

SYNERGISTIC EFFECT OF TRICHOSTATIN A AND THYMOQUINONE IN INDUCING ANTICANCER ACTIVITY IN BREAST CANCER

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DECLARATION

This is to certify that Thesis entitled **“SYNERGISTIC EFFECT OF TRICHOSTATIN A AND THYMOQUINONE IN INDUCING ANTICANCER ACTIVITY IN BREAST CANCER”** which is the result of the work carried out by me. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions. The work was done under the guidance of Dr. Samir Kumar Patra, Associate Professor, Department of Life Science, National Institute of Technology, Rourkela.

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Date: 09/May/2015

CERTIFICATE

This is to certify that the thesis entitled "*Synergistic effect of Trichostatin A and Thymoquinone in inducing anti-cancer activity in Breast Cancer*" which is being submitted by Miss Priti Patel, Roll No.413LS2045 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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ABSTRACT

The field of epigenetic has witnessed a recent breakthrough in our information and stands at the forefront of biomedical research. Epigenetic alterations may result in changes in chromatin structure leading to a deregulation of gene expression associated with various diseases. Cancer has been deliberated to be brought about by a wide variety of genetic and genomic alterations. However, cancer development is not limited to the genetic changes, but may also include epigenetic changes. Breast cancer is the most common cancer and the principal reason of cancer death in women. In this context, anticancer drug is effective in reversing the epigenetic modifications. Although, trichostatin A (TSA) is an excellent inhibitor of histone deacetylase (HDAC) activity, it is highly toxic in nature. In contrast, Thymoquinone (TQ), an active component of *Nigella sativa*, being phytochemical is potentially non-toxic exhibiting anti-cancer property. The principle of this study was to examine the conjoint effect of TSA and TQ in inducing anticancer activity in MCF-7 breast cancer cells. Our experiments clearly demonstrate that TSA and TQ synergistically exert more anti-cancer effect on MCF-7 breast cancer cells than TSA and TQ in isolation. It is also observed that the combination of both TSA and TQ can more efficiently rejuvenate the expression of tumor suppressor genes (p21 and Maspin). Further investigation in this direction might open a new therapeutic window for the treatment of breast cancer.

KEYWORDS: Breast Cancer, Trichostatin A, Thymoquinone, Anticancer drugs

1. INTRODUCTION

The field of epigenetic has observed a recent breakthrough in our information concerning into ribonucleic acid (RNA) (Connolly et al, 2012).The Significance of epigenetic mechanisms is moving to the forefront of biomedical research (Vaissiere et al, 2008). Epigenetics refers to modifications in gene expression that are not a result of changes in the primary nucleotide sequence of a gene in contrary to that of genetic mutations. Instead, epigenetic alterations may result in changes in chromatin structure leading to a repressive chromatin state and silencing of both gene expression and transcription of DNA.

1.1 CANCER

Cancer has been considered to be brought about by a wide variety of genetic and genomic alterations, such as amplifications, translocations, deletions, and point mutations. It holds a dramatic end-point the activation of oncogenes and the inactivation of tumour-suppressor genes. However, cancer development is not restricted to the genetic changes, but may also involve epigenetic changes. Epigenetics is concerned with the inheritance of information based on gene-expression levels, as opposed to genetics, whose realm is that of information transmitted on the basis of gene sequence(Santiago Ropero et al, 2007).Breast cancer is the most common cancer and the principal reason of cancer death in women[WHO reports for Breast Cancer]. Early detection is essential to reduce cancer mortality. Analyzing the participation in an organized breast cancer screening program is important in order to assess the program success. Breast cancer estimation both permits minimally invasive breast surgery and reduce cause-specific mortality (Padoan et al, 2014).

The examined patterns of cervical and breast cancer screening among Asian American women in California and considered their screening trends over time. The pooled weighted data from 5 cycles of the California Health Interview Survey (2001, 2003, 2005, 2007, and 2009) to examine breast and cervical cancer showing developments and analysts among 6 Asian nationalities. The calculated informative statistics, bivariate associations, multivariate logistic regressions, predictive margins, and 95% confidence intervals was analyzed that Multivariate analyses indicated that Papanicolaou test rates did not significantly change over time (77.9% in 2001 vs. 81.2% in 2007), but mammography receipt increased among Asian American women overall (75.6% in 2001 vs. 81.8% in 2009). Socio-demographic and health care access factors had varied effects, with education and insurance coverage significantly predicting screening for certain groups. Conclusions from the results that underscore the need for intervention and policy efforts that are targeted to specific Asian nationalities, recent immigrants, and individuals without health care access to increase screening

rates among Asian women in California (Chawla et al , 2014). Breast cancer is one of the common disorders in women. Frequency and mortality due to cancer, particularly breast cancer has been increasing for last 50 years, even though there is an interval in the diagnosis of breast cancer at early stages.

According to World Health Organization (WHO) 2012 reports, breast cancer is the leading cause of death in women, accounting 23% of all cancer deaths. In Asia, one in every three women faces the risk of breast cancer in their lifetime as per reports of WHO 2012. Here, different breast cancer markers, that is, tissue markers (hormone receptors, human epidermal growth factor-2, uro kinase plasminogen activator, plasminogen activator inhibitor, p53 and cathepsin D), genetic markers (BRAC1 and 2 and gene expression microarray technique, etc.), and serum markers (CA 15.3, BR 27.29, MCA, CA 549, carcino-embryonic antigen, onco proteins, and cytokeratins) used in present diagnosis, but none of the mentioned markers can diagnose breast cancer at an early stage. There is a disquieting need for the identification of best diagnosing marker, which can be able to diagnose even in early stage of breast carcinogenesis (Donepudi et al, 2014).

1.2 CAUSES OF BREAST CANCER

For years, scientists have been involved in sorting out the origins of human cancer, and the comparative roles of genetic against epigenetic abnormalities have been argued. An eruption of data indicating the importance of epigenetic progression, particularly those causing in the silencing of key regulatory genes, has directed to the awareness that genetics and Epigenetics facilitated all stages of cancer development (Jones et al, 2007).

Epigenetics is defined as transferable changes in gene expression that are not accompany by changes in DNA sequence (Jones et al, 2007).Recent studies have exposed that Epigenetics plays a crucial role in cancer biology, mode of action of transportable elements, gene therapy of somatic cell lines, transgenic technologies, genomic imprinting, viral infections, neurological disorders, developmental abnormalities, and X-chromosome inactivation. The three most important epigenetic signalling mechanism are: DNA methylation, histone modification and RNAi associated silencing or activation.

1.3 EPIGENETICS

Epigenetics is defined as heritable changes in gene expression that are, divergent mutations, not attributable to modification in the sequence of DNA. The main epigenetic mechanisms are DNA methylation, loss of imprinting, modifications to chromatin and non-coding RNA (Hamilton et al,

2011). An epigenetic feature is a steadily heritable phenotype resultant from changes in a chromosome without change in the DNA sequence. It involves no change in the original genetic program even though; non-genetic factors cause appearance of differential phenotypes in the organism's genes. Recent studies have exposed that epigenetics plays a vital role in cancer biology, mode of action of movable elements, viral infections, gene therapy of somatic cell lines, transgenic technologies, genomic imprinting, developmental abnormalities, neurological disorders, and X-chromosome inactivation. The three major epigenetic signaling tools are: DNA methylation, RNAi associated silencing or activation and histone modification (figure: 1)

An epigenetic alteration that silences a tumor suppressor gene — such as a gene that maintain the growth of the cell in check — could direct to unrestrained cellular growth. Another example might be an epigenetic change that "turns off" genes that help restoring damaged DNA, primary to an increase in DNA damage, which in turn, increases cancer risk.

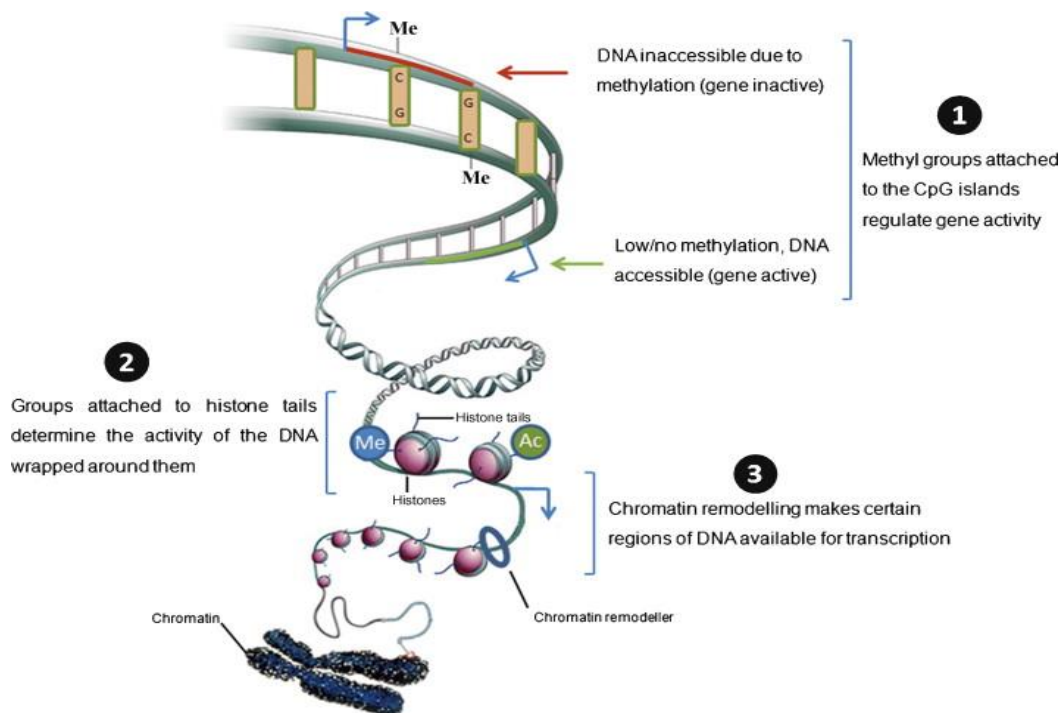


Figure 1: Epigenetics signaling of DNA sequence

1.3.1 DNA METHYLATION

DNA methylation is the covalent addition of a methyl group to the 5'-carbon (C5) position of cytosine bases in CpG dinucleotides. Cytosine methylation plays a significant role in a number of central cellular functions (Thomas et al, 2008).

Gene silencing may arise due to methylation of DNA at the promoter region of genes. Adenine, guanine, cytosine and thymine are the four bases which are the building blocks of our genetic make-up (Connolly et al, 2012). Of all epigenetic modifications, hyper methylation, have been most broadly

studied that causes suppression of transcription at the promoter area of tumours suppressor genes followed by gene silencing (Patra et al, 2008)

1.3.2 HISTONE MODIFICATIONS

Eukaryotic genomes are put together into a extremely dense chromatin, which imposes constriction on gene transcription and other chromatin-based development. To contract with this obstruction, histone acetylation has developed to open chromatin structure and make easy accessibility of transcriptional machinery to DNA templates in chromatin (figure 2). Histone acetylation is a reversible modification of specific residues in histone “tails” and is affected by histone acetylases (HATs) and histone deacetylases (HDACs) that normally act as transcriptional co-activators and co-repressors, respectively (Vaissiere et al , 2008) .An wide literature documents an detailed collection of reversible, dynamic post-translational modifications (PTMs) namely, acetylation, phosphorylation, ADP-ribosylation, methylation, ubiquitination, biotinylationm and SUMOylation occurring widely in the histone-tail and rarely in the core histones (Parbin et al,2014).

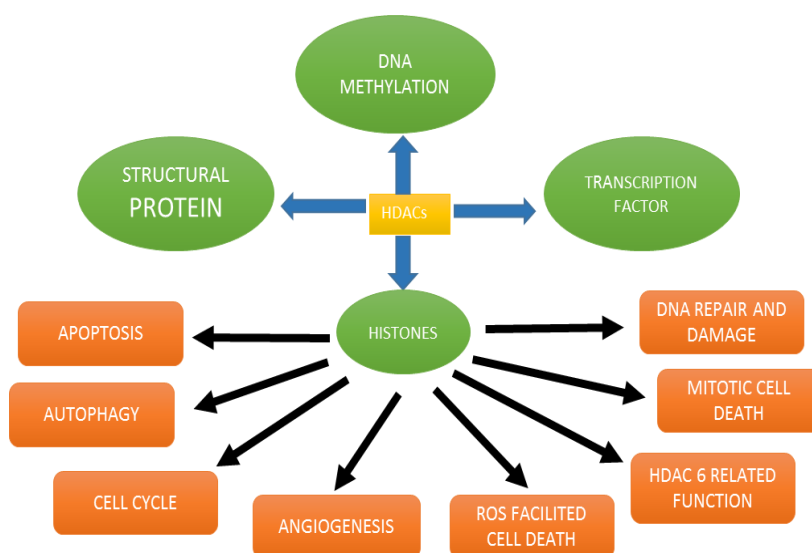


Figure 2: Role of HDAC inhibitor in cancer

1.3.3 HISTONE ACETYLATION, DEACETYLATION AND GENE REGULATION

The Acetyl-Coenzyme A is the most important source of the acetyl group in histone acetylation, and the acetyl group is transferred to Co-A in deacetylation. The negatively charged acetyl group neutralizes the positive charge on the histone, thus, decreases the relations of the histone with the phosphate group of DNA. This causes the transformation of folded structure into a relaxed structure (euchromatin) i.e., linked with greater levels of gene transcription (Figure 3). This can be inverted by HDAC activity that removes acetyl tags resulting in a more condensed structure of closely packed DNA referred to as heterochromatin. Histone modifications have various utilities in

diverse biological pathways ranging from gene regulation, chromosome condensation and DNA repair.

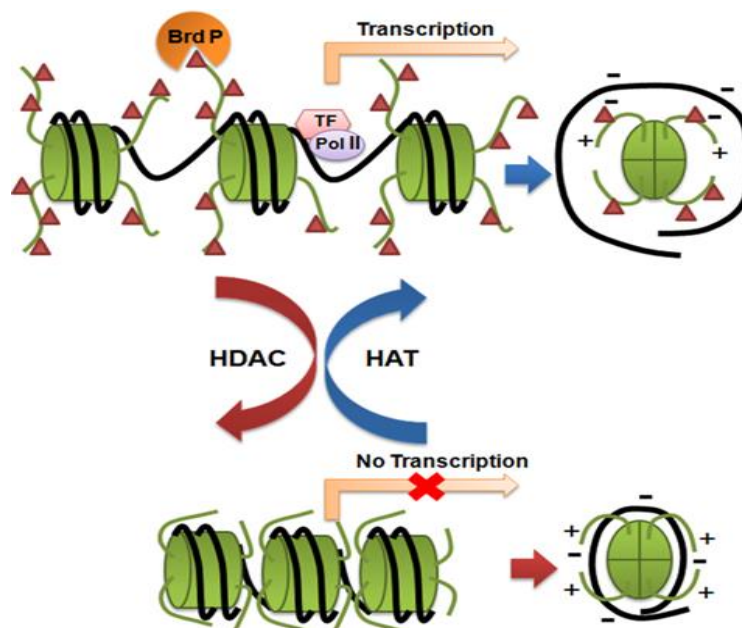


Figure 3: Histone acetylation and deacetylation (Parbin et al, 2014)

1.4 ANTICANCER DRUG

Anticancer drug, also called antineoplastic drug, is recognize by any drug that is effective in the conduct of malignant, or cancerous, disease. There are numerous major classes of anticancer drugs; these comprise alkylating agents, natural products, antimetabolites, and hormones. In addition, there are a number of drugs that do not fall within those classes but that reveal anticancer activity and thus are used in the treatment of malignant disease.

Trichostatin A (TSA), have a cytostatic and differentiating property in mammalian cell culture and inhibitor of histone deacetylase (HDAC) activity. The principle of this study was to estimate the anti-proliferative and HDAC inhibitory action of TSA in breast cancer cell lines (Vigushin et al ,2001) (figure 4(a)).Anticancer effects of TQ contains the inhibition of carcinogen metabolizing enzyme action and oxidative impairment of cellular macromolecules, reduction of inflammation, stimulation of cell cycle arrest and apoptosis in tumor cells, defend of tumor angiogenesis, and suppression of migration, invasion and metastasis of cancer cells. TQ shows synergistic and/or potentiating anticancer effects when combined with clinically used chemotherapeutic agents (Kundu et al, 2014) (figure 4 (b)).

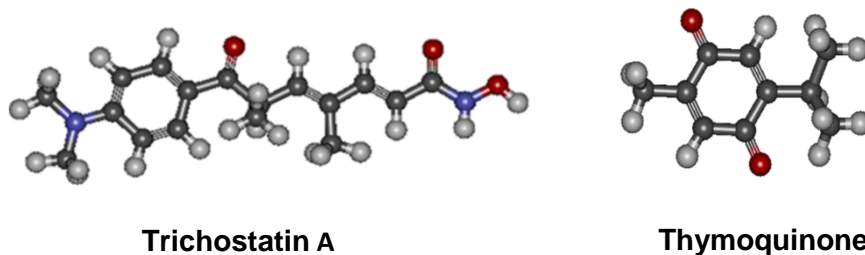


Figure 4: Molecular structure of (a) Trichostatin A and (b) Thymoquinone

1.4.1 TRICHOSTATIN A (TSA)

Trichostatin A is a selective and potent inhibitor of histone deacetylase (HDAC). It selectively inhibits the removal of acetyl groups from the amino-terminal lysine residues of core histones, resulting in chromatin relaxation and modulation of gene expression. Trichostatin A has been shown to inhibit both the G1- and G2- phases of the mammalian cell cycle and has been used to induce apoptosis in cancer cells with low toxicity to non-cancer cells

Histone deacetylase (HDAC) inhibitors have recently emerged as a new class of anti-cancer agents. Trichostatin A (TSA), a classical HDAC inhibitor, has been demonstrated to induce cell cycle arrest, promote cell apoptosis, and inhibit metastasis. However, the molecular mechanism underlying TSA function has not been fully elucidated. The histone deacetylase (HDAC) inhibitor Trichostatin A (TSA) is known to mediate the regulation of gene expression and anti-proliferation activity in cancer cells (Zohre et al , 2014).

Hydroxamic acid an important constituent of TSA, holds antifungal activity that was originally isolated from *Streptomyces hygroscopicus*. TSA is a specific and strong HDA-inhibitor (HDACi) which can alter gene expression and induce apoptosis in a variety of cancer cell sat very low concentration (Zohre et al , 2014).In the recent investigation, it has been identified that TSA treated HCT cells (colon-cancer cells) induces an altered expression of cell cycle-associated genes. Among the 84 genes related to cell cycle control, 34 genes were significantly altered by TSA treatment, with 7 genes upregulated and 27 genes down regulated. Interestingly, gene expression of minichromosomal maintenance protein-2 (MCM-2) was significantly down regulated by TSA treatment. This was confirmed by quantitative RT-PCR and Western blotting. Moreover, silencing of MCM-2 by siRNA led to cell cycle arrest and apoptosis in HCT116 cells. In addition, TSA caused an increase of phosphorylated JNK, which was involved in down regulation of MCM-2. Together, our results suggest that MCM-2 is a novel therapeutic target of TSA in colon cancer cells.

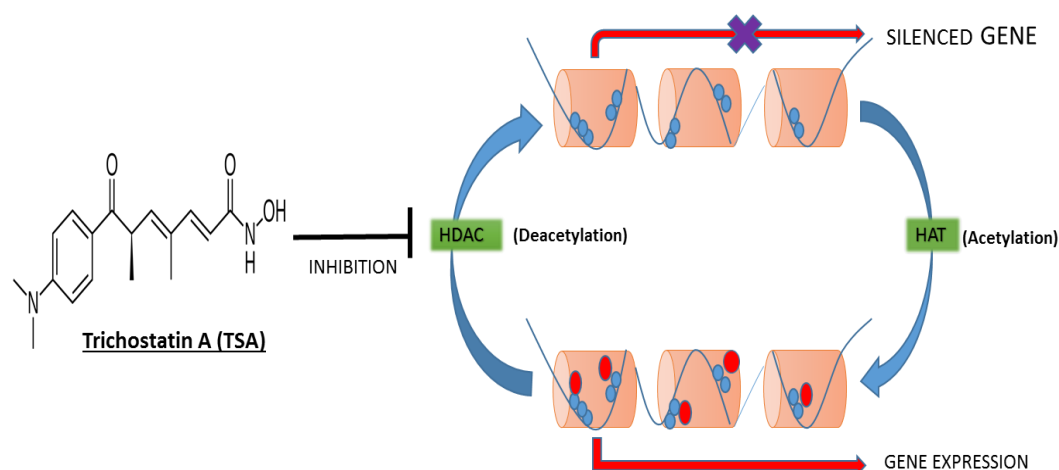


Figure 5: Inhibitory Effect of Trichostatin A on HDACs

1.4.2 THYMOQUINONE

Nigella sativa is an annual flowering plant that is native to Mediterranean countries, India, and Pakistan (Gali-Muhtasib et al, 2006). The seeds of *N. sativa* (often mentioned as black seed or black cumin) are used as a spice in Eastern cooking (Burits M et al, 2000, Hajhashemi V et al, 2004). Moreover, black seed has been used as a medicinal herb in Middle Eastern, Northern African, and Indian cultures for over 3000 years (Zohary D et al, 2000). Historically, the herb has been used to treat a number of illnesses associated with inflammation, including asthma, bronchitis, fever, arthritis, and rheumatism. More recently, it has been shown to have anticancer activity in animal and cellular models (Hasan et al, 2013). Thymoquinone (TQ) was shown to reduce tumor growth in several cancer models both in vitro and in vivo. So far there are only few targets of TQ including protein kinases have been identified. These kinases are promising candidates for targeted anticancer therapy, considered the complex kinase network regulated by TQ (Baba et al, 2014). Thymoquinone (TQ) has important roles in the prevention and treatment of cancer by controlling cell signaling pathways (Rahmani et al, 2014).

The antitumor activities of TQ, a compound isolated from *Nigella sativa* oil, were investigated in breast carcinoma in vitro and in vivo. TQ induced p38 phosphorylation and ROS production in breast cancer cells. These inductions were found to be responsible for TQ's anti-proliferative and pro-apoptotic effects. Moreover, TQ-induced ROS production regulated p38 phosphorylation but not vice versa. TQ treatment was found to suppress the tumor growth and this effect was further enhanced by combination with doxorubicin. TQ also inhibited the protein expression of anti-apoptotic genes, such as XIAP, survivin, Bcl-xL and Bcl-2, in breast cancer cells and breast tumor xenograft. Reduced Ki67 and increased TUNEL staining were observed in TQ-treated tumors. TQ was also found to increase the level of catalase, superoxide dismutase and

glutathione in mouse liver tissues. Overall, it has been demonstrated that the anti-proliferative and pro-apoptotic effects of TQ in breast cancer are mediated through p38 phosphorylation via ROS generation (Woo et al, 2012). *Nigella sativa* and TQ's anti-inflammatory potential account for the observed analgesic, antidiabetic, and antihistaminic effects, and ability to alleviate respiratory diseases, rheumatoid arthritis, multiple sclerosis, and Parkinson's disease(Salem et al, 2005).

2. REVIEW OF LITERATURE

cancer has been viewed as a disease that is determined by progressive genetic abnormalities, including mutations in oncogenes and tumour suppressor genes, and chromosomal abnormalities(Hanahan et al,2000 & Vogelstein et al, 2004).There are three epigenetic effects, DNA methylation, histone modifications and nucleosomal remodelling, mutually interact with each other to regulate gene expression(Pang-Kuo Lo et al,2008).The ultimate traits of cancerous cells, i.e. growth signals self-sufficiency, insensitivity to growth suppressing signals, apoptosis evasion, unlimited replicative potential, persistent angiogenesis, tissue incursion, and metastasis cooperatively lead to the alteration of a typical cell to a malignant phenotype. The chronological build-up of aberrations that arises during carcinogenesis exhibits considerable prospects for experimental interferences to avert cancer initiation and treat pre-neoplastic conditions. Carcinoma of the breast, prostate and colon are highly prevalent maladies in the Western nations and reports for roughly half of the total cancer-related mortalities among men and women (Jemal A et al, 2010).Introduction despite a remarkable progress in developing anticancer therapies, the incidence of various cancers and the number of cancer-related deaths are still on the rise (Mann et al, 2005). The increasing trend in chemotherapy failure, recurrence of certain tumours after primary cure, and the deterioration of patient's quality of life limit the real success of chemotherapy in fighting cancer (W. Baer et al, 2006).Genetic susceptibility, environmental factors and epigenetic modifications play a key role in carcinogenesis. Exposure of human beings to chemical compounds in the environment is the most critical risk issue in malignant transformation (Oliveira et al, 2007). The chronological build-up of aberrations that arises during carcinogenesis exhibits considerable prospects for experimental interferences to avert cancer initiation and treat pre-neoplastic conditions.

Living cells are constantly exposed to different sources of DNA damaging agents. In order to maintain their genomic integrity and survive, cells have evolved a complex response that either leads to apoptosis or cell cycle arrest. Several genes that are activated by stress responsive protein kinases regulate these two cellular events. In general, the genes that are activated by the stress response play a protective role against the cellular insults (Kimb et al, 2005).

2.1 EFFECT OF ANTICANCER DRUGS IN CANCER

2.1.1 TRICHOSTATIN A

The Histone deacetylase (HDAC) inhibitor Trichostatin A (TSA) is known to mediate the control of gene expression and anti-proliferation activity in cancer cells (Zohre et al, 2014).

According to the Xu S et al, Using cell lines representing different stages of endometrioid cancers the impact of DNMT inhibitor, ADC, and HDAC inhibitor, TSA, was examined on cell cycle and apoptosis. It was found that while both reagents were capable of preventing cell proliferation and bringing cell apoptosis, it was found that TSA appeared to be a more potent apoptosis inducer, but with a smaller effect on cell cycle. On the other hand, ADC exhibited strong effects on cell cycle regulation, but had smaller impact on cell apoptosis. It was subsequently confirmed the presence of a strong synergism between DNMT and HDAC inhibitors. Thus, ADC and TSA exhibited strong cytostatic and apoptotic effects in endometrial cancer cell lines and the combined application may deliver the highest response (Xu S et al, 2014).

According to the study by Sang-Min Jang et al, PUMA is a crucial regulator of apoptotic cell death mediated by p53-dependent and p53-independent mechanisms. In his study, it was found that the transcription factor Sox4 increased PUMA expression in reply to Trichostatin A (TSA), a histone deacetylase inhibitor in the p53-null human lung cancer cell line H1299. The protein and mRNA levels of PUMA were increased by TSA, resulting in apoptotic cell death (Jang et al, 2013).whereas according to Feng et al, PUMA was down regulated in gastric cancer cell lines and main gastric carcinoma tissues. Patients with low PUMA manifestation had significant decreases in complete survival. Ectopic PUMA appearance prevented the growth of gastric cancer cells whereas PUMA reduction encouraged cellular growth. It has been informed that the knockdown of limited HDAC3 effects upregulated PUMA expression. HDAC3 could bind to PUMA promoter, which was nullified after TSA treatment. In compare to TSA and SB, HDAC3 siRNA failed to upregulate p53 expression but encouraged the interaction of p53 with PUMA promoter. In other word it may be said that, PUMA was down regulated in gastric cancer. HDAC3 is significant to down regulate PUMA expression in gastric cancer and HDAC is, like TSA, helped PUMA expression through stabilizing p53 in addition to HDAC3 inhibition. In combination with chemotherapy, directing HDAC3 influence to be a capable approach to encourage apoptosis of gastric cancer cells.

According to Ruan et al, the effect of Trichostatin A (TSA) on the gastric cancer cell line BGC-823 was studied for growth inhibition and apoptosis.TSA can bring apoptosis, and increase acetylated histone H4 in BGC-823 cells. GPNMB expression is reduced in BGC-823 cells after TSA

action. GPNMB is highly expressed in gastric adenocarcinoma tissue. GPNMB involved in TSA-induced apoptosis might contribute in gastric cancer (Ruan et al, 2014).

According to Ma et al, ESCC cells were treated with Trichostatin A (TSA) and its antitumor properties and related mechanisms were examined. The results showed that TSA suppressed the propagation of ESCCs and caused G1 phase arrest by inducing the expression of p21 and p27. TSA also induced cell apoptosis by improving the expression of pro-apoptotic protein Bax and reducing the expression of anti-apoptotic protein Bcl-2. Moreover, TSA inhibited the expression of phosphatidylinositol-3-kinase (PI3K) and reduced the phosphorylation of Akt and extracellular signal-regulated kinase (ERK) 1/2 in EC9706 and EC1 cell lines. High intensities of acetylated histone H4 were identified in TSA-treated ESCC cell lines. Generally, it indicates that TSA overpowers ESCC cell growth by inhibiting the stimulation of the PI3K/Akt and ERK1/2 pathways. TSA also stimulates cell apoptosis complete epigenetic regulation of the appearance of apoptosis-related protein (Ma et al, 2015). Whereas, according to Wang et al, the Expression of HDAC2 was significantly higher in ESCC than in adjacent non-tumour tissues. Additionally, the in vitro invasion assay found that both down regulation of HDAC2 expression and TSA treatment inhibited ESCC cell invasion by approximately 75%. Also, an MMP2/9-specific inhibitor sharply suppressed ESCC cell invasion. Furthermore, both down regulation of HDAC2 and treatment with TSA decreased MMP-2 and MMP-9 protein levels in ESCC cells (Wang et al, 2013).

According to Tu et al, treatment with HDAC inhibitors Trichostatin A (TSA), but not suberoyl anilide hydroxamic acid (SAHA) or HDAC siRNA, can attenuate both protein and mRNA expressions of EGFR in lapatinib-treated triple-negative breast cancer cells, suggesting that TSA may suppress EGFR expression independently of HDAC inhibition. Nevertheless, TSA reduced EGFR 3'UTR activity and induced the gene expression of microRNA-7, a known EGFR-targeting microRNA. Furthermore, treatment with microRNA-7 inhibitor attenuated TSA-mediated EGFR suppression. These results suggest that TSA induced microRNA-7 expression to down regulate EGFR expression in an HDAC-independent manner (Tu et al, 2015).

2.1.2 THYMOQUINONE

Thymoquinone, a monoterpene present in black cumin seeds, shows pleiotropic pharmacological activities containing antioxidant, antidiabetic anti-inflammatory and anti-tumour effects. It is a compound obtained from Black Caraway seeds of *Nigella Sativa* and is active against various cancers (Syed et al, 2010). The bioactive natural yields (plant secondary metabolites) are extensively known to hold therapeutic importance for the inhibition and treatment of several chronic

diseases containing cancer. TQ has anti-inflammatory effects, and it inhibits tumor cell proliferation through modulation of apoptosis signaling, inhibition of angiogenesis, and cell cycle arrest (Banerjee et al, 2010). Several bioactive components of black seed have been identified, including thymoquinone, thymol, thymohydroquinone, and dithymoquinone. Among them thymoquinone (TQ) has been reported to exhibit antioxidant, anti-inflammatory, and chemo preventive effects (Attoub et al, 2012). TQ inhibits investigational carcinogenesis in a wide range of animal models and has been shown to arrest the growth of various cancer cells in culture as well as xenograft tumours in vivo (Juthika et al, 2014). Two different studies have conjugated TQ with fatty acids to enhance its membrane penetration capacity and antitumor activity. In both studies, the antitumor activity of TQ was significantly improved by attaching fatty acid derived groups to the molecule (Banerjee et al, 2010, Woo et al, 2012)

According to Yi et al, Thymoquinone shows inhibitory properties on cell proliferation of many cancer cell lines and hormone-refractory prostate cancer by overpowering androgen receptor and E2F-1. Thymoquinone repress cell proliferation and suppress the activation of AKT and ERK. It blocks angiogenesis in vitro and in vivo, avoid tumour angiogenesis in a xenograft human prostate cancer (PC3) model in mouse and repressed human prostate tumour growth at low amount with almost no insignificant toxin side effects. Besides, it was detected that endothelial cells were more profound to thymoquinone-induced cell proliferation, cell apoptosis and migration inhibition associated to PC3 cancer cells. It prevents VEGF-induced ERK stimulation, but revealed no inhibitory effects on VEGF receptor 2 activation. Generally, results specify that thymoquinone prevents tumour angiogenesis and tumour development, and might be used as a possible drug candidate for cancer therapy.

According to Woo CC et al, the anticancer properties of TQ on breast cancer cells, and its effect on the PPAR- γ beginning pathway. They establish that TQ employed strong anti-proliferative effect in breast cancer cells and, when joined with doxorubicin and 5-fluorouracil, enlarged cytotoxicity. TQ was found to increase sub-G1 growth and annexin-V positive staining, signifying apoptotic stimulation. Moreover, TQ activated caspases 8, 9 and 7 in a dose-dependent method. Migration and invasive properties of MDA-MB-231 cells were also reduced in the presence of TQ. Remarkably, for the first time that TQ was capable to increase PPAR- γ action and down-regulate the expression of the genes for Bcl-2, Bcl-xL and survivin in breast cancer cells. Moreover the rise in PPAR- γ activity was prevented in the presence of PPAR- γ specific inhibitor and PPAR- γ dominant negative plasmid, signifying that TQ may act as a ligand of PPAR- γ . Also, detected using molecular docking study that TQ indeed formed interactions with 7 polar residues and 6 non-polar residues

within the ligand-binding pocket of PPAR- γ that are described to be critical for its activity. Their different observations suggest that TQ may have potential effect in breast cancer prevention and treatment, and demonstration for that the anti-tumour effect of TQ may also be facilitated through variation of the PPAR- γ activation pathway (Woo et al, 2011).

2.1.3 CURCUMIN

Turmeric, a golden spice obtained from the rhizome of the plant *Curcuma longa*, has been used to give color and taste to food preparations since ancient times. Traditionally, this spice has been used in Ayurveda and folk medicine for the treatment of such ailments as gynecological problems, gastric problems, hepatic disorders, infectious diseases, and blood disorders. Various chemical constituents have been isolated from this spice, including polyphenols, sesquiterpenes, diterpenes, triterpenoids, sterols, and alkaloids. Curcumin, which constitutes 2-5% of turmeric, is perhaps the most-studied component (Gupta et al, 2012).

According to Dahmke et al, they pyrolysis curcumin with and without coconut fat or olive oil, and investigated the products by high-performance liquid chromatography (HPLC). A number of more hydrophilic curcumin isoforms and disintegration products, including a compound later identified by nuclear magnetic resonance spectroscopy (NMR) as "deketene curcumin" (1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadiene-3-one), formerly described as a synthetic curcumin derivative, were detected. Additionally, they proved that deketene curcumin, compared to curcumin, exhibits higher toxicity on B78H1 melanoma cells resulting in G2 arrest. In assumption to that, deketene curcumin is formed as a significance of pyrolysis during common household cooking, showing stronger anti-cancer effects than curcumin.

According to Goel et al, It binds to a variety of proteins and inhibits the activity of various kinases. Curcumin also down regulates cyclin D1, cyclin E and MDM2; and upregulates p21, p27, and p53.

According to Dang et al, Paclitaxel, isolated from *Taxusbrevifolia*, is considered to be an efficacious agent against a wide spectrum of human cancers, including human cervical cancer. Curcumin, a nontoxic food additive, has been reported to improve paclitaxel chemotherapy in mouse models of cervical cancer. In this study, two human cervical cancer cell lines, CaSki and HeLa, were selected in which to investigate the effect of curcumin on the anticancer action of paclitaxel and further clarify the mechanisms. The expression levels of p53 protein and cleaved caspase-3 were increased significantly in the curcumin plus paclitaxel-treated HeLa and CaSki cells compared with those in the cells treated with paclitaxel alone ($P < 0.01$). Significant reductions in the levels of

phosphorylation of I κ B α and the p53-NF- κ B subunit in CaSki cells treated with curcumin and paclitaxel were observed compared with those in cells treated with paclitaxel alone ($P < 0.05$). This suggests that the combined effect of curcumin and paclitaxel was associated with the NF- κ B-p53-caspase-3 pathway. In conclusion, curcumin has the ability to improve the paclitaxel-induced apoptosis of HPV-positive human cervical cancer cell lines via the NF- κ B-p53-caspase-3 pathway. Curcumin in combination with paclitaxel may provide a superior therapeutic effect on human cervical cancer (Dang et al, 2014).

According to Zeighamian et al, Poly (N-isopropyl acrylamide-co-methacrylic acid) (PNIPAAm-MAA) is one of the hydrogel copolymers utilized in the drug delivery system for cancer therapy. The aim of the study was to examine the cytotoxic potential of curcumin encapsulated within the NIPAAm-MAA nanoparticle, on the MCF-7 breast cancer cell line. Curcumin-loaded NIPAAm-MAA has more cytotoxic effect on the MCF-7 cell line and efficiently inhibited the growth of the breast cancer cell population, compared with free curcumin. Overall, it is concluded that encapsulating curcumin into the NIPAAm-MAA copolymer specifies that curcumin-loaded NIPAAm-MAA suppresses the growth of the MCF-7 cell line could open up new possibilities for breast cancer treatment (Zeighamian et al, 2015).

According to Choudhury et al, Apigenin and curcumin synergistically induced cell death and apoptosis and also blocked cell cycle progression at G2/M phase of A549 cells. The synergistic activity of apigenin and curcumin was also noticeable from their strong depolymerizing effects on interphase microtubules and inhibitory effect of reassembly of cold depolymerized microtubules when used in combinations, indicating that these ligands bind to tubulin at different sites. In silico modeling suggested apigenin binds at the interphase of α - β -subunit of tubulin. Binding studies with purified protein also showed both apigenin and curcumin can simultaneously bind to purified tubulin. Understanding the mechanism of synergistic effect of apigenin and curcumin could be helped to develop anti-cancer combination drugs from cheap and readily available nutraceuticals.

According to Yunos et al, Drug resistance remains an on-going challenge in ovarian cancer chemotherapy. The objective of this study was to determine the effect on synergism in activity from the sequenced combinations of cisplatin (Cis) with curcumin (Cur) and epigallocatechin-3-gallate (EGCG) in the human ovarian cancer cell lines. The drugs were added in binary combinations: Cis combined with Cur, and Cis combined with EGCG to the human ovarian A2780 and A2780 (cisR) cancer cell lines, using five different sequences of administration. When sequenced combinations of Cis with Cur and with EGCG are applied to human ovarian A2780 and A2780 (cisR) cancer cell

lines, lower concentrations and shorter time gap between the two additions seem to produce a higher cytotoxic effect (Yunos et al, 2011).

2.1.4 SULFORAPHANE (SFN)

TSA is a potent histone deacetylase inhibitor and can considerably enhance gene expression. Sulforaphane (SFN) is bioactive food component found in cruciferous vegetables and is a histone deacetylase inhibitor, leads to a transcriptional activation of gene expression. Sulforaphane (SFN), a compound found at high levels in broccoli and broccoli sprouts.

According to Gibbs et al, sulforaphane have the potential to reduce prostate cancer growth by inhibiting histone deacetylases, which are upregulated in cancer. Sulforaphane promotes HSP90 acetylation, thereby preventing its association with AR. Moreover, AR is frequently degraded in the proteasome, which leads to reduce AR target gene expression and reduce AR occupancy at its target genes. Finally, sulforaphane inhibits HDAC6 deacetylase activity, and the effects of sulforaphane on AR protein are reversed by overexpression of HDAC6 and mimicked by HDAC6 siRNA. The inactivation by sulforaphane of HDAC6- enabled HSP90 deacetylation and subsequent decline of AR signaling represents a newly defined mechanism that may help explain this agent's effects in prostate cancer (Gibbs et al, 2009).

According to Nagaveni et al , the interaction of SFN, an anticancer agent, with Amyloid beta (A β) was studied using ESI-MS. SFN is found to bind covalently and specifically with the free NH(2) group of N-terminal aspartic acid and the ϵ -amino group of lysine at positions 16 and 28. Aggregation assay studies showed a lesser tendency of A β to aggregate when SFN is present. Hence the present study helps in understanding the mechanism of the action of SFN on the A β peptide (Nagaveni et al, 2006).

According to Beaver et al, they utilized RNA sequencing and determined the transcriptomes of normal prostate epithelial cells, androgen-dependent prostate cancer cells, and androgen-independent prostate cancer cells treated with SFN. SFN treatment vigorously altered gene expression and resulted in distinct transcriptome profiles depending on prostate cell line. SFN also down-regulated the expression of genes that were up-regulated in prostate cancer cells. Network analysis of genes altered by SFN treatment revealed that the transcription factor Specificity protein 1 (Sp1) was present in an average of 90.5% of networks. Sp1 protein was significantly decreased by SFN treatment in prostate cancer cells and Sp1 may be an important mediator of SFN-induced changes in expression (Beaver et al, 2014)

According to Hussain et al, SFN showed cytotoxic effects on MCF-7 cells in a dose- and time-dependent manner by an apoptotic mode of cell death. Moreover, a combinational treatment of SFN and gemcitabine on MCF-7 cells resulted in growth inhibition in a synergistic manner with a combination index (CI) <1. SFN was found to significantly down regulate the expression of Bcl-2, an anti-apoptotic gene, and COX-2, a gene involved in inflammation, in a time-dependent manner. These results indicate that SFN induces apoptosis and anti-inflammatory effects on MCF-7 cells via down regulation of Bcl-2 and COX-2 respectively. The combination of SFN and gemcitabine might potentiate the efficacy of gemcitabine and minimize the toxicity to normal cells. Taken together, SFN may be a potent anti-cancer agent for breast cancer treatment (Hussain et al, 2013).

3. OBJECTIVES

- 1.** To study cytotoxic effect of Trichostatin A (TSA) and Thymoquinone (TQ) in isolation as well as in conjoint treatment to breast cancer cells (MCF-7) and normal skin keratinocytes (HaCat).
- 2.** To demonstrate apoptosis inducing activity of the combination treatment in contrast to single agent treatment in MCF-7 breast cancer cells.
- 3.** To examine anti-proliferative and anti-migratory efficiency of the combination treatment in MCF-7 cells.
- 4.** To demonstrate coordinated effect of the drugs in reactivation of tumor suppressor genes primarily p21 and Maspin in MCF-7 breast cancer cells.

4. MATERIALS AND METHODS

4.1 Cell Lines and Cell Culture:

Human breast cancer cell line MCF7 and normal human keratinocytes HaCat were procured from National Centre for Cell Science (NCCS, Pune) and were cultured in Minimum Essential Eagle's Medium (MEM) and Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Invitrogen) and 100 IU/mL Penicillin and 0.1 mg/mL streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. The cells were harvested by trypsinization and the number of living cells was calculated by Trypan blue staining (0.2% v/v) using haemocytometer.

4.2 Cell viability assay:

This assay was done to determine the sub-lethal concentrations (IC₅₀) of drug and proliferative activity of the cells in presence of drug. MCF 7 (80 % confluent) and HaCat cells were seeded into 96 well-plate at a density of 5×10^3 cells/well. After 24 h, the cells were treated with TrichostatinA (TSA), thymoquinone (TQ) and TSA+TQ at 7 different concentrations and incubated for 24 h. In parallel, the cells with the solvent control (DMSO) were also treated to assess its effect on cells. After completion of treatment period, to detect the cell viability MTT working solution was prepared by diluting the stock solution (stock 5mg/ml PBS, PH 7.2) in growth medium without FBS to the final concentration of 0.8mg/ml. 100 µl of MTT working solution was added to each well and incubated for 4 h in CO₂ incubator. After incubation, the media was removed without disturbing formazan precipitate and dissolved in 100 µl of 100% DMSO. An incubation of 15 minutes was carried out in dark and the colorimetric estimation of formazan product was performed at 570nm in a microplate reader (Perkin Elmer). The experiment was repeated thrice and the data (mean ± S.D) were plotted against drug concentration and non-linear regression curve fitting was performed.

4.3 Analysis of chromatin condensation by Hoechst 33342 stain:

For chromatin condensation assay, MCF 7 breast cancer cells (10^4 cells/well) were seeded in 6 well culture plates and allowed to grow for one day. Then cells were treated with TSA, TQ and TSA + TQ as mentioned above. After 24 hours of drug treatment, cells were stained with Hoechst 33342 (1 mg/ml) and incubated for 10 min at 37°C and images were taken under UV filter using Epi-fluorescent Microscope (Olympus IX71) at 400 X magnification. Condensed nucleus was counted against total number of nucleus in the field, and the percentages of apoptotic nuclei were analyzed.

4.4 Measurement of DNA Damage by Comet Assay

Comet assays were performed under alkaline conditions to determine the amount of double-strand DNA breaks. Two water baths were equilibrated at 40 °C and 100 °C respectively. Then 1% low-gelling-temperature agarose was prepared by mixing powdered agarose with distilled water in a glass bottle. The bottle was placed in the 100 °C water bath for several minutes and was transferred into a 40°C water bath. Agarose-precoated slides were prepared by dipping the slides into molten 1% agarose and wiping one side clean. Agarose was allowed to air-dry to a thin film. Slides were prepared ahead of time and stored with desiccant.

MCF-7 was subjected to treatment with TSA, TQ and TSA+TQ for 24 h. Then cells were harvested and added to preheated (37°C) low-melting point agarose. The solution was pipetted onto slides precoated with 1% agarose. The slides were allowed to lyse overnight at 4°C in alkaline lysis solution (1.2 M NaCl, 100 mM Na₂EDTA, 0.1% sodium lauryl sarcosinate, 0.26 M NaOH, pH > 13) prior to immersion in alkaline electrophoresis solution (0.03 M NaOH, 2 mM Na₂EDTA, pH ~12.3). After 30 min, slides were placed into a horizontal electrophoresis chamber samples for 25 min (0.6 V/cm). The slides were washed with deionized H₂O to remove the alkaline buffer, stained with propidium iodide (10 µg/ml stock) and incubate for 20 min. The slides were then washed with water and examined by Epi-fluorescent Microscope (Olympus IX71).

4.5. Clonogenic assay:

MCF 7 cells were plated at a low density (500 cells/well) in a 6 well plate, allowed to attach for 24 hours prior to treating with the drugs. The cells were treated with TSA, TQ at their respective IC₅₀ values and both TSA+TQ (50 nM TSA and 19 µM TQ). Cells were grown until distinct colonies were visible in untreated controls. Then they were fixed and stained with a mixture of 6.0% glutaraldehyde and 0.5% crystal violet, air dried, photographed and evaluated for colony estimation.

4.6 Cell migration assay:

MCF-7 were seeded to 6 well plate to create a confluent monolayer and incubated properly for approximately 6 hours at 37°C, allowing cells to adhere and spread on the substrate completely. The required number of cells for a confluent monolayer depends on both the particular cell type and the size of dishes and need to be adjusted appropriately. The cell monolayer was scraped in a straight line to create a scratch with a p200 pipette tip.

The debris was removed and the edge of the scratch was smoothed by washing the cells once with 1 ml of 1XPBS, pH7.2 and then replaced with 2 ml of MEM containing TSA, TQ and TSA + TQ. To obtain the same field during the image acquisition markings were created to be used as reference points close to the scratch. The first image of the scratch was taken and referred as 0 hour.

After the wound in the control was healed up the photographs were taken under a phase-contrast microscope.

4.7 Total Cellular RNA isolation

The total cellular RNA was extracted using TRI reagent (Sigma), following the manufacturer's instructions. MCF-7 cells were homogenized using TRI reagent i.e. 1 ml of the TRI Reagent per 10 cm² of glass culture plate surface area. After addition of the reagent, the cell lysate was passed several times through a pipette to form a homogenous lysate. Phase separation was done by allowing the samples to stand for 5 minutes at room temperature followed by addition of 0.2 ml of chloroform per ml of TRI reagent. Samples were covered and shaken vigorously for 15 seconds, allowing standing for 10 minutes at room temperature. The resulting mixture was centrifuged at 12,000g for 15 minutes at 4°C. Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA). The aqueous phase was transferred into a fresh tube and 0.5 ml of isopropanol was added per ml of TRI Reagent & mixed. The sample was allowed to stand for 5–10 minutes at room temperature and centrifuged at 12,000 g for 10 minutes at 2–8 °C. The RNA precipitate formed a pellet on the side and bottom of the tube. The supernatant was removed and RNA pellets were washed by adding a minimum of 1 ml of 75% ethanol per 1 ml of TRI Reagent. The sample was vortexed and then centrifuged at 7,500 g for 5 minutes at 4 °C. The RNA pellets were dried briefly for 5–10 minutes by air drying. An appropriate volume of nuclease free water was added and mixed by repeated tapping at 25 °C for 10–15 minutes.

4.8 Quantification of total cellular RNA

Final preparation of RNA was analyzed using a Nano-drop UV spectrophotometric analyzer. The standard preparation of RNA is expected to have a 260/280 ratio of 1.8-2.0 and a 260/230 ratio of <1.65, which indicates the preparation to be free from proteins and oligo-peptides contamination.

4.9 cDNA preparation

In a 1.5 ml tube, 1 µg of template RNA was taken and the volume was adjusted to 11 µl with nuclease free water. To it 1 µl of oligo-dT primer was added to make a total volume of 12 µl. The tube with the contents was mixed by centrifugation and incubated at 65°C for 5 minutes.

The tube was then snap cooled on ice and given a short spin. After that 4 µl of Reverse Transcriptase buffer (5X) , 1 µl of Ribolock RI, 1 µl of Reverse Transcriptase, and 2 µl of 10mM dNTP mix was added to make a total volume of the reaction mixture 20 µl .The tube was then snap spun for few minutes. The PCR was carried and the DNA was amplified under the following conditions- i.e. 42°C for 60 minutes and terminated at 70°C for 5 minutes. The amplified cDNA was stored at -20°C for

further processing. The synthesized cDNA was evaluated by performing PCR over the house keeping gene such as GAPDH.

GAPDH mRNA expression as internal control

The amount of cDNA of different samples was so adjusted to have the expression of housekeeping gene (GADPH) similar in both control and treated samples.

4.10 Gradient PCR

The PCR primers were chemically manufactured and procured from Sigma. A gradient PCR was done to optimize the annealing temperature of each set of primers. We used 3 sets of primers to amplify p21, Maspin and GAPDH genes having sequence as detailed in Table no.2. The annealing temperature for each set of primers was optimized from 50°C to 60°C in order to obtain the most suitable temperature for the primers to be annealed on the target sequence. The PCR were carried out using standard protocols in a thermo cycler (Bio-Rad) and the DNA was amplified under the following conditions: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 50°C to 56°C for 30 s, and 72 °C for 30 s, and the final extension of 72 °C for 5 min. The PCR products for each sample were electrophoresed on a 1.5% agarose gel to determine the optimum annealing temperature.

Table 1: Primers used for RT-PCR Analysis

SL. NO.	PRIMER NAME	PRIMER SEQUENCE	AMPLICON SIZE (in bp)
1	p21 – forward primer	5'--TGAGCCGCGACTGTGATG—3'	82
	P21 – reverse primer	5'--GTCTCGGTGACAAAGTCGAAGTT—3'	
2	Maspin – forward primer	5'--GGAATGTCAGAGACCAAGGGA—3'	139
	Maspin – reverse primer	5'--GGTCAGCATTCAATTCATCCTT—3'	
3	GAPDH– forward primer	5'—GGAGCGAGATCCCTCCAAAAT—3'	197
	GAPDH – reverse primer	5'—GGCTGTTGTCATACTTCTCATGG—3'	

4.11 Gene Expression Analysis of p21, Maspin by Semi-quantitative reverse-transcription PCR (Semi-q-RT-PCR)

MCF 7 cells were treated with sub lethal dosages of TSA, TQ& TSA +TQ for 24 hours. Total cellular RNA was extracted from treated samples and untreated control with TriReagent (Sigma) according to the manufacturer's instructions. cDNA was prepared using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit. cDNA from different treatment groups were used to analyze the expression of p21, Maspin and GPDH as a house keeping gene.

PCR conditions:

The PCR sample mixtures, in a 20 µl volume, contained 15.4 µl of nuclease free water, 2 µl of 10X PCR buffer (Thermo Scientific), 0.2 mM of dNTP (Thermo Scientific), 0.2 µM each of the forward and reverse primers of p21, Maspin, GPDH and 0.2 µl Taq DNA-polymerase (0.05U/µl, Pure gene) and optimized amount of each cDNA sample (in µl) was added. PCR amplifications of p21, Maspin and GPDH were performed in a thermal cycler by initial denaturation at 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s, and the final extension of 72 °C for 5 min.

4.12 Agarose Gel Electrophoresis of the PCR products:

The generated PCR products were analyzed by electrophoresis on 1.5% agarose gel. Agarose gel was prepared with 1X TAE (Tris Acetate EDTA, Sigma) buffer. Before casting 1.5 µl of ethidium bromide was added to the gel. 10 µl of sample (PCR product) was loaded to each well along with 2 µl of 5 X loading dye. 1 µl of ladder was loaded. The gel was run in TAE buffer at 80 volt for 35 minutes.

4.13 Analysis of the Relative Expression level of p21 and Maspin by quantitative Reverse-transcription PCR (q-RT-PCR)

qRT-PCR was performed using SYBR® Green JumpStart™ Taq Ready Mix in the Realplex4 Eppendorf system for p21 and Maspin gene. The mRNA level was normalized to GAPDH. The primer sequences for real time PCR are same as that of semi-q-RT-PCR and are given in Table 1.

5. RESULTS AND DISCUSSION

5.1 Determination of cytotoxic effect and IC₅₀ value of TSA , TQ and TSA +TQ by cell viability assay

Cell viability of MCF 7 was determined after TSA , TQ and TSA+TQ treatment by MTT Assay. The IC₅₀ values for the drugs were calculated. Both TSA and TQ treatment showed decrease in cell viability, but conjoint treatment with TSA +TQ showed higher decrease in cell viability indicating more cytotoxicity towards breast cancer cells.

We determined the sub-lethal concentration of different extracts (TSA, TQ and TSA+TQ) by MTT assay, which was taken as standardized concentration for further treatment. The optimized IC₅₀ values of TSA and TQ are found to be 180 µg/ml and 34 µg/ml for 24 h MCF 7 cells (Figure 6).

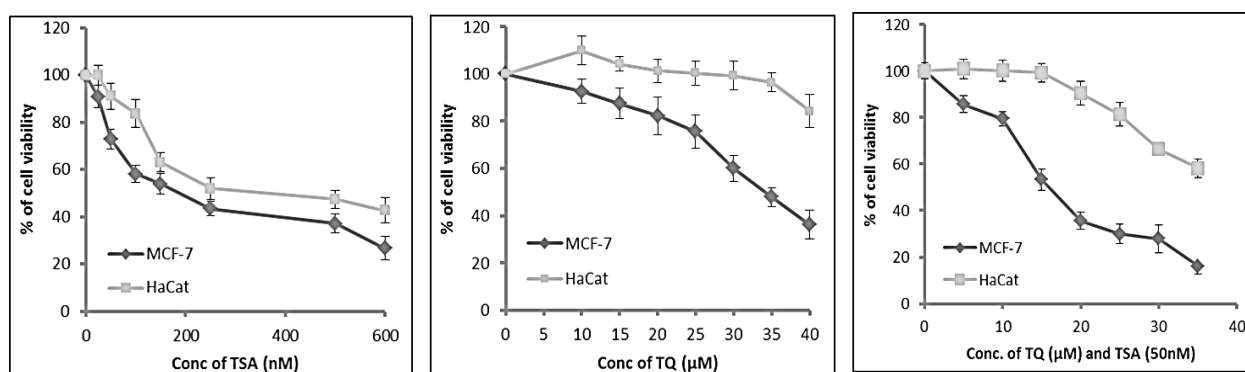
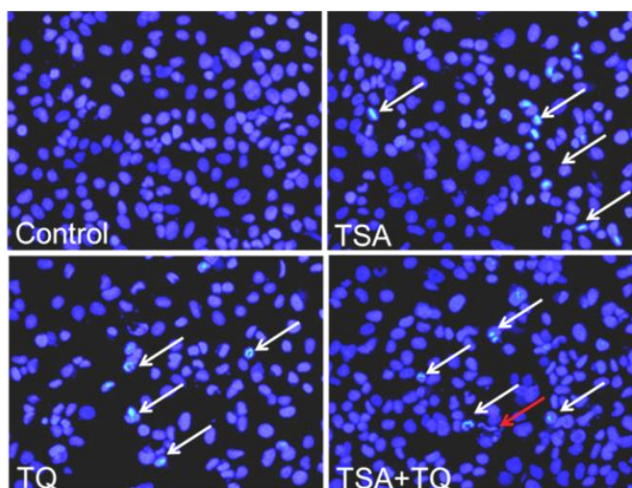


Figure 6.Graphical presentation of analysis of cell viability after TSA, TQ and TSA +TQ treatment

5.2 Detection of apoptosis induced of TSA ,TQ and TSA +TQ by Chromatin condensation assay

The cell death inducing ability of both combination of TSA and TQ is determined by chromatin condensation assay. This is indicated by formation of highly condensed and fragmented apoptotic bodies. Here, we found that the number of condensed nuclei in TSA + TQ treatment is comparatively more than that of TSA and TQ treatment separately in MCF-7 breast cancer cells. The untreated controls show no condensed or fragmented nuclei (Figure 7).



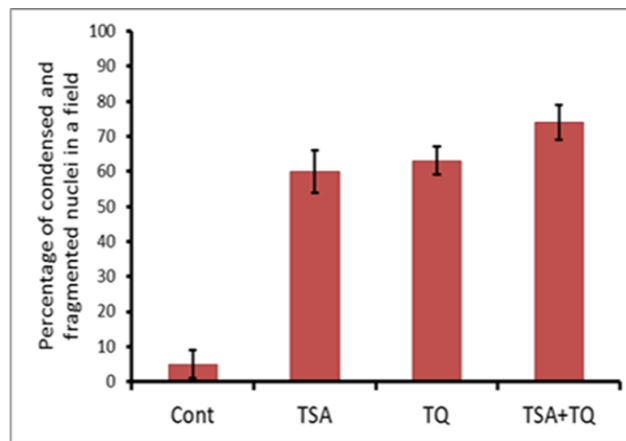


Figure 7: Fluorescence microscopic images representing condensed chromatin after TSA, TQ and TSA + TQ treatment in MCF 7 breast cancer cells.

5.3 Measurement of DNA damage induced by TSA, TQ and TSA + TQ treatment by Comet assay

Comet assay denotes the extent of DNA damage due to apoptosis on application of any insults to the cells. The tail moment denotes the apoptosis inducing ability of extracts. The tail moment of comet in case of TSA+TQ treatment is higher than TSA and TQ (Figure 8). The characteristic comet tail length suggested the amount of DNA damaged.

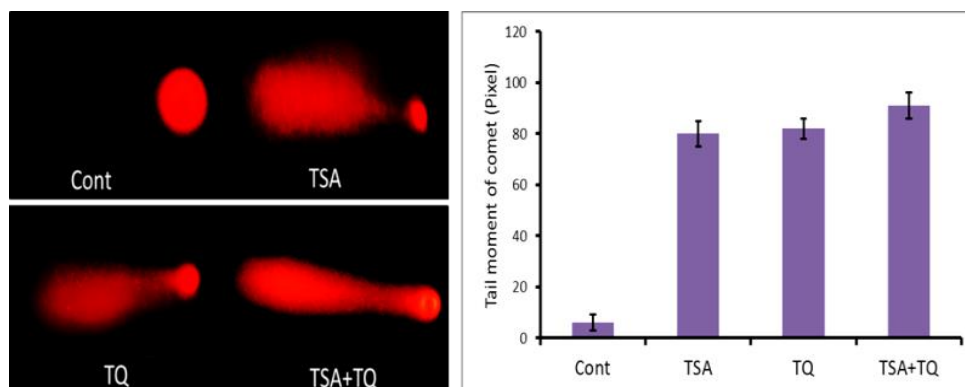


Figure 8: Fluorescence microscopic images of comets showing DNA damage after TSA, TQ and TSA + TQ treatment and their histogram analysis

5.4 Determination of anti-migratory activity of TSA, TQ and TSA + TQ on MCF 7 cells by Scratch assay

To determine the migratory property of the MCF 7 cells after TSA, TQ and TSA +TQ treatment for 72 h, the scratch assay was performed. While in control, the cancer cells migrate and close the gap in 72 h, in treatment groups very less number of cells migrate towards the gap. Moreover, the wound healing ability of TSA and TQ is found to be least among all. The results showed that there was more migration of cells towards the scratched area in TSA + TQ treated cells as compared to the TSA and TQ separately treated cells in comparison with the untreated

plates taken as control (Figure 9). This clearly demonstrates the efficient anti-migratory property of TSA + TQ on breast cancer cells.

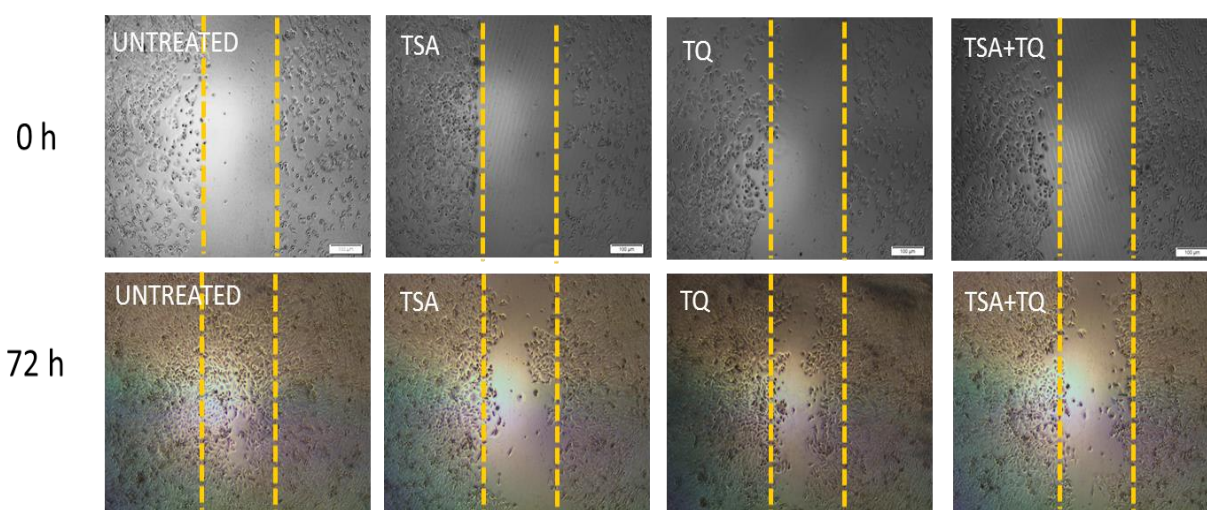


Figure 9: Microscopic images showing changes in the migratory property of MCF 7 cells after TSA, TQ and TSA + TQ treatment.

5.5 Determination of anti-proliferative activity of TSA, TQ and TSA + TQ on MCF 7 cells by colony formation assay

The colony formation assay also demonstrates the anti-cancer effect of conjoint treatment with TSA and TQ with respect to control. The number of colonies formed is less in case of conjoint treatment than that of TSA and TQ in isolation (Figure 10)

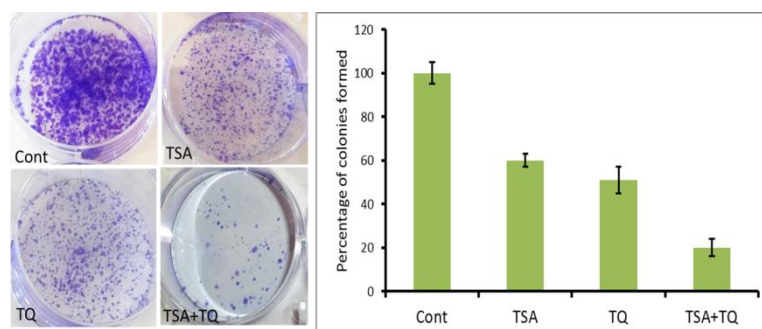


Figure 10: Representative images showing colony formation in MCF 7 cells after TSA, TQ and TSA + TQ treatment.

5.6 Gene Expression Analysis of p21, Maspin and GPDH by Semi-quantitative reverse-transcription PCR (Semi-q-RT-PCR)

Semi-q-RT-PCR data confirmed that the transcript level of p21 is not expressed whereas Maspin is slightly expressed in MCF 7 breast cancer cells. After treatment with TSA, TQ and TSA + TQ both p21 and Maspin expression is increased. Maspin and p21 is a tumor suppressor gene, which has

been reported to have low expression in breast cancer cells. Here in our study we also found the lower expression of Maspin and p21 at transcription level, on treatment with TSA, TQ and TSA + TQ, the Maspin and p21 is demonstrated to be up-regulated in breast cancer cells (Figure 10). The TSA+TQ can more effectively restore the expression of p21 and Maspin than TSA and TQ. This denotes that TSA + TQ may have HDAC inhibitory potential. The level of GAPDH remains same in all cases.

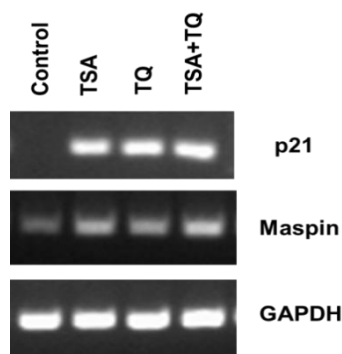


Figure 11: Representative images of semi-q-RT-PCR result depicting expression level of p21, Maspin and GDPH after TSA, TQ and TSA+TQ treatment.

5.7 Gene Expression Analysis of p21 and Maspin by quantitative reverse transcription PCR (q-RT-PCR)

The real time PCR analysis of mRNA level of Maspin and p21 shows that the p21 is up-regulated by five folds for TSA, three fold for TQ and four fold for TSQ with respect to control respectively (Figure 10). This supports our finding from semi-q-RT-PCR study. The Maspin is also demonstrated to be up-regulated in drug treated cancer cells. From the increase in transcript level of p21 and Maspin in treatment groups, it is apparent that the TSQ has HDAC inhibitory activity.

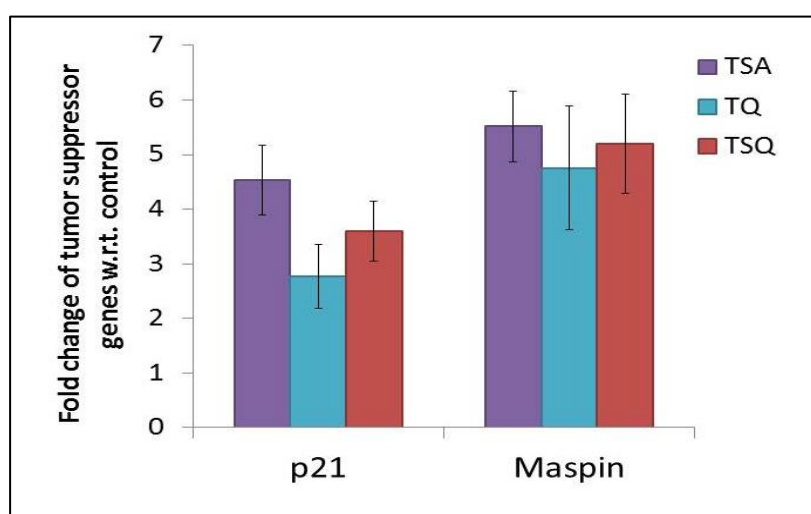


Figure 12: Representative images of q-RT-PCR result depicting expression level of p21, Maspin and GAPDH after TSA, TQ and TSA + TQ treatment

6. CONCLUSION

From the present investigation, it is apparent that the TSA + TQ have the anti-cancer potential. This is revealed from their anti-proliferative, apoptotic and anti-migratory activity. Our experiments clearly demonstrate that the TSA + TQ exert more anti-cancer effect on breast cancer cells than TSA and TQ separately. It is also observed that the TSA + TQ can more effectively restore the expression of p21 and Maspin with simultaneous increase in transcript level of HDAC.

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