Ocimum sanctum: A potential source of anti-cancer agent and its role in restoration of Maspin via Snail and HDACs in Breast cancer

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ABSTRACT

With cancer being a wide spread threat to humanity, plants have an important role in cancer prevention and in therapy. Medicinal plants provide new active chemo-preventive molecules. It is suspected that *Ocimum sanctum* would be a potent weapon discovered so far in fighting against all kinds of Cancer. There are many HDAC inhibtiors, but they are toxic in nature, this study aim at finding a phytochemical which is less toxic to human. For this ethanolic and methanolic extract of Tulsi leaves and inflorescence were detected to check whether they contain any components that can inactivate HDAC and can activate tumor suppressor genes. Further the anti- proliferative and anti-apoptoic effect of ethanolic and methanolic extract is studied. The role of theses extracts on the expression of tumor suppressor gene (Maspin), transcriptional factor (Snail), and epigenetic modulator (HDAC1, HDAC2) is investigated along with their anti-migratory effects on cancer cells. It is very much clear from the current investigation that the tulsi leaf and inflorescence extracts have the anti-cancer potential. This anti-cancer potential is due to the combinatorial effect of anti-proliferative, apoptotic and anti-migratory effect of the tulsi extracts. It is demonstrated from our experiments that the EETL exert more anti-cancer effect on breast cancer cells than METL and EETI. This investigation also demonstrates the higher efficacy of EENL in restoring the expression of maspin with concurrent enhancement of HDAC-1 and HDAC-2 mRNA levels, and reduced expression of snail. This reflects the HDAC inhibitory activity of tulsi extract.

1. INTRODUCTION

Medicinal plants have been in playing a vital role throughout human history. Plants have a number of chemically synthesised compounds that are used in many biological functions. About 12,000 such compounds have been isolated so far which constitute only 10% of the total chemical compounds in plants (Gupta et al., 2005). These chemical compounds mediate their effect on human body through processes identical to those conventional drugs in terms of how they work. Since time immortal, plants are used for maintaining health and also for curing many diseases. About 120 active compounds have been isolated from plants, which are being used as herbal medicine today. About 80% similarity is seen between their traditional use and modern therapeutic use. About two third of the total plant species in the world are estimated to have medicinal values. In modern era at least 7,000 medical compounds have been derived from plants. India is rich in medicinal herbs and spices consisting of about 2000 species. Traditional medicines such as Ayurveda, Unani, Siddha are studied for their chemical and pharmacological potential medicinal value[1] (Sandhu and Heinrich, 2005). Since thousand years human beings have been using medicinal plants for treating different diseases. Traditional medicines are still preferred by many people who can't afford pharmaceutical products for their physical and psychological requirements. In many of the developing countries mostly in rural areas people rely on traditional medicines for their primary health care. These are comparatively cheaper and safer than the modern synthetic medicines (Ammara et al, 2009)

With cancer being a wide spread threat to humanity, plants have an important role in cancer prevention and in therapy. Medicinal plants provide new active chemo-preventive molecules. A number of mechanisms are responsible for the derivation of plant compounds that have anti-tumor activities; these mechanisms involve effects on cytoskeletal proteins which play a key role in cell division, DNA topoisomerase enzymes inhibition, anti-protease or anti- oxidant activity, stimulation of the immune system etc. Plants can delay or prevent cancer on set and also can support the immune system, thereby improving body resistance to Cancer. Breast Cancer has been proved to be the fatal breed of cancers among female. It is the cancer originating from the tissues of breast, particularly from intramural lining of milk ducts which impart the ducts with milk. Cancers emanating from ducts are said to be ductal carcinomas, whereas cancers beginning from lobules are called as lobular carcinomas. The exposition of breast cancer is 22.9% among cancers in women. Breast cancer caused about 4,58,503 deaths worldwide which is at least 13.7% of deaths in women due to cancer. The

third most cancer after lung and stomach cancer is breast cancer. In US breast cancer accounts for one-third of all cancer incidents. It has become the second cause of death among cancer next to lung cancer. The American cancer society had estimated 1,82,000 new cases in 1993 in US and 46,000 deaths in women were due to cancer. In the past two and three decades long term increase in breast cancer have been observed similar to that occurs in US (Lipworth L,1994). A significant decrease in breast cancer is seen due to anti-cancer therapeutics

In the present day, 1.3 million women are detected with breast cancer each year. During the last 25 years, incidences related to breast cancer have risen to a greater number. Moreover, deaths because of breast cancer have also been declining constantly since 1990 due to earlier detection and advanced treatments. Breast cancer rate is relatively low in India, despite of its huge population size. In India, generally breast cancer is not detected earlier, in comparison with developing or developed country. Therefore breast cancer death rate is high in India.

1.1 Pharmacological importance of plants in cancer

In recent years, engrossment in cancer treatment by plants and their phytochemicals has been increased. Though different plant parts have prospects for curative use and chemoprevention, their mechanisms is very difficult to understand. Therefore extensive research has been recognised for many targeted molecules which can have potential to be used as an anti-cancer agent. There are instances of modifying abilities of many plants and plant products on many signalling pathways, along with their anti-inflammation and anti-apoptotic target for cancer therapy. There are several phytochemicals like resveratrol , allicin , lycopene, indole-3-carbinol , vitamin C , -gingerol , emodin , natural antioxidant mixture , sulphoraphan, ellagic acid , myrecitin, vanillin and eugenol that have anti-cancer property. They act through one or more signaling pathways.(AR Khuda-Bukhsh et al,2014)

1.2. *Ocimum sanctum* in Cancer treatment

One of such medicinal plant having the above character is *Ocimum sanctum* which is used traditionally as medicine in south-east Asia. It possesses anti-inflammatory, analgesic, antipyretic, antidiabetic, hepatoprotein, hypolipidemic, immune modulatory and anti-stress activity. It has been seen that *Ocimum sanctum* and some of its phytochemicals such as eugenol, linoleic acid, luteolin, β-sitosterol that prevents skin, liver, oral and lung cancers.

Their effects are mediated by increasing the anti-oxidant activity, inducing apoptosis, altering the gene expression, and inhibiting metastasis (Baliga et al).

Long ago some researchers tried to put some *Ocimum sanctum* leaves over tumour cells which resulted in the inhibition of the growth of tumour cells. From then, *Ocimum sanctum* has been suspected as a medicine to cure Cancer. *Ocimum sanctum* has abundant quantities of Eugenol, the anti-cancerous component. It is suspected that *Ocimum sanctum* would be a potent weapon discovered so far in fighting against all kinds of Cancer. Now scientists are trying to find new ways in which Eugenol can be produced in larger amount. If this succeeds, *Ocimum sanctum* will be officially declared as anti-Cancer medicine and can be used in curing Cancer. Many researches are going on to genetically modify *Ocimum sanctum* so as to obtain more anti-Cancerous compound. No reports have been found on the effects of *Ocimum sanctum* on Breast Cancer till now. (Makker et al, 2007)

The properties of *Ocimum sanctum* is because of the active components present in the plant, which includes eugenol, linoleic acid, oleic acid, rosmarinic acid, Ocimarin, isorientin, orientin, aesculectin, aesculin, chlorgrnic acid, galuteolin, gallic acid, Citronellal, Camphene, Sabinene, Dimethyl benzene , Ethyl benzene, Vitamin C, Calcium, and many more. Molecular structures of some of the components of *Ocimum sanctum* are as follows. (J Baby et al, 2013)

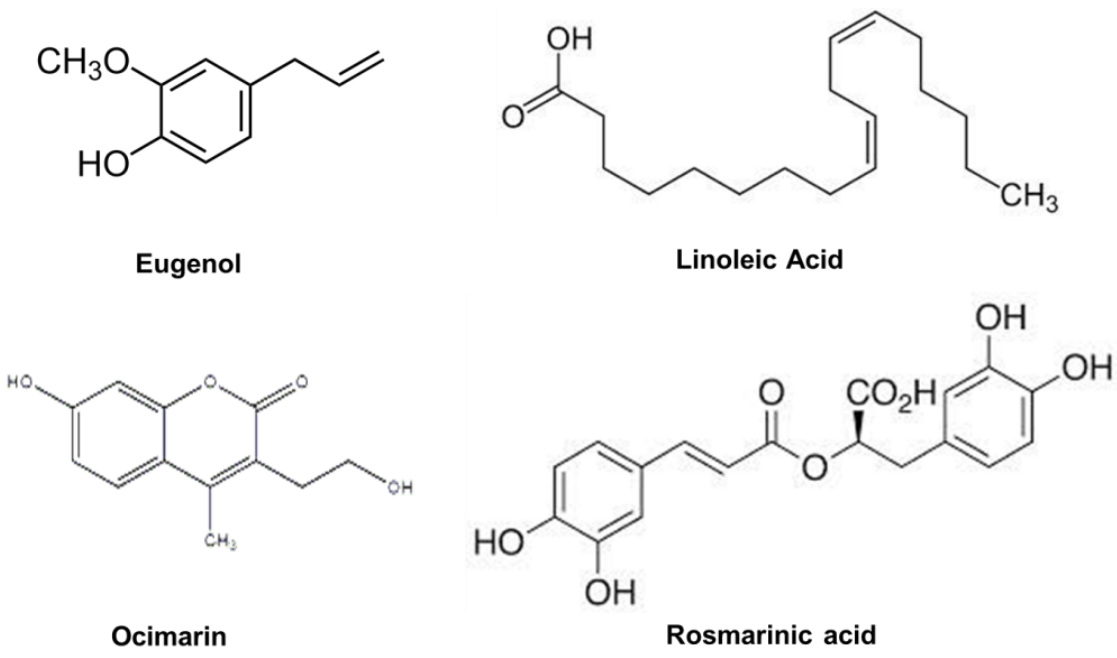

Figure 1: Molecular structures of some of the active components of *Ocimum sanctum*

1.3. Epigenetics and Cancer

Epigenetic is all about alterations in the accessibility of chromatin in trancscriptional regulation either by locally or globally via DNA modifications or in nucleosome rearrangement. Epigenetic is an important event in the development of mammals including the maintenance of tissues and their function. Epigenetic gene expression mainly involves the nucleosome present on the middle. It is made up of two turns of DNA wrapped around a histone octamer which consists of two subunits of H2A, H2B,H3 and H4. H1 is the linker histone that attaches and helps in the further compaction of histones. Besides core histones there are a variety of histone proteins that can be inserted within the nucleosome probably serving like landmarks for different cellular functions. Histone proteins have a N-terminal tail that protrude out of the nucleosomal core particle. The epigenetic signals bind on these tails which serve as a regulatory register. The covalent modifications of hictones include acetylation, methylation, phosphorylation, ubiquitination, ADP-ribosylation and sumoylation.

Acetylation is related to the activation of gene expression which is due to the balance between acetylation and deacetylation. Besides histones, transcriptional regulators, many chromatin modifiers including intracellular signal transducers and post translational modifications by acetylation. HAT and HDAC are mutated and silenced in many cancers. HDACs have opposite function in cancer development. HDAC play a vital role in gene silencing of tumor suppressor genes and also in their mechanism. HDACs are linked with many types of silencing mechanism such as histone methylation, DNA methylation, PcG mediated repression etc. Moreover HDAC activity is important in the preparation of templates for histone methyl transferases by the removal of the acetyl group and there by obstructing methylation (Lund and Lohuizen, 2014).

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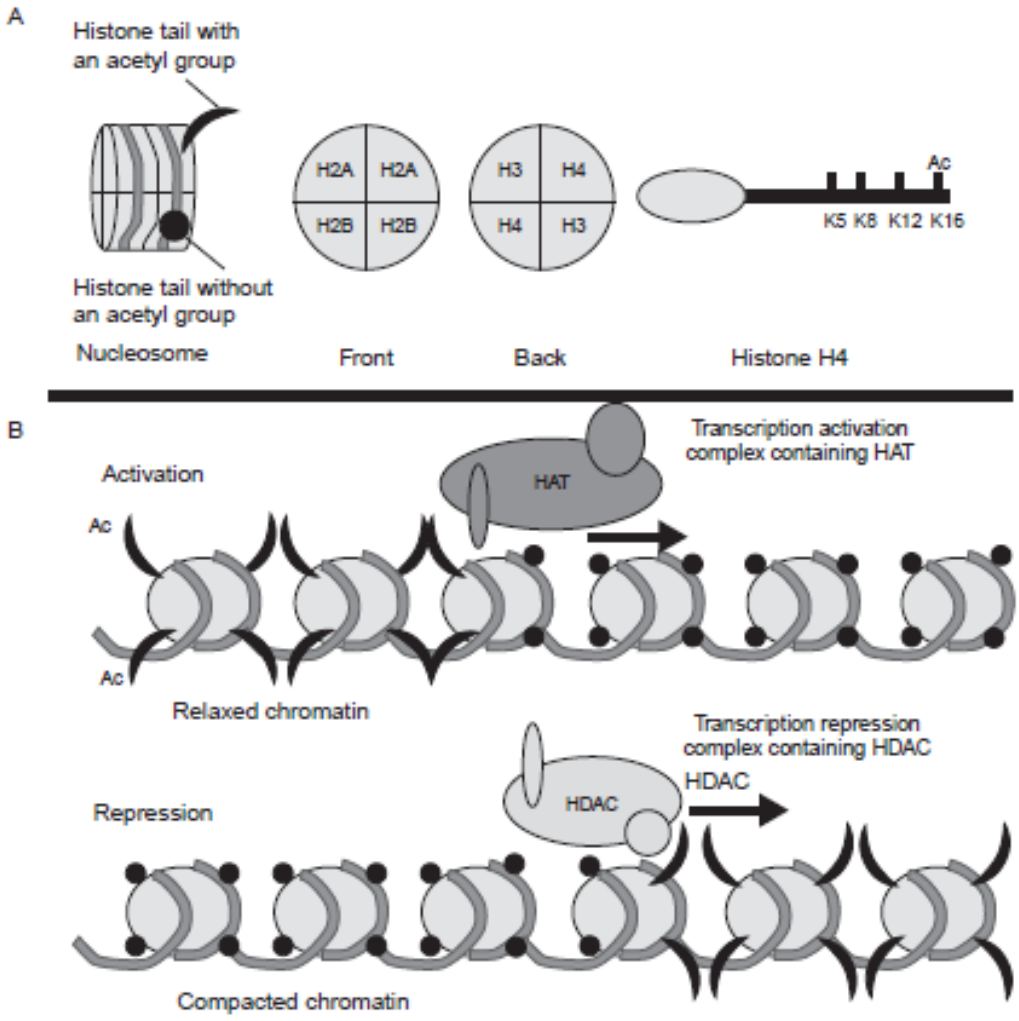

Figure 2: Schematic representation of (A) a nucleosome and (B) transcriptional activation and repression in chromatin (Adapted from Stumpp et al, 2005)

1.4. Role of histone deacetylases (HDACs) in cancer

Besides the modern development of better and productive anticancer drugs, resistance to some kind of chemicals has become a considerable cause for failure of treatments. Many evidences are there that proves that epigenetic has a vital role in developing resistance. HDACs have a key role in activation of gene expression. They are linked with different types of cancers and thus enhance cancer development. (Hrabeta J et al, 2013). The equilibrium between histone acetylation and histone deacetylation is an epigenetic level with an important role in the gene expression. Histone acetyltransferase (HAT) induces histone acetylation which is associated with transcription, whereas histone deacetylase induces hypoacetylation associated with gene silencing. Since both HAT and HDAC induces aberrant expression of genes, they regulate different functions such as apoptosis, cell proliferation etc. Thus HDACs

are one of the important therapeutic for treatment of cancer. Histone acetylation is also linked with chromatin remodelling along with gene transcription. During acetylation the negatively charged acetyl groups are bound to the positively charged lysine residues, as a result DNA is loosely bound to histones and a euchromatin structure is formed. This helps the transcription factors to bind to the DNA and thus transcription occurs. This is how HAT helps in the activation of gene expression. But in case of deacetylation catalysed by HDAC the acetyl groups are removed as a result of which the DNA and the histones which have a strong force of attraction form a heterochromatin structure. Therefore the transcriptional factors can't bind to the DNA and hence no transcription occurs. Thus HDAC helps in the gene silencing. Histone acetylation is associated with DNA repair, recombination and chromatin assembly. HDAC also regulates many cell processes like cell cycle progression, apoptosis etc. HDACs are recruited independently to DNA without DNA methylation. This is the result of the involvement of transcription factors and nuclear receptors. Basically HDAC1 and HDAC2 are involved in the gene silencing regulated by Rb, retinoblastoma protein. Deactivation of DNA methylation and histone acetylation has a fatal consequence of inactivation of gene expression. Hypomethylation is associated with the loss of acetylated lys 16 and trimethylated lys20in human cancer (Ropero S et al, 2007).

1.5. Role of maspin in cancer

Mammary serine protease inhibitor (Maspin) is one of the members of the serpin superfamily, whose expression is down-regulated prostate cancers as wel as in braest cancer but over-expressed in colo-rectal cancers which shows that maspin has activities depends on cell types. Cancer in breast and ovary has a maspin importance associated with its localization: maspin expression in nucleus indicates a good projection, whereas cancer in panceas predicts a poor one. An increase in the adhesion due to the maspin can lead to the inhibition of tumour metastasis, because tumor metastasis generally requires the invasion and detachment of tumor cells by the basement membrane. Moreover different location of maspin inside the cell or its epigenetic changes may explain the dissimilar behavior in the expression of maspin between tumors.(Berardi et al,2013).Structural considerations shows that maspin can act as a serine protein inhibitor to interact with serine protease like that of HDAC1. E-acetyl lysine is hydrolysed residues in its substrate proteins by the activity of HDAC1, which is the function of the catalytic domain resembling to metallo proteinases. Maspin is expressed in a manner that can act as the regulator of HDACs (Li X et al, 2013).

Maspin expressions in cancer cells are controlled by the methylation in CpG islands. However in Breast Cancer hypermethylation of CpG islands in Maspin promoter is linked with inactivation of transcription. Breast cancer cells have both Hypermethylation and deacetylation of histone that have shown to repress expression of maspin. (Dokras A et al, 2006)

1.6. SNAIL

The Snail is a superfamily of zinc-finger transcription factors. It is associated in cellular processes that indicate distinct cell movements, both in the embryonic development process and during the accession of invasive properties during progression of tumour. There are many different family members of snail superfamily who have also been compromised in the signalling cascade that are conferred with left–right identity, including the genration of appendages, cell division,cell survival, neural differentiation etc. One among the best-known functions of snail family is to induce epithelial to mesenchymal transitions (EMTs), which then convert epithelial cells directly into migratory mesenchymal cells. (Nieto et al, 2002) Snail is recognised as a component of pathways during early development of flies and mammals including metastasis. They basically act to suppress gens which encode important structural elements of cell-cell junction apparatus, like that of E-cadherin and ocdudin which results in a de-differentiated mesenchymal phenotypic characterization by increasing motility rate (Sugimach K et al, 2003).

2. REVIEW OF LITERATURE

It has been found that the antitumor mechanism of ethanolic extract of Tulsi is elucidated in A549 cells and also in Lewis lung carcinoma (LLC) animal model. It is seen that ethanolic extract of Tulsi exerts cytotoxicity against A549 cells, exhibits apoptotic bodies in A549 cells and increases the sub-G1 population. Furthermore Tulsi ethanolic extracts also cleaves poly Adenophosphate (ADP)-ribose polymerase (PARP), and release cytochrome C to the cytosol and activate caspase-9 and caspase-3 proteins. Ethanolic extract of Tulsi increases the ratio of apoptotic proteins like Bax protein with Bcl-2 and inhibits the phosphorylation of Akt and extracellular signal regulated kinase (ERK) in A549 cancer cells. In addition it was found that ethanolic extract of Tulsi can suppress the growth of LLC inoculated onto C57BL/6 mice in a dose- dependent manner. Overall, these results demonstrate that ethanolic extract of Tulsi induces apoptosis in A549 cells via a mitochondria caspase dependent pathway and also inhibits the in vivo growth of LLC, suggesting that ethanolic extract can be applied to lung carcinoma as a chemopreventive candidate.(V Magesh et al,2009)

Moreover, the anticancer property of tulsi leaves extract was verified, using Benzo pyrene induced fore-stomach and 7,12 dimethyl benzanthracene initiated skin papillomagenesis. The activities like hepatic glutathione S-transferase and DT-diaphorase were found to be increased above basal level by tulsi leaves (Anti-cancer effect was verified from decreased tumor, also from the lesser number of animals with tumors). Moreover, Tulsi leaves extract was seen to be highly efficient in inhibiting carcinogen-induced tumor instances in both the tumor models.

The anticancer property of Tulsi was studied in skin papillomagenesis using Benzo-pyrene-induced fore-stomach and 7,12 dimethyl benzanthracene (DMBA). Chemopreventive response of Tulsi was detected was confirmed from the lowering tumor , also from the reduction in the number of tumour bearing animals. Morever Tulsi leaves extract was much effcient in the reduction of carcinogen induced tumor instances in both the models. Tulsi leaves can be used as a potential anti-cancer agent because of its efficiency in the induction of drug detoxifying enzyme such as DST and DTD and also by checking carcinogenic phase I enzyme. Since tulsi has shown anti- toxic effect at all the tested doses, it may be applied in chemoprevention of cancer, in order to decrease the risk of cancer.(Dasgupta et al,2004)

Tulsi has been explored for its anti-cancer activity for 7,12-dimethylbenz-anthracene (DMBA)-induced buccal pouch carcinogenesis. Fresh leaf paste of Tulsi and its aqueous extract along with the ethanolic extract were either applied locally or orally administered to buccal pouch mucosa of animalsand they were allowed to be exposed to 0.5% of DMBA. It was observed that carcinomas of papillomas and squamous cell were significantly reduced, along with rise in the survival rate in the topically applied area and orally administered extracts to the animals. The orally administered aqueous extract was found to be more effective among other two forms. These findings were confirmed by the histological observations carried out on the mucosa. Further studies of fluorescent spectral at 405 nm excitation on the mucosa of control, DMBA and that of the extracts orally administered experimented animals were found to show prominent maxima at 430 nm for control, 628 nm for DMBA induced carcinomas. Furthermore the aqueous and ethanolic leaves extracts administered animals were found to show at 486 nm and 488 nm, respectively. The fluorescent absorbance at 630 nm was significantly reduced and the ratio of fluorescent intensities at 520 nm and 630 nm were significantly increased in orally administered extracts compared to DMBA treated animals. These observations suggest that the orally administered extract of Tulsi can have the ability to prevent the early events of cancer (Karthikeyan K et al,1999).

There are reports about the modulating effect of ethanolic leaves extract of Tulsi on the activities of aryl hydrocarbon hydroxylase enzymes and also in the cytochrome p-450, and in the liver and glutathione-S-transferase. Tulsi extract has also influence in reducing glutathione level in the lung, liver and stomach of the mouse. Oral treatment with the leaf extract at an amount of 400 and 800 mg/kg body wt for 15 days would significantly increase the activities of cytochrome p-450, aryl hydrocarbon hydroxylase, and glutathione S-transferase. All of these are important in the detoxification of carcinogens and mutagens. The leaf extract also elevated the reduced glutathione level in stomach tissues ($p < 0.01$, $p < 0.001$), liver and lung. Mice when fed with a diet that contain 0.75% butylated hydroxyanisole that revealed no change in the hepatic cytochrome p-450 and aryl hydrocarbon level, but alterations were observed in case of hepatic cytochrome b5 and also in glutathione S-transferase activity in hepatic and extrahepatic organs in a time-responsive manner. The observations suggest further exploitation of Tulsi leaf extract or its active principle involved in the chemoprevention of carcinogenesis in different animal model systems.(Banerjee S et al,2009)

The anti-metastatic activity of ethanolic extract of Tulsi leaves were investigated through activation of anti-oxidative enzymes. Ethanolic Extract of Tulsi Leaves exerts cytotoxicity against Lewis lung carcinoma (LLC) cells. Also, Ethanolic Extract of Tulsi Leaves significantly inhibits cell adhesion and invasion as well as activities of matrix metalloproteinase-9 (MMP-9). But it has no effect on MMP-2, which indicates the important role of MMP-9 in anti-metastatic regulation of Ethanolic Extarct of Tulsi Leaves. In addition, Ethanolic Extract of Tulsi Leaves significantly reduces the tumor nodule formation and lung weight in LLC-injected mice. Hematoxylin and eosin (H&E) staining confirms the inhibitory effect of EETL on metastasis. Notably, it was also found that Ethanolic Extarct of Tulsi Leaves enhances the activities of anti-oxidative enzymes in a concentration-dependent manner. This study supports that Ethanolic Extract of Tulsi Leaves can be a potent anti-metastatic candidate through inactivation of MMP-9 and enhancement of anti-oxidant enzymes (Kim Sun-Chae et al, 2010).

A study was done to determine the efficiency of flavonoid vicenin-2 (VCN-2), which is an active constituent present in the medicinal herb Tulsi. It was taken in combination with docetaxel (DTL) in carcinoma of prostate (CaP). It was observed that VCN-2 is able to effectively induce anti-proliferative, pro-apoptotic and anti-angiogenic effect in CaP cells (PC-3, DU-145 and LNCaP) irrespective of their androgen responsiveness or p53 status. VCN-2 inhibited EGFR/Akt pathway. Morever it also decreased c-Myc, cyclin B1, CDK4, PCNA and hTERT in vitro. VCN-2 was seen to reach a level of 2.6 ± 0.3 μmol/l in serum after oral administration in mice. This reflected that VCN-2 is orally absorbed. The i. v. administration of docetaxel (DTL), which is the current drug of choice in androgen-independent CaP, is found to be associated with dose-limiting toxicities like febrile neutropenia. This has led to characterization of different routes of administration and potential combinatorial regimens. In this regard, DTL in combination with VCN-2 synergistically inhibited the growth of prostate tumors in vivo with a greater decrease in the levels of AR, , pAkt, PCNA, cyclin D1, Ki67, CD31, and increase in E-cadherin. VCN-2 has been investigated for radioprotection and anti-inflammatory properties. This is the first study on the anti-cancer effects of VCN-2. In conclusion, these investigations collectively provide strong evidence that VCN-2 is effective against CaP progression along with indicating that VCN-2 and DTL co-administration is more effective than either of the single agents in androgen-independent prostate cancer.(Nagaprashanthaa L D et al,2011)

In an experiment aqueous extracts of mature Tulsi leaves were prepared related Processes related to tumour progression and angiogenesis like that of proliferation, apoptosis, chemotaxis 3D growth tumor growth were studied in the presence or absence of the tulsi leaves extract. In another experiments a comparative study was made with purified commercially available active components of Tulsi such as eugenol and ursolic acid. Aqueous Tulsi leaf extract was found to inhibit cell proliferation, anchorage independent growth of cells, migration, 3D growth and morphogenesis including the induction of COX-2 in breast cancer cells. A comparative study with eugenol, and ursolic acid found to show that the inhibitory effects on chemotaxis and 3D morphogenesis of breast cancer cells were confined to Tulsi extract. Morever, Tulsi extracts reduced tumor size and neo angiogenesis in a MCF10 DCIS.com xenograft model of human DCIS. This experiment shows that tulsi leaf extract might be effective as a breast cancer prevention and therapeutic agent and can be considered as additive in the arsenal of components aimed at combating breast cancer progression and metastasis.

In a study, eugenol the active component of Tulsi derived from Eugenol caryophyllata is found to inhibit gene expression activity of inducible cyclooxygenase (COX-2) in lipopolysacharide (LPS) activated mouse macrophage cells. Ten COX-2 is found to catalyze the conversion of arachidonic acid to prostaglandins11. This is the early response of gene when it is stimulated by serum, mitogens,cytokines and hormones. There were twelve Studies which have shown that selective COX-2 inhibitors help in the reduction of the human breast cancer. It is demonstrated by the inhibition of COX-1 and COX-2 levels by eugenol and six other similar phenolic compounds extracted from Tulsi leaves and stems. Ursolic acid another compound isolated from Tulsi has been shown to suppress nuclear factor-κB (NF-κB), which helps in the regulation of the expression of many genes whose products are involved in carcinogenesis. Thus, agents that are responsible to suppress COX-2, NF-κB activation have the potential to suppress tumorigenesis and have therapeutic effect.(Makker et al,2007)

While maspin occurs in normal breast tissue and normal breast cells in culture, it is not present in breast cancer tissue and breast cancer cells in culture. An experiment suggested a biologically relevant explanation about the inhibition mechanism of recombinant Maspin (rMaspin) in human breast cancer cell line MDA-MB-435 from invading in vitro. Addition of rMaspin to those cells resulted in a decrease in the in vitro invasiveness with an increase in

their cell surface expression of the α5-containing integrin, and an increase in their adherence to fibronectin. These results were confirmed by Northern blot analysis, which showed an increase in mRNA in rMaspin treated cell also by the observations of 1) treated cells showing inhibition of increased adhesion to fibronectin, and 2) facilitating their ability to invade through the fibronectin matrix in vitro at a rate equal to the untreated controlled cells. These observations supported previous observations that there is a competitive reversion of rMaspin action in breast cancer cells that were treated with an RGD peptide known to block integrin function. Together, these results indicated a functional change in response of MDA-MB-435 cells to their surrounding which could have been induced by rMaspin and includes both transcriptional and translational processes in the cells. A relevant observation from this study is invasive and metastatic cells appearance to assume a more benign, epithelial-like morphology in response to rMaspin. Whereas E-cadherin expression did not appear to increase with respect to rMaspin, this indicates that the actual junctional adhesion complexes had not been formed during the 24 hour period of these observations. Although rMaspin treatment also cause a decrease in the cell surface expression of the α2- and β1-containing integrins, a change was absent in the ability of the cells to adhere to any of the ligands that are associated with these integrins. Furthermore, the inability to block antibody to the α6-subunit to restore the invasiveness of the rMaspin treated cells through the laminin/gelatin matrix barrier in the in vitro invasion assay suggests that there is a specific relationship between the α5-containing integrin and rMaspin which apparently does not exist with the other integrins.

In brief these observations indicated that rMaspin reduced the invasive potential of MDA-MB-435 cells by altering their integrin profile which changes how they recognize and interact with their extracellular environment. rMaspin treated cells shows more adhesion to fibronectin-containing biological substrates and are subjected to phenotypic changes that resulted in the conversion from an invasive, fibroblastic phenotype to an epithelial-like, less invasive phenotype.(Richard E.B,)

In an experiment it is proved that aberration in cytosine methylation and chromatin condensation of the maspin promoter usually participates in the maspin suppression during neoplastic progression. For testing this, the cultured normal human mammary epithelial cells (HMECs) were compared to 9 cultured human breast cancer cell lines. It was observed that HMECs expressed maspin mRNA and displayed a non-methylated maspin gene promoter

with an euchromatin. It was seen that maspin expression was absent in seven out of nine breast cancer cell lines and six of these seven maspin-negative breast cancer cell lines displayed anaberrant pattern of cytosine methylation of the maspin promoter. Morever the maspin promoter was observed to be methylated in maspin-negative normal peripheral blood lymphocytes. This indicated that the maspin promoter is not a functional CpG island and the cytosine methylation of this region may help in the contribution to normal tissue-restricted gene expression. Chromatin accessibility studies with MCF-7 cells, that lack maspin expression and have a methylated maspin promoter, was found to show a heterochromatin compared to that of HMECs. Maspin gene expression can also be activated in MCF-7 cells when treated with 5-aza-2-deoxycytidine, a DNA demethylating agent. Thus, aberrant cytosine methylation and formation of hetero chromatin structure of the maspin promoter may silence maspin gene expression, which contribute to the progression of human mammary cancer. Although the maspin expression is silenced, its deletions and mutations have not yet been found .These observations proved the hypothesis that the maspin might be suppressed by any epigenetic mechanism which involves aberrant cytosine methylation. In this study, it is reported that aberrant cytosine methylation of the maspin gene promoter in human breast cancer cell lines is associated with an altered chromatin configuration of the promoter and repression of maspin gene expression. In addition, it is shown that the methylation inhibitor 5-aza-dC can re-activate maspin gene expression in a dose-dependent fashion in aberrantly methylated, maspin-negative breast cancer cells. (Domann E F et al, 2000)

3. OBJECTIVES

The Objectives of our study are:

1. To study the anti-proliferative effect of ethanolic and methanolic extract of tulsi leaves and inflorescence in MDA MB 231 breast cancer cells.
2. Deciphering the anti-apoptotic potential of ethanolic and methanolic extract of tulsi leaves and inflorescence breast cancer cells.
3. To study the anti-migratory effect of ethanolic and methanolic extract of tulsi leaves and inflorescence breast cancer cells.
4. To investigate the role of ethanolic and methanolic extract of tulsi leaves and inflorescence on expression of tumor suppressor gene (Maspin), transcriptional factor (Snail), and epigenetic modulators (HDAC1 and HDAC2) with establishment of correlation between them.

4. MATERIALS AND METHODS:

4.1. Preparation of Plant extract (EETL,METL,EETI):

Tulsi (*Ocimum sanctum*) leaves and inflorescence were obtained from tulsi plants in NIT campus. The leaves and inflorescence were washed with distilled water and air dried. Then the extracts were prepared according to Othman et al, 2011. 60 g of leaves and inflorescence were ground using a grinder to obtain the fine powdered form, and the powder was kept in a flask with 120 ml of 80% ethanol added to it. The mixture was mixed and kept overnight at room temperature. The other day, the mixture was filtered into a beaker while the residue was left in the borosilicate glass bottle. Another 120 ml of 80% ethanol was then poured into the borosilicate glass bottle to soak the remaining residue, which was then kept overnight at room temperature. These steps were then repeated for the next three consecutive days. Ethanolic extract of tulsi leaves (EETL) and inflorescence (EETI) was evaporated using a rotary evaporator (RV 10 Basic IKA Rotary Evaporator) at 60˚C. Same procedure was repeated for methanolic extracts of tulsi leaves (METL) preparation, but in this case 60 gm of powdered leaves were extracted in 120 ml of absolute methanol and evaporated at 40˚C under vaccum. After this the extracts were oven-dried for an additional 48 hours and then crude form stored at -20˚C. An aliquot of 200 mg of this dried extracts were dissolved in 500µL of dimethyl sulfoxide (DMSO) to prepare a stock of concentration 400 µg/µL. Secondary stock solutions of 40µg/µL were prepared by diluting the primary stock with ethanol and methanol for ethanolic and methanolic extract, respectively. The suspensions were then filtered with a 0.22µm filter and stored at -20˚C. The final concentration of DMSO never exceeded 0.01% in the culture medium.

4.2. Estimation of total phenol Content:

Total phenolic content of the methanol and ethanolic extracts of *Ocimum sanctum* was determined by using Folin–Ciocalteu reagent and gallic acid (GA) as standard. To develop the standard curve, 0.5 mL aliquots of 12.5, 25, 50, 100, 200, and 400 µg/mL concentrations of methanolic gallic acid solutions were prepared from a stock of 400 mg/ml solution and EETL and METL were diluted to the concentration 200 µg/mL. Then to each concentration of solution, 2.5 ml Folin's reagent (diluted 1:9) and 2.5 ml (7.5g/100 mL) sodium carbonate were added. All determinations were performed in duplicate. The reaction mixture was incubated at 25^0C for 30 min. Then phenolic contents were determined at 760 nm against reagent blank by UV Spectrophotometer (PerkinElmer). Total phenols were determined as gallic acid equivalents (GAE) (mg GAE/g extract) by the following formula:

C = c x V / m

where C - total content of phenolic compounds, mg/g plant extract, in GAE

c - the concentration of gallic acid established from the calibration curve, mg/mL

V - the volume of extract, mL;

m - the weight of pure plant methanolic/ethanolic extract, g.

4.3. Cell Line and Cell Culture:

MDAMB 231 cell line was purchased from National Centre for Cell Science (NCCS), Pune, India. The cells are known to be of epithelial breast adenocarcinoma origin and are triple negative. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin-streptomycin sulfate (Invitrogen). The cells were grown in 5% CO2 in humid condition at 37^{o} C.

4.4. Cell Viability Assay:

This assay was done to determine the sub-lethal concentrations (IC_{50}) of extracts and proliferative activity of the cells in presence of extracts. MDAMB 231 (80 % confluent) cells were seeded into 96 well plate at a density of 3×10^{3} cells/well. After 24 h, the cells were treated with tulsi extracts i.e ethanolic and methanolic extract of tulsi leaves (EETL, METL), and ethanolic extract of tulsi inflorescence (EETI) at 7 different concentrations (10µg/mL, 25 µg/mL, 50 µg/mL, 75 µg/mL, 100 µg/mL, 200 µg/mL, 400µg/ml) and incubated for 24 h and 48 h. In parallel the cells with the solvent control was also treated to assess its effect on cells. After completion of treatment duration, 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 hours in CO_2 incubator. After incubation, the media was removed carefully 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.

4.5. Clonogenic Assay:

MDA-MB-231 cells were plated at a low density (300 cells/well) in a 6 well plate, allowed to attach for 24 hours prior to treating with the extracts. Then cells were treated with METL, EETL, EETI at their respective IC_{50} values. 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. Analysis of chromatin condensation by Hoechst 33342 stain:

For chromatin condensation assay, MDA-MB-231 cells (10^4cells/well) were seeded in 6 well culture plates and allowed to grow for one day. Then cells were treated with METL, EETL, EETI at their respective IC_{50} values. After treatment with the extracts, the cells were stained with Hoechst 33342 stain (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 analysed.

4.7. 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.

MDA-MB-231 were subjected to treatment with METL, EETL, EETI at their respective IC_{50} values 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 Na_2EDTA, 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 H_2O 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).

4.8. Cell migration assay (Scratch assay):

MDA-MB-231 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 pipet tip.

The debris was removed and the edge of the scratch was smoothed by washing the cells once with 1 ml of 1X PBS, pH7.2 and then replaced with 2 ml of DMEM containing METL, EETL, EETI at their respective IC_{50} values. 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

4.9. Total Cellular RNA isolation:

The total cellular RNA was extracted using TRI reagent (Sigma), following the manufacturer's instructions. On the culture dish 1 ml of the TRI Reagent per 10 cm^2 of glass culture plate surface area was added. After addition of the reagent, the cell lysate was passed several times through a pipette to form a homogenous lysate. TRI Reagent is not compatible with plastic culture plates. To ensure complete dissociation of nucleoprotein complexes, samples were allowed to stand for 5 minutes at room temperature. 0.1 ml of 1-bromo-3-chloropropane or 0.2 ml of chloroform was added for per ml of TRI Reagent used. Samples were covered tightly, shaken vigorously for 15 seconds, and were allowed to stand for 2–15 minutes at room temperature. The resulting mixture was centrifuged at 12,000 g for 15 minutes at 2–8 °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 to a fresh tube and 0.5 ml of isopropanol was added per ml of TRI Reagent used in Sample Preparation, step 1 and 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 used in Sample Preparation. The sample was vortexed and then centrifuged at 7,500 ´ g for 5 minutes at 2–8 °C. The RNA pellets were briefly dried 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.

4.10. Estimation of total cellular RNA:

Final preparation of RNA was analyzed using a nano-drop UV spectrophotometric analyzer. The standard preparation of RNA should 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.

4.11. cDNA Synthesis:

In a 1.5 ml tube, 2 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 spinned 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 one of the house keeping genes such as β-actin.

β-actin mRNA expression as internal control

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

4.12. 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 five sets of primers to amplify Maspin, Snail, HDAC-1, HDAC-2 and β-actin genes having sequence as detailed in Table no.1. The annealing temperature for each set of primers was optimized from 50˚C to 58˚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 thermocycler (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 58˚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.

4.13. Gene Expression Analysis of Maspin, Snail, HDAC-1, HDAC-2 by Semi-quantitative reverse-transcription PCR (Semi-q-RT-PCR):

MDA-MB-231 cells were treated with sub lethal dosages of EETL, METL and EETL 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 maspin, Snail, HDAC1 and HDAC2 gene along with β-Actin as a house keeping gene.

PCR conditions:

The PCR sample mixtures, in a 20 µl volume, contained 15.4µl 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 maspin, Snail, HDAC1, HDAC2, β-actin (Sigma) 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 maspin, Snail, HDAC1, HDAC2, β-actin 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.

4.14. 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 1.5µl of ethidium bromide was added to the gel. 10 µl of sample (PCR product) was loaded to each well along with 2µl of 5 X loading dye. 1µl of ladder was loaded. The gel was run in TAE buffer at 80 volt for 35 minutes.

4.15. Analysis of the Relative Expression level of Maspin and HDAC-1 by quantitative reverse-transcription PCR (q-RT-PCR):

qRT-PCR was performed using SYBR® Green JumpStart™ TaqReadyMix in the Realplex4 Eppendorf system for maspin and HDAC1 gene. The mRNA level was normalized to β-Actin. The primer sequences for real time PCR are same as that of semi-q-RT-PCR and are given in Table 1.

Table 1.Table showing the sequence of the forward and reverse primers

SL. NO.	PRIMER NAME	TYPE	PRIMER SEQUENCE	AMPLICON SIZE (in bp)
1	HDAC1	*Forward*	5'-GGAAATCTATCGCCCTCACA-3'	168
		Reverse	5'-AACAGGCCATCGAATACTGG-3'	
2	HDAC2	*Forward*	5'-ATAAAGCCACTGCCGAAGAA-3'	245
		Reverse	5'-TCCTCCAGCCCAATTAACAG-3'	
3	MASPIN	*Forward*	5'-GGAATGTCAGAGACCAAGGGA-3'	139
		Reverse	5'-GGTCAGCATTCAATTCATCCCTT-3'	
4	SNAIL	*Forward*	5'-TCTAGGCCCTGGCTGCTACAA-3'	131
		Reverse	5'-ACATCTGAGTGGGTCTGGAGGTG-3'	
5	β-ACTIN	*Forward*	5'-TCTACAATGAGCTGCGTGTG-3'	250
		Reverse	5'-TCTCCTTCTGCATCCTGTC-3'	

5. RESULTS AND DISCUSSION

5.1.1. Estimation of total phenolic content

Total phenolic content of tulsi leaf extracts (EETL, METL and EETI) was determined using Folin-Ciocalteu assay (Khatoon et al., 2013). Samples comprising phenolic compounds are reduced by Folin-Ciocalteu reagent thereby generating a blue-coloured complex. The greater amount of phenolic complexes causes more potent free radical scavenging effects. Oxidation produces free radicals in food, drugs and often in living systems also (Pourmorad et al, 2006). These free radicals in turn cause various disorders in humans. Due to degradation of natural antioxidants in our immune system, consuming antioxidants that serve as free radical scavengers is indispensable. Antioxidant property of plants is most probably due to their phenolic compounds (Cook and Samman, 1996). *Ocimum sanctum* consists of flavonoids, a group of polyphenolic complexes associated with free radical scavenging activity. Phytochemicals with phenolic complexes have anticancer properties. Both mono and polyphenolic complexes in plant extracts suppress or inhibit the initiation, progression and invasion of cancers in cells in vitro (Wahle et al, 2010). Phenolic complexes can contribute to our body's immune system by recognizing and destroying cancerous cells as well as suppressing angiogenesis required for tumor growth. They are also associated with attenuating adhesiveness, invasiveness and propagation of cancer cells thereby weakening their metastatic potential. The higher phenol content of EETL than METL and EETI indicates its higher anti-oxidant potential, which in turn can be correlated with its anti-cancer potential. The higher anti-cancer property of EETL has been further evaluated by investigating its anti-proliferative, apoptotic and anti-migratory activity on MDA MB 231 breast cancer cells (Figure 3).

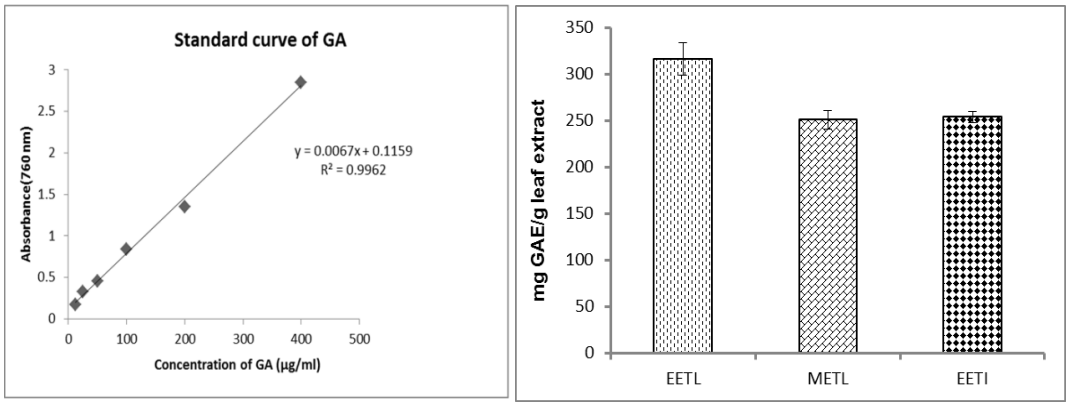

Figure 3. Standard GA calibration curve and total phenolic content in EETL, METL and EETI.

5.1.2. Determination of cytotoxic effect and IC_{50} value of EETL, METL and EETI by cell viability assay

Cell viability of MDA MB -231 was determined after EETL, METL and EETI treatment by MTT Assay. The IC_{50} values for both the extracts were calculated. Treatment with EETL, METL and EETI showed decrease in cell viability but EETL treatment showed higher decrease in cell viability indicating more cytotoxicity towards breast cancer cells. The sub-lethal concentrations of different extracts (EETL, METL and EETI) were determined by MTT assay, which was taken as standardized concentration for further treatment. The optimized IC_{50} values of EETL, METL and EETI are 50 µg/ml, 75 µg/ml and 100 µg/ml for 24 h in MDA-MB-231 cells (Figure 4).

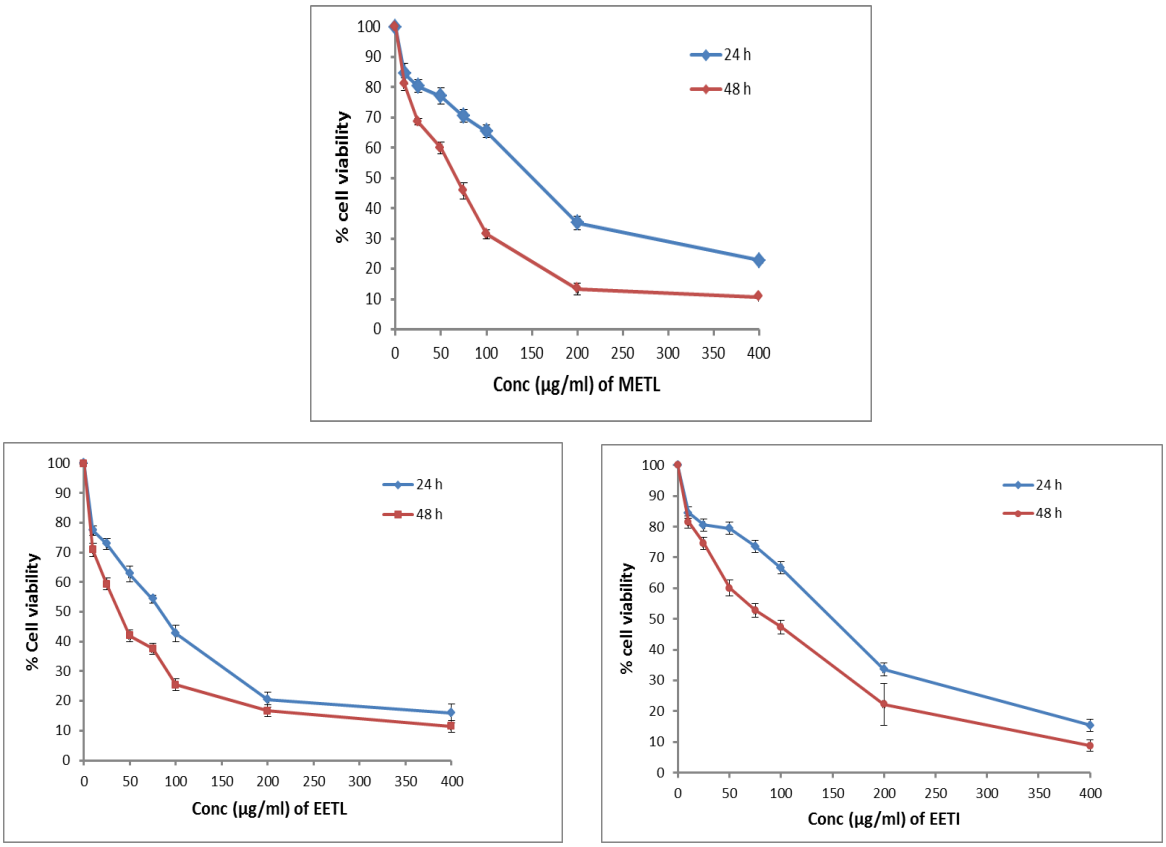

Figure 4. Graphical analysis of cell viability after EETL, METL and EETI treatment

5.1.3. Detection of apoptosis induced by EETL, METL and EETI by Chromatin condensation assay

The cell death inducing ability of neem leaf extracts 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 EETL treatment is

comparatively more than that of METL in MDA MB 231 breast cancer cells. The untreated controls show no condensed or fragmented nuclei (Figure 5).

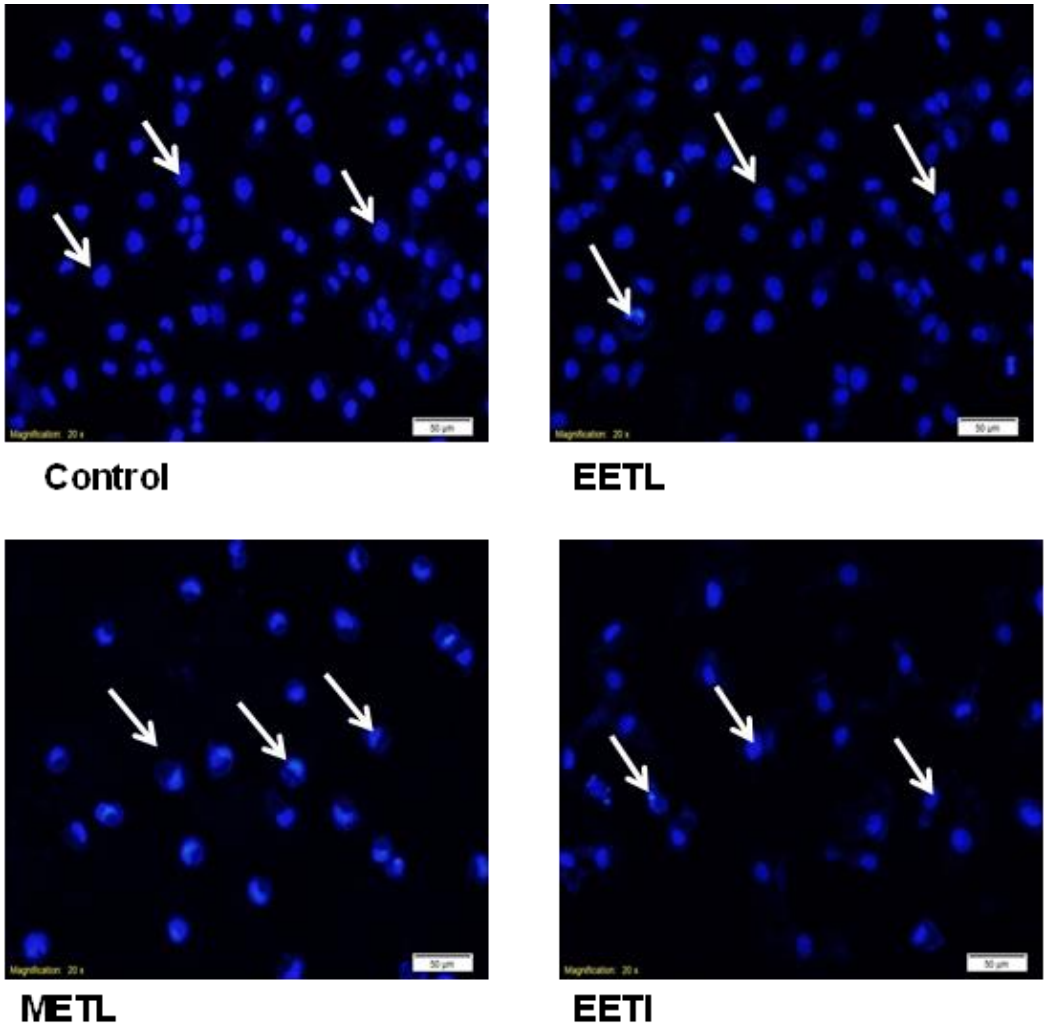

Figure 5. Fluorescence microscopic images representing condensed chromatin after EETL and METL treatment in MDA MB 231 breast cancer cells.

5.1.4. Measurement of DNA damage induced by EETL, METL and EETI 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 EETL treatment is higher than METL (Figure 6). The characteristic comet tail length suggested the amount of DNA damaged.

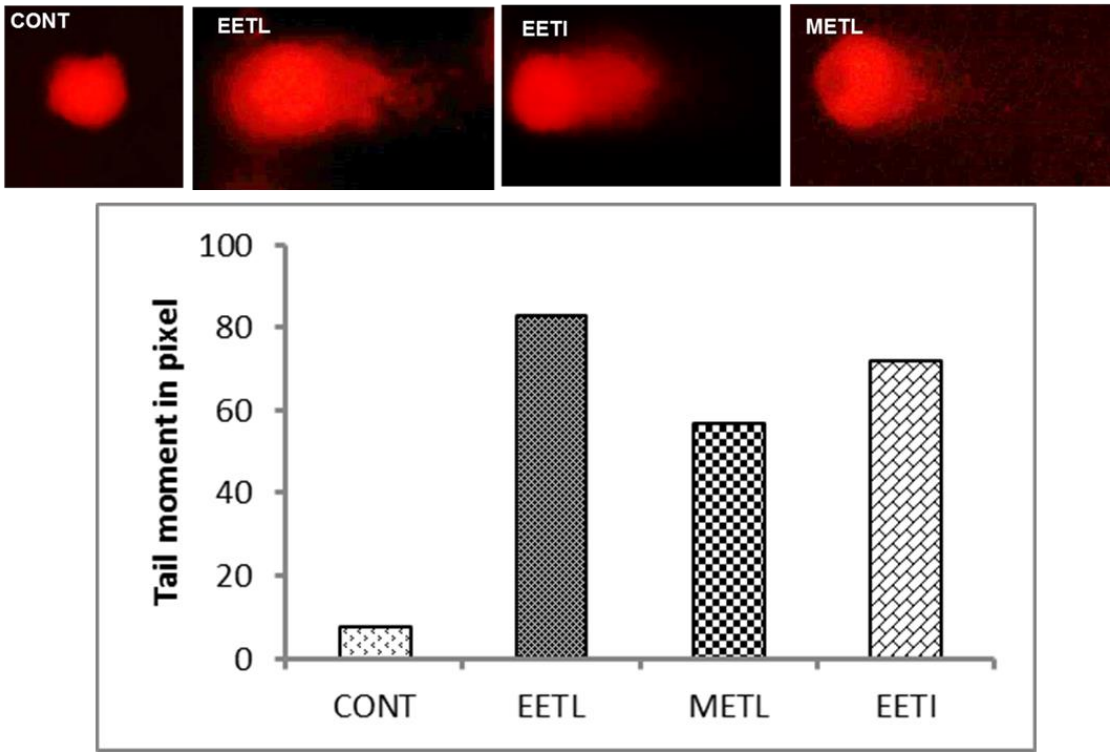

Figure 6. Fluorescence microscopic images of comets showing DNA damage after EETL, METL and EETI treatment.

5.1.5. Determination of anti-migratory activity of EETL, METL and EETI on MDA MB 231 cells by Scratch assay

To determine the migratory property of the MDA MB-231 cells after EETL, METL and EETI treatment for 24 h, the scratch assay was performed. While in control, the cancer cells migrate and close the gap in 24 h, in treatment groups very less number of cells migrate towards the gap. This indicates the anti-migratory property of tulsi extracts. Moreover, the wound healing ability of EETL is found to be least among all (Figure 7). The results showed that there was more migration of cells towards the scratched area in METL treated cells as compared to EETL treated cells in comparison with the untreated plates taken as control. This clearly demonstrates the efficient anti-migratory property of EETL on invasive breast cancer cells.

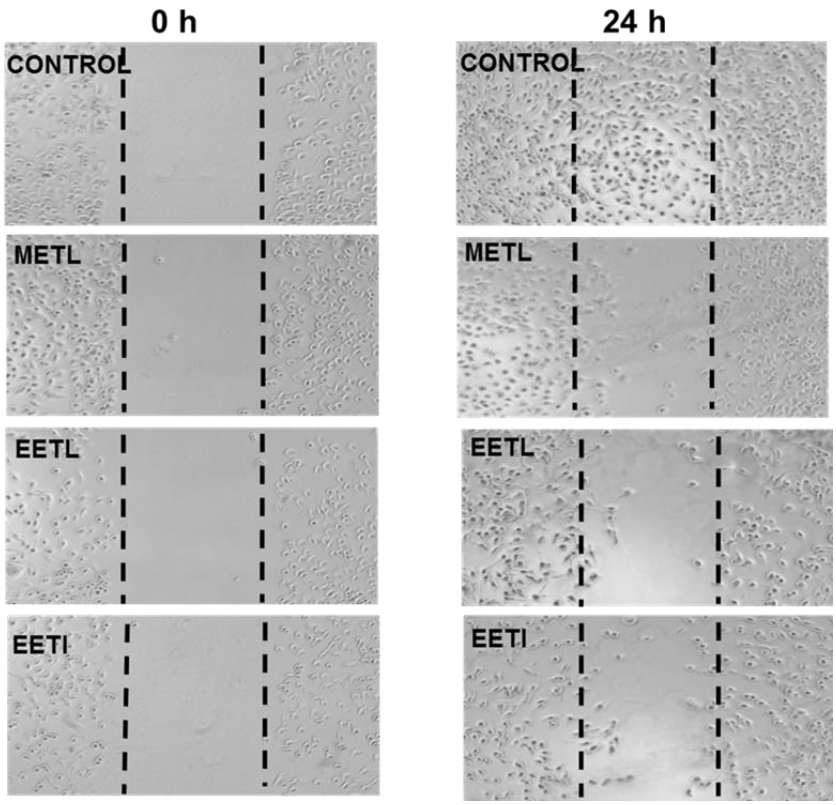

Figure 7. Microscopic images showing changes in the migratory property of MDA MB-231 cells after EETL, METL and EETI treatment.

5.1.6. Gene Expression Analysis of MASPIN, Snail, HDAC-1, HDAC-2 by Semi-quantitative reverse-transcription PCR (Semi-q-RT-PCR)

Semi-q-RT-PCR data confirmed that the transcript level of MASPIN is increased in MDA-MB 231 breast cancer cells after treatment with EETL and METL. MASPIN 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 at transcription level. On treatment with EETL and METL, the MASPIN is demonstrated to be up-regulated in breast cancer cells (Figure 10). The EETL can more effectively restore the expression of MASPIN than METL, with higher expression of both HDAC1 and HDAC2 and lower expression of Snail (Figure 8). This denotes that EETL and METL may have HDAC inhibitory potential. The level of β-actin remains same in all cases.

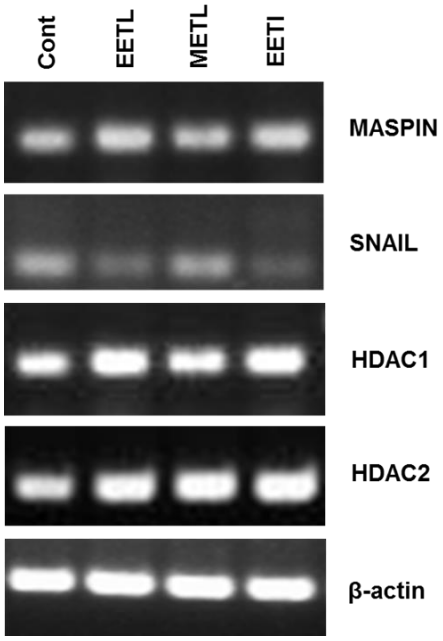

Figure 8. Representative images of semi-q-RT-PCR result depicting expression level of MASPIN, Snail, HDAC-1, HDAC-2 and β-actin after EETL and METL treatment.

5.1.7. Gene Expression Analysis of MASPIN and HDAC-1 by quantitative reverse-transcription PCR (q-RT-PCR)

The real time PCR analysis of mRNA levels of maspin and HDAC-1 shows that the maspin and HDAC-1 are up-regulated by 13 and 33 folds, respectively, in EETL than METL. This supports our finding from semi-q-RT-PCR study (Figure 9 and 10). The HDAC-1 is also demonstrated to be up-regulated in extract treated cancer cells (Figure 10). From the increase in transcript level of HDAC-1 in treatment groups, it is apparent that the EETL and METL have HDAC inhibitory activity.

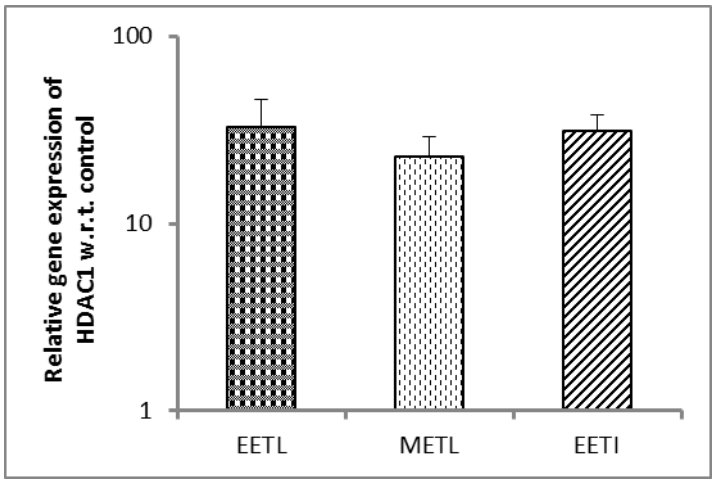

Figure 9. Graphical representation of RT-PCR results for expression level of HDAC1 after EETL, METL and EETI treatment

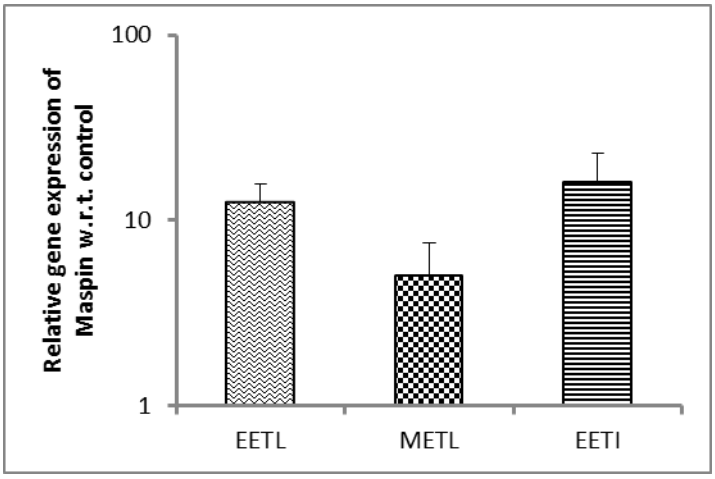

Figure 10. Graphical representation of RT-PCR results for expression level of MASPIN after EETL, METL and EETI treatment.

6. CONCLUSION:

It is very much clear from the current investigation that the tulsi leaf and inflorescence extracts have the anti-cancer potential. This anti-cancer potential is due to the combinatorial effect of anti-proliferative, apoptotic and anti-migratory effect of the tulsi extracts. It is demonstrated from our experiments that the EETL exert more anti-cancer effect on breast cancer cells than METL and EETI. This investigation also demonstrates the higher efficacy of EENL in restoring the expression of maspin with concurrent enhancement of HDAC-1 and HDAC-2 mRNA levels, and reduced expression of snail. This reflects the HDAC inhibitory activity of tulsi extract. Furher research in this regards will shed light on the HDAC inhibitory activity of tusi extracts, which can be used for the treatment of cancer.

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