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Comparative *In silico/In vitro* Study of Synergistic Effect of Curcuminoids as Inhibitors of Breast/ Liver Cancer Cells

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Abstract

In the present work curcumin, demethoxycurcumin and bisdemethoxycurcumin have been synthesized and their inhibitory effects were studied on three different human breast cancer cell lines ZR-75, MDA-MB-231, HepG2 (Hepatocellular Carcinoma) and one normal cell line MCF10A. Their effects were compared with the cumulative effect of natural curcumin (Curcuminoid Mixture) through *in vitro* and *in silico* study. For *in vitro* screening, cytotoxicity analysis of curcuminoids was done in breast cancer cell lines, while changes in cellular and nuclear morphology were examined using phase contrast microscopy and Hoechst staining. Results obtained were further validated through *in silico* study, via examining role of seven major key regulatory proteins of breast cancer as targets of curcumin and curcuminoids. The individual curcuminoids and natural curcumin appear to act via different pathways causing apoptosis and necrosis. Based on the present study, it has been observed that synthetic curcumin, curcuminoids and their naturally occurring mixture significantly affect the molecular pathways of apoptosis and necrosis.

Keywords: Apoptosis; Breast cancer; Curcuminoids; FACS; Liver Cancer; Necrosis

Abbreviations:

AKT	:	Protein Kinase B, MTOR- Mammalian Target of Rapamycin,
BCL-2	:	B-Cell Lymphoma-2
Est	:	Oestrogen
ER	:	Oestrogen Receptor
EGFR	:	Epidermal Growth Factor Receptor
FADD	:	Fas-Associated protein with Death Domain
GRB2	:	Growth Factor Receptor-Bound Protein 2
GAB1	:	GRB2-Associated Binding Protein1
HER2	:	Human Epidermal Growth Factor Receptor 2
IKK	:	Inhibitor of nuclear factor Kappa-B Kinase
MAPK	:	Mitogen-activated Protein Kinases
MEK	:	Mitogen-activated protein kinase Kinases
NF-κB	:	Nuclear Factor κappa B
NIK	:	NF-κB-inducing Kinase
PI3K	:	Phosphatidylinositide 3-Kinase
PDK1	:	Phosphoinositide-dependent kinase-1
PAK1	:	p21-activated kinases
RTK	:	Receptor Tyrosine Kinase
RAS	:	'Rat sarcoma' protein
RAF	:	Rapidly Accelerated Fibrosarcoma' protein
RAC1	:	Ras related C3 botulinum toxin substrate 1
SOS	:	Son of seven less protein

TNF : Tumour Necrosis Factor

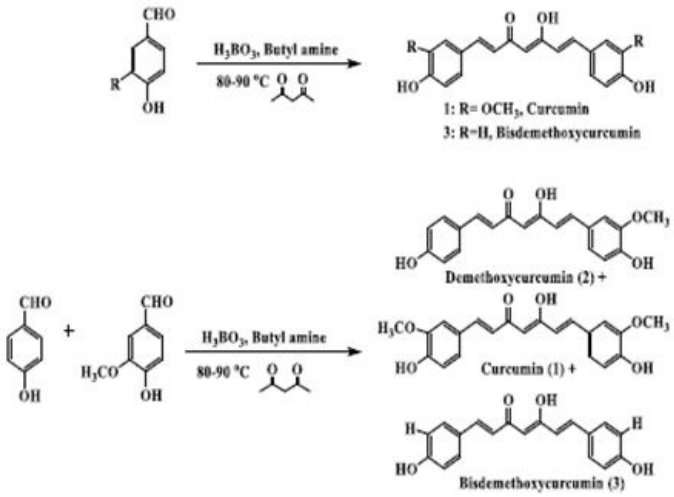
TNFR : TNF Receptor RIP Receptor Interacting Protein

Introduction

Breast cancer is a leading, recurrent cancer type comprising approximately 23% of all cancers in women [1]. Molecular alterations such as genetic abruptions and epigenetic mechanism like chromatin architectural changes or DNA methylation in breast cancer cells are currently exploited by target specific drugs [2]. Various drugs have been reported for single to multiple targets of breast cancer [3]. Curcumin, a bis-α, β-unsaturated β-diketone (Polyphenol), a major component of the rhizome of turmeric. Various experimental reports have also demonstrated its appreciable anti-cancerous activities in various types of cancers in which breast and liver cancers comprise a good percentage [4]. Curcumin (CUR) occurs in nature along with its analogues Demethoxycurcumin (DMC), Bisdemethoxycurcumin (BDMC) and cyclocurcumin, jointly classified as curcuminoid family. Nonetheless, majority of the reports about the therapeutic value of curcumin are actually based on the commercially available curcumin (~95%) which is actually a mixture of curcuminoids (CUR~75%, while DMC~10-20%, BDMC~<5%) [5-7]. Among various molecular modulators and pathways reported in breast cancer aetiology, Nuclear factor Kappa-B (NF-κB) pathway, Phosphatidylinositide 3-Kinase (PIK3) pathway, Mitogen Activated Protein Kinases (MAPK) pathway, comprise the most prominent candidates [8]. In addition, modulated activity of cancer markers and surface receptors like Epidermal Growth Factor Receptor (EGFR), Estrogen receptor-α (ER-α), Tumour necrosis factor receptor etc. is also notable. Interestingly, curcumin is reported as potent modulator of all these pathways and regulatory molecules in various studies, supporting its strong therapeutic candidature for different stages of breast cancer initiation and progression [9]. The role of each curcuminoid separately in biological activity was demonstrated by comparative analysis of inhibitory efficacy of synthetic CUR, BDMC and DMC, along with natural curcumin sample, on the growth of liver and breast cancer cell lines in a dose dependent manner via MTT assay, with IC50 values in the micro-molar range leading to cell death through apoptosis. Cellular and nuclear morphology was also observed by using phase contrast microscopy and Hoechst staining followed by Fluorescence Activated Cell Sorting (FACS) analysis for apoptotic and necrotic cell death induced by compounds. These molecular targets associated with breast cancer aetiology were analyzed employing molecular docking studies to draw a theoretical explanation of inhibitory mechanism of compounds in a comparative manner.

Materials and Methods Chemical synthesis

The melting points of all synthesized compounds were determined on a JSGW melting point apparatus and are uncorrected. 1H and 13C NMR spectra were recorded on a Bruker bio spin 400 MHz spectrometer, at 400 MHz and 100 MHz for 1H and 13C respectively. Chemical shifts are given in δ values and Tetramethylsilane (TMS) was used as internal standard. The 1H and 13C spectra are reported for all compounds. Value of Coupling constant (J) is reported in Hz. All the solvents and reagents were bought from Sigma, Merck or Loba Chemie companies and were of LR/AR grade. Dry solvents were either bought from Merck or were prepared as per standard methods. Aluminium based TLC (thin layer chromatography, UV254nm) plates were used to monitor reactions and were bought from Merck. To visualise spot of reactant and products either UV chamber (254 nm and 320 nm) or iodine or charring them at higher temperatures (100-120ºC) was used. Purification of products was carried out by either crystallization or silica gel column chromatography (60-120 or 100-200 mesh, Merck chemicals). Synthesis was carried out starting with corresponding aldehydes, acetyl acetone and catalytic amount of n-butyl amine according to the patent WO2007/110168 A1, as illustrated in Scheme 1[10].



Scheme 1: Synthesis of curcumin, demethoxycurcumin and Bisdemethoxycurcumin.

Computational Analysis

Target Preparation

3D coordinates of seven major key regulatory target molecules including Nuclear factor NF-κB p50 subunit (PDBID:1NFK, 2.3 Å), BCL-2 (PDBID: 4AQ3, 2.4 Å), MTOR (PDBID: 4JSX, 3.5 Å), ERK2 (PDBID: 2OJI, 2.6Å), PAK1(PDBID: 2HY8, 2.0 Å), EGFR (PDBID: 1M17, 2.6 Å) and ER- α (PDBID: 1ERR, 2.6 Å), were retrieved from Protein Data Bank (PDB). They were refined by proper bond order assignment, addition of missing disulfide bonds, proper hydrogen bond assignment, water removal (within 5Å vicinity of active site) and loop filling using OPLS2005 force field.

Ligand Preparation

All ligands used in study, were drawn using ChemDraw 14.0 and their two-dimensional structures were converted into three dimensional structures using LigPrep 2.4 (shipped by Schrödinger). All structures were also optimized and minimized using OPLS 2005 force field.

Docking Simulations

Docking simulations were performed using Glide program (Grid-based Ligand Docking with Energetics) of Schrödinger suit 2010.

Cell line Screening Analysis

All three synthesized curcuminoids (1-3) along with natural curcumin (CNAT, 4) were screened for their anticancerous property. For this purpose, three different human cancer cell lines ZR-75 (ER Positive Breast Cancer Cell Line), MDA-MB-231 (Breast Adenocarcinoma, Estrogen, Progesterone, Her2 Negative Cell Line), HepG2 (Hepatocellular Carcinoma) and one normal cell line MCF10A (Epithelial Breast Cells, ER Negative) were used. Cytotoxicity analysis was done via standard MTT assay and IC50 value were calculated after 24h incubation. Cell death was further confirmed through nuclear morphology and FACS analysis.

Results and Discussion

Yields, Melting Points and NMR Spectra of Synthetic Compounds

Compound (1) Curcumin, (1E, 6E)-1,7-bis(4-Hydroxy-3-methoxyphenyl)-hepta-1,6-diene-3,5-dione Yield 65%, m.p.: 181-183 ºC (lit 183-185ºC). 1H-NMR (Acetone D6) 3.83 (s,6H), 5.89 (s, 1H), 6.59 (d, J=15.6Hz, 2H), 6.63 (d, J=8Hz, 2H), 7.08 (dd, J=8.0 and 1.2Hz, 2H), 7.24 (d, 1.2Hz, 2H), 7.49 (d, J=15.6Hz, 2H); 13C-NMR (Acetone D6) 55.78, 101.10, 111.05, 115.70, 121.76, 123.26, 127.61, 140.85, 148.26, 149.50, 183.88.

Compound (2) DMC, (1E, 6E)-[1-(4-Hydroxy-3-methoxyphenyl)-7-(4-hydroxy)]-hepta-1,6-diene-3,5-dione Yield 30%, m.p.: 142-144 ºC (lit 146-147 ºC). 1H-NMR (Acetone D6) 3.81 (s,3H), 5.89 (s, 1H), 6.55 (d, J=15.6Hz, 2H), 6.60 (d, J=15.6Hz, 2H), 6.81 (m, 3H), 7.03 (d, 8.4Hz, 1H), 7.24 (s, 1H), 7.46 (d, J=8.8Hz, 1H) 7.49 (d, 3.2Hz, 1H), 7.53 (d, 2.8Hz, 1H); 13C-NMR (Acetone D6) 55.77, 101.19, 110.98, 115.70, 116.12, 116.24, 121.47, 121.72, 123.32, 127.09, 127.61, 130.43, 132.27, 140.54, 140.87, 148.26, 149.49, 160.01, 183.89.

Compound (3) BDMC, (1E, 6E)-1,7-bis(4-Hydroxyphenyl)-hepta-1,6-diene-3,5-dione Yield 50%, m.p.: 228-230 ºC (lit231-232 ºC). 1H-NMR (Acetone D6) 5.90 (s, 1H), 6.55 (d, J=16.0Hz, 2H), 6.81 (d, J=8.8Hz, 4H), 7.46 (d, J=8.8,4H), 7.50 (d, 16.0Hz, 2H); 13C-NMR (Acetone D6) 101.21, 116.25, 121.46, 127.09, 130.43, 140.55, 160.03, 183.93.

Docking Study on Target Proteins

Quantitative binding capacity of all compounds at p50 protein is tabulated in Table 1a. Best docking poses and protein-ligand hydrogen bonding interactions for test ligands are depicted in Figure 1a. They follow activity order of CUR>DMC>BDMC. Docking scores along with van der Waals, hydrogen bonding and electrophilic energy contributions made in docking at the active site of BCL-2 protein are tabulated in Table 1b and binding conformations of all three curcuminoids along with hydrogen bond interaction at active site as shown in Figure 1b. To explore curcumin's inhibition mechanistic insight all test ligands were docked at the active site of MTOR and docking scores are depicted in Table 1c and their binding conformations shown in Figure 1c. All compounds showed good binding affinity at active site of ERK2 owing to good hydrogen bond interactions with protein, shown by the docking scores of all compounds in Table 1d. In addition, binding conformations of ligands at the active site of ERK2 are shown in Figure 1d. Curcuminoids were docked at the active site of PAK1 and docking score with other energy contribution terms are tabulated in Table 1e. In addition, best docking conformations and hydrogen bond interactions between ligands and target residues are depicted in Figure 1e. Docking of curcuminoids was performed at the kinase domain of EGFR protein and comparative binding affinity of all compounds in terms of docking score is shown in Table 1f. Also, best binding conformations and hydrogen interactions made by corresponding ligands (1-3) with active residues of protein EGFR are shown in Figure 1f. Comparative docking scores of all curcuminoids, docked at ERα are tabulated in Table 1g and their best binding conformations attained by all curcuminoids are depicted in Figure 1g.

Title	XP GScore	XP LipophilicEvdW	XP Electro	XP HBond
CUR	-6.019	-1.848	-2.363	-2.009
DMC	-5.345	-1.66	-2.394	-1.31
BDMC	-4.829	-1.684	-2.218	-1.295

(a) p50

Title	XP GScore	XP LipophilicEvdW	XP Electro	XP HBond
CUR	-6.152	-4.358	-0.231	-1.743
DMC	-4.376	-2.27	-0.801	-1.31
BDMC	-4.143	-2.56	-0.731	-1.325

(b) BCL-2

Title	XP GScore	XP Lipophi-licEvdW	XP Electro	XP HBond
CUR	-9.246	-4.811	-0.421	-1.632
DMC	-7.434	-3.842	-0.308	-1.028
BDMC	-0.215	-4.527	-0.431	-1.27

(c) MTOR

Title	XP GScore	XP Lipophi-licEvdW	XP Electro	XP HBond
CUR	-8.225	-3.153	-1.808	-3.013
DMC	-7.926	-3.476	-1.597	-2.571
BDMC	-7.516	-3.092	-1.593	-2.545

(d) ERK2

Title	XP GScore	XP Lipo-philic EvdW	XP Electro	XP HBond
CUR	-9.767	-3.839	-1.28	-3.03
DMC	-6.521	-3.621	-0.881	-2.782
BDMC	-6.618	-3.157	-0.629	-2.651

(e) PAK1

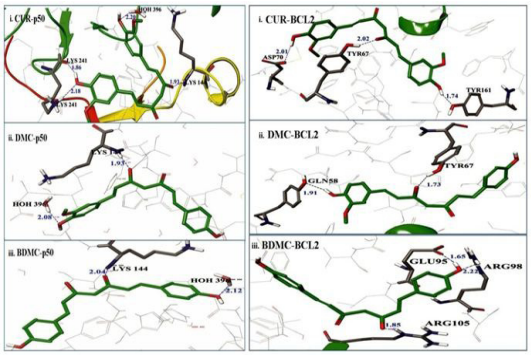
Title	XP GScore	XP Lipophi-licEvdW	XP Electro	XP HBond
CUR	-6.129	-2.785	-0.965	-1.745
DMC	-6.819	-2.746	-1.226	-1.969
BDMC	-6.447	-2.724	-1.227	-1.49

(f) EGFR

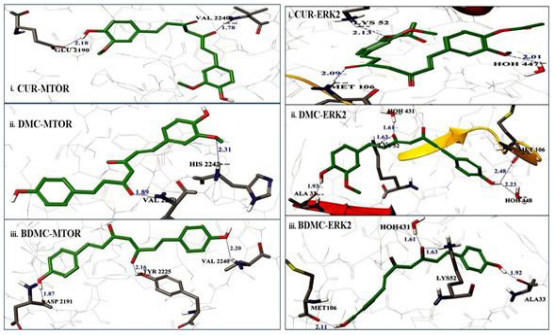
Title	XP GScore	XP Lipophi-licEvdW	XP Electro	XP HBond
CUR	-8.963	-4.741	-0.668	-1.812
DMC	-4.468	-4.157	-0.7	-2.048
BDMC	-3.552	-4.116	-0.17	-0.7

(g) ER-α

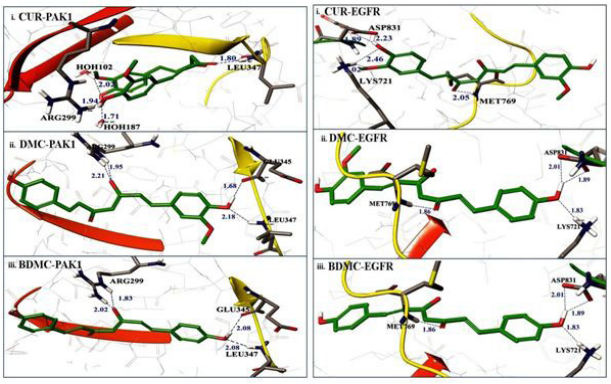
Table 1(a-g): Docking score and interaction parameters of test compounds with target proteins (a) p50; (b)BCL-2; (c)MTOR; (d)ERK2; (e)PAK1; (f)EGFR; (g)ER-α.



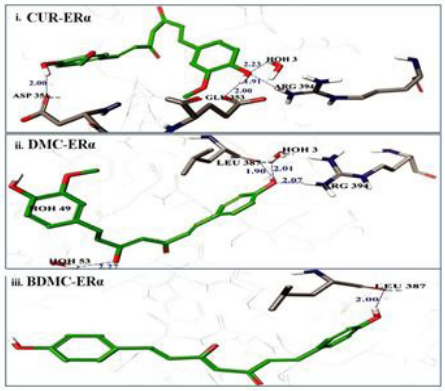
(a) p50 protein
(b) BCL-2 protein



(c) MTOR protein
(d) ERK2



(e) PAK1
(f) EGFR



(g) ER-α

Figure 1(a-g): Docking conformations of Curcuminoids at active site of target proteins (a) p50; (b)BCL-2; (c) MTOR; (d) ERK2; (e) PAK1; (f) EGFR; (g) ER-α.

In all three curcuminoids, viz., CUR BDMC and DMC central keto-enol moiety was found mainly involved in hydrogen bonding and salt bridge interaction with protein via LYS144 while phenolic hydroxyls were observed interacting with crystal embedded water HOH396. The important interacting residues participating in ligand binding are also tabulated in Table 2a. In case of BCL-2, CUR showed better binding than DMC and BDMC because of the presence of two methoxy groups which facilitate its better anchoring at hydrophobic pocket and better docking score while in case of DMC, presence of only one methoxy group lowers its binding than CUR while providing better binding over BDMC. Important interacting residues participating in ligand binding are also tabulated in Table 2b. All interacting residues at the active site of MTOR via different types of interaction with docked ligand is given in Table 2c. All ligands depicted hydrogen bonding with conserved LYS52 residue which is essential for catalytic function of ERK2. Detailed account of residues participating in various types of interactions of ERK2 with all three ligands is given in Table 2d. With PAK1 protein, curcumin is showing the highest binding affinity followed by DMC and BDMC. All residues interactions with corresponding docked ligand is enumerated in Table 2e. DMC and BDMC made two hydrogen bonds with ASP831 and one with LYS721 via their hydroxyl groups while CUR in addition, made an extra hydrogen bond with LYS721 via its methoxyl group, suggesting the crucial role of orthophenolic hydroxyl and methoxyl groups in curcuminoids for interacting with active site residues in EGFR. Crucial amino acids involved in ligand interaction are depicted in Table 2f. The active site residues of ER-α participating in interaction are enumerated in Table 2g.

S.N.	Ligand	Hydrogen Bonding	π-π Stacking	Hydrophobic Interactions	Charged/Polar Interactions
1	CUR	LYS144, LYS241, HOH396	HIS141	PRO243, ALA242, CYS59, VAL58, TYR57, LEU207	LYS241, HIS141, THR143, LYS144, LYS145, SER208, LYS241
2	DMC	LYS144, HOH396	TYR57	TYR57, LEU207, CYS59	ARG54, LYS241, LYS145, LYS144, THR143, SER208, HIS141,GLU60
3	BDMC	LYS144 (Hbond& Salt Bridge) HOH396		CYS59, LEU207, TYR57	THR143, SER208, HIS141, GLU60

(a) p50

S.N.	Ligand	Hydrogen Bonding	π-π Stacking	Hydrophobic Interactions	Charged/Polar Interactions
1.	CUR	TYR67, ASP70, TYR161	TYR67	ALA108, PHE157, TRP103, VAL107, LEU160, ALA59, TYR161, PHE63, TYR67, PHE71	ASP62, ARG66, ARG105, ASN102, ASP70
2.	DMC	TYR67, TYR161		ALA59, PHE63, TYR67, LEU96, TRP103, VAL107, PHE157, LEU160, TYR161	ASP62, ARG66, ASP70, ARG105

3.	BDMC	GLU95, ARG105 (Salt bridge)	-	PHE63, TYR67, PHE71, MET74, LEU96, ALA108, PHE112	ASP70, GLU95, ARG98, ASP99, ARG105

(b) BCL-2

S.N.	Ligand	Hydrogen bonding	π-π stacking	Hydrophobic interactions	Charged/polar Interactions
1.	CUR	ALA33, LYS52 MET106, LYS112, HOH431		LEU154,ILE82, CYS164, VAL37, MET36, ALA33, TYR34, ALA50, LEU105, ILE29, MET106	ASP109, ASP108, LYS112, GLN103, ASP104, GLU69, ASP165, GLU31, ARG65, LYS52, GLU107
2.	DMC	ALA33, LYS52,MET106, HOH431, HOH448,	TYR34	ILE29, MET106, LEU154, CYS164, ILE82, MET36, TYR34, ALA33, VAL37, ALA50, LEU105	LYS112, THR108, ASP109, GLN103, GLU69,LYS52, GLU31, ARG65,GLU107
3.	BDMC	LYS52, ALA33, MET106, HOH431		ILE29, LEU105, ALA50, VAL37, ILE82, CYS164, ALA33, TYR34, LEU154, MET106	THR108, GLN103, LYS52, GLU69, ASP165, ARG65, LYS112,

(c) MTOR

S.N.	Ligand	Hydrogen Bonding	π-π Stacking	Hydrophobic Interactions	Charged/Polar Interactions
1.	CUR	TYR67, ASP70, TYR161	TYR67	ALA108, PHE157, TRP103, VAL107, LEU160, ALA59, TYR161, PHE63, TYR67, PHE71	ASP62, ARG66, ARG105, ASN102, ASP70
2.	DMC	TYR67, TYR161		ALA59, PHE63, TYR67, LEU96, TRP103, VAL107, PHE157, LEU160, TYR161	ASP62, ARG66, ASP70, ARG105
3.	BDMC	GLU95, ARG105 (Salt bridge)	-	PHE63, TYR67, PHE71, MET74, LEU96, ALA108, PHE112	ASP70, GLU95, ARG98, ASP99, ARG105

(d) ERK2

S.N.	Ligand	Hydrogen Bonding	π-π Stacking	Hydrophobic interactions	Charged/Polar
1	CUR	ARG299, LEU347, HOH102, HOH187		VAL342,MET344,LEU347, TYR346,LEU396,VAL284, ILE276,ALA297	GLU345,GLN278,ASP393, SER351,THR406,ASP407, SER281, ARG299
2	DMC	ARG299, GLU345, LEU347		PHE410,VAL284,TYR346, LEU396,ILE276,LEU347, ALA297,MET344,VAL328, ALA280	GLU315, ASP407, SER281,GLN278, ARG299, GLU345, THR406

3	BDMC	ARG299, LEU347, GLU345		TYR346,LEU396,ILE276, LEU311,PHE410,ALA280, VAL284,NET344,VAL328, ALA297,LEU347	GLU345, THR406, ARG299, GUN278, SER281, ASP407, GLU315, GLU345

(e) PAK1

S.N	Ligand	Hydrogen bonding	π-π stacking	Hydrophobic interactions	Charged/polar Interactions
1.	CUR	GLU738, MET769	--	MET742, CYS751, LEU764, LEU768, LEU694, MET769, VAL702, ALA719, PRO770, CYS773, LEU820	LYS704,LYS721,GLU738THR766, THR830, ASP831,
2.	DMC	MET769,LYS721	--	LEU694, LEU768, MET769, VAL702, ALA719, PRO770, LEU820,	LYS692, LYS704,LYS721, THR766, GLN767
3.	BDMC	LYS721, MET769	--	LEU694, VAL702, LEU820, PRO770, MET769, LEU768, ALA719	LYS692, LYS704,LYS721, THR766, GLN767, ASP831

(f) EGFR

S.No.	Ligand	Hydrogen Bonding	π-π Stacking	Hydrophobic Interactions	Charged/polar Interactions
1	CUR	ASP351, GLU353, ARG394, HOH3	PHE404	LEU349, ALA350, PHE404, LEU384, LEU346, MET343, LEU525	ARG394, GLU353, ASP351, LYS529, THR347
2	DMC	LEU387, ARG394, HOH3, HOH53	PHE404	LEU346,ALA350,LEU349, LEU387, LEU391,MET388, LEU384, PHE404, MET421, LEU525, MET343,LEU354, TRP383, LEU539, LEU536	GLU353, ARG394, THR347, ASP351
3	BDMC	LEU387	PHE404	TRP383, ALA350, MET343, PHE404, LEU346, LEU349, LEU428, LEU391, LEU387, MET388, LEU384, LEU525, PRO535, LEU539, VAL533, LEU536,LEU354	ASP351, GLU353, ARG394, THR347, ASP351

(g) ER-α

Table 2(a-g): Interaction of Curcumin, Demethoxy and Bis-demedthoxy curcumin with active site residues of target proteins (a) p50; (b) BCL-2; (c) MTOR; (d) ERK2; (e) PAK1; (f) EGFR; (g) ER-α.

Cell Line Assays

For exploring differential behaviour of three curcuminoids, viz., CUR, BDMC and DMC, each was chemically synthesized separately and were studied against three cancerous and one non-cancerous cell lines, along with naturally extracted curcumin sample (CNAT) in comparative manner for the first time. Compounds exhibited activity in micro and milli molar level as depicted by their IC50value and % cell death histograms (Figure 2 and Table 3). Quantitative in vitro screening was performed by standard MTT assay and IC50 values were calculated after 24 h incubation and have been summarised in Table 3.

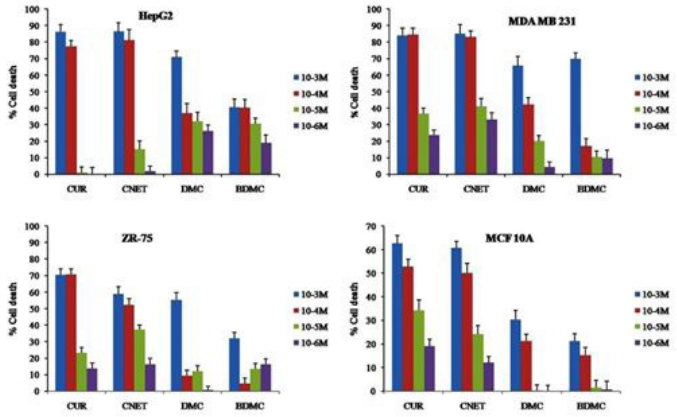


Figure 2: Bar graph showing percentage cell death after treatment of cells (HepG2, MDA-MB-231, ZR-75, MCF10A) with IC_{50} value with natural curcumin and synthetic curcuminoids.

S.N.	Compounds	Cancer Cell lines			Normal cells/Control
		ZR-75 cells	**MDA-MB-231 cell line** (Adenocarcinoma	**HepG2 cell line**	**MCF10A cell line** (ER^-/PR^-)
		(ER^+/PR^+ cells)	ER^-/PR^-)	(Hepatocellular Carcinoma)	
1	CUR	0.24µM	0.20 µM	0.43 µM	4.3mM
2	DMC	6 mM	12 mM	10.5 mM	20 mM
3	BDMC	27 mM	49 mM	3.39 mM	50 mM
4	CNAT	0.1 µM	0.15 µM	0.30 µM	8.3 µM

Table 3: Cytotoxicity (IC50 values in µM concentration) of synthesized curcuminoids and natural curcumin against human cancer cell lines.

CUR was more active (in micro molar range) than BDMC in all the three cancer cell lines. Among DMC and BDMC, former showed three to four-fold higher activity in both ER+ and ER- cells and approximately three-fold higher activity in hepatocarcinoma, while both depicted very moderate selectivity over normal cells.

Phase Contrast Analysis

Phase contrast pictures showing morphological details of cells after 24-hour incubation with each test ligand at the IC_{50} value are depicted for cell lines ZR-75, MDA-MB-231 and HepG2 in Figure 3a-c.

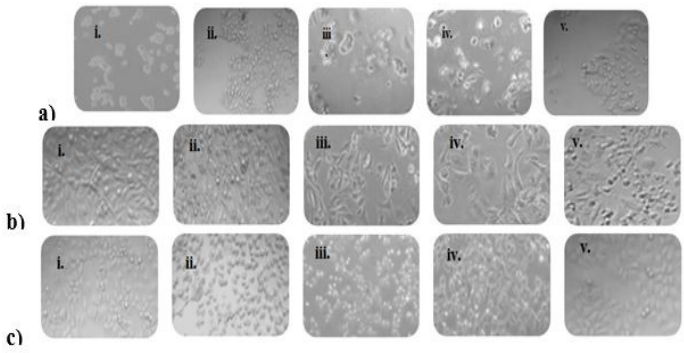


Figure 3(a-c): Phase contrast Photomicrograph of cell lines of: (a) ZR-75 (b) MDA-MB-231 and (c) HepG2. Compounds at IC_{50}; i. Control, ii. Curcumin, iii. Demethoxycurcumin, iv. Bisdemethoxycurcumin, v. Natural curcumin

Figure 3a depicts the control ZR-75 cells versus the cells treated with four test compounds 1: CUR, 2: DMC, 3: BDMC and 4: CNAT. Normal ZR-75 cells looked healthy glued to the substratum whereas treated cells appeared rounded which shows dead cells with maximum effect in compounds treated with test compounds conforming their anti-proliferative effect this cell line. Synthetic CUR and CNAT show more inhibitory effect as compared to Comp2 (DMC) and 3 (BDMC) in ZR-75 cells as depictive of their IC_{50} value. Figure 3b depicts the control MDA-MB-231cells versus the cells treated with compound 1-4 at IC_{50} respective value. Such morphology depicts dead cells with maximum effect in cells treated with compounds 1-4 which is the sign of anti-proliferative effect of these compounds in this cell line. Figure 3c depicts the control HepG2 cells versus the cells treated with compound 1-4 at IC_{50} respective value. BDMC and DMC compounds showed moderate dead cells while CNAT showed significant cell death which have acquired round morphology and are detached from the substratum. CUR along with BDMC and DMC depicted bilobic, fragmented and flattened nuclei with good signs of distorted nuclear morphology to confirm the cell death.

Hoechst staining (Figure 4).

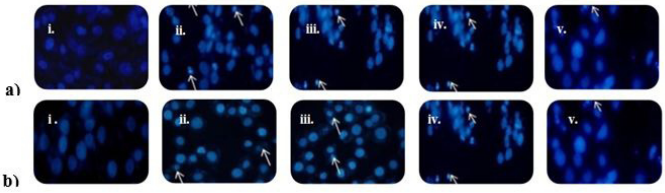


Figure 4: Hoechst staining (40 X) after 24 h of treatment with test compounds at IC_{50} value in cell lines of (a) MDAMB- 231 (b) HepG2; Compounds at IC_{50}: i. Control, ii. Curcumin, iii. Demethoxycurcumin, iv. Bisdemethoxycurcumin, v. Natural curcumin.

To further quantify the extent of apoptosis, HepG2 cells were co-labelled with annexin and PI after treatment with compounds 1-4 for 24 h. Compounds 1-3 showed maximum degree of apoptosis up to 95% as shown by annexin positive cells whereas compound 4 i.e. natural curcumin showed necrosis as a mode of cell death (Figure 5 and 6).

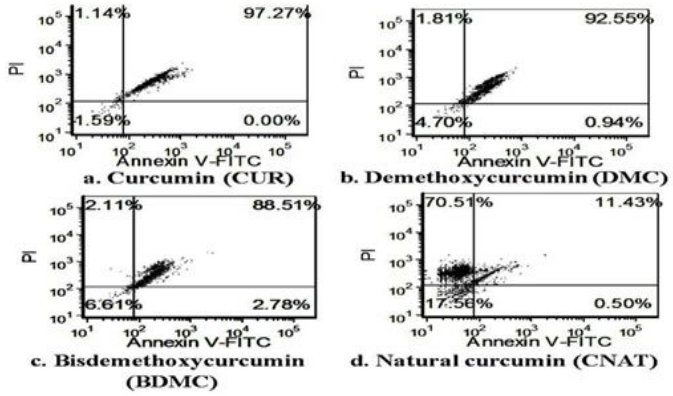


Figure 5: FACS labelling of test compounds (at IC_{50} value): a. Control, b. Curcumin, c. Demethoxycurcumin, d. Bisdemethoxycurcumin, e. Natural curcumin treated HepG2 cells with annexin and **PI.**

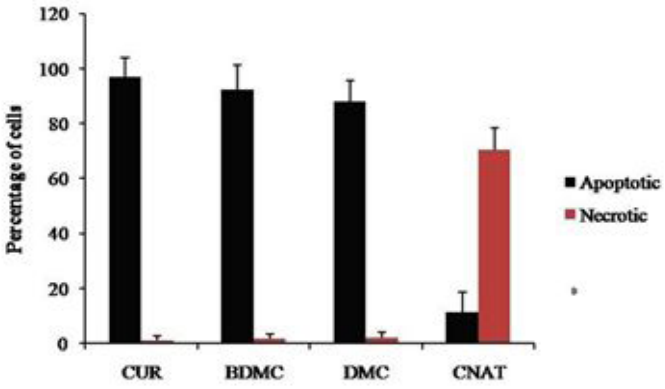


Figure 6: Histograms showing the quantitative analysis of annexin / pico- labelling at IC_{50} value in HepG2 treated with test compounds: a. Curcumin, b. Demethoxycurcumin, c. Bisdemethoxycurcumin, d. Natural curcumin; through FACS.

Conclusion

Synthetic curcuminoids have been tested individually for anti breast/liver cancer activity on human cell lines HepG2, MDA-MB-231, ZR-75 and MCF10A. As a result, DMC with only one methoxy group and BDMC having no methoxy group have been found to be relatively lesser active than curcumin in the sequence CUR>DMC>BMC. The results were validated with *in silico* studies. However, the results obtained from FACS analysis clearly indicate that synthetic curcumin and curcuminoids (DMC, BMC) independently cause apoptosis by mitochondrial pathway, but the mixture of all (Natural curcumin, CNAT) appears to cause necro-

sis indicating a different pathway of activity, possibly following TNFα and PAK1 mediated pathway as shown in computational study also.

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Conflict of Interest

All authors confirm that there is no conflict of interest amongst them.

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