidants and Antioxidants.” *Experimental Physiology* 82 (2): 291–95.

Simon, A., D. P. Allais, J. L. Duroux, J. P. Basly, S. Durand-Fontanier, and C. Delage. 1998. “Inhibitory Effect of Curcuminoids on MCF-7 Cell Proliferation and Structure-Activity Relationships.” *Cancer Letters* 129 (1): 111–16.

Singh, S., and B. B. Aggarwal. 1995. “Activation of Transcription Factor NF-Kappa B Is Suppressed by Curcumin (Diferuloylmethane) [Corrected].” *The Journal of Biological Chemistry* 270 (42): 24995–0.

Solecki, R. S. 1975. “Shanidar IV, a Neanderthal Flower Burial in Northern Iraq.” *Science* 190 (4217): 880–81. https://doi.org/10.1126/science.190.4217.880.

Sreejayan, null, and M. N. Rao. 1994. “Curcuminoids as Potent Inhibitors of Lipid Peroxidation.” *The Journal of Pharmacy and Pharmacology* 46 (12): 1013–16.

Srimal, R. C., and B. N. Dhawan. 1973. “Pharmacology of Diferuloyl Methane (Curcumin), a Non-Steroidal Anti-Inflammatory Agent.” *The Journal of Pharmacy and Pharmacology* 25 (6): 447–52.

Stohs, S. J., and D. Bagchi. 1995. “Oxidative Mechanisms in the Toxicity of Metal Ions.” *Free Radical Biology & Medicine* 18 (2): 321–36.

Strimpakos, Alexios S., and Ricky A. Sharma. 2008. “Curcumin: Preventive and Therapeutic Properties in Laboratory

Studies and Clinical Trials." *Antioxidants & Redox Signaling* 10 (3): 511–45. https://doi.org/10.1089/ars.2007.1769.

Sugiyama, Y., S. Kawakishi, and T. Osawa. 1996. "Involvement of the Beta-Diketone Moiety in the Antioxidative Mechanism of Tetrahydrocurcumin." *Biochemical Pharmacology* 52 (4): 519–25.

Sun, You-Min, Hong-Yu Zhang, De-Zhan Chen, and Cheng-Bu Liu. 2002. "Theoretical Elucidation on the Antioxidant Mechanism of Curcumin: A DFT Study." *Organic Letters* 4 (17): 2909–11.

Suśruta, Y.T. Ācārya, and N.R. Acharya. 1998. *Suśrutasaṃhitā*. Kṛshṇadāsa Āyurveda Sīrīja. Kṛṣṇadāsa Akādamī. https://books.google.co.in/books?id=4hXbAAAAMAAJ.

T. Masuda, T. Maekawa, K. Hidaka, H. Bando, Y. Takeda, and H. Yamaguchi. 2001. "Chemical Studies on Antioxidant Mechanism of Curcumin: Analysis of Oxidative Coupling Products from Curcumin and Linoleate." *Journal of Agricultural and Food Chemistry* 49 (5): 2539–47.

Tak, Paul P., and Gary S. Firestein. 2001. "NF-KB: A Key Role in Inflammatory Diseases." *Journal of Clinical Investigation* 107 (1): 7–11. https://doi.org/10.1172/JCI11830.

Tamvakopoulos, Constantin, Konstantinos Dimas, Zacharias D. Sofianos, Sophia Hatziantoniou, Zhiyong Han, Zhong-Li

Liu, James H. Wyche, and Panayotis Pantazis. 2007. “Metabolism and Anticancer Activity of the Curcumin Analogue, Dimethoxycurcumin.” *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 13 (4): 1269–77. https://doi.org/10.1158/1078-0432.CCR-06-1839.

Tangvarasittichai, Orathai, and Surapon Tangvarasittichai. 2019. “Oxidative Stress, Ocular Disease and Diabetes Retinopathy.” *Current Pharmaceutical Design* 25 (January). https://doi.org/10.2174/1381612825666190115121531.

Teymouri, Manouchehr, Nastaran Barati, Matteo Pirro, and Amirhosein Sahebkar. 2018. “Biological and Pharmacological Evaluation of Dimethoxycurcumin: A Metabolically Stable Curcumin Analogue with a Promising Therapeutic Potential.” *Journal of Cellular Physiology* 233 (1): 124–40. https://doi.org/10.1002/jcp.25749.

Valko, Marian, Dieter Leibfritz, Jan Moncol, Mark T. D. Cronin, Milan Mazur, and Joshua Telser. 2007. “Free Radicals and Antioxidants in Normal Physiological Functions and Human Disease.” *The International Journal of Biochemistry & Cell Biology* 39 (1): 44–84. https://doi.org/10.1016/j.biocel.2006.07.001.

Viña, Jose. 2019. “The Free Radical Theory of Frailty: Mechanisms and Opportunities for Interventions to

Promote Successful Aging." *Free Radical Biology and Medicine*, February. https://doi.org/10.1016/j.freeradbiomed.2019.01.045.

Vyas, Alok, Prasad Dandawate, Subhash Padhye, Aamir Ahmad, and Fazlul Sarkar. 2013. "Perspectives on New Synthetic Curcumin Analogs and Their Potential Anticancer Properties." *Current Pharmaceutical Design* 19 (11): 2047–69.

Wang, RuiBin, and Xue ChengHu. 2010. "Study on Interaction between Curcumin and DNA by Electrochemical Methods." *Progress in Modern Biomedicine* 10 (23): 4466–68.

Wang, Yan, Wei Qi, Li Zhang, Zhenguang Ying, Ou Sha, Chunman Li, Lanhai Lü, et al. 2017. "The Novel Targets of DL-3-n-Butylphthalide Predicted by Similarity Ensemble Approach in Combination with Molecular Docking Study." *Quantitative Imaging in Medicine and Surgery* 7 (5): 532–36. https://doi.org/10.21037/qims.2017.10.08.

Wei, Binqing Q., Larry H. Weaver, Anna M. Ferrari, Brian W. Matthews, and Brian K. Shoichet. 2004. "Testing a Flexible-Receptor Docking Algorithm in a Model Binding Site." *Journal of Molecular Biology* 337 (5): 1161–82. https://doi.org/10.1016/j.jmb.2004.02.015.

Wei, Xue-Qin, Juan-Fang Zhu, Xin-Bo Wang, and Kai Ba. 2020. "Improving the Stability of Liposomal Curcumin by

Adjusting the Inner Aqueous Chamber PH of Liposomes." *ACS Omega* 5 (2): 1120–26. https://doi.org/10.1021/acsomega.9b03293.

Wells, P, Y Bhuller, C Chen, W Jeng, S Kasapinovic, J Kennedy, P Kim, R Laposa, G Mccallum, and C Nicol. 2005. "Molecular and Biochemical Mechanisms in Teratogenesis Involving Reactive Oxygen Species." *Toxicology and Applied Pharmacology* 207 (2): 354–66. https://doi.org/10.1016/j.taap.2005.01.061.

Wong, Ee Tsin, and Vinay Tergaonkar. 2009. "Roles of NF-KB in Health and Disease: Mechanisms and Therapeutic Potential." *Clinical Science* 116 (6): 451–65. https://doi.org/10.1042/CS20080502.

Wright, James S. 2002. "Predicting the Antioxidant Activity of Curcumin and Curcuminoids." *Journal of Molecular Structure: THEOCHEM* 591 (1–3): 207–17. https://doi.org/10.1016/S0166-1280(02)00242-7.

Yang, Jiangke, and Zhenggang Han. 2018. "Understanding the Positional Binding and Substrate Interaction of a Highly Thermostable GH10 Xylanase from Thermotoga Maritima by Molecular Docking." *Biomolecules* 8 (3): 64. https://doi.org/10.3390/biom8030064.

Yen, Yu-Hsiu, Chi-Ming Pu, Chen-Wei Liu, Ya-Chun Chen, Yu-Chen Chen, Chan-Jung Liang, Jung-Hsien Hsieh, Hui-Fu Huang, and Yuh-Lien Chen. 2018. "Curcumin Accelerates Cutaneous Wound Healing via Multiple Biological

Actions: The Involvement of TNF-α, MMP-9, α-SMA, and Collagen." *International Wound Journal* 15 (4): 605–17. https://doi.org/10.1111/iwj.12904.

Yoon, M. J., Y. J. Kang, J. A. Lee, I. Y. Kim, M. A. Kim, Y. S. Lee, J. H. Park, et al. 2014. "Stronger Proteasomal Inhibition and Higher CHOP Induction Are Responsible for More Effective Induction of Paraptosis by Dimethoxycurcumin than Curcumin." *Cell Death & Disease* 5 (March): e1112. https://doi.org/10.1038/cddis.2014.85.

Yu, Yangyang, Qian Shen, Yihong Lai, Sun Y. Park, Xingmei Ou, Dongxu Lin, Meiling Jin, and Weizhen Zhang. 2018a. "Anti-Inflammatory Effects of Curcumin in Microglial Cells." *Frontiers in Pharmacology* 9: 386. https://doi.org/10.3389/fphar.2018.00386.

———. 2018b. "Anti-Inflammatory Effects of Curcumin in Microglial Cells." *Frontiers in Pharmacology* 9 (April). https://doi.org/10.3389/fphar.2018.00386.

Zaitsev, E. N., and S. C. Kowalczykowski. 1998. "Binding of Double-Stranded DNA by Escherichia Coli RecA Protein Monitored by a Fluorescent Dye Displacement Assay." *Nucleic Acids Research* 26 (2): 650–54.

Zhao, Huiying, Qingchun Liu, Saisai Wang, Fang Dai, Xiaofei Cheng, Xiaobin Cheng, Wenbin Chen, Min Zhang, and Dong Chen. 2017. "In Vitro Additive Antitumor Effects of Dimethoxycurcumin and 5-Fluorouracil in Colon Cancer

Cells." *Cancer Medicine* 6 (7): 1698–1706. https://doi.org/10.1002/cam4.1114.

Zhou, Hongyu, Christopher S. Beevers, and Shile Huang. 2011. "The Targets of Curcumin." *Current Drug Targets* 12 (3): 332–47.

Zhou, L. Z., A. P. Johnson, and T. A. Rando. 2001. "NF Kappa B and AP-1 Mediate Transcriptional Responses to Oxidative Stress in Skeletal Muscle Cells." *Free Radical Biology & Medicine* 31 (11): 1405–16.

Zsila, Ferenc, Zsolt Bikadi, and Miklos Simonyi. 2004. "Circular Dichroism Spectroscopic Studies Reveal PH Dependent Binding of Curcumin in the Minor Groove of Natural and Synthetic Nucleic Acids." *Organic & Biomolecular Chemistry* 2 (20): 2902–10. https://doi.org/10.1039/B409724F.

Zwart, L. L. de, J. H. Meerman, J. N. Commandeur, and N. P. Vermeulen. 1999. "Biomarkers of Free Radical Damage Applications in Experimental Animals and in Humans." *Free Radical Biology & Medicine* 26 (1–2): 202–26.