DECIPHERING THE ANTI-CANCER EFFICACY OF *Azadirachta indica* AND ITS ROLE IN RE-EXPRESSION OF E-CADHERIN IN BREAST CANCER VIA SNAIL AND HDACs

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DECLARATION

I hereby declare that this project report on, **"DECIPHERING THE ANTI-CANCER EFFICACY OF** *Azadirachta indica* **AND ITS ROLE IN RE-EXPRESSION OF E-CADHERIN IN BREAST CANCER VIA SNAIL AND HDACs"**, 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 and Head, Department of Life Science, National Institute of Technology, Rourkela.*

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ABSTRACT

Neem (*Azadirachta indica*), considered natural drug house, has been a source of numerous modern drugs in both developed and developing countries across globe. It provides with the active ingredients that act as a prophylaxis for the treatment of diverse ailments more specifically against cancer. The anti-cancer components of Neem comprising of azadirachtin, nimbolide, quercetin, limonin, glucopyranoside, azadirone, and deoxo nimbolide are targeted to the culprit enzymes accountable for tumor formation, subsequently combating cancer. Cancer, especially breast cancer has been responsible for the drastic mortality rate among all females. Cancer is characterized by genetic and epigenetic aberrations. These epigenetic aberrations are reversible thus accounting for the restoration of the functionality of tumor suppressor gene. In our study, we have carried out an effort towards checking the effect of neem extracts towards restoring the expression of tumor suppressor gene, E-cadherin (CDH1) via Snail and histone deacetylases (HDACs) in breast cancer cells MDA MB 231. Histone acetylation erasers, HDACs are the epigenetic manipulators playing a crucial role in silencing of tumor suppressor genes concerned with cancer initiation and progression. There are several HDAC inhibitors available but they have higher toxicity. Here, we have considered both the ethanolic and methanolic extracts of neem leaves (EENL and MENL), as they have been reported to be more effective than the aqueous extracts. The higher total phenolic content of EENL indicates its higher anti-oxidant property, which accounts for its anti-cancer potential. Also the cytotoxicity, anti-proliferative, apoptotic and anti-migratory potential of EENL are demonstrated to be more than that of MENL. Moreover, EENL restores more efficiently the expression of CDH1 and causes an enhanced transcript level of HDAC1 and HDAC2. Thus, it can be concluded that EENL may have HDAC inhibitory activity than MENL. Further research in this concern will shed more light on the HDAC inhibitory property of EENL and MENL on breast cancer cells.

1. INTRODUCTION

India has a diverse culture of medicinal herbs and spices, which includes about more than 2000 species. Plant & plant-derived compounds acted as a prophylaxis in the development of several anti-cancer agents. Plants provide the active ingredients including both primary & secondary metabolites such as-vincristine, vinblastine, flavonoids, terpenes, alkaloids, topotecan and irinotecan etc. These have been utilized by human beings for the treatment of diverse ailments for thousands of years [1]. There has been a great demand for herbal medicines in both developed and developing countries as a source of primary health care including ethno medicinal activities, high safety margins and lesser costs. Numbers of important modern drugs have been derived from plants inspite of the advent of modern or allopathic medicine, to be used by native people. Numbers of compounds used in mainstream medicine have been detected by researchers derived from "ethno medical" plant sources. There are numerous medicinal herbs that contribute a lot as anti-cancer agents. To name a few among them are, *Azadirachta indica*, *Camellia sinensis*, *Catharanthus roseus*, *Withania somnifera, Boswellia serrata*, *Abrus precatorius*, *Albizzia lebbeck*, *Asparagus racemosus*, *Plumbago zeylanica* and others.

Azadirachta indica (Neem) presents greenhouse treasure cultivated in Indian subcontinent. It's a member of the Meliaceae family. Neem because of its widespread domestic, therapeutic, agricultural &ethno medical applications is abbreviated as the "sacred gift of nature" or "natural drug house". All parts of neem including-leaves, barks, seed-oil, and twigs along with their purified products are widely used as anti-cancer agents. The phytochemicals being extracted from this plant include- nimbin, nimbidiol, azadirachtin, nimbidin and quercitin. These phytochemicals are of antiviral, antibacterial, antifungal, anti-malarial, antiseptic, anti-pyretic, anti-inflammatory, anti-oxidant, anti-ulcer and anticancer properties. The detailed study of bioactive components of Neem constitutes the following.

1.1 Bioactive components of Neem (*Azadirachta indica*)

A major crude extract, Nimbidin isolated from the oil of seed kernels of neem exhibits several biological activities. Several tetranortriterpenes, including nimbin, nimbinin, nimbidinin, nimbolide and nimbidic acid have been isolated from this extract (Figure 1). It shows considerable dose reliant anti-inflammatory activity against carrageenin stimulated acute paw oedema in rats and formalin stimulated arthritis [2, 3]. Nimbidin has also been reported to exhibit anti-pyretic activity. Nimbolide, component of nimbidin inhibits the growth of *Plasmodium*

falciparum thus confirming anti-malarial activity[4] (Figure 2a). Also it exhibits anti-bacterial activity against *S. aureus* and *S. coagulase* while gedunin (in neem seed oil) has been reported to have anti-fungal and anti-malarial activities [5]. Azadirachtin a well oxygenated C-secomeliacins extracted from seed of neem possess strong anti-feedant action (Figure 2b). In addition, it also exhibits anti-malarial property. It displays inhibitory activity against the maturity of malarial parasites[6]. Mahmoodin (deoxygedunin), possess moderate antibacterial activity against few strains of human pathogenic bacteria. Condensed tannins comprising gallic acid, catechin, epicatechin, gallo-catechin, , and epigallo-catechin, among which epicatechin, gallic acid, and catechin inhibit the chemi-luminescence production via stimulated human poly-morphonuclear neutrophil (PMN) [7], thus indicative of restraining oxidative burst of PMN through inflammation. Three tricyclic di-terpenoids margolone, iso-margolonone and margolonone isolated from neem stem bark are act against Klebsiella, Staphylococcus and Serratia species. Polysaccharides extracted from neem display diverse biological effects. One of the polysaccharide isolated from neem bark shows inhibitory action against carrageenin-stimulated inflammation in mouse. Two polysaccharides GIa and GIb (water-soluble) extracted from the bark of *Melia azadirachta*, cause complete deterioration of the tumors, 24 h after subcutaneous inoculation of Sarcoma-180 cell whilst administered in mice at a daily dose of 50 mg/kg for four days [8]. GIIa and GIIIa (polysaccharides) isolated from *M. azadirachta* bark induced considerable anti-inflammatory effect on carrageenin-induced oedema in mice [8]. The detailed biological significance has been illustrated in Table 1.
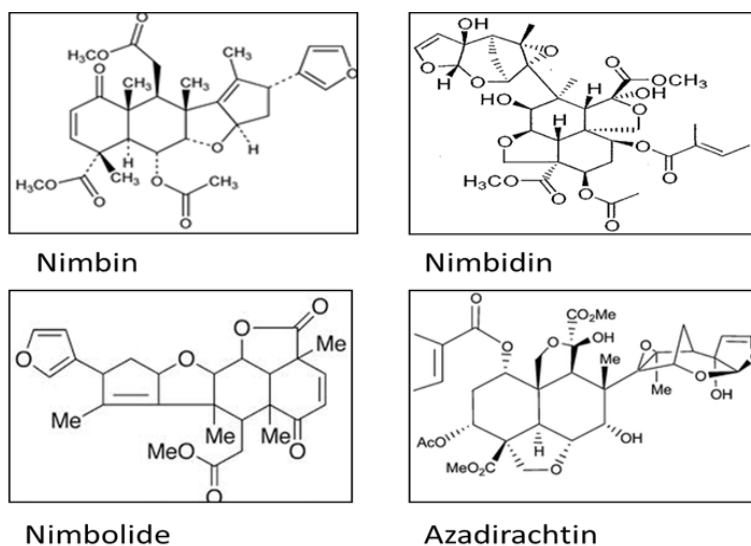

Figure 1. Molecular structure of some bioactive components of Neem (*Azadirachta indica*)

Table 1. Some bioactive components from Neem

NEEM COMPOUND	SOURCE	BIOLOGICAL ACTIVITY
Nimbidin	Seed oil	Anti-inflammatory, Anti-arthritic, Antipyretic, Hypoglycaemic, Anti-gastric ulcer, Spermicidal Antifungal, Antibacterial, Diuretic
Sodium Nimbidate		Anti-inflammatory
Nimbin	Seed oil	Spermicidal
Nimbolide	Seed oil	Antibacterial Antimalarial
Gedunin	Seed oil	Antifungal Antimalarial
Azadirachtin	Seed	Antimalarial
Mahmoodin	Seed oil	Antibacterial
Gallic acid, epicatechin,catechin		Anti-inflammatory immunomodulatory
Margolone , margolonone and isomargolonone	Bark	Antibacterial
Cyclic trisulphide and cyclic tetrasulphide	Leaf	Antifungal
Polysaccharides		Anti-inflammatory
Polysaccharides GIa , GIb	Bark	Antitumor
Polysaccharides GIIa , GIIIa	Bark	Anti-inflammatory
NB-II peptidoglycan	Bark	Immunomodulatory

The anti-cancer components of Neem comprises of azadirachtin, nimbolide, quercetin, limonin, glucopyranoside, azadirone, and deoxonimbolide. These anticancer agents are targeted to the enzymes responsible for tumor formation subsequently combating cancer.

1.2 Epigenetics and cancer

Epigenetics refers to the study of mitotically or meiotically heritable changes in gene function and/or phenotype without any changes in the underlying sequence of DNA. It involves no change in the underlying genetic program whilst; non-genetic factors cause expression of differential phenotypes in the organism's genes. Epigenetics has evolved as a rapidly emergent research area in the present scenario. Recent studies have revealed that epigenetics plays a vital role in cancer biology, mode of action of mobile 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 2).
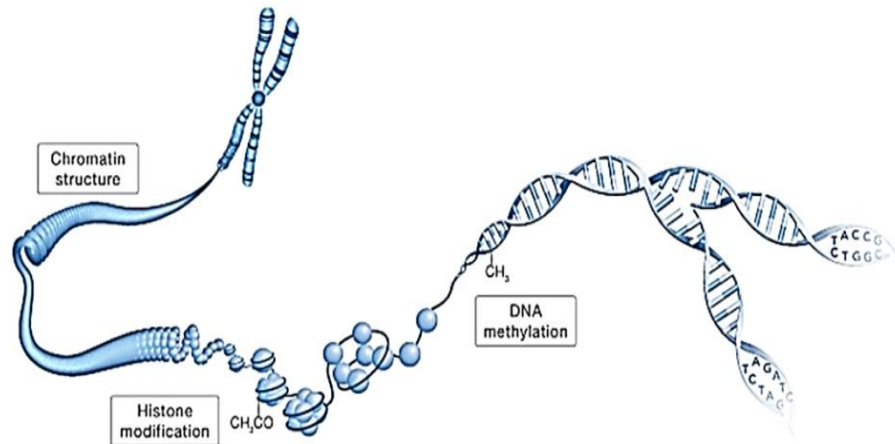
Figure 2. Epigenetic signaling tools.

1.2.1   DNA Methylation

It is a DNA level modification. This refers simply the addition of a methyl (CH_3) group to the 5' carbon of cytosine pyrimidine ring or the 6' nitrogen of the adenine purine ring. DNA methylation is one of the crucial regulators of transcription, and its role in inducing cancer has been a topic of significant interest in the past few years. Alterations in DNA methylation are familiar in a variety of tumors as well as in developmental abnormalities. Of all epigenetic modifications, hyper methylation, have been most extensively studied that causes repression of transcription at the promoter regions of tumors suppressor genes followed by gene silencing [9]. DNA methylation causes the silencing of genes whereas demethylation effects gene expression.

1.2.2. RNAi mediated gene silencing

It occurs at RNA level. RNAi is an RNA-dependent gene silencing pathway under the control of RNA-induced silencing complex (RISC). The process is initiated by short ds (double stranded) RNA molecules in a cell's cytoplasm where they unite at the RISC complex.

1.2.3. Histone Modifications

Histone proteins, that package DNA into chromatin, are subject to several post translational modifications that ultimately affect gene regulation. These post-translational covalent modifications (PTMs) occur in the ε-N-terminal tails of histone proteins that regulate the transcriptional state of the genome via chromatin structure resulting in differential expression of genes. The various PTMs include- acetylation, methylation, sumoylation, phosphorylation, ADP ribosylation, citrullination, proline isomerisation and ubiquitination. These alter the interaction of DNA and nuclear proteins with histones. Histones are acetylated by histone acetyl transferases (HAT) & deacetylated by histone deacetyltransferases (HDACs). Histone modification plays an important role in gene transcription, and its role in carcinogenesis has been a topic of great interest in the past few years.

1.3. Histone acetylation, deacetylation and gene regulation

The Acetyl-Coenzyme A is the major 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 interaction of the histone with the phosphate group of DNA. This causes the transformation of folded structure into a relaxed structure (euchromatin) i.e., associated with greater levels of gene transcription (Figure 3). This can be reversed by HDAC activity that removes acetyl tags resulting in a more condensed structure of tightly packed DNA referred to as heterochromatin. Histone modifications have numerous utilities in varied biological pathways ranging from gene regulation, chromosome condensation and DNA repair.
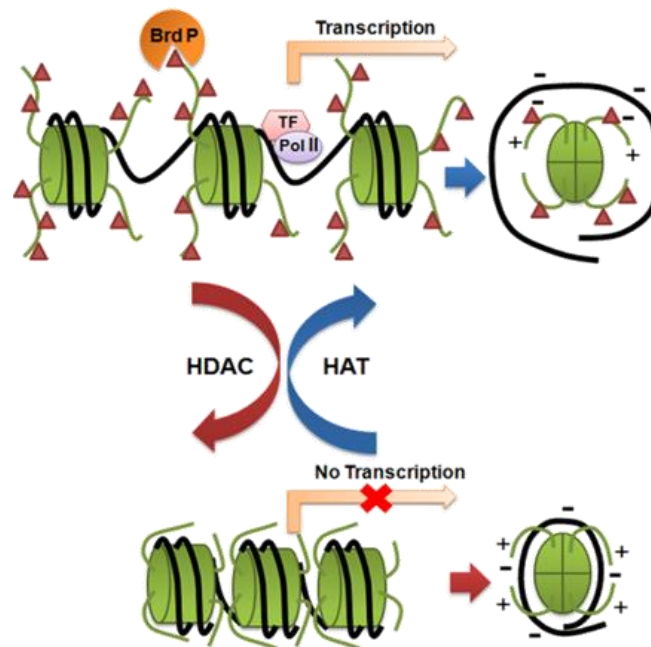
Figure 3. Histone acetylation and deacetylation (Parbin et al, 2014)

1.4 HDACs and cancer

As we know cancer in short; a malignant tumor is a fatal disease resulting from regulatory mechanisms breakdown ultimately governing normal cell behavior. The propagation, delineation, and survival of individual cells in organisms are regulated carefully so as to meet the needs of organism as a whole. But this regulation is misplaced in cancer cells, which grow and divide in an unrestrained manner and subsequently spreading throughout the body through metastasis thus interfering with normal cellular behavior. It may occur at any age in any part of the body representing the largest cause of mortality in the world claiming over 6 millions [11]. Amongst all cancers, breast cancer is the chief cause of mortality in women both worldwide & Malaysia.

Breast cancer contributes one of the most common malignancies in women estimated to be 182,460 new cases and reported for 40,480 deaths in 2008 in the United States (US)[12]. Despite advent in molecular mechanisms of breast cancer and evolution in early detection and treatment, cancer cells either are intrinsically resistant or quickly acquire resistance against various cytotoxic chemotherapeutic drugs in 50% of cases[13]. Moreover, approximately 30% of all patients with early breast cancer stages will develop recurrent disease, which is principally metastatic and resistant to treatment [14, 15]. Currently, cancer metastasis and drug resistance, intrinsic or acquired, are chief obstacles in the successful treatment of breast cancer [16, 17]. Cancer is characterized by genetic and epigenetic aberrations. The epigenetic aberrations are

reversible thus the functionality of tumor suppressor gene can be restored. Epigenetic modulators play significant role in cancer.

HDACs otherwise called histone acetylation erasers are one of such epigenetic manipulators which play a vital role in silencing of tumor suppressor genes concerned with cancer initiation and progression. Carcinogenesis includes the transition of normal cells into malignant cells which is characterized by uncontrolled cell proliferation, a lack of differentiation, including epithelial and hematopoietic differentiation, and the prevention of apoptosis. During cancer initiation, HDACs exert their silencing effects on cell-cycle checkpoint regulatory genes and genes associated with the differentiation process [10] (Figure 4).
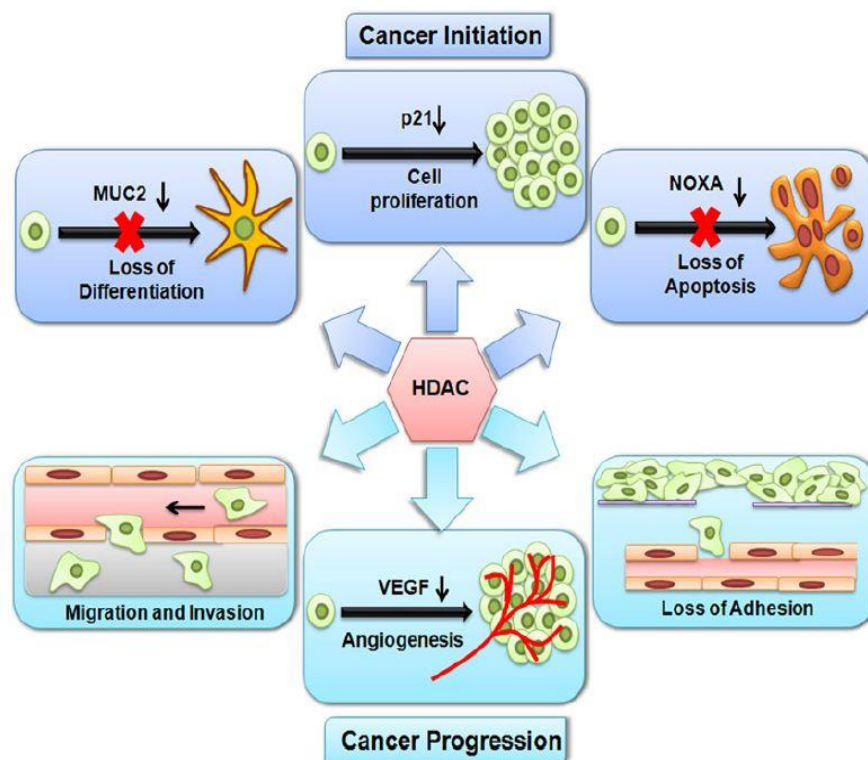
Figure 4. Role of HDACs in Cancer initiation and proliferation (Parbin et al, 2014)

1.4.1. Role of HDACs in cancer initiation and progression

HDACs are responsible for uncontrolled cell proliferation by repressing the expression of specific cyclin dependent kinase (CDK) inhibitors-p21 and p27[18].Higher levels of HDAC2 expression in Apc-deficient mouse adenomas are associated with loss of 15-hydroxyprostaglandindehydrogenase (15-PGDH) expression. HDAC2 interacts with the 15-

PGDH promoter, thereby causing its suppression. But, treatment with HDACi namely, Trichostatin-A (TSA),induces the expression of TGF- β RII and leads to formation of open chromatin conformation in lung cancer cells [19].HDACs are associated with loss of differentiation in cancer cells. Genes associated with differentiation elicits inappropriate cell proliferation on down-regulation. Pancreatic cancer cells that lack MUC2, upon treatment with TSA, were reported to inducing histone acetylation along with MUC2 mRNA and protein expression [20]. HDACs play crucial role in suppressing programmed cell death in various cancer cells. Noxa, member of the Bcl-2 family, up-regulated by p53 plays vital role in loss of apoptosis. It causes diminishing effects on apoptosis upon down-regulation. HDAC2 dictates epigenetic silencing of NOXA in pancreatic cancer cells [21]. Co-treatment with HDACi namely valproic acid (VPA) and TRAIL (TNF-α related apoptosis inducing ligand) causes enhanced apoptosis of pancreatic cancer cells [22]. HDACs are also involved in restraining the genes concerned with cancer progression. The two fundamental events associated with cancer progression are angiogenesis and metastasis. Angiogenesis refers to the development of new blood vessels from pre-existing ones. It comprises of two conditions i.e. normoxic and hypoxic. Hypoxic conditions in angiogenesis are interlinked with inducible expression of HDACs, includingHDAC1, 2, & 3[23, 24]. VEGF, platelet-derived growth factor produced by cells stimulates vasculogenesis and angiogenesis upon down-regulation. HDAC1overexpression leads to silencing of p53 and VHL but induces the hypoxia-responsive genes HIF-1 α and VEGF thus stimulating angiogenesis [23, 25]. A cell's lack of ability to interact precisely with its external environment and its neighboring cells is the universal feature of metastasis. Metastasis is characterized by invasion, migration and then loss of adhesion. HDAC1 knockdown reduces cellular invasion by overexpression of cystatin, a peptidase inhibitor [26]. Also, HDACs inhibit CDH1 expressions that associate with hypoacetylated histones H3 and H4 at its promoter[27]. HDAC1 and HDAC2 function to suppress the CDH1 expression. On the contrary, treatment of pancreatic cells with HDACi restores the CDH1 with diminished EMT [28, 29].

There are several HDACi known in the present scenario but some of them possess high toxicity and low specificity. Henceforth, our study focuses on identification of anticancer property of Neem and its effect on HDAC and the tumor suppressor gene (E-cadherin). E-cadherin (CDH1) is crucial mediator of cell-cell interactions, and playing a significant role in suppression of breast tumor. Its inactivation is mediated through instability at its chromosomal

locus and mutations as well as through epigenetic mechanisms namely hypermethylation of promoter and transcriptional repression. The transcription factor, Snail has been reported as a direct repressor of CDH1 expression during development and carcinogenesis by the recruitment of the Sin3A/Histone Deacetylase 1 (HDAC1)/HDAC2 Complex. Thus, upon targeting inhibitors to this complex will deactivate the functionality of the enzymatic complex which in turn will restore the expression of CDH1subsequently combating cancer.

2.   REVIEW OF LITERATURE

Cancer, a multi-faceted ailment, is the main cause of morbidity globally, reporting for 7.6 million deaths annually. Carcinogenesis is a multistep process involving three distinct stages namely- initiation, promotion, and proliferation [30]. 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[31]. The ideal 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 buildup 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 [32]. Clinical therapies along with radiation, chemoprevention, immune-modulation and surgery in curing cancer have accomplished limited success. This is evident from the high mortality rates, thus indicating the urge for the development of new cancer management system. Chemo-prevention includes the use of dietary bio-factors, pharmacological, phytochemicals and even whole plant extracts to check, arrest, or reverse the cellular and molecular processes of carcinogenesis due to its manifold interference strategies [33]. The quest for compounds with few or no adverse effects that will block the invasion of cancer and protect against the adverse biological effects of chemotherapeutic agents as compared with the agents currently in use is henceforth of greatest relevance. Plant herbs and plant-derived drugs have therefore proven as the source of potent anti-cancer agents in conventional cultures worldwide and are becoming rapidly popular in current society [34].

Natural phytochemicals derived from these herbs have shown noteworthy recognition in the management of several human ailments, including cancer [34, 35]. Much research has been oriented towards the plant extracts evaluation as prophylactic agents, presenting a great potential to repress the carcinogenic process. Simultaneously, the synergistic effects of the combination of plant metabolites and their multiple points of intervention offer higher efficacy during chemo-preventive regimens [36]. The preventive machinery of tumor induction by natural phytochemicals range from the inhibition of genotoxic effects, increased antioxidants and anti-inflammatory activity, inhibition of proteases and cell propagation, protection of intracellular communications to modulate apoptosis and signal transduction pathways. A number of new chemotherapeutic agents are being identified based on the ability to modulate one or more specific molecular events. The discovery of valuable herbs and elucidation of their underlying mechanisms could lead to the development of an alternative and corresponding method for cancer prevention and/or treatment. The Indian sub-continent has great botanical diversity and extensive usage of traditional medicine practice known as ayurveda; however, only a relatively small number of these plants have been subjected to accepted scientific evaluation for their potential anticancer effects [37].

The ability of neem extracts to remove cancerous phenotype was known long before to people in Asia, mostly in India. Researchers in India and abroad, during the last two decades, have put forwarded the opinion that the onset of cancerous phenotype resulted from mutagens and pro-carcinogens may be efficiently treated from extracts prepared from various parts of the neem tree. In vivo murine system, the chemotherapeutic property of dietary doses of aqueous neem leaf extract (ANLE) was observed against H-B-α-P(Benz-α-pyrene)-induced carcinogenesis in terms of 3H-B-α-P-DNA adduct formation. Their results showed that the metabolic activation of 3H-B-α-P is declined with subsequent decrease in the level of 3H-B-α-P-DNA adduct in presence of ANLE [38]. In this investigation, the molecular and biochemical modulations seen at the initial stage of carcinogenesis reflects the chemo-therapeutic significance of *A. indica.*

According to a study, conducted by Chaimuangraj et al., the neem leaf extract fed at doses of 20, 100, 250 mg/kg body weight drastically inhibit the azoxymethane-induced aberrant crypt foci (ACF) and also decrease the proliferating cell nuclear antigen (PCNA) labeling indices i.e. $p < 0.0006$ in the colon epithelium in rats. Several research groups have also verified the

preventive action of neem flowers against DMBA and BαP induced cancers [39-41]. These investigations showed that different parts of *A. indica* may be used in dietary form playing a crucial role in future drug discovery.

O6-methylguanine-DNA methyltransferases (MGMT) is an enzyme that detoxifies O6-alkylguanine, a potent mutagen and pro-carcinogen inducing agent, thus maintaining the genomic integrity. It has been demonstrated that the ethanolic and aqueous extracts of neem enhance the MGMT activity, thus resulting in time-dependent methylation of O6-methylguanine and consequently preventing its cytotoxic abrasions [42]. An enhanced elimination of these aberrations by increasing the MGMT activity is a beneficial chemotherapeutic strategy. Recently, it has been noted that the azadirachtin and nimbolide of neem extracts inhibit the development of DMBA-induced hamster buccal pouch (HBP) carcinoma in a dose-dependent manner via varied mechanisms, that include prevention of activation of pro-carcinogen, up-regulation of antioxidant and carcinogen detoxifying enzymes and oxidative DNA damage [43].

Extracts from different parts of neem can also be used as tumor suppressors as proved by the researchers. Researchers from India and Japan have reported that polysaccharides and limonoids from neem leaves, bark, and seed oil declined the progression of tumors and cancers, reflecting its efficacy in lymphocytic leukemia. Additionally, researchers from Japan have shown that hot water extracts from neem bark posseses notable effectiveness against different types of cancers. Many of these extracts were equally effective or better than the coventional anticancer agents, especially in solid tumors. Ethanolic extracts (80%) from neem leaves, when administered at doses of 250–500 mg/ kg, decreased the average count of papilomas induced by BαP and DMBA in the 7-week-old Swiss albino mice model [44]. Similarly, Japanese researchers have demonstrated that nimbolide, a triterpenoid present in edible parts of *A. indica*, arrested the human colon carcinoma cells in G2/M and G_0/G_1 stages apparently through up-regulation of p21. p21 is a well-known down-stream effector of the p53 gene, which is a very significant anticancer protein that monitors a number of genes involved in tumorigenesis.

Similarly, Cyclin D2 and Chk2 have been found to be up-regulated, while the expression of cyclin A, cyclinE, Cdk2 and Rad17 were reduced when treated with nimbolide [45]. Kumar et al extensively carried out their research on U937, HL-60, THP1 and B16 cell lines and observed that nimbolide exerted anti-proliferative and apoptotic effects with reduction of bcl-2/bax ratio

and induction of Apaf-1 and caspase-3 expression. These experiments implied that the neem ingredients function through different molecular pathways to exert their anticancer effects [46].

An experimental approach was followed by Subapriya et al. [47] in which they investigated the anticancer efficacy of ethanolic extracts of neem leaf (EENL) on DMBA-induced carcinogenesis in buccal pouch of hamster basically emphasizing on their mode of regulation. Their investigations revealed engrossment of the PCNA (Proliferating cell nuclear antigen), mutant p53 and bcl2 genes. These genes are generally up regulated during DMBA-induced cancer formation. EENL suppresses these genes as well as DMBA-induced cellular proliferation and differentiation. Bieberich et al, proposed that neem seed oil suppresses breast tumor formation in the ex vivo investigational models of female mice. They detected that 50 μl of neem seed oil inoculation at a dilution of 1:25 every week in MCF-7 cell line for roughly 50 days reduced tumor affliction by 50%. These estimations present emphatic evidence assisting to improvise working strategies thus making use of anticancer efficacy of neem extracts for cancer treatment.

2.1. Apoptotic activity of neem in cancer cells

Apoptosis is a genetically driven process that leads to a series of biochemical events. These are characterized by blebbing, nuclear fragmentation, cell shrinkage, chromosomal DNA fragmentation, and chromatin condensation. It involves caspases, natural biochemical blades meant for surgery required to wipe out the unwanted, wayward, redundant and irreparable body cells without shedding even a single drop of blood and any material loss to the body as a whole. Treatment with neem extracts followed by cell viability assays and DNA fragmentation, has reported to induce apoptosis in a dose-dependent manner in prostate cancer cells (PC-3) [46].These observations exhibit that there is suppression in the bcl-2 protein expression, a strong pro-survival factor in cancer cells and simultaneously enhanced the pro-apoptotic Bax protein level of expression. Neem extract could thus be a potentially effective treatment against prostate cancer. Working on the choriocarcinoma cells, Kumar et al[48] have shown that nimbolide, which is an active anticancer active component of the neem leaf and flowers, induces apoptosis through engagement of the mitochondrial pathway. This pathway is based on the fact that nimbolide mediates up-regulation of Apaf1 andcaspase3 and leads to decrease in the bcl2/bax ratio. Nimbolide has been observed to disrupt cell cycle check points (G2/M, G0/G1 and G/S) in correlation to induction of apoptosis on treatment with U937, HL-60,

THP1 and B16 cell lines [49]. The molecular correlation that exists between cell cycle disruption and apoptosis has not been clearly shown by them in these cells lines. Often, cell cycle disruption followed by arrest of cells at the cell cycle check points is attributed to immunity against apoptosis.

The anti-proliferative activity of ethanolic neem leaves extract (EENL) has been well demonstrated. EENL alone or in combination with cisplatin targets cell proliferation by cell viability assay on human breast (MCF-7) and cervical (HeLa) cancer cells. Nuclear morphological examination and cell cycle investigation were performed to establish the mode of cell death. Further, in order to identify its molecular targets, the expression of genes concerned with apoptosis, cell cycle progression, and drug metabolism was analyzed by RT-PCR. Treatment of MCF-7, HeLa, and normal cells with EENL differentially suppressed the growth of cancer cells in a dose and time dependent manner through apoptosis. Furthermore, lower dose combinations of EENL with cisplatin resulted in synergistic growth inhibition of these cells in comparison to the individual drugs. EENL modulated the expression of bax, cyclinD1, and cytochrome P450 mono-oxygenases (CYP 1A1 and CYP 1A2) drastically in a time-dependent manner in these cells. These results highlight the chemo-preventive ability of neem alone or in combination with chemotherapeutic treatment to minimize the cytotoxic effects on normal cells, while strengthening their efficacy at lower doses [50].Thus, neem may be a regarded as a forthcoming therapeutic agent to combat gynecological cancers.

Multiple active components are present in *Azadirachta indica* possessing potent anti-inflammatory and anticancer properties. This study investigates the novel targets of the anticancer activity of ethanol extract of neem leaves (EENL) in vitro and evaluates the in vivo efficacy in the prostate cancer forms. Analysis of the components in the EENL by mass spectrometry suggests the occurrence of 2', 3'-dehydrosalannol, 6-desacetyl nimbinene, and nimolinone. C4-2B and PC-3M-luc2 prostate cancer cell lines upon treatment with EENL show inhibitory activity on cell propagation. Genome-wide expression analysis, utilizing oligo-nucleotide microarrays, unveiled the differential gene expression with EENL treatment in prostate cancer cells. Their functional investigation revealed that most of the up-regulated genes were associated with drug metabolism and cell death, and the down-regulated genes were associated through cellular cycle, replication of DNA, recombination, and repair functions. Quantitative PCR analysis confirmed significant up-regulation of 40 genes and immunoblotting

disclosed increase in the protein expression levels of HMOX1, AKR1C2, AKR1C3, and AKR1B10. The growth of C4-2B and PC-3M-luc2 prostate cancer xenografts in nude mice was inhibited upon EENL treatment. Basically tumor growth suppression is associated with the formation of hyalinized fibrous tumor tissue and the induction of cell death by apoptosis. These results revealed that EENL containing natural bioactive compounds retain potent anticancer property and the regulation of multiple cellular pathways could exert pleiotropic effects in prevention and cure of prostate cancer[51].

It has been well documented in a study that ethanolic neem leaf extract displayed down regulation on c-Myc oncogene expression in 4T1 breast cancer BALB/c mice. c-Myc oncogene expression was down regulated in *in- situ* RT-PCR when stimulated by 500 mg/kg of ethanolic neem leaf extract[11]. This suggested that in physiological circumstances, neem retains the ability to minimize cellular propagation in response to the cancer in mice [52]. For tumor induction, c-Myc not only requires to promote cell propagation, but also concurrently restrains its inclination towards cellular death, in order to raise cell numbers to form a tumor mass [53]. The suppressed expression of c-Myc in neem treated cancer mice has not only shown a tendency towards inhibiting the cancerous cells proliferation but also to induce apoptosis against 4T1 cancer cells. A variety of chemotherapeutic drugs can initiate apoptosis in tumor cells, leading to the degeneration of a cancerous tumor.

Additionally another investigation has demonstrated that consumption of 5% of aqueous extract of neem leaves resulted in increase of antioxidant enzymes to a level of absolute inhibition of chemically induced hepato-carcinogenesis in rats. These studies revealed that neem may contribute its greatest effect against the 4T1 breast cancer model not only via apoptosis induction but also by its antioxidant bioactivity. The overall results exhibits that the ethanolic neem leaf extract has a significant effect against breast cancer in the 4T1 mouse model. But detailed study is needed to understand the mechanism of neem leaf extract on oncogene expression [11].

In the present scenario, no such research work has been oriented towards synergistic activity of both Neem and HDACS to combat cancer. But, interrelationship between HDACS and cancer has been well demonstrated as described below.

2.2 . HDACs and Metastasis

HDACs upon interaction with cystatin and e-cadherin effect invasion, migration and then loss of adhesion. HDAC1 knockdown reduces cellular invasion by overexpressing cystatin, a peptidase inhibitor [26]. Also, HDACs inhibit CDH1 expressions that associate with hypoacetylated histones H3 and H4 at its promoter [27]. CDH1 expression is often lost in prostate cancer cells in due to the high expressivity of HDACs, especially HDAC1, a key repressive enzyme for this silencing [54]. Silencing of CDH1is carried out by HDAC1 and HDAC2 during the metastatic process of pancreatic cancer cells. The underlying mechanism involves recruitment of transcriptional silencers, ZEB and Snail to the CDH1 promoter which, in turn recruit HDAC1 and HDAC2. These HDAC1 and 2 in turn functions to suppress the CDH1 expression. On the contrary, treatment of pancreatic cells with HDACi restores the CDH1 with diminished EMT [28, 29]. HDAC1 and HDAC2 are also essential components of the co-repressor complex— EZH2/HDAC1/2/Snail—concerned with the silencing of CDH1 causing EMT in nasopharyngeal cancer [55].

2.3. Synergistic effect of Snail & Slug on E-cadherin

Conversion of premature stage tumors into invasive malignancies constitutes one of the hallmarks in tumorigenesis [56]. It is basically associated with activation of the embryonic EMT program [57-59]. Thus, the early detection of EMT development or attenuation of the EMT phenotype in cancer cells may boost classical chemotherapy treatment and improve the clinical management of cancer. E-cadherin (CDH1) expression emerges as a critical step driving EMT in various human cancers, including invasive breast cancer [60, 61]. Several mechanisms have been proposed for transcriptional repression during EMT, as well as promoter CpG island hyper-methylation [62] and direct inhibition by transcriptional repressors such as Snail [63]. Transcription factor, Snail requires histone deacetylase (HDAC) activity to repress CDH1 promoter. Overexpression of Snail is correlated with deacetylation of histones H3 and H4 at the CDH1 promoter. It is also involved in epithelial to mesenchymal transitions(EMTs) where they are overexpressed in epithelial cell lines as well as in embryonic development thus acting as inducer of invasive breast cancer [64].

3. OBJECTIVES

a. To study the anti-proliferative effect of ethanolic and methanolic extracts of *Azadirachta indica* leaves on MDA MB 231 breast cancer cells.

b. Deciphering the apoptotic potential of ethanolic and methanolic extracts of *Azadirachta indica* leaves on MDA MB 231 breast cancer cells.

c. To study the anti-migratory effect of ethanolic and methanolic extracts of *Azadirachta indica* leaves on MDA MB 231 breast cancer cells

d. To investigate the role of ethanolic and methanolic extracts of *Azadirachta indica* leaves on expression of tumor suppressor gene (E-cadherin), transcription factor (SNAIL) and epigenetic manipulators (HDAC1, HDAC2) on MDA MB 231 breast cancer cells with establishment of correlation among them.

4. MATERIALS AND METHODS

4.1. Preparation of Plant extracts (EENL, MENL):

Neem (*Azadirachta indica*) leaves were collected from the National Institute of Technology (NIT), Rourkela campus. The leaves were washed with distilled water and air dried. Then the leaf extracts were prepared according to Othman et al, 2011. The leaves were ground using a grinder to obtain the fine powdered form. A total of 50 g of powdered neem leaf was transferred into a borosilicate glass bottle to which 100 ml of 80% ethanol was added. 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 100 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 neem leaves (EENL) was evaporated using a rotary evaporator (RV 10 Basic IKA Rotary Evaporator) at 60˚C. Same procedure was repeated for methanolic extracts of neem leaves (MENL) preparation, but in this case 50gm of powdered leaves was extracted in100 ml of absolute methanol. 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 di-methyl 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 phenolic content:

The phenolic content of plant extracts was determined from a gallic acid (GA) calibration curve. 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 EENL and MENL 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^{0}C for 30 min. Then phenolic contents were determined at 765 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:

$$C = c \times V / m$$

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:

MDA MB 231, a breast cancer cell line was procured from National Center for Cellular Sciences (NCCS), Pune. The cells are known to be of epithelial breast adeno-carcinoma origin and are triple negative. All culture works were carried out under strict aseptic conditions. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 units/ml penicillin-streptomycin sulfate (Invitrogen) in a humidified incubator supplied with 5% CO_2 at 37°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 neem extracts i.e ethanolic and methanolic extract of neem leaves (EENL, MENL) at 7 different concentrations (10-100μg/mL for EENL and 10-700μg/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 MENL and EENL 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 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 EENL and MENL at their respective IC_{50} values. 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.

4.7 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 was subjected to treatment with MENL and EENL 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 Na_2EDTA, 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 to6 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 DMEM containing MENL and EENL 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. MDAMB 231 cells were homogenized using TRI reagent i.e. 1 ml of the TRI Reagent per 10 cm^2 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.

4.10. 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.

4.11. cDNA preparation

In a 1.5 ml tube, 2 µg of template RNA was taken and the volume was adjusted to 11µl 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 E-cadherin (CDH1), Snail, HDAC-1, HDAC-2 and β-actin genes having sequence as detailed in Table no.2.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.

Table 2. Primers used for RT-PCR Analysis

SL. NO.	PRIMER NAME	PRIMER SEQUENCE	AMPLICON SIZE (in bp)
1	E-cadherin Forward primer	5'-CGAGAGCTACACGTTCACGG-3'	119
	E-cadherin Reverse primer	5'-GGGTGTCGAGGGAAAAATAGG-3'	
2	SnailForward primer	5'-TCTAGGCCCTGGCTGCTACAA-3'	131
	Snail Reverse primer	5'-ACATCTGAGTGGGTCTGGAGGTG-3'	
3	HDAC1Forward primer	5'-GGAAATCTATCGCCCTCACA-3'	168
	HDAC1 Reverse primer	5'-AACAGGCCATCGAATACTGG-3'	
4	HDAC2Forward primer	5'-ATAAAGCCACTGCCGAAGAA-3'	245
	HDAC2 Reverse primer	5'-TCCTCCAGCCCAATTAACAG-3'	
5	β- actinForward primer	5'-TCTACAATGAGCTGCGTGTG-3'	250
	β- actin Reverse primer	5'-TCTCCTTCTGCATCCTGTC-3'	

4.13. Gene Expression Analysis of CDH1, 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 EENL &MENLfor24 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 CDH1, 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 CDH1, 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 CDH1, 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 CDH1 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 2.

5.   RESULTS AND DISCUSSION

5.1.   Estimation of total phenolic content

Total phenolic content of neem leaf extracts (EENL and METL) 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). *Azadirachtaindica* consists of flavonoids, a group of polyphenoliccomplexes 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 EENL than MENL indicates its higher anti-oxidant potential, which in turn can be correlated with its anti-cancer potential. The higher anti-cancer property of EENL has been further evaluated by investigating its anti-proliferative, apoptotic and anti-migratory activity on MDA MB 231 breast cancer cells (Figure 5).
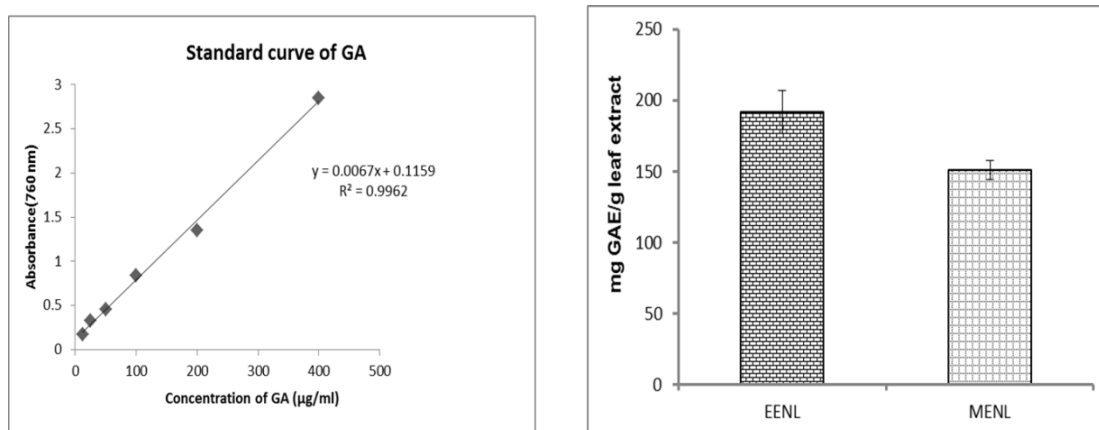

Figure 5. Standard GA calibration curve & total phenolic content in EENL & MENL.

5.2. Determination of cytotoxic effect and IC_{50} value of EENL and MENL by cell viability assay

Cell viability of MDA MB -231 was determined after EENL and MENL treatment by MTT Assay. The IC_{50} values for both the extracts were calculated. Both the EENL and MENL treatment showed decrease in cell viability but EENL treatment showed higher decrease in cell viability indicating more cytotoxicity towards breast cancer cells.

We determined the sub-lethal concentration of different extracts (EENL and MENL) by MTT assay, which was taken as standardized concentration for further treatment. The optimized IC_{50} values of EENL & MENL are found to be 35 µg/ml and 500 µg/ml for 24 h MDA-MB-231 cells (Figure 6).
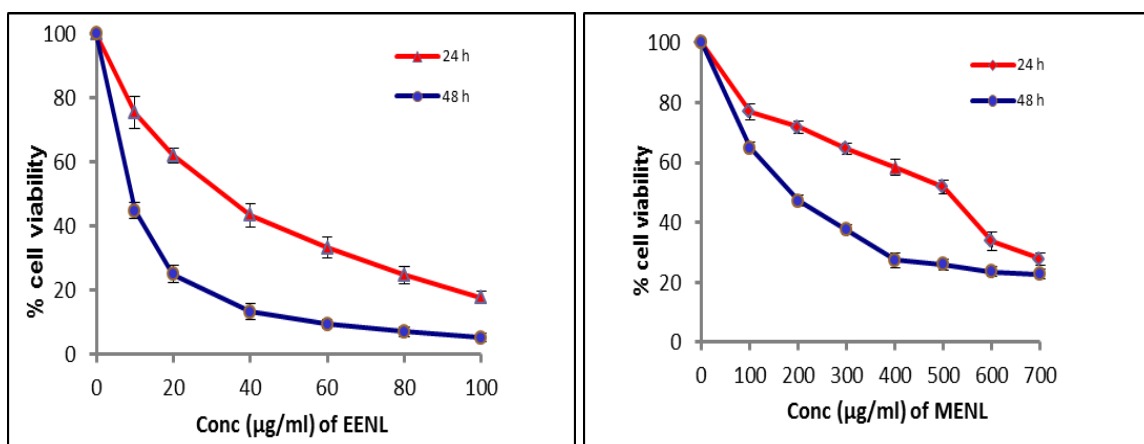

Figure 6.Graphical analysis of cell viability after EENL and MENL treatment

5.3. Detection of apoptosis induced by EENL and MENL 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 EENL treatment is comparatively more than that of MENL in MDA MB 231 breast cancer cells. The untreated controls show no condensed or fragmented nuclei (Figure 7).
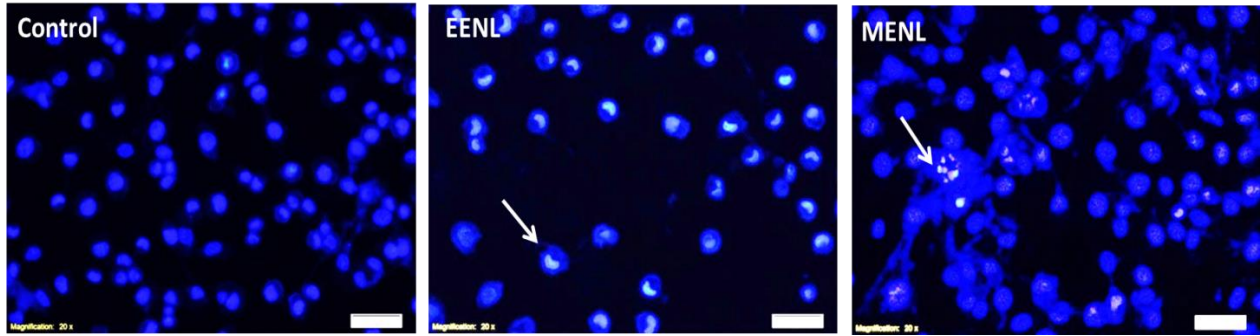
Figure 7. Fluorescence microscopic images representing condensed chromatin after EENL and MENL treatment in MDA MB 231 breast cancer cells.

5.4. Measurement of DNA damage induced by EENL and MENL 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 EENL treatment is higher than MENL (Figure 8). The characteristic comet tail length suggested the amount of DNA damaged.
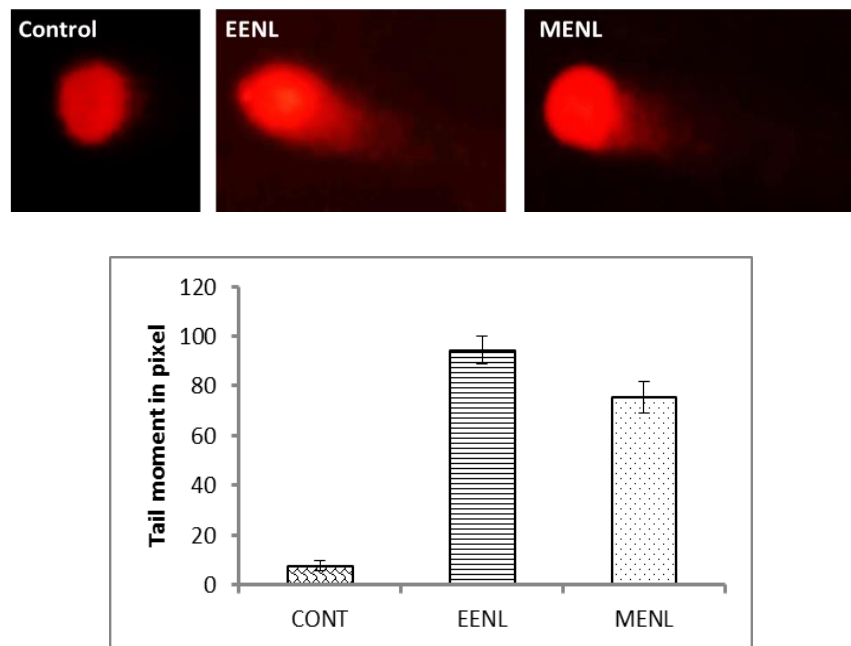

Figure 8. Fluorescence microscopic images of comets showing DNA damage after EENL and MENL treatment and their histogram analysis.

5.5. Determination of anti-migratory activity of EENL and MENL on MDA MB 231 cells by Scratch assay

To determine the migratory property of the MDA MB-231 cells after EENL and MENL 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 neem extracts. Moreover, the wound healing ability of EENL is found to be least among all. The results showed that there was more migration of cells towards the scratched area in MENL treated cells as compared to the EENL treated cells in comparison with the untreated plates taken as control (Figure 9). This clearly demonstrates the efficient anti-migratory property of EENL on breast cancer cells.
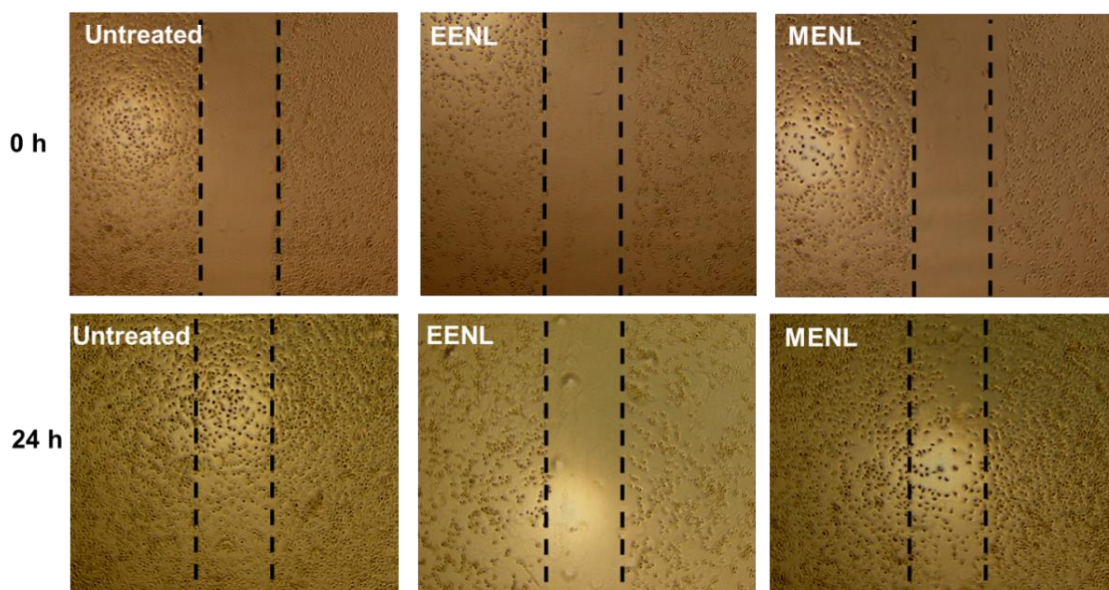

Figure 9. Microscopic images showing changes in the migratory property of MDA MB-231 cells after EENL and MENL treatment.

5.6. Gene Expression Analysis of CDH1, 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 CDH1is increased in MDA-MB 231breast cancer cells after treatment with EENL and MENL. CDH1 is a tumor suppressor gene, which has been reported to have low expression in breast cancer cells (Burstin). Here in our study we also found the lower expression of CDH1 at transcription level. On treatment with EENL and MENL, the CDH1 is demonstrated to be up-regulated in breast cancer cells (Figure

10). The EENL can more effectively restore the expression of CDH1 than MENL, with higher expression of both HDAC1 and HDAC2 and lower expression of Snail. This denotes that EENL and MENL may have HDAC inhibitory potential. The level of β-actin remains same in all cases.
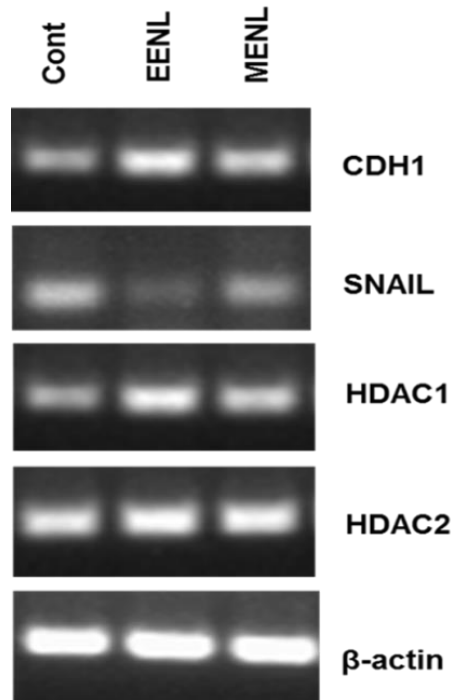

Figure 10. Representative images of semi-q-RT-PCR result depicting expression level of CDH1, Snail, HDAC-1, HDAC-2 and β-actin after EENL and MENL treatment.

5.7. Gene Expression Analysis of CDH1and HDAC-1 by quantitative reverse transcription PCR (q-RT-PCR)

The real time PCR analysis of mRNA level of CDH1 shows that the CDH1 is up-regulated by ten folds in EETL than MENL which is found to be six folds with respect to control (Figure 11). This supports our finding from semi-q-RT-PCR study. The HDAC-1 is also demonstrated to be up-regulated in extract treated cancer cells. From the increase in transcript level of HDAC-1 in treatment groups, it is apparent that the EENL and MENL have HDAC inhibitory activity.
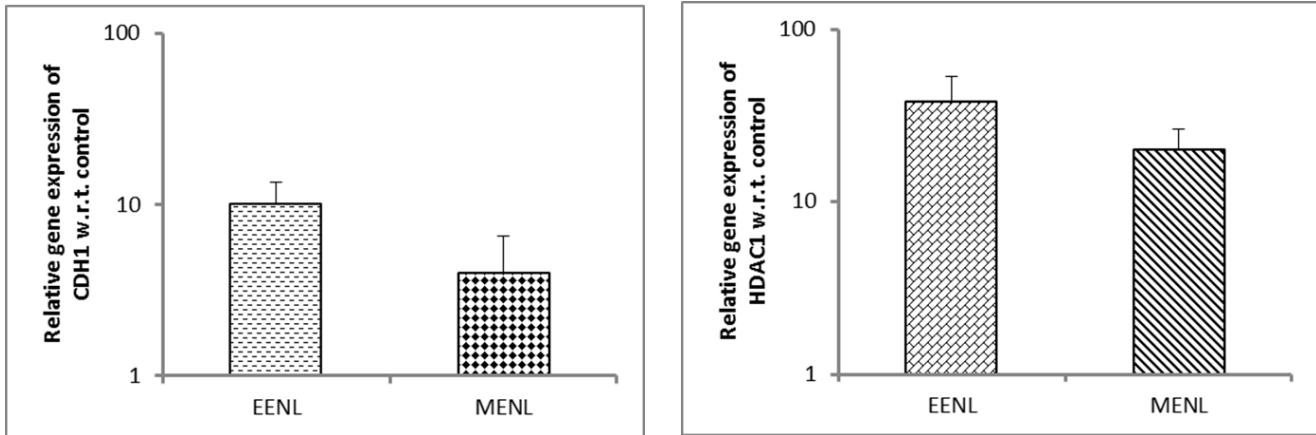
Figure 11.Graphical representation of expression levels of CDH1 & HDAC1 after EENL & MENL treatment.

6. CONCLUSION

From the present investigation, it is apparent that the neem leaf extracts have the anti-cancer potential. This is revealed from their anti-proliferative, apoptotic and anti-migratory activity. Our experiments clearly demonstrate that the EENL exert more anti-cancer effect on breast cancer cells than MENL. It is also observed that the EENL can more effectively restore the expression of CDH1 with simultaneous increase in transcript level of HDAC-1 and HDAC-2, and reduced expression of snail. This indicates the HDAC inhibitory potential of neem leaf extract. Further work is needed in order to reveal the HDAC inhibitory activity of neem leaf extracts.

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