INDUCTION OF APOPTOSIS BY SULFORAPHANE AND CURCUMIN AND EXPRESSION PROFILE OF Jmjd3, A KEY HISTONE DEMETHYLASE IN BREAST CANCER CELL LINE MDA-MB-231

THESIS SUBMITTED TO

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CERTIFICATE

This is to certify that the thesis entitled ***"Induction of apoptosis by sulforaphane and curcumin and expression profile of jmjd3, a key histone demethylase in breast cancer cell line MDA MB-231"*** which is being submitted by Ms. Rutusmita Mishra, Roll No-412LS2043, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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DECLARATION

I do hereby declare that the Project Work entitled ***"Induction of apoptosis by sulforaphane and curcumin and expression profile of jmjd3,a key histone demethylase in breast cancer cell line MDA-MB-231"***, submitted to the Department of Life Science, National Institute of Technology, Rourkela is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Samir Kumar Patra, Associate Professor and HOD, Department of Life Science, National Institute of Technology, Rourkela, Odisha.

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TO MY PARENTS

AND

BELOVED ONES……

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First and foremost I bow down before the almighty God who has made everything possible.....

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ABSTRACT

Breast cancer is one of the most common forms of cancer occurring in females resulting in to high rate of mortality worldwide. Uncontrolled cell proliferation leads to cancer which may be attributed to genetic mutations or epigenetic alterations in the genome. Deregulations of epigenetic mechanisms are highly associated with cancer progression. Uncontrolled cell proliferation and evasion of apoptosis are important hall marks of cancer progression. Hence in this study we have investigated the role of sulforaphane and curcumin in the induction of apoptosis in metastatic adenocarcinoma MDA-MB-231 cells. As aberrant Histone methylation and demethylation pathways are most commonly associated with breast cancer progression, we have also tried to investigate the expression profile of Jmjd3, a key histone demethylase, at transcriptome level after treatment with of sulforaphane and curcumin for 24 hours. This investigation also aimed at finding a correlation between Jmjd3 expression and induction of apoptosis in breast cancer cell line MDA-MB-231. The down regulation of anti apoptotic gene Bcl-2 and up regulation of pro apoptotic gene Bax along with formation of chromatin condensation, DNA fragmentation and DNA damage suggests that there is induction of apoptosis in response to the sulphoraphane and curcumin treatment for 24 hours at their corresponding IC_{50} values. From all these investigations we can presume that may be these natural polyphenols are mediating their effect on cancer cells at transcriptome level by inducing apoptosis related genes. Moreover the down regulation of Jmjd3 and down regulation of Bcl-2with treatment of sulphoraphane and curcumin may be correlated to have a conjoint effect on apoptosis induction in breast cancer cells. Further studies at protein level may unravel hidden dimensions of Jmjd3 in the induction of apoptosis in breast cancer cells which may create a better therapeutic avenue for treating cancer patients in future.

Key words: apoptosis, cancer, MDA-MB-231, sulforaphane, Curcumin, Bcl-2, Bax, Jmjd3

INTRODUCTION

Breast cancer in women has been a glooming issue in the medical history for decades. The prevalence of breast cancer patients reflects that it is the most common cancer in women in developing and developed countries. The morbidity and mortality rate of breast cancer patients indicates that one in ten of all new cancer cases diagnosed worldwide every year is a cancer in female breast. The modern unhealthy lifestyle makes women susceptible to reproductive and hormonal risks at their early ages. The occurrence of breast cancer is also dependent on such factors. The initiation and progression of breast cancer has been perceived to be caused by the accumulation of genetic mutations which lead to aberrant cellular functions. DNA is the genetic material which controls all the principal cellular functions. Aberrant genomic alterations result in deregulation of gene expression and disruption of signaling networks that control cell survivability and proliferation. Uncontrolled cell proliferation leads to cancer that arises in individuals through genetic mutations, either inherited or sporadic. This may result in the activation of oncogenes and the inactivation of tumor suppressor genes. There are some specific genes that control the cells to grow, divide in to new ones and then die after a particular time. Oncogenes are the genes that enhance the cell division where as tumor suppressor genes slow down cell division forcing them to die timely. Mutations in DNA that 'turn on' the oncogenes or 'turn off' the tumor suppressor genes are likely to be the impelling cause of transformation of a normal breast cell to a cancerous one. For example, certain inherited mutations like mutation in the *BRCA* genes (*BRCA1* and *BRCA2*) can cause breast cancer in females. The more recent discoveries claim that along with the genetic alterations, some reversible alterations in the genome play important role in cancer initiation and progression. These modifications occur at the histone tails and in the DNA which is known as the epigenetic modifications. Epigenetic modifications are the heritable changes in the gene expression without the change in DNA sequence. These epigenetic mechanisms are very crucial in developmental processes as well as in maintenance of specific gene expression patterns. Any abnormality in these regulatory mechanisms can lead to altered gene function and malignant cellular transformation. Aberrant changes in the epigenome are a hallmark of cancer. The component of the epigenetic machinery in cancer includes methylation in the promoters of DNA ,modifications in the histone proteins including acetylation at lysine residues, methylation at lysine and arginine residues,

phosphorylation at serine and threonine residues, ubiquitination and sumoylation at lysine residues. These modifications play essential roles in regulating chromatin structure and gene expression, mainly by altering stability and accessibility of nucleosome *(Kouzarides,2007).*Posttranslational modifications in the amino and carboxyl-terminal of histone tails as well as in the globular domains of histone protein are driven by authoritative association of several modulatory enzymes like Histone Methyl Transferase(HMT)/Histone Demethylase(KDM), Histone Acetyl Transferse(HAT)/Histone Deacetylase(HDAC) etc. Methylation at lysine residues can occur several times (mono, di or tri-methylation) and each modification results in to different functions. In general acetylation at lysine residue results in to transcription activation and methylation at lysine residue is associated with transcription activation or repression depending on the residue and degree of methylation. H3K9 or H3K27 methylation or H4K20 methylation, results in to gene silencing. In contrast, H3K4, H3K36 and H3K79 methylation is correlative with transcriptional activation. According to the Histone code hypothesis covalent modifications of histones have regulatory hold on gene expressions that have several functional consequences. Recent investigations shows that histone modifications have crucial roles in gene(oncogene/tumor suppressor gene) transcription and expression, DNA repair, cell proliferation, mitosis, meiosis ,cell survivability and apoptosis. Apoptosis or programmed cell death (PCD) is an important hall mark of cancer progression *(Hanahan et al., 2000).*It is a fundamental process to development and maintenance of cellular homeostasis.

APOPTOSIS IN CANCER:

Cells have the ability to grow, divide and differentiate according to the signals they get. Normal cells respond to both growth promoting and growth inhibitory signals that decide whether the cells should divide, differentiate or die. Cancer cells divide in an uncontrolled manner escaping from programmed cell death.Deregulation of certain gene expression or signalling mechanism appears to be the cause of initiation and execution stages of apoptosis that results in the insufficient elimination of tumor cells conferring acquired resistance to chemotherapeutics or enhancing the uncontrolled migration of cells leading to metastasis.Therefore it is essential to identify and target those disabled pathways which may effectively inactivate tumor progression and drive the cells towards the programmed cell death .To date apoptosis is thought to be regulated by two classical pahways;i.e. extrinsic and intrinsic pathways*(Elmore,2007).*Extrinsic

pathway is associated with transmembrane ligand induced activation of death receptors and activation of procaspase-8 which ultimately activate caspase-3, driving the cells towards programmed cell deth.The intrinsic pathway is triggered by the response of internal apoptotic stimuli that results in to the change in the mitochondrial transmembrane potential. Activated by this response several pro apoptotic proteins like cytochrome-c,Smac/DIABLO,serine protease HtrA2/omi are released which activate procaspase 9 and Apaf1 to form apoptosome or death complex. This in turn activates the procaspase 3 and induces apoptosis. Apoptosis is well characterized by typical morphological and biochemical hall marks including chromatin condensation, cell shrinkage, nuclear DNA fragmentation, membrane blabbing etc(*Hengartner,2000).*The intrinsic and extrinsic paths of apoptosis are described below in fig-1.

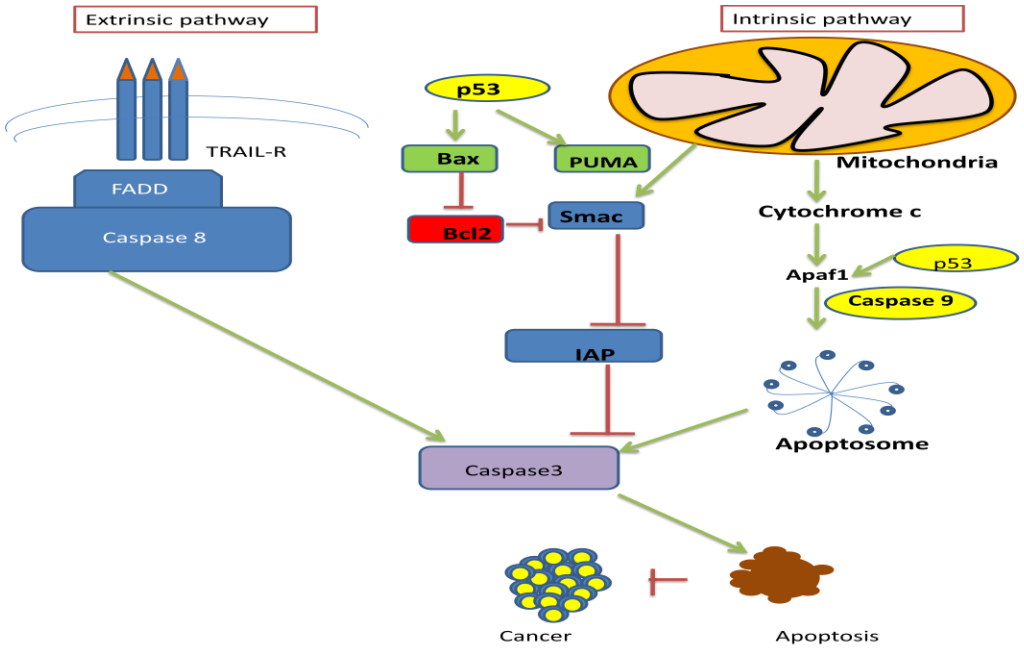


Fig1: Extrinsic and Intrinsic mechanisms of apoptosis

EPIGENETIC REGULATION OF APOPTOSIS:

Apoptosis is a cellular event that removes unwanted cells from the body during normal growth and development in multi cellular organisms and also is the consequence of reactivity of cells towards various DNA damaging agents or chemotherapy. A common feature to all the cells undergoing apoptosis is the chromatin condensation and fragmentation of nuclear DNA. These nuclear changes during apoptosis are associated with epigenetic modifications and apparently

regulated under the action of post translational histone modifications. Chromatin structures of transcriptionally active regions are characterized with greater accessibility than to the transcriptionally inactive heterochromatin structure. Histone modifications like phosphorylation, acetylation, deacetylation, methylation and demethylation have long been suggested to affect chromatin structure and accessibility. Epigenetic modifications are likely to have a commanding role over the nuclear changes experienced by apoptotic cells. Hence unraveling the hidden dimensions of the association of these epigenetic modulatory enzymes and their role in controlling the programmed cell death would certainly provide a comprehensive knowledge in context of breast cancer progression. Moreover activation or inhibition of these principle regulators in the presence of epigenetic modulatory drugs may entail the regulatory mechanisms of programmed cell death in cancer cells.

EPIGENETIC DRUGS:

Unlike the genetic mutations in cancer, epigenetic alterations can be reprogrammed by targeting the enzymes involved in the chromatin packaging and transcription of key regulatory genes. Hence drugs that restore the epigenetic balance by targeting such regulatory enzymes, show amazing therapeutic potential by reverting back the aberrant gene expression associated with different types of cancers. Researchers are finding ways to regress epigenetic changes by specifically targeting abnormal cells while minimizing damage to normal cells. In contrast with standard chemotherapeutics for cancer treatment that affect both abnormal and normal cells at higher dosages, epigenetic drugs more specifically target the chemical modifiers that bring heritable change in the gene expression even at lower dosages. Several epigenetic therapies including drugs targeting DNA methylation, Histone acetylation and deacetylation pathways have already been approved by the Food and Drug Association (FDA) and many more are in the preclinical trial phases for cancer treatment and prognosis. Environmental factors and diet directly influence epigenetic mechanisms in humans. Dietary polyphenols from green tea(epigallocatechin gallate-EGCG), soybeans(Genistein), turmeric(curcumin), broccoli(sulforaphane), grapes(resveratrol) and others have shown to possess several cell-regulatory activities within cancer cells*(Link et al.,2010).*Bioactive plant polyphenols mediating epigenetic modifications are often involved with reactivation of tumor suppressor genes, repression of oncogenes and induction of apoptosis in many types of cancer. They often alter the

DNA methylation pattern and histone modifications required for gene activation or silencing during cancer chemo prevention .In this study we target the natural polyphenols curcumin and sulforaphane which shows potential DNMT inhibitory, HAT inhibitory and also HDAC inhibitory activity. Moreover the role of these natural polyphenols in the induction of apoptosis in breast cancer cell will certainly create an avenue for effectively treating breast cancer patients.

SULFORAPHANE:

Sulforaphane (SFN) is an isothiocyanate organosulphuric compound that is derived from broccoli extracts and other crucifer vegetables like brussel sprouts, cabbage, cauliflower etc*(Zhang et al.,1992)*.It exhibits anticancer and antimicrobial properties in in-vitro and in-vivo applications. It is produced when the enzyme myrosinase transforms glucoraphanin, a glucosinolate, into sulforaphane .Glucoraphanin (GR) or 4-methylsulfinylbutyl glucosinolate is the major glucosinolate found in broccoli. The enzyme myrosinase gets activated, released upon physical damage to the plant and catalyzes the hydrolysis of GR to sulforaphane (SFN) *(van Poppel et al,1999)*.The structure of sulforaphane is shown below in fig-2.

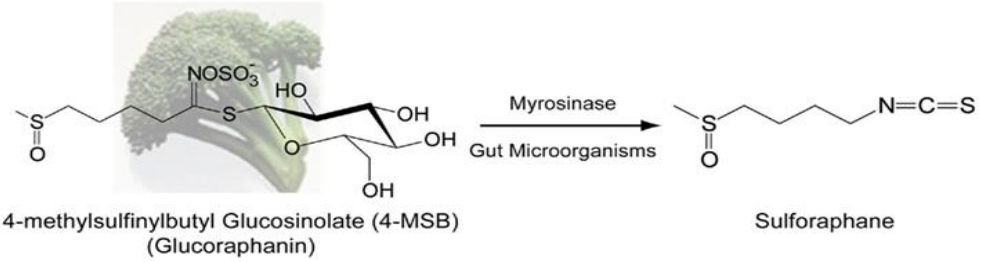


Fig-2:Metabolism and structure of Sulforaphane*(Boddupalli et al,2012)*

CURCUMIN:

Curcumin (1, 7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione)),a hydrophobic polyphenol is derived from the dietary spice turmeric: the dried ground rhizome of the perennial herb Curcuma longa Linn. It is commonly called diferuloylmethane and exhibits keto-enol tautomerism. Commercial curcumin is a mixture of curcuminoids, containing approximately 77% diferuloylmethane, 18% demethoxycurcumin, and 5%bisdemethoxycurcumin *(Anand et al., 2007)*. Curcumin is least soluble in water but quite soluble in organic solvents. The two tautomeric forms of curcumin are described below in fig-3.

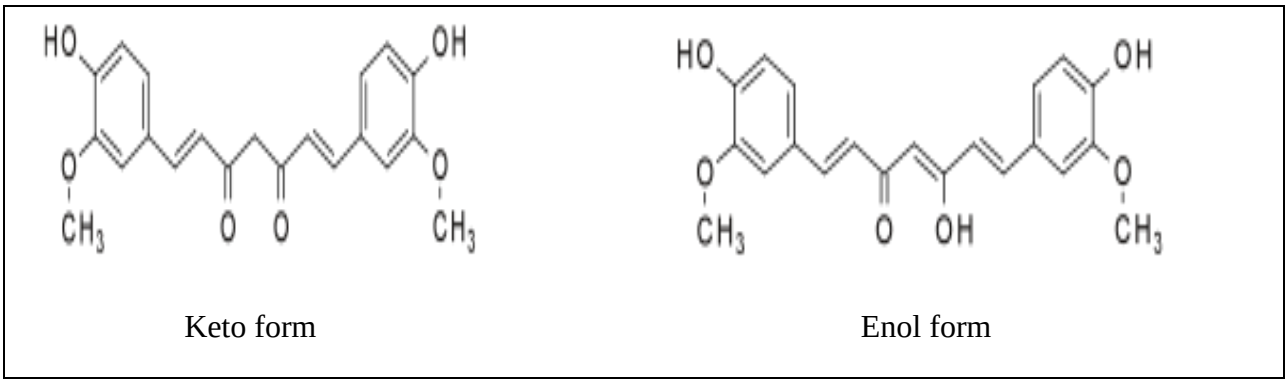


Fig-3: Tautomeric forms of curcumin (http://chemcool.tumblr.com/post/23659551189)

Curcumin has been well characterized for its anti-inflammatory, antioxidant, anti-cancerous, chemo preventive and potentially chemotherapeutic properties. Curcumin is found to modulate the activity of epigenetic modulatory enzymes like histone acetyltransferases, histone deacetylases, DNA methyltransferase I, and miRNAs*(Reuter et al.,2011)* controlling the 'on' and 'off ' switches of tumor inducing genes. It has a putative chemotherapeutic effect on killing cancer cells by inducing programmed cell death.

The epigenetic changes leading to the development of human cancer are accompanied by alterations in the chromatin architecture and function. These modification statuses of chromatin are instrumental in regulating gene expression and genome stability. Histone methylation and demethylation can influence chromatin condensation and poise genes for either transcriptional repression or activation, depending on how the modification is interpreted and translated in a specific circumstance. Histone methyltransferases and demethylase enzymes are essential for development and maintenance of cellular homeostasis whereas deregulated expression has been linked to human disorders such as cancer*(Albert et al.,2009).*H3 lysine (K) 27 methylation (mono-, di-, and tri-methylation) induces transcriptional silencing, and thereby controls gene expression patterns. Tri-methylation mark at H3k27 is reckoned as repressive histone modification mediated by the histone methyltransferase EZH2 (Enhancer of Zest Homologue 2), which is a component of the Polycomb group (PcG) complex 2 (PRC2). Aberrant histone methylation at lysine 27 of histone 3 protein (H3K27me3) derived by the abnormal EZH2 expression has been associated with various cancers including breast cancer *(Yoo et al.,2011)*. In contrast to this phenomenon, the lysine-specific demethylases (LSDs) and the Jumonji C (JmjC) domain containing histone lysine demethylases (KDMs), UTX and JMJD3 are responsible for

reverting the repressive state to an activated state by erasing the repressive methyl marks from the histone tail. Maintenance of histone methylation, required for growth, differentiation, gene expression and cellular proliferation counts a balance between methylases and demethylases. According to the recent findings, aberrant expression of methylases and demethylases are involved in course of tumor initiation and progression. DNA methylation/demethylation pathways, Histone methylation/demethylation pathways are aberrantly deregulated in cancer cells *(Patra et al., 2002)*. The Jumonji C (JmjC) domain containing lysine demethylase, JMJD3 belongs to the second and largest class of histone demethylase (KDMs) more specifically to KDM6 cluster in mammalian cells. These are Fe(II) and 2-oxoglutarate-dependent demethylase enzymes specific for H3K27me3/me2*(Shi et al.,2007)*.Aberrant expressions of these writer or eraser enzymes have been implicated in the course of tumor initiation and progression. Mis-erased or mis-written histone methyl marks are associated with many types of cancer. Hence mechanistic insight into the role of histone demethylase, JMJD3 enzyme in progression of cancer and its influence in presence of natural polyphenols Sulforaphane and Curcumin, on programmed cell death will pave the way for the development of new therapeutic intervention for the treatment of cancer.

REVIEW OF LITERATURE

Apoptosis, or programmed cell death occurs by two regulatory pathways. One may be through the activation of mitochondria caspase cascades or though the death-receptor caspase cascades. The important hallmarks of apoptosis are changes in Bcl-2 protein family ratios (both increased pro-apoptotic proteins and decreased anti-apoptotic proteins), poly (ADP-ribose) polymerase (PARP) cleavage and decrease in IAP, release of cytochrome C from the mitochondrial membrane, nucleosome DNA fragments, chromatin condensation, and activation of caspases. Apoptotic pathways are generally deregulated in almost all the cancers. Cells proliferate uncontrollably escaping from programmed cell death worsening the stage of cancer progression. Bcl-xl, Bcl-2, mcl-1, and Bax are the functional members of the Bcl-2 gene family. They function as repressors or promoters of apoptosis. The Bcl-2 oncogenes behave as a broad anti apoptotic factor and promote tumor cell survival inhibiting several cell death mechanisms induced by chemotherapy or radiation therapy. Over expression of anti apoptotic Bcl-2 is common in many types of human cancer and is very often correlated with decreased susceptibility to chemotherapeutics and inhibition of cell death induced by many stimuli, including hypoxia, nutrient deprivation, growth factor deprivation, and oxidative stress *(Yip et al.,2008)*. In contrast, with the oncogenes Bcl-2, the Bax gene is a promoter of apoptosis and helps in prognosis of cancer in humans *(Friess et al.,1998).*Anti-apoptotic molecule such as Bcl-2 or Bcl-xl can inhibit the activation of Bax following a repression of death signal*(Gross et al.,1999).*In patients with prostatic cancers, there is a high ratio of Bcl-2(apoptotic suppressor) to Bax(apoptotic activator) which increase their resistance towards radiotherapy. Hence the Bcl-2/Bax ratio serves as a potential molecular marker for predicting radio resistance of prostatic tumors *(Mackeya et al., 1998).*Bax acts s a tumor suppressor and activates tumorigenesis in a p53 dependent mechanism and stimulates apoptosis in vivo *(Yin et al.,1997).*

Cancer progression is concordant with aberrant activation or silencing of many targeted genes that allow cells to survive and multiply in an uncontrolled manner. Interestingly, SFN can modulate these targets and redirect their activities towards apoptosis or cell cycle arrest, thereby eliminating unwanted cells from the general population. At initial stages of cancer, SFN potentially blocks carcinogenesis by inhibiting phase I enzymes that are bioactivators of

chemical carcinogens and induce phase II enzymes which protect our cells or tissues from damages due to chemical carcinogens and enhance the transcription of tumor suppressor genes *(Fimognari et al., 2007).* The multi targeted ‘‘blocking” of SFN includes decrease in CYP1A1,CYP2B1/2,CYP3A4 phase I enzymes and increase in CYP4A10,CYP4A14 ,CYP39A1enzymes, increase in NQO-1,GST,c-GCS,UGT phase II enzymes, increase in level of anti-oxidants Glutathione peroxidase 3,Thioredoxin reductase 1 and 3,Proteasome subunits 26S ,alpha 1 and 3, beta 5, and ,increase in CDK 4, 7 and 9 ,cell cycle and cell growth Cyclins D1, E2 and T2, ,increase in transcription factors CREB binding protein etc*(Clarke et al.,2008).* SFN induces apoptosis in cancer cells by increasing caspase3 activity, increase in BAX:Bcl2 ratio, cytosolic activation of smac/Diablo, increase in p53 status ,increasing PARP activity, decreasing IAP level*(Karmakar et al.,2006).*SFN has been identified as a HDAC(Histone Deacetylase Inhibitor) inhibitor in human embryonic kidney 293 (HEK293) cell*(Myzak et al,2004)* . SFN is found to potentially inhibit the growth of prostate cancer xenografts by altering histone acetylation and HDAC activities in vivo. In human subjects, an intake of 68 g broccoli sprouts was found to inhibit HDAC activity in circulating peripheral blood mononuclear cells, with incidental induction of histone H3 and H4 acetylation *(Roderick et al., 2007).* SFN inhibits the progression of benign tumors to malignant transformation *(Conaway et al.,, 2005),* angiogenesis *(Bertel et al., 2006)*and metastasis*(Thejass et al.,2006).*HepG2 and HeLa cells showed apoptotic body formation and the accumulation of the cells in sub-G1 phase in presence of sulforaphane *(Park et al., 2007).*Induction of Fas ligand and activation of caspase-8,caspase-3,and poly(ADP ribose) polymerase are shown to be modulated by SFN. Also decreased Bcl-2 expression, release of mitochondrial cytochrome c, activation of caspase 9 and 3 into the cytosol are characteristically found in breast cancer cell lines treated with SFN*(Pledgie-Tracy et al.,2007).* In addition, it has been shown that down regulation of IAP family proteins including cIAP1, cIAP2, and XIAP, SFN-induced cell death also includes Bax activation and apaf-1(apoptotic protease activating factor-1) induction *(Choi et al., 2007).*In PC3 and DU145 human prostate cancer cell lines, SFN-induced apoptosis is demonstrated by formation of reactive oxygen species (ROS) and change in membrane potential of mitochondria leading to cytosolic release of cytochrome c *(Singh et al., 2005).* The various cellular responses in presence of sulforaphane (SFN) are described in fig-4 below. In skin cancer cells, sulforaphane is seen to suppress cancer

progression by reducing PcG(Poly comb Group) protein level through activation of a proteasome-dependent mechanism *(Balasubramanian et al.,2011).*

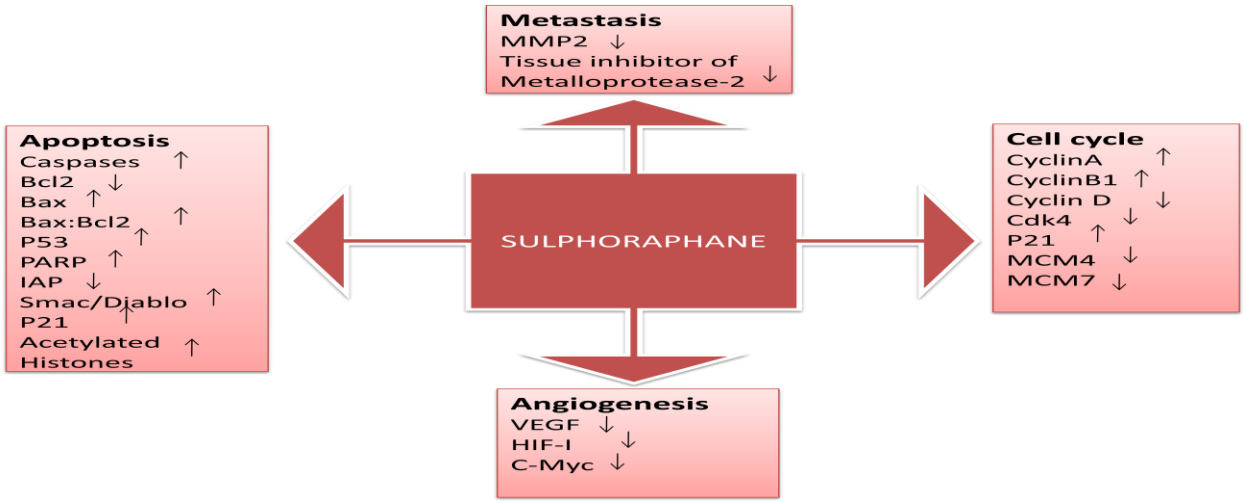


Fig-4: Role of SFN in various cellular processes involved in cancer progression

Due to the anti carcinogenic properties, SFN is a potential therapeutic agent that can act against any type of cancer at its early, intermediate or late phases and can reduce the process of carcinogenesis. It has already been proven its potential in phase II clinical trials and it will create a new avenue for clinical applicability to cancer patients in near future.

Curcumin has a wide array of molecular targets associated with numerous biochemical and molecular cascades regulated epigenetic modulation of gene expression. Curcumin specifically inhibits p300/CREB-binding protein (CBP*) (Marcu et al., 2006).* Curcumin inhibits the p300-mediated acetylation of p53 in vivo and significantly decrease acetylation of HIV-Tat protein in vitro as well as checks proliferation of the virus*(Balasubramanyam et al.,2004).*Curcumin activates caspase-3–mediated apoptosis and PARP cleavage in brain glioma cells as a mechanistic histone hypoacetylation *(Kang et al. 2006).* Curcumin is found to have potent DNA hypomethylation capacity, thrusting regulatory hold in various patho-physiological conditions like inflammation and cancer *(Liu et al.,2009).*In-silico studies showing molecular docking of the interaction between curcumin and DNMT1 suggests that the catalytic thiolate of DNMT1is covalently blocked by curcumin resulting in to inhibition of DNA methylation. Curcumin's effect on HDAC expression is prominently investigated in past years. One study has revealed that expression of HDAC 1, 3, and 8 were significantly reduced in curcumin treated

Raji cells compared to the untreated Raji cells. Also there was an increase in the levels of acetylated histone H4 *(Liu et al. 2005).* Similarly HDAC1 and HDAC3 were significantly decreased after treatment with curcumin (*Chen et al. 2007).* By contrast with treatment of curcumin, HDAC2 expression was restored as a critical component of corticosteroid anti-inflammatory molecule in impaired lungs of patients with chronic obstructive pulmonary disease *(Meja et al. 2008).* In acute myelogenous leukemia cell line HL-60, curcumin is seen to induce apoptosis through cleavage of Bid, activation of casapase 8, release of cytochrome c and its suppression is related with ectopic expression of Bcl-2 and Bcl-xl*(Anto et al.,2002).* Curcumin induces apoptosis in human breast cancer cell MCF7 through induction of Bax in a p53-dependent manner*(Choudhuri et al.,2002).*The various epigenetic modifications caused by the action of curcumin and its role in the induction of apoptosis are described below in fig-5.

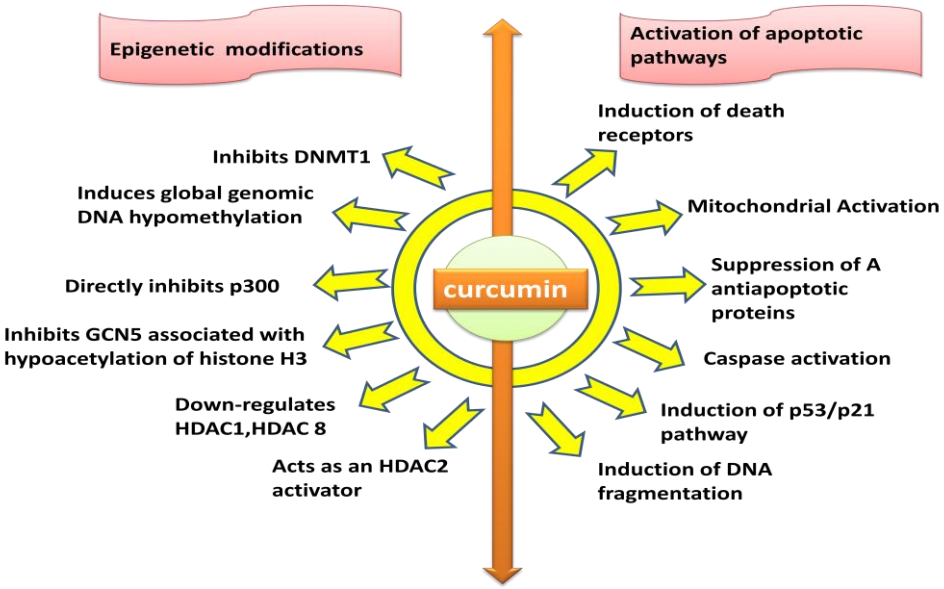


Fig-5: Effect of Curcumin on epigenetic modifications and apoptotic pathway activation

H3K27 demethylases are proposed to act as tumor suppressor genes with opposite to the action of oncogenic PcG proteins*(Rotili et al.,2011).*The Jmjd3gene is located on chromosome 17 near the p53 tumor suppressor gene. When there is an allelic loss at 17p13.1, including both p53 and JMJD3, more aggressive tumor are characteristically formed *(Agger et al., 1989).* Epigenetic silencing of the INK4A-ARF tumor suppressor locus encoding tumor suppressor genes p16INK4A and p14ARF is associated with the development of human cancers. JMJD3 imparts the reactivation of INK4A-ARF by eliminating repressive H3K27me3 marks during induction of stress induced senescence or oncogenesis *(Agger et al., 1989).* It has been suggested

that in breast cancer cell lines MCF7 (i.e. an estrogen positive cell line), the activation of BCL2 transcription factor is dependent on the simultaneous inactivation of the H3K27 methyltransferase, EZH2 at a poised enhancer by the ERα-dependent recruitment of histone demethylase JMJD3.Depletion of JMJD3 has resulted in the induction of apoptosis followed by decrease in Bcl2 expression and decrease in DNA content *(Svotelis et al., 2011)*. But in estrogen negative cancer cell lines, the regulatory mechanisms are yet not been well evidenced. Therefore in this study we have experimented whether the Bcl2 depletion and Bax induction by the natural polyphenols sulforaphane and curcumin is dependent on transcriptional expression of Jmjd3. Hence we can presume that JMJD3 could provide an important barrier to tumorigenesis and induce apoptosis through regulated expression of pro apoptotic and anti apoptotic genes.

OBJECTIVES

1. **To analyze the effect of natural polyphenolic compounds Sulforaphane and Curcumin as epigenetic modulators on breast cancer cell line MDA-MB-231.**

2. **To observe cellular and morphological changes in MDA-MB-231 cells as a sign of apoptosis induction**

3. **To analyze the transcriptional level of Jmjd3 a key Histone demethylase in response to the treatment with Sulphoraphane and Curcumin.**

MATERIALS AND METHODS

1. Maintenance of cell line

Human breast carcinoma cell line MDA-MB-231 was obtained from the National Centre for cell Science(NCCS), Pune, India. The cells are known to be of epithelial breast adenocarcinoma in origin .This cell line was maintained in Dulbecco's modified eagle's medium(DMEM) with 10% fetal bovine serum,2mM L-glutamine and 100 units/ml penicillin-streptomycin sulphate (invitrogen).This cells were seeded properly in flask with the growth media and left for 2-3 days to get confluency. Then these cells were trypsinized and counted to be reseeded in 6 well plates or 96 well plates with the presence of media. When the cells get confluent after 24 hours, drugs of a particular IC50 value were administered to the cells. The drug treatment was done with sulforaphane (SFN) and curcumin (all purchased from sigma), for a period of 0, 24, 48,72hrs.

2. MTT Assay:

To determine the cell proliferation capacity in presence of drugs, MDA-MB-231 cells were seeded in 96 well plates at a density (3000 cells per well) based on the doubling time in presence of 200µl growth media (10% FBS) and incubated for 24 hours in incubator containing 5% CO_2 at 37°C .Uniform cell seeding was done to obtain a perfect dose response effect of drugs. The drugs of interest were curcumin and sulforaphane, diluted at different concentrations in growth media. After 24 hours of seeding, the existing media was removed and replaced by fresh media along with various concentrations of drug. Then the plates were incubated for 24,48and 72 hours in the CO_2 incubator. To detect the cell viability, MTT working solution was prepared from a stock solution of 5mg/ml in growth medium without FBS to the final concentration of 0.8mg/ml.100 µl of MTT solution was added and incubated for 3-4 hours. Addition of MTT is done in the dark as this solution is light sensitive. After 4 hours of incubation the MTT solution was discarded from each well and 100 µl of DMSO solvent was added in each well under dark followed by an incubation of 20-30 minutes. The metabolically active cells reduce the yellow tetrazolium solution of MTT in to formazan complex which on addition of solvent turns in to purple color solution. Then the colorimetric estimation of formazan product is done at 565nm with a reference 595nm wavelength in a microplate reader. The data was plotted against drug

concentration and the percentage of viable cells was known. From the plotted graph the IC_{50} value was obtained which signifies the drug concentration at which 50% of the cells are viable.

3. Isolation of total cellular RNA:

The total cellular RNA was isolated from MDA-MB-231 cell lines by Trizol (Sigma-Aldrich,USA) method according to manufacturer's instructions. In brief, ($1x10^6$) cells were washed with 1 ml of 1xPBS (pH 7.4) and spun down at 1300 g for 5 min. Then the pellet was re-suspended in 1 ml of Trizol reagent to lyse the cells. In this lysed cellular mix, 200µl chloroform was added (for phase separation and disintegration of lipid bilayer), shaken vigorously for 15 sec and incubated at room temperature for 15 minutes. Contents were centrifuged at 12000g for 15 minutes at 4 °C to separate components in organic, interphase and in aqueous phase. Aqueous phase was transferred to a fresh eppendrof tube and RNA was precipitated using 500µl of isopropyl alcohol. RNA pellet was collected by centrifugation at 12000g for 10 min at 4 °C, washed with 70% ethanol then again centrifuged at 7500g for 5 minutes. Then it was dissolved in 20µl of autoclaved DEPC treated water.

4. Quantification of total cellular RNA:

The concentration of isolated total cellular RNA was measured using a nanodrop UV spectrophotometer analyzer. It was likely that a standard preparation of RNA should have a 260/280 ratio of 1.8-2 and 260/230 ratio 2-2.2 which indicates the preparation of RNA free of protein and oligopeptide contamination.

5. Complementary DNA (cDNA) preparation:

First strand cDNA was synthesized using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit. In PCR tube, 1µl of oligo (dT) 18 primer, 1 µg of total RNA(drug treated) was taken and the volume was made up to 12 µl with the addition of nuclease free water. The mixture was gently mixed with pipetting and was briefly centrifuged. Then the mixture was incubated at 65°C for 5 minutes. After 5 minutes, the tubes containing mixture was snap cooled on ice. Then to this 12 µl mixture, 4 µl of 5X Reaction Buffer, 1 µl of RiboLock RNase Inhibitor (20 u/ µl), 2 µl of 10 mM dNTP mixture and1 µl of Revert Aid M-MuLV Reverse Transcriptase (200 u/ µl) was added. The total volume of the mixture now became 20 µl. All the steps were performed

while kept on ice. The total mixtures were mixed thoroughly with gentle pipetting and spin down for few seconds to ensure proper mixing. Then the mixture was set for reverse transcriptase-PCR with incubation at 42°C for 60 minutes followed by end incubation at 70°C for 5 minutes. The cDNA synthesized from the RNA was stored in ice at -20°C for further use.

6. Real Time-PCR:

Real time Polymerase Chain Reaction or qRT PCR is a method that allows exponential amplification of DNA sequences and simultaneously quantitates differences in mRNA expression. This system includes a DNA binding cyanine dye such as SYBR green that specifically binds to the major groove of double strand DNA but not to the single stranded DNA.SYBR green binds to the amplicons accumulated during PCR process which is proportional to the fluorescence emission of the dye .One reference dye(ROX dye) was used to serve as an internal reference for normalization of the SYBR green fluorescent signals.ROX dye allows for correction of well-to-well variation due to pipetting inaccuracies and fluorescence fluctuations. Four genes were taken for the experiment, three of which were test genes and one was housekeeping gene as a reference. Housekeeping gene are generally taken as reference genes to check for the expression of test genes i.e. how much fold the test gene's expression has increased or decreased with respect to normal gene expression. For this experiment the test genes were Bcl-2, Bax and Jmjd3 and reference gene used was β-actin.

The total reaction volume prepared was 5 µl .

Calculation:

4 genes were taken, therefore,

4 genes x 3 replicates×2 treatment = 24 reactions~26 reactions (to avoid inaccuracy due to pipetting error)(12 reactions for each treatment)

26 reactions x 5 µl = 130µl total volume was to be prepared.

SYBR ® Green master mix dilution:

The stock SYBR ® Green master mix solution was of 2X concentration(containing optimized mixture of SYBR green dye+$MgCl_2$ +dNTPs+ Taq DNA Polymerase+ qPCR Reaction buffer) and from this working solution of 1 X concentration was taken.

2X x (? µl) = 1X x 130µl

=> (? µl) = 1X x 130µl / 2X

= 65µl

SYBR ® Green master mix= 65µl

Reference dye:

For 100 µl of mixture reference dye taken, is 1 µl

Hence for 130 µl reaction mixture reference dye taken was,130 µl/100=1.3 µl

cDNA was diluted at 1:20 ratio with addition of nuclease free water.

For each reaction we required 1µl of diluted cDNA(SFN treated and curcumin treated).

Therefore, for 26 reaction= 1x26=26µl (for each treatment 13 µl template was needed).

Primer:

The stock solution of primer contained 10µM, from which we required 500nM for each reaction

10 µM x (? µl) = 500x10 $^{-3}$µM x100

=> (? µl) = 500x10 $^{-3}$µM x100 / 10 µM

= 5µl(each for forward and reverse primer)

Reference dye=1.3 µl

Forward Primer=5 µl

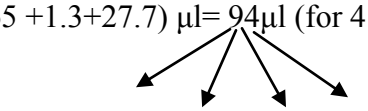
Reverse primer= 5 µ

Template=26 µl

Autoclaved distilled water=27.7µl

Total=65 µl

Then a semi master mix was prepared with the addition of SYBR® Green master mix + Reference dye +water= (65 +1.3+27.7) µl= 94µl (for 4 genes)

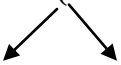


23.5µl 23.5µl 23.5µl 23.5µl

Forward Primer= +1.25

Reverse Primer= +1.25

Total= 26(for 2 treatment)



13µl 13µl (for 5 µl of reaction 1 µl template is needed,

for 13µl of reaction template needed= 13/5=2.6)

Template= + 2.6 + 2.6

Total= 15.6 µl (for each triplicate)

5.20 µl 5.20 µl 5.20 µl

For Real time PCR analysis mainly 2 types of primers are used: Forward primer and reverse primer. The primers were specifically designed with the help of NCBI primer blast tool. The primer sequence, their melting temperature and product length is shown below in table-1

Table-1: Table showing the sequence of the forward and backward primers:

Name of the primer	**Type**	**Sequence**	**Product length**
Bcl-2	Forward	5'CCTGTGGATGACTGAGTACC 3'	128bp
	Reverse	3'GAGACAGCCAGGAGAAATCA 5'	
Bax	Forward	5'TTCATCCAGGATCGAGCAG 3'	94bp
	Reverse	3' CGCTCAGCTTCTTGGTGG 5'	
Jmjd3	Forward	5'GGAGACCTTTATCGCCTCTG 3'	116bp
	Reverse	3' TCCCTTTCACCTTGGCATT 5'	
β actin	Forward	5'CTGGAACGGTGAAGGTGACA 3'	140bp
	Reverse	3'AAGGGACTTCCTGTAACAACGCA5'	

Real-time PCR was carried out in Eppendorf Masterplex Real Time PCR. The experiment was set up with the following PCR program. The threshold frequency taken was 33%. The cycle temperatures taken were as follows described in table-2:

Table-2: Cycle temperature and time for qRT-PCR:

Stage	Temperature° C	Time	Cycle
Stage 1	95	20sec	1
Stage 2	95 55 68	15sec 15sec 20sec	40
Stage 3	95 60 95	15sec 15sec 15sec	1

The melting curve was analyzed by the inbuilt software and the change in relative gene expression with respect to β actin was detcted as fold change at logarithmic scale.

7. Clonogenic Assay for measuring Cell survivability:

In brief, media form stock culture of cells actively growing in monolayer was removed, then washed with 1x PBS for 2 times for removal of dead cells. Then 500µl of trypsin was added and incubated for 5-10 minutes to detach the cells. Trypsin was neutralized with the addition of 500µl media. Then the cells were collected in a suspension. The number of cells per milliliter in this suspension is then counted using a hemocytometer . From this stock culture, approximately 600 cells were seeded into each well of a 6 well plate. The plate was incubated for approximately 2 weeks. Each single cell divides many times and forms a colony that is easily visible with the naked eye, especially if it is fixed and stained. Hence after 2 weeks 1ml of 1x ice cold PBS was added and left for 10minutes followed by incubation in absolute ice cold methanol for 10-15 minutes. Then it was stained in 0.05%crystal violet and kept for 10-15 minutes undisturbed. To avoid excess staining it was de stained in normal water for four times. The plates were seen under UV filter using Epi-fluorescent Microscope (Nikon TE 2000E). All the cells that make up the colony are the progeny of a single cell. For the 600 cells seeded into the dish, the number of colonies counted should be at least 50 or above.

8. Soft agar colony forming assay:

Anchorage-independent growth is one of the hallmarks of malignant transformation of cells .The Soft Agar Assay for Colony Formation is an anchorage independent growth assay, which is considered the most stringent assay for measuring proliferation of cells in a semisolid culture media and its sensitivity towards carcinogenic inhibitors. The soft agar plate provides a 3D system which mimics our cellular environment. Standard soft agar assays are usually performed in 35mm dishes, cells pretreated with carcinogenic inhibitors or drugs are cultured with appropriate controls in soft agar medium for 14-21 days. Following this incubation period, formed colonies can either be analyzed morphologically using cell stain and quantifying the number of colonies formed per well.

8.a. Preparation of Base Agar:

0.7% agar was melted in a microwave. Equal volumes of 2X DMEM with 10% FBS and 0.7% agar were mixed thoroughly. Then 1.5ml of this mixture was added to each 35mm petri plates and set aside for 5 minutes to solidify.

8.b. Preparation of Top Agarose :

Cells pretreated with drugs were trypsinized and the number of cells per ml was counted.5000 cells were taken for each plate.0.8% agarose was melted in microwave.3ml of 2X DMEM+10% FBS and 3ml of 0.8% agarose was added to the cell suspension in a 10ml tube. The mixture was gently swirled for appropriate mixing.1.5ml of this mixture was added to each of the agar dishes made previously. To be in the safer side 3-4 replicates were prepared. Negative control without cells and positive controls without treatment were taken. The plates were incubated at 37°C in humidified incubator for 14-21 days. The cells were fed 1-2 times per week with DMEM. All the steps were done aseptically to avoid any microbial contamination.

8.c. Staining:

After 21 days, the plates were stained with 0.5mi of 0.005% crystal violet for 30-60 minutes. The excessive stains were removed by washing with normal water for 2-3 times. The colonies were counted using a dissecting microscope.

9. Comet Assay to measure DNA damage:

Comet assay is a gel electrophoresis with fluorescence microscopy based method used to visualize migration of DNA strands and to measure DNA damage from individual eukaryotic cells. Circular comet head contains undamaged DNA having high molecular weight and the comet tail represents damaged DNA. Longer and brighter tail shows higher level of DNA damage.

9.a. Agarose preparation

Two water baths were equilibrated at 40 °C and 100 °C respectively. Than 1% low gelling-temperature agarose was prepared by mixing powdered agarose with distilled water in a glass beaker or bottle. The bottle was placed in the 100 °C water bath for several minutes and was transferred into a 40 °C water bath.

9.b. Slide Percolating

Agarose-precoated slides were prepared by dipping the slides into molten 1% agarose and wiping one side clean. It is best to work in a low-humidity environment to ensure agarose adhesion. Agarose was allowed to air-dry to a thin film. Slides can be prepared ahead of time and stored with desiccant.

9.c. Sample Preparation

A single-cell suspension was prepared using enzyme disaggregation or mechanical dissociation. The cells were kept in ice-cold medium or phosphate-buffered saline to minimize cell aggregation and inhibit DNA repair. Using a hemocytometer or particle counter, cell density was adjusted to about 2×10^4 cells/ml in phosphate-buffered saline lacking divalent cations. Slides were labeled on frosted end using a pencil. 0.4 ml of cells into a 5 ml plastic disposable tube was pipette out. 1.2 ml 1% low-gelling-temperature agarose at was added at 40 °C. 1.2 ml of cell suspension onto the agarose-covered surface of a pre-coated slide was mixed by vigorous pipetting. Agarose was allowed to be gel for about 2 min.

9.d. Lysis and Electrophoresis

After agarose has gelled, slides were submerged in a covered dish containing A1 lysis solution [1.2M NaCl, 100mM Na_2EDTA, 0.1% sodium lauryl sarcosinate, 0.26M NaOH (pH.13)]. Samples were lysed overnight (18−20 h) at 4 °C in the dark. After overnight lysis, slides were removed carefully and submerge in A2 rinse solution [0.03M NaOH, 2mM na_2EDTA (pH 12.3)] for 20 min at room temperature (18−25 °C). The process was repeated two times to ensure

removal of salt and detergent. Care was taken for not allowing DNA to renature even briefly (i.e., by lowering pH below 12.3) until after electrophoresis, as this will result in DNA tangling and reduced migration. After these three rinses, slides were submerged in fresh A2 solution in an electrophoresis chamber. The chamber was filled with a consistent volume of buffer that is about 1–2 mm above the top of the agarose. It was ensured that the chamber is level using a bubble leveling device. Electrophoresis was conducted in solution A2 for 25 min at a voltage of 0.6 V/cm. The current was about 40 mA using 20 V.

9.e. Slide Staining

Slides were removed from electrophoresis chamber and were rinsed and neutralized in 400 ml of distilled water. Slides were placed in staining solution containing 2.5 μg/ml of propidium iodide in distilled water for 20 min. Finally the slides were rinsed with 400 ml distilled water to remove excess stain.

9.f. Slide Analysis

Analyzes of cells were done by examining at least 50 comet images from each slide. Analyzing doublets or comets at slide edges should be avoided. Image j analysis software was used to analyze individual comet images.

10. Analysis of chromatin condensation by Hoechst 33342 staining:

An average of 1×10^4 cells were plated in 60mm petri plates. After 24 hours of seeding when cells get properly adhered to the plate surface, they were treated with sulforaphane and curcumin at their respective IC_{50} values. The respective controls were left against each treated samples. After 24 hours of drug treatment the cells were stained with Hoechst 33342 stain (1mg/ml) and incubated for 10 minutes at 37°C and images were taken under UV filter using Epi-fluorescent Microscope(Nikon TE 2000E).Condensed nucleus were counted against total number of nucleus in the field and the percentage of apoptotic nuclei were analyzed.

11. DNA fragmentation assay:

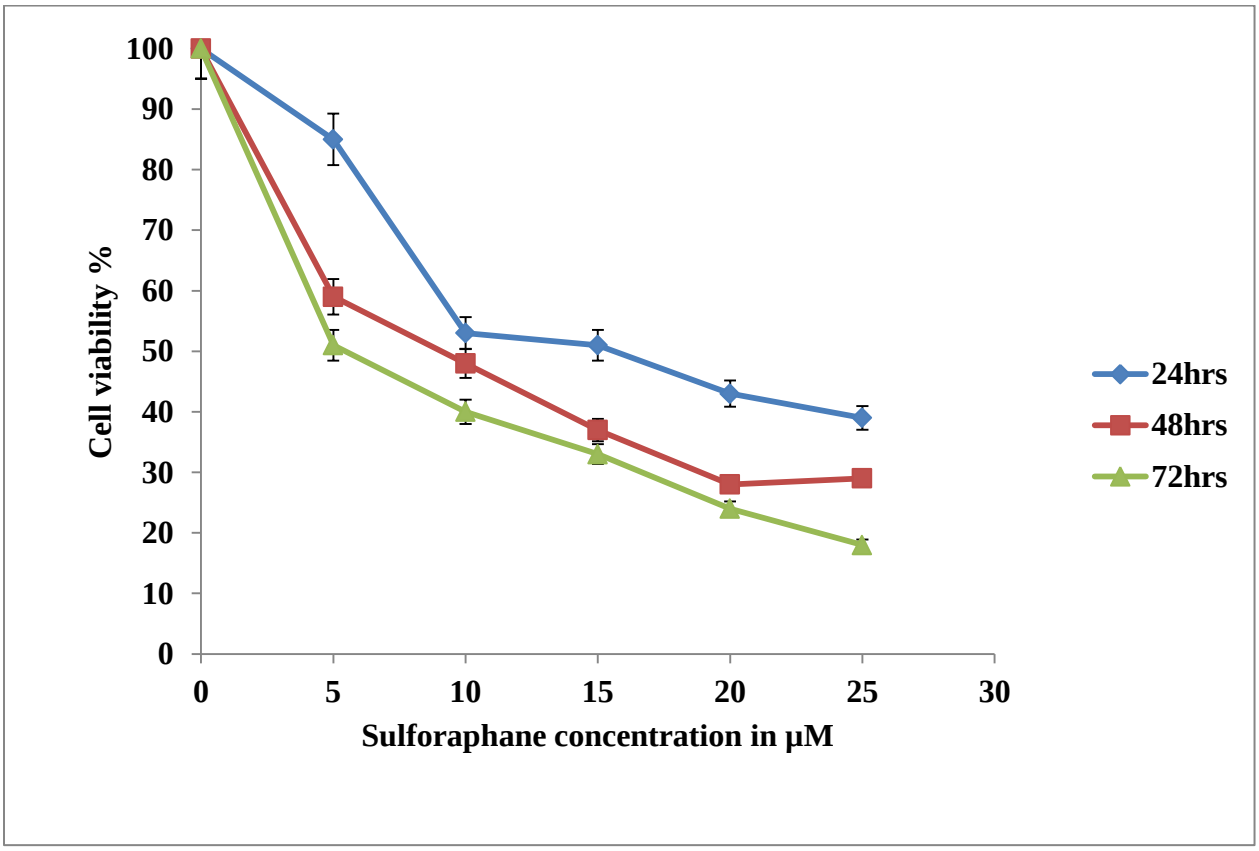
Fragmentation of DNA and chromatin is an integral process during apoptosis, and has been reported to occur in more than one distinct stage. During initial stages, high molecular weight DNA fragments of 50 kbp or longer size have been observed in morphologically normal cells

committed to undergo apoptosis. The low molecular weight DNA fragments are associated with late events such as formation of apoptotic bodies. The extensive nucleosome fragmentation or DNA fragmentation induced during apoptosis can be detected using techniques such as DNA ladder assay followed by agarose gel electrophoresis. For this purpose,2×10^{4} cells were seeded in petriplates. The next day it was treated with 15µM of sulforaphane and 20 µM of curcumin and left for 24 hours of incubation in 5% CO_2 incubator at 37°C.Then the next day cells were dislodged by trypsinization and washed with 1X PBS twice. 100µl of lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl, and pH 7.5) was added and kept for 10 sec in order to lyse the cells. For the Preparation of 1ml of lysis buffer 10% NP-40 100 µl, 200 mM EDTA 100 µl , 0.2 M Tris-HCl (pH 7.5) 250 µl , D.W. 550 µl were added together. After adding lysis buffer the solution was centrifuged at 3,000 rpm for 5 min to obtain the supernatant 10 µl of 10% SDS solution was added to pooled supernatant (final: 1% SDS), then it was treated with 10 µl of 50 mg/ml RNase A (final 5 mg/ml) and incubated for 2 h at 56 °C. After that 10 µl of 25 mg/ml Proteinase K (final 2.5 mg/ml) was added and again incubated for 2 h at 37 °C. To this 1/2 vol. (65 µl) of 10 M ammonium acetate was added. 2.5 vol. (500 µl) of ice-cold ethanol was then added and mixed thoroughly and allowed to stand for 1 h in – 80 °C freezer so that ethanol was precipitated. The solution was centrifuge for 20 min at 12,000 rpm, and then the white pellet was washed with 200 µl 80% ice cold ethanol and allowed to air-dry for 10 min at room temperature. The pellet was dissolved with 50 µl of TE buffer. DNA concentration was determined by taking absorbance at 260 and about 4 µg of the same concentration of DNA was run in 2% agarose gel electrophoresis. The gel picture was taken in a gel doc system. The densitometric calculations of the DNA bands were done using image j analysis software.

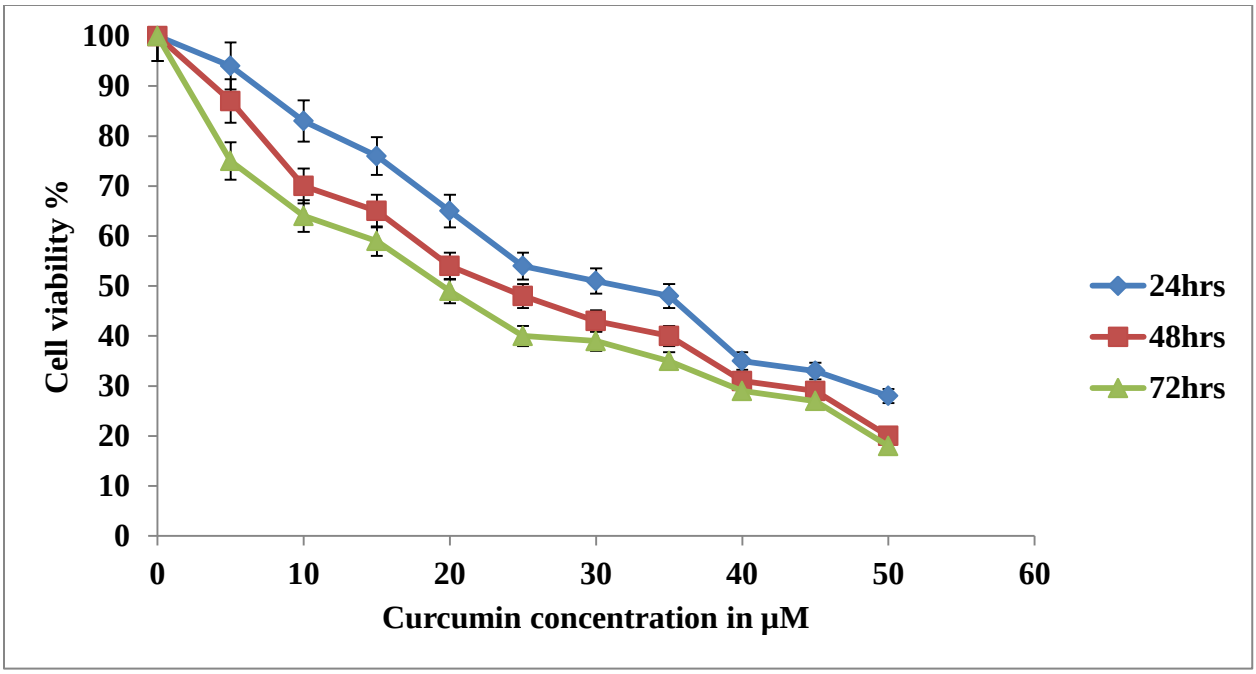
RESULT AND DISCUSSION

1. Cell Viability assay by MTT method:

The viability of MDA-MB-231 cell line, treated with Sulforaphane and Curcumin, was determined by analyzing the spectroscopic reading of MTT assay. The MTT assay was done for 24,48and 72 hours of time period. The IC_{50} values for both the drugs were calculated against the cell viability percentage. The IC_{50} value of sulphoraphane was calculated at 15µM and curcumin at 25 µM concentration .The corresponding graphs are shown in graph-1 &2.



Graph 1: MTT assay result of Sulforaphane (24, 48, 72 hours of treatment)



Graph 2: MTT assay result of Curcumin (24, 48, 72 hours of treatment)

2. Isolation of Total Cellular RNA by Trizol method:

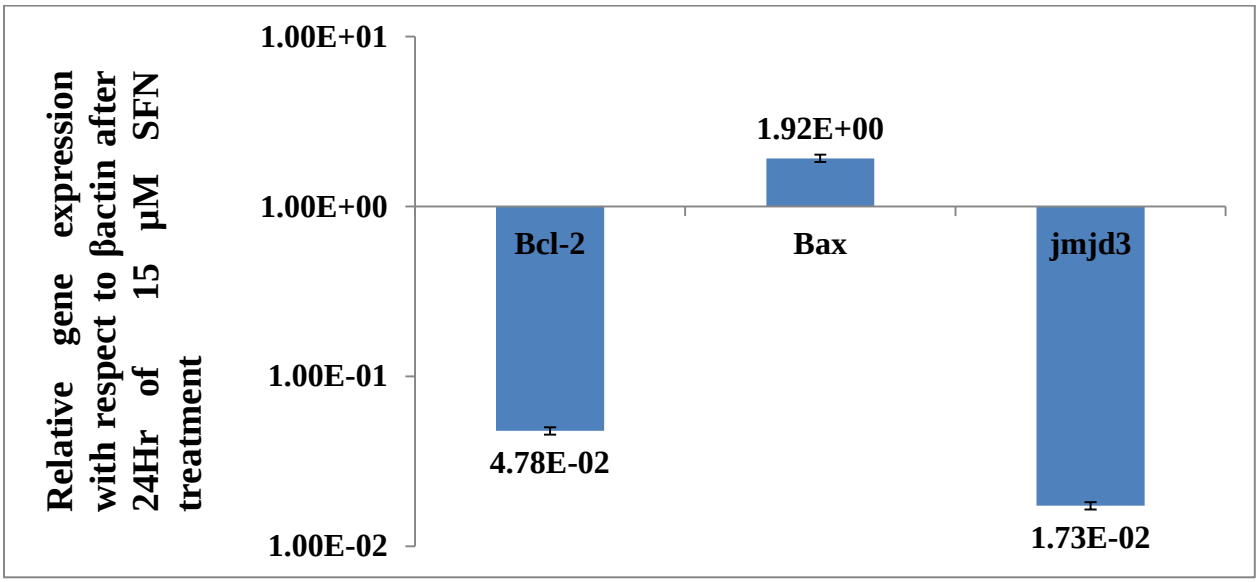
The total cellular RNA was isolated following the manufacturer's instruction with the help of Tri-reagent(Sigma).The isolation was carried out from the cultured MDA-MB-231 cells treated with sulforaphane and curcumin for 24 hours. The RNA was quantified with the help of Nano drop spectrophotometer. The 260/280 ratio and 260/230 ratio was measured to be in the range of 1.8-2.0 and 2.0-2.2 respectively. The yield of RNA was good. The values of RNA yield, 260/280 ratio,260/230 ratio are listed in the table-3 below.

Table-3: Quantification of total cellular RNA

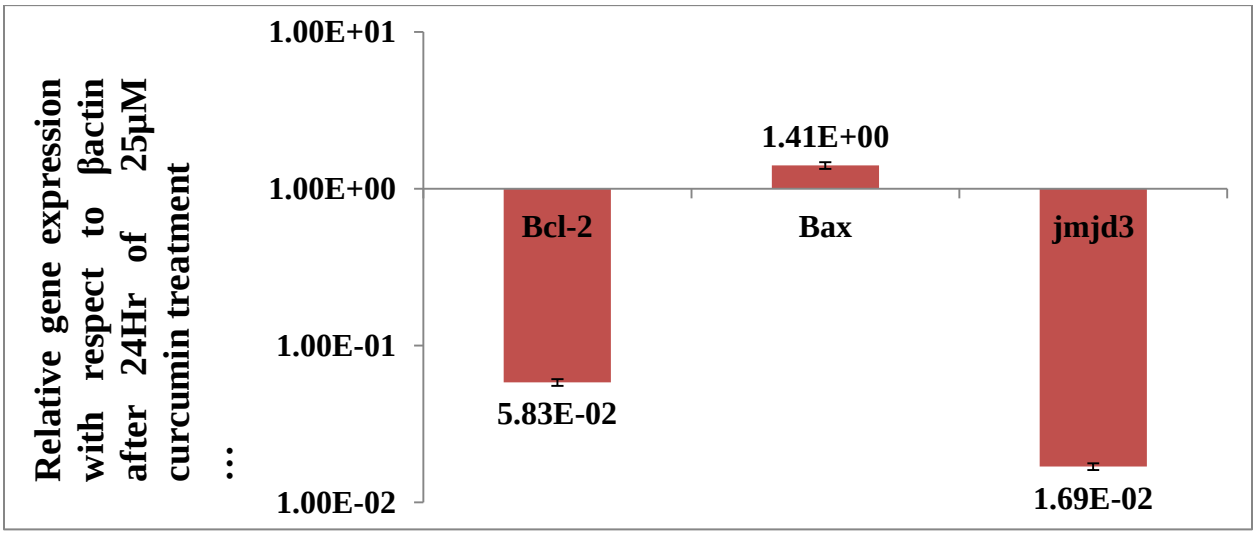
Treatment	**Concentration(yield)**	**260/280**	**260/230**
Control	909.30µg/µl	1.90	1.98
Sulforaphane	388 µg/µl	1.86	1.99
Curcumin	617.00 µg/µl	2.01	2.05

3. Relative gene expression analysis by RT-PCR:

From previous literature studies, it has been known that the anti apoptotic Bcl-2 is found to be up regulated and pro apoptotic Bax is down regulated in breast cancer cells. With our investigation we found that Sulphoraphane and curcumin treated MDA-MB-231 cells show a down regulation of anti apoptotic bcl-2 gene and an up regulation in pro apoptotic Bax gene expression. The status of Jmjd3 is also found to be down regulated with the treatment of sulforaphane and curcumin. The relative gene expression data is shown below in graph-3&4.



Graph-3: Graphical representation of RT-PCR results for the expression of Bcl-2,Bax and Jmjd3 after treatment with sulforaphane.



Graph-4: Graphical representation of RT-PCR results for the expression of Bcl-2, Bax and Jmjd3 after treatment with curcumin.

4. Analysis of Clonogenic assay for measuring cell survivability:

Cells ability to divide and form colony as a measure of survivability potential can be measured by clonogenic assay. The MDA-MB-231 cells treated with sulforaphane and curcumin for 24 hours showed reduced cell proliferation capacity as compared to the untreated samples. SFN treated samples showed more reduced number of colony formation. The pictures of the plates were taken and colonies were counted with the help of colony counting software. A graph was plotted taking the number of colonies formed with the treatment of sulforaphane and curcumin. The fig-6 below shows the clonogenic potential of MDA-MB-231 in response to drug treatment and the graph-5 shows quantification of clonogenic potential of MDA-MB-231 in response to sulforaphane and curcumin treatment.

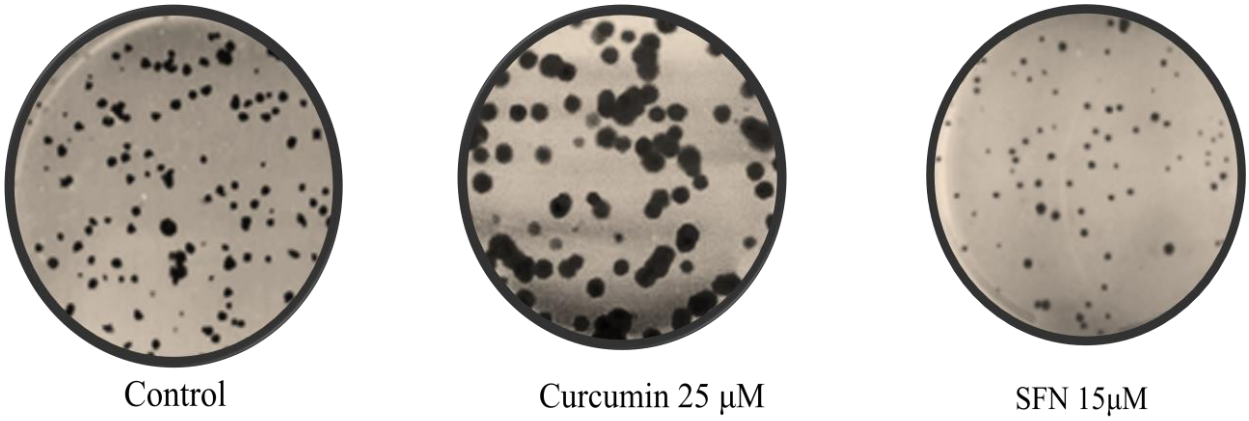
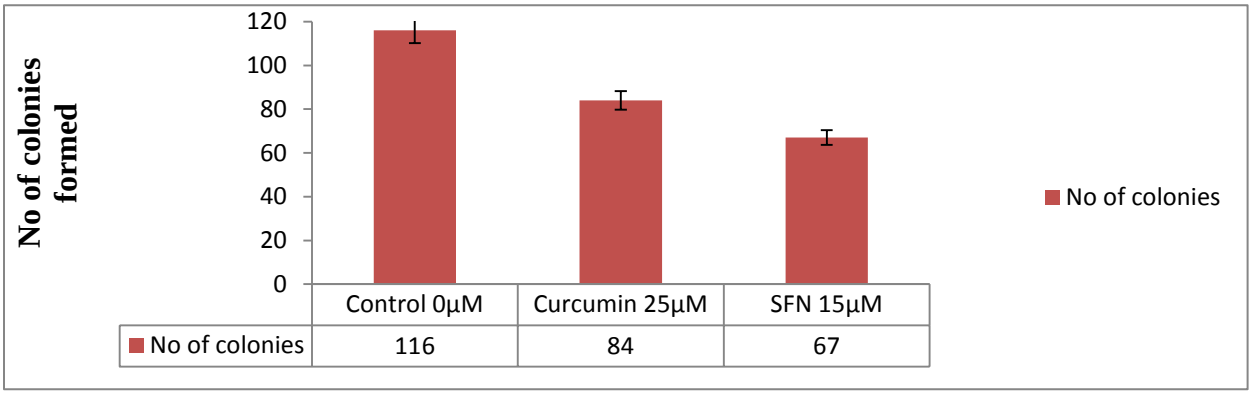


Fig-6:Clonogenic potential of MDA-MB-231 cells in response to 24hrs treatment of sulforaphane and curcumin



Graph-5: Analysis of clonogenic assay of MDA-MB-231 cells with a 24 hours treatment of sulforaphane and curcumin.

5.Analysis of soft agar colony forming assay:

Soft agar colony forming assay was done in an anchorage independent manner to know the survivability potential of MDA-MB-231 cells treated with sulforaphane and curcumin for 24 hours .The colony forming ability was seen to be reduced in presence of sulforaphane and curcumin as compared to the control.The image of the soft agar colony and the graph showing number of colonies in presence of sulphoraphane and curcumin is shown below in fig-7 and graph-6.

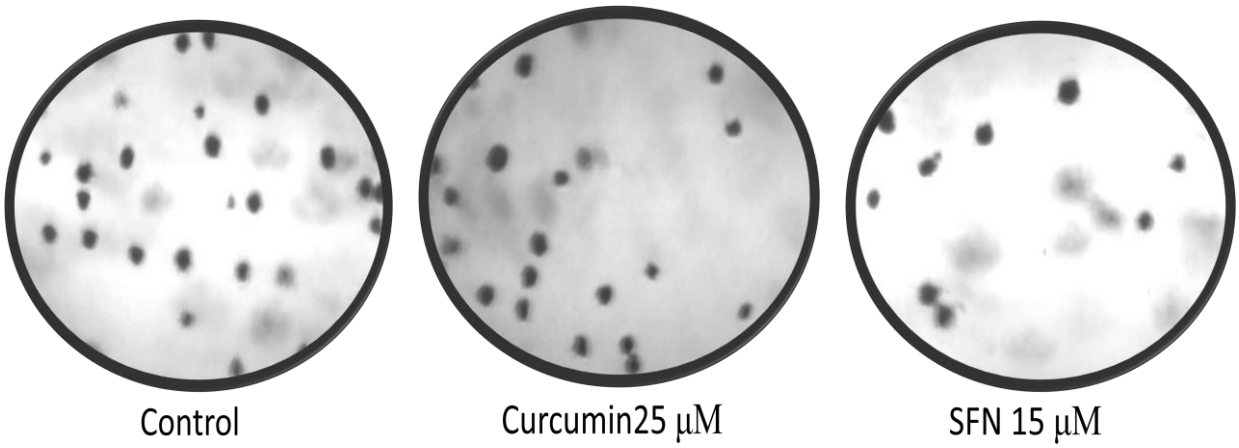
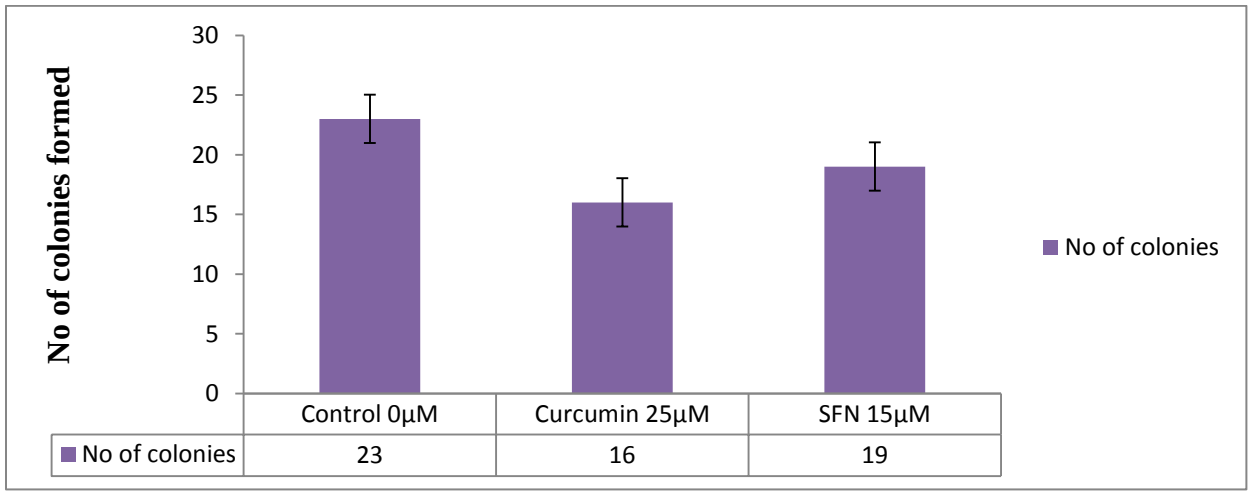


Fig-7:Soft agar colony forming assay of MDA-MB-231 cells in response to 24 hours of treatment with sulphoraphane and curcumin



Graph-6: Analysis Soft agar colony forming assay of MDA-MB-231 cells in presence of sulphoraphane and curcumin

6. Analysis of chromatin condensation by Hoechst 33342 staining:

After treatment of MDA-MB-231 cells with sulforaphane and curcumin for 24 hours, the plates were analyzed for any change in the morphology of nuclei. The formation of granulations in the nuclei is a measure of chromatin condensation which is well documented in the drug treated samples. The result indicated that there were formations of more condensed chromatin after drug treatment. The images were taken and sown in the fig-8 below.

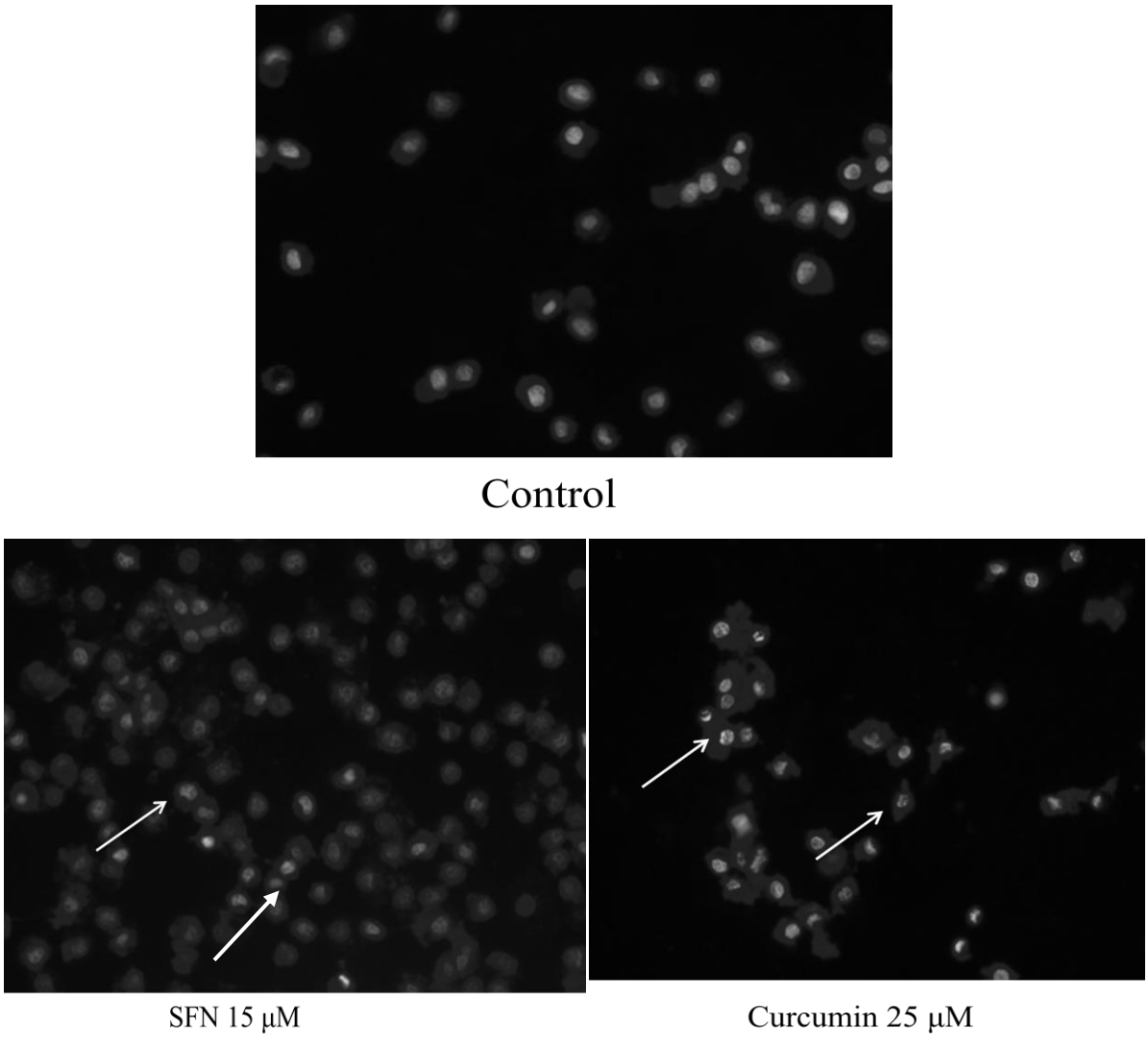


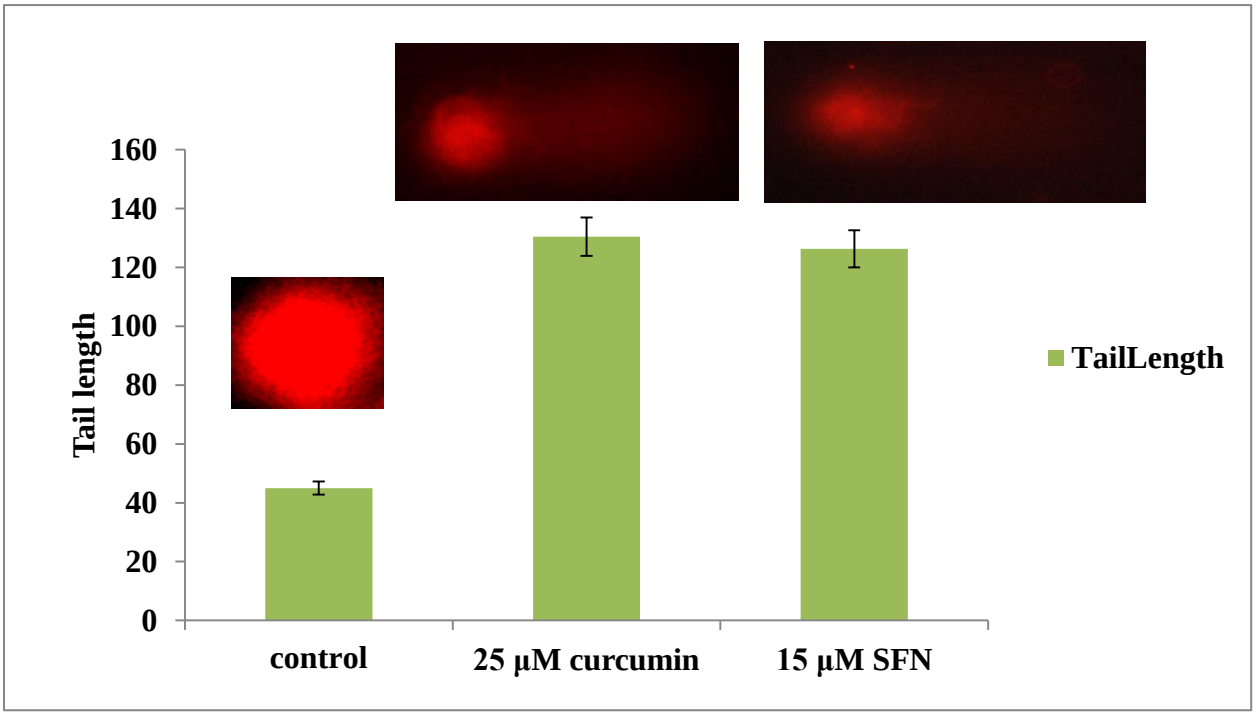
Fig-8:Fluroscence microscopic image of condensed chromatin of MDA-MB-231 cells treated with sulforaphane and curcumin for 24 hours with respect to control.

7. DNA damage measured by Comet assay:

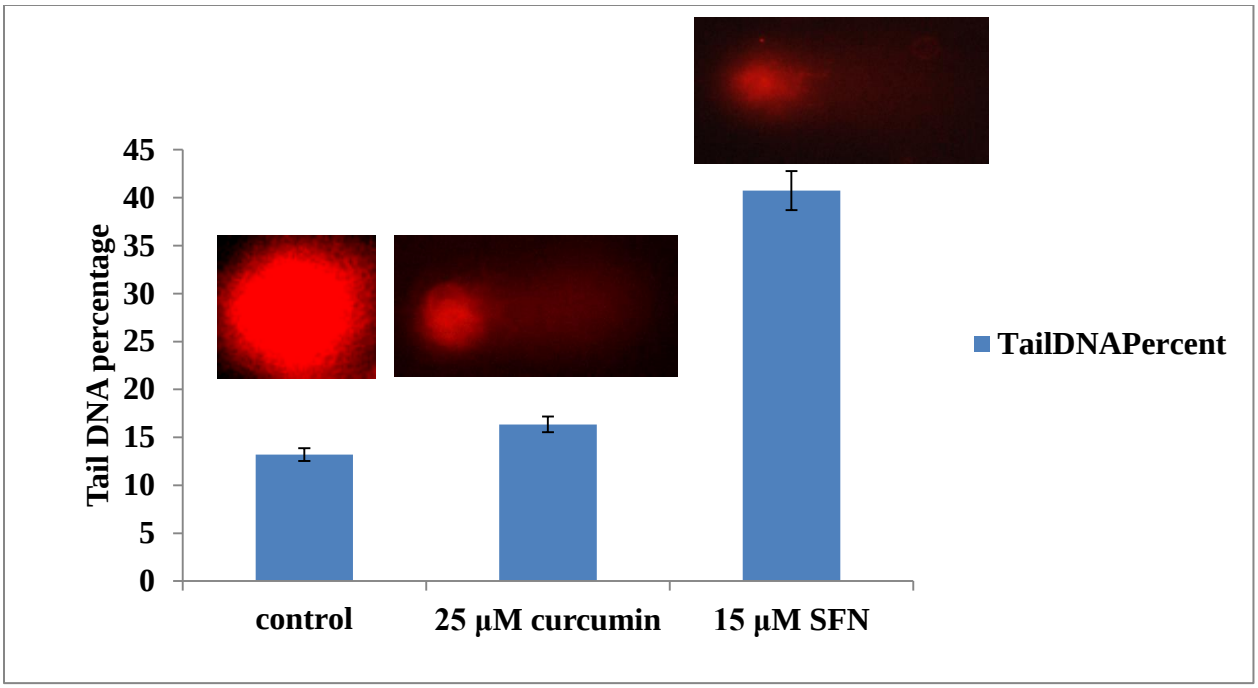
MDA-MB-231 cells after treated with sulforaphane and curcumin were analyzed for DNA damage.The characteristic formtion of comet tail in drug treated cells confirmed that DNA damage occurred due to the drug treatment,whereas the control showed the presence of a round head with no tail formation. Both curcumin and sulforaphanetreated cells were found to have prominent tail formation .The images of comet DNA were taken and analyzed with imagej software.The tail length, tail DNA percentage and tail moment were calculated with the help of software ang graphs were plottedwhich is shown below in graph-7,8,9 and the corresponding values are listed in the table-4.

Table-4:Quantification of comet parameters(tail length,tail moment and tail DNA %)

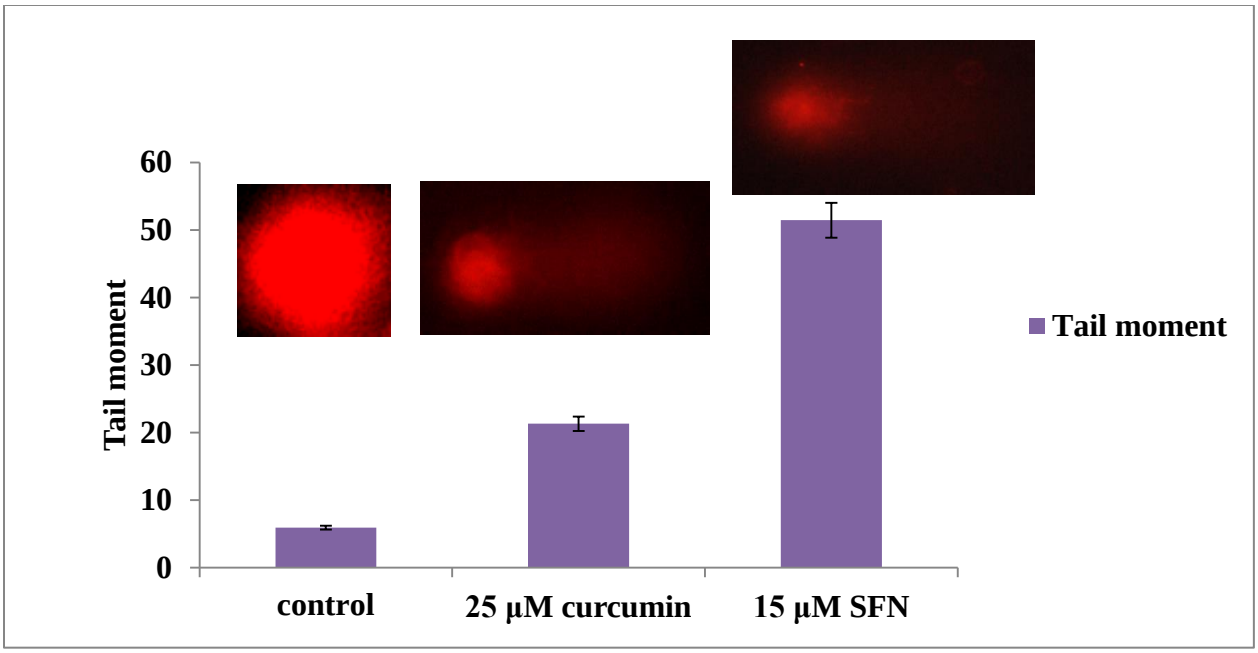
Drug	Tail length(in pixels)	Tail moment	Tail DNApercentage
Contro	45	5.936982331	13.19329407
25 μM curcumin	130.4	21.32449134	16.35313753
15 μM SFN	126.3	51.45276905	40.73853448



Graph-7: Tail length of the comet formed due to the effect of sulforaphane and curcumin treatment for 24 hours



Graph-8: Tail DNA percentage in the comet formed due to the effect of sulforaphane and curcumin treatment for 24 hours



Graph-9: Tail moment of the comet formed due to the effect of sulforaphane and curcumin treatment for 24 hours

8. Analysis of DNA fragmentation assay:

The gel picture showed the fragmented DNA in case of sulphoraphane and curcumin treated MDA-MB-231 cells but in the untreated control cells, DNA remained intact near the lane. The fragmented DNA was visualized as separated bands. Densitometric analysis of ladder lanes were done using Image-J software which showed distinct peaks representing a single band on the gel, ranging from high to low molecular weight. The gel picture and densitometric graph is shown in fig-9 and graph-10 respectively.

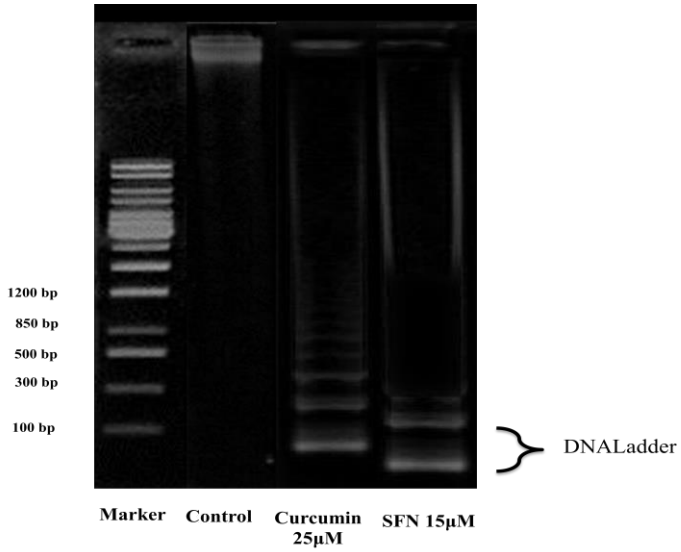
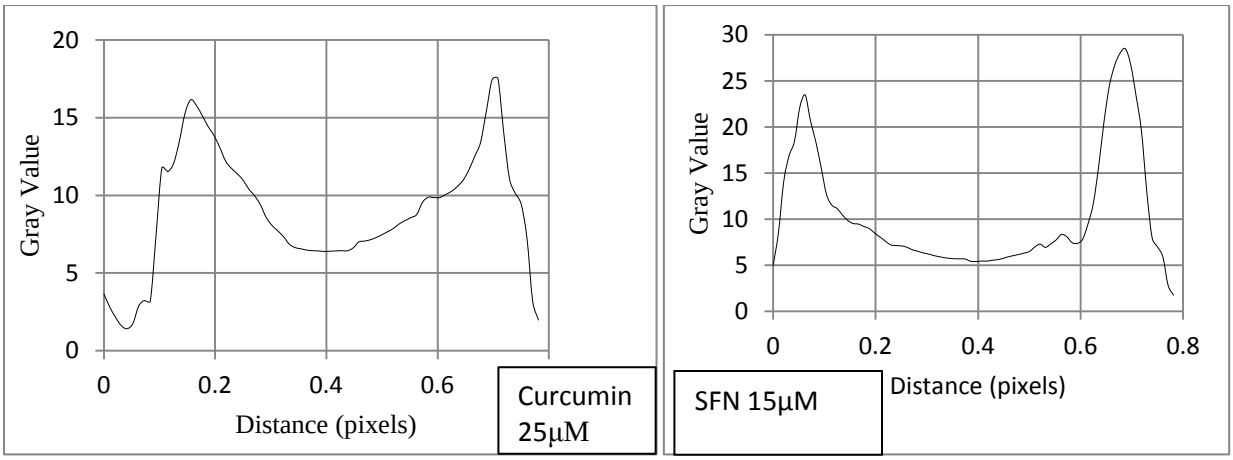


Fig-9: Fragmented DNA ladders of MDA-MB-231 cells treated with sulforaphane and curcumin for 24 hours.



Graph-10:Densitometric analysis of DNA fragmentation data

CONCLUSION

Cancer progression is a bewildering combination of genetic and epigenetic alterations associated with principal cellular dysfunctions. The relentless proliferation potential and suppressed cell death mechanisms provide the underlying mechanisms of neoplastic progression. Hence researchers are now a day trying to target those deregulated pathways with the help of epigenetic modulatory drugs which are potent enough to drive the cancer cells towards programmed cell death from which they generally escape out. The balance between pro apoptotic and anti apoptotic gene expression is needed for the regulation of cell death mechanisms. Additionally maintenance between methylation and demethylation patterns is tightly associated with cancer progression. Histone demethylase JMJD3 is having critical regulatory functions in cancer progression and its role in regulation of apoptosis is yet to be cleared. Here in this study, the down regulation of anti apoptotic gene Bcl-2 and up regulation of pro apoptotic gene Bax along with formation of chromatin condensation, DNA fragmentation and DNA damage suggests that there is induction of apoptosis in response to the sulphoraphane and curcumin treatment for 24 hours at their corresponding IC_{50} values. From all this investigations we can presume that may be these natural polyphenols are mediating their effect on cancer cells at transcriptome level by inducing apoptosis related genes. Moreover the down regulation of Jmjd3 and down regulation of Bcl-2with treatment of sulphoraphane and curcumin may be correlated to have a conjoint effect on apoptosis induction in breast cancer cells. Further studies at protein level may unravel hidden dimensions of JMJD3 in the induction of apoptosis in breast cancer cells which may create a better therapeutic avenue for treating cancer patients in future.

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