Epigenetic Modulation in Cancer Cell by the Bioactive Compounds

Thesis submitted to Jawaharlal Nehru University

for the award of degree of

DOCTOR OF PHILOSOPHY

Saad Mustafa

Biochemistry and Toxicology Laboratory

School of Environmental Sciences

Jawaharlal Nehru University

New Delhi-110067, India

2017

जवाहरलाल नेहरू विश्वविद्यालय
Jawaharlal Nehru University
SCHOOL OF ENVIRONMENTAL SCIENCES
New Delhi-110067
Tele. 011-26704303, 4304

CERTIFICATE

The research work embodied in this thesis; "***Epigenetic Modulation in Cancer Cell by the Bioactive Compounds***" has been carried out in the SCHOOL OF ENVIRONMENTAL SCIENCES, Jawaharlal Nehru University, New Delhi-110067, India. The investigation is original and has not been submitted in part or full for any degree or diploma for any other university/ institution elsewhere.

Saad Mustafa

Saad Mustafa

(Candidate)

Ilora Ghosh

Dr. Ilora Ghosh

(Supervisor)

26/7/2012

Prof. S. Mukherjee

(Dean SES)

Prof. S. Mukherjee
Dean
School of Environmental Sciences
Jawaharlal Nehru University
New Delhi-110067

In the name of **Allah**, the most Gracious, the most Merciful

ACKNOWLEDGEMENT

First praise is to Allah, the Almighty, on whom ultimately we depend for sustenance and guidance. "we seek assistance and forgiveness from Allah. And we seek refuge in Allah from our lower selves and from the wrongs we commit.I prostrate before my creator who has granted this precious life to me and made me a human being. MAY ALLAH'S CHOICEST BLESSINGS be ON HIS LAST MESSENGER AND APOSTLE OF PEACE WHO IS MERCY FOR THE WHOLE MANKIND and who showed the light of truth to humanity.

Second, my sincere appreciation goes to my supervisor Dr. Ilora ghosh whose guidance, careful reading and constructive comments was valuable. His timely and efficient contribution helped me shape this into its final form and I express my sincerest appreciation for his assistance in any way that I may have asked. I am indebted to my parents and (my dadi and nani) and my brother for their encouragement and support. Each and every member of my family and my relatives and my all neighbors' truly deserves my deepest appreciation for their continuous supplications for me. I am also deeply indebted to Professor Kasturi Datta (Baddi Mam) for his invaluable advice and supervision at every stages of this study.. I also wish to thank the School of environmental Science, itsprevious and present Dean and the staff for providing me with an academic base, which has enabled me to take up this study. I am particularly grateful to Professor Khilare, prof Akhtar Husain, prof Tasneem fatima prof Moshahid prof Sudha bhattacharya, prof kasturi mukha uppadhayya Associate Prof.Rais , Assoc prof Sudesh yadv, Assistant Prof Vijay pal and Assistant Prof Ram avtar .

I would also like to thanks my school teachers and my coaching teachers and my Venky college teachers and JMI teachers.

I greatly appreciate the help and co-operation extended by nonteaching and technical staff especially Devii singh ji during the course of work.

“ There are some Islamic quotes that motivates me especially during Ph.D work

Abu Hurayrah narrates that The Prophet pbuh said: “There is no disease that Allah has created, except that He also has created its remedy.”

Sadness weakens the heart; weakens the aspirations; harms the determination; Therefore be happy and have glad tidings.....and have best expectations from Allah and be grateful in whatever state you are in!

Prophet Muhammad (ﷺ)peace and blessings be upon him, said, "He who does not thank people, does not thank Allah"

Allah has tailor made the test for each and every one of us and none of us will be given something which we can’t bear. I am given something that I can bear and you are given something that YOU can bear. The tests won’t be the same for you and me.”

I would also like to acknowledge all my senior for unconditionally supporting me Dr .Raghib bhai Dr Abhijeet Mishra bhai, , Dr. Ausaf bhai Dr. Razique bhai, Dr Ahswani bhai Dr.Sandeep bhai,,Dr.R.L.A.Prsasad ,,Dr.P. Saha ,Dr N.R. Aggarwal , Dr N. Maurya, ,Dr.Gyan gupta,Dr.Naseem bhai, , Dr Sajjad bhai,, Dr.Ikraam, bhai , Dr,Noor bhai, Dr Mustaque, Dr Zeeshan bhai and Dr.Arif bhai for their help, co-operation and valuable suggestions related to my research work.

I am extremely grateful to my dearest colleagues Jogendra yatendra, Suraj anil , shashi chandershekhar, manoj, sughosh,,arif, Vikas ,maroof Naveen,vineet aijaz, madan bikram and rohit, for all the love, help and co-operation which they extended during my Ph.D. tenure. Their humorous and friendly sarcasm lightened my tension. They were always by my side during difficult situations and together we shared wonderful moments.

I would also like to thank my juniors,Debarpan Abhinav ,Mannet ,vikas mukesh and Amit for their help and kind co-operation during the course of researchWork

I owe a deep sense of gratitude to my friends Aasif khan, shoby,ayaz Asad, Faez ,Ahmad, Danish parvez, Farhan, Mughees,Kamal,Moiz,hishaam ,rahat,shahnaz and zubair anas,iqbal, tauseef , mudassir and hammad for their constant moral support and encouragement. Special thanks to My school friends arshad, faisal, Hassan ,tanveer , Kamran tauseef saad They pushed me out through the difficult moments of the study and always motivated me. I gained a lot from them, through their personal and scholarly interactions,their thoughtful suggestions at various points of my research programme.

Special thanks, tribute and appreciation to all those their names do not appear here who have contributed to the successful completion of this study.

I thank and acknowledge each and every thing on the biosphere who helped me, supported me or is associated with me in any way.

UGC and JNU are gratefully acknowledge for their financial support.

SAAD MUSTAFA

ABBREVIATIONS

μg	Microgram
μl	Microlitre
μM	Micromolar
μm	Micrometer
Å	Angstrom
AP	Alkaline phosphatase
APS	Ammonium persulfate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminiscence
kb	kilobase
kDa	Kilodalton
M	Molar
mg	Milligram
ml	Millilitre
mM	milimolar
NBT	Nitro Blue Tetrazolium
ng	Nanogram
nm	Nanometre
nM	Nano molar
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered saline
pI	Isoeletric point

PMSF	Phenyl methyl sulfonyl fluride
RNA	Ribonucleic acid
RNase A	Ribonuclease A
ROS	Reactive oxygen species
rpm	rotations per minute
SDS	Sodium dodecyl sulphate
TAE	Tris acetate EDTA
TEMED	N, N, N', N'-tetramethylene diamine
Tris	Tris-(hydroxymethyl) amino methane
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl Sulphoxide
dNTP	Deoxyribonucleotide triphosphate
FBS	Foetal Bovine Serum
R123	Rhodamine 123
DAPI	4',6-diamidino-2-phenylindole
JC-1	5,5',6,6' - tetrachloro - 1,1',3,3' - tetraethylbenzimidazolylcarbocyanine iodide
TQ	Thymoquinone
SAHA	Suberoylanilide hydroxamic acid
HDAC	Histone deacetylase
LC-MS	Liquid chromatography–mass spectrometry
ESI -MS	Electrospray ionization - mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
H2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
AO	acridine orange
X-Gal	5-Bromo-4-chloro-3-indolyl β-D-galactoside,

CONTENT

Review of Literature

1.0 Introduction

Few reports revealed that cancer is a multifactorial disease attributed to genetic moderations. But increasingly people are becoming aware that cancer has a strong epigenetic background as well. Epigenetics could be understand as heritable modifications in the expression of gene without any changes in genetic code. There are three key categories of epigenetic occurrences including methylation of DNA, histone acetylation, and RNA interference.(Falkenberg & Johnstone, 2014) These epigenetic phenomena play an important role in the maintenance and balance of common tasks and functions of the cell during all critical stages. In addition, it also help to adaptation to different changes at environmental level, such as chemicals, radiation, variation of nutrition or exposure to smoke of cigarette and/or industry, and changes at hormonal level . Nevertheless, if cells could not adapt these adaptations lead to alterations of different epigenetic targets that may also causes several diseases, such as cancer(Kanherkar, Bhatia-dey, & Csoka, 2014).Current research is aimed on investigating the individual and collective effects of these epigenetic mechanisms. Up-regulation of transcriptional activity is observed due to histone acetylation. Further, degree of acetylation levels are maintained by actions of two different enzymes: histone acetyl transfereases as well as histone deacetylases. In the light of current knowledge HDACs are central class of enzymes which can modify the protein that are further involved in many physiological phenomena such as cell proliferation, chromosome remodelling, and gene transcription. Different types of tumours were found to associate with deregulation or up-regulation of HDAC activity, thus plausible explanation of the use of HDAC inhibitors for cancer therapeutics and management. Effect of HDAC inhibitors shows dose-dependent effect in several cancer cells. However in some cases, inhibition of growth was found at low concentrations, while apoptosis was observed at higher doses; while in other conditions, apoptosis may induced before growth arrest. Outstandingly, normal are more resistant than tumour cells to HDAC inhibitors(Koeneke, Witt, & Oehme, 2015). Mutation cause loss or gain in gene functions which may linked to most human diseases including cancer. Gene expression is regulated by a complex process. Chromatin structures contained most eukaryotic genes, which attributed to high condensations of the genes that involve process of dynamic chromatin remodelling to assist their transcription process. DNA methylation and modifications of histone

signify the major chromatin remodelling processes. Further, they also assist to incorporate environmental signals for the cancer cells to moderate the purposeful production of their genome function. Complexity of human diseases such as cancer and type II diabetes are supposed to have a strong environmental signals in addition to genetic causes. Changes in chromatin remodelling are linked with together genetically as well as environmentally related diseases(Romani, Pistillo, & Banelli, 2015).

Human genome encodes around thirty thousand genes. It is predicted that more than eight thousand human diseases are mainly caused by errors in single genes. Cystic fibrosis and haemophilia are example of unifactorial or monogenic diseases, which are individually rare and touches roughly 1% of the total human population. In compare, the major causes of common diseases, such as cancer and diabetes, encompass both susceptibility of genes and their interactions with the environment(Liu et al, 2008). Gene and its environment interactions are found to be facilitated by epigenetic modifications of the genome cause increase in response to modifications in the environment. Epigenetic changes are usually reversible, and relying on the presence or elimination of the activating factors. Interactions of gene and environment can change activities of gene of interest and bring about cascades of many cellular events to aid the adaptation of an individual cell to its surrounding environment(Alegría-torres & Baccarelli, 2013). Amongst numerous cellular actions, regulation of gene at transcription level is a central mechanism, which controls the RNA and following protein manufacturing that are vital at many cellular functions. The interactions between genes and cellular protein factors can be regarded as an endogenous gene-environment interaction. *In vitro*, animal and human investigations have suggested various categories of environmental chemicals that transform epigenetic marks, comprising heavy metals such as cadmium, arsenic, nickel, chromium and methylmercury, peroxisome proliferators such as tri-chloroethylene, di-chloroacetic acid, tri-chloroacetic acid), air pollutants including particulate matter, black carbon and benzene, and reproductive poison for example diethylstilbestrol, bisphenol and organic pollutants. In animal investigations, many hazardous chemicals such as alloxan, cyclophosphamide, orthoaminoasotoluol, benzopyrene, diethylstilbestrol and vinclozolin have been shown to encourage phenotypic effects at trans-generational level Transmission of trans-generational phenomena due to of different chemicals leads to epigenetic changes((Pogribny and Rusyn, 2014).

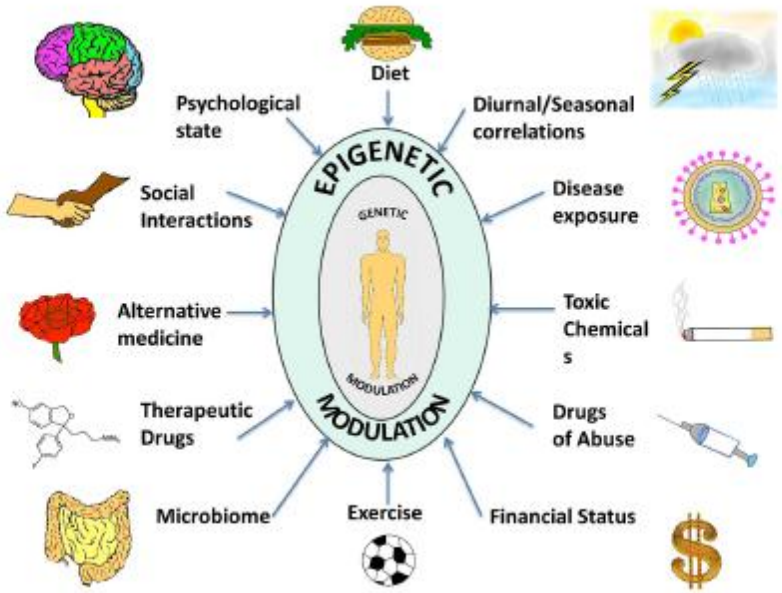

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Fig: Image 1 (Environment-Epigenetics interactions Figure adapted from (Kanherkar et al., 2014).

This process have been speculated as a probable mechanisms for these effects environmentally caused epigenetic alterations and it may be sufficient to cause abnormalities in physiological functions proposed as a potential mechanisms for these effects. There are many exposures, has been established that chemicals toxicants can change epigenetic marks. The impression of lifestyle consist of different aspects like nutrition, stress, daily exercise, working routines, smoking and alcohol intake. While, cumulative signal revealed that different environmental and lifestyle factors may deeply impact epigenetic mechanisms as shown in Fig (Image 1) .for example methylation of DNA, histone acetylation and expression at microRNA level. Many lifestyle factors have been found which may change epigenetic patterns including junk food, fatness, lack of physical activity, smoking, alcohol drinking, various environmental pollutants, stress, and working on night shifts. (Kanherkar et al., 2014).

Foods: Epigenetic mechanisms may influenced by nutrition. For example, polyunsaturated fatty acids rich diet could be linked with mutation causing free radicals and oxidative stress. Furthermore, diets including seasonal fruits and vegetables, which are rich in natural fibre and anti-oxidants, may protect from cancer.

Obesity: Obesity and lazy lifestyle are serve as major risk factors for many diseases, including diabetes, cancer and cardiac problem. While, body weight of human is controlled by genes regulating energy homeostasis.

Tobacco smoke: Smoke of tobacco contains many group of organic and inorganic compounds, many of which have potent carcinogenic and pro-inflammatory properties. However, specific effects of individual compounds have been investigated through different epigenetic studies and found to associate with several diseases.

Abuse of drugs: Few reports shown abuse of some drugs such as cannabinoids, heroin, and cocaine shown to change particular epigenetic factors. The complete investigation ofepigenetic changes caused by drug abuse such as cocaine. Administration of cocaine in maternal mice is identified to change in DNA methylation and gene expression in hippocampus neurons of offsprings. (Kanherkar et al., 2014).

Psychological and external stress: Now a days, social experience may have an explanatory effect on stress comebacks future in life through epigenetic mechanisms. The living area and/or workplace can regulate the degree of exposure to different environmental factors that are possibly capable to change epigenetic marks.

Shift work: Working schedule on night shift might have negative impact on the healthy workers due to incompatibility between the endogenous diurnal timing systems such as light/dark cycle. Circadian genes linked with epigenetic reprogramming which have been served as a possible response which may altered circadian rhythms(Alegría-torres & Baccarelli, 2013).

1.1 Epigenetics: Fundamentals

The term epigenetics was coined by Conrad Wadington in the 1940s. He defined it as a branch of biology that studies casual interactions within the genes and their products and brings the phenotypes into being. Epigenetics refers to mechanisms that can modify gene expression and phenotype without changes in the underlying DNA sequence(Romani, Pistillo, & Banelli, 2015). Transcription in eukaryotic cells is infuenced by the manner in which DNA is packaged. In resting cells, DNA is tightly compacted to prevent accessibility of transcription factors. DNA is packaged into chromatin, a highly organized and dynamic protein plus DNA complex. (Ali Khan et al., 2015)The fundamental subunit of chromatin, the nucleosome, is composed of an octamer of four core histones, i.e. an H3, H4 tetramer and two H2A,H2B dimers, surrounded by 146 bp of DNA. De Ruijter et al During activation of gene transcription, this compact, inaccessible DNA is made available to DNA binding proteins via modification of the nucleosome. This architecture of chromatin is strongly influenced by posttranslational modifications of the histones. Acetylation and deacetylation of histones play a very important role in transcription regulation of eukaryotic cells. The acetylation state of histones and non histone proteins are determined by histone deacetylases and histone acetylases. Initial reports suggested that cancer is a disease caused due to genetic reasons. But increasingly people are becoming aware that cancer has a strong epigenetic background as well. Epigenetics refers to heritable changes in gene expression without alteration of the genetic code. The three main kinds of epigenetic phenomena include: DNA methylation, histone acetylation, and RNA interference. Ongoing research is focused on dissecting the individual and cumulative effects of these epigenetic mechanisms. (Dienstbier, R. et al 2014).

Histone Deacetylase As An Epigenetic Tool

Histone deacetylase (HDAC) enzymes involves removing the acetyl group from the histones comprising the nucleosome Hypoacetylation results in a decrease in the space between the nucleosome and the DNA that is wrapped around it. Tighter wrapping of the DNA diminishes accessibility for transcription factors, leading to transcriptional repression.

1.2 Types Of HDAC And Their Location And Function

Depending on sequence similarity and co factor dependency HDAC is divided into four classes and two families: the classical includes class I, II and IV the silent information *r*egulator 2 (Sir2) lies under class III. In humans, 18 HDAC enzymes have been identified and classified, based on homology to yeast HDACs. (Blander and Guarente, 2004; Bhalla, 2005; Marks and Dokmanovic, 2005). HDAC have both histone and non-histone targets. (Greene, M. L., et al,2007) Non histone targets include p53, STAT3, GCCR, BCL6 etc. Non-histone protein targets of HDACs include transcription factors, transcription regulators, signal transduction mediators, DNA repair enzymes, nuclear import regulators, chaperone proteins, structural proteins, inflammation mediators and viral proteins.

The classical family of HDAC (having Zn^{2+} as cofactor)

CLASS	TYPES	LOCALISATION	FUNCTION(S)
HDAC CLASS I	HDAC1,HDAC2, HDAC 3, HDAC8	Nucleus	cell proliferation and survival regulator of chromatin compaction with cell cycle progression and repair. Branzei, D et al (2008)
HDAC CLASS II	HDACIIa HDAC4,5,7, HDACIIb HDAC 6,10	HDAC 9, 10 can shuttle between nucleus and cytoplasm .The rest remains in the nucleus. HDAC 6 occurs in the cytoplasm	Regulator of the HSP90 chaperone. Regulates chondrocyte hypertrophy , endochondral bone formation, muscle development, and cardiovascular diseases Cellular functions coordinating with microtubule network Haberland, M et al 2009).
HDAC CLASS IV	HDAC 11	Nucleus	Molecular targets that influence immune function. Yu, H et al (2004)

The sirtuin family (having NAD^+ as cofactor) Hirschey, M. D. (2011).

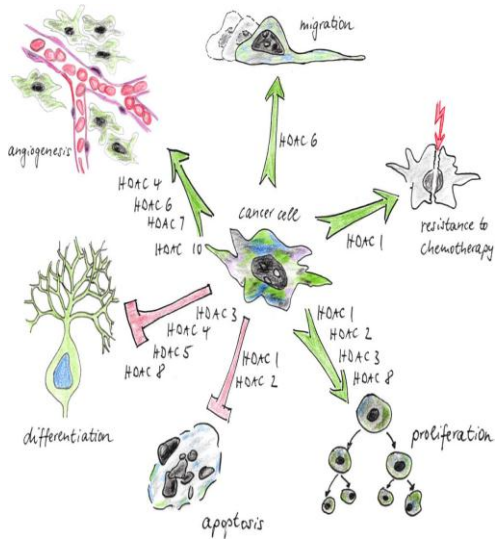
CLASS	TYPES	FUNCTION(S)
CLASS III HDAC	SIRTUINS 1-7	Ubiquitously expressed in humans There are both histone (H1,H3,H4) as well as non histone (FOXO,NF-KB) targets of sirtuins. Campos, E. I., et al . (2010). Deactetylation of transcription factors by sirtuins a) alters their sub cellular location. b) changes their expression level c) alters their binding to DNA d) changes their interaction with regulatory protein. Qi, Z., & Ding, S. (2012)

1.3 HDACs In Cancer

Epigenetic modifications of genes are a central part of various physiological phenomena processes such as cellular development and differentiation. Among various epigenetic regulation at HDACs level they have major contribution in the survival of cancer via epigenetic silencing of several tumour suppressor genes as well as deacetylation of tumour suppressor proteins. Many reports have revealed that some HDAC family members are abnormally expressed in several tumours(Witt, Deubzer, Milde, & Oehme, 2009)(Grabarska, Łuszczki, Nowosadzka, & Gumbarewicz, 2017). Abnormally selection of HDACs has been observed to play an instrumental role in tumour development such as ovarian, pancreatic, liver, colon and cervical cancer(Xie et al., 2012). Furthermore, as discussed HDACs regulate several crucial cellular events as shown in (fig:Image2), which is via through epigenetic control as well as deacetylation of non-epigenetic protein. Thus, Classical HDACs might be encouraging novel class of target of anti-cancer drugs. (Butler, K. V., et al)

1.3.1 Expression And Function Of Classical HDAC In Cancer

HDAC Class	Cancer type	Knock down result
HDAC1 EXPRESSION : associated with dedifferentiation, enhanced proliferation, invasion, advanced disease and poor prognosis.	upregulated in Cervical,gastric cancer.	Mice gene knockdown studies of HDAC1 in several cancers
	Upregulated in pancreatic cancer together with HIF1a expression.	• HDAC1 knockdown in HeLa cells caused inhibition in cell proliferation.
	• Significantly high levels of HDAC1 in colorectal, breast and renal cancer reduced patient survival.	
	• In hepatocellular carcinoma , high HDAC1 expression was associated with cancer cell invasion in the portal vein.	• G1 or G2/M transition arrest in breast cancer cells.
	• HDAC1 overexpression led to undifferentiated phenotype in prostrate cancer cells.	• Sensitization of CLL cells for TRAIL induced apoptosis.



Role of HDAC in cancer fig:Image2 adapted from (Witt et al., 2009)

HDAC2 EXPRESSION : associated with advanced stage disease and poor prognosis in gastric, colorectal and prostrate cancer.	• Upregulation of HDAC2 in colorectal cancers in early polyp stage.	Gene knockdown studies of HDAC2 in several cancers
		• HDAC2 knockdown caused differentiated phenotype, increase in apoptosis associated with increased P21 expression in cervical cancer cells.
	• High level of HDAC2 in cervical dysplasia and invasive carcinoma.	• Increases functional DNA binding activity of p53 associated with induction of cellular senescence in breast cancercells.

HDAC3 EXPRESSION	Upregulated in large number of gastric, prostrate and colorectal cancer samples.	Gene knockdown studies of HDAC3 in several cancers
		In acute promyelocytic leukemia cells HDAC3 regulates the PML-RARA expressing cells. Knockdown of HDAC3 in such cells restores expression of retinoic acid dependent genes.

HDAC8 EXPRESSION	Upregulated mostly in childhood neuroblastoma cells and is associated with advanced stage disease and poor prognostic marker.	Gene knockdown studies of HDAC8 in several cancers
		• Proliferation of cervical, lung and colon cancer reduced after HDAC8 knockdown. • HDAC8 knockdown induces apoptosis in T cell derived lymphoma and leukemic cells but not solid cancer cell lines.

HDAC4 EXPRESSION	Upregulated in breast cancer samples.	Gene knockdown studies of HDAC4 in several cancers
		• HDAC4 was found to interact with leukemic PLF-RARa fusion protein and repress differentiation of associated genes.
		• HDAC4 regulates HIF-1a transcriptional activity in renal carcinoma cells.

HDAC5 EXPRESSION	Upregulated in colorectal cancer.	Gene knockdown studies of HDAC5 in several cancers.
		HDAC5 interacts with transcription factor GATA1 and shuttles from the nucleus to the cytoplasm upon erythroid differentiation of mouse erythroleukemia cells.

HDAC7 EXPRESSION	Highly expressed in colorectal cancer cells.	Gene knockdown studies of HDAC7 in several cancers
		HDAC7 silencing in endothelial cells altered their morphology, migration and ability to form capillary tube like structures in vitro but did not affect cell adhesion, proliferation or apoptosis.

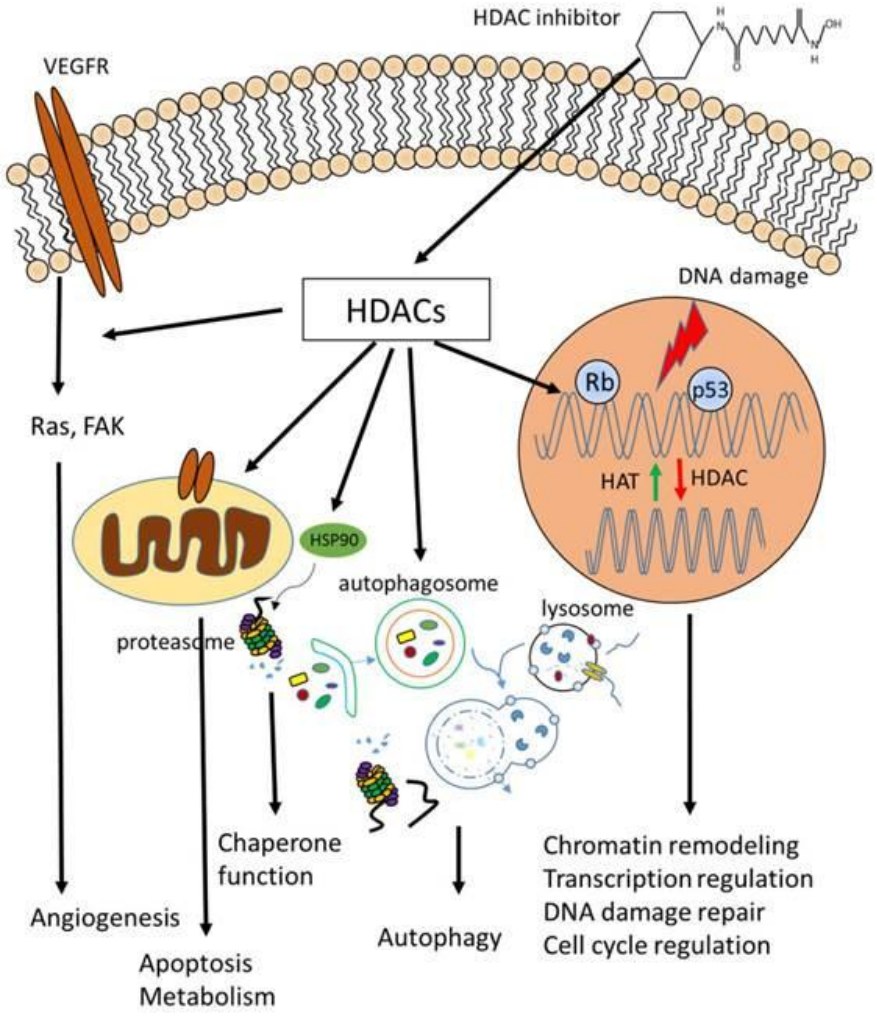
HDAC6 EXPRESSION	High levels of HDAC6 in oral aquamous cell carcinoma	Gene knockdown studies of HDAC6 in several cancers
	HDAC6 expression correlated with better survival and was higher in small tumors and in estrogen-progesterone receptor positive tumors.	Targeted inhibition of HDAC6 leads to acetylation of HSP90 and disruption of its chaperone function resulting in depletion of progrowth proteins in K562 leukemic cells.

HDAC10 EXPRESSION	Expression of HDAC10 in cancer samples not yet reported to data.	Gene knockdown studies of HDAC10
		Knockdown of HDAC10 (and HDAC6) reduces VEGF receptor 1 and 2 expression in cancer cells.

HDAC11 EXPRESSION	No published data on expression of HDAC11 in tumor tissues available.	

1.4 HDAC Inhibitors

HDAC inhibitors (HDACi) alter gene transcription, in part, by chromatin remodeling and by changes in the structure of proteins in transcription factor complexes. (Xu, W. S et al)HDAC inhibitors act through binding into the active site pocket and chelation of the catalytic zinc ion located at its base.(ref).Due to the highly conserved nature of the enzymatic pocket, most HDACinhibitors do not selectively inhibit individual HDAC isoenzymes and either inhibit all HDACs or at least several members simultaneously. Hence there are three classes of HDACinhibitors: panHDACi,class I HDACi and Class II HDACi. HDACi have been discovered with different structural characteristics, including hydroximates, cyclic peptides, aliphatic acids and benzamides. The FDA approval of SAHA and FK-228, two HDACi for the effective HDACi-based therapies.(Thaler, 2012) HDACi Induces autophagic cell death as shown in (Fig:image 3) , cell cycle arrest, senescence, mitotic cell death, Activates extrinsic and intrinsic apoptotic pathways, Accumulates HSP90 and disrupts of protein function. Activates protein phosphatase I and disrupts HDAC-ppi complexes leading to inactivation of Akt and it also Inhibitis angiogenesis(Xu, Parmigiani, & Marks, 2007).



HDAC inhibitors in various process, Fig:image 3 adapted from (Li & Zhu, 2014)

HDAC inhibitor	MECHANISM	EXAMPLES
PAN HDAC INHIBITOR	Induction of p27 which inhibits CDK4 and CDK 2 containing complexes, causing dephosphorylation of Rb protein , which blocks E2F activities in the transcription of genes for G1 progression and G2/S transition. (Neganova, I., et al, 2009).	TSA, vorinostat, belinostat, panbinostat, PCI-24781
HDAC CLASS I INHIBITORS	Induces cell cycle block and inhibition of	MS 275, dipepsipeptide(romidespin),

(HDAC1 and 2 selective inhibitors)	proliferation by induction of cyclin dependent kinase inhibitor (p21 waf/cip1)(ref)in lower concentrations and induction of Reactive oxygen species(ROS), Mitochondrial damage, caspaseacticvation and apoptosis in high concentration. Boonstra, J et al . (2004).	Valproic acid, apicidintrapoxin, Cpd 2,
HDAC CLASS II INHIBITORS		Tubacin ,APHA derivatives, mercaptoacetamide.

1.5 HDAC Relation With Senescence

HDACs have a significant role in controlling cell physiology throughout the aging process, and contribute to the cellular modulation linked with transformation. While, HDACi induces various phenotypes in several cancer cells such as growth arrest and premature senescence.HDAC1 inhibition leads to senescence in WI 38 cell (Place, Noonan, & Giardina, 2005) and treatment with HDAC inhibitors such as TSA and SAHA induces senescence in gall bladder cancer cells..(Yamaguchi et al., 2010)Therefore HDAC plays a crucial role for controlling tumorogenesis and metastasis. Cellular senescence is an irreversible cell growth arrest and senescence. (Herbig, U et, al 2006). Unlike dead cells, senescent cells remain viable and metabolically active. Senescence can be the consequence of various stimuli such as DNA damage, telomere attrition, and oncogene activation. (Ben-Porath et ,al. 2005). Senescence may be categorised into 3 kinds: premature senescence, replicative senescence and oncogene-induced senescence. Premature senescence is brought about by stress signals such as DNA damage.(Freund,A et al 2010). Replicative senescence is prompted by the successively shortened telomeres. Oncogene-induced senescence is triggered by aberrant activation of oncogenes, for instance, overexpression of Ras can trigger senescence and prevent unnecessary proliferation. Because senescent cells

cannot re-enter the cell cycle, it acts as a checkpoint for the immortalization and transformation of cells. (Allday, M. J. et al2009),Senescent cells are identifiable by distinctive cell morphology with enlarged cytoplasm and stain positive for beta-galactosidase at pH6. Several cell cycle check point proteins are involved in and up-regulated during senescence, such as p53, p21cip1 and p16INK4a. Senescence is also associated with formation of senescence-associated heterochromatic foci (SAHF). (Deursen, 2014)

1.5.1 Regulation Of Senescence

Overexpression of oncogenes, for exapmle Ras, may trigger senescence to neutralise the unnecessary pro-proliferation signals. INK family proteins p14ARF, p15INK4b, p19 in mouse, p19ARF and p16Ink4a are involved in helping oncogene mediated senescence. (Venza et al., 2015) Oncogene activation can induce p19ARF; moreover p19ARF inhibits mouse double minute 2 homolog (MDM2), whose function is to inhibit p53 accumulation through proteasomal degradation. Located on chromosome 9p21 in the human and in 4 mouse. ARF is a small protein that binds with MDM2. Oncogene activation can also activate p16Ink4a. p16Ink4a inhibits cyclin-dependent kinase CDK4/6 and causes cell cycle arrest by regulating the tumour suppressor protein Rb. Rb is activated in its hypophosphorylated state, E2F target genes are transcriptionally silenced, and cells enter senescence. Moreover Rb can is involved in the formation of heterochromatin by methylation of histone H3 lysine 9 (H3K9me) and thereby induces senescence. DNA damage when induced by chemotherapeutic drugs (e.g. etoposide) can cause DNA double strand break. In this case p53 is activated and it in turn activates downstream p21cip1. DNA damage- induced senescence can also be mediated through p16Ink4a.

Replicative senescence is caused by progressive telomere shortening. Telomere attrition can cause chromosomal end-to-end fusions. These are recognized as double-strand breaks and trigger the DNA damage repair machinery. P53-p21 and p16INK4a-Rb signalling are key regulators in mediating replicative senescence. It has been seen in many human tumours that cell cycle checkpoint proteins such as p53 and INK4 families are silenced

by mutations or promoter methylation which compromise the senescence barrier. (Shay & Roninson, 2004)(Correia-melo et al., 2016)

1.5.2 Role Of Senescence In Cancer

Senescence is a vital suppression mechanism in the premalignant lesion step. In prostate cancer, cellular senescence caused by cell cycle check point protein p27kip1 up-regulation has been shown to be a barrier during the transition from prostatic hyperplasia and prostatic intraepithelial neoplasia to invasive tumour. (Tsantoulis, P. K., et al . 2005). Senescence is a preliminary block during lymphoma development. Histone methyltransferase Suv39h1 is necessary for Rb-mediated methylation of histone H3 lysine 9 and senescence-associated heterochromatin formation. Braig*et al.* have reported that Eμ-N-Ras transgenic mice with Suv39h1 heterozygous lesions developed lymphoma at a significantly accelerated stage than N-Ras-transgenic wild-type mice. It was established that the senescence gatekeeper can delay the onset of oncogenic Ras-induced malignancy.

However, senescence can also be a significant cancer supporting mechanism. Senescence may lead to therapeutic resistance in response to anti-cancer drugs. Senescent cells resting in G1 cell cycle will be unaffected to anti-cancer drugs suitable for proliferating cells. Furthermore, senescent cells may produce a sympathetic microenvironment for tumour progression. It was demonstrated in the co-culture situation that senescent fibroblasts can promote the propagation of prostate epithelial cells. Conditioned medium from the senescent fibroblasts also facilitates the growth of prostate epithelial cells. It is hypothesised that the tumorigenic effect of senescent fibroblasts is mediated by paracrine factors like growth factors or inflammatory factors released by these growth-arrested fibroblasts.(Deursen, 2014)

1.5.3 Tumour Suppression Mechanisms

Very rarely does a single mutagenic event promote complete transformation of a cell; rather it is a build-up of mutations that result in genomic instability and heightened predisposition to cancer development. The inability of tumour cells to check their own multiplication originates from compromised

mechanisms of self-surveillance: apoptosis and senescence. A master gene orchestrating both of these responses is the *TP53* gene. Programmed cell death, particularly apoptosis, is a tightly controlled mechanism of cellular suicide involving many steps of signalling cascades which lead to nuclear fragmentation, cell shrinkage, membrane disruption and clearance by the immune system. This mechanism controls unregulated cellular proliferation and can be triggered by the damaged cell itself (intrinsic pathway) or by signals from surrounding cells (extrinsic pathway) (Ouyang*et al.*, 2012). Senescence also checks the clonal propagation of pre-tumour cells, but in contrast to apoptosis, the cells are not eliminated and their physiological processes are maintained. Senescent cells constantly execute a strong and irreversible programme of tumour suppression that a cancer cell needs to subvert in order to attain tumourigenic characteristics.

1.54 An Intrinsic Tumour Suppression Mechanism : Senescence

Oncogenic events that trigger mitogenic signals and the extent of cell growth stimulation sometimes cannot be prevented by regular, reversible cell cycle checkpoints. In case a cell receives enhanced and uncontrolled proliferation signals, it can be restrained by a built-in, fail-safe mechanism that will prevent the cell from failing and will render it insensitive to growth stimuli. This mechanism is termed cellular senescence.

1.5.5 Replicative Senescence: Hayflick Limit

The first observation that vertebrate fibroblasts could grow indefinitely in culture suggested that there exist *in vivo* mechanisms of growth control that are lost when the cells are extracted and subcultured. These results were never validated and were linked to experimental error During this time, Hayflick and Moorhead reported difficulties in maintaining fibroblasts in cultures for a prolonged period of time (Hayflick & Moorhead, and it was hypothesized by Hayflick that cells possess finite replicative property which is correlated with ageing. According to his observations, after reaching the “Hayflick limit” of approximately 50-60 rounds of division, cellular growth is inhibited but the cells remain metabolically active. Hayflick further suggested

that cancer cells are initially mortal and need to undergo multiple physiological changes in order to attain their immortality(Shay & Roninson, 2004)

1.5.6Relative Importance Of Senescence Pathways In Human And Mouse

In normal mouse fibroblasts, stress-mediated senescence signals depend mostly on the ARF/p53 pathway. In human, they are transmitted via both the ARF/p53 and p16INK4A/RB pathways, (Shay & Roninson, 2004) and there is a cross talk between p53/p21 and RB. Replicative potential of human fibroblasts is enhanced with optimal culture conditions as well as in co-culture onto a feeder cells layer, denoting the importance of extracellular matrix context in the contribution of *in vitro* senescence delay. Cancer development and tumourigenesis are limited chiefly by apoptosis and the induction of senescence. In human, senescence is regulated by three pathways that act in parallel or in collaboration: p19ARF/p53/p21, p16INK4A/pRB and the DNA damage response. (Networks, 2016) Most importantly, the activation of senescence blocks tumourigenesis*in vivo* as shown in figure(Deursen, 2014)., which suggests that triggering a senescence response as a check to cancer progression may be a potential therapeutic prospect.(Afanas, 2014)

1.5.7 Upregulation Of Tumour Suppressor Pathways

The induction of senescence is mediated by the activation of p19ARF/p53 and p16INK4A/pRB pathways. Phosphorylation of p53 at serine 15 by ATM mediates the response to DNA damage generated by activated oncogenes. Activated p53 triggers the transcriptional activation of numerous target genes, including *CDKN1A* (p21), *MDM2*, *PAI-1*, *GADD45A*, and *PML*, and also stimulates the appearance of PML nuclear bodies (NBs). Increased levels of hypophosphorylated pRB are also noticeable due to the increased expression of its upstream positive regulator, p16INK4A. Increased p16INK4A/pRB signalling induces chromatin compaction which forms DNA foci detectable under microscopy by DAPI staining(Pawlikowski et al., 2013) In response to *RAS*, these senescence-associated heterochromatin foci (SAHF) are produced (Park, Lim, & Jang, 2011) partly through stimulation of H3K9 methylation and co-localization of chromatin condensation factors.

1.5.8 Induction Of The DDR Pathway

Enhanced DNA damage signalling resulting either from replicative stress or activated oncogenes is also a well-characterized hallmark of senescent cells (Fumagalli et al., 2012; Mallette et al., 2007; Bartkova et al., 2006; di Fagagna et al., 2003; Di Micco et al., 2006; Moiseeva et al., 2006). Activation of ATM upon DNA damage triggers the phosphorylation of histone H2AX (□H2AX) which can be readily observed by immunostaining. An appreciation of the levels of activation of many DNA damage response proteins, such as ATM, ATM, CHK1, CHK2 and p53 also further characterize a senescence phenotype.

DNA Damage

Chemotoxic agents such as doxorubicin or etoposide inhibit the functions of topoisomerase II enzyme, which indirectly generate double strand breaks. It is still uncertain how the extent of DNA damage dictates the choice between senescence or apoptosis, but low levels of sustained DNA damage preferentially causes senescence(Networks, 2016)

vivo Exposure to ionizing radiation directly induces DNA double strand break, while that to UV radiation indirectly produces single strand damage by generation of covalently bound pyrimidine dimers. Low dose of either ionizing radiation or UV generally produces senescence, while high dose stimulates apoptosis;;. (Yang, Wang, Ren, Chen, & Chen, 2017)

DNA damaging agents involved in recent radiotherapy or chemotherapy promote senescence both *in vitro* and *in De-repression of Tumour Suppressors* (Sulli & Micco, 2012)

The signalling of tumour suppressors is usually kept in check in unstressed cells. Ectopic activation of p21 induced senescence in human fibrosarcoma cells (Demidenko et al., 2010). Restoration of p53 in sarcomas (Rozan & El-deiry, 2007) or hepatocarcinomas also led to cell cycle arrest and senescence. In breast cancer, inactivating p53 mutations occur in only < 20% of the cases where transcriptional inhibition of *TP53* is a prevailing feature as

shown in (Fig: image 4) These data suggests that restoration of *TP53* expression or other tumour suppressor genes might participate in the restitution of senescence in cancer cells. Abbas and Dutta 2010)

1.5.9 Phenotype Features Of Senescent Cell

In vitro and *in vivo* identification of senescent cells is possible through the observation of distinct features which, collectively, serve as biomarkers. These features may vary among different tissue types or experimental settings. It is therefore advisable to visualize senescence markers in their entirety rather than to rely on a particular marker).

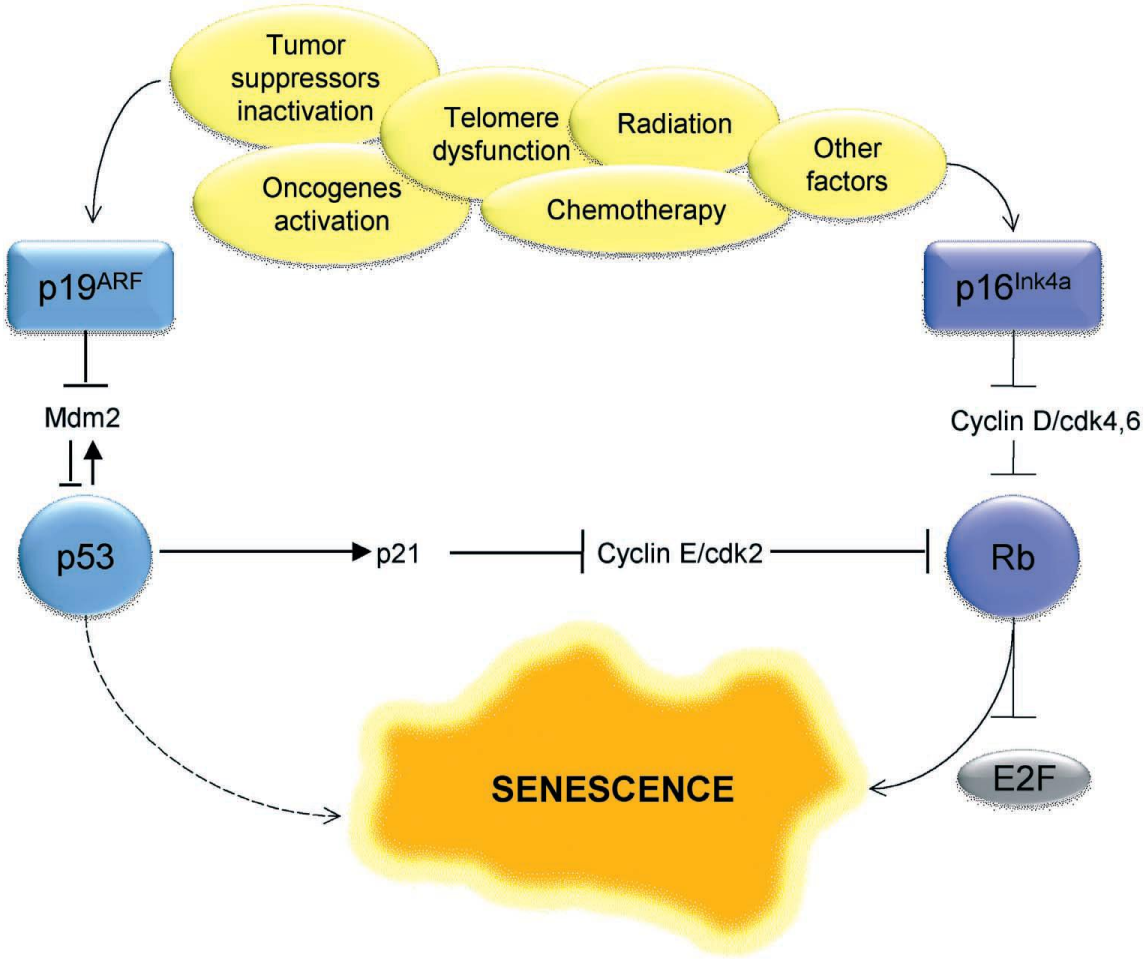


Fig: image 4 adapted from(Chuaire-noack, S, Roc, Rez-clavijo, & The, 2010)

1.5.10 Cell Cycle Arrest During Senescense

The most easily and directly observable characteristic of senescent cells is the marked growth arrest. Senescent cells are principally arrested in G1/S (Tsai, Hsu, Huang, Lin, & Pan, 2016)(but can also senesce during the G2/M transition (Gewirtz, 2013)((The irreversibility of the cell cycle exit is somewhat unclear, as the levels of expression of p16INK4A), or the extent of activation of *KRAS* prior to the entry in senescence appear to dictate the strength of senescence irreversibility. (Coppe´ et al., 2008).

1.5.11 Morphological Analysis Of Senescent Cell.

Microscopic analysis of senescent cells *in vitro* also reveals a flat morphology with extended spindle-shaped cytoplasm) and SAHF (Narita et al., 2003) but this feature does not appear *in vivo*The misshapen cytoskeleton and altered membrane organisation appear to be dependent upon CDK5 activation by pRB *RAS*-induced senescent cells often exhibit a vacuolated cytoplasm caused due to endoplasmic reticulum stress in response to protein misfolding (Chan et al., 2005). And also due to disruptiontion of F-actin.(Aifuwa, Giri, Longe, Lee, & An, 2015)

1.5.12 Increased SA-β-gal Activity

Another characteristic of senescent cells is the increased senescence-associated β-galactosidase (SA-β-gal) activity at at pH 6 is probably the most widely utilized method foridentification of senescent cells, which can be assessed biochemically (Dimri et al., 1996). Cycling cells express the β-galactosidase encoded by the *GLB1* gene which displays normal activity at pH 4. Expansion of the lysosomal compartment and expression of a senescence-related isoform of *GLB1* may be responsible for increased SA-β-gal activity at pH 6 (Again, it is of utmost importance to measure the general level of activation of other senescence markers, in combination with SA-β-gal activity, since this activity increases in aged, confluent, but non-senescent cells as well (Yang & Hu, 2005).)

1.6 ROS Cross Talk With HDAC

ROS works on histone and DNA, by working in organized epigenetic phases, during regulation of mitochondrial and nuclear DNA. Cumulative of ROS occurs in transformed cells, cultured with HDACi such as TSA, vorinosatat and butyrate. In several tumour cells, ROS oxidation –reduction pathway are important. Thiredoxin (Trx) acts in antioxidant pathway which working on scavenging ROS(Uttara, Singh, Zamboni, & Mahajan, 2009). HDACi works on Trx binding protein (TBP2) which leads to increase in ROS linked to downregulation of Trx in cancer cells but not in normal cells. Which serves in senescence, autophagy and apoptosis. analyses (Yang and Hu, 2005).

1.6.1 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are evolved through different pathways that occur both inside as well as outside the cell. Reactive oxygen species (ROS) is produced as the unavoidable consequence of aerobic metabolism. Mitochondria catalysed electron transport reactions, UV light irradiation, X-rays, gamma rays, chronic inflammation, lipid peroxidation, and environmental pollutants are the common stimuli for ROS induction. These molecules are of considerable interest because they behave as novel signal mediators that have a role to play in growth, differentiation, progression, and death of the cell. Under a physiological state, a dynamic balance is established in the level of cellular ROS, and cellular processes that generate ROS and destroy them maintain this stability.(Circu & Aw, 2010)

1.6.2 Types of Reactive Oxygen Species

Atomic oxygen has two unpaired electrons in separate orbits in its outer electron shell. This electron structure makes oxygen susceptible to radical formation. The sequential reduction of oxygen through the addition of electrons leads to the formation of a number of ROS. ROS comprise of chemical species that include at least one oxygen atom in each molecule but are more highly reactive than diatomic oxygen. Examples of ROS include free radicals such as superoxide, hydroxyl radical, and singlet oxygen, besides non-radical species such as hydrogen peroxide which is generated by the

partial reduction of oxygen.(Di Dalmazi, Hirshberg, Lyle, Freij, & Caturegli, 2016)

1.6.3 Intrinsic Sources of ROS

Production of the highly reactive ROS is mainly because of the reduction of molecular oxygen (O2) through high-energy exposure, or electron-transfer reactions. There are two main sources of cellular ROS: first, there are those biological processes, in particular the mitochondrial oxidative metabolism, which release ROS as a by-product, or a waste product, of other different necessary reactions. The primary product of the mitochondrial respiratory chain is chiefly evolved by complexes I and III and could be rapidly converted into H_2O_2 by the enzyme superoxide dismutase (SOD) and then could be reduced to water by catalase or glutathione peroxidise.(Marchi et al., 2012) Second, there are those processes, in response of a cell to xenobiotics, cytokines, and exogenous bacteria, that produce ROS as necessary intermediates intentionally, either in molecular synthesis or in breakdown, as part of a signal transduction pathway, or as part of a cell defence mechanism. NADPH oxidases (Nox) including Nox1 to Nox5 and Duox1 and Duox2, which are grouped into three classes, according to the presence of domains in addition to the gp91phox (NOX2) domain, are another important source of cellular ROS.(Poljsak, Šuput, & Milisav, 2013)

Besides, external stimuli including tumour necrosis factor-α (TNF-α), epidermal growth factor (EGF), Interleukin-1β (IL-1β), and hypoxia and irradiation also stimulate the production of ROS.

1.6.4 ROS And Cell Signalling

The Classical view held that ROS were host cell defence molecules produced by only phagocytic cells like neutrophil with purpose of eliminating invading pathogens such as bacteria; however, the vast majority of recent work has demonstrated that ROS have a role in cell signalling, including; apoptosis; gene expression; and the activation of cell signalling cascades. It is indeed remarkable that ROS can have pivotal roles as both intra- and inter-cellular messengers. ROS can modulate Bcl-2 expression levels, thereby influencing

the role of Bcl-2 to induce cell death through the necrotic or apoptotic pathway. Apoptotic regulation involves receptor activation, a change in the expression levels of the Bcl-2 family of proteins, caspase activation, and mitochondrial dysfunction. C-Jun N-terminal kinase (JNK), or stress-activated protein kinase (SAPK), members of the mitogen-activated protein kinase superfamily (MAPK), are also involved in ROS-mediated cell death. In tumor cells, at high dose, ROS induced Bax, caspase-3 , 8 and 9, activation of PARP-1, TNF-α, NF k B, HIF-1 α, Nrf2 and HABP1/P32 while, Bcl-2 and HDAC 1 and 2 were downregulating. When at low to moderate concentrations, ROS induces cellular senescence by upregulating p16 and p21 and autophagy and play a beneficial physiological role as anti-tumourigenic species.(Saha, Chowdhury, Dutta, Chatterjee, & Ghosh, 2013)

1.6.5 Oxidative Stress And Cancer

Oncogenic stimulation has been associated with elevated levels of ROS, and thus oxidative stress can be considered a significant category of carcinogen. Chronic inflammation is linked with an increased risk of various types of human cancers, and inflammation is associated with the induction of oxidative stress and LPO, which generate excess ROS and DNA-reactive aldehydes. Cancer development is characterized by the cumulative action of multiple events in a single cell with initiation, promotion, and progression stages; the ROS are involved in all stages. (Reuter, S., et al 2010).

Cells cultured *in vitro* are exposed to non-physiological nutrients and O_2 levels, and are maintained in a completely distinct extracellular environment. These culture stresses lead to premature senescence. Mouse cells are more sensitive than human cells to O_2, but their culture can be prolonged by incubation at physiological O_2 levels In addition, MEFs constitutively express telomerase but are still readily sensitive to oxidative stress Dienstbier, R. (2014)(Deursen, 2014). One interesting characteristic of senescent MEFs is that they can spontaneously bypass the replication arrest barrier imposed by senescence. It is thought that prolonged exposure to oxygen generates cumulative mutations that lead to the inhibition of tumour suppressor pathways(Mazo, Lleonart, Castellvi, & Cajal, 2011)(Shay & Roninson, 2004).

Indeed, disruption of the p19ARF/p53/p21 pathway is observed in immortalized MEFs, and the downregulation of miRNAs associated with tumour suppressor functions seems to be partly responsible for this senescence bypass (Rizzo et al., 2011).

When the level of ROS exceeds the anti-oxidant defence mechanisms, a cell is said to be in a state of “oxidative stress.” Since oxygen free radicals exhibit such strong reactivity, they possess the potential to cause a number of deleterious events such as peroxidation of lipids, damage to nucleic acids, enzyme inhibition, oxidation of proteins, activation of programmed cell death (PCD) pathway, and ultimately leading to cell death.

Cumulatively produced ROS/RNS in the body induce a cellular red-ox imbalance and subsequent bio-molecular damage. Oxidative stress is a common mechanism in senescence, and the emergence of various kinds of cancers and neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS).

1.7 HDAC And Mechanism Of Cell Death

Histone acetylation is known to cause upregulation of transcriptional activity. Acetylation levels are maintained by concerted actions of two enzymes: histone acetyl transfereases (HATs) and histone deacetylases (HDACs). Gong, F.,et al (2013). Accumulating knowledge has brought to light that HDACs are important class of protein-modifying enzymes that are instrumental in cell proliferation, chromosome remodelling, and gene transcription. Many tumours are known to involve deregulated or upregulated HDAC activity, thereby justifying the use of HDAC inhibitors for cancer treatment. HDAC inhibitors have dose-dependent effect. In a few cases, growth arrest is brought about at low doses, while apoptosis is brought about at higher doses; in other circumstances, growth arrest come before apoptosis. Strikingly, normal are almost always considerably more resistant than tumour cells to HDAC inhibitors.(Rikiishi, 2011)

1.8 Apoptosis

There are two types of programmed cell death: Type I, apoptosis; and Type II, cytotoxic autophagy. Type I, apoptosis, involves an energy dependent process that is controlled by the transcription and translation of proteins involved in DNA repair and regulation of cellular homeostasis. Propagation of this response can occur via the extrinsic or intrinsic pathways(Maiuri, M. C.,et al . 2007).(Circu & Aw, 2010)

1.8.1Extrinsic Pathway Of Apoptosis

The extrinsic cellular death pathway involves activation of receptors belonging to the death receptor subclass of the TNF receptor family such as CD95 (cell death receptor 95/APO-1/ Fas) and TNF-related apoptosis-inducing ligand receptor I (TRAIL/ DR4) (144).(Mary, B.et al 2012). These receptors are pre-associated homotrimers located within lipid rafts on the plasma cell membrane and, upon binding of their respective ligands, undergo a conformational change enabling binding of death domain adaptor proteins to form the death-inducing signalling complex (DISC) The DISC complex is composed of the activated receptor bound to its respective ligand and the corresponding death domain-associated adaptor proteins, such FADD; which recruits and specifically binds to the effector caspase, procaspase-8 (Sessler, T et al 2013) Upon its recruitment, procaspase-8 undergoes self-cleavage into its active form, caspase-8. Caspase-8 activation is suppressed by expression of cellular FLICE-like inhibitory protein 1 (c-FLIP-1) and cellular FLICE-like inhibitory protein s (c-FLIP-s) (Mary, B.et al 2012). Activated caspase-8 then can either: cleave BID to truncated-BID (tBID), which by way of the mitochondrial pathway leads to cell death; or directly lead to the cleavage and activation of caspase-3, promoting cell death.(Rahal et al., 2014)

Caspase-3 may then translocate to the nucleus and deactivate DNA fragmentation factor-45 (DFF45) through cleavage of the protein, disabling it from binding to and inhibiting the DNA fragmentation factor-40 (DFF40) enzyme. DFF40, otherwise referred to as caspase-activated DNase, is then able to perform its enzymatic duty of DNA fragmentation, thus leading to a cell's demise Mary, B. Et al (2012).

1.8.2 Intrinsic Pathway Of Apoptosis

The intrinsic apoptosis pathway involves intracellular insults which promote activation of proapoptotic Bcl-2 family member proteins (Czabotar, P. E et al 2014). The mitochondrial component of the extrinsic pathway is termed the intrinsic apoptosis pathway. Extrinsic apoptotic pathway events leading to the cleavage of BID into its active form, tBID, by caspase-8 enables its translocation to the mitochondrial membrane. tBID associates with anti-apoptotic Bcl-2 family member proteins, causing their release from association on the outer mitochondrial membrane with Bax and Bak. Bax and Bak then undergo conformational changes which enable permeabilization of the mitochondrial membrane and the release of factors, in particular cytochrome c, cytochrome c interacts with Apaf-1 and together, this complex causes activation of procaspase-9. Active caspase-9 induces cleavage and activation of caspase-3 and eventual cell death).Sessler, T et al 2013 show Inhibition of the intrinsic apoptosis pathway has previously been shown to suppress the cytotoxicity of several thymidylate synthase (TS) inhibitors, including pemetrexed. (Bareford, M. D., et al 2011) The intrinsic apoptotic events occurring at the mitochondrial membrane resemble the signalling events that take place in the case of autophagy induced cellular death Type II.

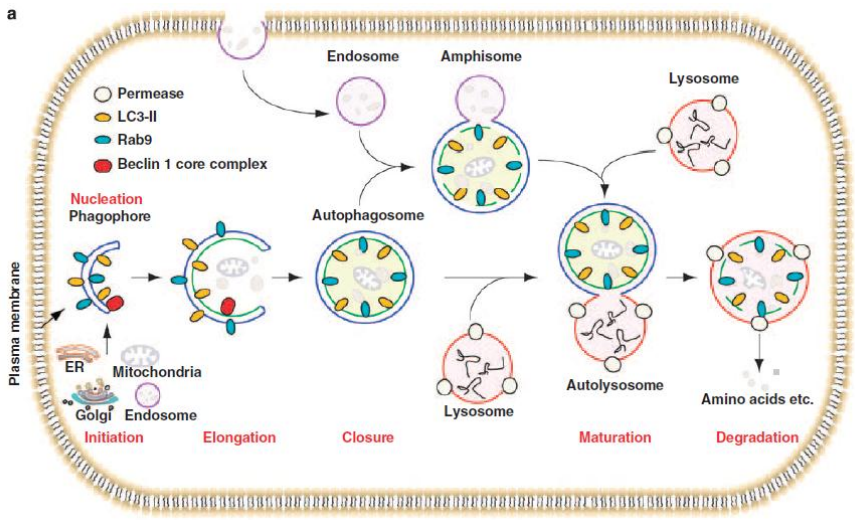
1.8.3 Apoptotic Cell Death Induced By Histone Deacetylase (HDAC) Inhibitors

(HDAC) inhibitors can trigger apoptosis in cancer cells, although the detailed mechanism is yet to be established. Cell death mediated by apoptosis is achieved by a family of cysteine proteases and caspases. There are two main caspase activation pathways are categorised, first one is death receptor pathway, and another one is the mitochondria or cytochrome-c mediated pathway. Death receptor pathway is associated with binding of protagonists to members of thesuperfamily of TNF or nerve growth factor receptor can activate the caspase (caspase-8), which further activates effector caspase (caspase-3).(Sznarkowska, Kostecka, Meller, & Piotr, 2017) While, in cytochrome c regulated pathway, several provocations come together at mitochondria and induce release of cytochrome-c to the cytoplasm, a process which is regulated by the Bcl-2 family of proteins. After release of cytochrome-

c induces the main facilitatorApaf-1 which allow to form a multimeric protein complex i.e. apoptosome. Further, the apoptosome activates caspase-9, which afterward activates the downstream other caspases. HDACi is known for downregulation of Bcl-2,and upregulation of Bax, p53 and other tumorsuppressor involve in apoptosis(Westphal, Dewson, Czabotar, & Kluck, 2011).

1.9 Autophagy

Autophagy is a natural process occurs in normal dormant cells for the purpose of defence to removing pathogens. In order to maintain and restore and homeostasis, autophagy process have important role. Autophagy is a self-digestive phenomenon which make sure the lysosomal degradation of unnecessary and/or injured organelles and mis-folded proteins. Defects in autophagy have been associated with many diseases and health problems such as, neurodisorder, early aging, microbial infection, Crohn's disease and cancer. In addition, the key role of autophagy in cancer is reasonably complicated. Autophagy helps in tumour suppression during cancer development, on other hand it also contributes to tumour survival during progression of cancer. Autophagy can be used to resist tumour cells against several anti-cancer agents. In several reports, therapeutic advantages against several cancer could be achieved by inhibition of autophagy.



stages of autophagy (Fig image 5)adapted from(Kang, Zeh, Lotze, & Tang, 2011)

1.9.1 Cell Death By Autophagy

Continuous activation of autophagy lead to increase of turnover of proteins and organelles more than survival threshold, thus improving the efficacy of treatment. In this regard, cell death occurred via autophagy could provide an alternative platform for therapeutics. Aautophagy is apparently induced after dying of cells after failed effort to cope with tremendous stress. autophagic markers such as accumulation of autophagosome and up-regulation of the LC3 II. Cell death by autophaghy should be associated with the following conditions: 1) Death of the cell without apoptosis; 2) Autophagic flux should be increase and 3) Cell death can be stopped or prevented by either pharmacological inhibitors.

1.9.2 HDAC And Autophagy Relationship

Recently, histone deacetylases (HDACs) is an important factor in cancer biology, and this enzyme family has been shown to promising role in autophagy.(Witt, O., et al 2009). Further, HDAC inhibitors (HDACi) are being used for cancer therapy and clinical trials. West, A. C., et al (2014). Along with the rapid development of HDACi which are able to target selectively each and individual HDAC isozymes. This shows great prospective for targeted specific therapy that has more well-defined effects on cancer biology and lower toxicity. HDAC enzymes are evolutionarily conserved enzyme family are involved in several cellular processes including proliferation, differentiation, and autophagy. Further, their established roles has been explored in both cytoplasmic signalling as well as epigenetic regulation of gene expression. Abnormal expression of HDAC family member enzymes leads to various types of cancer such as lung cancer, breast cancer, colon cancer, and pancreatic carcinoma. (Croce, C. M.et al 2009). HDAC enzymes are crucial on different levels for the change of autophagy. Reducing cells of class I HDACs encouraged autophagic flux, which is further proved accumulation by of the LC3-II which is an autophagosomal marker. Class I and IIa HDACs are linked with inhibition as well as induction of the beginning stage of autophagy.(Lee et al., 2012) (Koeneke, Witt, & Oehme, 2015). However, changes in the transcription or expression of autophagy related proteins such as Beclin1 for nucleation, or as ATG7 or LC3B for elongation. Autophagy

initiation could be prevented by these family members. On the other hand, Class IIb HDACs have been observed to support the later phase of autophagy. (Ana Gonzalez-Polo, R., et al 2015).

1.10 Crosstalk Of ROS/RNS And Autophagy

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) play significant role in regulating cell survival as well as death. (Fubini, B., et al (2003)). However, several research papers has been revealed on the role of ROS/RNS in breast cancer cells. In order to maintain redox balance, cells generate and eliminate of ROS/RNS species. However, judicious levels of ROS/RNS using for signals promoting cell proliferation and survival, while several fold increases of ROS/RNS induce cell death(Y. Zhang, Wu, Tashiro, Onodera, & Ikejima, 2011). There is an intricate and conjoint relationship between different pathways that control degree of ROS and autophagy (L. Zhang et al., 2016). Autophagy is induced by several stimuli in cells and among several stimuli ROS are one of these autophagy activators. (Maiuri, M. C et al (2007). Accumulation of ROS in the cells activates autophagy, which regulates the accumulation of ROS in cells by stimulating the clearance of damaged mitochondria and oxidized cellular substrates.(D'Aquila P, Bellizzi D, 2015)

1.11 Bioctive Compounds

Bioactive compounds are necessary and unnecessary compounds (e.g., flavonoids or polysaccharides) that can be isolated naturally, are part of the food chain, and can be shown to have an impact on human health.

Bioctive Compound are common secondary metabolites in plant produce such as fruits, vegetables, and spices that have been proved to be favourable for human health due to properties like antioxidant effect, inducing detoxification enzymes, inhibiting nitrosamine formation, binding/diluting carcinogens in the digestive tract, altering hormone metabolism, and modulating carcinogenic cellular and signalling events. Recently, the ability of bioactive compounds to prevent and treat human cancer through genetic and epigenetic modifications has been validated by gathering results. Crozier, A et al (2009). The marine

organisms also provide a rich source of bioactive compounds for the treatment of several human diseases and also used as traditional medicine.

1.11.1 Bioactive Compounds And Action Mechanism Of Active Compounds In Cancer Prevention

The growth of cancer cells, migration and invasion can be prevent by bioactive compounds such as apigenin, reservatol, lycopene,Thymoquinone, querecitin, silibinin etc shows its effect in a dose and time dependent manner. The bioactive compounds induced apoptosis by the activation of caspases up-regulation of anti-apoptotic Bcl-2], an increase in p53 expression] and a decrease in cyclins B1 and D1. NF-κB is a target which is associated with cell growth inhibition and apoptosis inducton in cancer cells[106]. Thus, the effects of bioactive compounds on the cell cycle explains the anti-tumor activity or cell growth inhibition.

It was also observed that the administration of bioactive compounds helped activation of caspase-3,-7, and -9 (Sznarkowska et al., 2017) and cause the cleavage of poly-ADP-ribose polymerase (PARP), with reduction of constitutive phosphorylation, nuclear localization and the reporter gene activity of STAT3 (signal transducer and activator of transcription-3). It is also weakening the expression of STAT3 target gene products like survivin, c-Myc, and cyclin-D1, -D2, and increases the expression of cell cycle inhibitory proteins p21 and p27(Woo et al., 2013).After administration of bioactive compounds, it has shown controlled phosphorylation of upstream kinases like Janus-activated kinase-2 (JAK2), epidermal growth factor receptor (EGFR), tyrosine kinase, and Src kinase,which cause apoptosis in cancer cells through blocking of STAT3 signalling via inhibition of JAK2 and Src-mediated phosphorylation of EGFR tyrosine kinase . (Darakhshan, Bidmeshki Pour, Hosseinzadeh Colagar, & Sisakhtnezhad, 2015)

It was also seen that bioactive caused an inhibition of angiogenesis by suppressing AKT/ERK signaling pathway *in vitro* and *in vivo* and a prevention of tumor angiogenesis in xenograft human prostate cancer (PC3) model of mouse.(de Blas et al., 2016) Moreover it also showed no chemotoxic side effects, inhibiting human prostate tumor growth at low dosage. The ability of

bioactive compounds to facilitate telomere attrition by inhibiting the activity of telomerase was also observed. The induction of apoptosis by bioactive compounds is through the modulation of multiple targets and is supposed to be extremely useful for apoptosis in cancerous cells. The induction of apoptosis is p53-dependent and p53-independent pathway . Bioactive compound is known to exert anti-proliferative effects on Akt mediated mTOR signaling and its inhibitory effect on molecular crutches of translational machinery involved in cyclin D1 expression. It is an Akt suppressor which inhibits breast cancer cell survival, and prevents Akt induced therapeutic resistance as an adjuvant in combinational therapies of combatting breast cancer(Farrand, Oh, Song, & Tsang, 2014)

Bioactive compound also arrests cancer cells and canine osteosarcoma cells (COS31), in the G_1 phase It was seen that in cancer cells, an arrest is associated with a decrease in cyclin D1 and an increase in p16, whereas in cancer cells, there is an increase in p21 and p53 and a decrease in anti-apoptotic Bcl-2 protein and upregulation of Bax after treatment with bioactive compounds With an increase in p53 and a decrease in cyclin B1, bioactive compounds also caused a G_2-M phase arrest in spindle carcinoma cells. Bioactive compounds also caused apoptosis by activating caspase-3, caspase-9 and caspase-8 in p53-null myeloplastic leukemia HL-60 cells]it has shown inhibition in proliferation of canine osteosarcoma (COS31) and the cisplatin-resistant variant (COS31/rCDDP), human ovarian adenocarcinoma (BG-1) cells and human breast adenocarcinoma (MCF7), but had very little effect on Madin-Darby canine kidney cells. These data suggest the anticancer potential of bioactive compounds(Cirmi et al., n.d.).

1.11.2 Role Of Some Bioactive Compound In Epigenetic Modulation

Allium Vegetables

Reports suggest that Allium vegetables such as garlic (*Allium sativum*), onions (*A. cepa*), leeks (cultivar of *A. ampeloprasum*), and chives (*A. schoenoprasum*) protect against stomach, prostate, bladder, and colorectal cancer owing to the presence of organosulphur compounds and mainly allyl

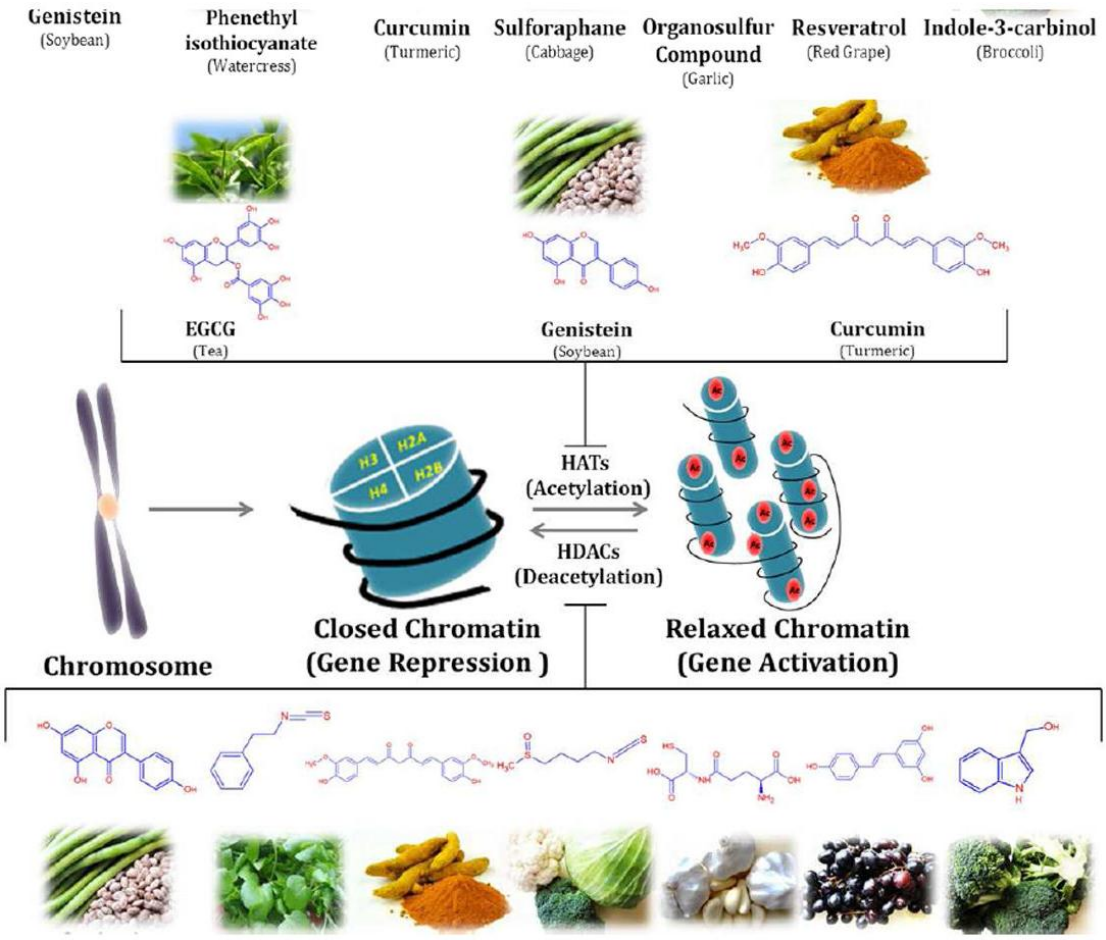
derivatives.as shown in (fig image 6) The garlic compounds, in particulardiallyl disulphide, allylmercaptan, allylisothiocyanate, butanethiol, and their metabolites, are shown by present data to inhibit HDAC activity and increase the acetylation of core nucleosomal histones and thereby favour cell differentiation *in vitro* as well as in vivo. Upadhyay, R. K. Et al (2017).

Apigenin

Apigenin is a flavonoidfound in abundance in common fruits such as grapefruit (*Citrus paradisi*), and vegetables such as parsley (*Petroselinumcrispum*), onions (*A. cepa*), oranges (*C. sinensis*), tea (*Camellia sinensis*), camomile, and wheat sprouts. It is known to prompt cell cycle arrest and apoptosis in several solid tumours and haematological cancers. And also inhibit HDAC activity Crozier, A et al (2006).

Chrysin

Chrysin is a polyphenolic compound that is present in fruits, vegetables, olive oil, tea (*C. sinensis*), and red wine. It was found to be cytotoxic to breast, colon, and prostate cancer cells.(Huang, W. Y et al 2009).



(Fig Image -6)Phytochemicals targeting HDAC figure adapted from (Shankar, Kumar, & Srivastava, 2013)

Cruciferous Vegetables

Broccoli, Brussels sprouts, cabbage, cauliflower, collard greens, kale, kohlrabi, mustard, rutabaga, turnips, bokchoy, and Chinese cabbage are examples of cruciferous vegetables (family Brassicaceae, also called Cruciferae). They contain in plentyglucosinolates and their hydrolysis products, including indoles and isothiocyanates such as indole-3-carbinol (I3C) and sulphoraphane (SFN), which have been extensively studied in experimental *in vitro* and *in vivo* carcinogenesis models. Especiallynotable results have been obtained in animal models of lung, colorectal, oesophageal, prostate, and bladder cancer. Dias, J. S. Et al (2012).

Curcumin

Curcumin, the biologically active component of the Asian spice turmeric (*Curcuma longa*), shows anticancer effect in many human cancer cell lines. Several ongoing clinical trials have tested the efficacy of curcumin for the treatment of pancreatic cancer, multiple myeloma, and colorectal cancer it also induces HDAC inhibition as shown in (fig image 6).(Thakur, Deb, Babcook, & Gupta, 2014)(Anand, P et al . 2008).

Ginger

The active chemicals contained in ginger (*Zingiberofficinale*) are reported to be phenylpropanoid-derived compounds including gingerols and shogaols. As dehydrated products of gingerols, 6-shogaols are found in fresh ginger at low levels but exist in larger amounts in dried ginge. 6-Shogaol is one of the most bioactive components of ginger rhizomes. Zam, W., et al. (2017).

Grape Polyphenols

Resveratrol is a polyphenolic chemical present in grapes (*Vitisvinifera*) and wine that has shown great potential for the prevention and treatment of cancer. It has been shown that resveratrol has the chemical structure to inhibit the activity of different human HDAC enzymes. Baur, J. A.,et al . (2006).

Green Tea

It has been reported that green tea and its major active component, epigallocatechin-3-gallate (EGCG), have anticancer activity on different cancers by downregulating HDAC. Khan, N., et al (2006).

Organoselenium Compounds

Se-methyl-L-selenocysteine and selenomethionine are amino acid components of selenium-enriched yeast and seleniferous plants such as garlic (*A. sativum*), onions (*A. cepa*), and broccoli (cultivar of *Brassica oleracea*). Epidemiological data and laboratory investigations provide strong evidence of the crucial role of organoselenium compounds possessingwide-

rangingchemopreventive properties against different human cancer cells (bladder, prostate, breast, ovarian, colon, liver, lung, and leukemic).

Quercetin

Quercetin is a dietary flavonoid commonly found abundantly in fruits, vegetables, and beverages, is known to exert powerful anticancer effects against a broad spectrum of tumours By upregulating ac-H3 in lung cancer both *in vitro* and *in vivo.* (Chan, Yang, Huang, Liao, & Yeh, 2013)

Soy Isoflavone

Genistein is the chief constituent of soy isoflavones found in soybeans. Genistein treatment decreased cell growth and proliferation in colon cancer cell lines. Genistein has also been shown to inhibit cell proliferation in prostate cancer, breast cancer, and colon cancer upregulate histone H3K9 acetylation in prostrate cancer cell(Phillip et al., 2012)

Aims & objectives

“Cancer is a generic term for a large group of diseases characterized by the growth of abnormal cells beyond their usual boundaries that can then invade adjoining parts of the body and/or spread to other organs” (WHO, 2017). It occurs due to modulation of signalling pathways leading to loss of cell cycle checkpoint control in genetic and epigenetic structure. It is a disastrous condition occurs mainly by different environmental factors that mutate genes encoding critical cell-regulatory proteins. Among all types of cancer, cervical cancer is the fourth most common cancer in women, and the seventh overall, worldwide. Approximately 528,000 new cases and 266,000 deaths from cervical cancer are reported yearly worldwide and the majority of deaths occur in the less developed regions of the world (Cancer & Sheets, 2016). In Indian women (aged 30–69 years), cervical cancer is the leading fatal cancer (Dikshit et al., 2012) in both rural and urban areas, with somewhat higher rates in rural areas, and approximately 132,000 new cases and 74,000 deaths reported annually (Kaarthigeyan, 2016) in spite of successful Papanicolaou smear-based screening and treatment course. It is also reported that during aggressive tumor malignancies cancer cell show chemo-radiation resistance and epigenetic/phenotype changes.

Epigenetic modifications are an integral part of cellular development and differentiation. Out of various epigenetic regulations, it is known that HDACs contribute to cancer survival through epigenetic silencing of tumor suppressor genes and deacetylation of tumor suppressor proteins. Upregulation of HDAC isoforms has been observed in many cancer types and In cervical cancers, some HDACs such as HDAC1 and HDAC2(Glaser et al., 2003) (Barneda-zahonero & Parra, 2012), have been found over expressed. During advanced malignancy transcriptional repression are seen which is mainly due to epigenetic regulation of Histone deacetylases (HDACs). Aberrant recruitment of HDACs has been found to have a causal role in tumorigenesis for nearly every type of cancer (e.g. ovarian neuroblastoma, pancreatic, liver, colon and cervical cancer. Moreover, as HDACs regulate many essential cellular

processes, not only through epigenetic control, but also through non-epigenetic protein deacetylation.

Histone deacetylase inhibitors (HDACi) have shown great promise as cancer therapeutics through their ability to arrest proliferation in many transformed cell lines through cell cycle arrest, apoptosis, cellular differentiation, and inhibition of angiogenesis, antagonize tumor growth, augment chemotherapy, reverse chemoresistance ,attenuate metastasis, halt epithelial-mesenchymal transition and block tumor immune evasion and cell migration. SAHA (Vorinostat, Zolinza) have shown synergistic killing of HPV-positive, cervical cancer cell lines. Similarly, treatment of HeLa cells xenografts with the combination of Bortezomib and TSA retarded tumor growth combining HDAC inhibitors with radiotherapy—a vital method for the treatment of a variety of cancer types as an adjuvant, neoadjuvant and palliative modality.(Li & Zhu, 2014) Several HDAC inhibitors enhance the radiosensitivity of cancer cells, including TSA,SAHA, and valproic acid HDAC inhibitor-mediated radiosensitization has been shown in various cancer cell lines including breast, prostate, lung, colon, cervical and head and neck(Thaler, 2012) (Kortenhorst, Carducci, & Shabbeer, 2006). There are also side effects of synthetic HDAC inhibitors including nausea, vomiting, diarrhoea, and dehydration. More severe side effects also occurs, specifically neutropenia (decrease in blood neutrophils) and thrombocytopenia (decreased platelets) were observed(Subramanian, Bates, Wright, Espinoza-delgado, & Piekarz, 2010).

To address these problems, the cancer treatment of the future will incorporate, within a single molecule, elements that allow for simultaneous targeting of multiple therapeutic targets, while maintaining lower or no side effects. Furthermore, epigenetic defects may eventually lead to genetic defects which are not reversible, appropriate time of interventions using bioactive compounds, which might be effective in slowing down cancer progression may be critical. Hence, it is important to design and perform

appropriate experiments needed to address these issues and to analyse the data obtained in an efficient manner. Recent years has seen greater attraction towards natural medicine in the form of plant derived bioactive compound and is widely used as a substitute for synthetic chemical drugs as they are compatible in curing a wide range of diseases.(Farrand, Oh, Song, & Tsang, 2014)

Bioactive compound are a vast group of natural compounds that not only provide much of the flavour and colour of our plants and algae but also responsible for remarkable biological and medicinal activity. Bioactive compounds and its derived active compounds may be achievable alternatives for the treatment of cancer and its complications without any adverse effects. Bioactive compounds may act as pro-oxidant or anti-oxidant. Pro-oxidant refers to those bioactive compounds that induces oxidative stress either by generation of ROS or by suppressing antioxidant systems It can include all reactive, free radical containing molecules in cells or tissues. Antioxidant are those compound that inhibited ROS generation and Bax translocation and led to a significant protection against oxidative stress induced apoptosis (Manuscript, 2013).

In recent years, the role of epigenetics in the development and treatment of cancer has gained interest and the effects of internal and external factors on the epigenetic profile are under investigation. Recent reports indicate that bioactive compounds can prevent or reverse these epigenetic modifications in cell culture studies and in some animal models of cancer. Appropriate exposure time is critical for bioactive compounds to intervene the epigenetic process. (Rajendran, Ho, Williams, & Dashwood, 2011)Design of novel HDACi has become a popular research avenue in recent years a database (HDACiDB) has been established to accommodate this wealth of information(Pharmacy, Sciences, & Florida, 2017)

In view of the present reports that even crude extracts from some plant are highly active, and relatively more active than some of the hitherto known

secondary metabolites from the same plant,. However, it should also be considered in that way the active compounds in the extracts may act synergistically in such a way that the bioactive potential of the extracts are higher than that of their pure components(Aiyelaagbe et al., 2011) and the reason for such better response from crude may be due to some Significant interactions occur which are not evident when single constituents are studied in isolation, whereas some herbal medications may produce a more favourable response when administered in pure form.(Search & Tutorial, 2017)

Therefore, there is a need to establish a therapeutics, whose cytotoxic activity is selective (i.e. causes minimal damage to healthier tissues and eradicated tumour cells). In this regard, natural substances with minimal toxicity is proposed to be a solution to the problem In the present study three bioactive compounds were selected.

Fucoidans are a complex series of sulfated polysaccharides found widely in the cell walls of brown seaweeds. In recent years, different brown algae were analyzed for their content of fucoidans including Pelvetia canaliculata Fucus vesiculosus , F.evanescens, F.serratus , F.distichus, Sargassumstenophyllu, Ascophyllum nodosum and other brown algae). Such polysaccharides do not occur in other divisions of algae and in land plants(Atashrazm, Lowenthal, Woods, Holloway, & Dickinson, 2015). Thymoquinone is found in herbs and spices. Thymoquinone is a major constituent of seed oil of black cumin, Nigella sativa (Woo et al., 2013). Diosmin, a bioflavonoid (3 ,5,7-trihydroxy-4 -methoxyflavone 7-rutinoside) present in Citrus fruits, (Hsu, Lin, Cheng, & Wu, 2017). Yet these compounds are rarely studies in Epigenetic modulation to prevent the cancer.

Thus keeping all the above stated literature in mind, the following objectives have been proposed.

To identify the effect of bioactive compounds e.g. Fucoidan, Thymoquinone, Diosmin on HeLa cell line.

To study the modulation in epigenetic signalling cascade and In-silico studies to establish their effects.

Comparative analysis of the effects of crude and pure Fucoidan both from Fucus vesiculosus species on survivability and toxicity on cervical adenocarcinoma HeLa cells.

Materials and Methods

3.0Materials

Fucoidan F5631 and Fucoidan pure≥ 95% F8190 ,Thymoquinone, Diosmin and SAHA, High Glucose DMEM, cell culture antibiotics, monodansylcadaverine, acridine orange, propidium iodide (PI), DMSO, antibodies HDAC1, p16, p21,β-Actin and secondary antibodies from sigma , (DAPI) nuclear stain were purchased from Sigma–Aldrich (USA). BECN, p53,Bax and Bcl-2 from Santa Cruz Biotechnology. FBS was obtained from Invitrogen (Gibco). Alexa-Fluor-488 and H2DCFDA,mitosox red, JC-1 , rhodamine 123 and rhodamine-conjugated phalloidin were obtained from Molecular Probes, Inc. HDAC Activity/Inhibition Assay kit (epigenetex), ATP bioluminescent assay kit (Sigma–Aldrich). PVDF membrane from Millipore . All other chemicals used were standard molecular grade. water used for media preparation and other reagent preparation was either triple disttiled or autoclaved Milli –Q (Millipore purification system USA) .

Methods

3.1 Maintenance Of Mammalian Cell Culture

HeLa cells were grown in high glucose DMEM supplemented with 10% FBS, 100µg/ml streptomycin and 50 µg/ml fungizone in tissue culture dishes and plates. Cells were grown in at 37º C with 5% CO_2 humid condition. Cells were cultured in monolayer, and sub-cultured routinely by trypsinization. Cells were grown for 24 hrs prior treatment to attain 60-70% confluency and treated with bioactive compounds, for 24 hrs.

3.2 MTT Assay For Cell Viability

For cell viability, 2×10^3 cells were seeded in 96 well plates for 24 hours and then treated with Fucoidan at various concentrations from 0 to 300µg/ml for 24 hours, then 15µl MTT (5mg/ml) was added to the cells and kept in the incubator for 3-4hr, then media was discarded and 150µl of DMSO was added to the solubilizing the formazan crystals. Absorbance was measured on Spectramax M5 Spectrophotometer at 570nm. And all the other experiment are performed at below IC 50 that is low doses, 60 and80µg/ml for Fucoidan,30 and 40µM Thymoquinone, 75 and 100 µM Diosmin and with

500nM SAHA whereas for pure Fucoidan µg/ml 150 and 200 µg/ml and NAC N-Acetyl-L-cysteine 2mM used only in ROS assay .

3.3 Assessment Of Reactive Oxygen Species (ROS) Generation

HeLa cells were grown for 24 hours in 6 well plate for FACs analysis and in 12 well plate for microscopic analysis. After 24 hours cells were treated with 60 and80µg/ml for Fucoidan,30 and 40µM Thymoquinone, 75 and 100 µM Diosmin and with 500nM SAHA whereas for pure Fucoidan µg/ml 150 and 200 µg/ml, for 24 hours and then incubated with H2DCFDA for 20 mins. Cells were harvested in PBS and DCF fluorescence was detected using a FACs VERSE flow cytometer. ROS levels were expressed as mean fluorescence intensity as performed by BD FACS Verse System and data was analysed by BD FACS Suite Software and similarly fluorescent microscopic analysis was done under CarlZiess epifluorescence fluorescence microscope at excitation (488 nm) and emission (530 nm), equipped with Axiocam camera system with axiovision software (Carl Zeiss, Germany) (Banerjee & Mandal, 2015).

3.4 Assessment Of Mitochondrial ROS (Super Oxide anion)

HeLa cells were grown in 12 well plate and were treated with for 24 hours and then incubated with MitoSOX™ Red for 20 mins. Cells were harvested in PBS and MitoSOX™ Red excitation at 510 and emission 580nm, fluorescence were detected using a FACs VERSE flow cytometer. Superoxide anion were expressed as mean flourescense intensity as performed by BD FACS Verse System and data was analysed by BD FACS Suite Software. (You & Park, 2014).

3.5 ATP assay

HeLa cells were grown in 12 well plate and were treated with Fucoidan 80 µg/ml, TQ 40µM ,Diosmin 100 µM and SAHA500nM, which were then analysed for ATP content using an ATP bioluminescent assay kit (Sigma–Aldrich), as per the mentioned protocol.

3.6 HDAC Activity/Inhibition Assay

HeLa cells were grown in six well plate, And Subsequently after treatment with Fucoidan 80 µg/ml, TQ 40µM ,Diosmin 100 µM and SAHA500nM . HDAC activity/inhibition was evaluated by HDAC Activity/Inhibition Assay kit (EpiGentek), as per the manufacturer's protocol.

3.7 Mitochondrial Membrane Potential Detection (JC-1 & Rhodamine 123)

JC-1 a cationic dye which is lipophilic in nature was used to measure change in Mitochondrial membrane potential in Live cells using fluorescent microscopy and spectroscopy. JC-1 dye accumulates in the mitochondria and forms aggregates which appears red at high MMP but at low MMP, JC-1 emits green fluorescence., HeLa cells were grown over cover slip for 24 hr, after the treatment for 24 hrs the cells were incubated with 5 µM JC-1 in dark for 35 min at 37º C. After incubation cells were washed three times with PBS and immediately viewed and captured under CarlZiess fluorescence microscope, excitation at 488 nm (blue) and emission at 510-530 nm (green) and 650 nm (red), equipped with Axiocam camera system with axiovision software (carlzeiss,Germany)(Banerjee & Mandal, 2015). Similarly FACS analysis was done after culturing HeLa cell in 6 well pates after treatment of cell were incubated with Rhodamine123 solution at 10µM concentration in dark for 30 min at 37° C .and Cells were harvested in PBS and analysed by BD FACS verse system at excitation 488 and emission at 525 nm(Sun, Chen, Wang, Chen, & Wei, 2011).

3.8 Monodansylcadaverine (MDC) assay:

HeLa cells were grown over cover slip for to 24 hr, after the treatment f(Fucoidan 80µg/ml, TQ40µM Diosmin 100 µM and SAHA 500nM)or 24 hr, the cover slips were washed with PBS three times then incubation of the live cells with 50 µM MDC in PBS for 10 min at 37º C for Autophagic vacuoles staining. after incubation cells were washed again four times with PBS and immediately viewed and captured under CarlZiess epifluorescence fluorescence microscope excitation/emission at 335/525 nm, fitted with

axiocam camera system with axiovision software (carlzeiss, Germany) (Saha et al., 2014).

3.9 Autophagosomes Detection By Acridine Orange:

HeLa cells were grown over cover slip and after incubation the coverslips are washed thrice with PBS and incubated with acridine orange 20µg/ml for 15 min at 37º C. Incubated cells were washed again four times with PBS and immediately analyzed under CarlZiess epifluorescence fluorescence microscope excitation at 488 nm (blue) and emission at 510-530 nm (green) and 650 nm (red), fitted with AxioCam camera system with AxioVision software (CarlZiess, Germany) at similarly at same wavelength analysis was performed by BD FACS Verse System and data analysed by BD FACS SuiteSoftware(Di, Shiu, Newsham, & Gewirtz, 2009).

3.9 Detection Of F-Actin

HeLa cells were grown over cover slip for 24 hr after the treatment for 24 hrs the cells were then fixed in 2% paraformaldehyde in PBS for 10 min and then wash with 0.1M glycine for 5min to quench excess aldehyde. Cells were permeabilised using 0.1% Triton X-100 for 1min , wash with PBS and incubate with rhodamine conjugated phalloidin then wash with PBS , viewed and captured under the CarlZiess fluorescence microscope, excitation at 540 nm and emission at 565 nm, equipped with Axiocam camera system with axiovision software (carl Zeiss, Germany(Yang, Lee, Lee, & Moon, 2014).

3.10 Cell Cycle Analysis Using Flow Cytometer.

For DNA content Cell cycle profile was assessed using flow cytometry.Briefly, 2×10^6 cell were trypsinized, fixed in 70% chilled ethanol for 30 minutes at -20°C, then rehdyrate with PBS and resuspended in PBS containing 125 U/ml RNase A then add 50 µg/ml propidium iodide.For cell cycle profile,DNA content was analyzed by flow cytometry using the (Beckman countler) at AIRF JNU(You & Park, 2014).

3.11 Autoflourescense Analysis By Flow Cytometry

HeLa cells were grown in 6 well plate were harvested in PBS after 24 hour treatment and were assessed immediately to avoid cell aggregation for changes in AF (488–530 nm), cell size (FSC-A)and Granularity (SSC-A) .Flow cytometric analysis was performed on 10,000 cell events cell debris with using BD FACS Verse System and data analysed by BD FACS SuiteSoftware.(Maurya, Agarwal, & Ghosh, 2015)

3.12 SAHF Assay And Apoptotic Nuclei Staining By DAPI

As for for SAHF(senescence-associated heterochromatin foci) staining, HeLa cells after treatment (Fucoidan 80µg/ml TQ40µM Diosmin 100 µM)for 24 hours were harvested in PBS were incubated with ethanol for 1 h. After centrifugation, the cells were incubated in 500 µl PBS containing 1 µg/ml of 406-diamidino-2-phenylindole (DAPI) at room temperature for 5 min. was analyzed under CarlZiess epifluorescence fluorescence microscope, excitation at 350 nm, equipped with Axiocam camera system with axiovision software (carlzeiss, Germany)(Park, Lim, & Jang, 2011).

3.13 Senescence Associated β-Galactosidase Activity Assay

HeLa Cells were grown on 12 well plate on coverslips for 24 hours then treated with low dose of Fucoidan 80µg/ml ,TQ40µM,Diosmin 100 µM and SAHA 500nM for 24 hours were fixed with 2% formaldehyde, washed with PBS and then stained with solution containing 5mM Potassium hexacyano-ferrate (III) solution, 40mM citric acid/Na phosphate buffer,5mM Potassium hexacyano-ferrate (II) trihydrate solution 150 mM NaCl, 2mM Magnesium chloride hexahydrate solution and 1mg/ml X-gal solution for max 16hr. at 37°C washed 3 times with PBS and viewed under microscope(Luo, Yang, Schulte, Wargovich, & Wang, 2013)

3.14 Morphological Analysis By Haematoxylin-Eosin Staining

HeLa Cells were grown on 12 well plate on coverslips for 24 hours then treated with Fucoidan (80µg/ml and 150 µg/ml) and Fucoidan pure 200 µg/ml, TQ40µM Diosmin 100 µM and SAHA 500nM for 24 hours then cell were wash

twice with PBS and then fixed with chilled methanol followed by rinsing twice with 99% ethanol for 2 min, similarly with 95% ethanol for 2 min. Thereafter cells were washed with water followed by two minutes staining with Hematoxylin. After thorough washing in distilled water, Eosin was added for 1 min. Cells were again rinsed in double distilled and then dehydrated by 2 changes of 95% ethanol then followed by 2 changes of 99% ethanol for 2 min. each. Finally, coverslips were mounted with 50% glycerol and observed under light microscope(Saha, Ghosh, & Datta, 2014)..

3.15 Immuno Cytochemical Analysis

Sub-cellular localization of HDAC1 and p21 proteins in normal and treated cells grown on cover slips in 12-well plates, was done following the standard process of our lab. the cells were fixed on coverslips with paraformaldehyde (1%) for 3 min, wash with PBS and then permeabilized with Triton X-100 (0.1%). After blocking with 3% BSA in PBS pH 7.2, for 60 min at 37˚C, the cells were incubated with the specific primary antibody for 3 h at 37˚C and were then washed with PBS (thrice for 10 min). Then, the cells were keep with the fluorescently tagged secondary antibody dilution in the dark for 45 min at 37˚C in a humid chamber followed by three to four washing with PBS. Thereafter, the cells were stained with DAPI, washed, and mounted in 50% glycerol in PBS. viewed and captured under CarlZiess fluorescence microscope, excitation at 540 nm and emission at 565 nm , equipped with epifluorescence Axiocam camera system with axiovision software (carlzeiss, Germany) (Saha et al., 2014).

3.16 Western Blotting

Hela cells were grown in 100 mm culture dishes and after appropriate treatment cells were harvested by scrapping. Cell lysate was prepared by sonication supplemented with protease inhibitors. Protein concentration was measured by Bradford protein estimation method. Equal concentrations of protein were loaded and seprated by SDS-PAGE was run and the proteins and transferred onto PVDF membrane using Bio rad semidry gel protein transfer. Following this Membranes were then incubated with antibodies against HDAC1, p16, p21 (sigma), BECN1, Ac-Histone H3 Antibody (Lys9/14)

(Santa Cruz) for over night at room temperature and subsequently with the appropriate alkaline phosphatase-conjugated secondary antibodies for 3 hour and then the blots were developed using NBT and BCIP solution. And atleast three experiment was done and analyzed the bands intensity using image studio software(Saha et al., 2014).

3.17 Apoptosis Assay

Apoptosis of HeLa cell was determined by Flow cytometry.after cell were treated and then stained witth FITC- conjugated Annexin V and PI . and were analysed usinf BD FACS verse system excitation at 488 nm and emission at 525nm and 575 nm respectively result were analysed BD FACS suite software (Sun et al., 2011).

.3.18 Protein Quantification By Flow Cytometry

Cell were grown in 6 well plate and after the appropriate treatment of Fucoidan,TQ,Diosmin and SAHA for 24 hrs the cell were trypsinized and collected in PBS and after washing with PBS twice cell were fixed in 70% ethanol and then the cell were incubated with primary antibody for 3-6hours followed by washing twice with PBS and further incubated with alexa flour 488 for 1-3hour. Cells were analyzed immediately flow cytometrically using BD FACS Verse system,at excitation 488 nm and emission at 525 nm, analyzed with BD FACS Suite software(Ronzoni, Faretta, Ballarini, Pelicci, & Minucci, 2005)(Friedrich, Ray, Laffin, & Lehman, n.d.)(Chung et al., 2005)

3.19 Molecular Docking

The binding association of Fucoidan (PubChem CID: 92023653),Thymoquinone (PubChem CID: 10281), Diosmin (PubChem CID: 5281613) andSAHA(5311) and HDAC1 (PDB code 4BKX) was performed on molecular dock modelling software Auto Dock (4.2) and pyMOL were employed to validate the possible protein-ligand association.**Auto Dock:**The first step is to retrieve required Ligand and Target.pdb files from major databases. The second step is preparing PDBQT format files for Target protein and Ligand (Target.pdbqt, Ligand. pdbqt) and Grid and Docking Parameter file (a.gpf and a.dpf) using AutoDock 4.2. The

Final step is to perform molecular docking using ***Cygwin*** http://www.cygwin.com/install.html and finally the results are visualized using Pymol . The HDAC1 (PDB ID:4BKX) crystal structure was obtained from RCSB protein data bank. Based on the research, the SAHA was used as a control(Shakil, 2013)

3.20 Liquid Chromatography-Mass Spectrometry Of Crude And Pure Fucoidan

LC-MS analysis was performed for both crude and pure Fucoidan compound using an electrospray ionization source was Performed at AIRF JNU, using LCMS - *Waters* SYNAPT G2 with 2D nano ACQUITY System it was also attached to a Synapt G2 Q-TOF system (Waters) fitted with an electrospray ionization (ESI) source.

LC-MS and MS/MS spectra were analysed using software, https://xcmsonline.scripps.edu/landing_page.php?pgcontent=mainPage

to tentatively identify peaks and bioactive compounds in both crude and pure Fucoidan.graphical representation on Easyplot software.(Iqbal et al., 2014)

3.21 Statistical Analysis.

All the result analysed as mean value ± the standard deviation (SD). Student t-test or ANOVAs analysis for variables of different groups that were compared. Alevel of *P< 0.05 and** p<.001 was accepted as being statistically significant.

Effects Of Fucoidan, Thymoquinone And Diosmin Treatment On The Hela Cells

4.0 Introduction

Cancer is a multifactorial disease that can affect any part of the body. Several factors either from inside and outside, which contribute to the development of cancer. For a long time, cancer has been considered to be the result of a wide variety of genetic and genomic changes, such as translocations, amplifications, deletions, and point mutations.(Ropero & Esteller, 2007) These have shown dramatic and contrasting end-point phenomena leads to activation of oncogenes and the inactivation of tumor-suppressor genes(Moradzadeh, Tabarraei, & Sadeghnia, 2015). Currently, development of cancer is not restricted to the genetic modulations as described above, but also includes epigenetic modulations. Epigenetics is concerned with the inheritance of information based on gene-expression levels, as opposed to genetics, whose realm on the information transmitted on the basis of gene sequence. The main epigenetic modifications in mammals, and particularly in human being, are DNA methylation and posttranslational histone protein modifications such as methylation, acetylation, phosphorylation, etc(Y. Li & Seto, 2016).

Till the last decade, DNA methylation, particularly promoter hypermethylation using silencing of tumor-suppressor genes has been the most widely reported for epigenetic modification in human tumors But now the present scenario, lysine residues become acetylated in histone 3 and 4 has been studied widely. Levels of acetylation as a result of the balance of the activities of histone acetyltransferase (HAT) and histone deacetylase (HDAC). Histone acetylation levels play an important role in chromatin remodeling as well as in the regulation of gene transcription(Y. Li & Seto, 2016). The presence of acetylated lysine in histone tails is associated with more relaxed chromatin state and gene-transcription activation, while the deacetylation of lysine residues is associated with a more condensed chromatin state and transcriptional gene silencing (Johnstone, 2002; Iizuka and Smith, 2003).

Histone deacetylation increases the ionic interactions between the positively charged histones and negatively charged DNA, which gives a more condensed chromatin structure and represses gene transcription by limiting

the accessibility of the transcription assembly. In addition, histone acetylation has been associated with other functions such as chromatin assembly, DNA repair, and recombination (Polo and Almouzni, 2005; Vidanes et al., 2005).

Furthermore, HDACs regulate gene expression in other different ways. For example, HDACs form corepressor complexes with the nuclear receptor in the absence of a ligand. Other studies indicate that HDACs may regulate the expression of a large number of factors by direct interaction with transcription factors such as E2f, Stat3, p53, the retinoblastoma protein, NF-κB, TFIIE, etc. (Lin et al., 2006). Moreover, HDACs are involved in the deacetylation of chromatin proteins, which can lead to altered gene-transcription regulation. In addition, deacetylation of non-histone proteins also occurs, which regulate important cellular functions to regulate cellular homeostasis cell-cycle progression, differentiation, and apoptosis (Minucci and Pelicci, 2006) Several studies have shown that certain HDAC family members are aberrantly expressed in many tumors (Stubbs et al., 2015). Whereas inhibition of HDACs upregulated the H3K9/14ac expression and overexpression of HDAC1 is reported in several cancer including cervical cancer cell (HeLa). HDAC inhibitors are key regulators of crucial biological processes in tumor inhibition that are targeted by either synthetic as well as by natural bioactive compound by various mechanisms including ROS.

Normal physiological conditions generate free radicals and other ROS in a broad range. ROS act as an intracellular signaling molecule regulates various physiological and biological cell processes. However, ROS also play a crucial role in many pathological situations including cancer, autoimmune diseases, cardiovascular diseases, and neuro-degenerative diseases. ROS also control the epigenetic regulation of both mitochondrial and nuclear DNA in an interconnected manner(Davalli et al., 2016). Some reports reveals that Mitochondrial ROS also upregulated during HDACi treatment in different cancer cells that sometimes result in autophagy and senescence process. (Rikiishi, 2011)

Autophagy is a catabolic process in response to starvation or other stress conditions to sustain cellular homeostasis. At present, histone deacetylase

inhibitors (HDACIs) for example TSA, romidepsin are known to induce autophagy in cells. Cellular senescence is an irreversible block of cellular division, and induction of senescence is being considered for treatment of many cancer types, mainly those resistant to classical pro apoptotic therapies. HDAC inhibitors (HDACis) NaB, resveratrol and quercetin induce senescence and/or apoptosis in many types of tumor cells For example, treatment with HDAC inhibitors induces the expression of the p16 and cyclin-dependent inhibitor $p21^{WAF1}$.Suberoylanilide hydroxamic acid (SAHA), a well-known HDAC inhibitor have strong antiproliferative effects on various cancer cell lines in the ROS-dependent manner (Kato et al., 2009) and is currently in clinical trial for the treatment of solid and hematological tumor (Y. J. Lee et al., 2012) and it is used as positive control in our studies. There are several therapies have been applied for cancer treatment and management including chemotherapy and radiotherapy but have severe side effects on human body. On this note, use of non-toxic bioactive compounds have no or lower side effects which make it suitable to be used as a food supplement so that it can provide a relief against cancer along with the on-going therapies.

Bioactive compounds

Bioactive compounds are majorly secondary metabolites in plant product such as fruits, vegetables, and spices that have been proved to be favourable for human health due to properties like antioxidant effect, inducing detoxification enzymes, inhibiting nitrosamine formation, binding/diluting carcinogens in the digestive tract, altering hormone metabolism, and modulating carcinogenic cellular and signalling events. Recently, the ability of bioactive compounds to prevent and treat human cancer through genetic and epigenetic modifications has been validated by gathering results(Phytochemicals, 2016)(Khan et al., 2015).

Fucoidan is a sulphated polysaccharide predominantly found in cell wall of brown algae(H. Lee, Kim, & Kim, 2012) and used as natural food ingredient with remarkable therapeutic potential comprising of anti-inflammatory, anti-thrombotic, anti-angiogenic (Xue et al., 2012), immunomodulatory, anti-

obesity , type 2 diabetes inhibition(Shan et al., 2016),nephro-protective, neuroprotective and anti-tumor activities. It is also used in clinical trials mainly for its anti-coagulant and anti-viral properties(Irhimeh, Fitton, & Lowenthal, 2009). However, its Epigenetic mechanisms have not been explored so far in cancer cells.

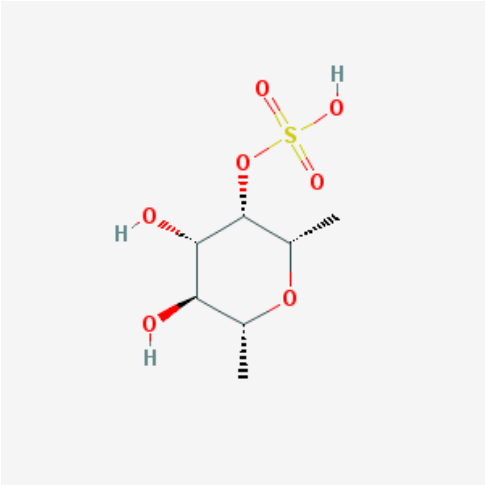


Figure 4.1 Fucoidan (pubchem)

Structure of the fucoidan molecule. For the brown algae *Fucus vesiculosus* , R is usually L -fucose, but it can also include other monosaccharides such as xylose and galactose.

Thymoquinone (TQ), an active component of *Nigella sativa* or black seed is used as a cure to all forms of illness dates back to a long time. Thymoquinone is an important bioactive compound extracted from the seeds of *Nigella*

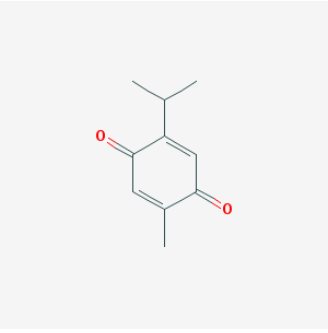


Figure 4.2 Thymoquinone (pubchem)

Sativa, known as the black seed, which has been used as a natural medicine for the treatment of several diseases and has been referred in the religious scriptures. Several active components isolated from *N. sativa*, includes compounds like thymoquinone, , dithymoquinone, thymohydroquinone thymol,

nigellimine-N-oxide, carvacrol, nigellidine nigellicine and alpha-hederin. Thymoquinone (TQ) with the chemical name of 2-isopropyl-5-methyl-1,4-benzoquinone and a molecular mass of 164.201 g/mol. Molecular Formula of Thymoquinone $C_{10}H_{12}O_2$(Darakhshan, Bidmeshki Pour, Hosseinzadeh Colagar, & Sisakhtnezhad, 2015)

Its well established properties such as antioxidant, hepatoprotective, anti-diabetic, nephroprotctive, neuroprotective anti-inflammatory, analgesic, anti-microbial , anti-cancerous, TQ also play protective role complex diseases autoimmune diseases, thrombosis and other cardiovascular complications . TQ also provide relief in all stress induced,radiation induced stress, heavy metal toxicity, optical disorders, female mid-age disorders and reproductive infertility. TQ, as a constituent of Nigella sativa seed showed positive effect against several inflammatory disorders like eczema, splenitis, rheumatoid arthritis, allergic asthma, allergic lung inflammation, experimental colitis.(Schneider-Stock, Fakhoury, Zaki, El-Baba, & Gali-Muhtasib, 2014)

Diosmin is a citrus fruit flavonoid present in rutinoside form of glycoside belonging to a flavone subclass and its flavanone analog is hesperidin (El-shafae & El-domiaty, 2001). Natural occurrence of diosmin

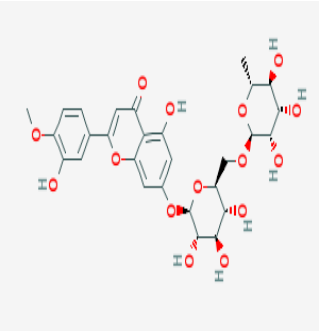


Figure 4.3 Diosmin (pubchem)

is in the form of glycoside which after ingestion by intestinal flora quickly transform to its glycone form, diosmetin . Despite being found in low concentration in *Citrus* fruits, diosmin display greater biological activity (Ali, 2013).Due to its activity as an important venotonic agent, It is present in a number of pharmaceuticals.

Diosmin has been reported to promote inhibition of cell growth in Caco-2 and HT-29 colon cancer cell lines.In HA22T hepatocellular carcinoma cells(Ali (Buddhan & Manoharan, 2017) , it displayed reduction in cell viability and cellular proliferative proteins, and induction of cell cycle arrest in the G2/M phase through activation of p53 and inhibition(Cirmi et al., n.d.) **.**Molecular Formula of Diosmin $C_{28}H_{32}O_{15}$, Molecular Weight**:** 608.549 g/mol (pubchem)

The aim of the present study is to assess the cytotoxic and in-vitro redox potential of the bioactive compound in Histone deacetylase inhibition, induction of Autophagy, and premature senescence, against human Cervical Epithelial Carcinoma HeLa cell and the modulation of associated signalling pathways by the bioactive compounds.

4.1Results

Throughout our study we have used HeLa cells as model system for studying the epigenetic modulation by bioactive compounds. Suberoylanilide hydroxamic acid (SAHA) histone deacetylase (HDAC) inhibitor, was used as an positive marker and Fucoidan used as in this chapter as crude form.

4.1.1Bioactive Compound Inhibits HeLa Cell Viability

Evaluation of the effects of Thymoquinone , Fucoidan, Diosmin on the growth of Hela cells showed a decrease in cellular viability in concentration dependent manner, by MTT assay the cells were found to show slower growth, distorted shape and are detached from the bottom of the plate .Furthermore, the numbers of viable cells decrease with increasing drug concentration. and IC50 value was calculated using linear regression formula and was found to be 242.11µg/ml ,77 µM, and 222µM respectively as shown in Fig (4.4)..

4.1.2Bioactive Compound Treatment Induces Reactive Oxygen Species (ROS) Generation In HeLa Cells.

ROS, a by-product of cellular metabolism play an important role in the various biological phenomenon. When Hela cells were treated with Thymoquinone ,Fucoidan, Diosmin and SAHA, they all showed an increase in total ROS generation in concentration dependent manner which was confirmed by the increase in green fluorescence intensity observed with both FACS analysis and fluorescent microscopy which demonstrated the gradual surge in ROS production at low dose of 60 and 80 µg/ml Fucoidan 2.4 and 2.6 fold increase and 30 and 40 µM Thymoquinone, with 1.67 and2.1 fold increases with and 75 and 100 µM diosmin treatmentwith 1.2 and 2.17 fold respectively, whereas SAHA at 500nM concentration showed 3.1 fold increase in ROS levelas shown in(fig 4.5, fig 7, fig 9 and fig4.12).

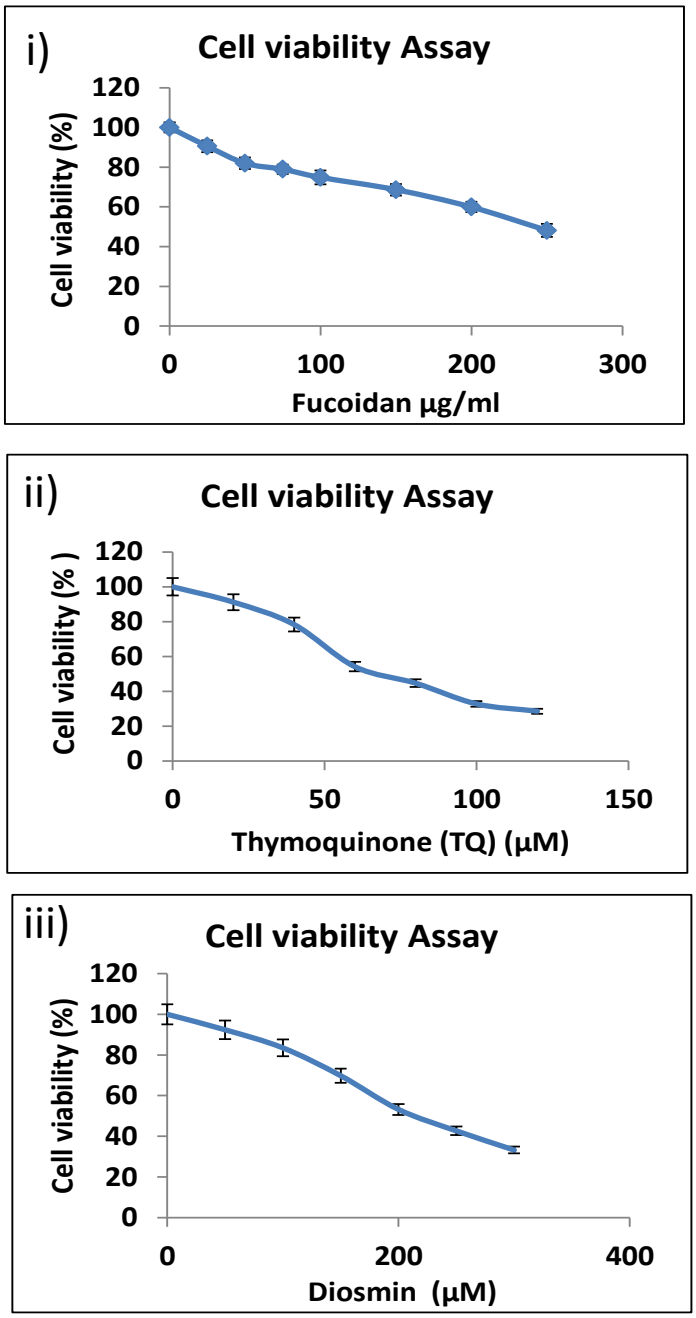


Fig.4.4 Bio active compound induced cytotoxicity in HeLa cells line was evaluated by determining cell viability by MTT assay. After treatment of i) Fucoidan concentration ranging from 0 to 250 µg/ml ,ii)Thymoquinone (TQ) 0 to 120 µM ,iii) Diosmin 0 to 300 µM in HeLa cells lines for 24 hrs. IC50 value was calculated using linear regression formula and was found to be 200µg/ml,77 µM, and 222µM respectively Values are means ± S.E.M. of at least three independent experiments

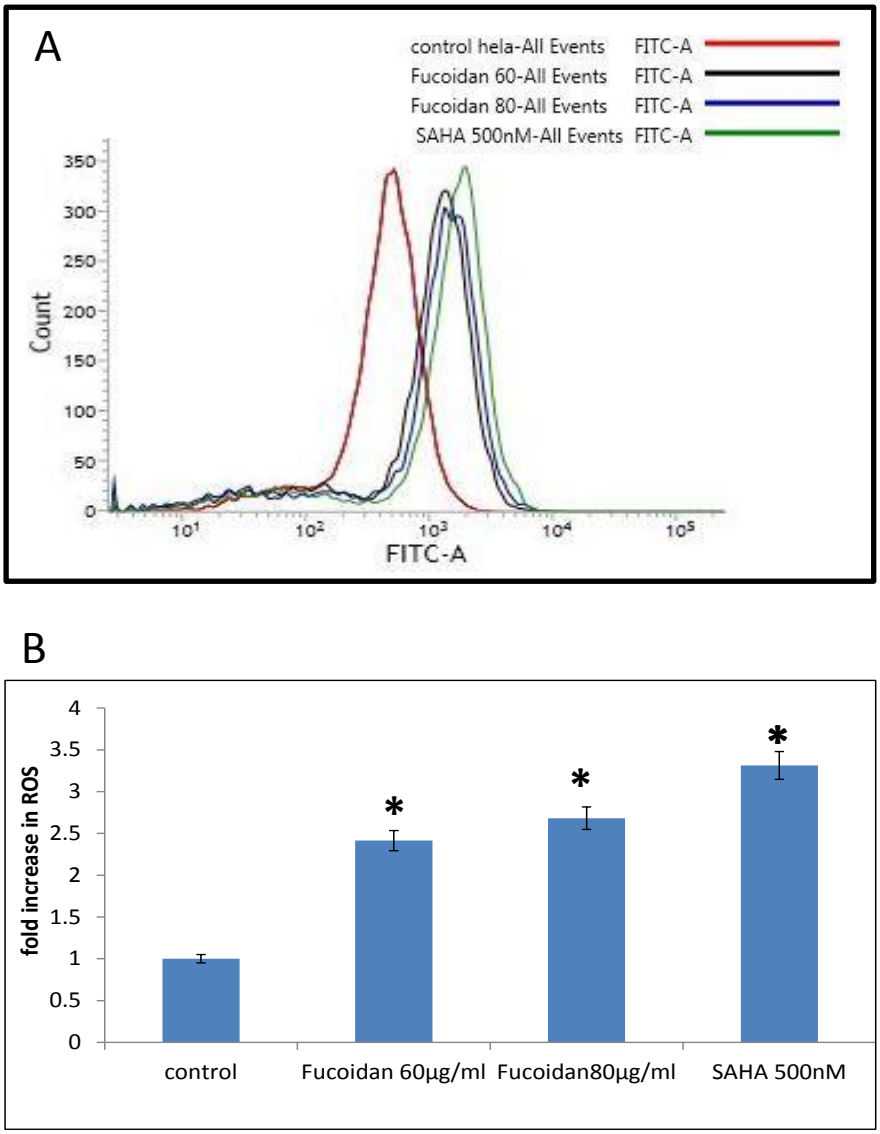
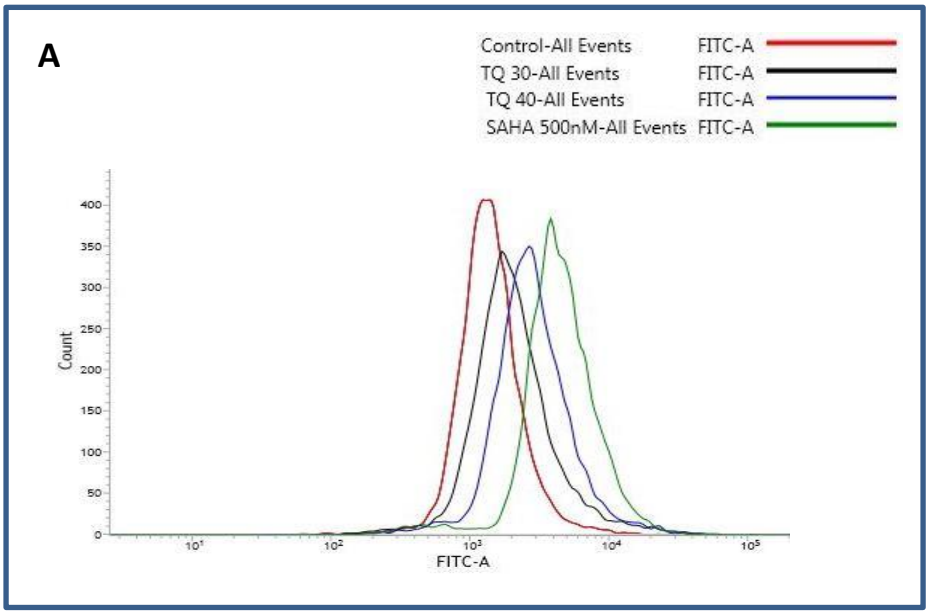


Fig.4.5 . ROS Analysis : HeLa cells were incubated with H2DCFDA and ROS generation potential were measured after 24hour of treatment, relative mean fluorescence intensity was analyzed by flow cytometry at a concentration of 60 and 80 µg/ml of Fucoidan and 500nM SAHA show enhanced ROS in comparison to control. B) Histogram profile showing fold change in ROS. (2.4 and 2.6 fold increase in Fucoidan and 3.3 fold in case of SAHA Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05



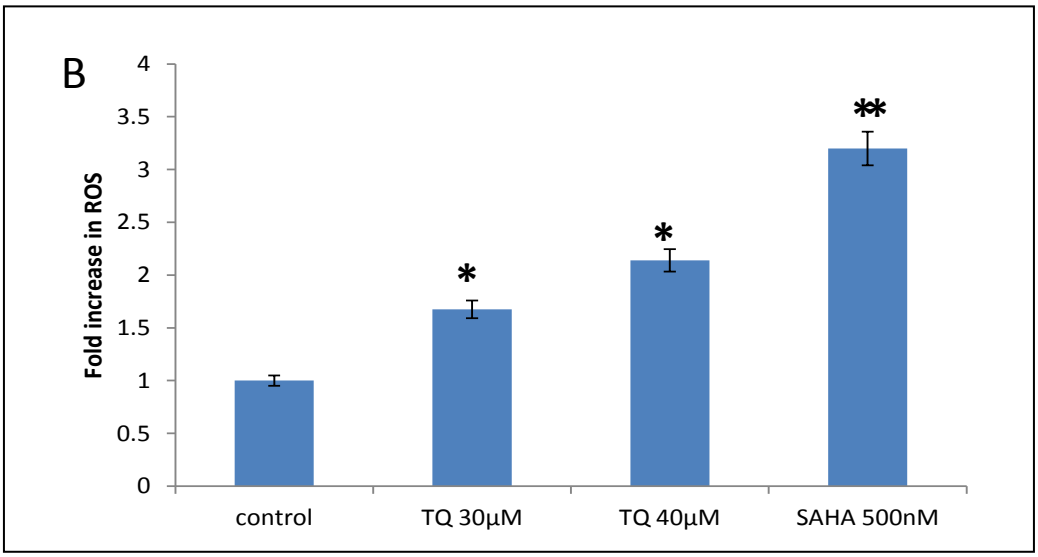
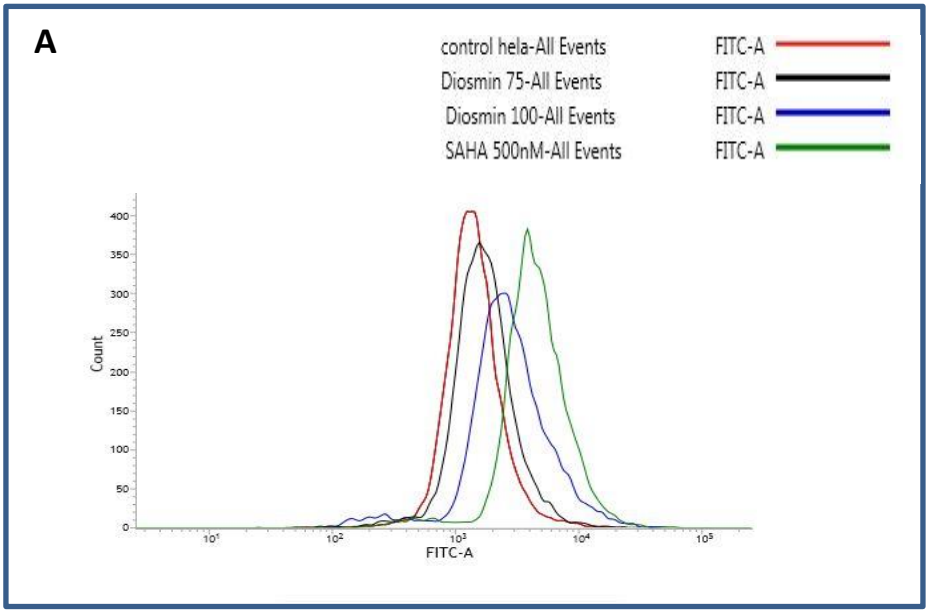


Fig 4.7 ROS Analysis : HeLa cells were incubated with H2DCFDA and ROS generation potential were measured after 24hour of treatment, relative mean fluorescence intensity was analyzed by flow cytometry at a concentration of 30 and 40µM of thymoquinone (TQ) and 500nM SAHA show enhanced ROS in comparision to control . B) Histogram profile showing fold change in ROS. (1.67 and 2.16 fold increase in TQ and 3.1 fold in case of SAHA Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05 ** denotes p<0.001



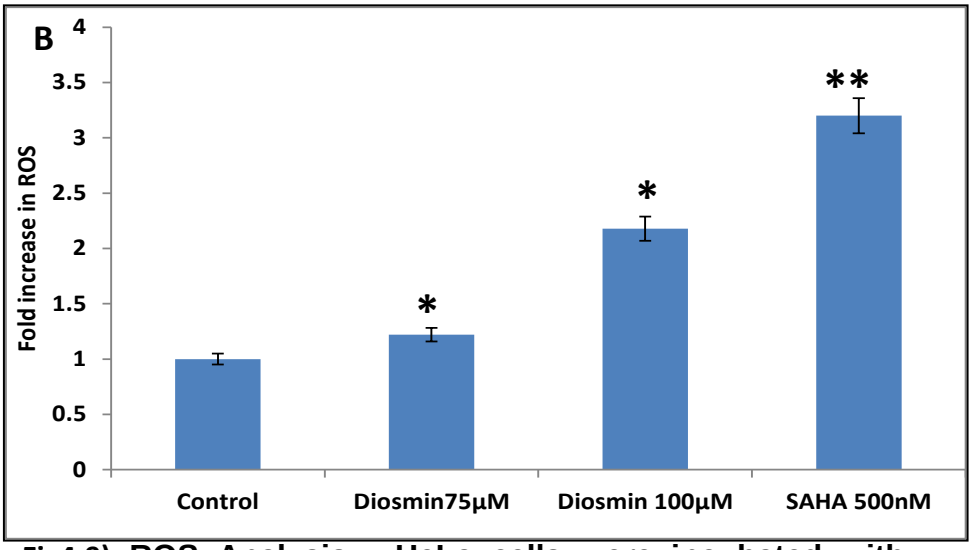


Fig4.9) ROS Analysis : HeLa cells were incubated with H2DCFDA and ROS generation potential were measured after 24hour of treatment, relative mean fluorescence intensity was analyzed by flow cytometry at a concentration of 75 and 100µM of Diosmin and 500nM SAHA show enhanced ROS in comparision to control . B) Histogram profile showing fold change in ROS. (1.22 and 2.17 fold increase in Fucoidan and 3.19 fold in case of SAHA Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05 ** denotes p<0.001

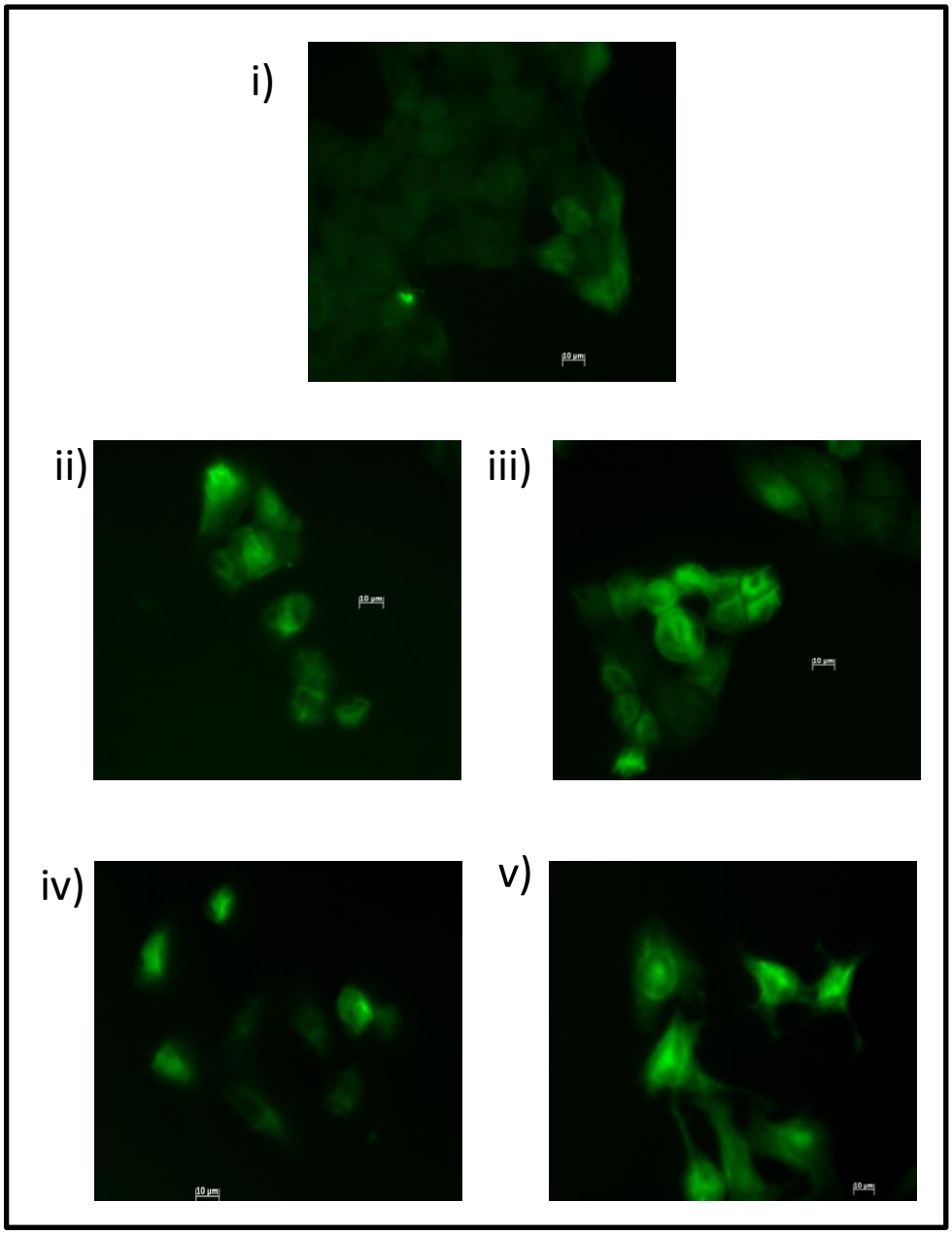


Fig 4.12) Fluorescence microscopy of H2DCFDA-stained cells Showing the total intracellular ROS change in Cell after treatment in HeLa cells (i). Control ((ii). Fucoidan 80 µg/ml (iii) TQ 40µM iV) Diosmin 100µM V) SAHA 500 nM

4.1.3Effect Of Pre-Teatment Of NAC With Bioactive Compounds In HeLa Cells

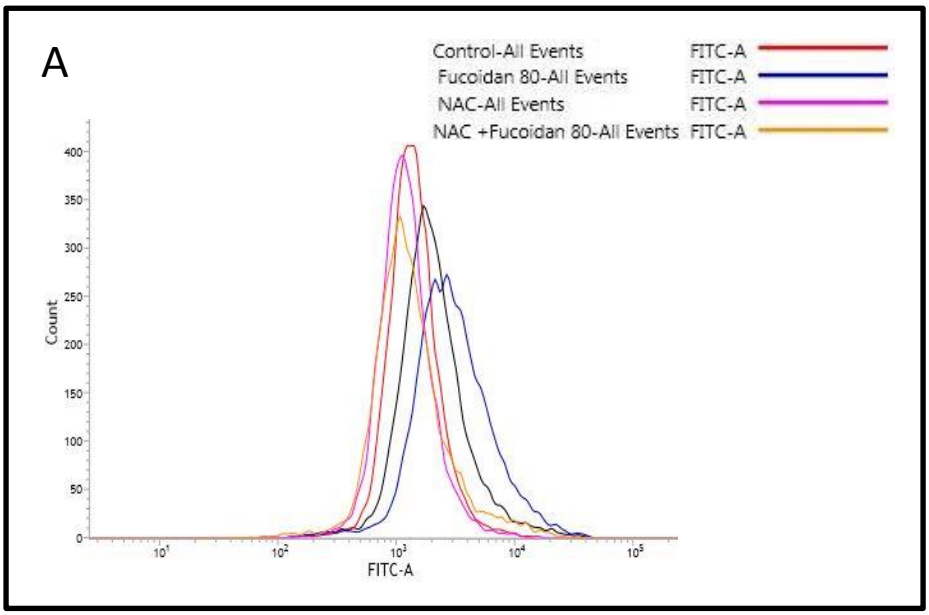
To confirm the generation of ROS is mainly due to bioactive compounds. HeLa cells is pretreated with the strong antioxidant NAC 2mM in the presence and absence of these bioactive compounds. FACS analysis in case of Fucoidan 80 µg/ml with NAC show 55% decrease in ROS, Thymoquinone 40 µM with NAC show 36% decrease ROS and Diosmin 100 µM show 46 % decrease in ROS similarly SAHA pretreated with NAC show 43 % decrease in ROS.as shown in(fig 4.6 ,4.8, fig4.10 and 4.11)

4.1.4Bioactive Compounds Treatment Induces Mitochondrial Superoxide Generation In HeLa Cells

Recent studies on mitochondria reported that mitochondria generate reactive oxygen species (ROS) in the form of superoxides (Ziegler, Wiley, & Velarde, 2015) which act as biological important molecule. When Hela cells were treated with Fucoidan and SAHA, they showed an increase in mitochondrial superoxide generation in concentration dependent manner which was confirmed by the increase in red fluorescence intensity observed by FACS analysis. At low dose of 60 and 80 µg/ml Fucoidan treatment shows 1.3 and 1.8 fold increase, TQ at 30 and 40µM shows 1.93 and 2.5fold increase Diosmin at 75 and 100µM shows 2.2 and 2.8 fold increase respectively, whereas SAHA at 500nM concentration showed 3.86 fold increases in superoxide level.as shown in (fig4.13 to 4.15)

4.1.5Effect Of Bioactive Compound On Mitochondrial Membrane Potential (Δψm) Of HeLa Cells

Change of mitochondrial membrane potential has been used to study the mitochondrial health. Mitochondrial is considered as a primary source of ROS in the cells under stress and this can cause damage to the mitochondria itself as well as the whole cell [15]. Rhodamine123 (R123) is used to detect membrane disruption. R123 is a cell-permeant, cationic, green-fluorescent dye that is readily sequestered by active mitochondria without cytotoxic effects. This product has been used to assay mitochondrial membrane



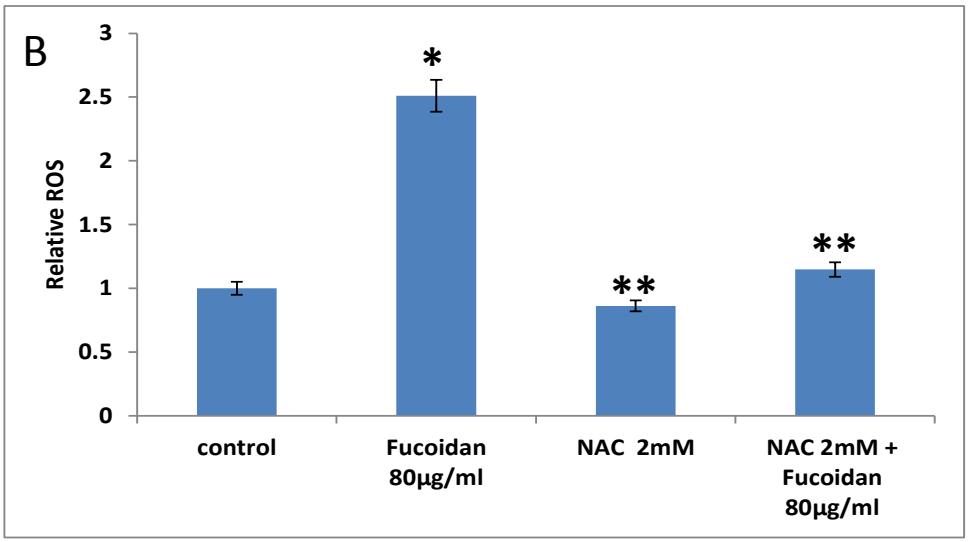
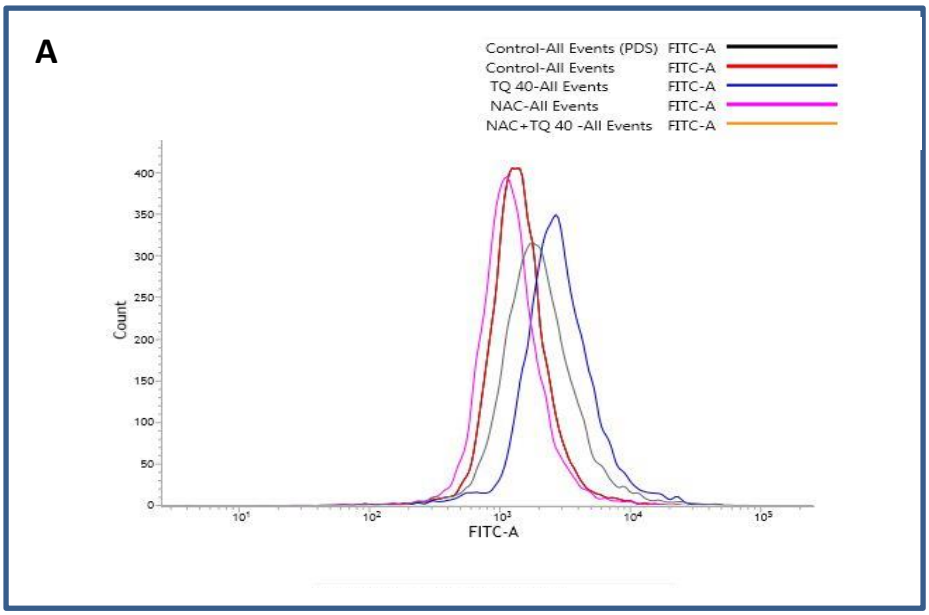


Fig. 4.6 ROS Analysis of HeLa cells pre-treatment with NAC 2mM for 1hr after which Fucoidan was added, FACS analysis was done after 24hour NAC pre-treatment diminish the ROS generation which confirms the pro-oxidant nature of Fucoidan. Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05 ** denotes p<0.001



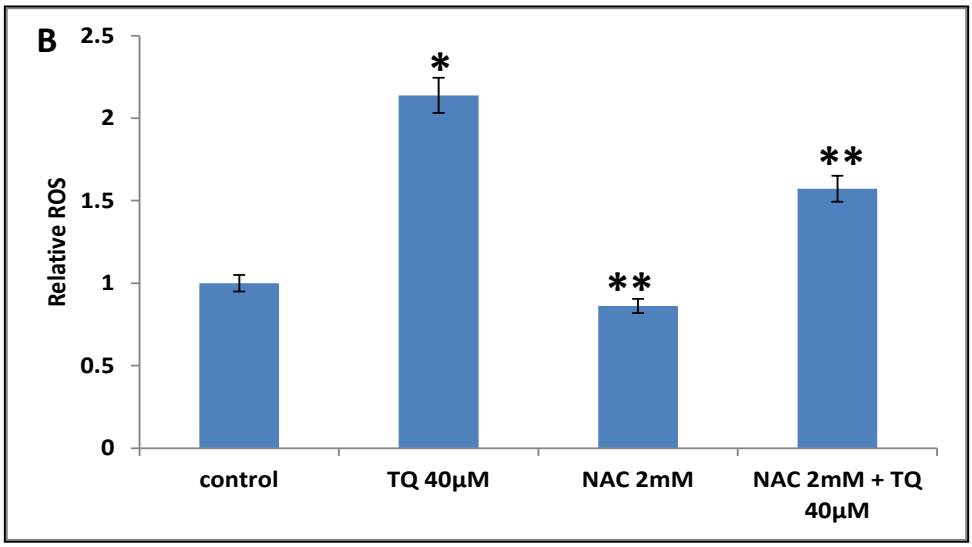
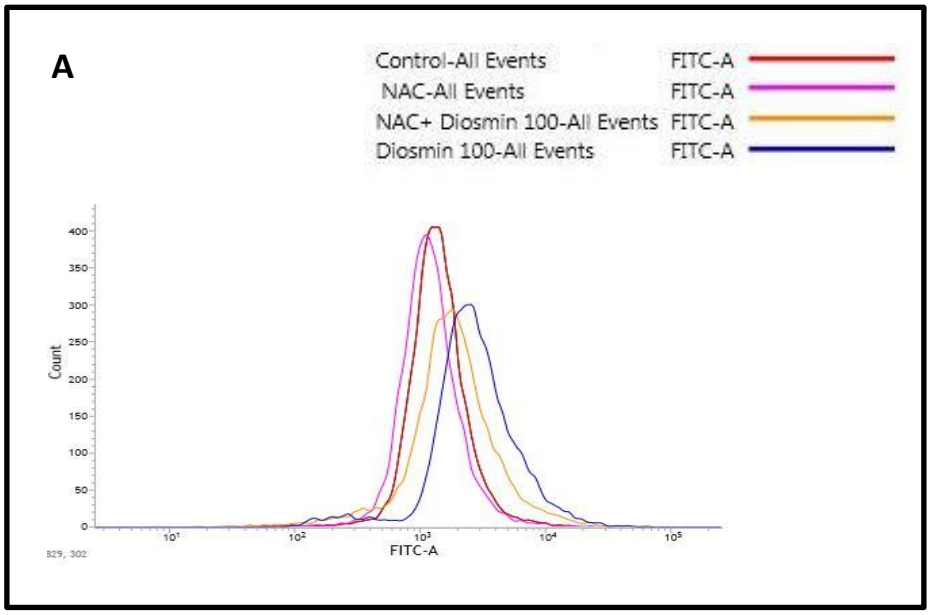


Fig. 4.8 ROS Analysis of HeLa cells pre-treatment with NAC 2mM for 1hr after which Thymoquinone was added, FACS analysis was done after 24hour NAC pre-treatment diminish the ROS generation which confirms the pro-oxidant nature of Fucoidan. Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05 ** denotes p<0.001



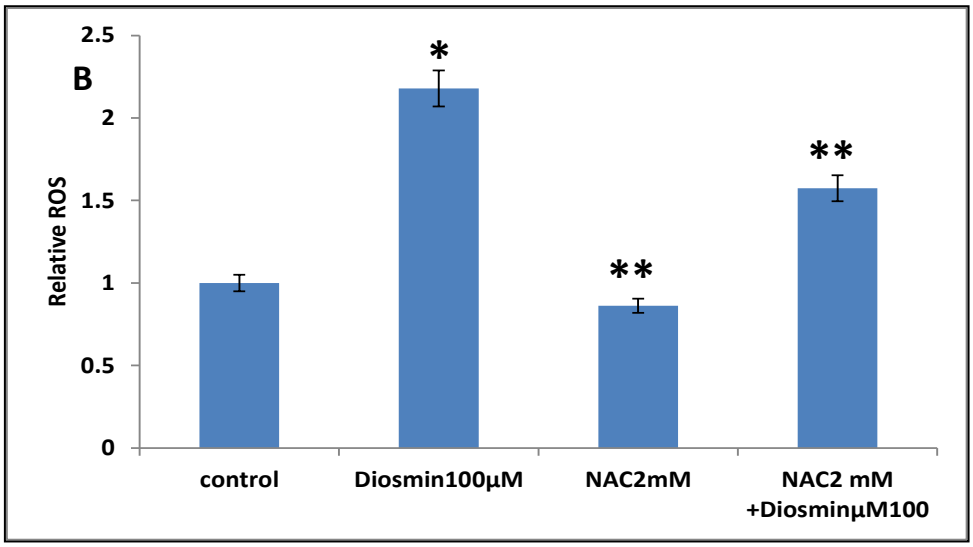


Fig 4.10)ROS analysis HeLa cells pre-treatment with NAC 2mM for 1hr after which Diosmin was added, FACS analysis was done after 24hour, NAC pre-treatment diminish the ROS generation which confirms the pro-oxidant nature of Fucoidan. Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05 ** denotes p<0.001

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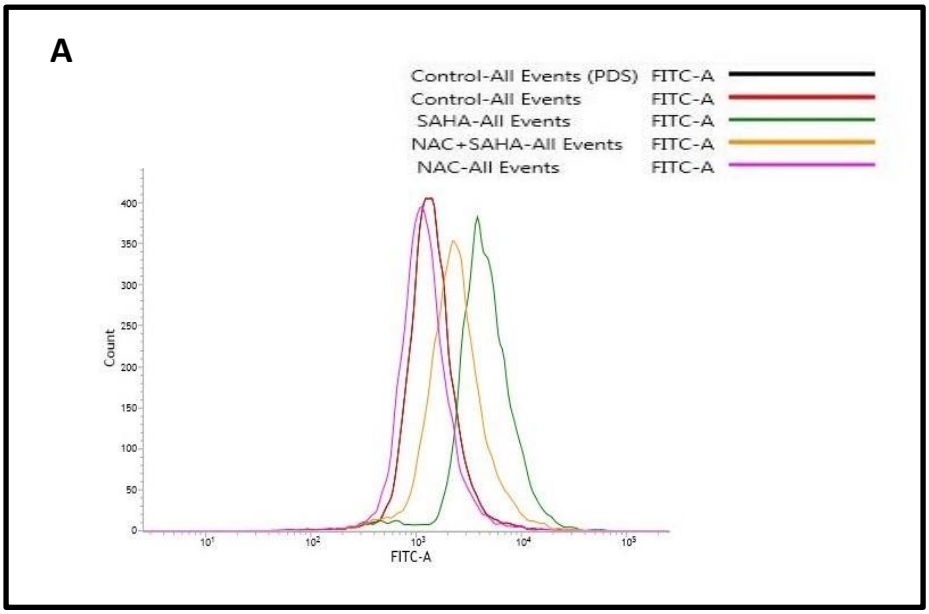


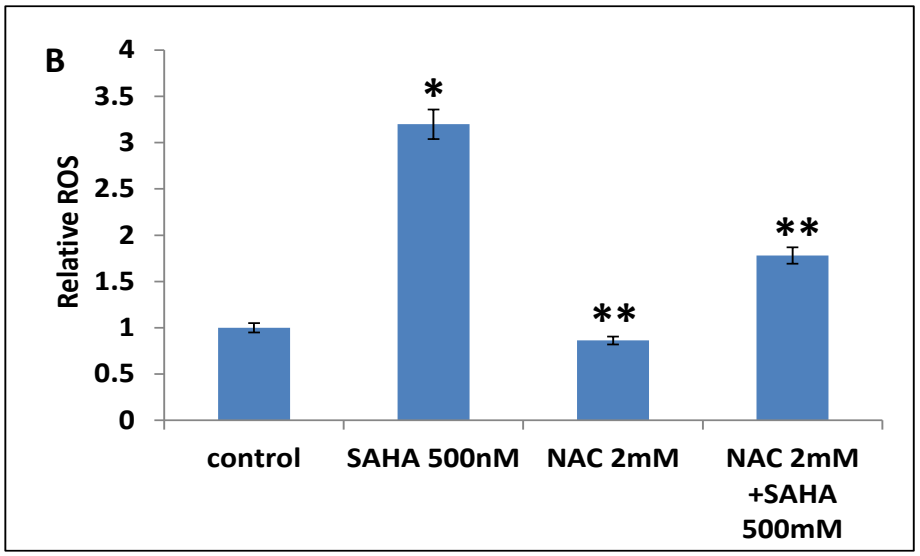
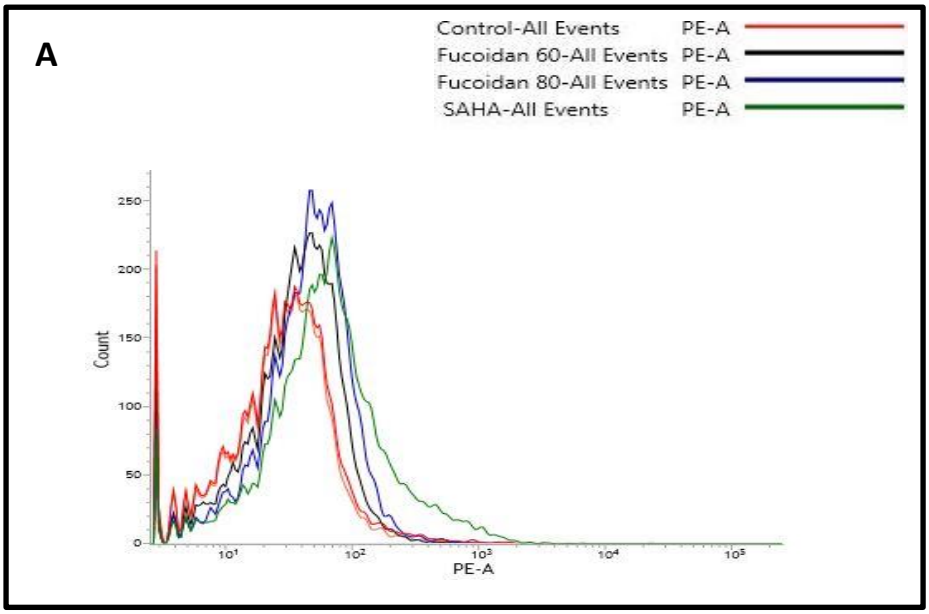


Fig4.11)ROS analysis HeLa cells pre-treatment with NAC 2mM for 1hr after which SAHA was added, FACS analysis was done after 24hour, NAC pre-treatment diminish the ROS generation which confirms the pro-oxidant nature of SAHA. Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05 ** denotes p<0.001



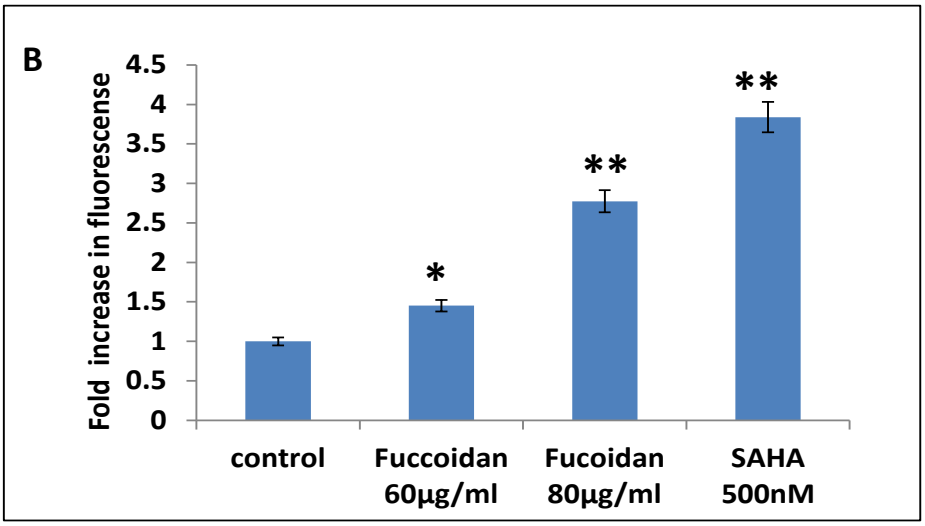
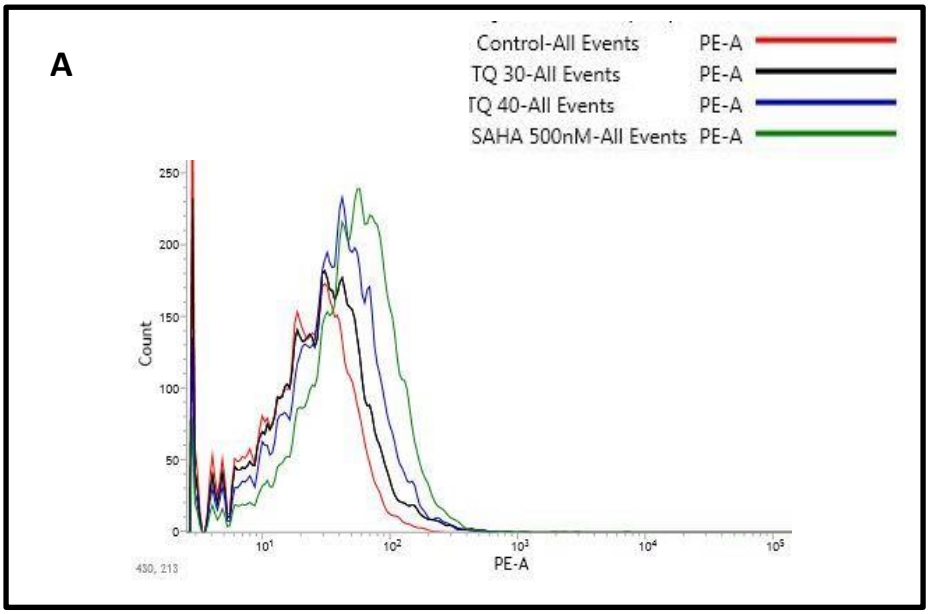


Fig 4.13)Mitochondrial ROS: HeLa cells were incubated with mito sox red and superoxide generation were measured after 24hour of treatment, relative mean fluorescence intensity was analyzed by flow cytometry at a concentration of 60 and 80 µg/ml of Fucoidan and 500nM SAHA show enhanced mitochondrial superoxide anion in comparision to control . B) Histogram profile showing fold change in. (1.453 and 2.7 fold increase in Fucoidan and 3.82 fold in case of SAHA.Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05 ** denotes p<0.001



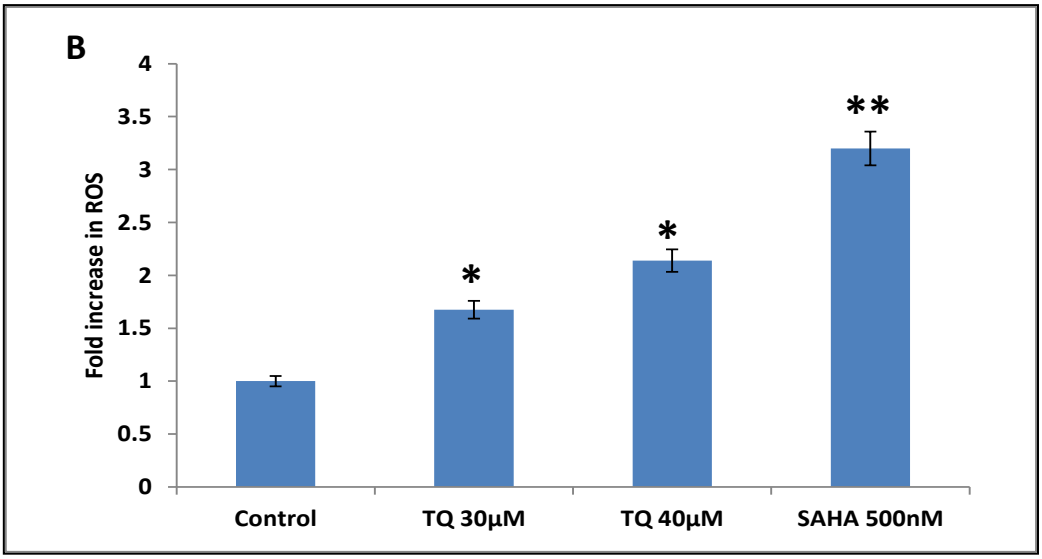
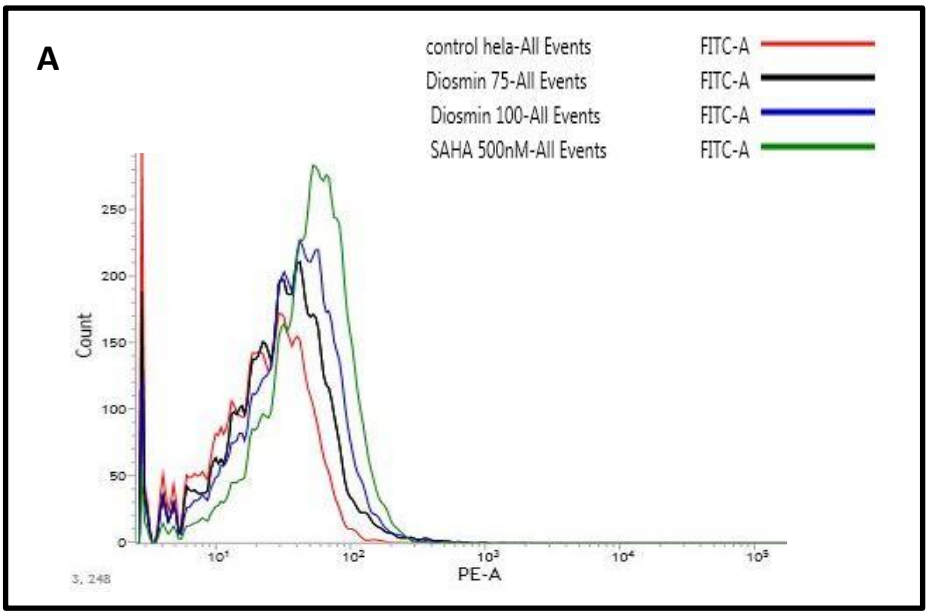


Fig 4.14 Mitochondrial ROS: HeLa cells were incubated with mito sox red and superoxide generation were measured after 24hour of treatment, relative mean fluorescence intensity was analyzed by flow cytometry at a concentration of 30 and 40 µMl of Thymoquinone and 500nM SAHA show enhanced mitochondrial superoxide anion in comparision to control . B) Histogram profile showing fold change in. (1.9 and 2.5 fold increase in TQ and 3.82 fold in case of SAHA. Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05 ** denotes p<0.001



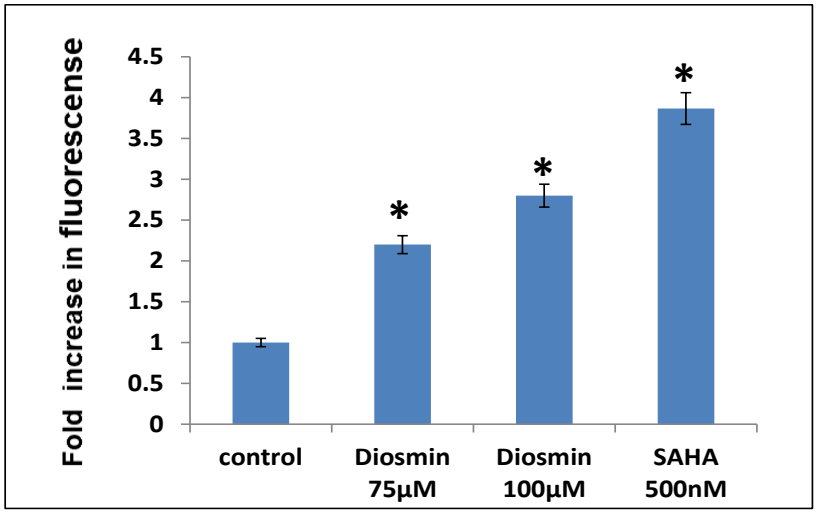
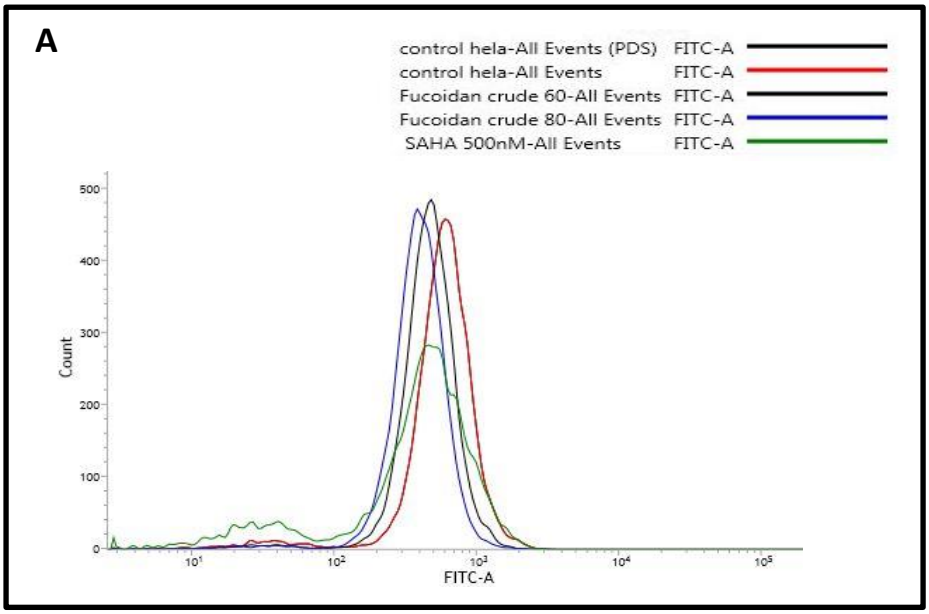


Fig4.15) Mitochondrial ROS : HeLa cells were incubated with mito sox red and superoxide generation were measured after 24hour of treatment, relative mean fluorescence intensity was analyzed by flow cytometry at a concentration of 75 and 100 µM of Diosminand 500nM SAHA show enhanced mitochondrial superoxide anion in comparision to control . B) Histogram profile showing fold change in. (2.2 and 2.8 fold increase in Diosmin and 3.82 fold in case of SAHA. . Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05 ** denotes p<0.001



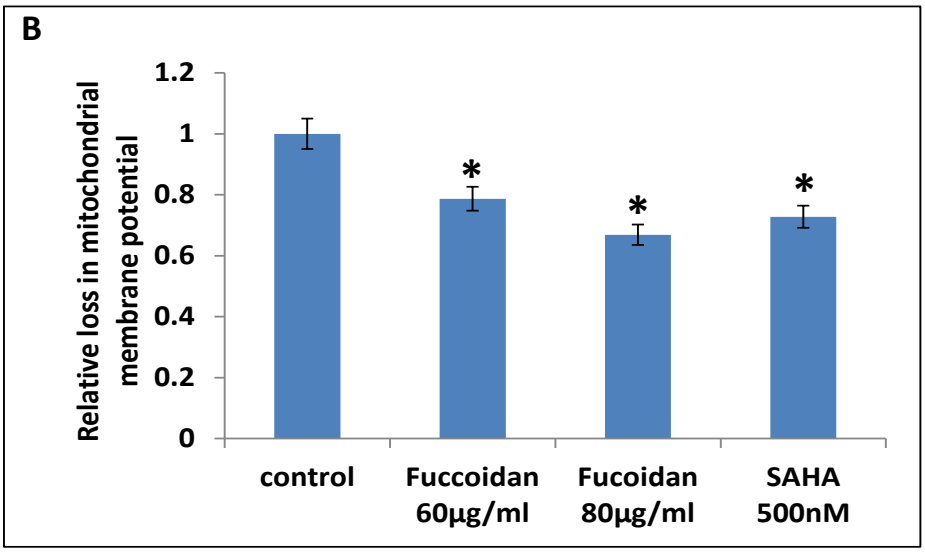
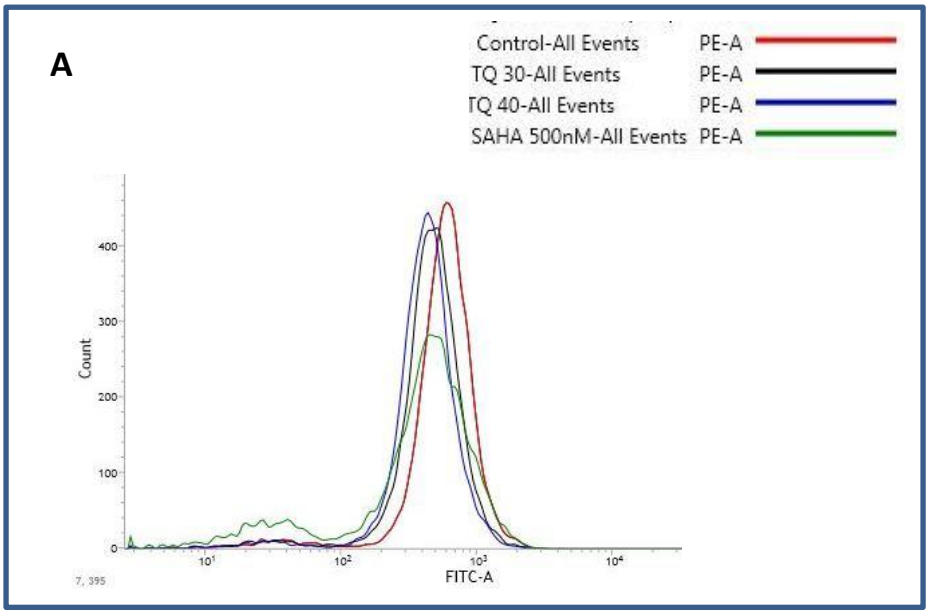


Fig 4.18)Showing the Mitochondrial Membrane Potential change in Cell after treatment with various concentrations of Fucoidan(µg/ml) in comparison with Known HDAC inhibitor SAHA. B) Histogram profile of relative Mitochondrial Membrane Potential change compared with the Control* denotes $p<0.05$



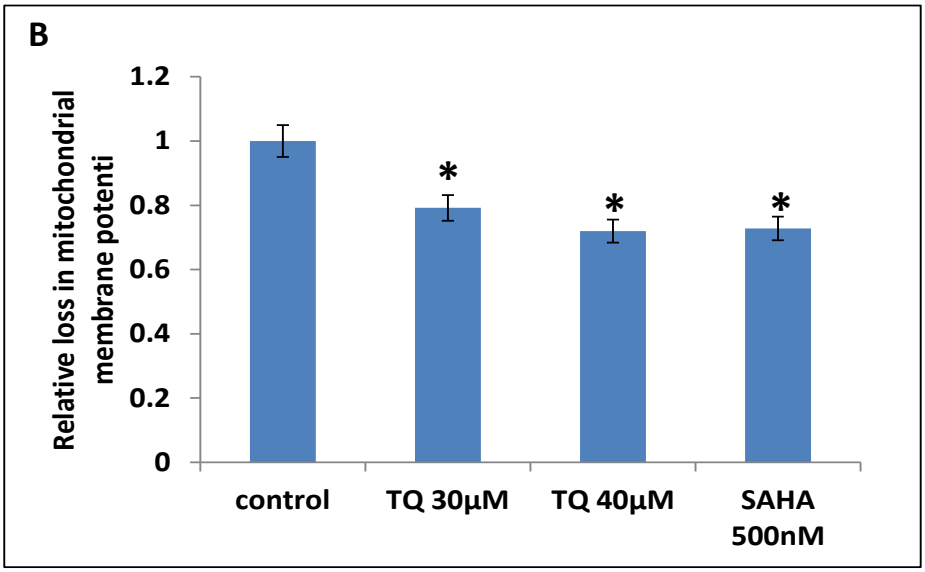
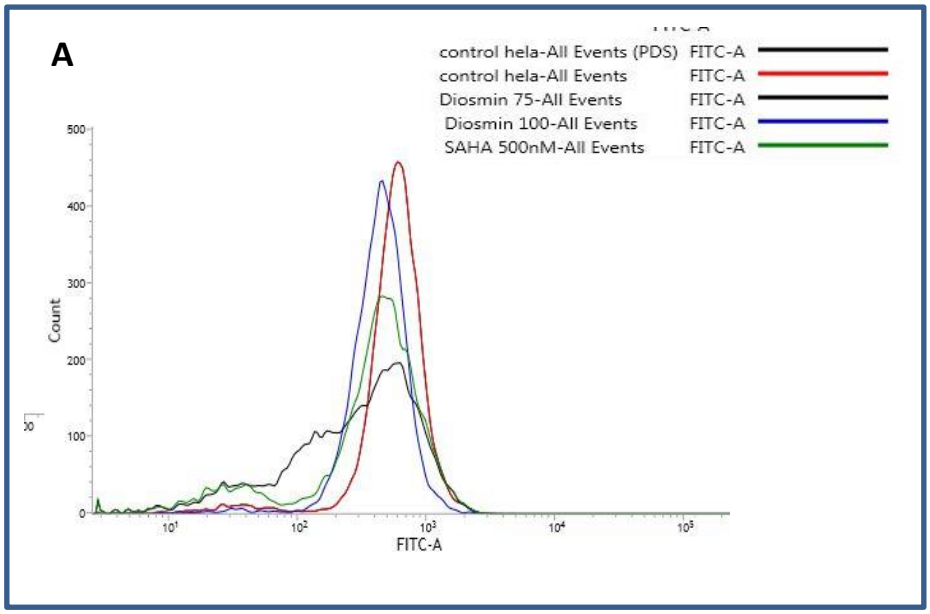


Fig 4.19)Showing the Mitochondrial Membrane Potential change in cells after treatment with various concentrations of Thymoquinone(µM) in comparison with Known HDAC inhibitor SAHA. B)Histogram profile of relative Mitochondrial Membrane Potential change compared with the Control. * denotes $p<0.05$



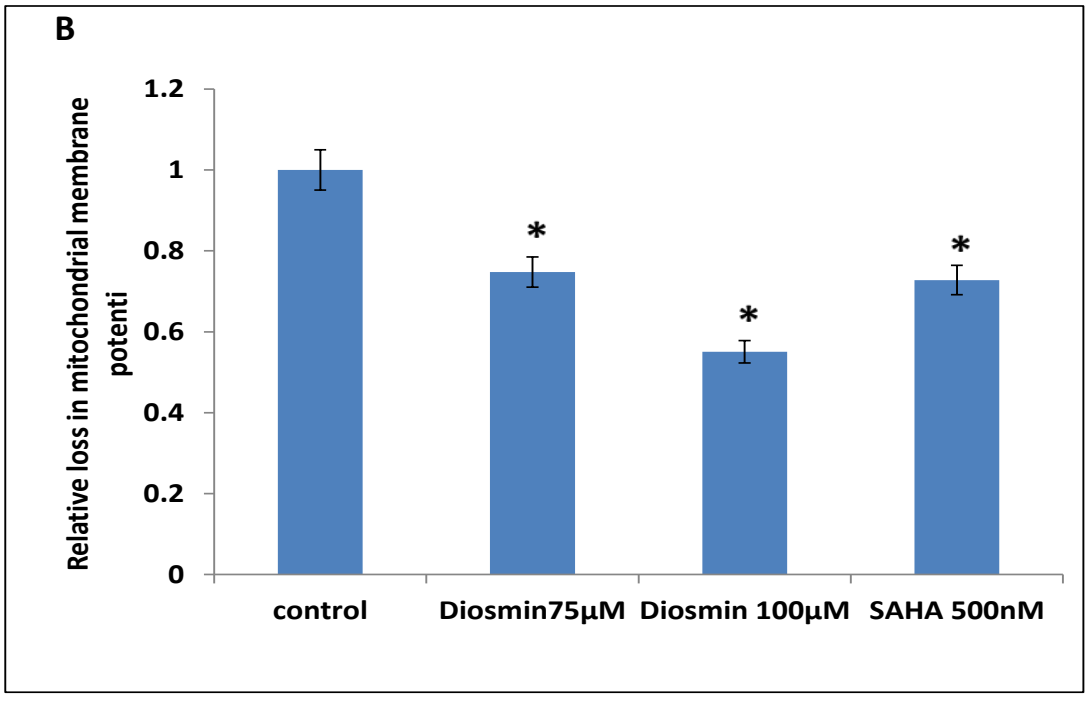


Fig4.20)Showing the Mitochondrial Membrane Potential change in cells after treatment with various concentrations of Thymoquinone(µM) in comparison with Known HDAC inhibitor SAHA. B)Histogram profile of relative Mitochondrial Membrane Potential change compared with the Control. . * denotes $p<0.05$

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potential in populations of cells showing oxidative stress. Mitochondrial membrane potential was found to be decreased as the green fluorescence intensity decrease significantly at a concentration-dependent manner as compared to control. This showed a loss in mitochondrial membrane potential because of the high amount of ROS generation by HeLa cells after treatment (Fig18-20) Fucoidan treatment at dose of 60 and 80 µg/ml shows 22% and 34% decrease on mitochondrial membrane potential,TQ at 30 and 40µM shows 21and 29% decrease while, Diosmin at 75 and 100µM shows 26 and 45% decrease respectively, whereas SAHA at 500nM concentration showed 28% decrease mitochondrial membrane potential.

4.1.6HDAC Activity Down Regulation By Bioactive Compounds

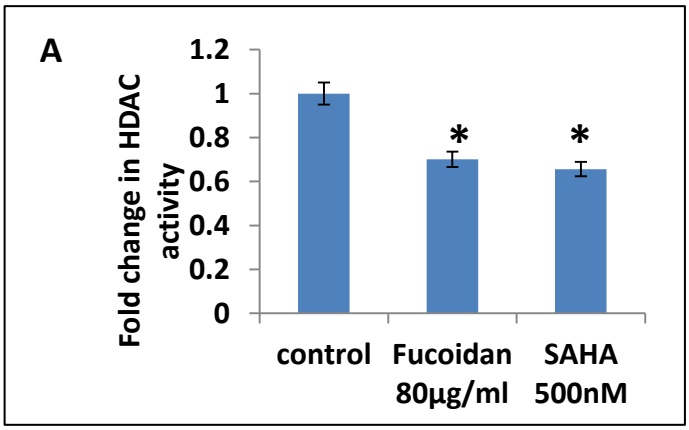
HDAC's activity was measured by colorimetric assay. Histone Deacetylase show 30%decrease in HeLa cells HDAC activity at 80µg/ml Fucoidan , 21% decrease in 40 µM thymoquinone treatment, , 27% decrease in 100 µM Diosmin 35% decresase in HDAC's activity at 500nM of SAHA in comparision to control HeLa cells as shown in figure (fig4.17).

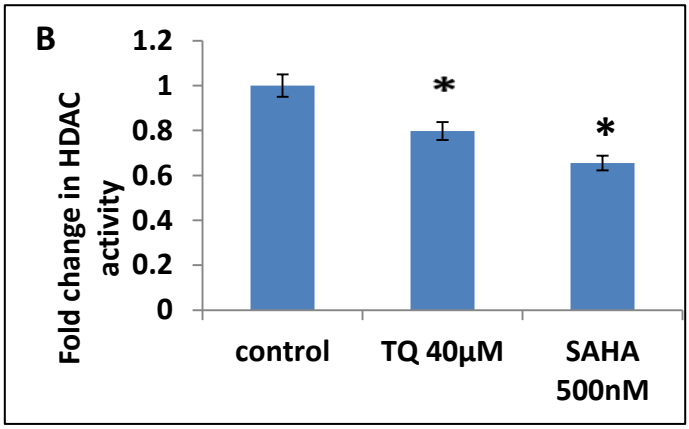
4.1.7Bioactive Compounds Induces Autophagosome Formation

MDC binds to intracellular autophagosomes, specifically phosphatidylethanol amine present in the autophagosome membrane, emits fluorescence. After Staining with 50 µM MDC HeLa cells were treated with Fucoidan, TQ Diosmin and SAHA gives high fluorescence as compared to control this gives an idea of induction of autophagy with the formation of autophagosome (Fig4.24).

4.1.8FACS Analysis Of Autophagy Induction By The Bioactive Compounds

We have also analysed the cells with Fucoidan TQ and SAHA against control HeLa cells for autophagosomes formation. Quantification with acridine orange staining by FACS. as shown in (fig.4.21to 4.23), and its histogram profile shows At low dose of 60 and 80 µg/ml Fucoidan treatment shows 1.3 and 1.43 fold increase in red/green fluorescence,TQ at 30 and 40µM shows 1.32 and 1.44 fold increase in red/green fluorescences Diosmin at 75 and





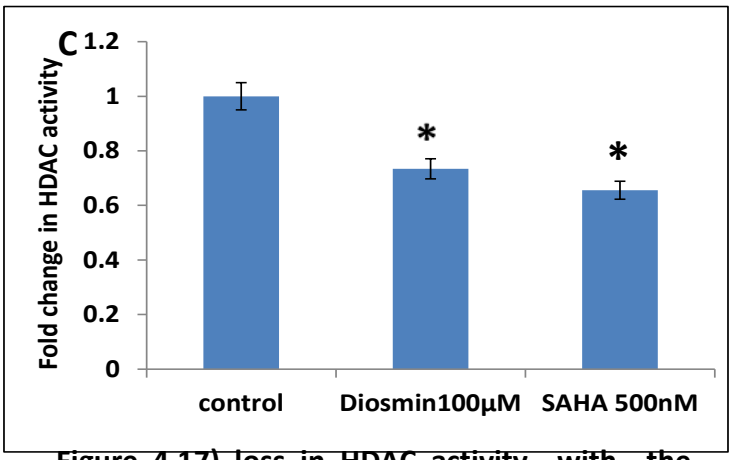
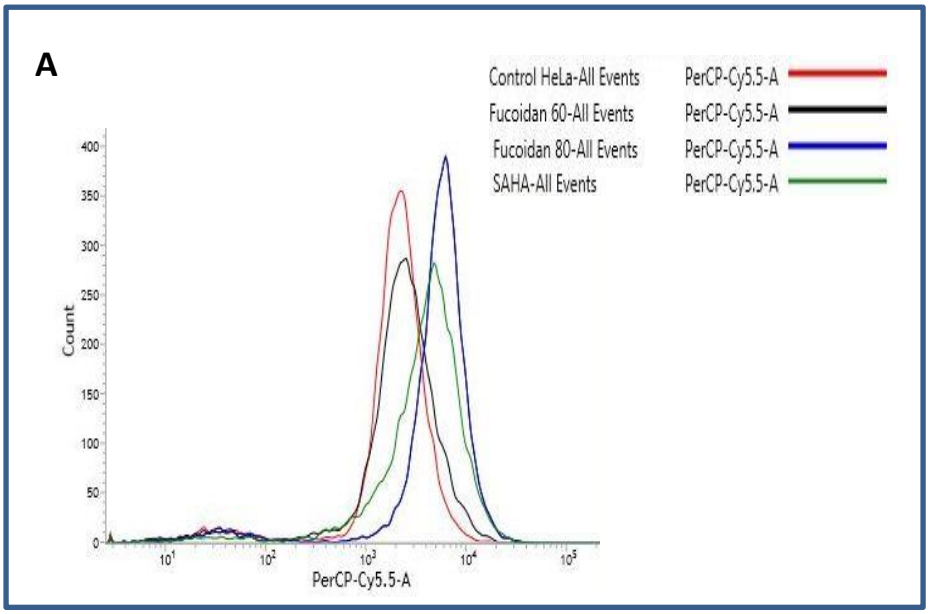


Figure 4.17) loss in HDAC activity with the treatment of A)Fucoidan, B)TQ, C)Diosmin and in compare to SAHA at 24 hour. Values are means ± S.E.M. of at least three independent experiments. * denotes $p<0.05$



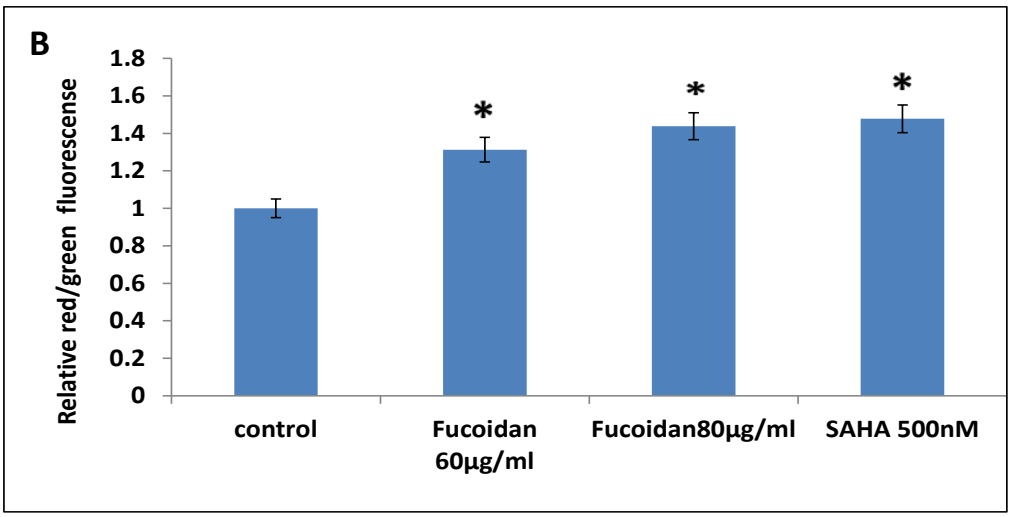
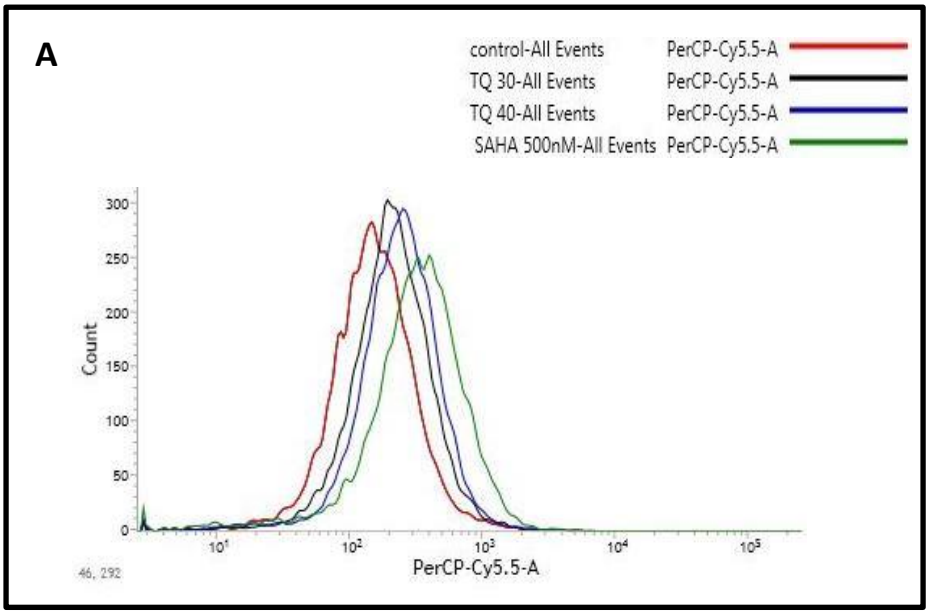


Fig 4.21)Autophagy analysis:Flow cytometry analysis of the acridine orange staining with Fucoidan and SAHA shows enhanced red/green ratio of fluorescence relative to control HeLa cells and B). Histogram profile to show red/green fluorescence (1.3 and 1.43 fold increase in Fucoidan and 1.47 fold in case of SAHA) * denotes $p<0.05$



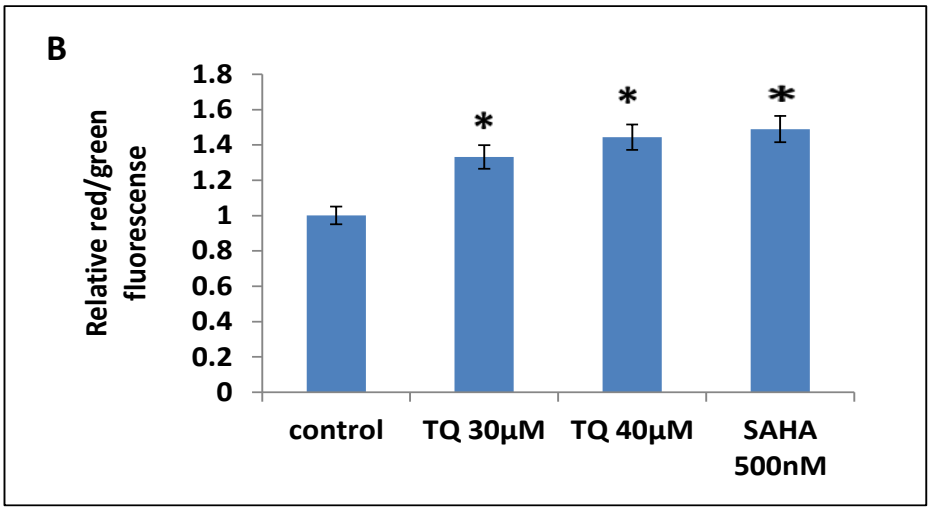


Fig4.22)Autophagy analysis:Flow cytometry analysis of the acridine orange staining with Thymoquinone and SAHA shows enhanced red/green ratio fluorescence relative to control HeLa cells and B) Histogram profile to show red/green fluorescence(1.33 and 1.44 fold increase in TQ and 1.48 fold in case of SAHA) . * denotes p<0.05

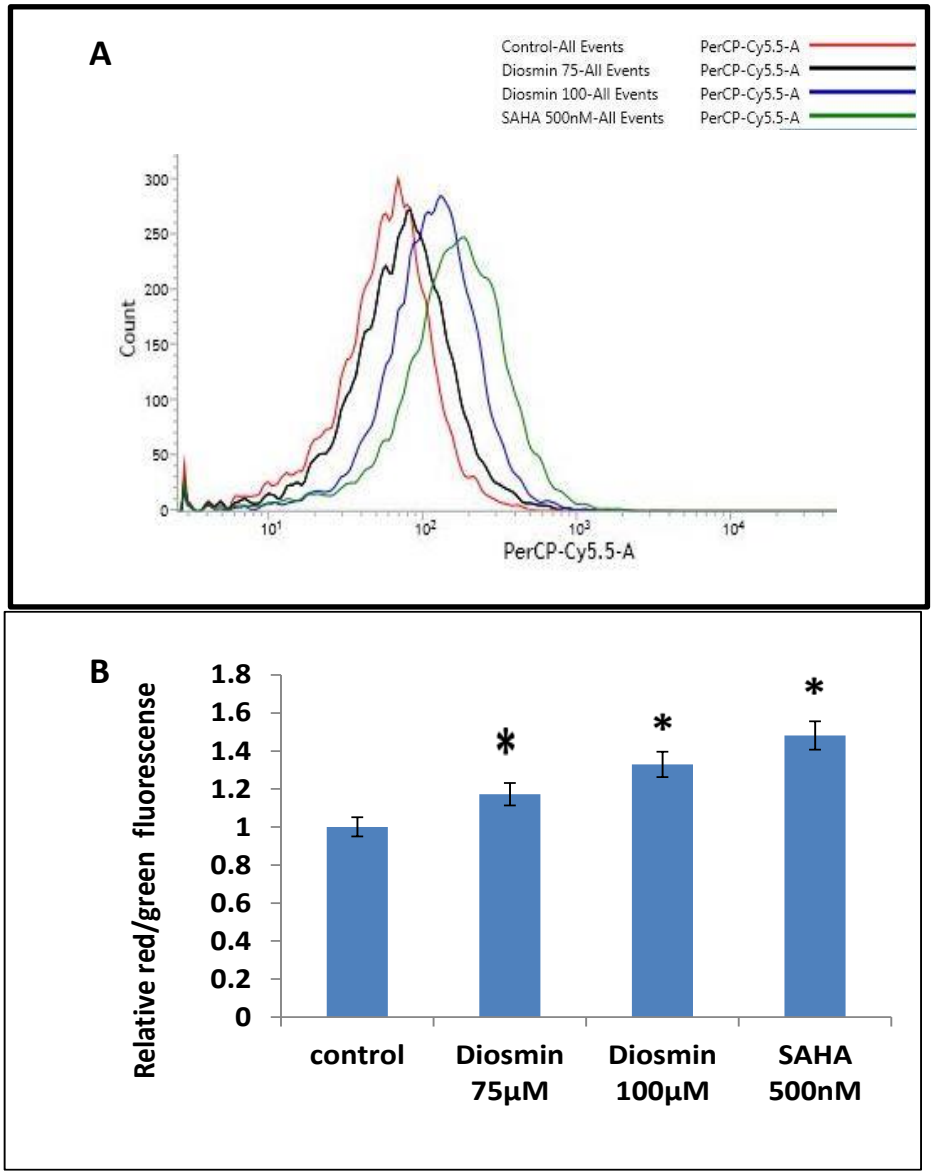


Fig 4.23Autophagy analysis:Flow cytometry analysis of the acridine orange staining with Diosmin and SAHA shows enhanced red/green ratio fluorescence relative to control HeLa cells and B. Histogram profile to show red/green fluorescence(1.17 and 1.32 fold increase in Diosmin and 1.48 fold in case of SAHA) * denotes $p<0.05$

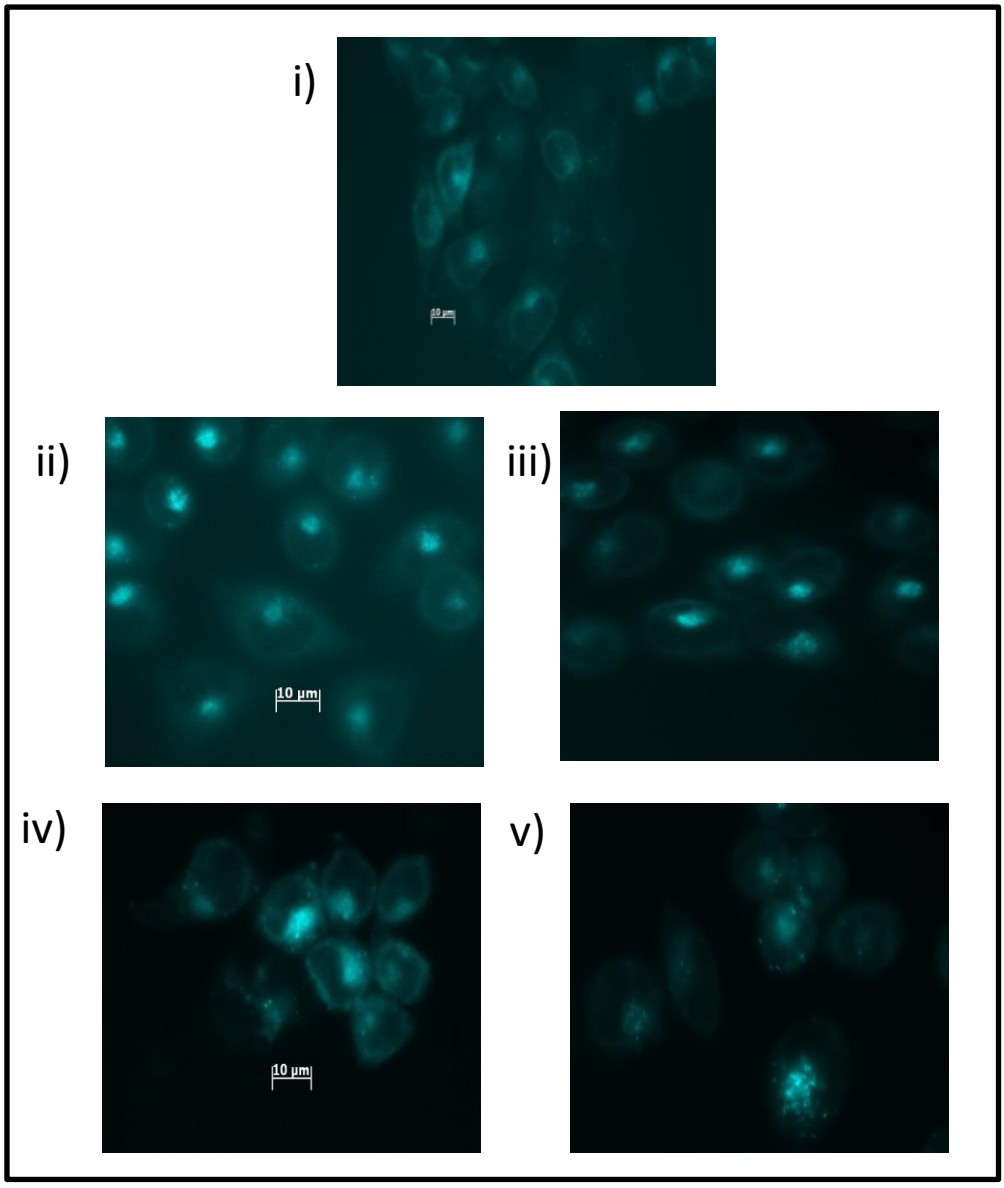


Fig 4.24) After treatment with bioactive compounds MDC staining in HeLa cells to confirm the autophagy induction or visualize the autophagosomes of cells against the control. (i) Control, (ii) Fucoidan 80 µg/ml, iii) Thymoquinone(40µM), , iv) Diosmin100µM (v) SAHA 500 nM

100µM shows 1.17 and 1.32 fold increase respectively, whereas SAHA at 500nM concentration showed 1.48 fold increase in comparison to control which showing autophagosome formation or enhanced acidic vesicular organelles confirming Autophagy compared with control.

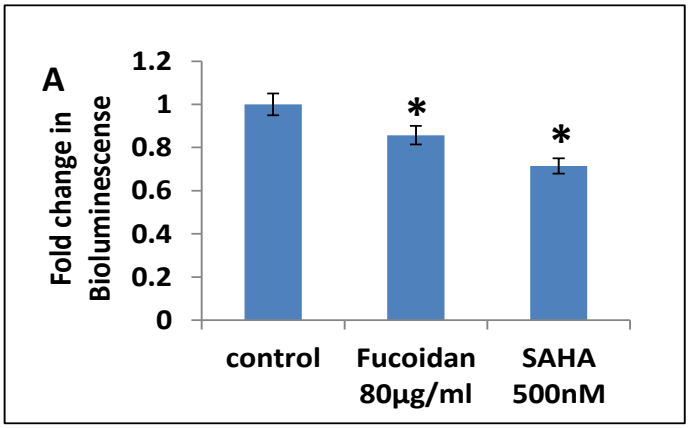
4.1.9Analysis Of Bioactive Compounds On ATP Activity Of HeLa Cells

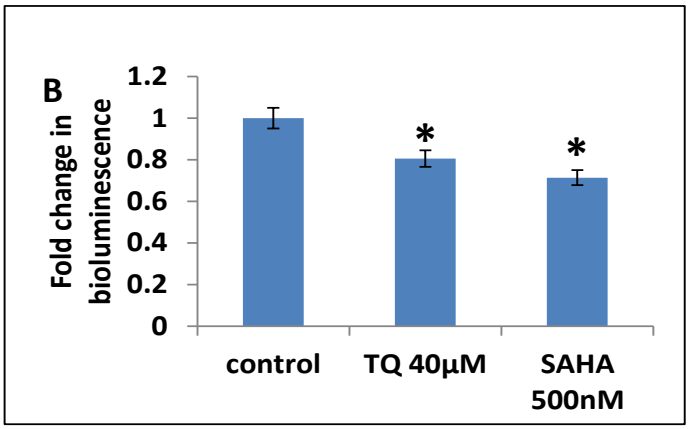
ATP activity was measured by bioluminescence assay . ATP activity show 15% decrease in HeLa cells ATP activity at 80µg/ml Fucoidan, show 20% decrease in 40 µM thymoquinone treatment, 19% decrease in 100 µM Diosmin 29% decresase in ATP activity at 500nM of SAHA in comparison to control HeLa cells as shown in figure (fig 4.16).

4.1.10Bioactive Compound Induced Premature Senescence In HeLa Cells

Several studies also show that ROS also plays a critical role in determining lifespan and cellular senescence of mammalian cells. To analyse towards which cellular process the HeLa cells were prompted due to oxidative stress generated by Fucoidan ,TQ, Diosmin and SAHA compared to control cells, Senescence-associated β-galactosidase (SA–β-Gal) staining for ageing pigment,cell autofluoresence,and DAPI for SAHF and cell-cycle analysis were performed. Positive β-galactosidase staining was observed after 24 hr. of treatment .The intense blue SA–β-Gal staining was observed in treated cells with flattened and enlarged cell morphology (Fig4.31).

Cells that undergone senescence show increase intrinsic fluorescence, called autofluorescence, which is caused by the accumulation of oxidatively damaged proteins and lipids. FACS analysis for autofluorescence and cell size under these bioactive compounds in compare SAHA a HDACi was done was done to check the cells undergoing senescence. The flow cytometric analysis done after 24 hours of treatment showed (Fig4.26 to 4.28) that most of the cells, shifted to higher level of autofluoresence (AF) in compare to control cells. Increase in FSC and SSC was also observed for cells showing increased AF showing that most of the cells grown with bioactive compound were senescent and also the SAHA treated cell show senescence





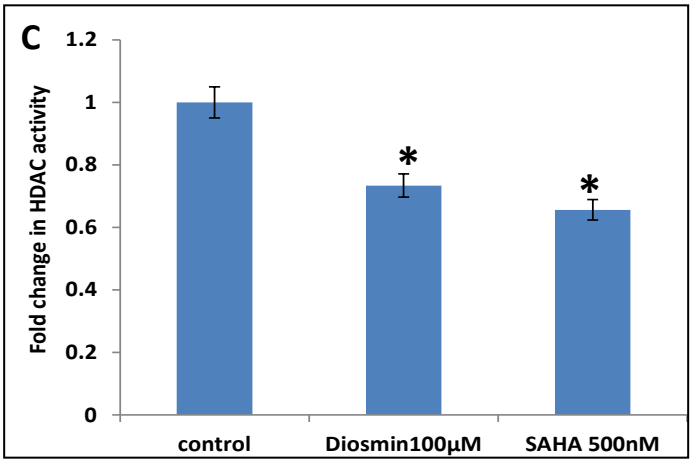


Fig 4.16)ATP generation by measuring bioluminescense showed a continuous decrease with treatment of A)Fucoidan, B)TQ C)Diosmin and in compare to SAHA at 24 hour,. Values are means ± S.E.M. of at least three independent experiments. * denotes $p<0.05$

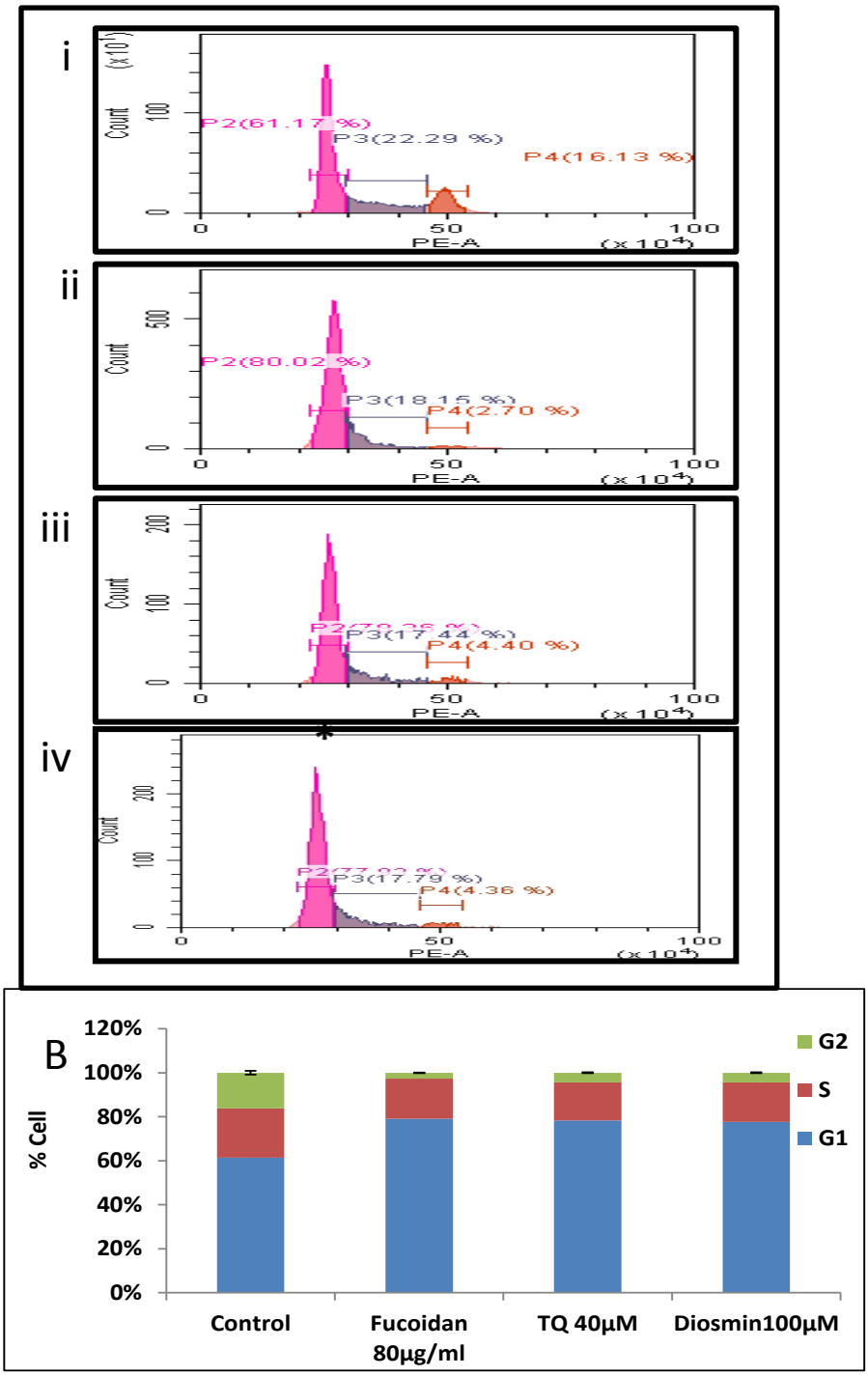
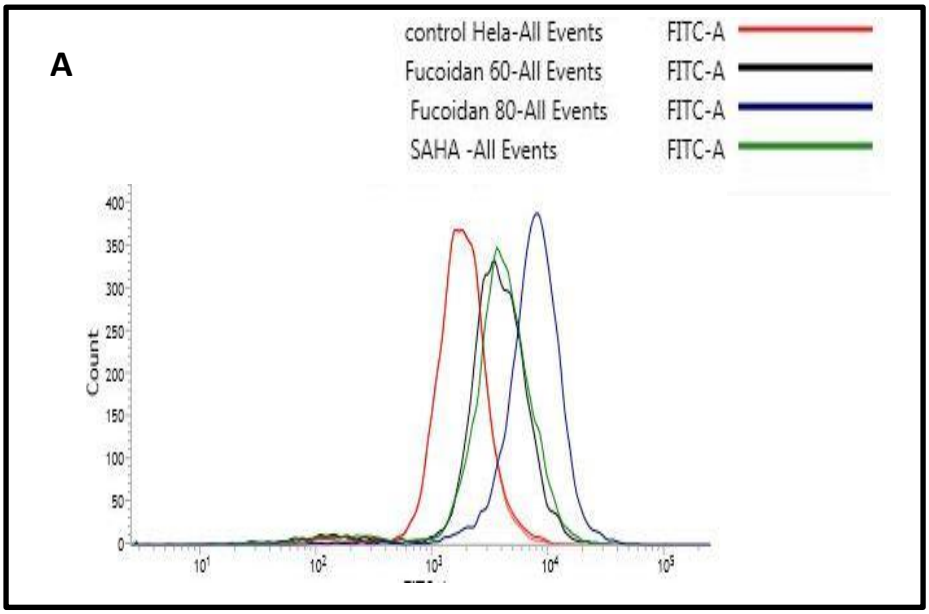


Fig4.25) Cell cycle by FACS analysis in Hela Cells (i) control, 61.17%cells in G1 phase (ii)Fucoidan 80 µg/ml,80.02% cell in G1 phase, iii) Thymoquinone (78.23%) in G1, iv) Diosmin 77.16%in G1 phase. B)Histogram profile to show the cell percentage in each phase.Values are means ± S.E.M. of at least three independent experiments.



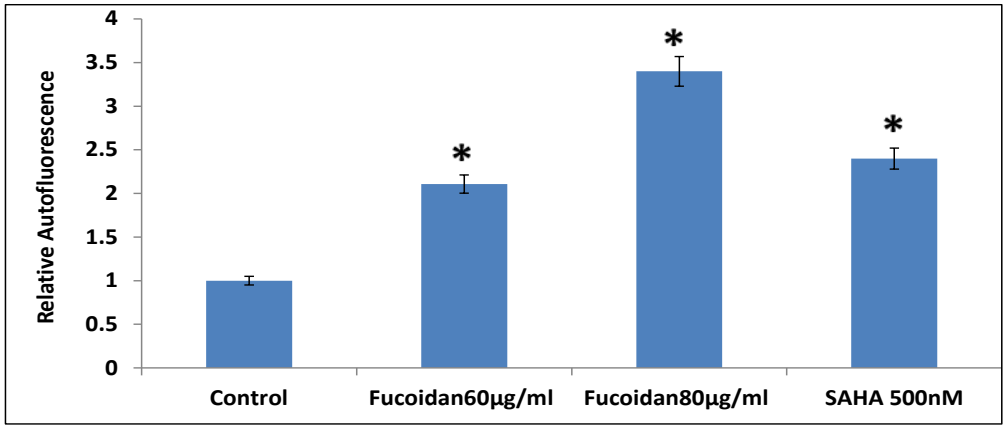
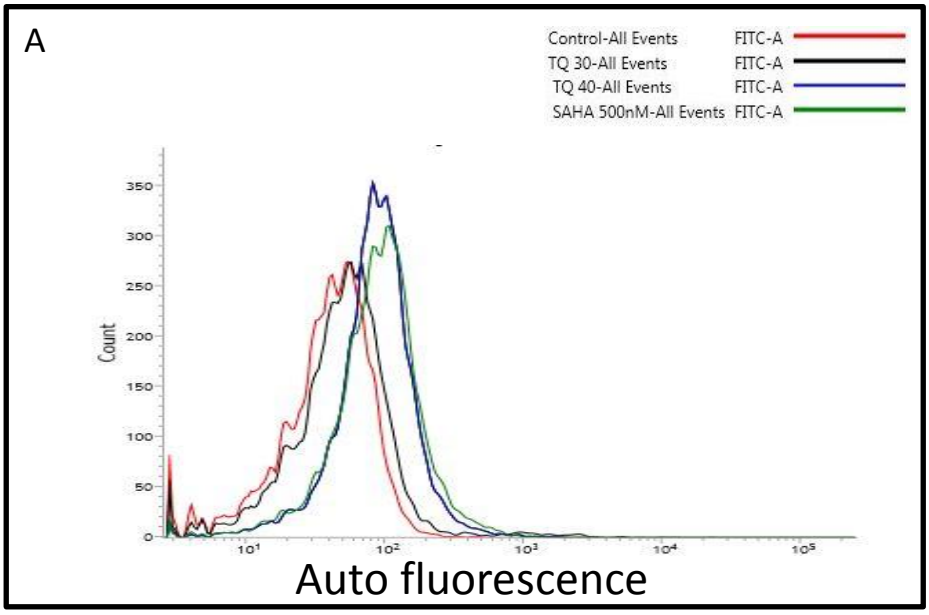


Fig4.26) Senescense analysis was done by measuring autofluorescense after treatment of Fucoidan and SAHA in HeLa cells.. B)Histogram profile showing fold change in autoflorescense Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05



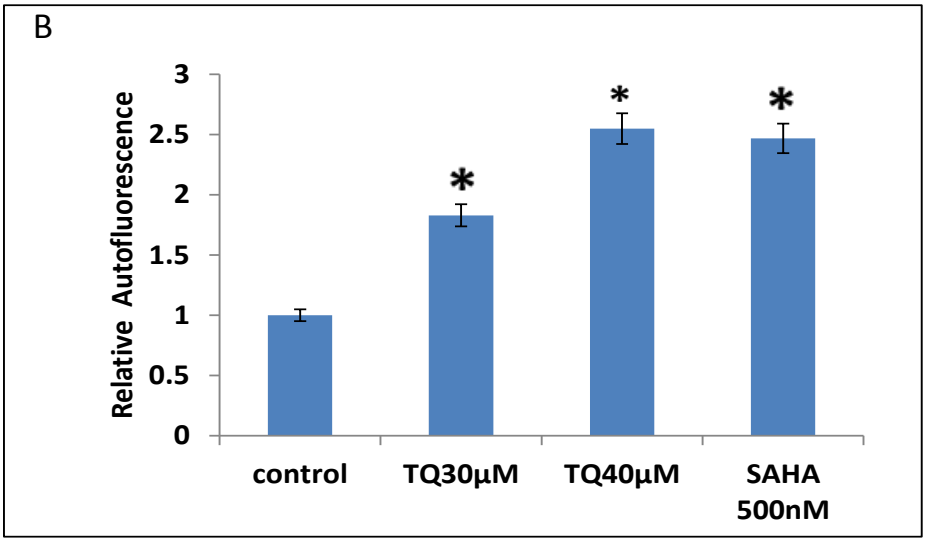
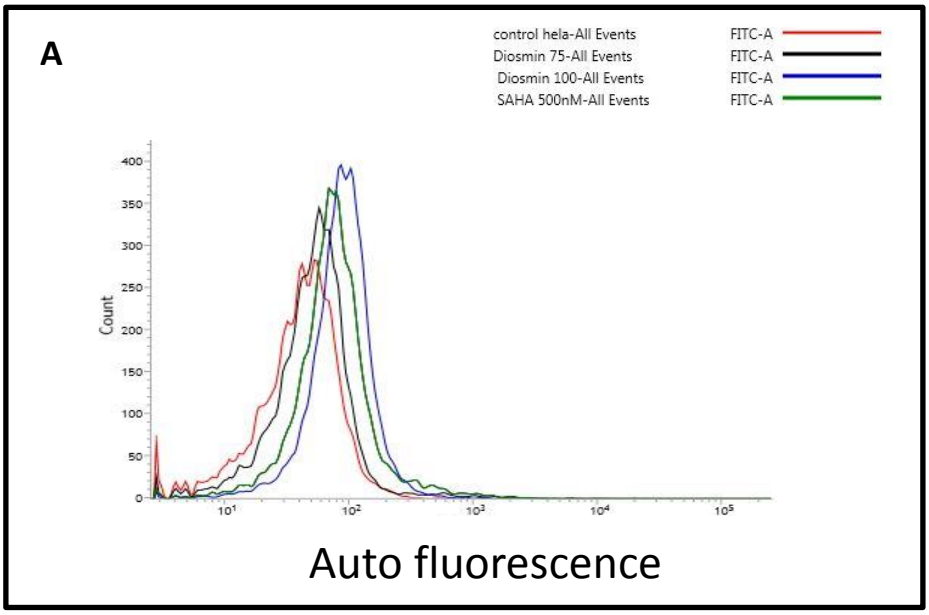


Fig4.27)Senescense analysis was done by measuring autofluorescense After treatment of TQ and SAHA in HeLa cell) B)Histogram profile showing fold change in autoflorescenseValues are means ± S.E.M. of at least three independent experiments. *denotes p<0.05



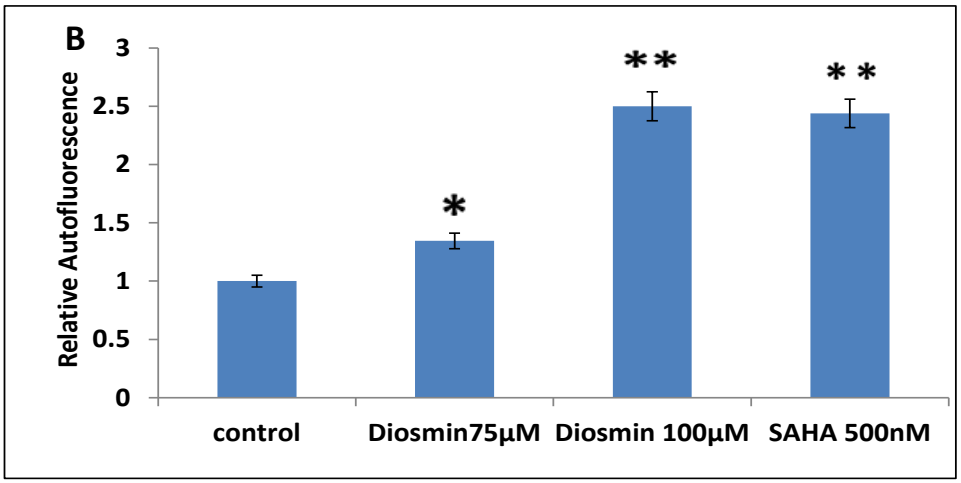


Fig4.28)Senescense analysis was done by measuring autofluorescense After treatment of Diosmin and SAHA in HeLa cell) B)Histogram profile showing fold change in autoflorescense. * denotes p<0.05 ** denotes p<0.001

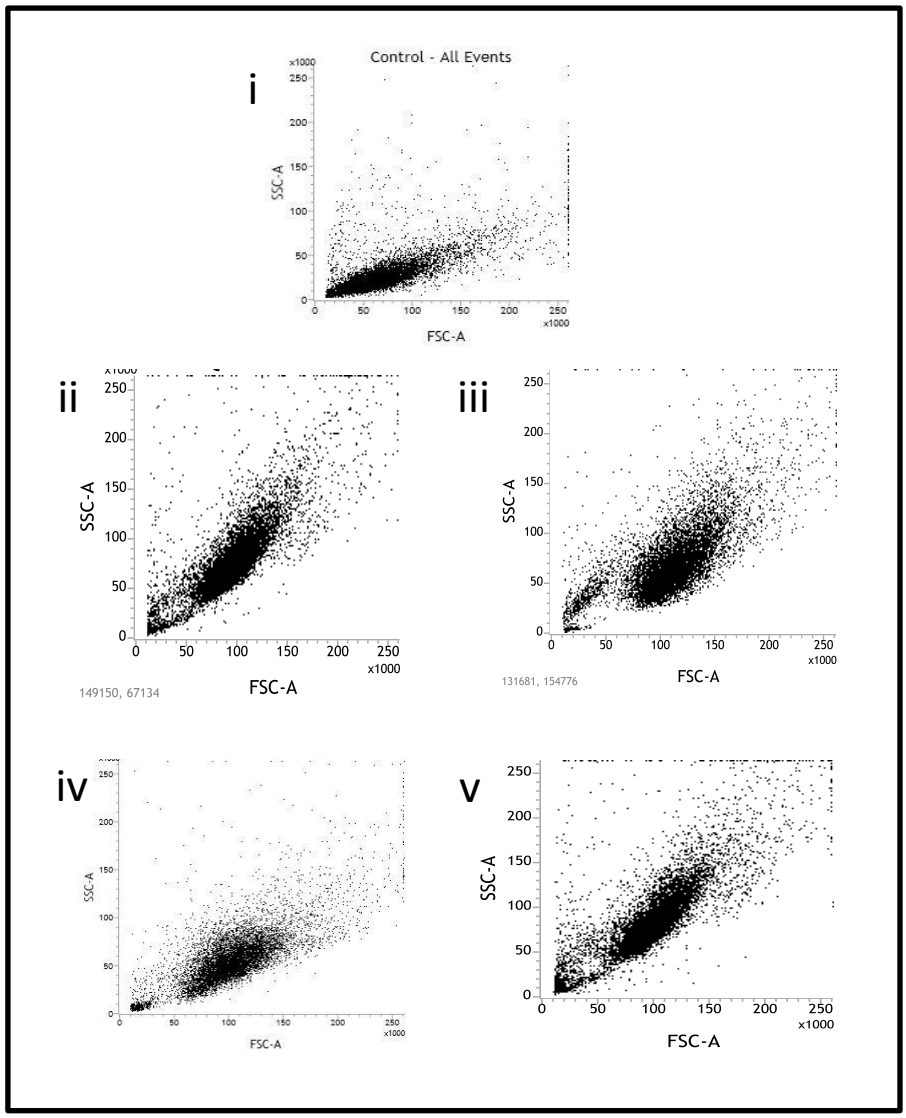


Fig 4.29)Senescensce study was carried out in HeLa cells.FACS analysis of cell size(FSC) and granularity(SSC) in control and treated HeLa i)control ii) Fucoidan80µg/ml,iii) TQ 40µM iv) Diosmin,v)SAHA

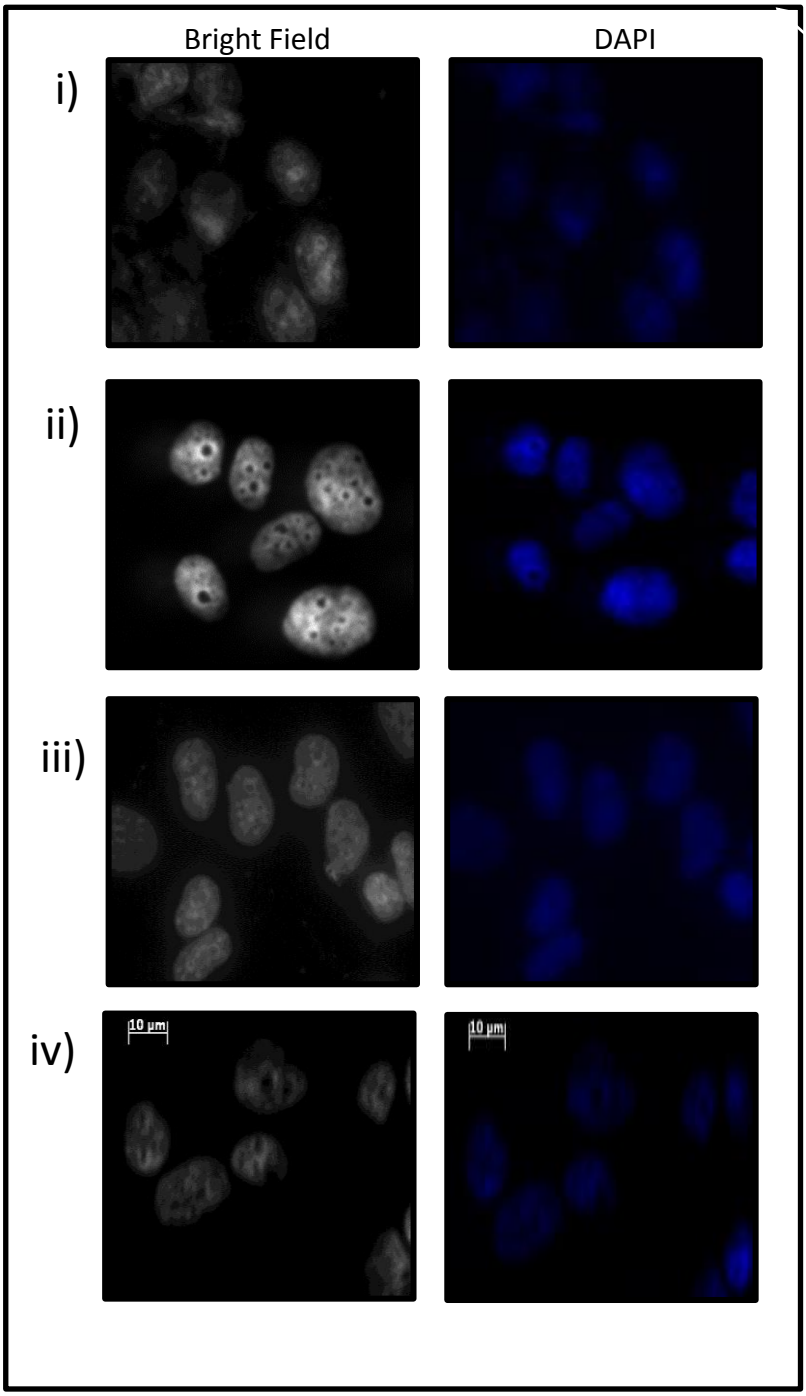


Fig 4.30)Senescensce study was carried out in HeLa cells using DAPI staining depicting the senescence-associated heterochromatin foci (SAHF) in HeLa cells (i) Control , (ii) Fucoidan 80 µg/ml (SAHF), iii)Thymoquinone(40µM) , iv) Diosmin 100µM.

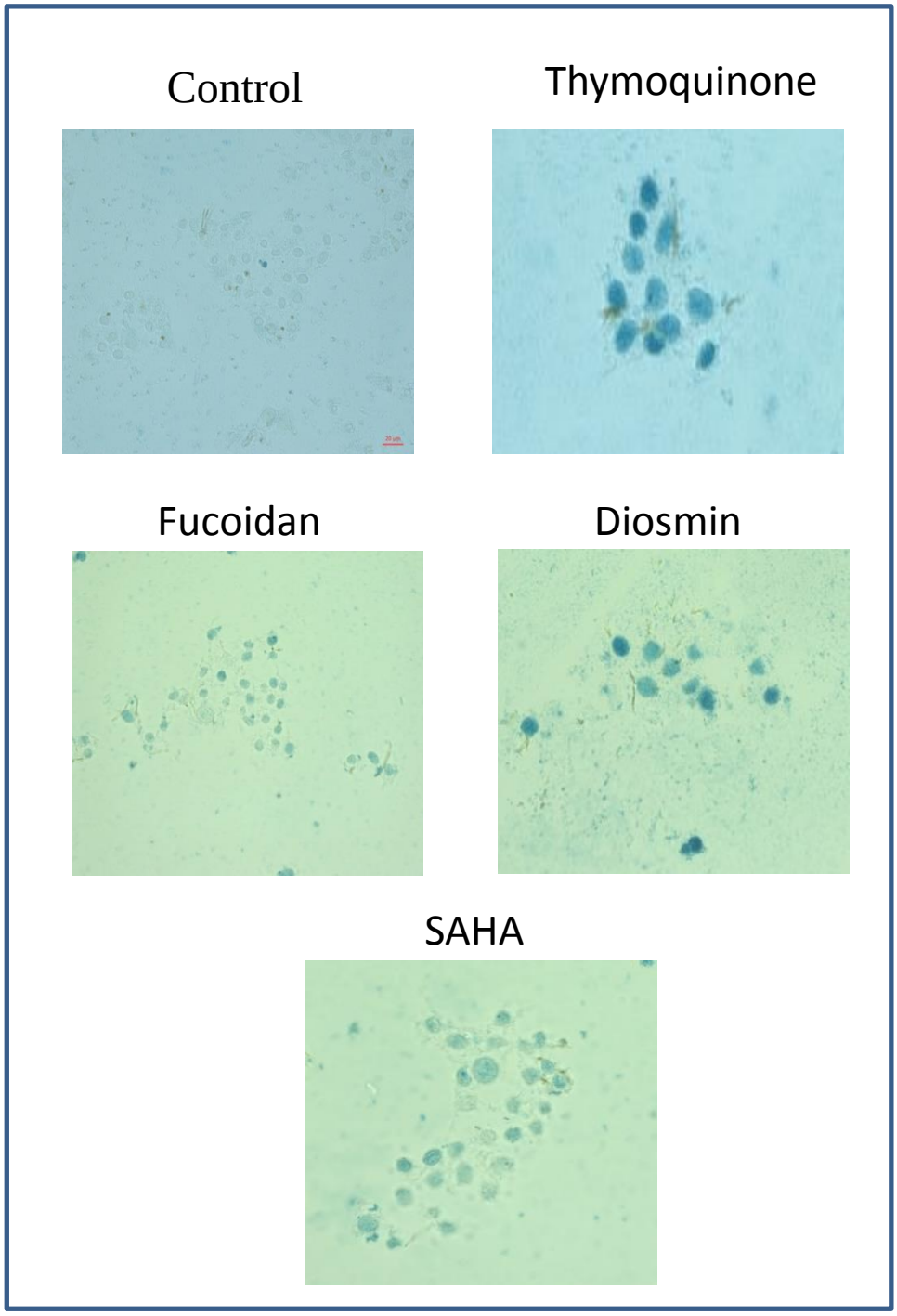


Fig 4.31)Senescensce study was carried out in HeLa cells.Senescence-associated β-galactosidase (SA–β-Gal) staining for senescent cell showing blue coloration of SA-β-gal-positive cells. in HeLa cells (i) Control , (ii) Fucoidan 80 µg/ml iii)Thymoquinone(40µM) , iv) Diosmin100µM and V) SAHA500nM.

,as shown in (fig 4.29). Further analysis with DAPI staining showed the formation of various heterochromatin domains in the form of Dark black spot called senescence-associated heterochromatin foci (SAHF) after the treatment of Bioactive compounds, as shown in (fig4.30)

4.1.11Effect Of Bioactive Compounds On The Protein Involved In Epigenetic Modulation

HDAC1 is a class1 Histone deacytlase is recruited as transcriptional corepressor complexes to regulate chromatin structure. It acts at DNA break sites during DNA repair and to chromatin around forks during DNA replication and in genome maintenance(Bhaskara, 2015). HDAC inhibitors directly target regulators of genome stability in order to kill the rapidly dividing cancer cells. In this study, Fucoidan, Thymoquinone, Diosmin and SAHA generate the ROS which in turn down-regulate the expression of HDAC1. Fucoidan treatment in HeLa cells for 24hours show maximum 40 % reduction , in case of TQ it shows at most 19% reduction, in case of Diosmin show highest decrease is 38%, and SAHA show40% reduction in HDAC1 expression as analysed by FACS As shown in (fig 4.36, 4.38 and fig 4.40.) as well as immune blot analysis. As shown in (fig 4.41and fig 4.42) and imunocyto of Fucoidan treated HDAC1 down regulation fig 4.32

4.1.12Effect Of Bioactive Compounds On Acetylated Histone 3 Lysine9/14 (Ac-H3lys9/14)

Acetylated Histone protein play an important role in chromatin remodelling and in the regulation of gene transcription .During HDAC1 inhibition it is found to upregulated.(X. Li, Li, & Sun, 2016).Next, we sought to determine whether bioactive compound treatment can depress the downstream target genes of HDAC1 that is Ac-H3lys9/14. Treatment in HeLa cell for 24 hour with Fucoidan show maximum 1.43 fold increase in case of TQ maximum 1.40 fold increase, Diosmin show 1.32 fold increase and SAHA 1.48 fold increase which shows these compound behave as epigenetic drug ,. As shown in (fig 4.36, 4.38 andfig 4.40.)

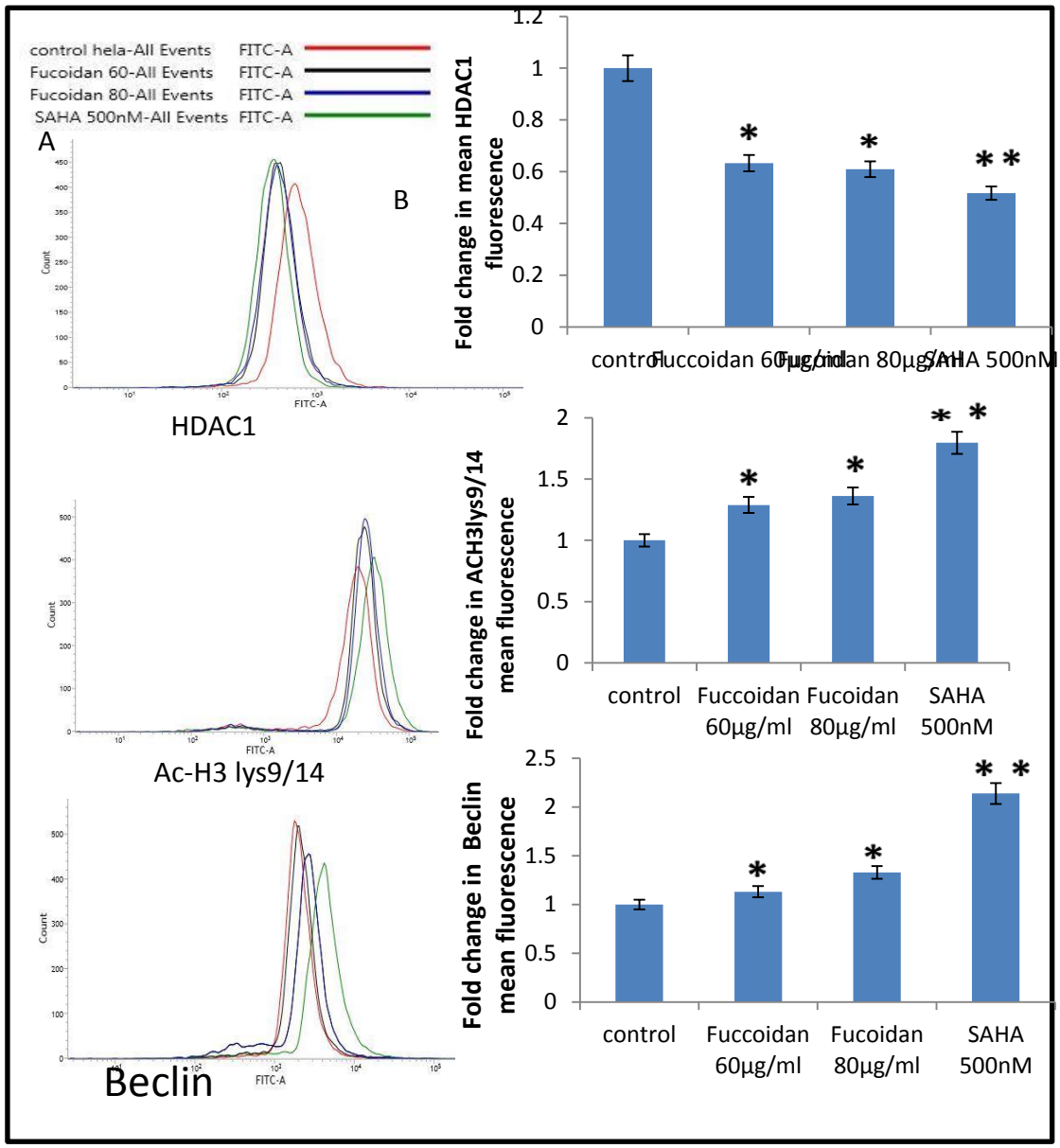


Fig4.36) A) Epigenetic modulator protein quantification by FACS Analysis indicates After Fucoidan treatment in HeLa cell causes down regulation in the level of HDAC1and upregulation of AcetylH3 Lys9/14 and it also shows increase in Beclin expreession show autophagy induction B)Histogram profile of protein Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05 ** denotes p<0.001

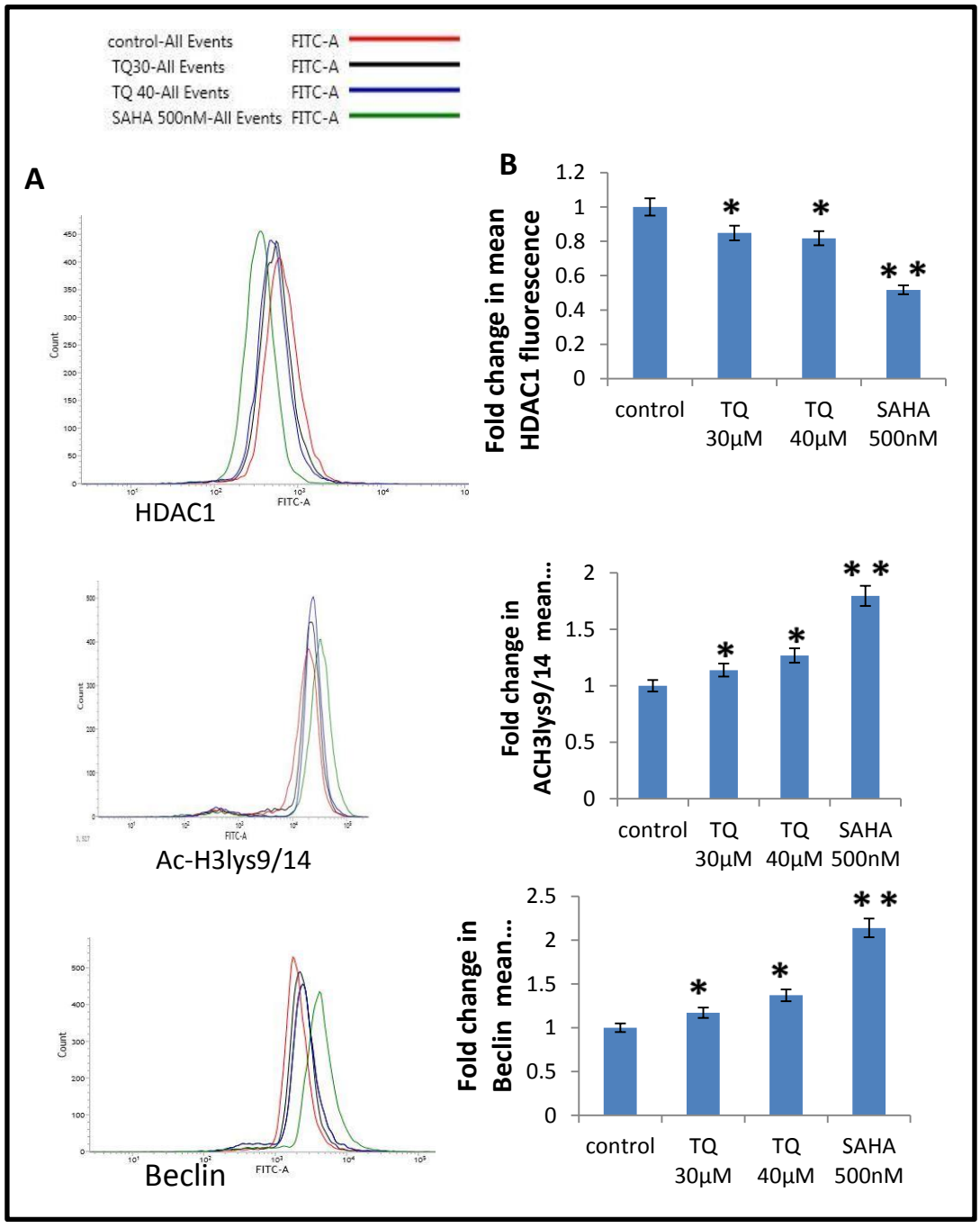


Fig4.38) A) Epigenetic modulator protein quantification by FACs analysis of TQ treatment in HeLa cell causes down regulation in the level of HDAC1and upregulation of AcetylH3 Lys9/14 and it also shows increase in Beclin expression show autophagy induction B)Histogram profile of protein Values are means ± S.E.M. of at least three independent experiments* denotes p<0.05 ** denotes p<0.001

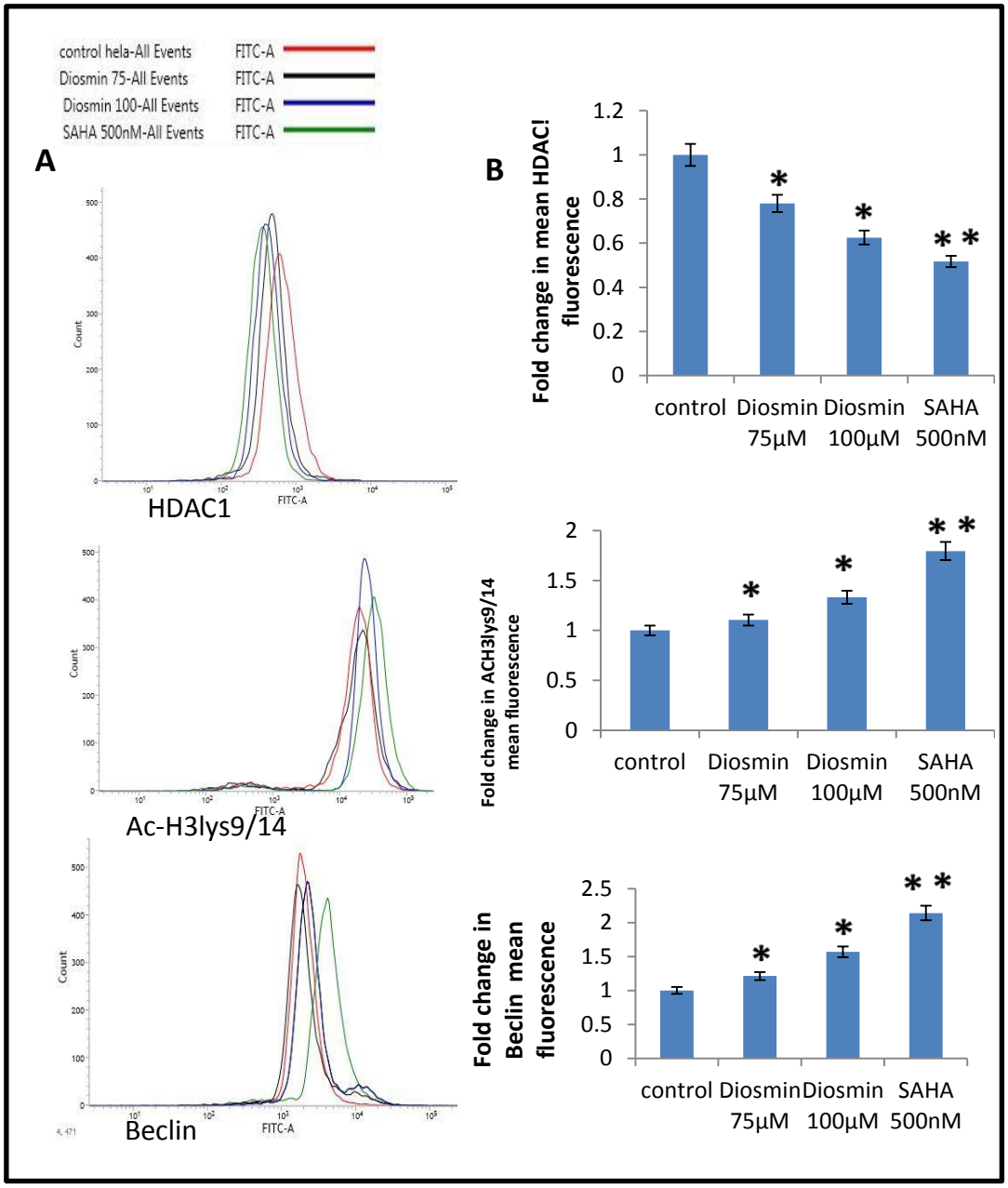


Fig4.40) A) Epigenetic modulator protein quantification FACS analysis of Diosmin treatment in HeLa cell causes down regulation in the level of HDAC1and upregulation of AcetylH3 Lys9/14 and it also shows increase in Beclin expreession shows autophagy inductionB)Histogram profile of protein Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05 ** denotes p<0.001

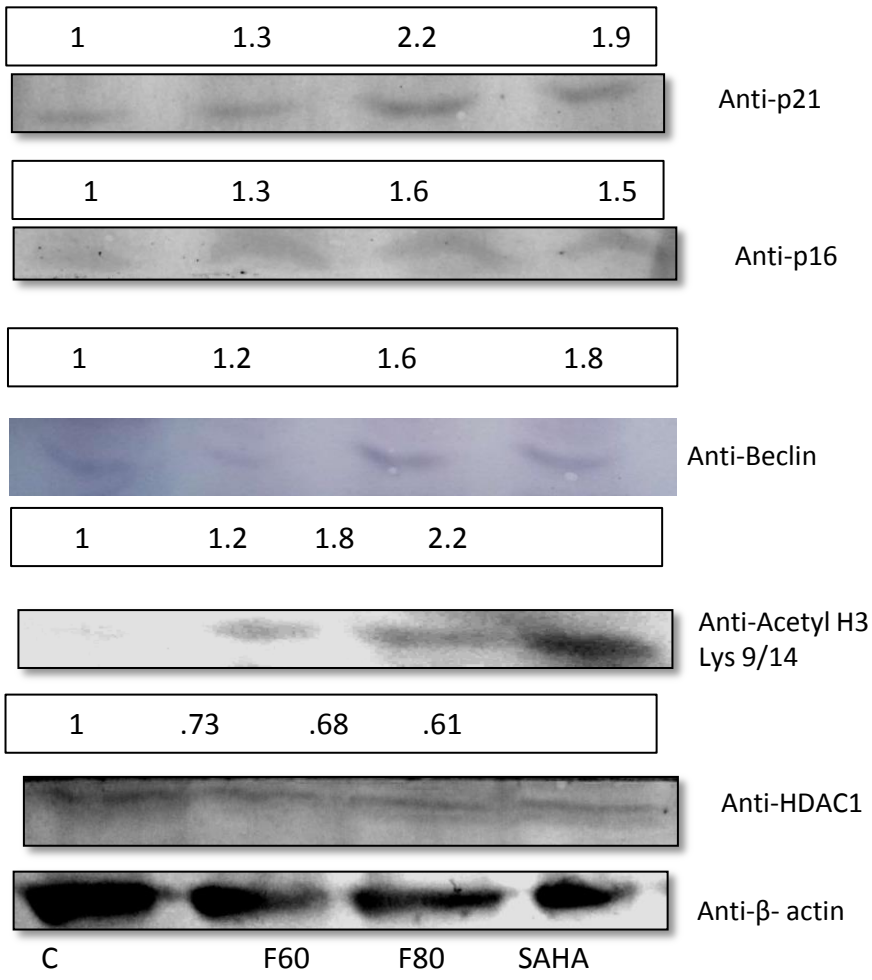
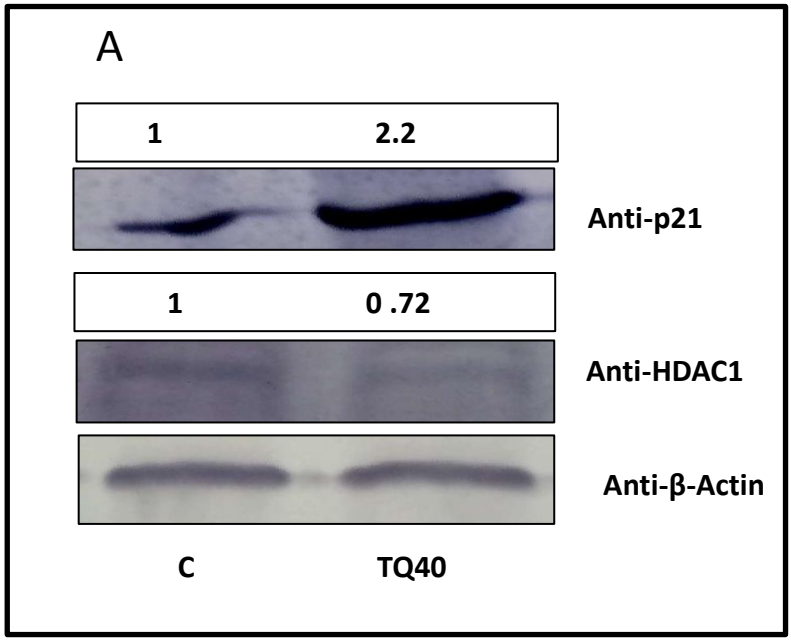


Fig 4.41)Immunoblot analysis after treatment with Fucoidan (60 and 80µg/ml and SAHA 500nM in HeLa cell show down regulation of HDAC1 and upregulation of p21 ,p16 beclin and AcetlH3 lys9/14,, Fold increase in p21 , p16 and beclin has been estimated against their respective β- actin expression level (not provided) data representative of the mean value of (n.3) experiments with ±SE



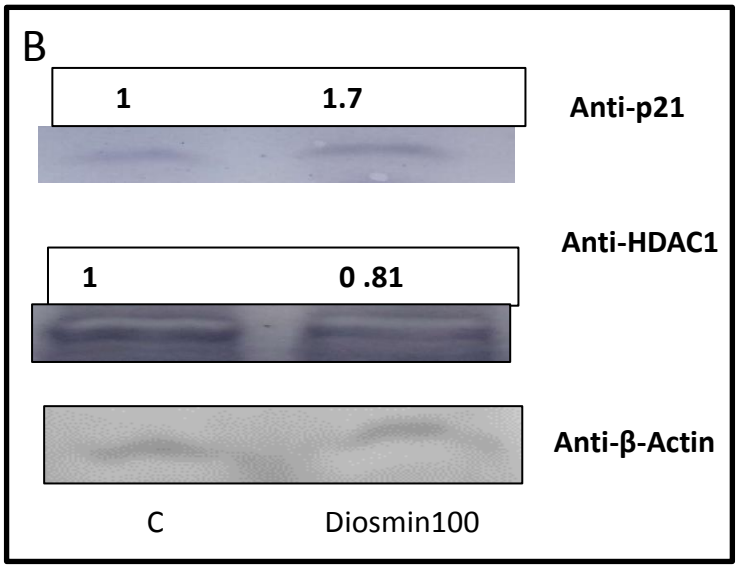


Fig 4.42)Immunoblot analysis of HDAC1 and p21 expression level: Western blots showing decrease in HDAC1 and increasein p21 expressionA) thymoquinone 40μM B) Diosmin 100 μM.All data representative of the mean value of (n.3) experiments with ±SE.

4.1.13Effect Of Bioactive Compounds On p21 Expression

Senescence p21 it is called as p21WAF1/Cip1 one of most common cyclin-dependent kinase (CDK)inhibitor play important role in cell cycle regulation and respond to anti -proliferative signals.(Gui, Ngo, Xu, Richon, & Marks, 2003) Treatment in HeLa cell for 24 hour with Fucoidan show maximum 2.24 fold increase in case of TQ maximum 1.80 fold increase, Diosmin show 2.1 fold increae and SAHA 3.5 fold increase which shows these compound behave as antiproliferative compound. similarly in case of Fucoidan immune-cytochemical (fig 4.33) and immunoblot analysis showed the decrease in the expression levels of HDAC1 and p21 over expression . As shown in (fig 4.35, 4.37 andfig 4.39.)

4.1.14Effect Of Bioactive Compounds On p16 Expression

Senescence associated protein $p16^{Ink4a}$ is a member of Ink4 family of CDK inhibitor, it is repoted to be involved in G1cell cycle arrest and tumor suppression. .Level of p16 was down regulated in cancer cell(Mazo, Lleonart, Castellvi, & Cajal, 2011). Treatment in HeLa cell for 24 hour with Fucoidan show maximum 1.55 fold i increase in case of TQ maximum 1.73 fold increase, Diosmin show 1.69 fold increase and SAHA 2.1 fold increase which shows that these compound arrest cell cycle in G1 phase. . As shown in (fig 4.35, 4.37 and fig 4.39.)

4.1.15Effect Of Bioactive Compounds On HABP1/ p32 Expression

HABP1, is member of the ‘hyaladherin’ family of proteins, a 34 kDa glycosylated phosphoprotein, which interacts with hyaluronan (HA) and is implicated in cell signaling. Studies have reported that, there is an increase expression of HABp1/p32 during autophagy and apoptosis. In the present study, it was observed that treatment with Fucoidan show 1.29 and 1.82 fold increase, in case of TQ it shows 1.36 and 1.95 fold increase, in case of Diosmin 1.04 and 1.80 fold increase, and SAHA show 2.53 fold increase in HABP1 expression level. . As shown in (fig 4.35, 4.37 andfig 4.39.)

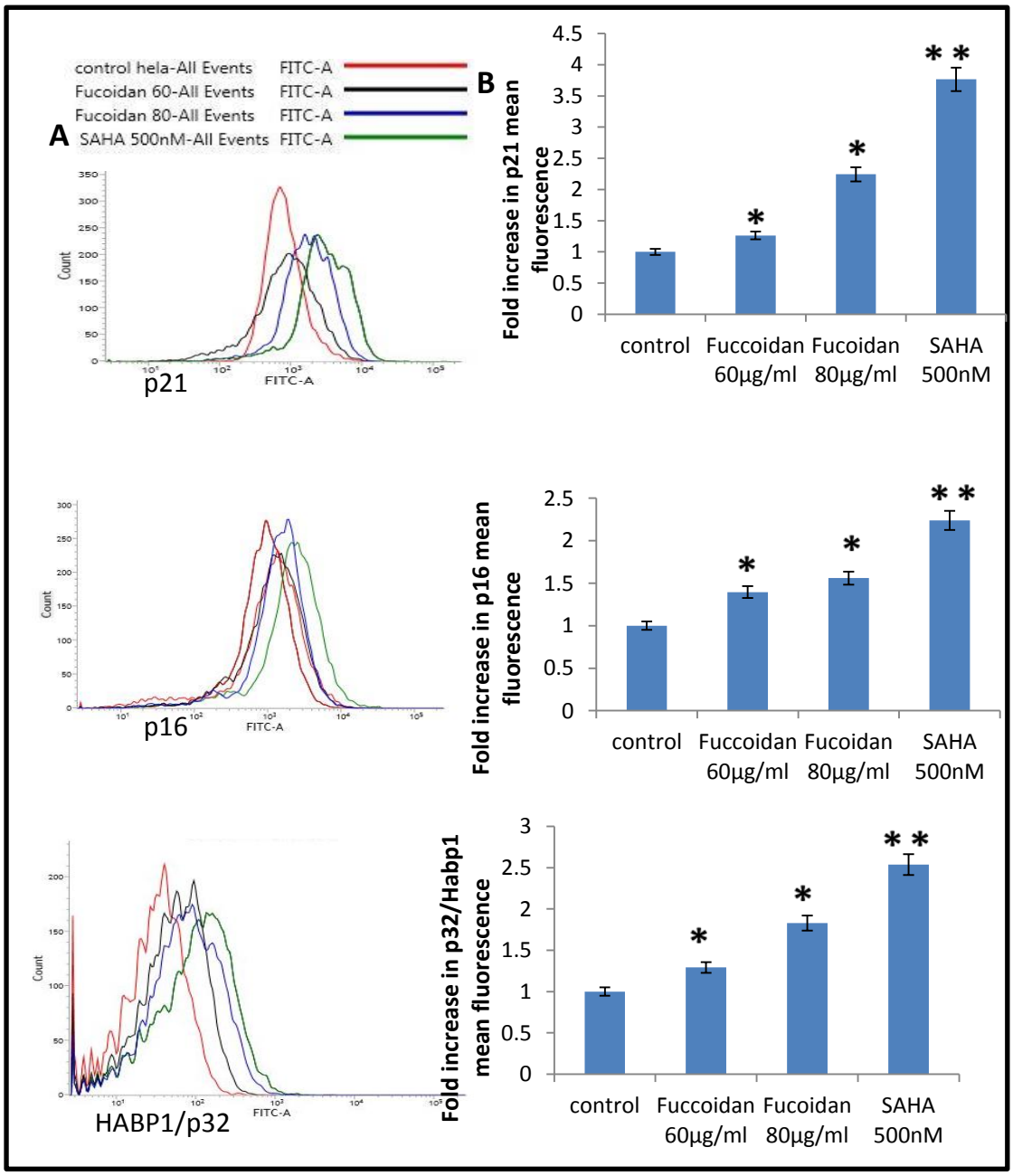


Fig 4.35) A) Protein quantification by FACS analysis of 1)P21, 2)p16and 3)p32(HABP1) found to be upregulated after treatment of Fucoidan indicates senescence induction B)Histogram profile of protein Values are means ± S.E.M. of at least three independent experiments * denotes p<0.05 ** denotes p<0.001

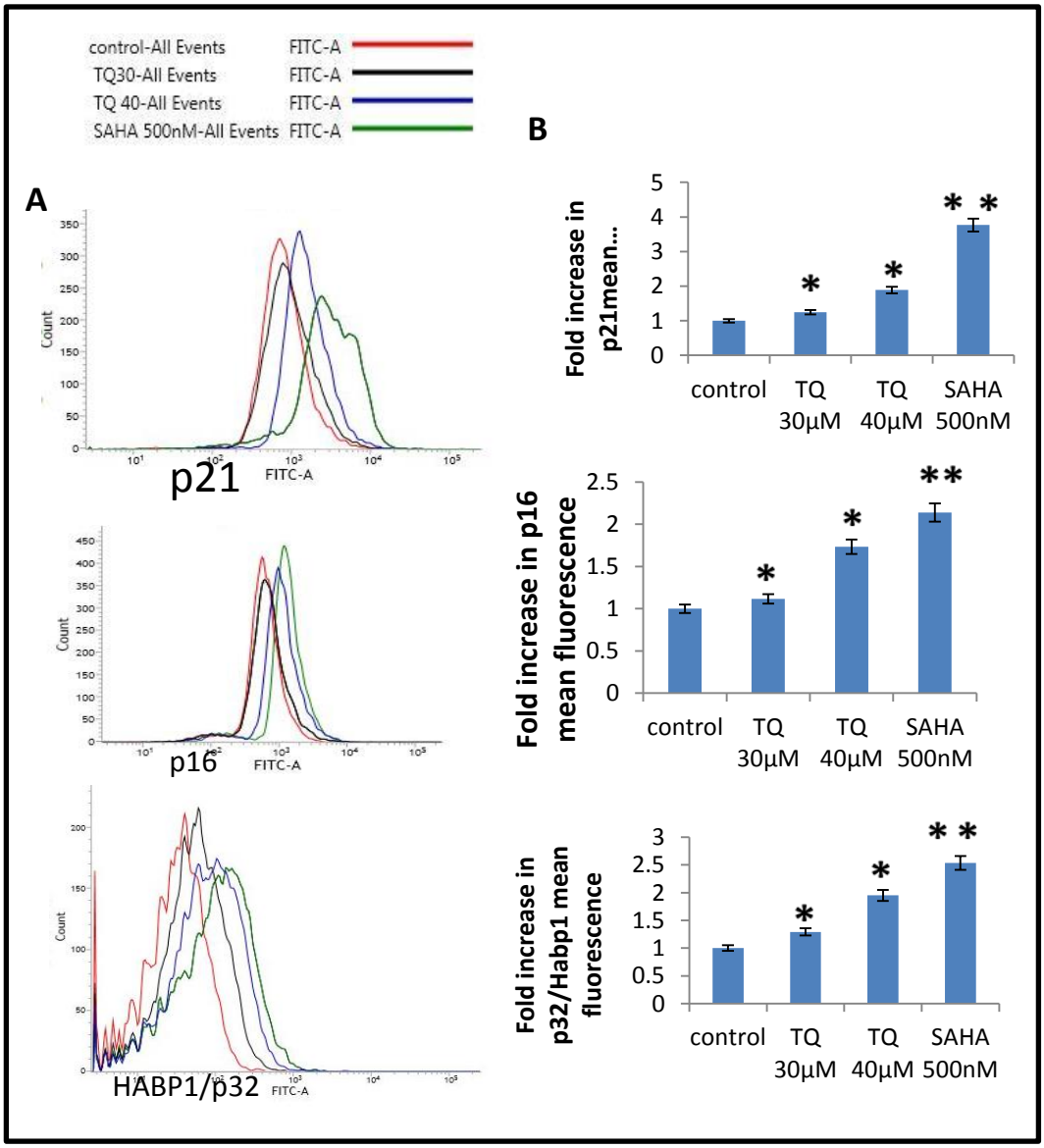


Fig4.37) A Protein quantification by FACS analysis of P21, p16and p32(HABP1) found to be upregulated after treatment of TQ indicates senescence induction B)Histogram profile of protein Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05 ** denotes p<0.001

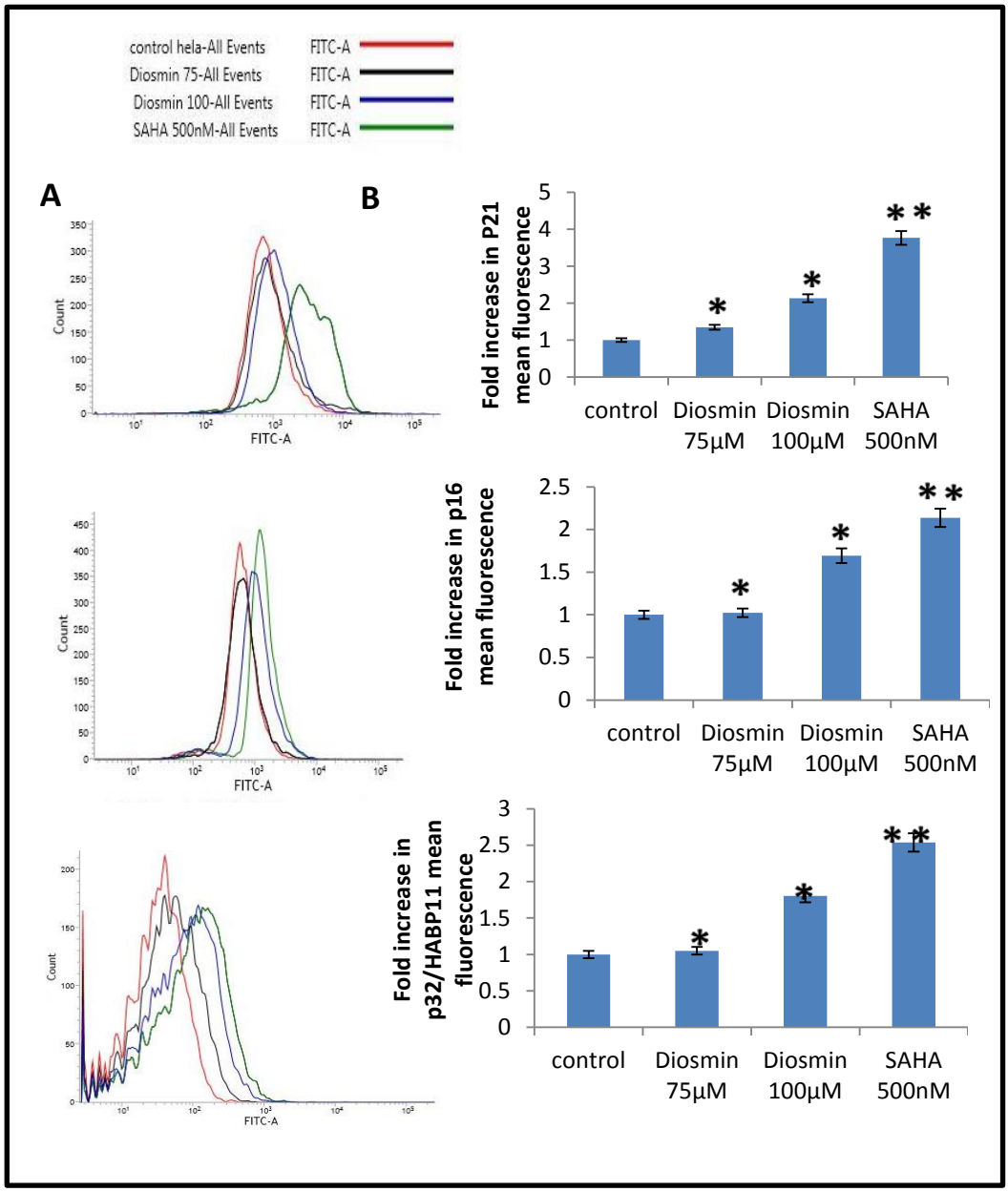


Fig4.39) A) Protein quantification by FACS analysis FACS analysis of P21,p16and p32(HABP1) found to be upregulated after treatment of Diosmin indicates senescence induction B)Histogram profile of protein Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05 ** denotes p<0.001

4.1.16Effect Of Bioactive Compounds On Beclin-1 Expression

ROS brings about the upregulation of autophagosome formation associated protein Beclin1 which is needed for nucleation of autophagosomes (Saha, Chowdhury, Dutta, Chatterjee, & Ghosh, 2013). Treatment in HeLa cell for 24 hour with Fucoidan show maximum 1.32 fold increase in case of TQ maximum 1.37 fold increase, Diosmin show 1.5 fold increae and SAHA 2.13 fold increase which shows these compound induces autophagy. As shown in (fig 4.36, 4.38 and fig 4.40.)

4.1.17Docking Analysis

HDAC1 Molecular docking with Fucoidan have binding energy -7.4kcal/mol TQ -6.4,Diosmin -8.4 whereas SAHA has -5.6kcal/mol and binding site are Lys331 and Tyr 327 as shown in fig .(fig 4.34) .which shows that bioactive compound have a strong affinity with HDAC1 then SAHA a well-known HDAC inhibitor

4.2Discussion

Possible reasons behind the use of these bioactive compounds namely Fucoidan Thymoquinone and Diosmin main interest lies in their natural origin, widespread availability, lesser side-effects and the possibility of inclusion in the routine diet. However, most of the synthetic HDAC inhibitors have shown adverse side effects, narrow in their specificity and also expensive. Hence, bioactivecompounds, which are widely available with lesser toxic effects, have been tested for their role in epigenetic modulatory activities in cancer prevention and therapy. Encouragingly, many bioactive compounds potentially altered the expression of key tumor suppressor genes, tumor promoter genes and oncogenes through the modulation of HDAC involve in cancer. These bioactive compounds showed promising results against various cancers.

In the present study we have assess the cytotoxic potential of different bioactive compounds derived from ocean and plant sources on Human epithelial cervical carcinoma cell line HeLa. Results suggest that these

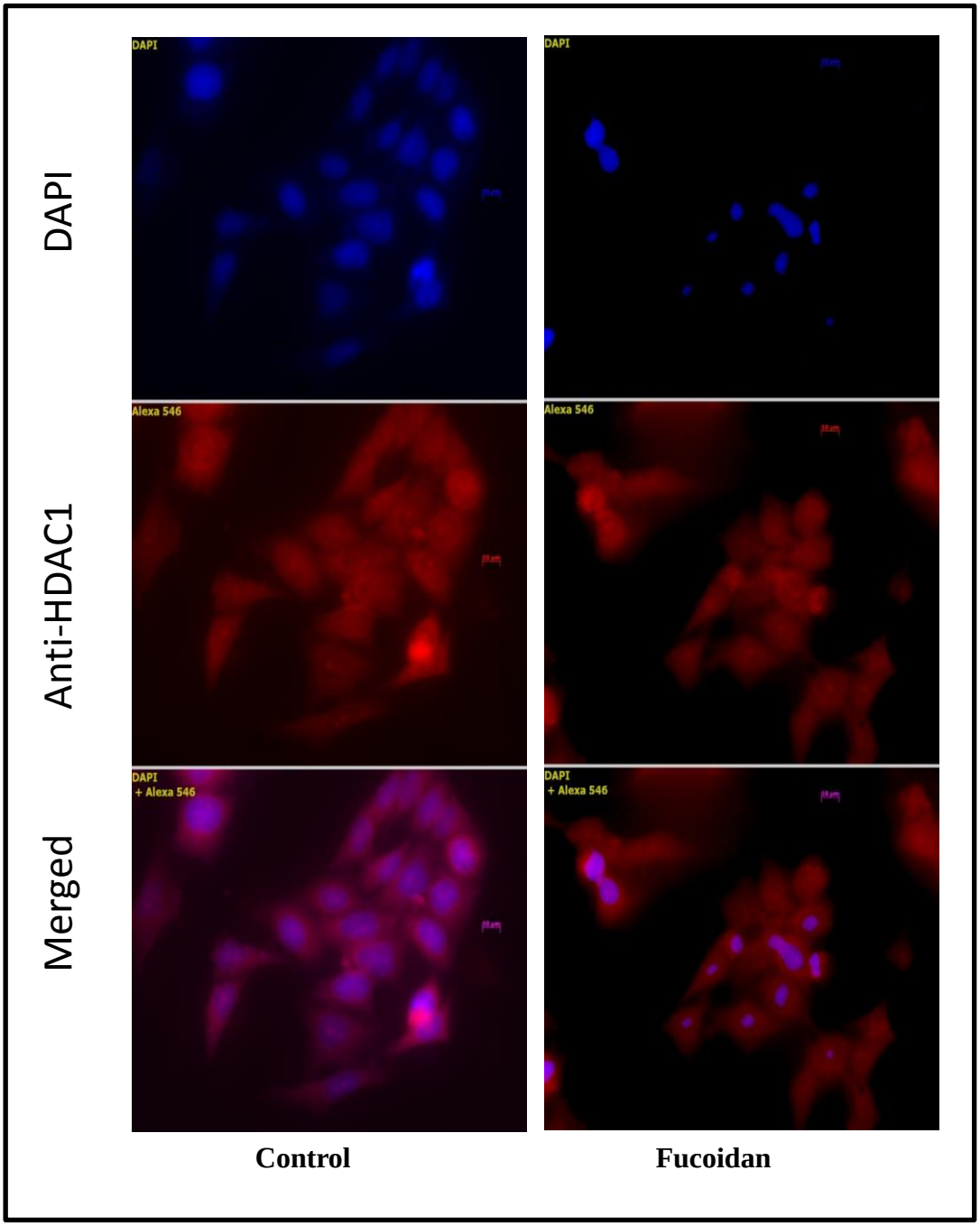


Fig4.32) HeLa cells after treatment with Fucoidan80µg/ml showed the change in the expression of HDAC1 was observed upon visualization under Fluorescence microscope after immunocytochemical analysis was performed .

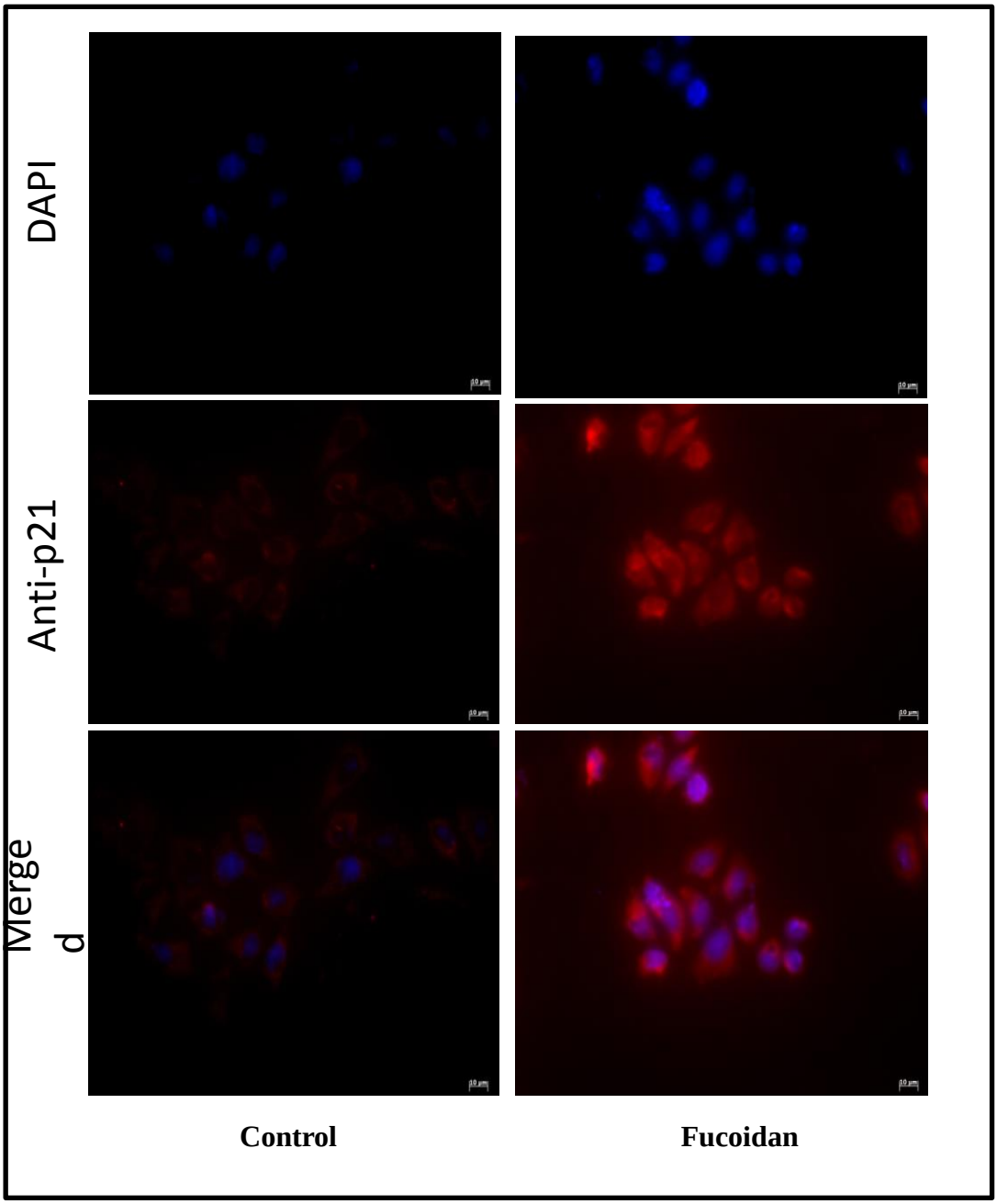
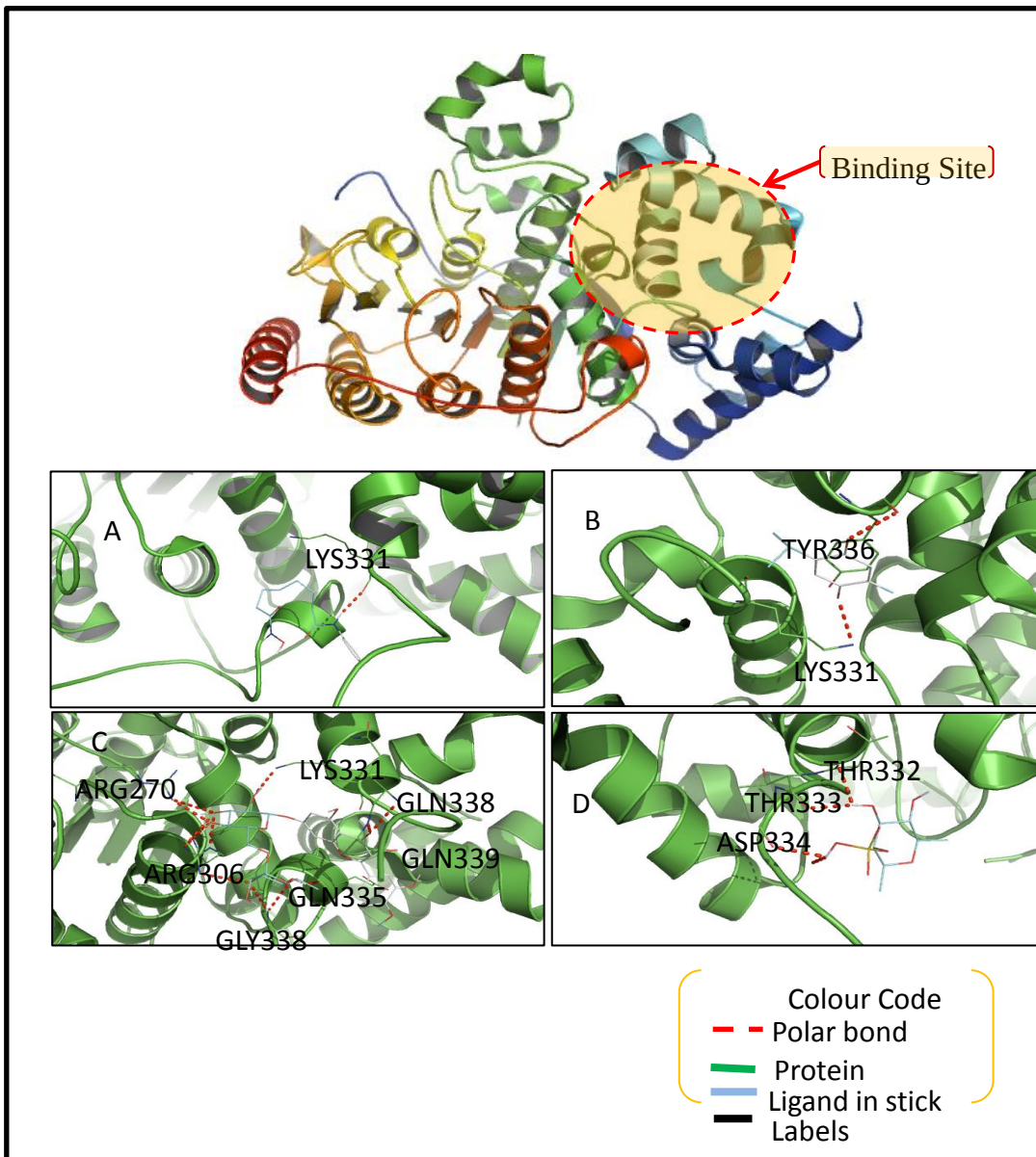


Fig.4.33) HeLa cells after treatment with Fucoidan80µg/ml showed the change in the expression and localization of p21 upregulation was observed upon visualization under Fluorescence microscope after immunocytochemical analysis was performed



Serial No.	PubChem ID	Drug Name	Energy (Kcal/mol)
A	5311	SAHA	-5.6
B	10281	Thymoquinone	-6.4
C	5281613	Disomin	-8.5
D	92023653	Fucoidan	-7.4

Fig 4.34) showing the HDAC1 docked structure with different ligand A) SAHA B) Thymoquinone , C) Diosmin and D) Fucoidan binding site are Lys331 and Tyr 327: showing strong affinity with HDAC1.

bioactive compounds effects the viability of HeLa cells and show cytotoxicity to HeLa cell in concentration dependent manner. Autophagy and senescence share a lot of common features which suggest that these processes could assist the similar cell fate as reported during stress condition like nutrients deprivation, radiation exposure, and phytochemical treatment. Moderate increase in ROS level, which is mainly responsible for regulating HDAC inhibition, autophagy and senescence phenomena. In the present study we have investigated the redox potential of different bioactive compounds while treating HeLa cells with bioactive compounds at low doses Fucoidan Thymoquinone, Diosmin and SAHA a known HDAC inhibitor, these compounds was found to enhance ROS generation in dose-dependent manner(fig 4.5 to4.11) and this increase in ROS results in the induction of Autophagy(fig 4.21 to 4.24).and premature senescence and HDAC inhibition .

Further a well known antioxidant N acetyl L cysteine (NAC) an aminothiol and synthetic precursor of intracellular cysteine and GSH is applied in this study to describe the role of ROS in bioactive compound induced effect. NAC play its role as an antioxidative as well as free radical scavenger by increasing intracellular GSH (glutathione)level, GSH, plays an important role by act as a substrate of ROS scavenging enzymes Fucoidan, Thymoquinone, Diosmin and SAHA show the decrease in ROS in NAC pre-treated HeLa cells which confirms the proxidant nature of these compounds.

After that we analysed the mitochondrial super oxide anion,Mitochondria being the major source and target of ROS that result in DNA damage and mitochondrial dysfunction, ROS activates pro oxidant genes and imbalances antioxidant genes induction. The set of alterations caused by ROS lead to induction of cell senescence which in turn develop as both positive and negative effects. with the treatment of Fucoidan, Thymoquinone and Diosmin treated Hela cells show enhanced Mitochondrilal super oxide anion as measured by mitosox red similarly HDAC inhibitor SAHA show enhanced superoxide anion It is also reported by other research group that ROS/ RNS are required for sustaining Autophagy, mitochondria being the main source of ROS(Filomeni, Zio, & Cecconi, 2014). In our study, we have also shown that

the low dose treatment of bioactive compounds causes the depolarization of mitochondrial membrane potential leading to the induction of Autophagy and senescence due to enhanced ROS and mitochondrial superoxide anion generation .we have also analyse the ATP generation in HeLa cell after 24 hours of treatment with bioactive compounds shows the loss in ATP generation due to the mitochondrial ROS generation which impairs the ability of mitochondria to synthesize ATP and to perform the wide range of metabolic functions,(fig 4.16).

Many synthetic HDAC inhibitor such as LAQ-824 sodium butyrate and SAHA triggered an early reactive oxygen species (ROS) peak to trigger cancer cell apoptosis through diverse cytotoxic actions including glutathione depletion and oxidative stress(Rosato et al., 2008). Chemotherapeutic drugs cause cell death through releasing cytochrome c and also disrupt the mitochondrial electron transport chain which leads to ROS increase (Sun, Chen, Wang, Chen, & Wei, 2011). Similar mechanism was seen in these bioactivecompounds

Similarly pre mature senescence was found after treatment with low dose of bioactive compounds and SAHA in HeLa cell as indicated by morphological changes such as higher FSC and SSC in treated cells, positive senescence-associated β-galactosidase activity , and heterochromatin foci as studied by DAPIstaining, HDAC inhibitor such as SAHA arrest cell cycle in G1 phase,in our study we have found that bioactive compounds shows irreversible G1 cycle arrest.p16^{ink4A} is inactivated during epigenetic changes HDAC inhibitors regulates p16^{ink4A} in epigenetic manner increase in expression which in turn reduces the cell proliferation as shown by us in HeLa cells and by others in Colon and liver cancer cell lines (Banerjee & Mandal, 2015)(Park, Lim, & Jang, 2011). The treatment also showed a higher level of Autofluorescence (or increased intrinsic fluorescence) implying the accumulation of oxidatively damaged proteins (Goodwin & Dimaio, 2001) and lipids in the Hela Cells. Our results matched with the recently explained role of mitochondria in cellular senescence induced by pro-oxidant signals (Correia-melo et al., 2016). It is also reported by other research group that ROS/ RNS are required for

sustaining Autophagy, mitochondria being the main source of ROS(Filomeni et al., 2014). In our study, we have also shown that the low dose treatment of bioactive compounds causes the depolarization of mitochondrial membrane potential leading to the induction of Autophagy and senescence due to enhanced ROS and mitochondrial superoxide anion generation.(fig4.13 to 4.15)Similarly these compounds display the feature of early events of autophagosome formation and cell cycle arrest as observed by us (Haritunians et al. 2008) and also loss in ATP is a signal of ROS generation that causes reduced actin dynamics resulted in aberrant F-actin aggregates ("The actin cytoskeleton : a key regulator of apoptosis and ageing and induction of Autophagy(Cho, Yen, Shim, Kang, & Kang, 2016).

Bioactive compounds treatment shows increased in ROS production. Enhanced ROS generation plays the primary role in HDAC inhibitor-induced responses (Falkenberg & Johnstone, 2014). HDAC1 inhibition leads to many changes at the molecular and cellular levels and is currently under target for drug designing (Coderch, Panchuk, & Skorokhyd, 2016). ROS signaling might be inducing the acetylation of lysine residues on the N-terminal tails of core histones by histone acetyltransferases (HAT) and the deacetylation of histones by histone deacetylases (HDAC) employing nucleophilic mechanism(Afanas, 2014). During normal condition targeted disruption of HDAC1 causes early embryonic lethality in the cancer cell. HDAC1 represses the Function of tumor suppressive genes such as p21WAF1/CIP1 to maintained their proliferation, and there is an Overexpression of HDAC1 in cervical cancer cells in vivo and in vitro(Lin et al., 2010). while knockdown through HDAC1 siRNA and treatment with SAHA in HeLa cells gives direct evidence to oxidative stress(Kato et al., 2009) and autophagic induction(Oh, Choi, & Kwon, 2008)whereas SAHA reduced HDAC1 expression levels in WI-38 cells(Place, Noonan, & Giardina, 2005) and in gallbladder carcinoma(Yamaguchi et al., 2010) induces the senescence-associated marker,(fig4.34 4.35 4.37and 4.39) we got the similar result in HeLa cells after treatment with Fucoidan Thymoquinone and Diosmin and this shows that these bioactive compounds may act in same direction to HDAC inhibitor. SAHA also known to induces autophagy by raising the ROS level which in

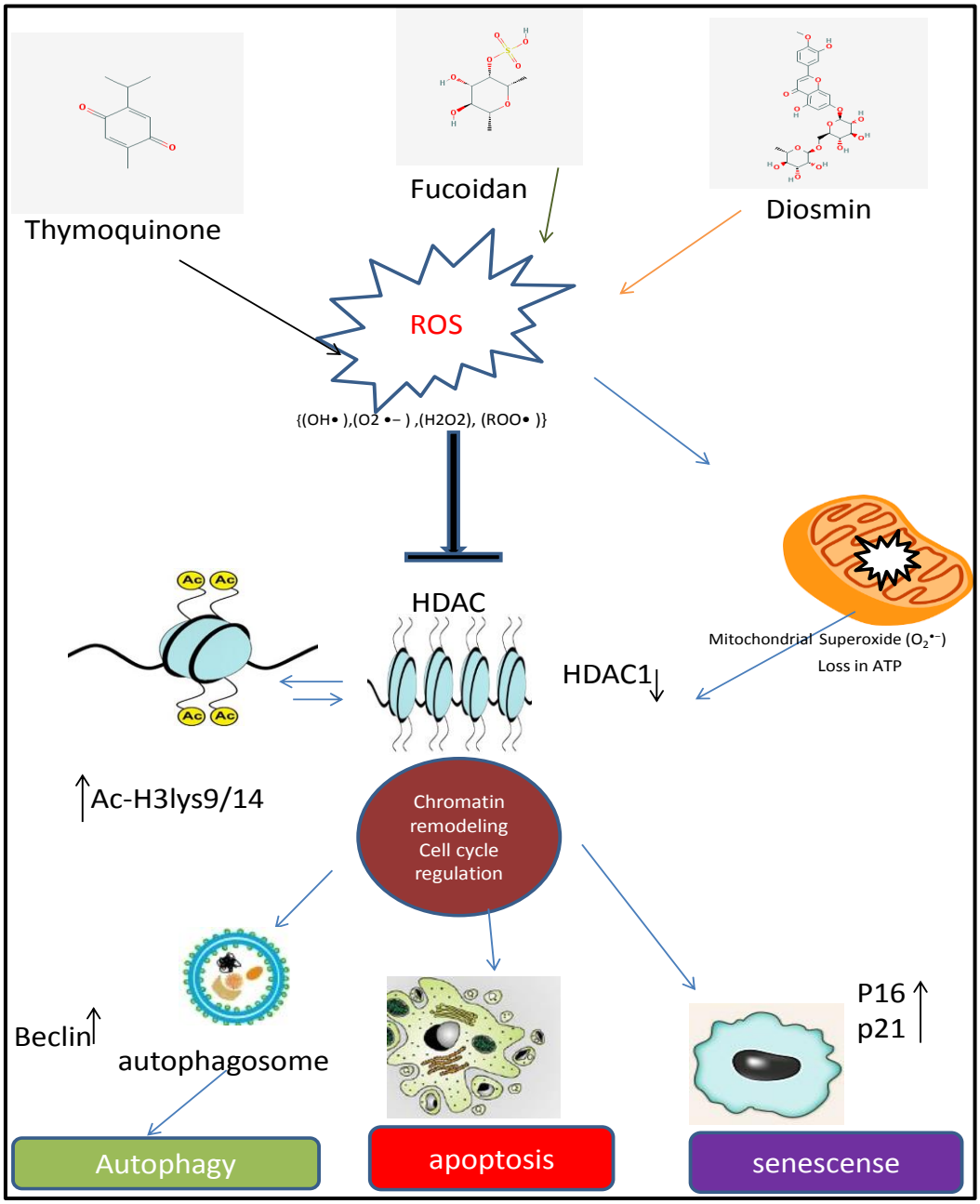


Fig 4.43) Schematic representation of ROS dependent Autophagy induction and senescence involving HDAC Inhibition.

turn down regulates HDAC1 in HeLa cells(Oh et al. 2008) and the initiation of Autophagy takes place with the formation of Autophagosomes (He & Klionsky, 2009) which upregulates the expression p32/HABp1 and Beclin1 component of the nucleation complex during autophagy (Saha et al., 2013) (Saha et al., 2013) these bioactive compounds gives the similar results. The HABP1/p32 protein remains localised predominantly in the cytoplasm with very little amounts in the nucleus in normal HeLa cells, Its Increased intracellular HABP1 levels during cisplatin induced apoptosis was reported in HeLa cell(Kamal & Datta, 2006) and also over expression of HABP1/p32 induces autophagy in ROS dependent manner,with the treatment of Bioactive compounds and SAHA in HeLa cell show upregulation of HABP1/p32 that further confirms autophagy induction in ROS dependent manner

Finally in our study, we have explored the computational method to precdict the noncovalent binding of protein with the ligand (fig4.34) . Here we analyse all the possible binding modes and key active site residues for each bioactive compounds with HDAC1 in comparison to SAHA, Fucoidan Thymoquinone and Diosmin show much stronger affinity with HDAC1 than SAHA . .

This study further confirmed the persistence of Autophagy and premature senescence during HDAC1 inhibition at lose dose of both bioactive compounds and SAHA respectively that gives an idea about potential relationship(s) between HDAC inhibition, autophagy and senescence in cancer prevention.

Comparative Analysis Of Crude Fucoidan And Pure Fucoidan From Brown Algae Fucus Vesiculosus On HeLa Cell

5.0 Introduction

In Asia, the edible seaweeds have remained a stable food for thousands of years. Being a rich source of fiber, they have been used both as diet and traditional medicines. However, in the past decade, only the brown seaweed has been the focus of the research. Fucoidan was isolated and named by Kylin in 1913(Fitton, 2011). It is an intracellular sulfated water-soluble and highly hygroscopic polysaccharide The principal function of fucoidan is to protect the seaweed surface from dryness and to contribute to the formation of a gel network (B. Li, Lu, Wei, & Zhao, 2008)The dietary fiber found in brown seaweed consists of soluble polysaccharides (like fucans, alginates and laminarans) and insoluble fibres (consisting of cellulose). Fucans are the cell wall polysaccharides which can be classified into three groups: ascophyllans, fucoidans and glycuronofuco-- galactans sulfate. The fucoidan is a kind of complex carbohydrate or sulfated polysaccharides (FCSPs) composed mainly of a backbone of (1-3)- and (1-4)-linked α-Lfucopyranose residues which can be substituted with single L-fucosyl residues, sulfate, and/or short fucoside chains.(Atashrazm, Lowenthal, Woods, Holloway, & Dickinson, 2015)

Fucoidan also reported as Biological response modifiers (BRMs) which augment immune response by scavenger receptor (SRs) that are expressed by macrophagesand dendritic cells (DCs). The characterized pattern recognition receptors PRRs which recognize polysaccharide Fucoidan are Class A SR, endogenously produced BRMs in our body are cytokines which are displayed by immune cells. Derivatives of bacteria, fungi, brown algae, *Aloe vera* and photosynthetic plants are called exogeneous BRMs(Leung, Liu, Koon, & Fung, 2006). Earlier studies have shown crude Fucoidan from *F. vesiculosus*, contains majorly of fucose (45.9%) and sulphate (32.1%) galactose (3.2%), xylose (3.6%) and with traces of uronic acid (8.7%) weheras the pure Fucoidan from F. vesiculosus consist of around 43.1 % fucose, 17.96% sulfate, 2.2% galactose, 8.8% xylose and 3.1% uronic acid(Zayed et al.,2016.). However, research on Fucoidan has increased greatly in the last decade, due to interest in its varied potentially beneficial biological activities such as anti-coagulant, anti-thrombin (Fitton, 2011), anti-

HIV and anti-tumor activities(Banafa et al., 2013) Despite this, the mechanisms of fucoidan, and the particular structures or fractions that are responsible for different biological activities, are still unclear, owing to a lack of standardized extraction and purification protocols.

Previous studies reported that crude Fucoidan from *F. vesiculosus* induces strong cytotoxic activity against human lymphoma HS-sultan cells human acute leukemia NB4 and HL-60 cells(Kwak, 2014), human colon cancer HT-29, HCT116 cells(S. Chen, Zhao, Zhang, & Zhang, 2014), human breast cancer MCF-7 cells,AGS human gastric adenocarcinoma(Banafa et al., 2013) and human bladder carcinoma 5637, T-24 (Cho et al., 2014). Fucoidan shows anti-metastatic potential on A549 lung cancer cells by down regulating of ERK1/2 and Akt-mTOR as well as NF-kB signaling pathways.Whereas pure Fucoidan from *F. vesiculosus* reported as supplementation in mice that protects against ethanol-induced liver injury, by down regulating hepatic production of the inflammatory cytokines, such as TGF-β1, COX-2 and enhancing the defence systems by the upregulating the HO-1 expression(J. Li & Liu, 2016). Bioactive compounds from different plant sources either purified compound and crude extract may have different activity. In some cases a similar group of purified compounds have more activity. There are possibility of pure compound may be inactive. Some formulations of algal crude extract have more activity than the purified compound. In the same there are chances for pure compound is more active than crude(Bauer et al.,2011.).

Crude fucoidan has been documented to possess strong anticancer activity, with minimal or negligible toxicity on healthy tissues. Owing to the selective nature of fucoidan, it is a potential natural substance, to be considered as great cancer therapeutics. Although fucoidan has been tested against different types of cancer, but not enough research has been done yet regarding the anti-cancer effect of fucoidan on cervical cancer.

In the present study, we used Fucoidan, sulphated polysaccharides from seaweed. Commercial crude and pure Fucoidan from *Fucus vesiculosus* (bladder wrack) were procured from sigma and their comparative analysis were done for analyzing their cytotoxic potential on human cervical carcinoma

HeLa cell line and also their Redox potentials and the impact of crude and pure Fucoidan on mitochondrial membrane potential. However in this study it is also comparatively analyse for the effect of crude and pure Fucoidan on senescense ,autophagy and apoptosis in HeLa cells using Flow Cytometer.

5.1 Results

5.1.1 Effect Of Crude And Pure Fucoidan On HeLa Cell Viability

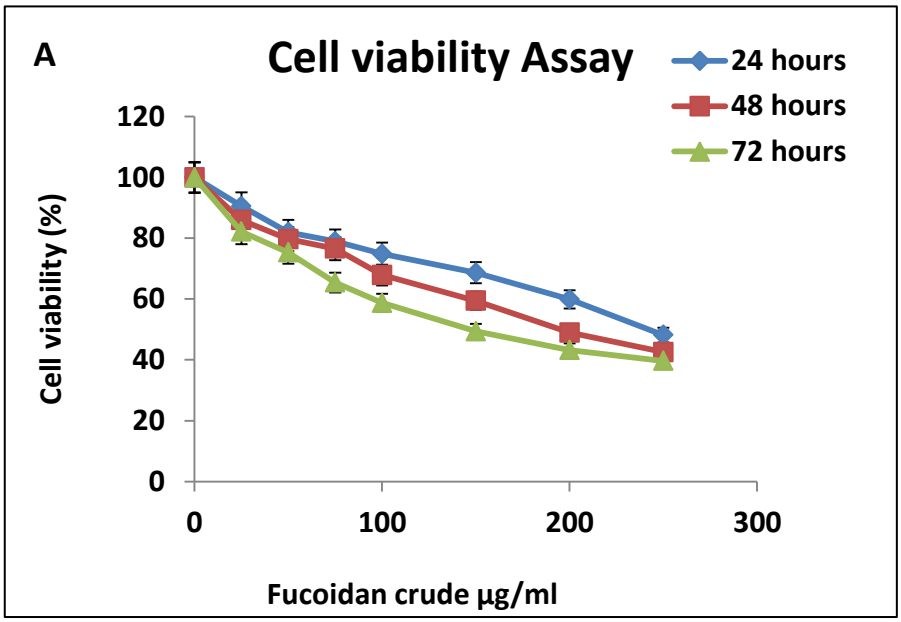
Evaluation of the effects of crude and pure Fucoidan on the growth of Hela cells after treatment with various concentration of Fucoidans in both dose and time dependent manner crude Fucoidan showed a significant decrease in cellular viability in concentration and time dependent manner, by MTT assay and IC50 value was found to be 242.11,205.70 and169.53 for 24,48 and 72 hour treatment respectively as shown in Fig (5.1). This indicates that the crude Fucoidan has the ability to inhibit cancer cell growth, whereas pure fucoidan show non-cytotoxic behaviour after treatment for 72 hour even at high dose.

5.1.2 Fucoidan Treatment Induces Reactive Oxygen Species (ROS) And Mitochondrial Superoxide Generation In HeLa Cells

ROS, a by-product of cellular metabolism play an important role in the various biological phenomenons. FACS analysis of ROS was done in HeLa cell after 24 hour of treatment to know the reason of cytotoxic and non-cytotoxic nature of compounds (In fig 5.2)Results shows that there is increase in ROS generation at dose of 60 and 80 µg/ml in the presence of crude Fucoidan 2.4 and 2.6 fold increase whereas in the case of pure Fucoidan at 150 and 200 µg/ml shows 0.98 and 0.84 fold which indicates slight decrease in ROS. This suggest that crude Fucoidan may act as an pro-oxidant and pure Fucoidan as an antioxidant in dose dependent manner which was further confirmed by analysing mitochondrial super oxide anion, mitochondria membrane potential and ATP analysis, and Whether cells moving towards apoptosis in the presence of crude Fucoidan.

5.1.3 Effect Of Crude And Pure Fucoidan On Mitochondrial Super Oxide Generation

Recent studies on mitochondria reported that mitochondria generate reactive oxygen species (ROS) in the form of superoxides (Ziegler, Wiley, & Velarde, 2015) which act as biological important molecule. When HeLa cells were treated with crude Fucoidan, they showed an increase in mitochondrial



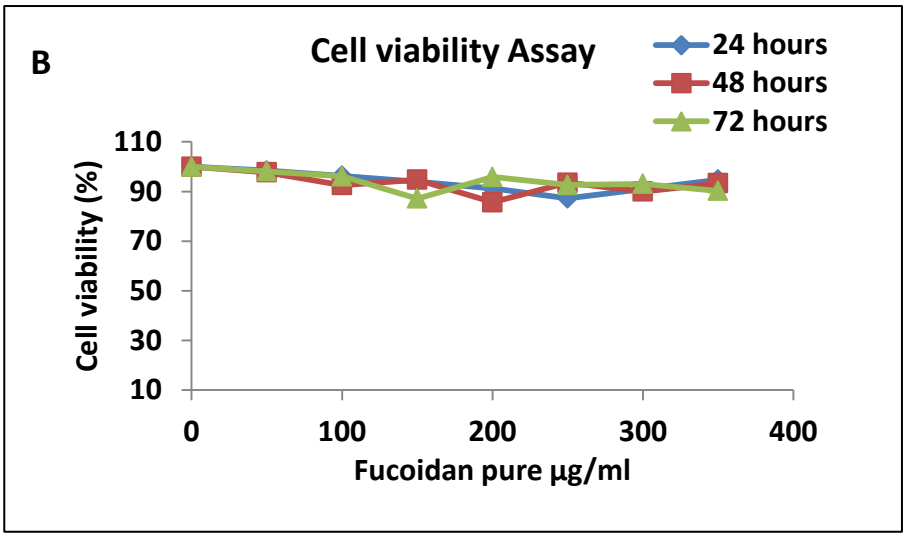
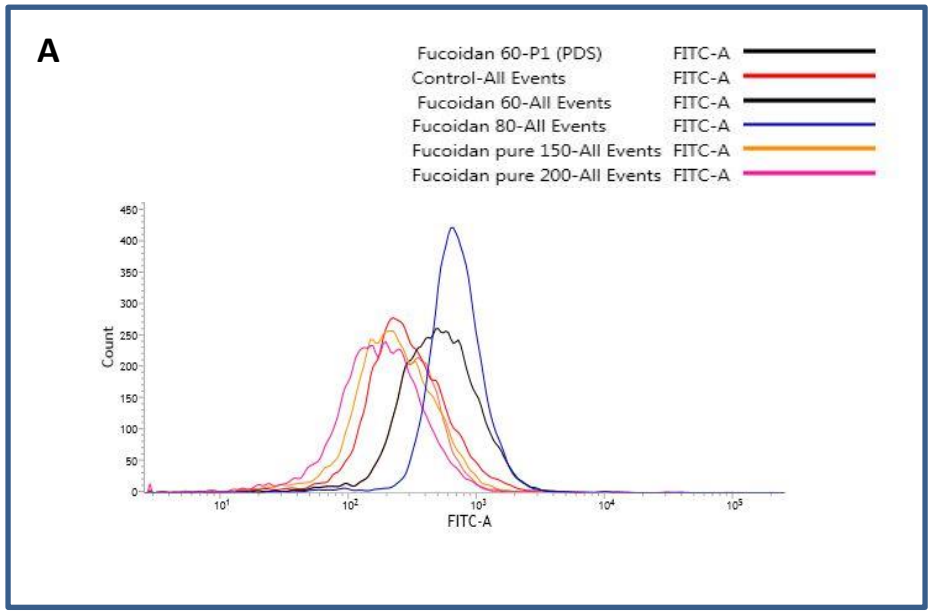


Fig 5.1) Fuucoidan crude induce cytotoxicity in HeLa cells line was evaluated by determining cell viability by MTT assay. After treatment of i) Fucoidan crude concentration ranging from 0 to 250 µg/ml ,ii)Fucoidan pure 0 to 350 µg/ml is non-cytotoxic in HeLa cells lines for 24 hrs, 48 hrs.and 72 hours. Values are means ± S.E.M. of at least three independent experiments.



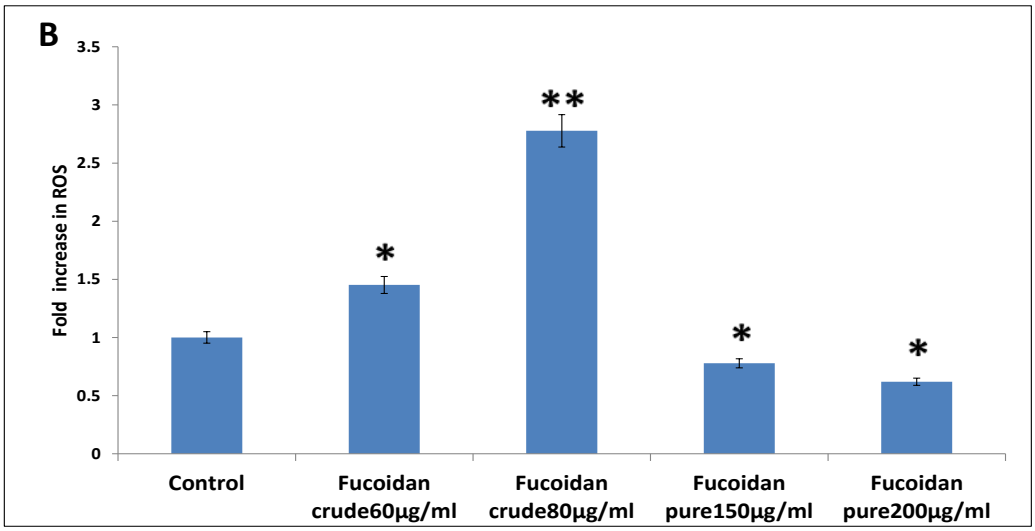
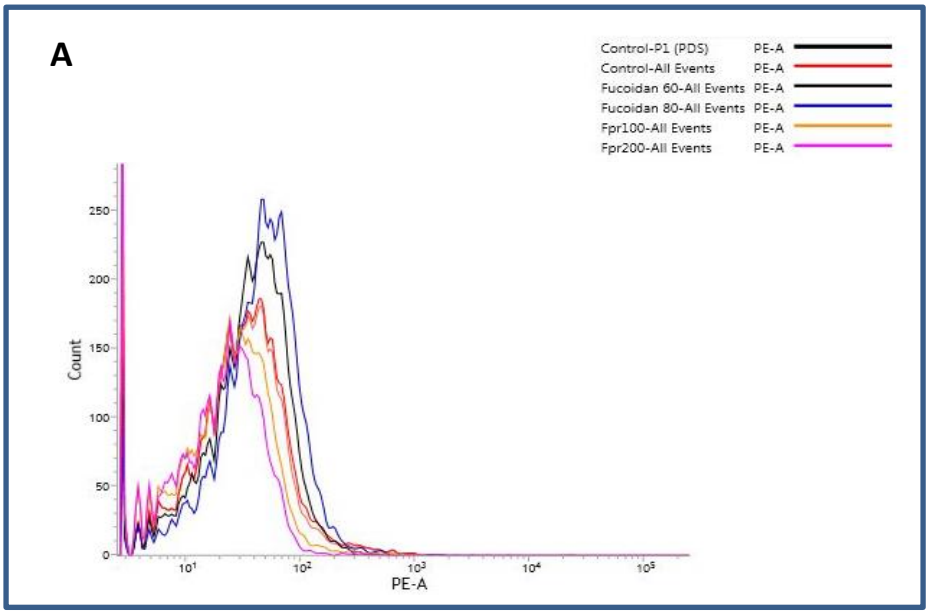


Fig.5.2 Showing the total intracellular ROS change in Cell after treatment with various concentrations .ROS analysis of Crude Fucoidan shows concentration dependent increase and pure Fucoidan show decrease in ROS concentration dependent manner B) Histogram profileshowing fold change ,Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05 ** denotes p<0.001



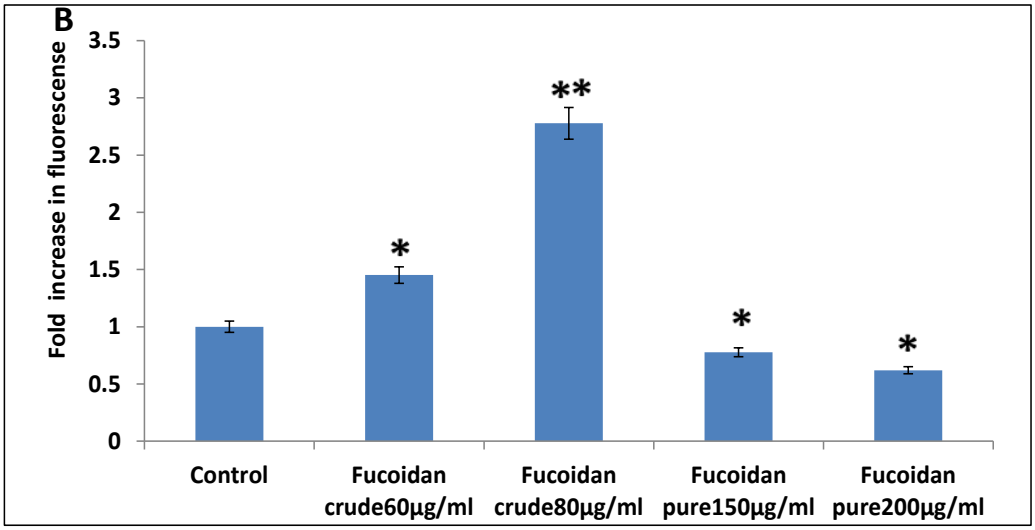


Fig 5.3 Mitochondrial superoxide anion analysis of Crude Fucoidan shows concentration dependent increase and pure Fucoidan show decrease in concentration dependent mannerB) Histogram profile showing fold change,Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05 ** denotes p<0.001

superoxide generation in concentration dependent manner which was confirmed by the increase in red fluorescence intensity observed by FACS analysis as shown in (fig 5.3.) At dose of 60 and 80 µg/ml crude Fucoidan treatment shows 1.45 and 2.7 fold increase respectively, whereas Pure Fucoidan at 150 and 200 µg/ml showed 0.7 and 0.61 fold decrease in superoxide level that further support antioxidant nature of pure Fucoidan.

5.1.4 Effect Of Crude Fucoidan And Pure Fucoidan On Mitochondrial Membrane Potential (Ψm) In HeLa Cells

Mitochondria are the major site and target of ROS generation. Enhanced ROS results in loss of mitochondrial membrane potential. The cationic dye JC-1 measured the mitochondrial potential by assessing red/green fluorescence intensity as shown in Fig(5.4). HeLa cells treated with crude Fucoidan 80µg/ml gave low red/green fluorescence intensity as compared to control condition that confirms the depolarization of mitochondria after treatment of 24 hours due to enhanced ROS generation whereas pure Fucoidan 200 µg/ml gave high red/green fluorescence.

And also FACS analysis with R123 is a cell-permeant, cationic, green-fluorescent dye show loss in mitochondrial membrane potential when HeLa cells treated with crude Fucoidan at dose of 60 and 80 µg/ml show 0.8 and 0.75 fold decrease whereas pure Fucoidan at 150 and 200 µg/ml show 1.03 and 1.12 fold increase mitochondrial membrane potential as visible from(fig 5.5).

5.1.5 Effect Of Crude And Pure Fucoidan On ATP Generation

ATP activity decreases with increase of ROS and loss in mitochondrial membrane potential in HeLa cells at 80µg/ml crude Fucoidan treatment but in case of pure Fucoidan no loss in ATP due to is decrease in ROS after its treatment. As depicted by (fig 5.6)

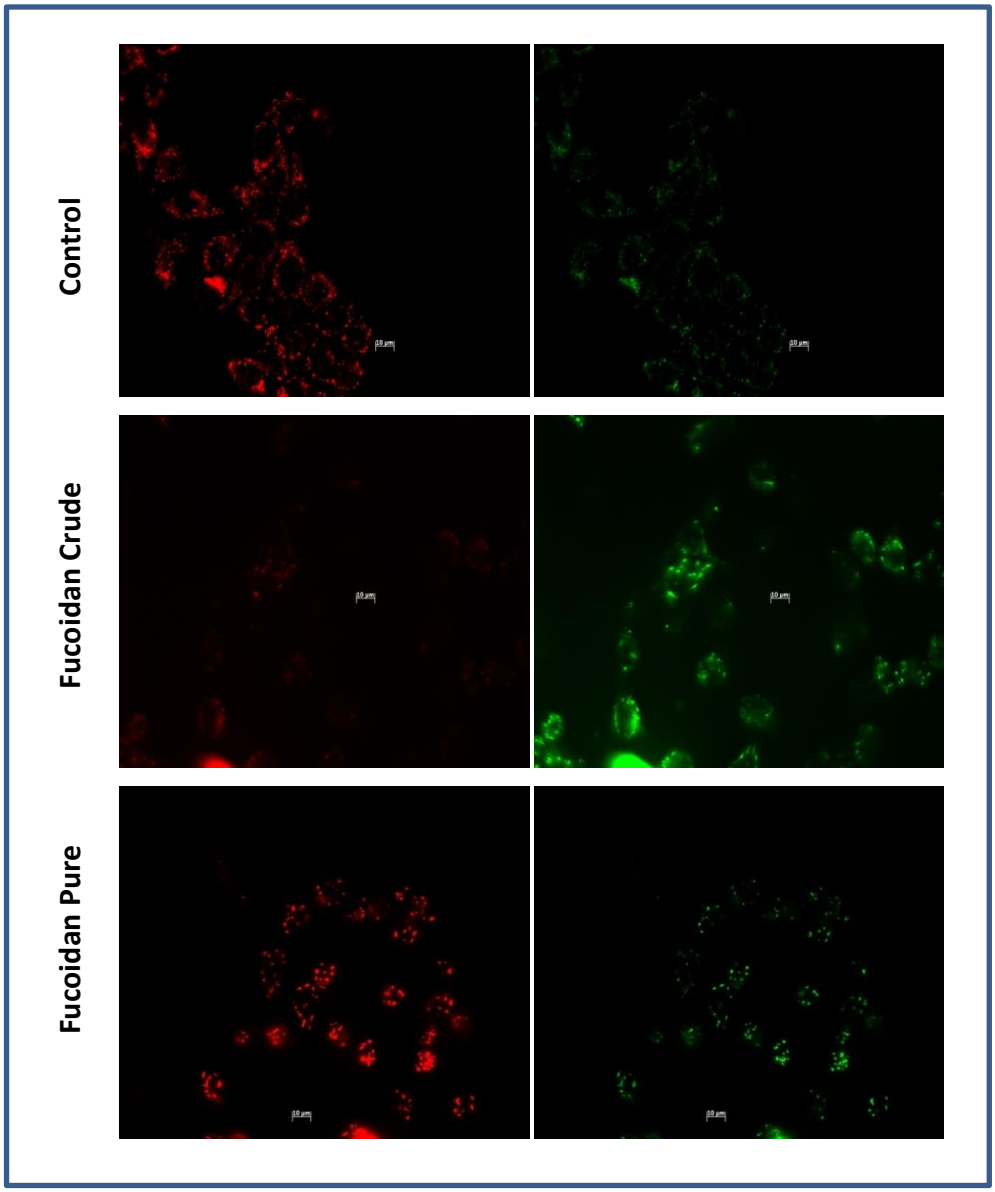
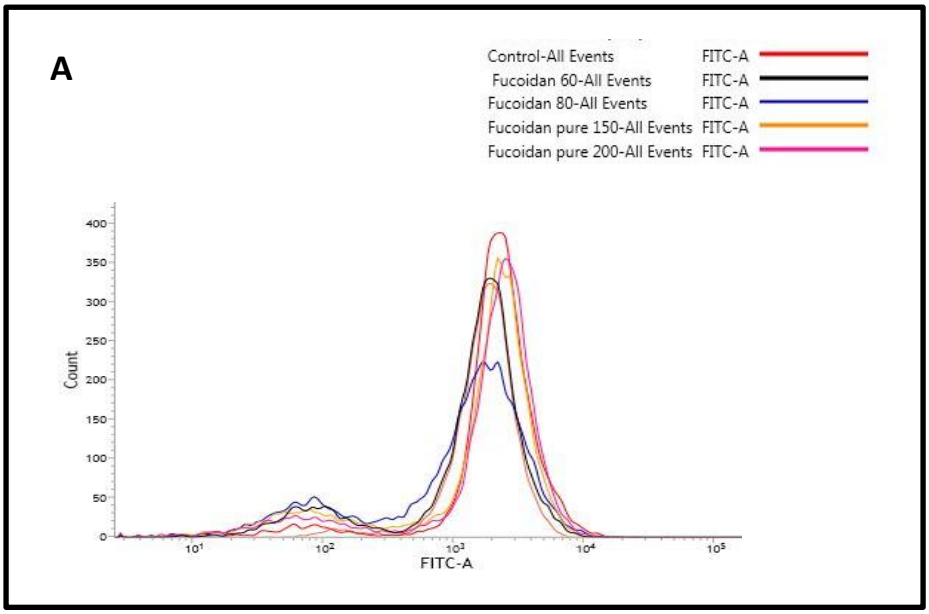


Fig5.4.)Depicting JC-1 staining to assess mitochondrial membrane potential in HeLa cells over a time period of 24 hour(i) Control, cells display red fluorescence and minimum green fluorescence showing basal Mitochondrial membrane potential. (ii) Fucoidan at 80µg/ml , display high red fluorescence and minimum green fluorescence, indicating loss of Mitochondrial membrane potential similarly(iii)Fucoidan pure 200µg/ml , shows higher red fluorescence compared with control which indicate hyper polarization of mitochondrial membrane potential



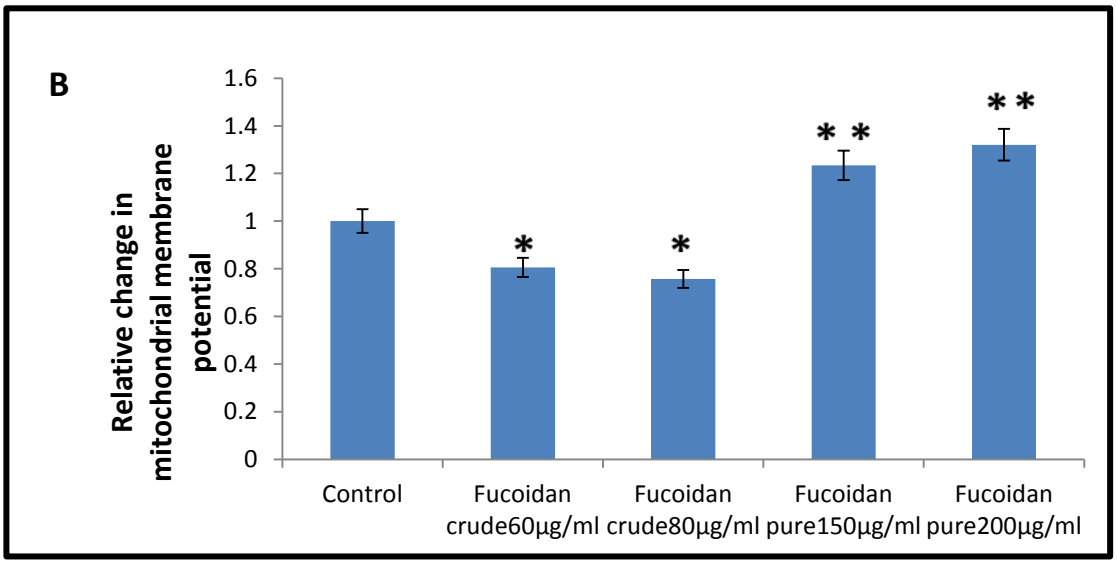


Fig 5.5) Showing the Mitochondrial Membrane Potential change in HeLa cells after treatment with various concentrations of crude and pure fucoidan by FACS analysis. B) Histogram profile showing fold change Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05 ** denotes p<0.001

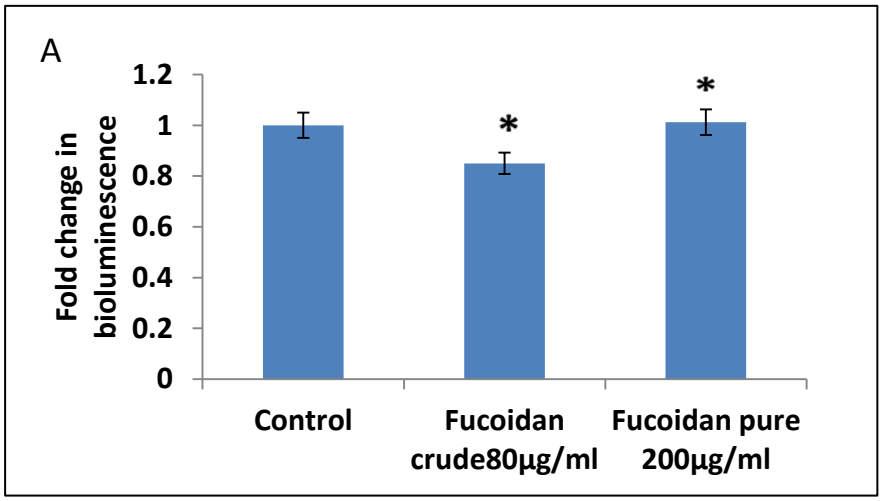


Fig (5.6) ATP generation by measuring bioluminescense showed loss in in ATP with treatment of crude Fucoidan slight change in case of pure Fucoidan .

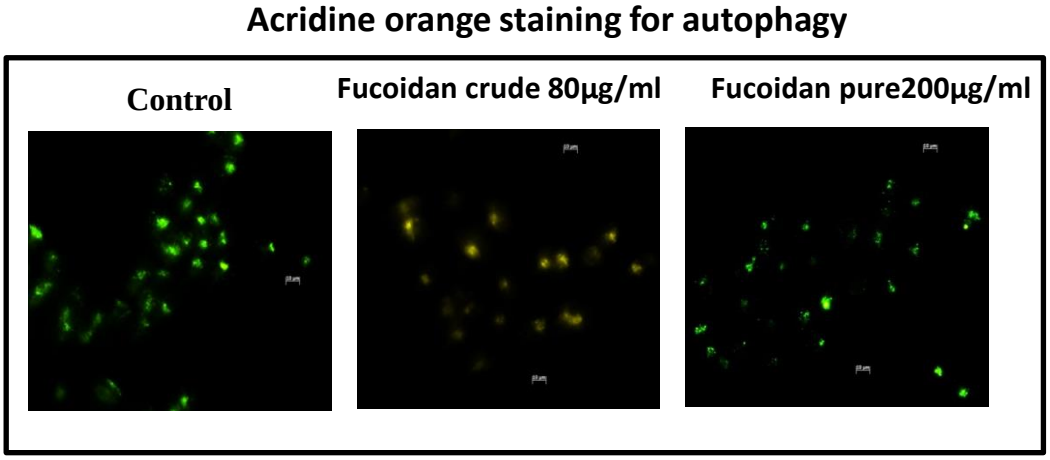


Fig (5.7) Autophgy analysis: HeLa cells were stained with Acridine orange and merged orange- red and green fluorescence were analysed (i) Control, cells display green fluorescence and minimum red fluorescence, indicating no autophagosomes(ii) Fucoidan crude shows Orange- red fluorescence compared with control because of accumulation of acridine orange dye in acidic autophagosome, (iii) Fucoidan Pure shows highly green fluorescence similar with control indicating no autophagosome underFluorescence microscopy

5.1.6 Effect Of Crude Fucoidan And Pure Fucoidan F-Actin Rearrangement

A dynamic balance exists between the actin cytoskeleton and the release of ROS from mitochondria. The release of ROS from mitochondria causes the change in F actin. Crude Fucoidan 80 µg/ml treated Hela cells showed aberration in F-actin, but treatment with pure Fucoidan 200 µg/ml show no aberration in F-actin which was visibile in rhodamine conjugated phalloidin stainining Fig (5.10).

5.1.7 Analyssis Of Hematoxylin-Eosin (H&E) And DAPI Staining

To know the effects of these ROS generation by crude Fucoidan we visualize HeLa cell at a high dose of 150 µg/ml of crude fucoidan treatment showed distorted shape and are detached from the bottom and pure fucoidan show no change in morphology on DNA fragments and chromatin condensation, we have done DapI staining of HeLa cells. Results are shown in fig(5.11). It shows that the in presence of crude fucoiden chromatin get condensed and DNA get fragmented. However HeLa cell remains unaffected in the presence of pure Fucoidan.show similarity with control cells may be due to its antioxidant effect.

5.1.8 Annexin – FITC Staining With Crude And Pure Fucoidan

We also assess the apoptosis by Flow cytometer using Annexin FITC dye in case of HeLa cell crude Fucoidan at high dose of 150 and 200 µg/ml show 35 and 59 percent apoptotic cell whereas pure Fucoidan 150 and 200 µg/ml at show no sign of apoptosis. As represented by fig (5.13)

5.1.9 Effect Of Crude And Pure Fucoidan On Autophagosome Formation

We have also visualized the cells with Crude and pure Fucoidan against control HeLa cells for autophagosomes formation under the fluorescence microscope with acridine orange staining. As shown (fig.5.7 and 5.8), control cells displayed green fluorescence and minimal red fluorescence, indicating no autophagosomes or inadequate acidic vesicular organelles. Crude Fucoidan treated cells on the other hand, showed highly red fluorescence

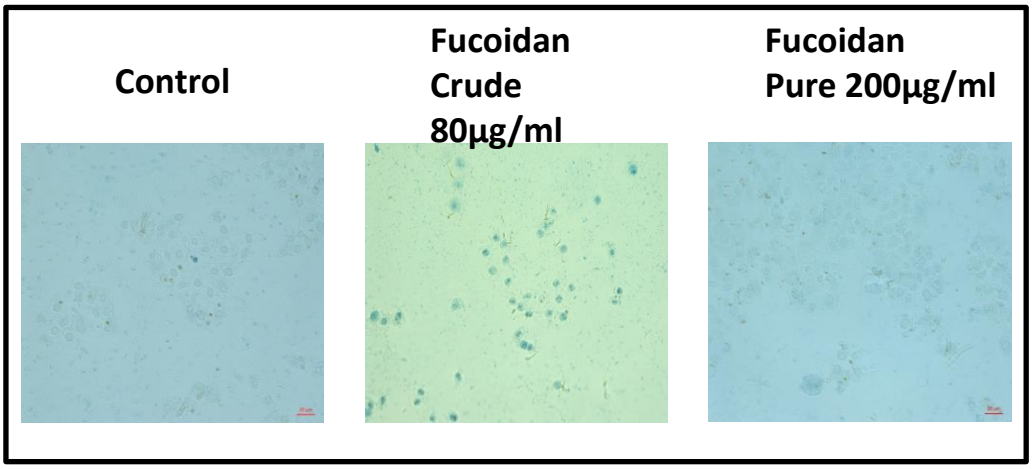


Fig 5.9)Senescence-associated Positive β-galactosidase staining in HeLa cells after treatment with crude fucoidan show senescence induction, whereas pure fucoidan show no sign of senescense.

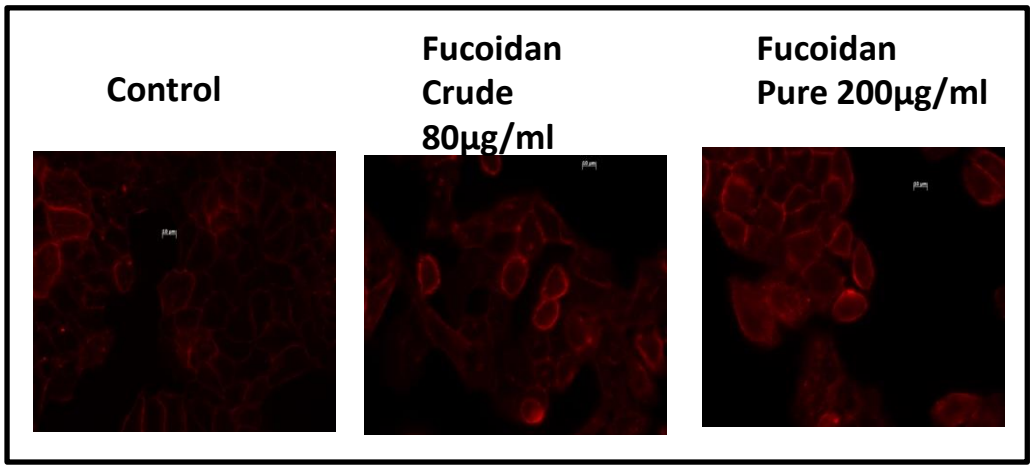


Fig 5.10) F-actin staining by Rhodamine phalloid depicting the F-actin after treatment of crude and pure Fucoidan in HeLa cells (i) Control (ii) Fucoidan 80 µg/ml show disrupted F-actin(iii) Fucoidan pure 200µg/ml show no disruption

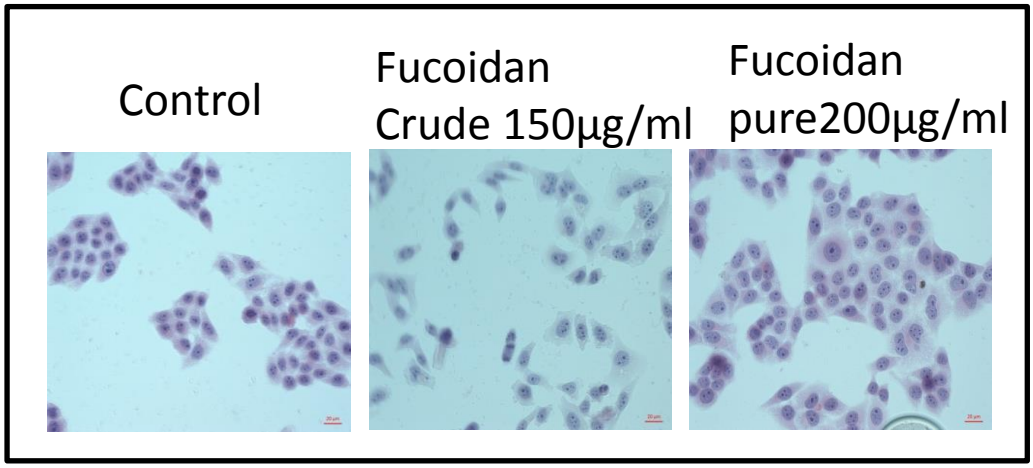
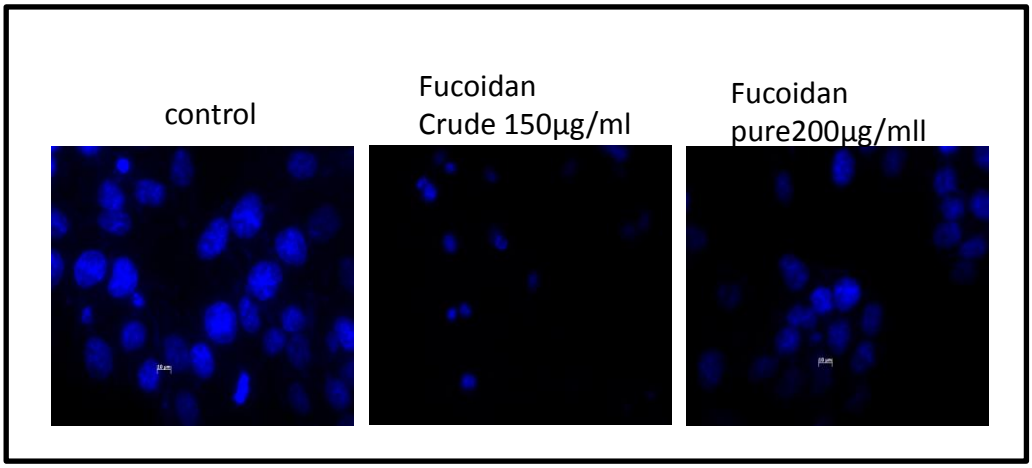
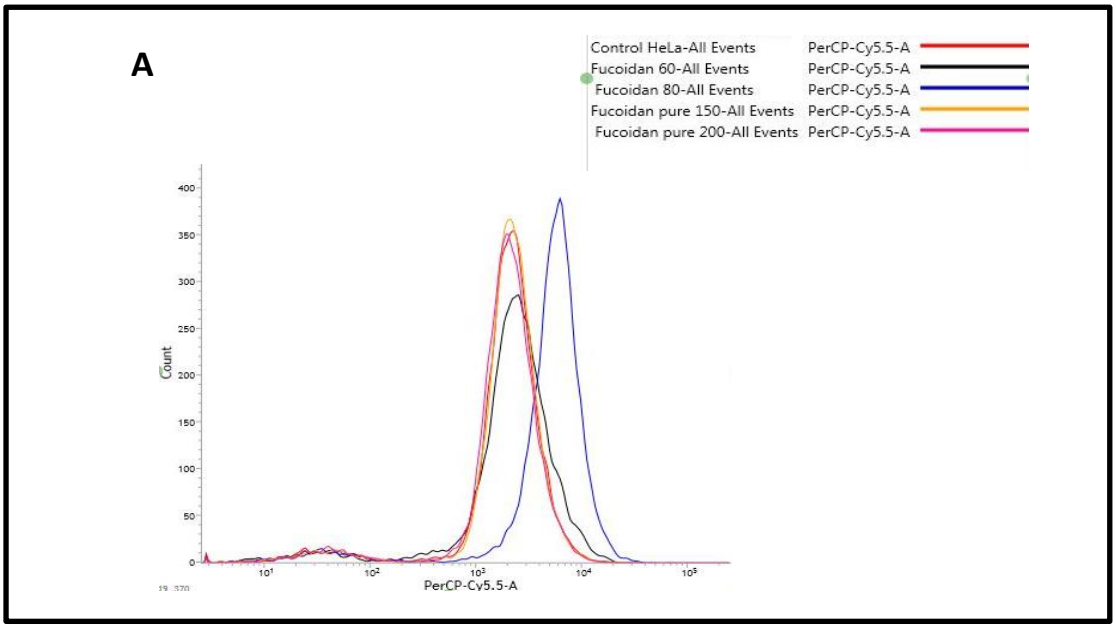
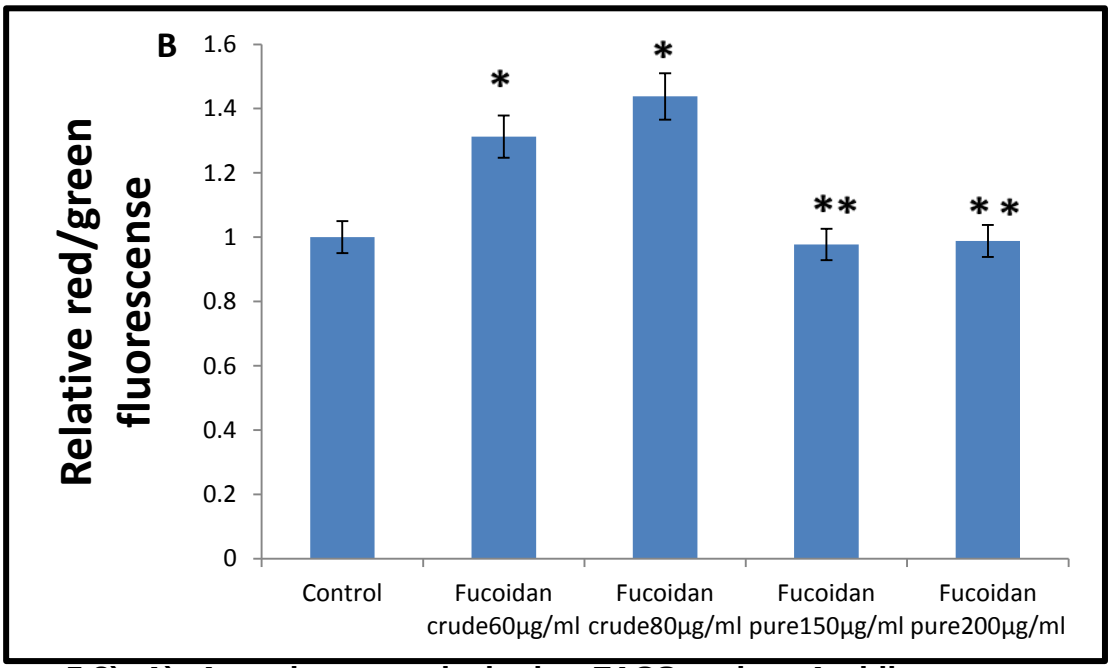


Fig 5.11A) Morphological analysis was done by H-E staining, depicting the morphology before and after treatment of crude and pure Fucoidan in HeLa cells



5.11B) After treatment of crude and pure Fucoidan in HeLa cells Dapi staining show apoptosis in crude Fucoidan whereas pure Fucoidan show no sign of Apoptosis





5.8) A) Autophagy analysis by FACS using Acridine orange staining after treatment with crude Fucoidan and pure Fucoidan in HeLa cells. Ratio of red/green fluorescence relative to control HeLa cells. B) Histogram profileshowing fold change in red/green fluorescence Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05 ** denotes p<0.001

compared with control, showing autophagosome formation or enhanced acidic vesicular organelles showing induction of Autophagy whereas pure Fucoidan treated cell display green fluorescent similar to control cell.

5.1.10 Effect Of Crude And Pure Fucoidan On Cellular Senescence

Oxidative stress induce by the exposure of crude Fucoidan 80 µg/ml at low dose causes premature cellular senescence in human cervical carcinoma HeLa which was further confirmed by morphological changes such as enlarged, flattened cells, increased SA β-gal activity and an increase in autofluorescence. Whereas pure Fucoidan at 200 µg/ml show no significant effect while assessing SA β-gal activity and autofluorescence.as shown in (fig 5.9)

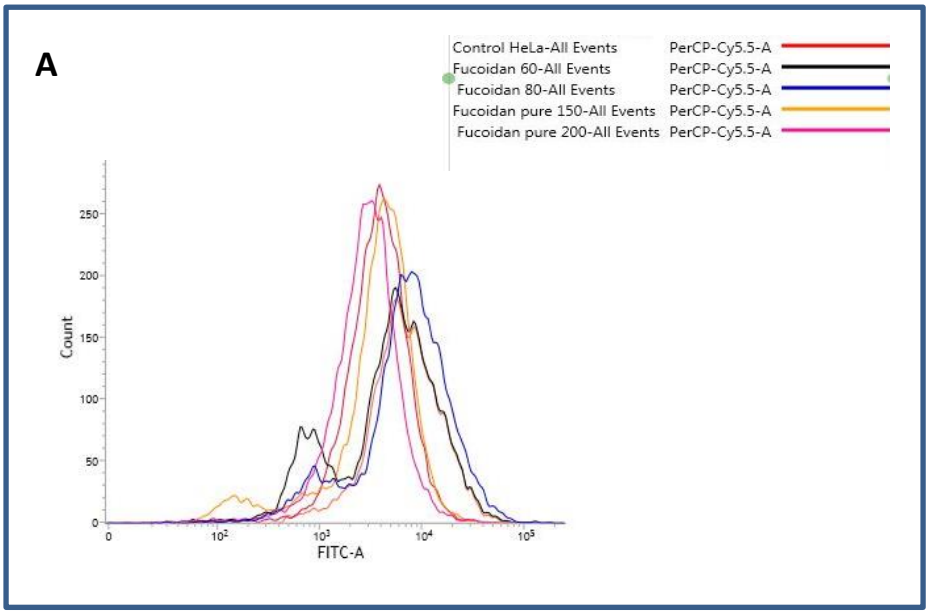
5.1.11 Modulation In Signaling Pathways Involving Crude And Pure Fucoidan

Crude Fucoidan treatment in HeLa cells generate ROS which in turn induce oxidative stress that result in up-regulation in the expression of P53 in response to cellular stresses FACS analysis of P53 expresion show 1.5 fold increase whereas in case of pure Fucoidan shows 9% decrease in p53 expression (fig5.14) . P53 downstream targets are p21 and BaX, it has been reported that the enhanced expression of p21 due to increase in ROS level in cancer cells which induces premature senescence. In case of crude Fucoidan p21 shows 2.2 fold increase but in pure Fucoidan show 4% decrease in p21 expresion.BAX a pro-apoptotic protein member show1.2 fold increase and Bcl-2 show 36% decrease in HeLa cells during crude Fucoidan treatment whereas pure Fucoidan show negligible effect in BAX expression, and narrow increase in BCL-2 expression as analysed by FACS (fig 5.15), which implies that during crude treatment cell is moving towards apoptosis due to continuous enhanced ROS.

5.1.12 LC-ESI MS/MS Analysis Of Crude And Pure Fucoidan

To compare the composition of the crude and pure samples we carried out LC-MS analysis. Fig. (5.16i), (5.17i), (5.18 A)and (5.19i) and pure Fucoidan Fig(5.16ii),(5.17ii), (5.18B) and (5.19ii) the LC data for crude and pure samples respectively. As can be seen from the LC spectrum there are multiple

Autofluorescense



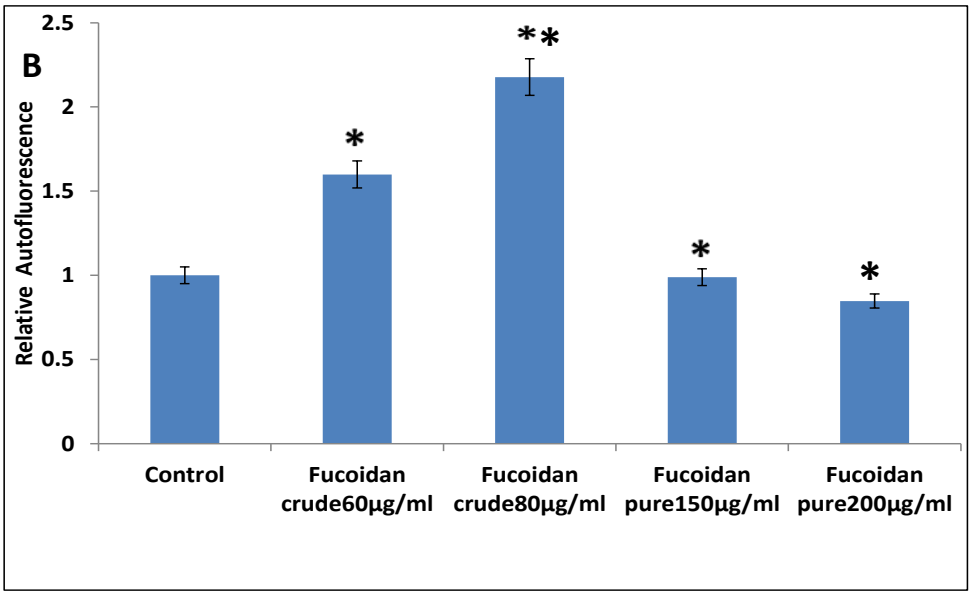


Fig 5.12)After treatment of crude and pure Fucoidan in HeLa cell Autofluorescence was analysed by flow cytometry as a measure of senescense. B) Histogram profile showing fold change in autoflorescense * denotes p<0.05 ** denotes p<0.001

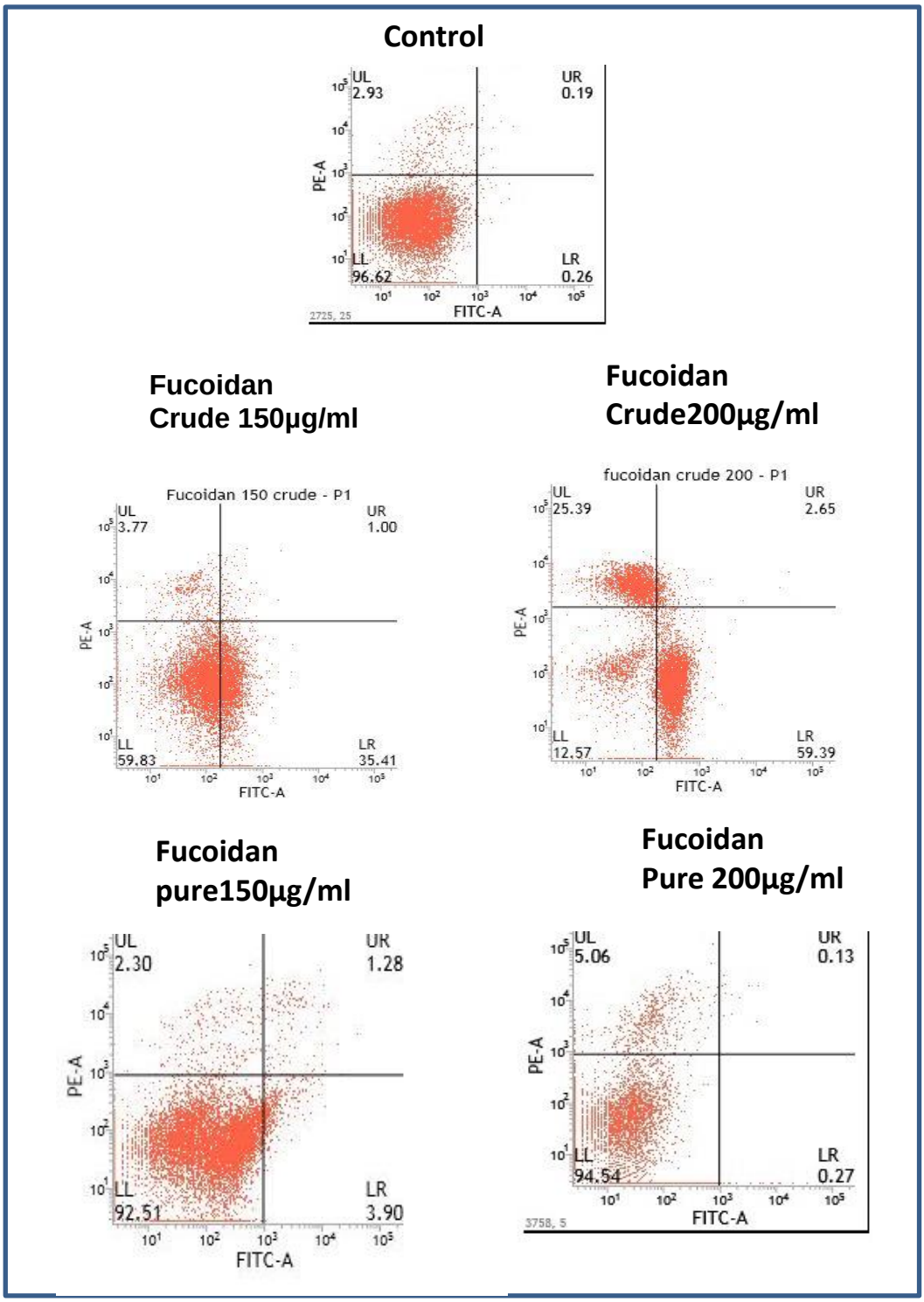


Fig 5.13) Annexin FITC and PI staining of crude fucoidan show apoptotic cell whereas pure fucoidan show no sign of apoptosis

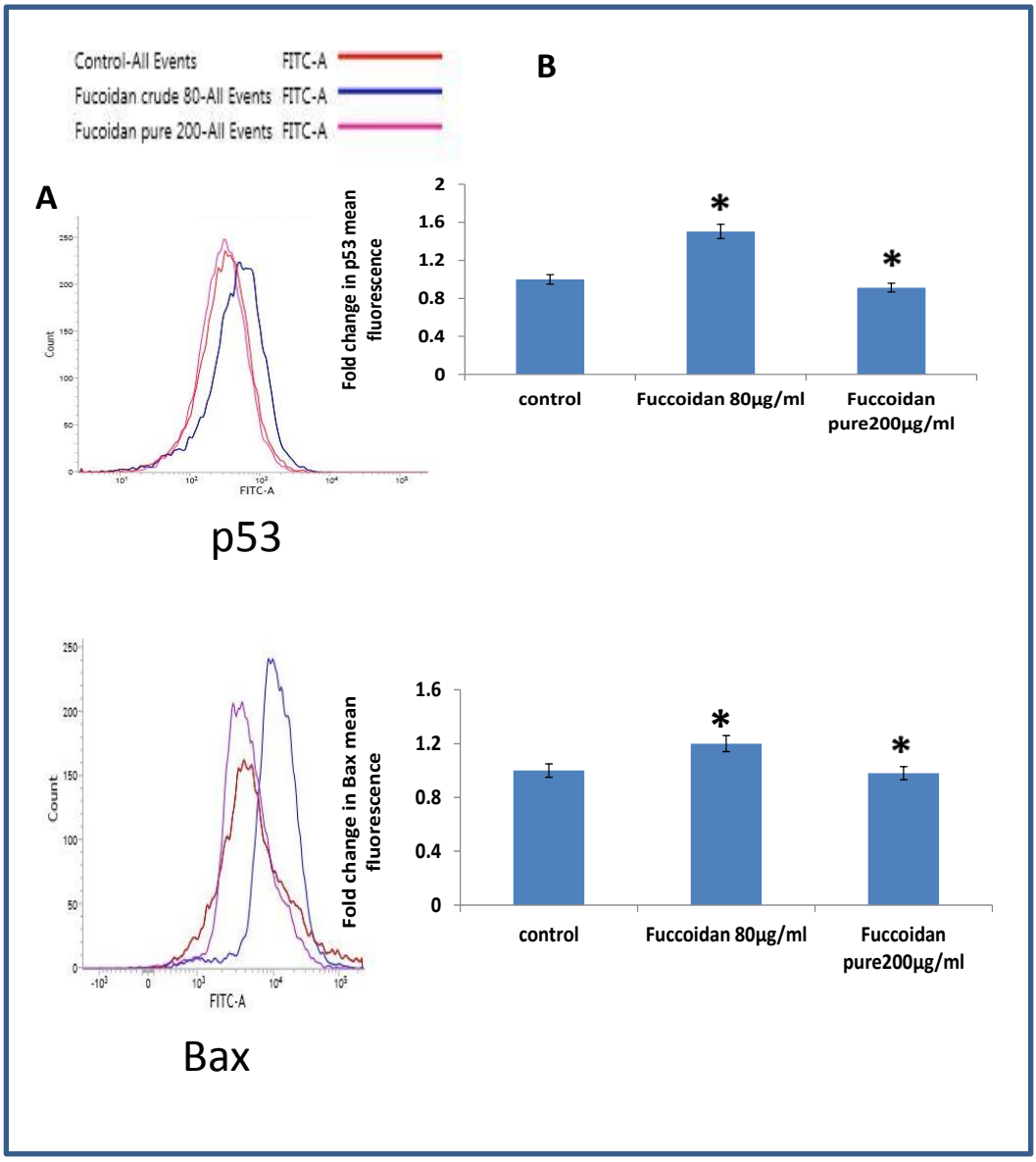


Fig 5.14) A) Protein quantification done by using FACS analysis of crude Fucoidan at 80µg/ml treatment in HeLa cells shows 1.5 fold increase in p53 while 1.2 fold increase in Bax as compare to control whereas, pure fucoidan shows slight decrease in both 9% in p53 and 2%in Bax. B) Histogram profile of protein Values are means ± S.E.M. of at least three independent experiments. *denotes p<0.05

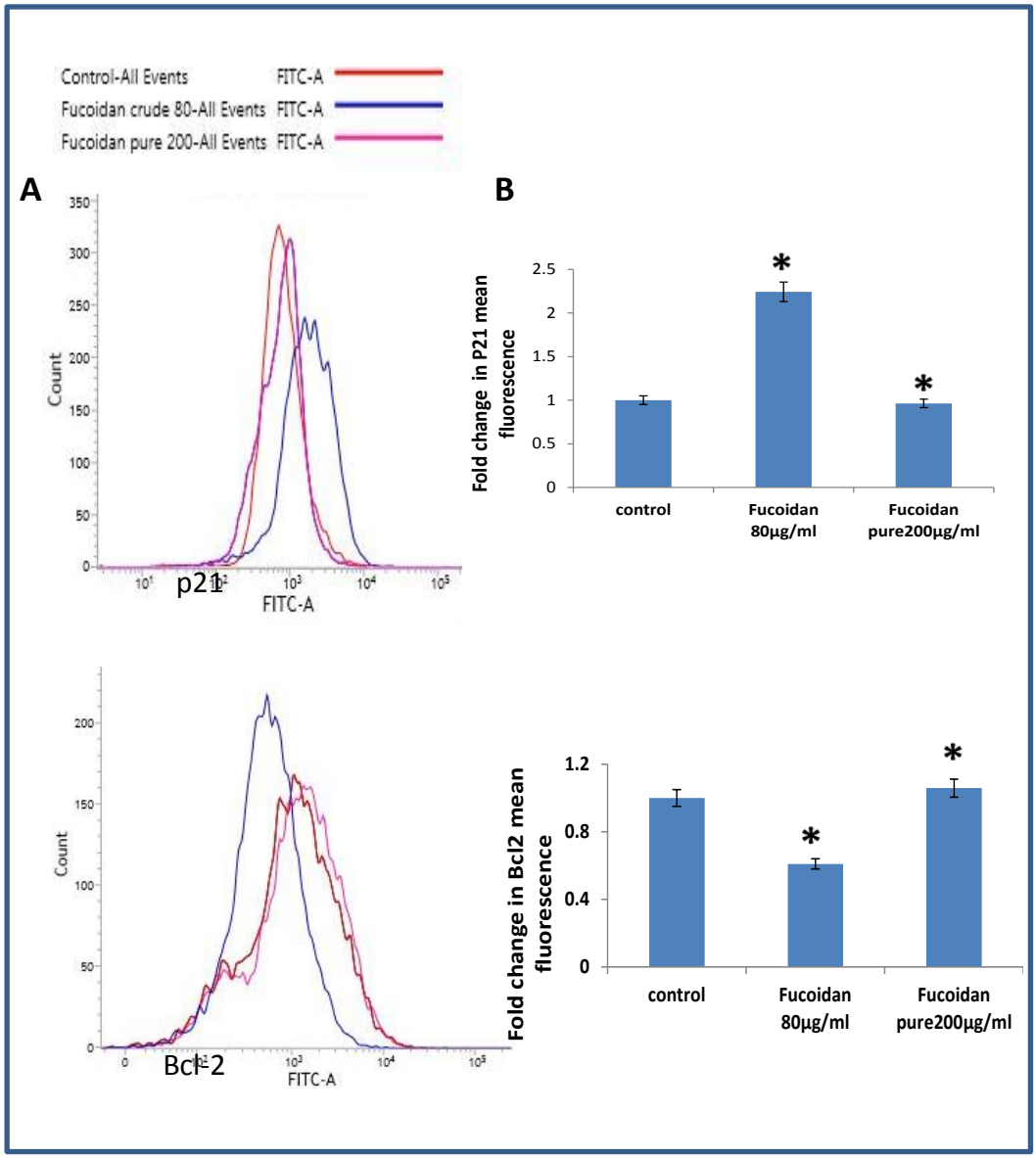
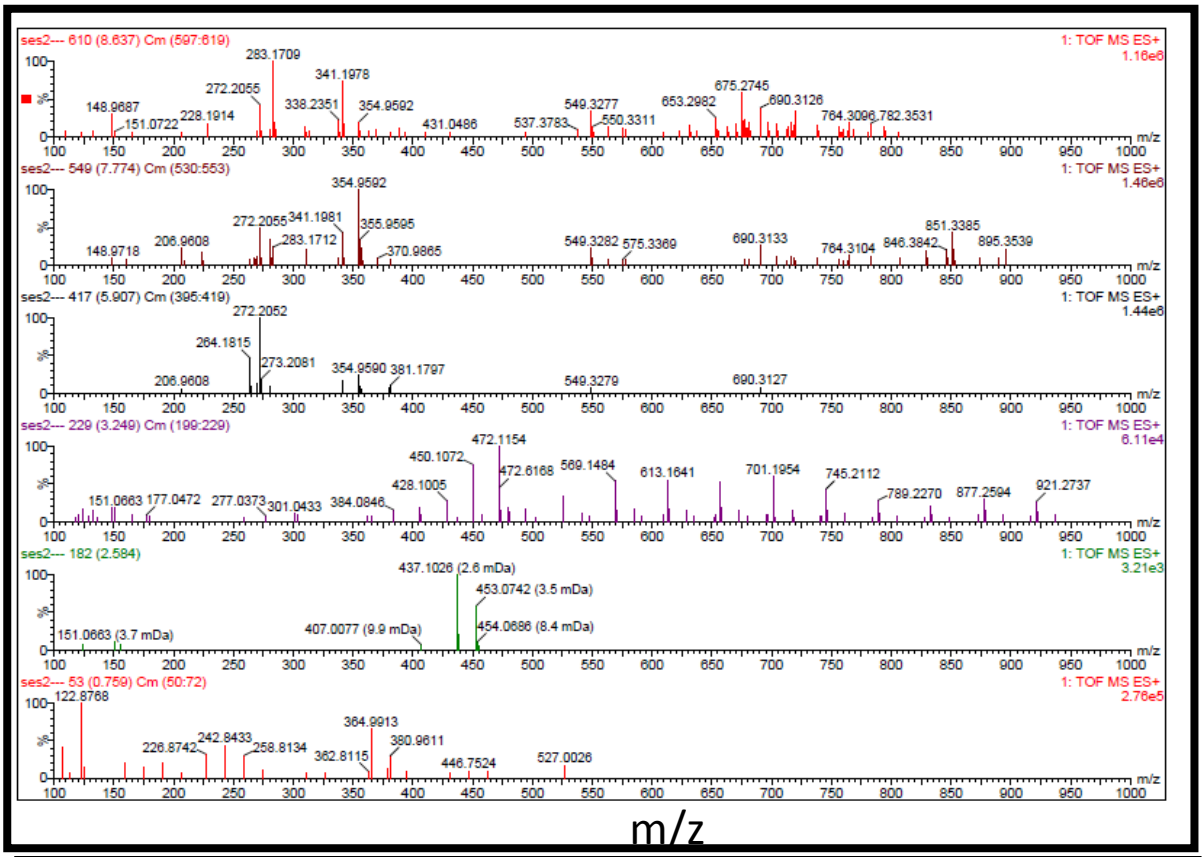


fig 5.15) A) Protein quantification done by using FACS analysis of crude Fucoidanat 80µg/ml treatment in HeLa cells shows in 2.2 fold in p21 and 36% decrease in Bcl2 in compare to control whereas pure fucoidan shows slight decrease in 4% in p21and 1.05 fold increase in Bcl2 and B)Histogram profile of protein Values are means ± S.E.M. of at least three independent experiments. * denotes p<0.05.

i)



ii)

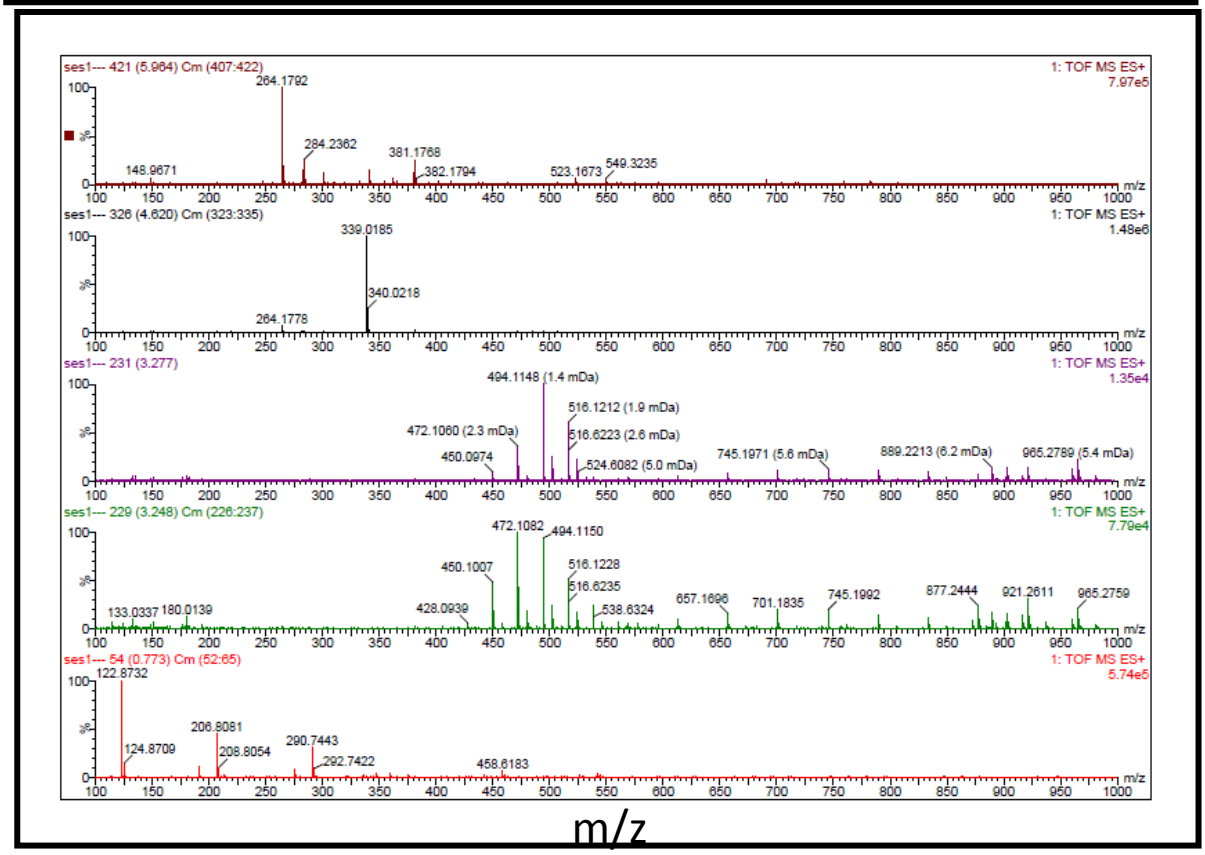
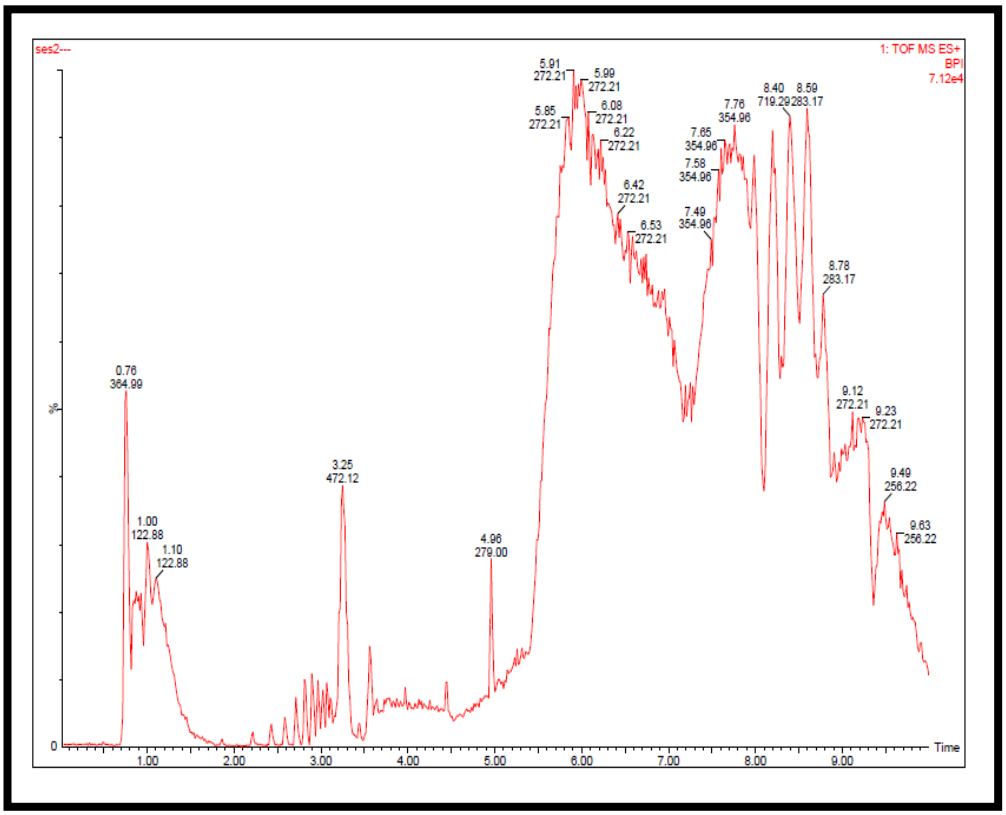


Fig (5.16) LC- ESI-MS spectrum of i)crude and ii) pure fucoidan at different retention time

i)



ii)

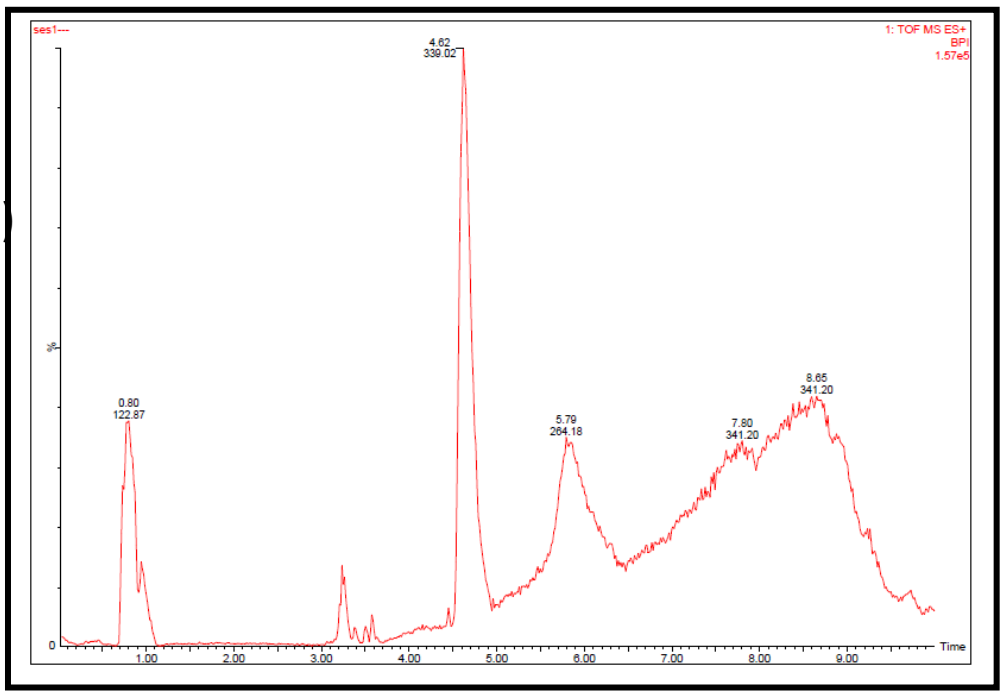
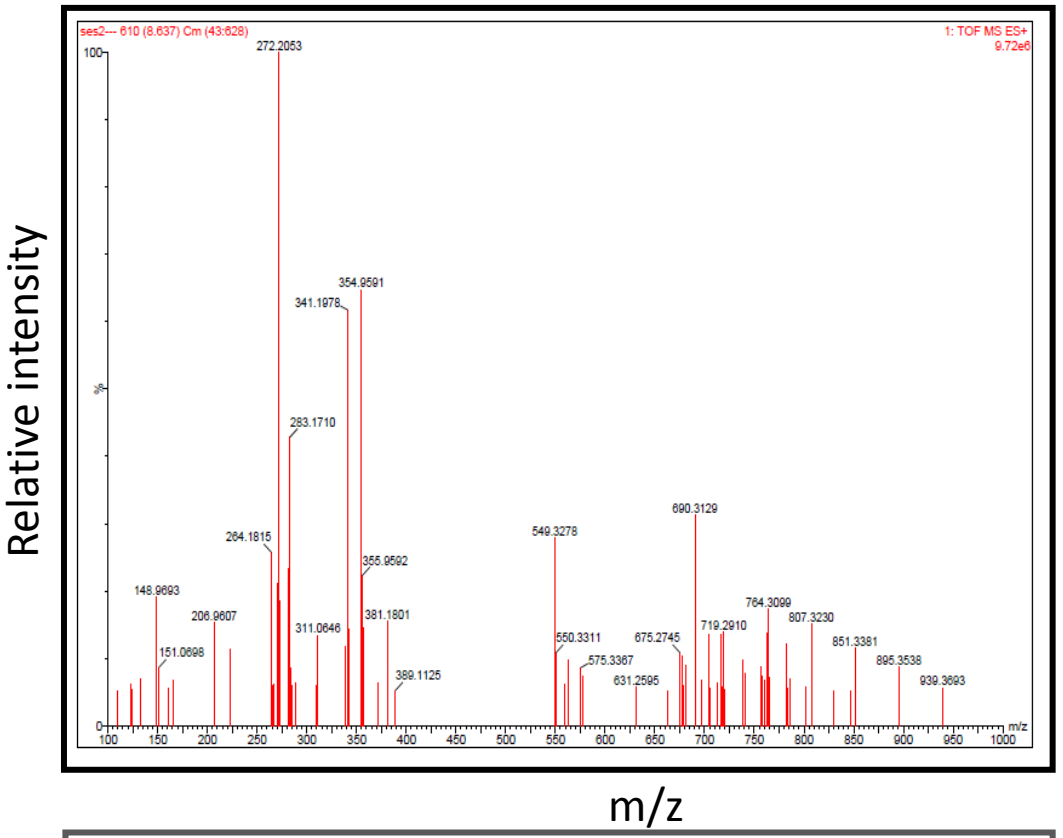


Fig (5.17) LC/MS total ion current (TIC) spectra of i)crude Fucoidan and ii) pure Fucoidan at different retention time.

A



B

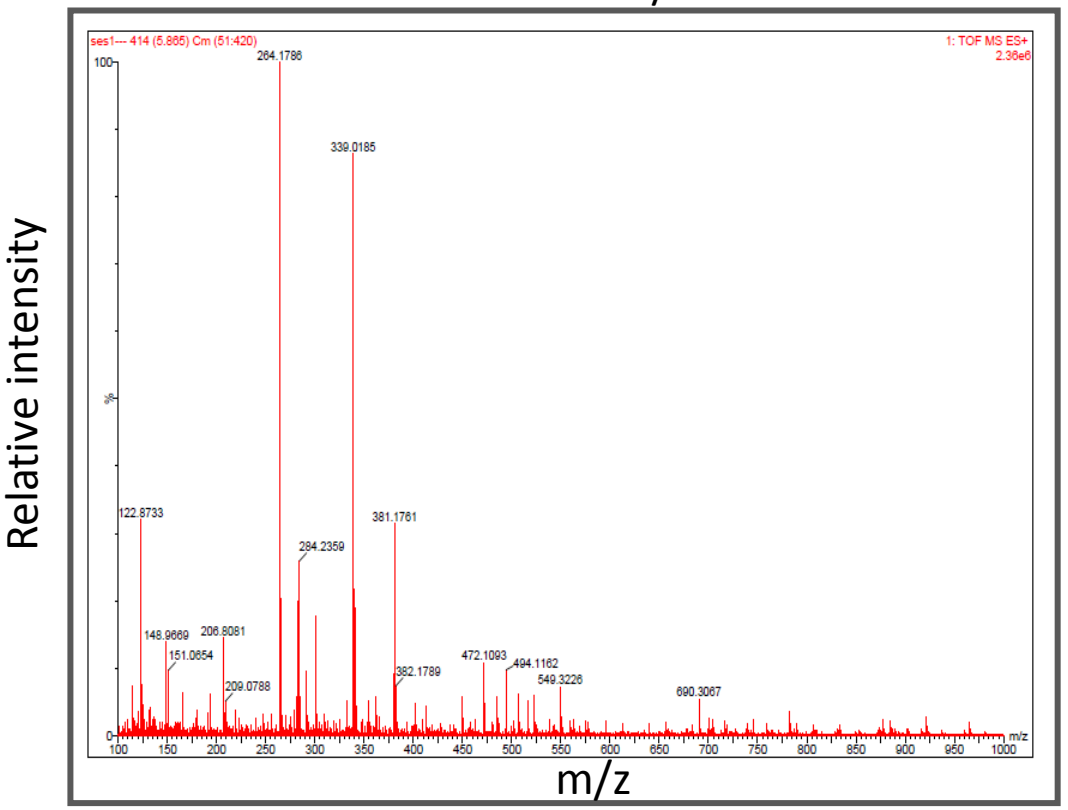
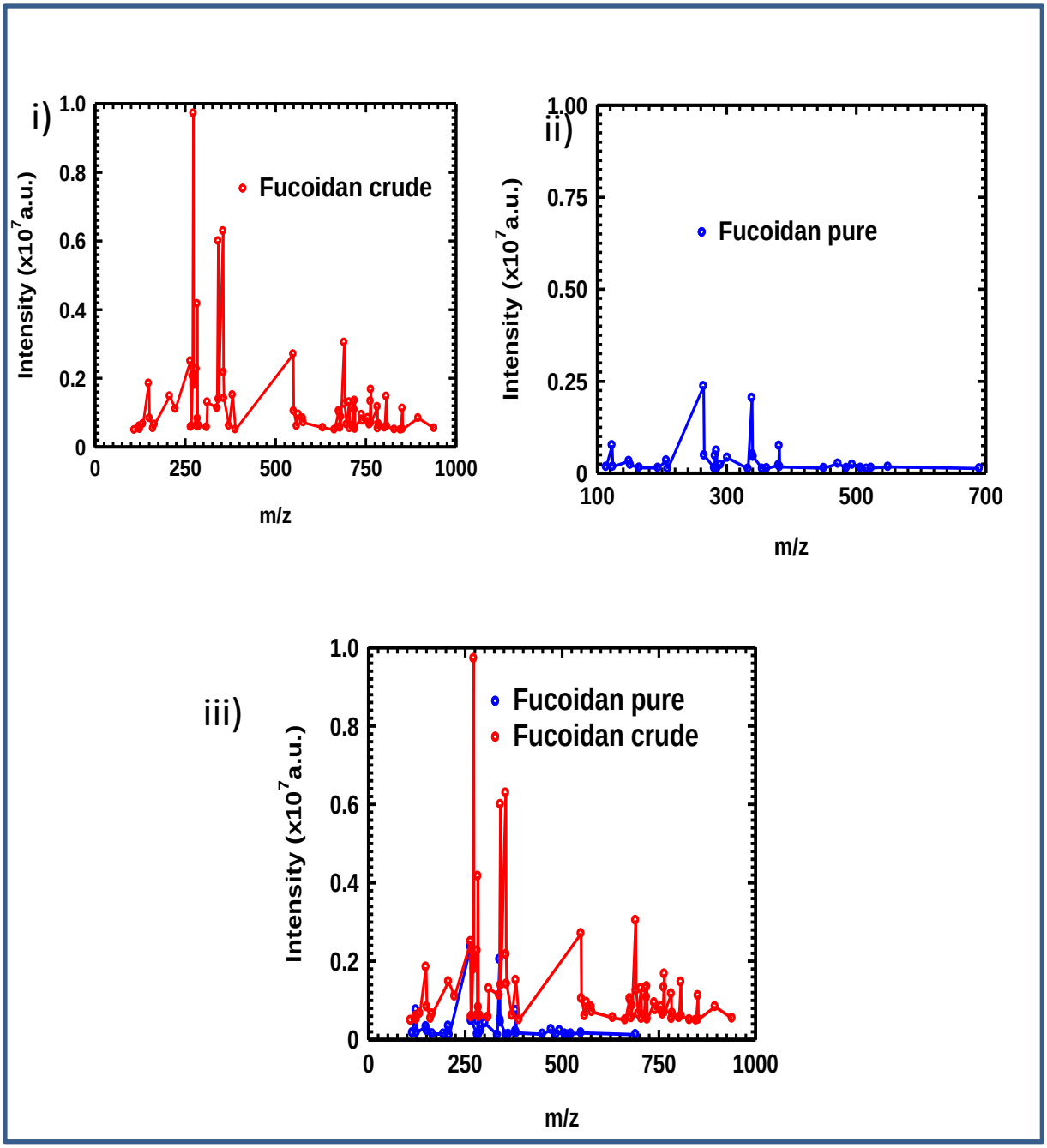


Fig (5.18) Electrospray ionization (ESI-MS)spectrum of A)crude Fucoidan and B)pure Fucoidan



Fig(5.19) LC-ESI -MS data plot showing intensity of different peak in i)crude Fucoidan ,ii) pure Fucoidan and iii) overlay of both crude and pure Fucoidan

peaks in LC of crude mixture with compared to pure sample. For the crude sample at higher hydrophobicity (retention time > 6 min) a number of peaks with high intensity were present 364.99m/z,122.88m/z 472.12m/z and 719.29 m/z etc .In comparison the pure sample showed 3 major peaks 122.87m/z,339.02m/z and 2.64m/z lesser number of peaks after 6 min. Electrospray ionization (ESI) was used for the mass spectrometric analysis show higher molecular mass in case of crude Fucoidan and low molecular mass in pure Fucoidan and LC-MS and MS spectra were analysed to the XCMS software and literature review to preliminary identify peaks and bioactive compounds peaks show different (Disaccharides, polysaccharides and oligosaccharides with various intensities are not assigned yet and further, need a detail research for structural classification and elucidation).

5.2 Discussion

We compared the effect of both crude and pure Fucoidan from *F.vesiculosus* on HeLa cells. The MTT cell proliferation assay was employed to determine the viability of cells and the results reveal that crude Fucoidan were more effective in inhibiting growth of HeLa cancer cells in a time and dose dependent manner better than the pure forms of Fucoidan .

Crude Fucoidan were found to induce more cell death in a dose as well as time dependent manner of treatment. It was demonstrated that crude Fucoidan was cytotoxic as it inhibited the growth of HeLa cell to the greatest extent (IC50 value for 24, 48, and 72 hours were 242.1µg/ml, 205.7µg/ml and169.5µg/ml respectively. However, inhibition by pure Fucoidan have no deleterious effect. Once the antiproliferative effect of Fucoidans were evaluated, the mechanism behind this effect was elucidated by flow cytometric analysis

ROS as an intracellular signaling molecule regulates various physiological and biological cell processes such as cell viability, mitochondrial health, autophagy, senescence and apoptosis.

Cells have necessary means to maintain redox balance through generation and elimination of reactive oxygen during physiological conditions. Excess ROS in cells were eliminated by the action of various enzymatic and non-

enzymatic antioxidant systems. Any alteration in the balance between the generation and elimination of ROS would leads to oxidative stress. However, an increased oxidative stress plays an important part in the pathology of various diseases including aging, cancer, and neurodegenerative diseases. So we observed the Change in the level of cellular ROS after exposure of crude and pure Fucoidan after 24 hours of treatment in HeLa cells.

All forms of life sustain a reducing environment within their cells. Disturbances occurring in this cell's normal redox state in response to stress because of the production of peroxides and of free radicals cause toxic effects and damage all components of the cell. Severe oxidative stress even causes cell death. Under normal physiological conditions, at moderate levels, the reactive nature of ROS allows their incorporation into macromolecules.. However, excessive ROS constantly attacks proteins, lipids, and DNA, under severe oxidative stress which ultimately causes oxidative damage. The property of ROS to induce cell death has been exploited in chemo and radiotherapy to enforce cell death in various cancers (Yang et al, 2013, Ryter et al, 2007).After the administration of crude Fucoidan HeLa cell show enhanced ROS generation whereas pure Fucoidan show decrease in basal level of ROS in Hela cell. We further analyse the mitochondrial ROS after 24 hour of treatment show enhanced mitochondrial superoxide anion generation in case of Crude Fucoidan treatment as measured by Mitosox red dye but in case of pure Fucoidan slight decrease mitochondrial superoxide anion. Now we measure the mitochondrial membrane potential by using JC-1 and R123dye in both crude and pure Fucoidan,HeLa cell show loss in mitochondrial membrane potential in case of Crude Fucoidan and show slight higher mitochondrial membrane potential in pure Fucoidan treatment.

Premature or stress-induced senescence occurs through exposure of the cells to exogenous cytotoxic agents generally associated with oxidative stressand DNA damage. The senescent cells are able to diversify constantly, like cancer cells, but missing proliferation as a motivator but metabolically active. Large and flat shape, rich cytoplasmic and vacuolar granularity, high levels of lysosomal *β*-galactosidase activity (SA-*β*gal), p21, are the most common senescent cells features in *invitro* cancer cells studies. Intrinsic and extrinsic

events can induce either the cell senescence or the apoptosis process, depending on the level of the impairment of the cell homeostasis and the p53 activity (Karrasch et al, 2008, Anversa et al, 2006, we observed senescence at low concentration of crude Fucoidan treatment 80 μg/ml but at high dose of 150 and 200 μg/ml crude Fucoidan show apoptosis in Hela cell wheras pure fucoidan show no sign of apoptosis even at high dose. The crude Fucoidan treatment also showed a higher level of Autofluorescence (or increased intrinsic fluorescence) implying the accumulation of oxidatively damaged proteins (Goodwin & Dimaio, 2001) and lipids in the Hela Cells.whereas in case of pure Fucoidan no such change was observed.

In case of crude Fucoidan treatment in HeLa cell level of p53 was upregulated and its down stream target p21 was also increase but in case of pure Fucoidan both the protein showed a slight decrease in expression.However intracellular level of BAX was also upregulated and Bcl-2 was down regulated in case of crude Fucoidan which signifies that Hela cell is moving towards ROS mediated mitochondrial apoptosis,whereas in case of pure Fucoidan intracellular level of BAX and Bcl-2 were at untreated level as observed by us similar result was shown by other research group in case of NAC(Halasi et al., 2013)(Luo, Yang, Schulte, Wargovich, & Wang, 2013).

In the present study, HeLa cells were morphologically analysed by H-E staining (Fig.5.11) for pure Fucoidan treatment has no visible impact on cellular morphology after treatment with 150μg/ml and 200 μg/ml concentration. Whereas crude Fucoidan at lower doses treatment cells showed rounding up of cell contours, condensed and vacuolated nuclei, morphological features of senescence, and at the higher dose of 150 μg/ml shrinkage of cell size, along with the unique feature of apoptosis were visible indicating the multiplicity of the insulting actions. A high level of lysosomal *β*-galactosidase activity (SA-*β*gal) was also observed in cells treated with crude Fucoidan. And senescence-associated heterochromatin factors (SAHF) were also observed with DAPI staining at lower dose treatment of crude Fucoidan at low doses as shown in previous chapter (fig4.30) and whereas at high dose treatment with crude Fucoidan shows apoptotic nuclei wheras pure fucoidan

show no such effect even at high concentration as observed with DAPI staining(H. Chen et al., 2017).

Autophagy is the process by which cellular constituents is transported to lysosomes for degradation and recycling so to provide an alternate energy source during stress condition. Under normal conditions in cell usually show low autophagic activities in the cell are but can get upregulated by several stress stimuli or by nutrient starvation. In our studies crude Fucoidan induces autophagy whereas pure Fucoidan treatment show the basal level of autophagy similar to control HeLa cells.

A dynamic balance exists between the actin cytoskeleton and the release of ROS from mitochondria. The release of ROS from mitochondria causes the change in F actin. Crude Fucoidan treated Hela cells showed significant aberration in F-actin, but treatment with pure Fucoidan show no aberration in F-actin which was visibile in rhodamine conjugated phalloidin stainining.Similarly crude fucoidan disrupt F-actin as feature of early events of autophagosome formation and cell cycle arrest is observed by us and reported by other researcher.(Haritunians et al. 2008) and also loss in ATP is a signal of ROS generation that causes reduced actin dynamics resulted in aberrant F-actin aggregates (“The actin cytoskeleton : a key regulator of apoptosis and ageing , 2005) and induction of Autophagy(Cho, Yen, Shim, Kang, & Kang, 2016).we assess the ATP in HeLa cell after treatment of crude and pure Fucoidan, it was observed in the loss of ATP in crude Fucoidan treatment and no effect on ATP generation in case of pure Fucoidan.

In the present studies crude forms were more effective in inhibiting the growth of HeLa cells than the pure forms and also In this study we have performed LC MS analysis which show crude Fucoidan have high molecular weight as seen by peak intensities by us and other researcher group(Fletcher, Biller, Ross, & Adams, 2017) and pure Fucoidan is of low molecular weight it may be due to various purification steps it needed and chemical hydrolysis was done to obtained purer form. Fucoidans bioactivities have been said to be related to their structural make-up, monosaccharide composition, sulfate content, position of sulfate ester groups, and molecular weight (B. Li et al., 2008).

Similarly to various polymers, Fucoidans are also polydisperse, which do not have a discrete molecular weight, but have a mixture or range of molecular weights. Fucoidans are also highly branched molecules, hence, even though a particular Fucoidan consists of similar molecular weight mixture(Fletcher et al., 2017) .(Zhurishkina, Stepanov, Shvetsova, Kulminskaya, & Lapina, 2017)

Fucoidan structure and monosaccharide composition itself is dependent upon various aspects like, the source of Fucoidan, the part of seaweed from which it is extracted, the time and location of harvesting and the extraction methods. As reported in the literature(Kim, 2012) (Wilfred Mak et al., 2014)reported that the sulfate groups in the Fucoidan molecule play a key role in the biological activities. Crude Fucoidan have high content of sulphate group in compare to pure Fucoidan.

This indicates that the crude Fucoidan might act as a natural food based anticancerous compound in cervical cancer cell, and pure Fucoidan act as antioxidant agent can play protective role in oxidative stress condition. In order to establish their anticancer activities in *in vivo*, further animal model experiments are required.

Summary

Cancer is a multifactorial disease that can affect any part of the body. Several factors either from inside and outside, which contribute to the development of cancer. Gene and its environment interactions are found to be facilitated by epigenetic modifications of the genome cause increase in response to modifications in the environment. Epigenetic changes are usually reversible, and relying on the presence or elimination of the activating factors. Interactions of gene and environment can change activities of gene of interest and bring about cascades of many cellular events to aid the adaptation of an individual cell to its surrounding environment (Alegría-torres & Baccarelli, 2013). Important cellular functions and cell signalling pathways are deregulated in cancer by epigenetic deregulation of critical tumor suppressor genes by methylation of on the promoters, abnormal post translation modifications of histone and some non-histone proteins by deregulation of acetylation/methylation.

Epigenetic changes involve functionally relevant changes to the genome without involving changes in its sequence. The basic idea to overcome cancer is not by killing the cancer cells but to correct their histone deacetylation or histone acetylation, there by reprogramming them for a proper functioning.

Epigenetic modifications are an integral part of cellular development and differentiation. Out of various epigenetic regulations, it is known that Histone deacetylases (HDACs) contribute to cancer survival through epigenetic silencing of tumor suppressor genes and deacetylation of tumor suppressor proteins. Upregulation of HDAC isoforms has been observed in many cancer types and In cervical cancers, some HDACs such as HDAC1 and HDAC2 (Glaser et al., 2003) (Barneda-zahonero & Parra, 2012), have been found over expressed. During advanced malignancy transcriptional repression are seen which is mainly due to epigenetic regulation of HDACs. Aberrant recruitment of HDACs has been found to have a causal role in tumorigenesis for nearly every type of cancer (e.g. ovarian neuroblastoma, pancreatic, liver, colon and cervical cancer. Moreover, as HDACs regulate many essential cellular processes, not only through epigenetic control, but also through non-epigenetic protein deacetylation.

HDAC inhibitors (HDACi) have shown great promise as cancer therapeutics through their ability to arrest proliferation in many transformed cell lines through cell cycle arrest, apoptosis, cellular differentiation, and inhibition of angiogenesis.

Bioactive compound are a vast group of natural compounds that not only provide much of the flavour and colour to plants and algae but also responsible for remarkable biological and medicinal activity. They may act as an achievable alternatives for the treatment of cancer and its complications without any adverse effects. They may act as pro-oxidant or antioxidants. Pro-oxidant refers to those bioactive compounds that induces oxidative stress either by generation of ROS or by suppressing antioxidant systems.

ROS as an intracellular signalling molecule regulates various physiological and biological cell processes, excessive generation of reactive oxygen species (ROS) stress in cancer cells has always been recognized and often considered as an undesirable event associated with carcinogenesis and cancer progression. However, increase in reactive oxygen species (ROS) induces autophagy, senescence and apoptosis as reported in the number of recent studies against the cancer cells. Autophagy and senescence share a lot of common features which suggest that these processes could assist the similar cell fate as reported during stress condition like nutrients deprivation, radiation exposure, and phytochemical treatment.

In recent years, the role of epigenetics in the development and treatment of cancer has gained interest and the effects of internal and external factors on the epigenetic profile are under investigation. Recent reports indicate that bioactive compounds can prevent or reverse these epigenetic modifications in cell culture studies and in some animal models of cancer. Appropriate exposure time is critical for bioactive compounds to intervene the epigenetic process.

In the present study, the impact of bioactive compounds on cancer by treating HeLa cells with three bioactive compound namely Fucoidan, Thymoquinone and Diosmin and their role in signalling cascade involve in epigenetic modulation was investigated. Fucoidan is a sulphated polysaccharide predominantly found in cell wall of brown algae(H. Lee, Kim, & Kim, 2012) and used as natural food ingredient with remarkable therapeutic potential comprising of anti-inflammatory, anti-thrombotic,

anti-angiogenic (Xue et al., 2012), immunomodulatory. Thymoquinone (TQ), is an active component of *Nigella sativa* or black seed is used as a cure to all forms of illness dates back to a long time. Diosmin is a kind of plant flavonoids found mainly in citrus fruits. It has also shows anti-apoptotic and anti-inflammatory functions. Role of these drug are still unexplored as an epigenetic modulator.

In order to confirm the role of these compounds as an epigenetic modulator, human cervical adenocarcinoma HeLa cell lines were treated with these compounds. After treatment with these 3 compounds for 24 hours they have shown significant growth inhibition due to increase in ROS generation, mitochondrial superoxide anion generation that leads to loss in ATP and mitochondrial membrane potential. It was also observed that the administration of these compounds result in the cell cycle arrest, HDAC1 inhibition and upregulation of acetylated Histone 3 lysine9/14. Our study also showed that low dose treatment result in premature senescence and induction of autophagy in ROS dependent manner as indicated by the upregulation of p16, p21 anHABP1/p32 and beclin-1. This indicates that these bioactive compounds have the ability to inhibit cancer cell growth and might act as a natural food based drugs.

Furthermore, comparative analysis of commercially available crude and pure Fucoidan from *Fucus vesiculosus* (bladder wrack), a sulphated polysaccharides from seaweed for their cytotoxic potential on human cervical adenocarcinoma HeLa cell lines was done. In case of crude Fucoidan enhanced ROS leads to loss in mitochondrial membrane potential of cells and energy of cells in the form of ATP, furthermore it causes pre-mature senescence and autophagy and at higher doses causes cellular apoptosis. Whereas in case of pure Fucoidan level of ROS decreases and no sign of senescence and apoptosis were observed. Further protein analysis have shown higher expression of p53, Bax and p21 down regulation of Bcl-2 as the marker of apoptosis but in case of pure Fucoidan treated HeLa cells expression level are some what similar to control cell. This indicates that the Crude Fucoidan can act as a pro-oxidant natural food based anticancerous compound, whereas pure Fucoidan act as an antioxidant. Adding to this, the crude and pure Fucoidan were examined using LC-ESI-MS/MS, and it was observed that crude Fucoidan has additional compounds which may be imparting it the abilities to affect

the HeLa cell lines whereas in pure Fucoidan we observed very few additional compounds.

Thus in agreement with the previous reports and the present study targeting the epigenetic modulation using bioactive compounds could be an effective strategy in controlling the cancer cell. HDAC inhibitor like Fucoidan, Thymoquinone and Diosmin shown here from natural sources can be advanced to the clinical stages for the treatment of cancer.

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