Elucidation of anticancer activity of *Andrographis paniculata* and its role in the expression of E-cadherin and p53 in prostate cancer

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Submitted by

HARSITA BISOYI

ROLL NO – 413LS2026

Under the guidance of

Dr. SAMIR KUMAR PATRA

ASSOCIATE PROFESSOR

DEPARTMENT OF LIFE SCIENCE

NATIONAL INSTITUTE OF TECHNOLOGY

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DECLARATION

I hereby declare that this project report on, "Elucidation of anticancer activity of *Andrographis paniculata* and its role in the expression of E-cadherin and p53 in prostate cancer", 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 (Life Science department), National Institute of Technology, Rourkela.

Date: 10-05-2015 *Harsita Bisoyi*

Place: Rourkela *Roll no-413LS2026*

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NATIONAL INSTITUTE OF TECHNOLOGY
राउरकेला ROURKELA - 769008, ओडिशा ODISHA

RIGHT TO INFORMATION

Dr. Samir Kumar Patra
Associate Professor

Date: 09/May/2015

CERTIFICATE

This is to certify that the thesis entitled ***"Elucidation of anti-cancer activity of Andrographis paniculata and its role in expression of E-Cadherin and p53 in prostate cancer"*** which is being submitted by Miss Harsita Bisoyi, Roll No.413LS2026 for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

DR. SAMIR KUMAR PATRA
Associate Professor,
Department of life sciences,
National institute of Technology, Rourkela
Rourkela-769008, Odisha, India
Phone no: 0661-2462683
Email: samirp@nitrkl.ac.in

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ABSTRACT

Plant-derived compounds act as a prevention within the development of many anti-cancer agents. Natural drugs have less toxicity than the commercially available drugs. So now a days they are widely used for curing many diseases including cancer. Among cancer especially prostrate cancer has been responsible for drastic mortality rate in males. Cancer is mainly studied by genetics and epigenetic modification. Mostly drugs are used to restore the function of tumor supressor genes..In our study, we have carried out an effort towards checking the effect of Kalmegh extracts towards restoring the expression of tumor suppressor gene, E-cadherin (CDH1) and p-53 in PC-3 cell line. HDACs are the epigenetic manipulators which plays an important role in the gene silencing which leads to the initiation of cancer and rapid proliferation.. There are several commercially available HDAC inhibitors but they are highly toxic in nature. So, here we are using the natural drugs from phytochemicals to reduce the toxicity level. As in cancer cells the tumor suppressor genes E-cadherin and P-53 shows the down regulation but while treating with the leaf extract of Andrographis paniculata the genes show up-regulation.

Keywords: E-cadherin, p-53, HDAC, *Andrograhis paniculata*, prostate cancer

1.Introduction

Epigenetics literally means that "above" or "on high of" genetic science. It refers to external modifications to DNA that flip genes "on" or "off." These modifications don't amendment the DNA sequence, but instead, they have an effect on however cells "read" genes. Epigenetic changes alter the physical structure of DNA. One example associate to epigenetic change is DNA methylation — the addition of an alkyl radical, or a "chemical cap," to a part of the DNA molecule, that prevents bound genes from being expressed. Another example is simple protein modification. DNA wraps around Histone protein (Without histones, DNA would be too long to attire within cells). If histones squeeze DNA tightly, the DNA can't be "read" by the cell. Modifications that relax the histones will create the DNA accessible to proteins that "read" genes. Epigenetics is the reason why a vegetative cell appearance totally different from a neuron or a vegetative cell. All the cells contain a similar DNA, however their genes are expressed otherwise (turned "on" or "off"), that creates the various cell sorts. Epigenetic modification may be a regular and natural prevalence, however can even be influenced by many factors as well as age, the environment/lifestyle, and disease state. Epigenetic modification will have additional damaging effects that may end in diseases like cancer. Epigenetics can play a role in the development of some cancers. For instance an epigenetic alteration that silences a tumor suppressor gene such as a gene that keeps the growth of the cell in check could lead to uncontrolled cellular growth. Another example might be an epigenetic alteration that turns off genes that assist repair damaged DNA leading to an increase in DNA damage which in turn increases cancer risk .

Epigenetics refers to the study of mitotically or meiotically transmitted changes in factor perform and/or composition with none changes within the underlying sequence of DNA. It involves no modification within the underlying genetic program whilst; non-genetic factors cause expression of differential phenotypes within the organism's genes. Epigenetics has evolved as a rapidly emerging analysis space within the contemporary affairs. Current studies have exposed that epigenetics plays an important role in cancer biology, mode of action of mobile components, microorganism infections, factor medical aid of vegetative cell lines, transgenic technologies, genomic learning, organic process abnormalities, medical specialty disorders, and X-chromosome inactivation. Epigenetic processes may additionally be concerned in cancer initiation. It's potential that epigenetic amendment might lead on to cancer initiation. As an alternative, changes already elicited among the epigenome might

'prime' cells in such the simplest way on promote cellular transformation upon a future DNA agent event. During this case the epigenetic part of the cancer initiation is elaborately entwined with the genetic part. The involvement of epigenetic amendment in cancer initiation is in fact not reciprocally exclusive thereto having additionally a job in cancer progression.The 3 major epigenetic sign tools are: DNA methylation, RNAi associated silencing or activation and simple protein modification.

India features a various culture of medicative herbs and spices, which has regarding quite 2000 species. Plant ; plant-derived compounds acted as a prevention within the development of many anti-cancer agents.These are used by individuals for the treatment of various ailments for thousands of years. There has been an excellent demand for flavoring medicines in each developed and developing countries as a supply of primary health care as well as ethno medicative activities, high safety margins and lesser prices. Numbers of vital fashionable medication are derived from plants inspite of the appearance of recent or medical care drugs, to be utilized by native folks. Numbers of compounds employed in thought drugs are detected by researchers derived from "ethno medical" plant sources. There are varied medicative herbs that contribute plenty as anti-cancer agents. Certain phytochemicals may be called flavonoids, flavanols, flavanones, antioxidants, isoflavones, catechins, epicatechins, anthocyanins, anthocyanidins, proanthocyanidins, isothiocyanates, carotenoids, alkyl sulfides, polyphenols, phenolic acids, and many other names. The term “phytochemicals” refers to a good form of compounds created by plants, however is especially accustomed describe those compounds that will have an effect on human health. Phytochemicals measure found in plant-based foods like fruits, vegetables, beans, and grains. Scientists have known thousands of phytochemicals, though solely a tiny low fraction are studied closely. A number of the known phytochemicals include beta carotene and alternative carotenoids, water-soluble vitamin (vitamin C), folic acid, and E.Phytochemicals measure promoted for the anticipation and treatment of the many health conditions, together with cancer, cardiovascular disease, diabetes, and high pressure level. There's some proof that sure phytochemicals might facilitate forestall the formation of potential carcinogens (substances that cause cancer), obstruct the action of carcinogens on their target organs or tissue, or act on cells to repress cancer development.

2. Review of literature

Epigenetics is that the study of the stable inheritance of constitution while not sterilization the genotype manifested by changes in organic phenomenon [1]. Epigenetic changes in organism biology are best ascertained throughout cellular differentiation [1, 2, and 3]. This process is regulated by activating some genes whereas silencing several others by advanced processes of epigenetic laws. It involves a novel modification of deoxyribonucleic acid at the pyrimidine 5-carbon position (hereafter, DNA-methylation), and various modifications in histones for the activation or repression of sure genes [3]. Varied proteins related to the chromatin granule folding and dynamics is also activated or silenced . This implies that each cell in our body has an equivalent programming, however totally different cell varieties are victimization different parts. Most epigenetic changes that are committed the chromatin granule modifications associated with organic phenomenon solely occur among the course of 1 individual organism's time period, however some epigenetic changes are heritable from one generation to consequent. Epigenetic processes embrace paramutation (the results of ancestral changes in organic phenomenon that occur upon interaction between alleles), factor bookmarking (a mechanism of epigenetic memory that functions to transmit through cellular division the pattern of active genes and/or genes which will be activated to girl cells), imprinting, factor silencing, sex chromosome inactivation, result, reprogramming, transfection (an epigenetic development that results from associate interaction between associate gene on one body and therefore the corresponding gene on the homologous chromosome), maternal effects, the progress of carcinogenesis, and lots of effects of teratogens, regulation of simple protein modifications and heterochromatin formation, and technical limitations touching parthenogenesis and biological research [1,2, 3 and4].

2.1 Andrographis paniculata

Andrographis paniculata is additionally referred to as “King of Bitters”. This plant belongs to the family of Acanthancea.It is mostly found in the hills of tropical india, from uttar Pradesh to kerala and also in Bangladesh, Pakistan and all south east asia countries. Andrographis is also used for a wide assortment of other conditions. It is mostly used for digestive complaints including diarrhea, constipation, intestinal gas, colic, and stomach pain; for liver conditions including an enlarged liver, jaundice, and liver damage due to medications; for infections including leprosy, pneumonia, tuberculosis, gonorrhea, malaria, cholera, leptospirosis, rabies,

sinusitis, and HIV/AIDS; and for skin conditions including wounds, ulcers and itchiness. However some people use andrographis for sore throat, coughs, swollen tonsils, bronchitis, and allergies. It is also used for "hardening of the arteries" (atherosclerosis), and prevention of heart disease and diabetes. Other uses include treatment of snake and insect bites, loss of appetite, kidney problems (pyelonephritis), hemorrhoids, and an inherited condition called familial Mediterranean fever.

2.2 Medicinal properties of *Andrographis paniculata*

Andrographis paniculata has several medicinal properties like Antibacterial, antifungal,Antiviral, antipyretic, adaptogenic, anti-inflammatory,Improves immunity, Liver protecting, Carminative, diuretic, gastric and liver tonic, Choleretic, hypoglycemic, hypocholesterolemic, Bitter tonic, Blood purifying. Traditional Uses of Andrographis paniculata is blood purification and it is also used for treating leprosy, gonorrhoea, scabies, boils, skin eruptions etc due to its blood purification properties. Its decoction prevents and treats liver diseases and fever. The brew is also used in sluggish liver, indigestion, bowel irregularity, anorexia, abdominal gas and diarrhoea. Fresh leaf juice is given to prevent excessive bleeding during periods. Whole plant is used in general ineffectiveness, during restoration after fevers. The plant is also useful for preventing and treating respiratory illness. Brew of leaves is given in general weakness and indigestion. Since history, its been employed in Asia to treat completely different quite infections. Its found within the Indian collection and it's incorporated in a minimum of twenty six principal ayurvedic formulas.The most active ingredients of this plant area unit andrographolides, that because of their chemical structure, belong to the diterpene lactones cluster. The largest quantity of andrographolides is found within the leaves so giving the plant a bitter style.

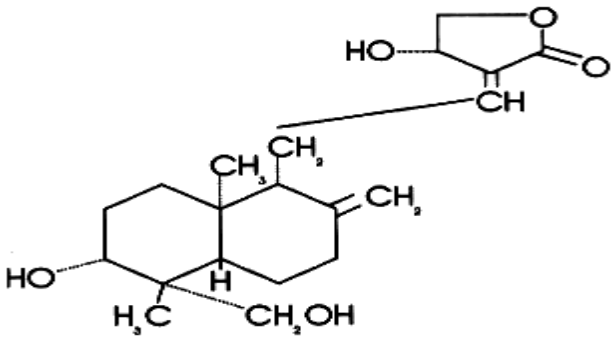


Fig.1(A)Andrographolide ,(B) Andrographis paniculata

The genus *Andrographis* consists of 28 species of small annual shrubs essentially distributed in tropical Asia. Only a few species are medicinal, of which *Andrographis paniculata* is the most popular. *Andrographis paniculata* grows erect to a height of 30– 110 cm in moist, shady places. It is also known as *Bhui-neem*, meaning "neem of the ground", since the plant, though being a small annual herb, has a similar strong bitter taste as that of the large Neem tree (*Azadirachta indica*). The herb is also available in northern parts of India, Java, Malaysia, Indonesia, West Indies and whereas in America where it is probably an introduced species. (Abhishek *et.al*.2010) The species also occurs in Hong Kong, Thailand, Brunei, and Singapore, etc

Other constituents: The leaves additionally contain different diterpene lactones, such as: neoandrographolide, andrographiside, 14-doexy-11, 12-didehydro andrographolide, etc., flavonoinds viz., oroxylin, wogonin, andrographidines A, B, C, D, E, F. Therapeutically important active principle of kalmegh found in aerial parts is andrographolide. It is colorless, crystalline, bitter in taste and known as diterpenelactone.Other reported compounds include 14-deoxy-11- oxoandrographolide ;14-deoxy-11, 12- didehydroandrographolide/andro-graphlide D ; 14-deoxy andrographolide ; non-bitter compound neoandrographolide; homoandrographolide ; andrographosterol ;andrographane; andrographone ; andrographosterin; andro-grapanin; α-sitosterol; stigmasterol; apigenin- 7, 4'-di-O-methyl ether; 5-hydroxy 7, 8, 2', 3'-tetramethoxy flavone ; monohydroxytrimethyl flavones; andrographin; dihydroxy-di-methoxyflavone; panicolingrapholide; 12S-hydroxyandrographolide and andro-graphatoside from the aerial parts of plant. These compounds showed inhibitory activity against several bacterial and fungal strains13. Four

xanthones 1,8-di-hydroxy-3,7-dimethoxy-xanthone; 4,8-di-hydroxy-2,7-dimethoxy-xanthone;-1,2-dihydroxy-6,8-dimethoxy-xanthone and 3,7,8 trimethoxy-1-hydroxyxanthone are reported from the roots. Structures of some major compounds are given in figure.

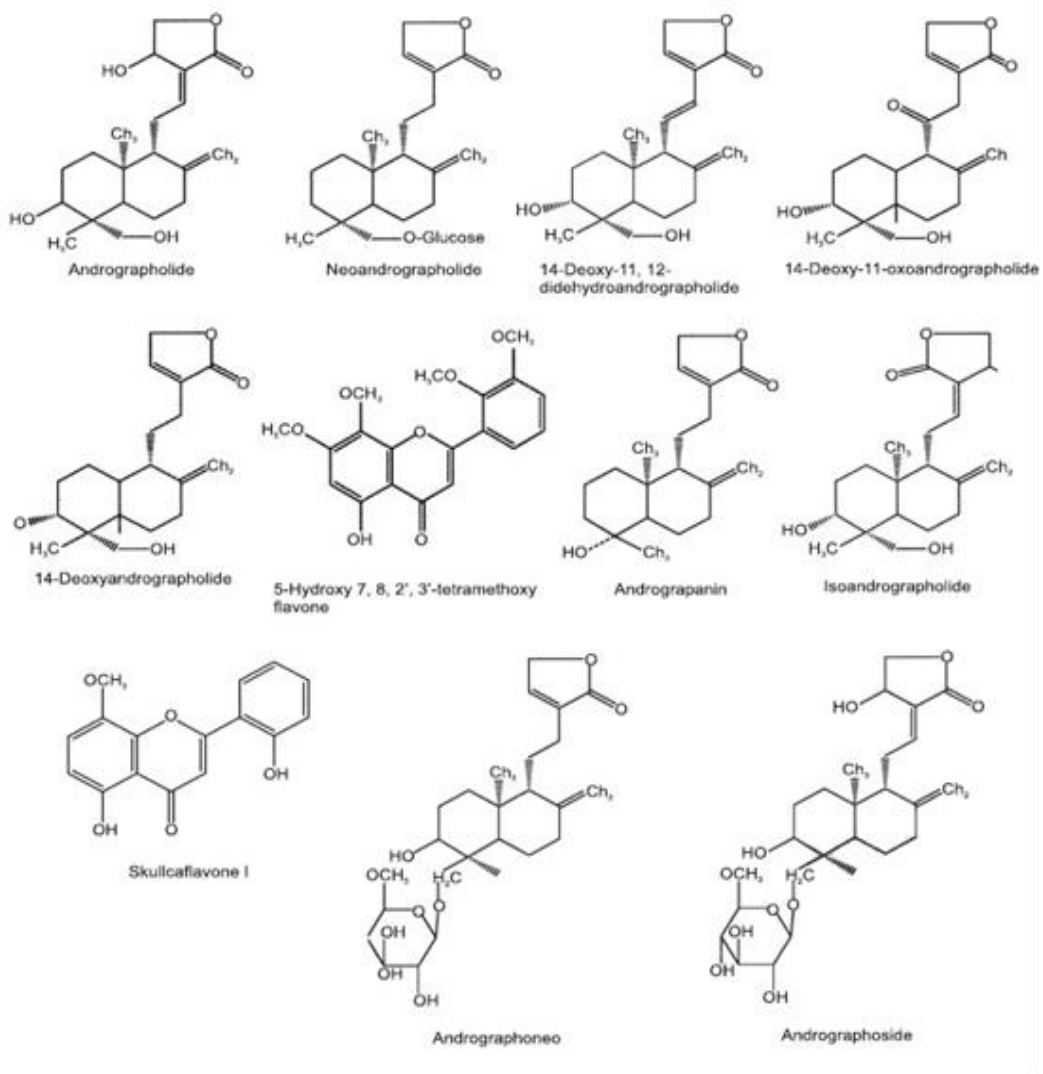


Fig.2 Molecular structure of some bioactive components of *Andrographis paniculata*

2.3 Anticancer property of *Andrographis paniculata*

As its been noted, malign cells don't reply to the_signals due to which their growths were restricted. Once cells mature properly, they outline their specialization so as to perform their individual functions. The malignant neoplastic sickness cells are people who don't grown-up, and are kind of like the embryonal cells. The terpenes from the plant caused the differentiation in malignant neoplastic disease cells, creating them to be a lot of mature, and so, less aggressive. The extract of *Andrographis paniculata*, still as inflicting the differentiation in cancerous cells, is additionally cytotoxic to those cells. This was incontestible once it acted against the epidermidal malignant neoplastic disease cells of the skin still as some leukemia cells. In 1977, a clinical analysis was done on sixty patients that were affected with Skin basalioma, forty one of that had metastasis. In keeping with "The Journal of Chinese Medicine", twelve patients, UN agency simply received extracts of *Andrographispaniculata*, recovered and in 1996; some tests showed that the extract blocked, in an exceedingly very secure approach, the event of adenocarcinoma, carcinoma and Non Hodgkin's cancer. The extract most likely inhibits the synthesis of polymer within the malignant neoplastic disease cells. *Andrographis paniculata* have side effects so it should not to be used during pregnancy or during the lactation period.

2.4 Antioxidant and anti inflammatory activities of *Andrographispaniculata*

The antioxidant and anti inflammtory activity of *Andrographis paniculata* has been reported by various investigators. *According to* Das et al, nicotine-induced inhibition of mitochondrial electron chain complexes and also the resultant increase in nitrous oxide (NO) in numerous elements of rats' brains was prevented by simultaneous treatment with the water and grain alcohol extracts of *Andrographis paniculata* or andrographolide; the water extract exhibited greater inhibitor activity than the grain alcohol extract. Phytochemical analysis showed higher flavonoid however lower phenol contents in water extract than in grain alcohol extract. Verma and Vinayak compared the inhibitor effects of the liquid extract on liver defense systems in lymphoma-bearing AKR mice. "e -aqueous extract considerably augmented the activities of enzyme, enzyme, and glutathione-S-transferase enzymes and reduced lactase dehydrogenase activity [5].A methanol extract inhibited formation of reactive oxygen species (ROS) in vitro and fully inhibited Carrageenan-induced inflammation [6].Andrographolide pretreatment considerably attenuates accumulation of phorbol-12-myristate 13-acetate (PMA)-induced formation of ROS and N-formyl-methionyl-leucylphenylalanine (fMLP)-induced adhesion of rat neutrophils. However, PMA-induced formation of ROS and fMLP-induced adhesion and transmigration of peripheral human neutrophils was solely part

reversed by andrographolide. "is study suggests that avoidance of ROS production was partially mediated by the direct activation of supermolecule kinase C by PMA and partially mediate by down-regulation of surface Mac-1 expression, an essential integrin for neutrophils adhesion and reincarnation. Excessive amounts of NO and autacoid E2 (PGE2), expression of inducible isoforms of nitric oxide synthase and Cox-2 (COX-2) from activated macrophages, play a significant role in inflammatory processes. Lipopolysaccharide (LPS) stimulates and promotes secretion of pro-inflammatory cytokines from macrophages and causes induction of iNOS, leading to augmented production of NO. Incubation of macrophages with alcohol extract, andrographolide, and NO production in a very concentration dependent manner. Andrographolide-induced reduction of iNOS activity could also be attributable to reduced expression of iNOS supermolecule. Andrographolide also absolutely restores the maximal contracted response of aorta to adrenergic after incubation with LPS, and attenuates the fall in mean blood pressure of an synthetized rats attributable to LPS. Distinct andrographolide, neoandrographolide was also effective ex vivo in suppressing NO production once macrophages were collected after oral administration of neoandrographolide and subjected to LPS stimulation. Andrographolide repressed LPS-induced increase in neoplasm gangrene factor-alpha (TNF) and granulocyte-macrophage colony stimulating issue. Neoandrographolide conjointly inhibits PGE2 synthesis and TNF-in LPS stimulated macrophages and its oral administration to mice considerably suppresses dimethylbenzene induced ear hydrous and acetic acid-induced tube porosity.

2.5 DNA methylation

DNA methylation is one amongst the foremost intensely studied epigenetic modifications in mammals. In natural cells, it assures the correct regulation of organic event and steady cistron silencing. DNA methylation is related to simple protein modifications and also the interaction of those epigenetic modifications is crucial to manage the functioning of the ordering by dynamic body substance design. The valency addition of an alkyl group happens usually in C inside CpG dinucleotides that area unit focused in giant clusters known as CpG islands. It is unremarkably famed that inactivation of bound tumor-suppressor genes happens as a consequence of hypermethylation inside the promoter regions and a varied studies have incontestable a broad vary of genes silenced by DNA methylation in numerous cancer varieties. On the opposite hand, hypomethylation, causing genomic instability, additionally contributes to cell transformation. Excluding DNA methylation alterations in promoter regions and repetitive DNA sequences, this development is associated additionally with

regulation of expression of noncoding RNAs like microRNAs which will play role in tumour suppression. DNA methylation appears to be promising in purported change of site use in patients and hypermethylated promoters could function biomarkers. The importance of DNA methylation alterations in tumorigenesis.5-methylcytosine-based DNA methylation happens by the valency addition of a methyl (CH3) group at the 5-carbon of the pyrimidine ring leading to 5-methylcytosine (5-mC). These alkyl group teams project into the key groove of DNA and inhibit transcription. In human DNA, 5-methylcytosine is found in or so 1.5% of genomic DNA [7]. In somatic cells, 5-mC happens nearly solely within the context of paired symmetrical methylation a CpG island, during which a pyrimidine ester is found next to a guanidine ester. Associate exception to the current is seen in embryonic stem (ES) cells, wherever a considerable quantity of 5-mC is additionally ascertained in non-CpG contexts. Within the bulk of genomic DNA, most CpG sites area unit greatly alkyl whereas CpG islands (sites of CpG clusters) in germ-line tissues and situated close to promoters of traditional bodily cells, stay unmethylated, therefore permitting organic phenomenon to occur. Once a CpG island within the promoter region of a factor is alkyl, expression of the factor is inhibited (it is turned off). The addition of alkyl group teams is controlled at many completely different levels in cells and is administered by a family of enzymes known as DNA methyltransferases (DNMTs). 3 DNMTs (DNMT1, DNMT3a and DNMT3b) area unit needed for institution and maintenance of DNA methylation patterns. Two further enzymes (DNMT2 and DNMT3L) can also have a lot of specialised however connected functions. DNMT1 seems to be liable for the upkeep of established patterns of DNA methylation, whereas DNMT3a and 3b appear to mediate association of DNA methylation patterns. Unhealthy cells like cancer cells is also completely different therein DNMT1 alone is not liable for maintaining traditional factor hypermethylation (an increase in international DNA methylation) and each DNMTs one and 3b could collaborate for this control.

Equally necessary and paired with DNA methylation is DNA demethylation, the removal of a alkyl radical. The demethylation method is necessary for epigenetic reprogramming of genes and is additionally directly concerned in several important illness mechanisms like tumour progression. Demethylation of DNA will either be passive or active, or a mix of each. Flaccid DNA demethylation sometimes takes place on recently synthesized DNA strands via DNMT1 throughout replication rounds. Active DNA demethylation primarily happens by the exclusion of 5-methylcytosine via the sequent modification of pyrimidine bases that are born-again by day enzyme-mediated reaction. The quantification of 5-mC content or global

methylation in unhealthy or environmentally compact cells may offer helpful info for detection and analysis of illness. Moreover, the detection of the DNA demethylation intermediate 5-fC in numerous tissues and cells can also be used as a marker to point active DNA demethylation.

This modification of the DNA alters the genes expressed in cells when they divide and differentiate from embryonic stem cells into cells of a particular tissue. The change in gene expression is constant and the cell does not regress back to a stem cell or another type of cell. The process of DNA methylation is blocked when a zygote is being formed but is restored as cell division occurs during development.

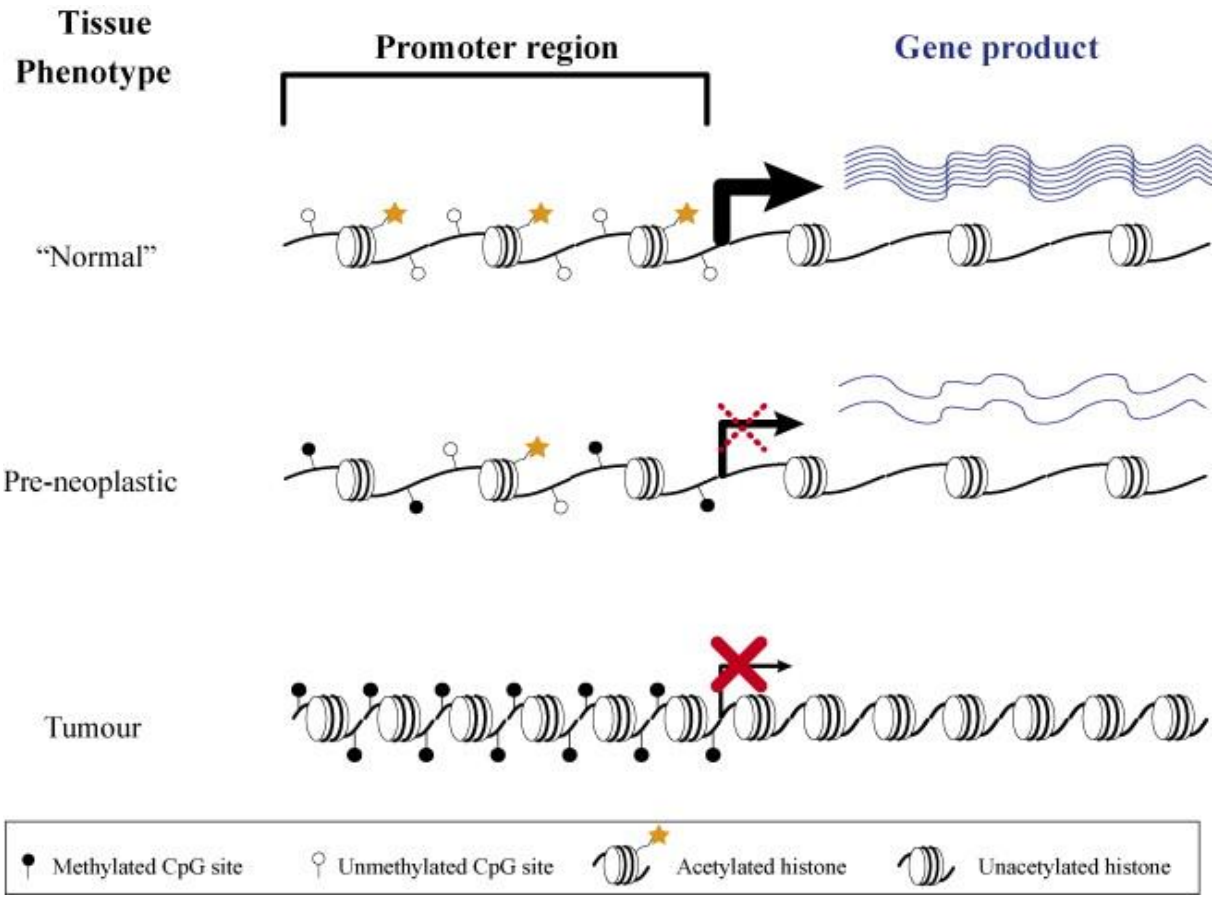


Fig.3.. *MEpigenetic gene silencing during tumourigenesis proceeds gradually.*

When a gene is expressed in normal tissue the CpGisland in the promoter region is typically unmethylated and histones at local chromatin are acetylated. As tissue progresses from normal to pre-neoplastic and tumour state, gradual deacetylation of histone tails and spread

of cytosine methylation at CpGisland is accompanied by a progressive loss of gene expression.(Epigenetic interplay between histone modifications and DNA methylation in gene silencing, Thomas Vaissière, Carla Sawan, Zdenko Herceg)

2.6 Histone modification

Histone proteins, that package DNA into chromatin, are subject to many post translational modifications that ultimately have an effect on cistron regulation. These post-translational valency modifications (PTMs) occur within the ε-N-terminal tails of simple protein proteins that regulate the transcriptional state of the ordering via body substance structure leading to differential expression of genes. The assorted PTMs include- acylation, methylation, sumoylation, phosphorylation, ADP ribosylation, citrullination, aminoalkanoic acid changeover and ubiquitination. These alter the interaction of desoxyribonucleic acid and nuclear proteins with histones. Histones are acetylated by histone acetyl transferases (HAT) &deacetylated by histone deacetyltransferases (HDACs). Simple protein modification plays a vital role in cistron transcription, and its role in carcinogenesis has been a subject of nice interest within the past few years.

2.7 Histone acetylation

Acetylation of the lysine residues at the N terminus of histone proteins removes positive charges, thereby reducing the affinity between histones and DNA. This makes RNA polymerase and transcription factors easier to access the promoter region. Therefore, in most cases, histone acetylation enhances transcription while histone deacetylation represses transcription.

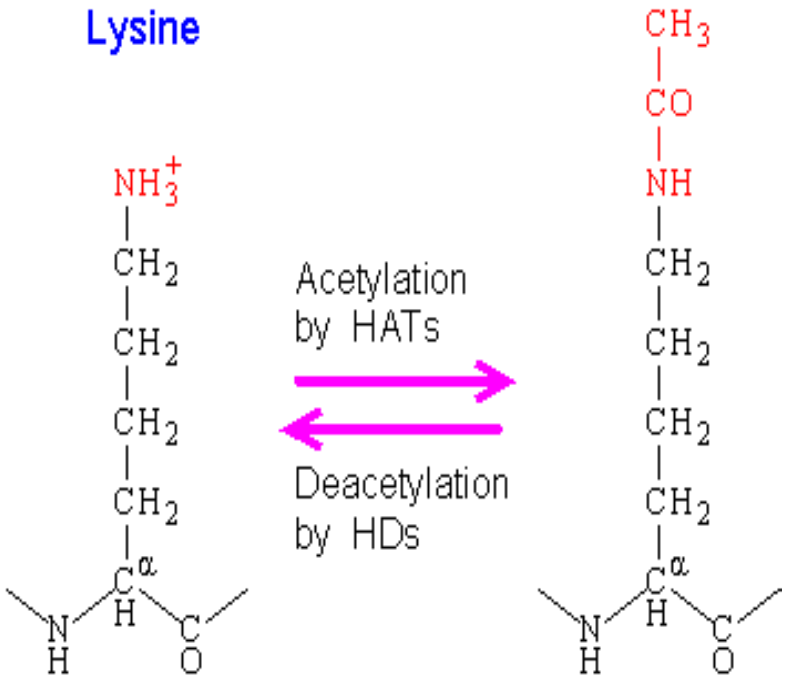


Fig.4 Acetylation and deacetylation of the lysine residue.

Histone acetylation is catalyzed by histone acetyltransferases (HATs) and histone deacetylation is catalyzed by histone deacetylases (denoted by HDs or HDACs). Several different forms of HATs and HDs have been identified. Among them, CBP/p300 is probably the most important, since it can interact with numerous transcription regulators.

Subsequently, acetylated core histones were shown to preferentially go together with transcriptionally active chromatin granule [7]. Acylation happens at essential amino acid residues on the amino-terminal tails of the histones, thereby neutralizing the electric charge of the simple protein tails and decreasing their affinity for deoxyribonucleic acid [8]. As a consequence, simple protein acylation alters nucleosomal conformation [9], which may increase the accessibility of transcriptional restrictive proteins to chromatin granule templates [10]. Taken along, these observations urged however simple protein acylation might end in hyperbolic transcriptional activity in vivo. However, there was basically no data concerning

the cause and impact relationship between simple protein acylation and transcriptional activity or concerning the underlying molecular mechanisms

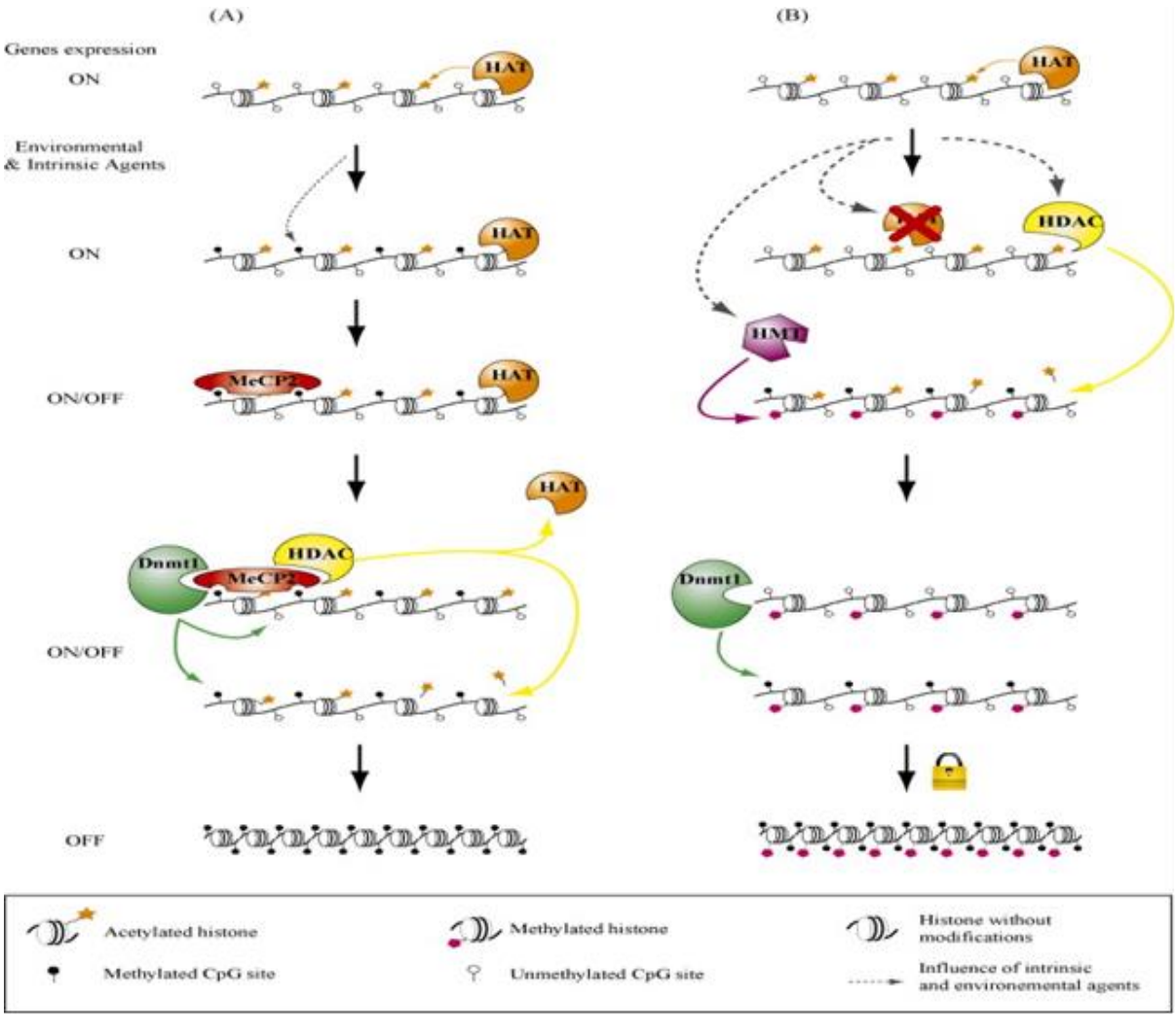


Fig.5. Models of the hierarchical order of events during epigenetic gene silencing.

(A) Environmental and intrinsic signals may trigger a partial methylation of the DNA at CpG sites. Methylated cytosines are bound by methyl-binding proteins (MePC2) that recruit other histone deacetylases (HDAC) resulting in deacetylation of histones. The presence of methyl-binding proteins and deacetylated histones may reinforce and amplify silencing signal by further recruitment of DNA methyltransferase (e.g. Dnmt1). The outcome of this is induction of an inactive state. (B) Histone acetylation states may induce DNA methylation during gene silencing. In this model, induction of hypoacetylation state, due to the loss of equilibrium

between HAT and HDAC activities (likely caused by adverse environmental or endogenous effects), is the primary event in epigenetic gene silencing. [Epigenetic interplay between histone modifications and DNA methylation in gene silencing, Thomas Vaissière, Carla Sawan, Zdenko Herceg]

The balance of simple protein acetylation associated deacetylation is an epigenetic layer with an important role within the regulation of organic phenomenon. Histone protein acylation elicited histone protein acetyl group transferases (HATs) is related to factor transcription, whereas simple protein hypoacetylation elicited by histone protein deacetylase (HDAC) activity is related to factor silencing. Altered expression and mutations of genes that encode HDACs are joined to growth development since they each induce the aberrant transcription of key genes control necessary cellular functions like cell proliferation, cell-cycle regulation and programmed cell death. Thus, HDACs area unit among the foremost promising therapeutic targets for cancer treatment, and that they have galvanized researchers to review and develop HDAC inhibitors.

The presence of acetylated essential amino acid in histone protein tails is related to the relaxed chromatin granule state and gene-transcription activation; whereas the deacetylation of essential amino acid residues is associated with the condensed chromatin granule state and transcriptional factor silencing [11]. Histone protein deacetylation will increase ionic interactions between the charged histones and charged DNA that yields a lot of compact chromatin granule structure and represses factor transcription by limiting the accessibility of the transcription machinery. Additionally, histone protein acylation has been related to alternative order functions like chromatin granule assembly, DNA repair, and recombination [12, 13].

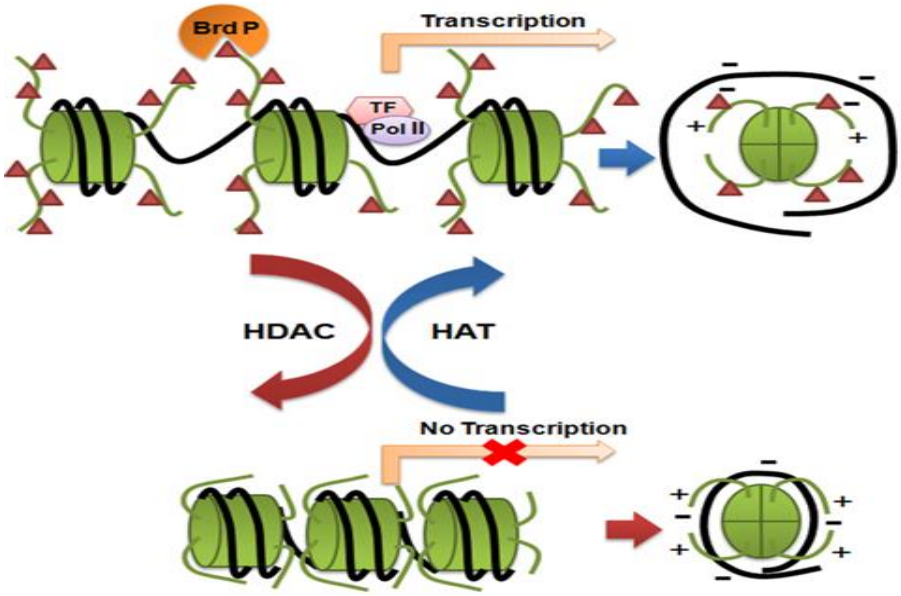


Fig.6 : Histone acetylation and deacetylation (Parbin et al, 2014)

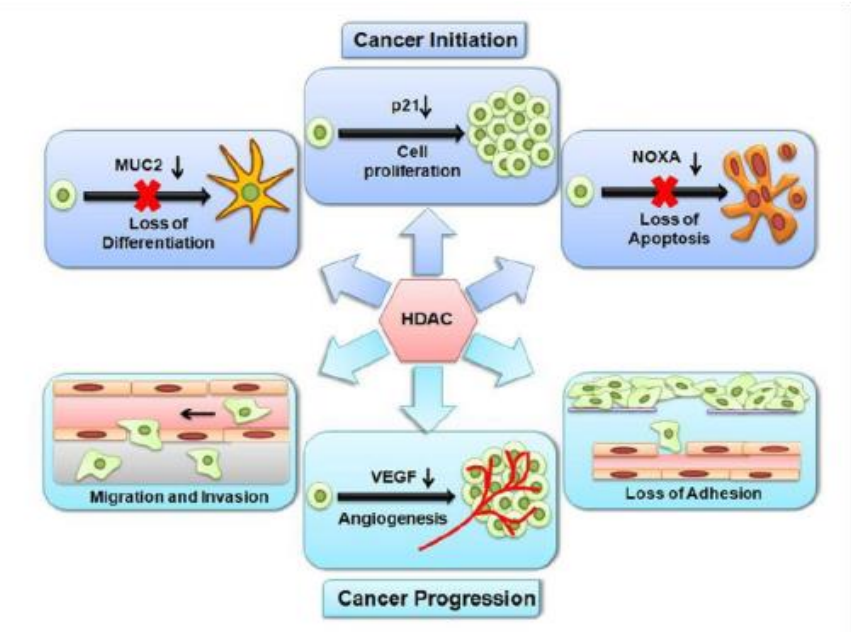


Fig.7.Role of HDACs in Cancer initiation and proliferation (Parbin et al, 2014)

2.8 E-Cadherin

The management of cellular adhesion and motility is one among the crucial mechanisms to blame for growth initiation and progression. The genes concerned also are contributors to malignancy beside genes to blame for cell proliferation and survival. Tumor cells adhere to one another less passionately than do other non-tumor cells. Cadherin the cell-cell adhesion receptors are regulators of tissue architecture during development and tissue homeostasis provided molecular candidates to link cell-cell adhesion, morphogenesis and cancer.As sort one membrane glycoproteins, they function as dynamic membrane-spanning molecule complexes. Metastasis could be a complicated and multi-step method within the progression of malignant cancer [14]. Cell migration ends up in the spreading of cancer that is the leading reason behind cancer-related mortalities. Within the method of cancer progression, bound cell adhesion molecules (CAMs) play a crucial role within the development of repeated, invasive and distant metastasis [15]. A loss or reduction within the expression of CAMs, together with cadherins, facilitates the detachment of single cancer cells from the growth bulk [16, 17]. one in every of the key molecules essential for cell-to-cell adhesion is E-cadherin, a membrane conjugated protein situated at cell adherent junctions [18,19]. E-cadherin aids the assembly of animal tissue cells and maintains the quiescence of cells inside sheets by forming adherent junctions with adjacent animal tissue cells [20]. Variety of studies hasincontestable that raised expression of E-cadherin is in a position to inhibit invasion and metastasis, whereas a reduced expression potentiates these phenotypes [20–23]. So as for animal tissue cells to become cancer cells, activation of the epithelial-mesenchymal transition (EMT) is needed (24). EMT causes the vegetative cell layers to lose polarity and cell-cell contacts. It thus triggers the transforming of the cellular skeleton [25, 26]. Upregulation of E-cadherin is concerned by the activation of EMT [27], and E-cadherin is thought to be a main indicator of epithelial/mesenchymal composition change [28]. The metastasis-associated cistron one (MTA1) was originally known by the differential screening of a DNA screening library victimisation extremely pathologic process duct gland glandular cancer cell lines [29]. MTA1 seems to move with, or might even be a member of, the simple protein deacetylase (HDAC) complicated, and acts as a co-activator of this complicated [30]. Studies have incontestable that MTA1 overexpression is related to the adhesion, invasion and metastasis of bound cancer

cells [31-33] and with a better growth grade, the event, microvascular invasion and poor prognosis in a very range of malignant cancer varieties [34]. Through repression of the steroid receptor α (ERα), hypoxia-inducible factor-1α (HIF-1α) and p53 macromolecule, MTA1 converts cancer cells into an additional aggressive composition [35]. Moreover, MTA1 has been known to see EMT phenotypes chiefly through downregulating the expression of E-cadherin that results in EMT [36, 37]. E-cadherin will be upregulated victimisation MTA1 tiny busy RNA (siRNA) in malignant melanoma cells that was conjointly confirmed in our previous study in cervical cancer cells [38, 39]. MTA1 and E-cadherin area unit concerned within the EMT method [40] since the loss of E-cadherin expression has been incontestable to extend cancer metastasis progresses [41] and growth cells with raised expression of MTA1 indicate additional invasive phenotypes. Feedback regulation is crucial for cells to see their fate and maintain operate throughout cistron regulation.

2.9 Role of E-cadherin on tumor suppression:

The onset of quality needs a relaxation of static simple protein structures on order to make pliable membrane protrusions. Rigid simple protein fibers square measure disassembled upon dorsal circular ruffle formation going a fine simple protein network from that cell wall protrusions (lamellipodia) fair measure fashioned. Formation of a stable, polarized animal tissue needs tight cell-cell and cell-matrix connections. E-cadherin is that the major part of animal tissue adherens junctions (AJ), that mediate animate thing adhesions. EMT could be a cellular method that permits a polarized somatic cell to assume a mesenchymal composition and therefore the start during this method is that the loss of E-cadherin and therefore the collapse of cell-cell communications. The downregulation of E-cadherin not solely ends up in a mechanical disruption of adherene junction(AJ), however it additionally liberates proteins from the protoplasm cell adhesion complicated, that exert ambivalent functions reckoning on the cellular localization. EMT has been known as a crucial mechanism in organization of cells inside the developing embryo, forming mesenchymal cells following tissue injury, and initiating the invasive and pathological process nature of animal tissue cancers. EMT and mesenchymal to animal tissue transition changes (MET) play crucial roles within the development of the prostate, the seminal vesicles and excretory organ. Many studies have established that EMT facilitates malignant transformation of cells and plays a crucial role in cells' ability to metastasise. Mesenchymal cells give support associated structure to animal tissue cells through the assembly of a living thing matrix (ECM) and square measure

extremely mobile and invasive, in contrast to their animal tissue counterparts. But, it's believed that insult to cells re-activates these organic process mechanisms out of context in adult cells to trigger oncogenesis. However, EMT isn't the sole example of somatic cell physical property. Another method entails the movement of animal tissue cells in a very physically and functionally collected cluster, termed collective migration. It's doable that collective migration falls on a spectrum somewhere between EMT and MET. This might be within the context of cancer that lacks the orderly and coordinated induction of EMT. In EMT throughout development, E-cadherin is replaced by N-cadherin and Vimentin and fibronectin replace cytokeratins. These changes additionally occur in duct gland tumors undergoing EMT. EMT permits a polarized epithelial cell that normally interacts with a basement membrane via its basal surface to undergo multiple changes that allows it to assume a mesenchymal phenotype. This mesenchymal cell has an elevated resistance to apoptosis, an amplified production of ECM components and has the capacity to migrate and invade.Some metastatic cancer cells have shown the ability to re-express E-cadherin after migration and colonization morphological profiling of EMT consists of several cellular markers. For instance, mesenchymal markers that are increased in EMT include: N-cadherin, Vimentin, Fibronectin, Snail, Slug, Twist, FoxC2, and MMP's-2, 3, 9. subsequently, epithelial markers that are decreased in EMT include: E-cadherin, B-catenin, Cytokeratin, and Desmoplakin. Snail and Twist are transcription factors which act as repressors of E-cadherin.TGF-β superfamily members induce Snail1 and Snail2. Moreover microRNAs recently emerged as potent regulators of EMT-MET inter-conversions, with their abilities to target multiple components involved in epithelial integrity or mesenchymal traits, thus impacting tumor progression, metastasis and colonization.

Cadherin expression is regulated in each physiological and pathological process, like embryonic ontogenesis and tumorigenesis. Tissue and organ formation is regulated in a very spatio-temporal manner involving cell proliferation, death, cell-cell adhesion, cell-substrate adhesion, polarization, and migration. One example of this extremely regulated method is blastodermic vesicle differentiation. E-Cadherin has a vital operate within the formation of the blastodermic vesicle throughout mouse embryonic development. Another example of the traditional physiological processes related to E-cadherin regulation is that the formation of fluid area in development of murine tube. During this embryonic method, E-cadherin is downregulated on the lateral membranes of cancellated plate. This down-regulation permits the method of fluid area gap within the organ of Cortex. Wound healing may be a third

example wherever a physiological event involves regulation of E-cadherin expression. Injury of the somatic cell layer within the skin signals the discharge of cytokines and different factors, like cuticular protein (EGF). These signals scale back cell adhesion and stimulate cell motility, providing wound repair. Beyond wound repair, cell adhesion is upregulated to revive the animal tissue layer to its traditional state. Therefore, E-cadherin must be extremely regulated within the on top of traditional physiological processes. Conversely, aberrant growth and differentiation result once E-cadherin isn't tightly regulated, like in cancer. Association of E-cadherin with neighboring cells acts to inhibit cell quality and to take care of traditional somatic cell makeup. Tumorigenesis is associate in nursing example of an organic process that involves E-cadherin regulation. The loss or down-regulation of E-cadherin expression has been represented in many tumors. E-cadherin acts as associate in nursing matter of the invasive and pathologic process makeup of cancer cells. Since tumour invasion and metastasis may be a multistep method, E-caderin could play a major role in control invasion and metastasis at the initial steps within the method by promoting homotypic cell-cell adhesion. Various mechanisms moving E-cadheirn-catenin advanced formation are related to a discount in cell adhesion. Whereas point mutation is answerable for inactivating E-cadherin-mediated cell adhesion in some breast cancers and stomachic adenocarcinomas, the precise mechanism of E-cadherin down-regulation in different extremely invasive tumors continues to be underneath investigation. Mechanisms that regulate homophilic cell adhesion embody reduction or loss of E-cadherin expression, reduced transcription of genes secret writing catenin proteins, distribution of E-cadherin to completely different sites among the cell, shedding of E-cadherin, cleavage of E-cadherin, and competition of proteins for binding sites on E-cadherin.

The proximal E-cadherin promoter contains multiple restrictive components together with 3 E-boxes, one CCAAT box, and a GC-rich part. Therefore, the E-cadherin promoter contains quite one website for transcription factors to bind and regulate cistron transcription in cancers. These factors embody AP-2, SNAIL,SLUG,dEF1/ZEB-1, SIP1/ZEB-2, E12/E47 and LEF/TCF whereas the metastatic tumor cistron and c-mycprotooncogene product transactivate the E-cadherin promoter in animal tissue cells through interaction with AP-2 transcription factors, transcription of E-cadherin is down-regulated by overexpression of ErbB2. SNAIL and SLUG transcription factors are shown to repress E-cadherin expression in carcinoma cell lines via all 3 E-box components, however notably, via EboxA and EboxC, situated within the proximal E-cadherin promoter. Moreover, SLUG may be a supposed in

vivo agent of E-cadherin in carcinoma. The E-cadherin promoter conjointly contains binding sites for the humour attention issue one (LEF1)-β-catenin transcription issue advanced; this complex down-regulates E-cadherin expression Overexpression of integrin-linked macromolecule enzyme (p59ilk) stimulates LEF1-β-catenin communication and causes downregulation of E-cadherin expression with a concomitant decrease in cell adhesion. One ester polymorphism within the E-cadherin promoter has conjointly been related to a better risk of glandular cancer in bound ethnic populations with a attainable role in transcriptional regulation of E-cadherin organic phenomenon in these people

2.10 p53:

This gene encodes a tumor suppressor protein containing transcriptional activation, DNA binding, and oligomerization domains. The encoded protein responds to diverse cellular stresses to regulate expression of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. Mutations in this gene are associated with a variety of human cancers, including hereditary cancers such as Li-Fraumeni syndrome. Alternative splicing of this gene and the use of alternate promoters result in multiple transcript variants and isoforms. Additional isoforms have also been shown to result from the use of alternate translation initiation codons.The p53 sequence just like the Rb gene, could be a tumour suppresser, i.e., its activity stops the formation of tumors. However, mutations in p53 are found in most tumour sorts, and then contribute to the advanced network of molecular events resulting in tumour formation. p53 (also referred to as supermolecule 53), is one amongst the chief tumour suppressor supermolecule that's encoded by the TP53 factor in humans. p53 is crucial in cellular organisms, wherever it regulates and maintain the cell cycle and so functions as a tumour suppressor that helps in suppressing cancer. p53 has many role in antineoplastic perform, and plays a essential role in cell death, inhibition of growing, and genomic stability. In anti-cancer role, p53 works through many mechanisms:

- It will activate the DNA repair proteins once the DNA has continuing to wreck.
- It will stimulate growth arrest by stop the cell cycle at the G1/S regulation purpose DNA injury recognition (if it holds the cell here for long enough, the DNA repair proteins can have time to mend the injury and therefore the cell are allowed to continue the cell cycle).
- It will initiate programmed cell death, if DNA injury proves to be irreparable

p53 supermolecule could be a stress activated transcription issue so activated p53 will either induce or repress transcription of the many target genes. Proteins encoded by these target genes square measure involved within the regulation of various biological functions together with growing, cell senescence, DNA repair, apoptosis, cell cycle, cell migration [42]. Diverse stressors, including DNA damage, oncogene activation, hypoxia/anoxia, ribonucleotide depletion and loss of support, stabilize the p53 supermolecule and enhance its activity [43]. The p53 supermolecule possesses the standard structural domains of a transcription issue additionally as many distinctive domain. This feature embodies the DNA binding domain, basic regulative region, the aminoalkanoic acid – made domain, transactivation domain. Central DNA binding domain mediates sequence specific binding to chromatin granule [44, 45]. MDM2 could be a nuclear protein that possesses various necessary functional domains, together with the p53-binding domain, a central acidic region in conjunction with a C4 atomic number 30 finger, and a C-terminal RING domain, that confers MDM2's E3 ligase activity. Mutation of the p53 in tumors is additionally suspected to induce resistance to cancer chemotherapy [46]. One response to genotoxic stress involves the p53 tumor suppressor product [47]. The p53 accumulates once the DNA injury and conjointly controls cellular proliferation preponderantly through its activity as transcription issue. Expression of downstream genes contributes to the tumor suppression either by activating cell arrest; presumably to grant the cell time, to repair the damage and evade genetic instability. Since p53 factor has been found to be mutated in additional than five hundredth of human cancers, it has fascinated the interest of diverse researchers. Ability of p53 for many biological functions is attributed to its ability to act as a sequence-specific transcription issue to control expression of over 100 totally different targets and so to remodel numerous cellular processes like programmed cell death, cell cycle arrest, DNA repair, etc. p53 supermolecule with its specific C- and N-terminal structures is severely modulated by many biological processes like acylation, phosphorylation and ubiquitination through that it effectively regulates the cell growth and death. p53 mutations could lead either to loss or amendment of p53 binding activity to its downstream targets and should so induce abnormal cell proliferation, with resultant malignant cellular transformation. Supported p53's essential role in carcinogenesis, scientist have developed many effective methods for treating cancer by enhancing perform of wild- type p53 or increasing p53 stability. p53 was antecedently viewed as associate degree transforming gene, however throughout these past many decades it has come back to be understood as a tumour suppressor. Till date, several p53 family transcriptional targets are recognized for having the flexibility to adapt numerous cellular processes together with

growth arrest, apoptosis, senescence, differentiation, and DNA repair. In fact, it's evident that this tiny 53- kDa tumour suppressor could be a molecular node at the crossroads of associate degree widespread and complicated network of stress response pathways. Deregulation of p53 has Brobdingnagian influence on carcinogenesis as a result of p53 will provoke associate degree increased epigenetic instability of tumour cells that facilitate and accelerated the evolution of the tumor. The perform of p53 is, presently a significant challenge in p53 analysis field, and such knowledge could eventually afford to novel targets and approaches to therapeutic manipulation of the p53 pathway within the healing of cancer. Challenge within the future are to use the knowledge of p53 and to develop a lot of extraordinarily effective strategy and novel medicine for cancer bar and treatment with less aspect effects. The p53 sequence has been mapped to chromosome seventeen. Within the cell, p53 macromolecule bind DNA , that successively stimulates another sequence to provide a macromolecule known as p21 that interacts with a cell division-stimulating macromolecule (cdk2). Once p21 is

complexed with cdk2 the cell cannot experience to subsequent stage of cellular division. Mutant p53 will not bind DNA in a good manner, and as a consequence the p21 macromolecule isn't created accessible to act because the 'stop signal' for cellular division. therefore cells divide uncontrollably, and leads to the development of cancer.. Help with unravelling the molecular mechanisms of cancerous growth has return from the utilization of mice as models for human cancer, during which powerful 'gene knockout' techniques may be used. The number of data that exists on all aspects of p53 traditional operate and mutant expression in human cancers is currently huge, reflective its key role within the pathologic process of human cancers. It's clear that p53 is simply one element of a network of events that culminate in tumour formation.

Objectives

- *Evaluation of cytotoxicity activity of methanolic leaf extract of Andrographis paniculata.*
- *To find out the apoptotic activity of leaf extract of Andrographis paniculata*
- *To examine the anti-migratory activity of Andrographis paniculata*
- *To evaluate the role of methanolic leaf extract of Andrographis paniculata on e-cadherin and p53 tumor suppressor gene*

3. MATERIALS AND METHODS:

3.1 Preparation of Plant extracts:

kalmegh (*Andrographispaniculata*) leaves were collected from the ISPAT HERBAL GARDEN,ROURKELA. The leaves were washed with distilled water and air dried. The leaves were ground using a grinder to obtain the fine powdered form. A total of 50 g of powdered kalmegh leaf was transferred into a borosilicate glass bottle to which 150 ml of methanol 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 150 ml of 80% methanol 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. Methanolic extract of kalmegh leaves was evaporated using a rotary evaporator (RV 10 Basic IKA Rotary Evaporator) at 60˚C. After this the extracts were oven-dried for an additional 48 hours and then crude form stored at -20˚C. An aliquot of this extracts were dissolved in PBS to prepare a stock solution. The suspensions were then filtered with a 0.22µm filter and stored at -20˚C.

3.2 In vitro cell culture:

Human prostrate carcinoma cell line PC-3 was cultured and maintained in Ham's F-12 Nutrient Mixture (F-12, Invitrogen) supplemented with Fetal Bovine Serum (FBS, Invitrogen) in a humified atmosphere of 5% CO2 at 37°C. The cells were harvested by trypsinization and the number of living cells was calculated by Trypan blue staining (0.2v/v) using haemocytometer.

3.3 Treatment with epigenetic drugs –AZA, TSA, SFN, Curcumin, crude leaf extract of *Andrographis paniculata* for Cell Viability Analysis by colometric MTT Assay:

Drugs	Concetration
curcumin	10,15,20,25,30,35,45(µM)
AZA	10,12,15,20,30,45(µM)
crude leaf extract of *Andrographis paniculata*	10,20,50,60,70 (mg/ml)
SFN	2,4,6,8,10,15(µM)
TSA	50, 100, 120,150, 200,300, 350(Nm)

Table 1: Different concentrations of the various epigenetic drugs considered for MTT assay

Stock solutions of AZA, TSA, SFN, EGCG and Curcumin (Sigma) were prepared in dimethylsulphoxide (DMSO, Sigma) whereas crude leaf extract of *Andrographispaniculata*is dissolved in 1X PBS. Cells were harvested by trypsinization and cell number was counted by haemocytometer. For determining the concentration of drug that inhibited cell proliferation by 50% (IC50), 5 X 103 cells per well were seeded in 96-well microtiter plate and after 24 h incubation, were treated with the epigenetic modulators at different concentrations (Table1) mixed in F-12 supplemented with 5% FBS. Control cells were treated with media F-12 and FBS only. The cells were then incubated for 24 h. briefly; the drug treated cells in each of the 96 wells were washed twice with PBS. After completion of treatment duration, to detect the cell viability (3-(4, 5- Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium) 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. The MTT assay is based on the observation that mitochondria in living cells can catalyze MTT molecules to a colorimetrically detectable dye. 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

3.4 Chromatin condensation analysis by Hoechst staining:

After treatment with epigenetic modulators at the IC50 concentration, cells were stained with Hoechst 33342 stain (1 mg/ml, Invitrogen) followed by incubation for 10 mins at 37o C. Images were taken under UV filter using Epi-fluorescent Microscope (Olympus IX71) at 100 X magnification with an excitation wavelength of 355-366 nm and an emission wavelength of 465-480 nm. Condensed nuclei were counted against total number of nuclei in the field, and the percentage of apoptotic nuclei were calculated and plotted graphically.

3.5 Scratch assay:

PC-3 cells 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 200µl 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 F-12 containing different drugs 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.

3.6. Total Cellular RNA isolation

The total cellular RNA was extracted using TRI reagent (Sigma).PC-3 cells were homogenized using TRI reagent. 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; allow that to stand 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 colourless 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 again 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.

3.7 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.

3.8 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 12µl. 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 GAPDH.

3.9 GAPDH mRNA expression as internal control

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

3.10 Gradient PCR

The PCR primers were chemically manufactured and acquired from Sigma.A gradient PCR was done to optimize the annealing temperature of each set of primers. We used two sets of primers to amplify E-cadherin (CDH1), p-53 and GAPDH genes whose sequence details are in Table no.The annealing temperature for each set of primers was optimized from 55˚C to 60˚C in order to obtain the most suitable temperature for the primers to be annealed on the target sequence. The PCR were carried out using standard protocols in a thermocycler (Bio-Rad) and the DNA was amplified under the following conditions: 95 ℃ for 5 min, 35 cycles of 95 ℃ for 30 s, 50˚C to 58˚C for 30 s and 72 ℃ for 30 s, and the final extension of 72 ℃ for 5 min. The PCR products for each sample were electrophoresed on a 1.5% agarose gel to determine the optimum annealing temperature.

3.11 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) bufferand350mg agarose. 1.5µl of ethidium bromide was added to the gel before casting it. 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.

3.12 Analysis of the Relative Expression level of E-cadherin and p-53 after drug treatment by Real Time PCR (q-RT-PCR)

qRT-PCR was performed using SYBR® Green JumpStart™ TaqReadyMix in the Realplex4 Eppendorf system for CDH1 and P-53 gene. The mRNA level was normalized to GAPDH. The primer sequences for real time PCR are are given in Table 2.

Table.2 Primers used for q-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.	**P-53 forward primer**	5´-TAACAGTTCCTGCATGGGCGGC-3´	121
	P-53 reverse primer	5´-AGGACAGGCACAAACACGCACC-3´	
3.	**GAPDH forward primer**	5'—GGAGCGAGATCCCTCCAAAAT—3'	**197**
	GAPDH reverse primer	5'—GGCTGTTGTCATACTTCTCATGG—3'	

4.Results and discussion:

4.1 Determination of cytotoxic effect and IC50 value of leaf extract of *Andrographis paniculata* by cell viability assay

Cell viability of p-53 was resolute after treatment with methanolic extract of *Andrographis paniculata by MTT assay. The* IC50 value for the extract was calculated. The treatment shows decrease in cell viability which indicates that the cytotoxicity towards prostate cancer cells.

We concluded the lethal concentration of the extract by MTT assay, which was taken as the standardized concentration for the further treatments. The optimized IC50 value found for the extract is 10mg/ml for PC-3 cell line.

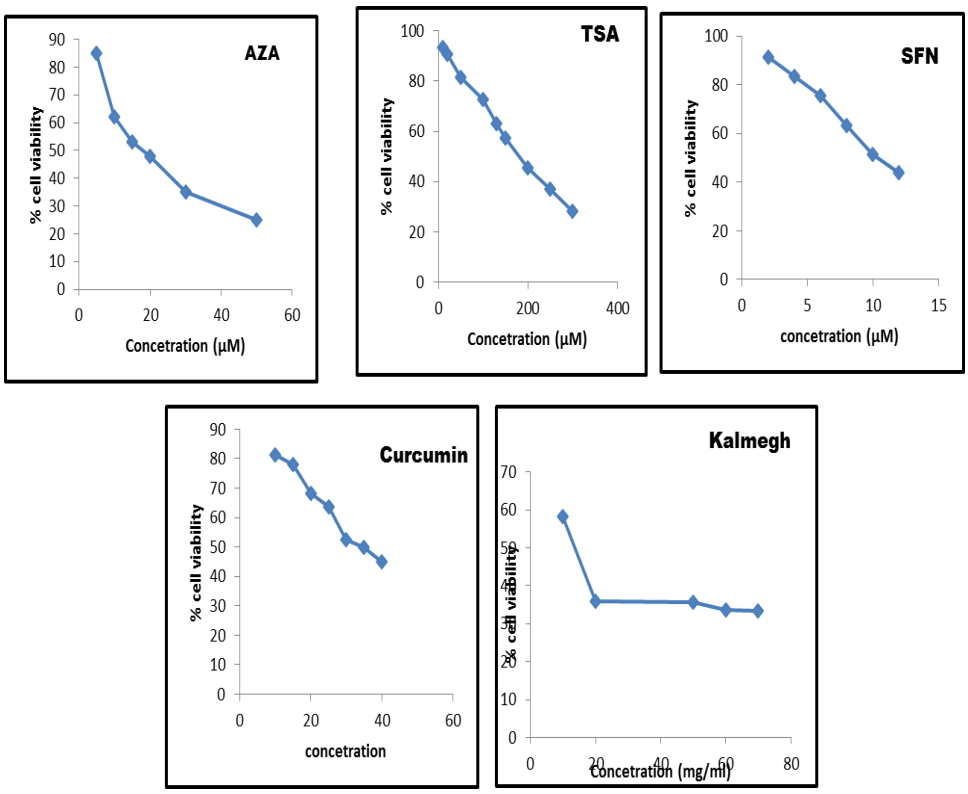


Fig.8: MTT Assay showing cytotoxicity activity and IC50 value of AZA, TSA , SFN, Curcumin, *Andrographis paniculata* on PC3 prostate cancer cell line.

4.2 Detection of apoptosis induced by leaf extract of *Andrographis paniculata* by Chromatin condensation assay

The apoptotic activity of the extract is determined by the chromatin condensation.This is indicated by formation of highly condensed and fragmented apoptotic bodies. Here we found that the untreated controls show no condensed or fragmented nuclei but the treated cells show condensed nuclei. The cells treated with TSA shows the morphological change in their shape.

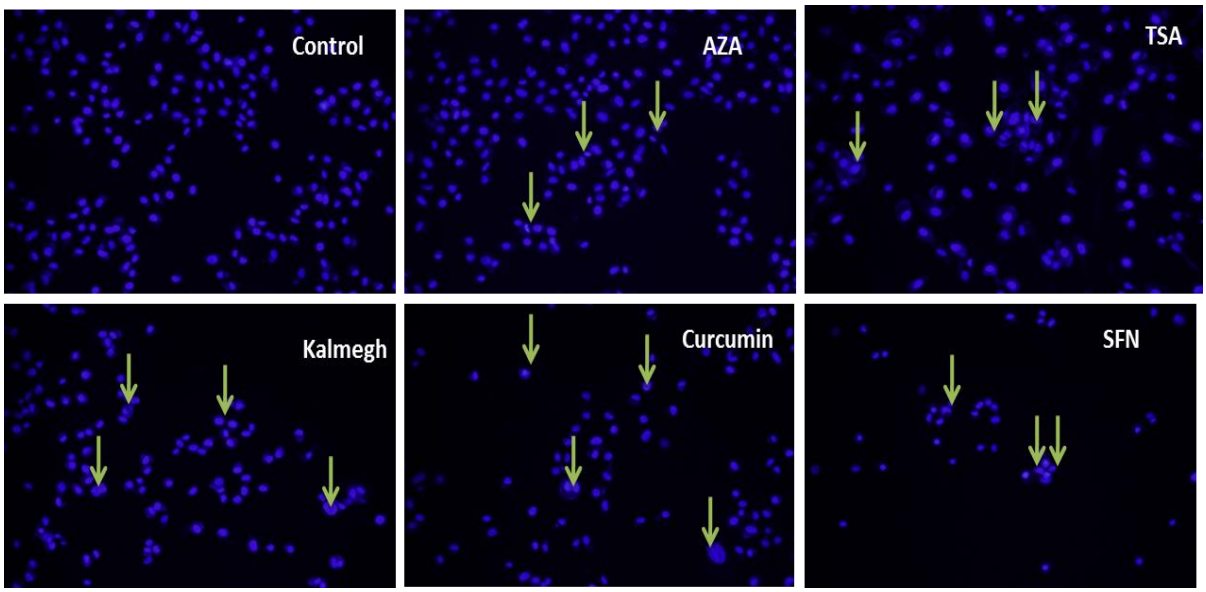


Fig 9: Flurosence microscopic images showing condensed chromatin after drug treatment on PC-3 prostate cancer cell line

4.3 Detection of anti-migratory activity by leaf extract of *Andrographis paniculata* by scratch assay

Scratch assay in performed to determine the migratory property of the PC-3 cells after treatment with crude extract for 24 h. While in control, the cancer cells migrate and close the gap in 24 h, but in treated groups very less number of cells are migrated towards the gap. This indicates the anti-migratory property of *Andrographolide paniculata* extracts.

The results showed that there was less migration of cells towards the scratched area as comparison with the untreated cells taken as control. This clearly demonstrates the efficient anti-migratory property of crude extract on prostrate cancer cells.

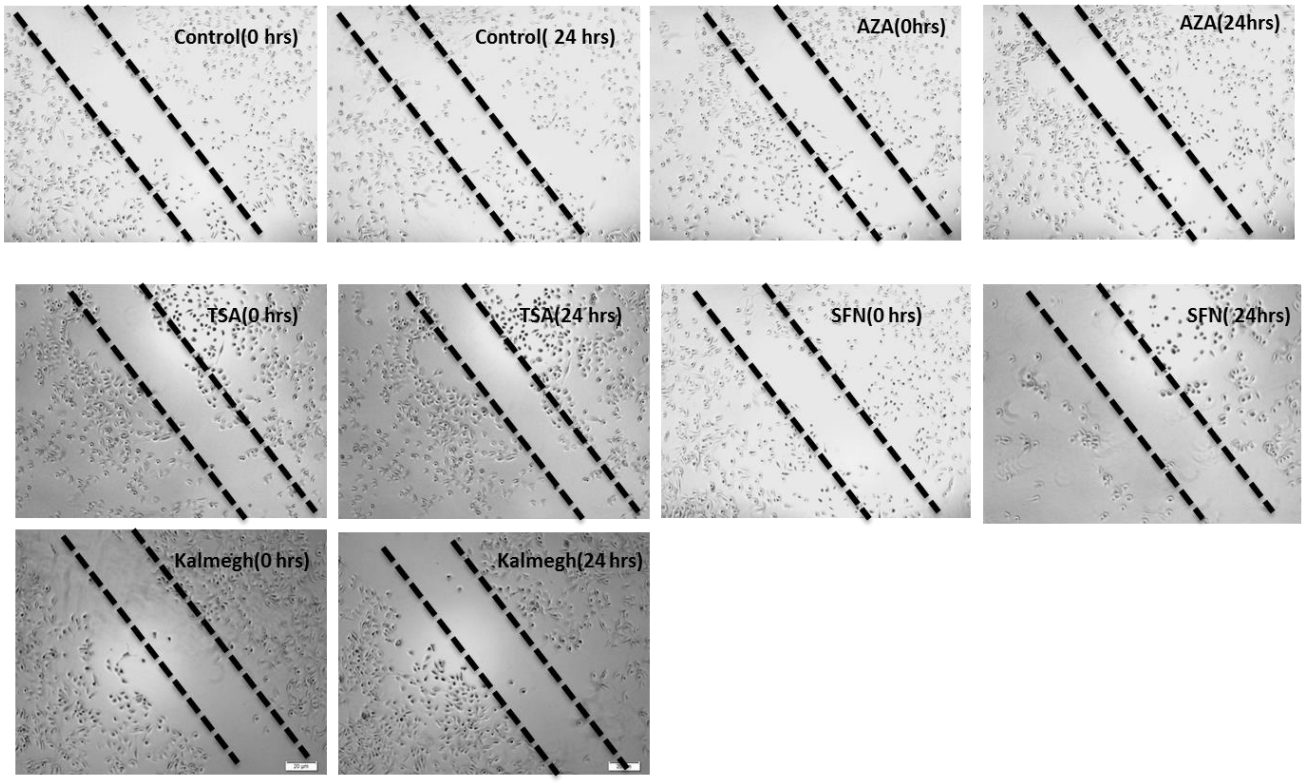


Fig 10: Scratch assay showing the changes in Antimigratory activity of PC3 cells after drug treatment

4.4 Measurement of DNA damage induced epigenetic regulators along with crude methanolic leaf extract of kalmegh

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 drug treatment is higher than the control. The characteristic comet tail length suggested the amount of DNA damaged and thus the intensity of comet tail relative to the head reflect the number of DNA breaks.

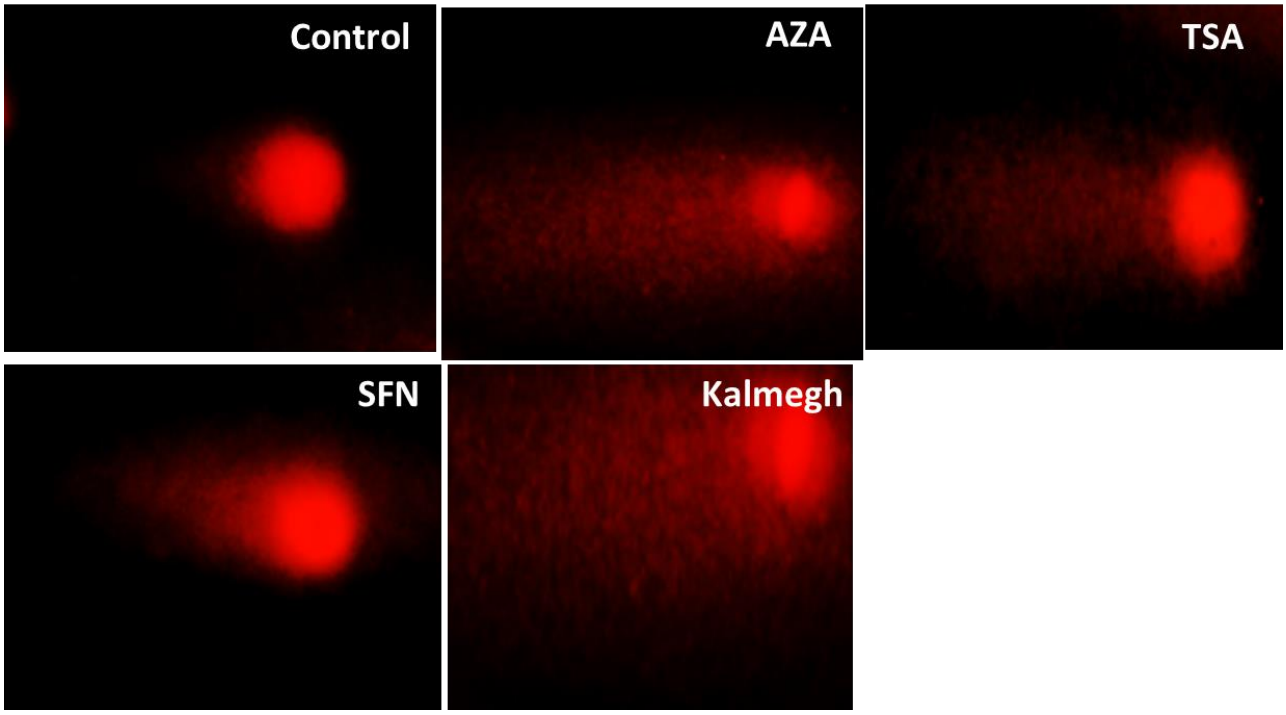
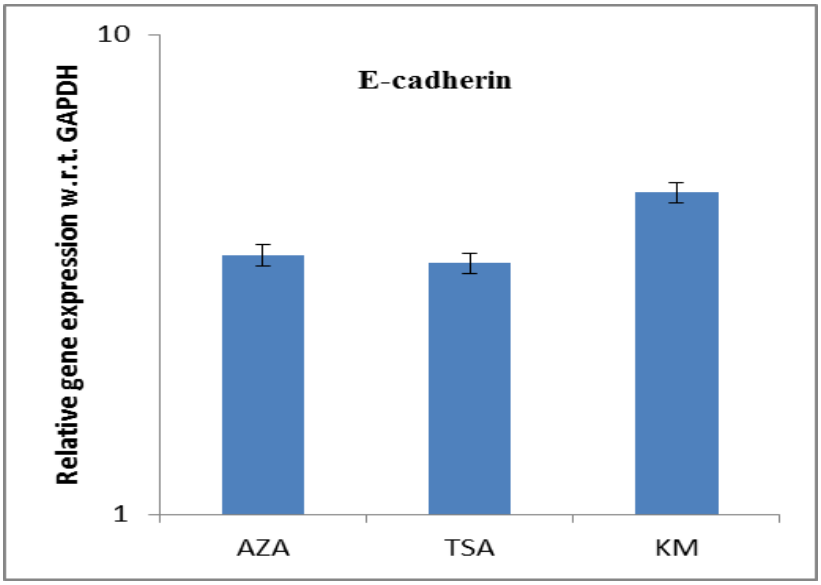


Fig 11:Fluorescence microscopic images of comets showing DNA fragmentation after drug treatment

4.5 Gene Expression Analysis of CDH1and p53 Real time PCR (q-RT-PCR)

The q-RT-PCR denotes the, the level of transcript expression of E-cadherin and p53 has consistently increased after drug treatment. E-cadherin shows up-regulation by 3 fold in AZand TSA treatment whereas 17 fold increase expression in case of crude extract of kalmegh treatment with respect to control (Figure).



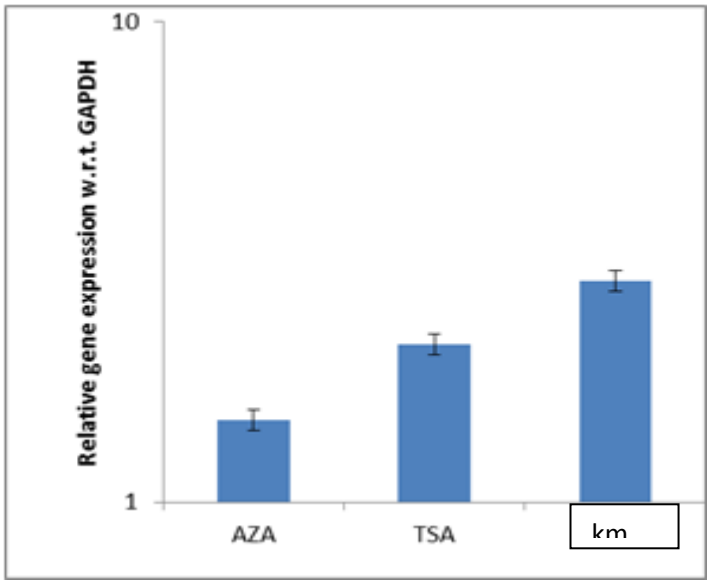


Fig 12: q RT PCR analysis denotes the gene expression of tumor suppressor gene E-cadherin and p53 after drug treatment.

5.Conclusion:

From all this experiments it may be concluded that, the plant Andrographis paniculata posses anti cancer activity. This is revealed from their anti-proliferative, apoptotic and anti-migratory activity. Our experiments clearly demonstrate that the methanolic leaf extract of Andrographis paniculata exert anti-cancer effect on prostate cancer cells . It is also detected that the crude extract of leaves can more effectively restore the expression of tumor suppressor gene E-cadherin and p53 in p53 prostrate cancer cell .

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